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Adherence of *Candida albicans* to a Fibrin-Platelet Matrix Formed In Vitro

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The adherence of *Candida albicans* to a fibrin-platelet matrix formed in vitro was studied. Platelet-rich plasma obtained from rabbits was incubated with thrombin and $CaCl_2$ to form a clot in tissue culture dishes. Such clots were then infected with 3×10^7 *C. albicans* cells per 0.3 ml prelabeled with [U^{14}C]-glucose, and the percent adherence was measured after 30 min of incubation by counting the radioactivity in saline washes of the clot as well as a streptokinase-streptodornase digest of the corresponding clot. Heat- and formaldehyde-killed cells did not adhere as well as viable cells. Pretreatment of *C. albicans* with trypsin, chymotrypsin, and pronase reduced adherence to the clots. Normal rabbit serum and anti-*Candida* antiserum also inhibited adherence 40 and 100%, respectively. Diethylaminoethyl-purified anti-*Candida* gamma globulin (1:8) completely inhibited adherence, whereas purified normal serum gamma globulin did not. Several *Candida* spp. and *Saccharomyces cerevisiae* showed differences in their ability to adhere to clots. *C. albicans* and *C. stellatoidea* presented the highest adherence, whereas *C. krusei*, *C. guilliermondii*, and *S. cerevisiae* adhered less readily. Other species were intermediate in their ability to adhere.

Infective endocarditis is characterized by the colonization of heart valves by microorganisms resulting in the production of an endothelial vegetation. Although *Candida albicans* accounts for only about 1 to 2% of all endocarditis cases (unpublished data), the clinical course of untreated *Candida* endocarditis is a fatal one (10). In a study of 87 patients with *Candida* endocarditis, only 20% recovered (13). The major reason for this low recovery rate is that early diagnosis is difficult because the classic signs and symptoms of an infection are not always present (11, 13).

The pathogenesis of the disease has been studied with a rabbit model (1, 11). The infection was established in rabbits precatheterized via the carotid artery. *C. albicans* cells could be seen adhering to the traumatized valves within 30 to 90 min after infection. It was demonstrated that the initial colonization by *C. albicans* occurred in areas of endothelial trauma on platelet-fibrin deposits.

Scheld et al. (12) studied the adherence of *Streptococcus sanguis* to a fibrin-platelet matrix (clot) formed in vitro. By using this fibrin-platelet method, we have initiated studies on the colonization of clot tissue by *C. albicans*.

MATERIALS AND METHODS

Cultures. The *C. albicans* (clone 4) strains used throughout this study have been previously characterized (7). Isolates of *C. krusei*, *C. guilliermondii*, *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis*, *C. stellatoidea*, and *C. albicans* A-1 were kindly donated by R. D. King. *Saccharomyces cerevisiae* was obtained from The American Type Culture Collection (ATCC 9763).

Preparation of cells. Yeast cells of the above-mentioned species were grown and labeled according to the method of Lee and King (personal communication). An overnight culture (0.2 ml) of cells grown in 25 ml of Phytone peptone broth (BBL Microbiology Systems, 10 g/liter, supplemented with 1 mg of glucose per ml after autoclaving) at 25°C and 150 rpm was used to inoculate 25 ml of fresh Phytone peptone broth containing 0.05 μCi of [U-^{14}C]glucose (316 mCi/mmol) per ml. The cultures were incubated overnight at 25°C and 150 rpm. The cells were then collected by centrifugation (1,700 rpm, 4°C), washed three times with phosphate-buffered saline (PBS) and standardized to 1×10^7 to 5×10^7 cells/ml with a hemacytometer.

Preparation of fibrin-platelet clots. Blood (4.5 ml) was collected from the marginal ear vein of female New Zealand rabbits (5 to 6 lb [ca. 2.2 to 2.7 kg]) in 3.5% sodium citrate (0.5 ml) and centrifuged at 2,000 rpm for 3 min, and the supernatant was collected (13). This fraction was designated as the platelet-rich plasma. In petri dishes (35- by -10-mm tissue culture dish, Falcon plastics), 1 ml of platelet-rich plasma was mixed with 0.4 ml of thrombin (500 U/ml, Sigma Chemical Co.) and 0.4 ml of 0.2 M calcium chloride. The clots were incubated at 37°C for 30 min to insure complete solidification and then stored at 4°C. Generally, the clots were used 2 days after preparation.

Infection of clots. Preliminary studies had indicated that a volume of 0.3 ml of radiolabeled cells (at various cell concentrations) permitted adequate coverage of the clots. After infection with this cell volume, the clots were incubated at 37°C, 125 rpm, for 30 min. For other experiments the incubation time was varied from 0 to 120 min. To enumerate the number of adhering *Candida*, the clots were washed five times with PBS (5×, 1 ml), and the washes were collected. After the saline washes, the clots were incubated with 2 ml of streptokinase-streptodornase (SK-SD, Varidase, Lederle Laboratories; 10,000 U/ml of PBS) at 37°C, 150 rpm for 4 h (12). Radiolabeled *C. albicans* in the dissolved clots (adhering *Candida*) and their corresponding washes (nonadhering *Candida*) were collected on GF/A filters, digested in NCS tissue solubilizer and counted for counts per minute as previously described (8).

Zero-time adherence was determined by infecting clots incubated on ice. The washes were immediately collected. The percent of total counts per minute obtained at zero time usually ranged from 5 to 10% of the inoculum counts per minute. The percent adherence in all experiments was then calculated as a percent of total counts per minute in the inoculum minus the percent adherence at zero time.

Adherence of viable and nonviable *Candida*. *C. albicans* (clone 4) strains were grown, labeled, washed, and standardized as described previously. A 5-ml volume of this suspension was either heat-treated (63°C, 2 h) or centrifuged and suspended in 5 ml of 0.5% formaldehyde in saline (overnight, 4°C) (4). Treated or untreated cells (preincubated in PBS) were washed twice with PBS, standardized to 5×10^7 cells/ml, and added to fibrin-platelet clots. Viability of heat- or formaldehyde-killed cells was determined by plating cells on brain heart infusion agar (Difco). Plates were incubated at 37°C for 48 h.

Effect of proteolytic enzymes on adherence of *C. albicans*. *C. albicans* clone 4 strains (3×10^8 cells per 0.3 ml) were treated with 100 and 250 μg of trypsin (Sigma) per 10^7 cells (final volume, 2 ml) for 1 h at 37°C and 150 rpm. The reaction was stopped by adding trypsin soybean inhibitor (Sigma) (1 μg of trypsin:1.5 μg of inhibitor). Two controls in PBS were used, one of which received inhibitor as described above. The final volume in all treatments was 3.0 ml.

All cell suspensions were restandardized and used to infect clots. Percent adherence was calculated as described earlier. Viability of treated and nontreated cells was determined as described above. Other *Candida* cells (3×10^8 cells/0.3 ml) were incubated with 100 or 250 μg of either chymotrypsin or pronase (Sigma) per 10^7 cells (final volume, 3 ml) for 1 h at 37°C, 350 rpm. The cells were washed, restandardized, and used to infect clots. Viability was measured by plating portions of cells on brain heart infusion agar.

Effect of serum on adherence by *C. albicans*. Antiserum to *C. albicans* (clone 4) was prepared in female New Zealand rabbits (5 to 6 lb [ca. 2.2 to 2.7 kg]) by using formaldehyde-killed cells by the method of Ellsworth and Reiss (3). Briefly, 0.5 mg (wet wt) per 0.5 ml of saline was injected intravenously three times weekly for 4 weeks. Rabbits were subsequently boosted with six additional intravenous injections over a 2-week period. Charcoal agglutination titers (11) from boosted animals were 1:256. Normal serum was collected from nonimmunized rabbits. Twofold dilutions of both immune and normal sera (total volume of 0.5 ml) were incubated with 3×10^7 cells (in 0.1 ml) of *C. albicans* overnight at 4°C. Subsequently, cells were centrifuged, washed several times with PBS, and reacted with goat anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate (GIBCO Laboratories, Santa Clara, Calif.) at 37°C for 30 min. The cells were then washed several times with PBS and examined under a Nikkon fluorescent microscope for fluorescence. Fluorescence was not obtained at a titer of 1:8 or greater with normal serum, whereas strong fluorescence was observed with *Candida* treated with antiserum at the same titer and at higher titers.

To determine the effect of these sera on adherence, a 1:8 titer of normal or immune sera (3 ml) was used in overnight incubations (4°C) with *C. albicans* (3×10^8 cells/0.1 ml). Subsequently, cells were washed twice with PBS, restandardized to 3×10^7 cells/0.3 ml and added to fibrin-platelet clots. Adherence was compared with that of cells incubated overnight in PBS at 4°C.

Preparation of the gamma globulin fraction of normal and anti-*Candida* sera. A 10-ml portion of each serum was dialyzed with 100 volumes of 0.01 M KPO_4 (pH 8) for 18 h. A column was packed with 8 g of diethylaminoethyl cellulose (Whatman DE52, 2.2 by 18 cm) and washed and equilibrated with 0.1 M KPO_4 (pH 8) (starting buffer). The dialyzed serum was applied to the top of the column and eluted with the starting buffer. Ten fractions of 10 ml each were collected (9). Protein distribution in the fraction was measured by optical density at 280 nm (Gilford Instrument Laboratories, Inc., spectrophotometer 2400). The fractions were concentrated five times with a Diaflo ultra-filter PM10 (Amicon Corp.). Immunoelectrophoresis was carried out in a Gelman apparatus in Veronal buffer (0.05 M, pH 8.6) at 10 mA per frame for about 2 h. Fractions with gamma globulin were pooled and adjusted to 500 μg of protein per ml by the Lowry et al. procedure. Twofold dilutions were made in PBS and then incubated with *Candida* and examined for fluorescence as described before. *Candida*, pretreated with gamma globulin from normal or immune sera, was tested for adherence in comparison to cells pretreated with PBS.

Scanning electron microscopy. Fibrin-platelet clots incubated for 30 min with *C. albicans* were washed with PBS and fixed with 3.5% glutaraldehyde in 0.01 M sodium phosphate buffer (pH 7.6). Subsequently, the infected clots were washed twice with phosphate buffer and dehydrated with a graded series of ethanol. The clots were critically point dried and coated with gold (200 to 300 nm) as described previously (1). Clots were viewed with an ETEC Autoscan electron microscope.

RESULTS

Standardization of assay. The following parameters were standardized during the initial

experimentation: number of saline washes required after incubation to remove nonadhering *Candida*, time of incubation of *C. albicans* with the clots, inoculum size (number of cells per ml), and volume. Table 1 shows the results of an experiment in which adherence of *C. albicans* was measured as a function of time. Fibrin-platelet clots were incubated with 5×10^7 cells of ^{14}C-labeled *C. albicans* per ml, and at the designated times, triplicate clots were removed and washed five times with saline (washes collected), and the clots were dissolved with SK-SD. The combined counts per minute of the washes and dissolved clots at 5, 15, and 30 min after incubation were greater than 90% of the original inoculum counts per minute. Recovery of counts per minute at 1 and 2 h, however, was lower (69 to 74% of inoculum counts per minute) and, therefore, an incubation time of 30 min was used throughout the rest of the experiments. The data in Table 1 indicate that adherence does increase to approximately 30% by 30 min as evidenced by an increase in counts per minute per clot or a decrease in counts per minute per five washes. Because of the requirement for an additional 4 h of incubation of the infected clots with SK-SD, we decided to quantitate adherence by counting the counts per minute in the pooled saline washes. Also, radiolabeled *C. albicans*, incubated with SK-SD only (no clots), lost some radioactivity over a 4-h incubation. In other experiments, clots were washed with saline as many as 10 times. However, the total amount of radioactivity removed by the additional washes was less than 3% of the total inoculum (data not shown).

When examined by scanning electron microscopy (Fig. 1), *C. albicans* cells could be seen enmeshed within a fibrin matrix similar to that seen on endocardial vegetations of infected rabbits. The *C. albicans* appeared to have formed germ tubes.

TABLE 1. *Adherence of C. albicans as a function of time*[a]

Time (min)	cpm (%)	
	Wash	Clot
0	88.1	ND[b]
5	83.1	13.9
15	71.9	20.1
30	63.8	28.2
60	38.4	31.0
120	25.0	59.4

[a] Radiolabeled *Candida* (5×10^7 cells/ml) were incubated with clots. At each time interval, triplicate clots were washed five times and the washes were collected and the clots were dissolved with SK-SD. Clot-associated counts per minute (cpm) (adhering) and wash-associated counts per minute (nonadhering) are presented as a percentage of the total counts per minute in the inoculum.

[b] Not done.

In the experiments described above, clots were infected with an inoculum volume of 1.0 ml (3×10^7 to 5×10^7 cells/ml of PBS). However, the volume could be reduced to 0.3 ml without a decrease in adherence (data not shown). This volume also seemed to reduce any attachment of *Candida* to the sides of tissue culture dishes. The adherence of *Candida* to clots was also a function of cell number (Fig. 2). Maximal adherence of *C. albicans* yeast-phase cells was observed at a concentration of 5×10^7 cells/ml or 0.3 ml. This concentration was used in all subsequent experiments.

Adherence of nonviable versus viable cells. To determine whether or not cell viability was essential for adherence to fibrin-platelet clots, *C. albicans* yeast cells (5×10^7 cells/ml) were killed with 0.5% formaldehyde (4°C, overnight) or heat (63°C, 2 h). The ability of such cells to adhere was compared with that of viable cells. The results of this experiment are presented in Table 2. Both heat- and formaldehyde-killed cells adhered less readily than control cells although inhibition of adherence was not complete. Heat- and formaldehyde-killed cells, prelabeled with [*U*-^{14}C]glucose and incubated at 37°C for 30 min in PBS alone, did not lose a significant amount of radioactivity.

Influence of proteolytic enzymes on adherence. *C. albicans* cells, when pretreated with trypsin, chymotrypsin, or pronase (100 or 250 $\mu g/10^7$ cells), did not adhere as readily as control cells (treated with PBS), although adherence was not completely blocked at any concentration of enzyme (Table 2). Adherence of control cells receiving inhibitor only was slightly depressed. At a concentration of 500 μg of trypsin, no additional decrease in adherence was observed (data not shown). Viability was not affected by the pretreatment of cells with these enzymes.

Influence of serum on adherence. The influence of both normal and immune sera (prepared against formaldehyde-killed cells) was investigated by preincubating *Candida* (3×10^8 cells/0.3 ml) with 3.0 ml of a 1:8 dilution of each serum at 4°C for 18 h. The adherence under each condition was compared with that of cells incubated overnight in PBS (4°C). As can be seen in Table 3, adherence was totally inhibited by anti-*Candida* antiserum (1:8), whereas adherence of cells incubated in normal serum was about 60% of control cells (PBS only). In addition, *Candida*, preincubated with a 1:8 dilution

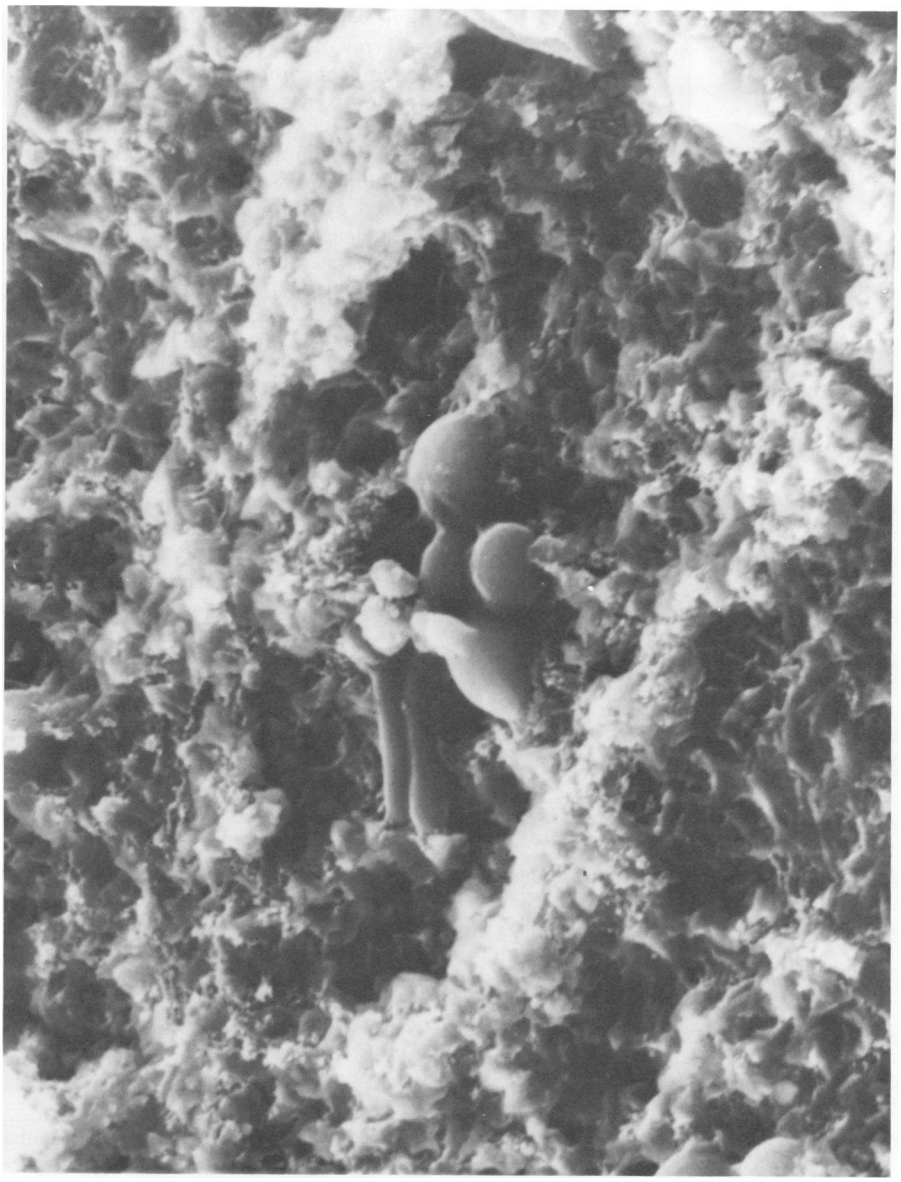

FIG. 1. *Scanning electron micrograph of C. albicans adhering to a clot (×24,000).*

of anti-*Candida* gamma globulin, did not adhere, whereas adherence of cells incubated with normal serum gamma globulin was above control (PBS) levels (Table 3). At a titer of 1:64, adherence was approximately 60% of controls with the anti-*Candida* gamma globulin, and at a titer of 1:512, adherence was actually above control levels. With normal serum gamma globulin, adherence was equal to control at a titer of 1:64. The decrease in adherence was not due to a killing of the *Candida* by the anti-*Candida* gamma globulin (as determined by the trypan blue dye exclusion procedure).

Adherence of *Candida* spp. and *S. cerevisiae*. *Candida* spp. and *S. cerevisiae* were compared with *C. albicans* clone 4 for their ability to adhere to clots. As shown in Table 4, *C. albicans* A-1 and *C. stellatoidea* adhered as well to clots as *C. albicans* clone 4. *C. tropicalis*, *C. parapsilosis*, and *C. pseudotropicalis* represented an intermediate group with regard to adherence since their adherence was 58.6, 48.5, and 40.5% of control. Finally, *S. cerevisiae*, *C. guilliermondii*, and *C. kursei* showed a lower adherence ability as compared with that of *C. albicans* clone 4.

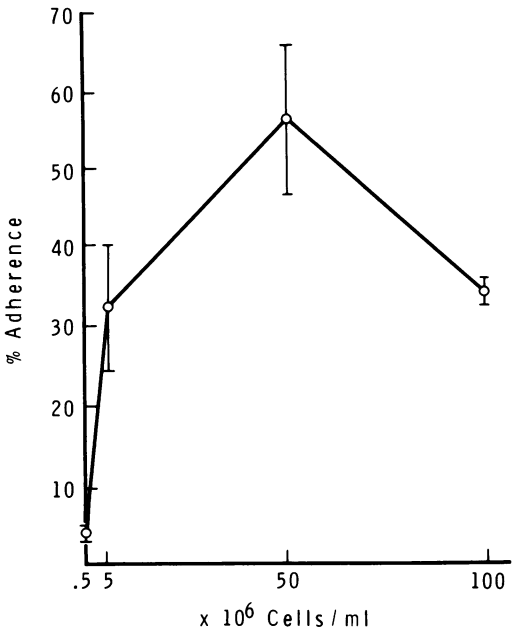

FIG. 2. *Adherence of C. albicans as a function of cell number.*

TABLE 2. *Effect of heat, formaldehyde, and proteolytic enzymes on the adherence of C. albicans clone 4 to clots*

Treatment	% of control[a]
Heat (63°C, 2 h)	53.6
Formaldehyde (0.5%, 4°C, overnight)	56.2
Trypsin, 0 μg + inhibitor, 375 μg	84.0
Trypsin, 100 μg	89.3
Trypsin, 250 μg	68.7
Chymotrypsin 100 μg	72.8
Chymotrypsin, 250 μg	63.4
Pronase, 100 μg	66.3
Pronase, 250 μg	72.8

[a] Control is the percent adherence of *C. albicans* clone 4 preincubated with PBS.

DISCUSSION

The factors which predispose individuals to fungal endocarditis include prolonged antibacterial chemotherapy and intravenous catheterization, drug addiction, and a previous history of heart disease. The infection is also common in recipients of prosthetic cardiac valves. The pathogenesis of infective endocarditis appears to be consistent in spite of the fact that a number of microorganisms can cause the disease. The primary lesion is most likely a thrombus (vegetation) which forms on the endothelium of the heart valve (5). The vegetation can be colonized by a number of microorganisms, with *Candida* spp. and *Aspergillus* the most commonly encountered fungi. Rabbit models of endocarditis have verified the importance of the vegetation in terms of disease development. Once the vegetations become colonized, they increase in size as the disease progresses. Conversely, a thrombus which can be induced in noninfected rabbits quickly becomes covered with endothelia.

Endocarditis caused by *C. albicans* has been studied by using a rabbit model which mimics the disease in humans in a number of ways (1, 11). In the rabbit model, catheterization via the carotid artery traumatizes the endocardium of the aortic valve. Subsequently, an intravenous injection of *Candida* results in the formation of a typical massive vegetation. When examined by electron microscopy at 30 to 90 min after catheterization and infection, *Candida* were seen adhering to fibrin-platelet-erythrocyte deposits (1). Phagocytes (more commonly mononuclear) were abundant and, within the vegetation, could be seen with ingested *Candida*. Many of the *Candida* appeared capable of surviving phagocytosis and had germinated. These surviving fungal elements presumably developed into the large colonies of abundant pseudohyphae and blastospores, now protected within the vegetation by the continued deposition of host elements (1).

Because of the association of the fibrin-platelet deposits and yeast cells during the early

TABLE 3. *Adherence of C. albicans preincubated with normal or immune sera and gamma globulin fractions of each*

Treatment (titer)	% of control[a]
Normal serum (1:8)	59.2
Anti-*Candida* serum (1:8)	0
Normal gamma globulin (1:8)	168.8
Normal gamma globulin (1:64)	106.0
Anti-*Candida* gamma globulin (1:8)	0
Anti-*Candida* gamma globulin (1:64)	59.4
Anti-*Candida* gamma globulin (1:512)	149.5

[a] Cells were treated as described and adherence was compared to PBS-treated *Candida*.

TABLE 4. *Adherence of Candida spp. and S. cerevisiae to fibrin-platelet clots*

Species	% Adherence of control[a]
C. albicans A-1	110.9
C. stellatoidea	105.6
C. tropicalis	56.5
C. parapsilopsis	48.5
C. pseudotropicalis	40.5
S. cerevisiae	30.0
C. guilliermondü	28.4
C. krusei	24.2

[a] Percent adherence of each strain as compared to that of *C. albicans* clone 4.

stages of the disease, this interaction was studied by using an in vitro system. PRP was combined with thrombin and $CaCl_2$ to form clots in tissue culture dishes. Scheld et al. (12) have used this system to study the adherence of *S. sanguis* to a fibrin-platelet matrix (clot). Adherence of *S. sanguis* was enhanced when clots were infected with the bacterium grown in media promoting the formation of surface dextrans (12).

Standardization of the assay was undertaken during early experimentation. Some problems were encountered with the variability in adherence, which probably was a function of the use of different rabbits. After addition of *Candida* to the fibrin-platelet clot, adherence was quantitated by counting the radioactivity (*Candida* cells had been labeled with [*U*-^{14}C]glucose) in pooled washes and SK-SD digest of the clots. Since these numbers were additive (recovery of total inoculum was usually greater than 90% within 30 min of incubation), the assay was quantitated in most experiments by counting the wash counts per minute only. This modification eliminated a 4-h incubation of infected clots with SK-SD, which resulted in some loss of radioactivity. The optimum conditions for adherence of *C. albicans* were a 30-min incubation with 5×10^7 cells per 0.3 ml.

The nature of the surface component of *Candida* which promotes adherence to the fibrin-platelet clots is not known at this time. Preincubation of *Candida* with proteolytic enzymes such as trypsin, pronase, and chymotrypsin resulted in some decrease in adherence. In this regard, Lee and King (J. C. Lee and R. D. King, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, F36, p. 319; Lee and King, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, F50, p. 371) have shown that *C. albicans* blastospores treated with 4×10^{-4} M trypsin lose their ability to adhere to human vaginal epithelial cells. Also, *C. albicans* attachment to phagocytes is inhibited significantly by pretreatment of fungal cells with chymotrpysin (0.25%), whereas *Candida* mannan, at concentrations of 1 and 10 mg/ml, inhibited attachment (2). Viability appears to be essential for adherence to vaginal epithelial cells (Lee and King, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, F36, p. 319), oral epithelial cells (4, 6), and, in this study, to fibrin-platelet clots. Formaldehyde- or heat-killed cells adhered less readily than nontreated cells.

Our data also indicate that normal serum and anti-*Candida* antiserum can block adherence to fibrin-platelet clots. At a 1:8 titer, adherence was completely blocked by whole antiserum and by anti-*Candida* gamma globulin purified by diethylaminoethyl column chromatography. Purified gamma globulin from normal serum did not block adherence and, in fact, adherence was higher than that in PBS-treated cells at a titer of 1:8 (normal gamma globulin). This in vitro observation implies that antibody may play some role in protection, most likely in association with an adequate T-cell response and normal clearance responses.

C. albicans (clone 4 and strain A-1) and *C. stellatoidea* adhered to fibrin-platelet clots to a greater extent than the other species of *Candida* and *S. cerevisiae*. Interestingly, *C. stellatoidea* is very similar to *C. albicans* serologically and physiologically and, therefore, it is not suprising that it adheres better than the other species tested. *C. tropicalis*, *C. parapsilosis*, and *C. pseudotropicalis* were of intermediate ability, whereas *C. krusei*, *C. guilliermondii*, and *S. cerevisiae* adhered the least of all species tested. These data are similar to those obtained by Lee and King (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, F36, p. 319) with human vaginal epithelial cells. The adherence values for *Candida* spp. correlates fairly well with the incidence of endocarditis caused by various *Candida* spp., although *C. stellatoidea* has not been reported as frequently as *C. albicans*.

C. albicans, when examined by scanning electron microscopy, appeared to have germinated. However, whether or not germination is essential for adherence is uncertain since there is not a great amount of germination after a 30-min incubation time.

The surface components of *Candida albicans* responsible for adherence are being investigated.

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