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Yeast Adherence in the Pathogenesis of *Candida* Endocarditis[1] (41261)

W. MICHAEL SCHELD, RICHARD A. CALDERONE,* GEORGE M. ALLIEGRO, AND MERLE A. SANDE[2]

*Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908, and *Department of Microbiology, School of Medicine and Dentistry, Georgetown University, Washington, D.C. 20007*

Abstract. The role of yeast adherence in the pathogenesis of *Candida* endocarditis was examined with both *in vitro* and *in vivo* techniques. *Candida albicans* consistently adhered better than *C. krusei* to the constituents of nonbacterial thrombotic endocarditis (NBTE), fibrin, and platelets, *in vitro*. *C. krusei* adherence to NBTE was 14–24% of that observed with *C. albicans* ($P < 0.001$) by two different methods. These differences correlated directly with the propensity of each organism to produce endocarditis *in vivo*. The infectious dose for 50% of the animals (ID_{50}) for experimental endocarditis in rabbits by *C. albicans* was $10^{5.24}$, this was significantly lower ($P < 0.001$) than the ID_{50} observed with *C. krusei* ($10^{7.47}$). Enhanced yeast adherence to NBTE may be an important virulence factor in fungal endocarditis as it is in streptococcal endocarditis.

Bacterial adherence to the constituents of nonbacterial thrombotic endocarditis (fibrin and platelets) is an important early event in the pathogenesis of infective endocarditis. The adherence of certain bacterial species to heart valves directly correlates with their frequency of isolation from endocarditis cases (1). Extracellular dextran production by viridans streptococci promotes adherence to artificial fibrin–platelet matrices (2) or damaged heart valves (3) *in vitro*. In addition, anti-whole-cell antiserum to *Streptococcus sanguis* prevents adherence to fibrin and platelets *in vitro*, which directly correlates with protection from endocarditis due to this organism in preimmunized rabbits *in vivo* (4).

Fungal endocarditis is increasing in frequency and usually appears in three clinical settings: after insertion of a cardiac valvular prosthesis, in heroin addicts, or as a complication of prolonged intravenous antibiotic therapy or hyperalimentation (5). Various species of *Candida* or *Aspergillus* cause the vast majority of these infections. Within the *Candida* genus, certain species, such as *C. albicans* or *C. parapsilosis*, are isolated much more frequently than other species from endocarditis cases (5, 6). The reasons for this interesting distribution of etiologic agents is unknown, and the role of fungal adherence in the pathogenesis of this disease remains unexplored. In a well-characterized rabbit model of *Candida* endocarditis (7), yeast adherence to the surface of the traumatically induced aortic valve vegetations (composed primarily of fibrin and platelets) was observed by scanning electron microscopy within 30 min after intravenous inoculation (8). Preliminary observations suggest that *Candida* strains may differ in their relative adherence to the constituents of nonbacterial thrombotic endocarditis *in vitro* (9). The purpose of the present studies was: (i) to determine the adherence of two species of *Candida* to a fibrin–platelet matrix *in vitro* by two independent techniques. The strains chosen represent species that commonly (*C. albicans*) or rarely (*C. krusei*) cause endocarditis in man, (ii) to determine if adherence differences between these two or-

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[2] Present address: Department of Internal Medicine, San Francisco General Hospital, San Francisco, Calif. 94110.

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ganisms correlates with the ability to produce experimental endocarditis *in vivo*.

Materials and Methods. *Organisms.* The source of the *C. albicans* (clone 4) and *C. krusei* isolates used throughout this study was described previously (9, 10). The *C. krusei* was originally kindly donated by Dr. R. D. King.

Preparation of yeast cells for adherence assay. The organisms were grown overnight in 25-ml phytone peptone broth (BBL microbiology systems, 10 g/liter supplemented with 1 mg/ml glucose) at 25° and 150 rpm. In one adherence assay (method I) the cells were labeled as described previously (9); 0.2 ml of the above culture (after 18 hr of incubation) was inoculated into 25 ml fresh phytone peptone broth containing 0.05 μCi [C-^{14}C]glucose (316 mCi/mmole)/ml and incubated overnight at 25° and 150 rpm. Prior to use in the adherence assays, the cells were centrifuged (1700 rpm, 4°) and washed with phosphate-buffered saline (PBS) three times, and standardized to $1-5 \times 10^7$ yeast cells/ml in a hemacytometer.

Adherence assay, method I. This has been described in detail previously (9). Platelet-rich plasma (PRP) was collected from rabbit blood by centrifugation and clotted by the addition of thrombin and $CaCl_2$ in plastic petri dishes at 37° for 30 min. The fibrin–platelet clots were stored at 4° prior to use (always within 2 days). Radiolabeled cells were added to the clots (5×10^7 cells in 0.3 ml) and incubated at 37°, 125 rpm, for 30 min. These conditions provided optimal adherence in this system (9). The clots were washed five times with PBS and dissolved by incubation in 2-ml streptokinase–streptodornase (SK–SD, Varidase, Lederle Laboratories, 10,000 U/ml PBS) at 37°, 150 rpm for 4 hr. The adherent candida (in dissolved clots) and nonadherent cells (in washes) were collected on GF/A filters, digested in NCS tissue solubilizer, and counted as described previously (9, 11).

Adherence assay, method II. This assay was a modification of techniques used previously to delineate bacterial adherence (2, 4). The yeast cells were prepared as above, washed thoroughly, filtered (8-μm Millipore), and verified as single organisms in a Petroff–Hauser chamber. Human blood (4.5 ml) was collected in tubes containing 0.5 ml 3.8% Na-citrate, and centrifuged at 2000 rpm for 3 min to obtain PRP; further centrifugation (3000 rpm $\times$ 15 min) was used to obtain platelet-poor plasma (PPP). Platelet-free plasma (PFP) was obtained by sequential filtration (8, 1.2, and 0.45 μm) of PPP; all manipulations were performed in siliconized glassware. One milliliter of PRP, PPP, or PFP was combined in 65-mm tissue culture dishes with 0.4 ml bovine thrombin (500 U/ml) and 0.4 ml 0.2 *M* $CaCl_2$ for 30 min at 37°, as described previously (2, 4). These clots contained either fibrin alone (PFP), or increasing concentrations of platelets in an evenly spread fibrin matrix.

The yeast cells were poured over the clots at an inoculum of approximately 10^3 cells/ml (in 5-ml volume) and incubated for 15 min at 37° during continuous agitation (120 cycles/min). Nonadherent organisms were removed by three sequential washes with PBS and the clot was then overlain with Sabouraud's dextrose agar (BBL) and incubated overnight at 37°. The adherent organisms were enumerated by colony count; the adherence ratio (AR) was defined as the number adherent to the fibrin–platelet clot surface (cfu) divided by the initial inoculum (cfu).

Production of endocarditis. Endocarditis was produced in 2- to 3-kg New Zealand white rabbits by modifications of techniques developed in this laboratory (7, 8, 12). A polyethylene catheter (Intramedic, Clay-Adams, PE-90) was introduced through the right carotid artery and threaded across the aortic valve into the left ventricle. The catheter was left in place for 30 min; this period of trauma uniformly results in the production of nonbacterial thrombotic endocarditis (NBTE, e.g., the deposition of fibrin and platelets) on the aortic valve (2, 13). The catheter was withdrawn 1 cm and the yeast inoculum injected directly through it. The yeast cells were grown for 24 hr in Phytone peptone broth at 25° and 125 rpm, standardized by OD readings in a Gilford spectrophotometer and verified by direct counting in a hemacytometer and 10-fold dilutions in

Sabouraud's dextrose agar pour plates. Groups of 8–10 animals were inoculated with either *C. albicans* or *C. krusei* at the following inocula (cfu in 1 ml volume injected): 10^4, 10^5, 10^6, 10^7, and 10^8. The catheter was then removed and the wound closed. Quantitative blood cultures (1 ml) were obtained 24 hr later. The presence or absence of endocarditis was confirmed 7 days later when the animals were sacrificed and the aortic valve and vegetations removed by wide excision, homogenized, and counted in pour plates (Sabouraud's dextrose agar) as described previously (7, 12). Identical procedures were performed on sections obtained from the left kidney. The infectious dose for 50% of the animals (ID_{50}) was calculated for each organism (based on the number of vegetations with positive cultures in each group), and statistical analysis performed by probit techniques as previously described (4).

Results. *In vitro adherence.* Both methods demonstrated that *C. krusei* adhered less readily to the constituents of NBTE *in vitro* than did *C. albicans* (Table I). When high inocula were used (method I), the mean adherence ratio for *C. albicans* to a rabbit fibrin–platelet clot was 0.345; this was only 0.083 for *C. krusei* ($P < 0.001$). *C. parapsilosis*, a common cause of fungal endocarditis in intravenous drug abusers (5) produced an intermediate adherence ratio (0.195), whereas *C. guilliermondii*, an infrequently isolated pathogen, developed a lower adherence ratio (0.098) similar to *C. krusei* (data not shown). The adherence of *C. krusei* was only 24% of that observed with *C. albicans* under these conditions.

These results were confirmed by viable count techniques evaluating relative adherence to human fibrin or fibrin plus platelet matrices (Method II, Table I). The mean adherence ratio ($\times 10^4$) for *C. krusei* was 400 to 500 for all three experimental surfaces. This value is only 14 to 20% of that observed with *C. albicans* under identical conditions ($P < 0.001$). The adherence ratios were significantly increased ($P < 0.01$) for each yeast when platelets were present in the surface when compared to fibrin alone (PFP); similar to results obtained with the viridans streptococci (2). However, increasing the platelet concentration found in the matrix from approximately 3000/ml (PPP) to 300,000/ml (PRP) did not enhance adherence further for either organism.

Production of endocarditis in vivo. The relative adherence *in vitro* correlated with the ability of *C. albicans* or *C. krusei* to cause endocarditis in rabbits (Table II). The mean ID_{50} for *C. albicans* was $10^{5.24}$, similar to values obtained with organisms commonly implicated in endocarditis such as *Staphylococcus aureus* or viridans streptococci (14). The mean ID_{50} for *C. krusei* was significantly ($P < 0.001$) higher at $10^{7.47}$ which exceeds the ID_{50} for all other organisms previously investigated in this experimental model ((14), W. M. Scheld, and M. A. Sande, unpublished data). The differences between the two *Candida* species are quite apparent when individual inocula are examined. For example, at an inoculum of 10^6 (log 6.078 cfu), *C. albicans* produced

TABLE I. ADHERENCE OF *C. albicans* AND *C. krusei* TO FIBRIN-PLATELET MATRICES *IN VITRO*

Organism	Method I[a] Adherence ratio[b]
C. albicans	0.345 ± 0.015
C. krusei	0.083 ± 0.020

Organism	Method II[a] Ratio ($\times 10^4$) of adherence[b] To PFP[c]	To PPP[c]	To PRP[c]
C. albicans	2015 ± 67	3135 ± 60	3468 ± 75
C. krusei	411 ± 25	516 ± 20	582 ± 42

[a] For details, see text.
[b] Adherence ratio = No. cfu adherent/No. cfu original inoculum. Mean ± SD.
[c] For definition, see text.

TABLE II. PRODUCTION OF EXPERIMENTAL ENDOCARDITIS IN RABBITS BY TWO *Candida* SPECIES

Organism	No. animals	ID_{50} (mean ± SD)
C. albicans	43	$10^{5.24} \pm 0.07$
C. krusei	39	$10^{7.47} \pm 0.05$*

* $P < 0.001$.

positive aortic valve cultures in seven of eight animals. In contrast, none of eight animals injected with *C. krusei* in similar concentrations (log 6.37 cfu) developed endocarditis ($P < 0.001$, F.E.T.). Endocarditis could not be reliably produced with *C. krusei* until the inoculum exceeded 5×10^7 cfu. This contrasts with *C. albicans* where an inoculum of 2.2×10^4 produced endocarditis in three of eight rabbits.

Discussion. These studies demonstrate that various *Candida* species differ markedly in their ability to adhere *in vitro* to the constituents of NBTE, the presumed nidus for colonization in infective endocarditis. In addition, adherence of *C. albicans* or *C. krusei* to fibrin–platelet surfaces correlated directly with the organisms' propensity to produce experimental endocarditis *in vivo*. *C. albicans* adhered avidly to a fibrin–platelet matrix and produced endocarditis readily ($ID_{50} = 10^{5.24}$), whereas *C. krusei* adhered poorly *in vitro* and rarely produced endocarditis in rabbits ($ID_{50} = 10^{7.47}$). The adherence of yeast cells to NBTE must represent an important step in the pathogenesis of fungal endocarditis; similar to the situation that exists for viridans streptococcal endocarditis (2, 4). In the latter infection extracellular dextran production of a high molecular weight by the streptococcus appears essential for optimal adherence (2–4, 15). In contrast, the relative contribution of cell surface factors in *Candida* adherence remains unknown. The presence of NBTE appears crucial to this interaction *in vivo*, since the intravenous inoculation of *Candida* species does not produce endocarditis unless the valve surface has been traumatized and fibrin–platelet deposition has occurred (7, 8, 12, 16, 17).

The precise factors that mediate fungal adherence are poorly understood. The yeast cell surface is complex (18) and consists primarily of protein and mannan components. The latter is prevalent since polysaccharide constitutes 80 to 90% of the fungal cell wall dry weight. The role of these components, or the mucus layer (19) in mediating *in vivo* attachment requires further study.

Several experimental manipulations alter the adherence of *C. albicans* to the constituents of NBTE *in vitro*. Adherence to fibrin–platelet surfaces is decreased by formalin-killing, low temperature, proteolytic enzymatic digestion, or specific antibody (9). Since specific antibody inhibits *C. albicans* adherence to NBTE *in vitro*, preimmunization of animals may protect them from the development of endocarditis upon subsequent challenge. This hypothesis is currently under investigation; a protective role for antibody was demonstrated in streptococcal endocarditis (4, 20). A potential role for surface mannan in the adherence of *C. albicans* to the constituents of NBTE was suggested in recent experiments (21) where alkali-soluble extracts of *C. albicans* cell walls complexed to sheep erythrocytes promoted the adherence of these particles to fibrin–platelet matrices *in vitro*. The extract was 72% polysaccharide with a mannose to glucose ratio of 3:1.

The adherence of *C. albicans* to other surfaces, including buccal or vaginal epithelial cells, has been investigated. Adhesion to buccal epithelial cells was decreased by incubation in PBS (less than incubation in saliva), low temperature, nonviability, manipulations that decreased germ-tube formation, or interference by oral bacteria (22–25). It is doubtful that these alterations are relevant to the interaction of *Candida* with NBTE *in vivo*. Although germ tube formation appears to enhance candidal colonization of buccal epithelial cells (22, 23) these structures were never observed by scanning electron microscopy of *Candida* on the vegetation surface *in vivo*, and the short incubation periods of the *in vitro* assays (15–30 min) would not encourage significant germ-tube formation.

Some species of *Candida* adhere avidly to vaginal epithelial cells (25). *C. albicans* was the most adherent of the species, followed by *C. tropicalis* and *C. stellatoidea*. Adherence was virtually absent for *C. guilliermondii*. This distribution parallels exactly our results shown previously (9) and in the present experiments. The adherence to vaginal epithelial cells was in-

creased for stationary (rather than log) phase organisms, with increasing blastospore to epithelial cell ratios, with increasing temperatures (37° > 25° > 4°), and with time of incubation (maximum at 30 min) (25). Similar findings obtain with the two adherence assays in the present experiments ((8), unpublished data).

C. albicans also attaches to the surface of phagocytic cells (26, 27). This interaction is not influenced by mannose, chitin, concanavalin A, dextran, or highly charged polyamino acids, but is reduced by mannans (in high, unphysiologic concentrations) and chymotrypsin (but not trypsin or protease) (26). This is in contrast to the results with fibrin–platelet surfaces, where all three proteolytic enzymes reduce adherence (9) and mannan pretreatment of the organisms is without effect (unpublished data).

Morphologic studies have not delineated the attachment mechanisms to NBTE. In experimental endocarditis in rabbits, yeast cells are apparent by scanning electron microscopy in areas of fibrin–platelet deposition 30 min after iv injection (8). Intact organisms were not seen on the vegetation surface when examined later (e.g., 2 or 7 days) in this study, but were noted in the chronic phase of infection by others (28). Early experiments suggested that blood cellular elements, particularly monocytes, carried streptococci to the vegetation (29). This type of interaction was never observed in our experiments (8) and monocytopenia does not alter the course of experimental endocarditis (30). Morphologic studies of *Candida* adherent to oral epithelium of rat or rabbit origin in organ culture have revealed five distinct layers of the fungal cell wall within the epithelia (31). Interestingly, adherence appeared to be mediated by the deeper layers and epithelial invasion was associated with local enzymatic lysis (31). Similar studies have not been performed in endocarditis and the role, if any, of preferential enzymatic lysis and penetration of the vegetation by certain species remains unknown.

The present experiments suggest that yeast adherence to NBTE is an important event in the pathogenesis of endocarditis. Definite cause and effect proof of this association awaits further manipulations of the adherence factors and correlation of endocarditis production *in vivo*. Other factors may also be relevant. *C. albicans* is the predominant member of this genus which colonizes mucous membranes of humans; *C. krusei* is infrequently isolated (32). This may explain the predominance of *C. albicans* infections if these regions are traumatized. In addition, strains may differ in invasiveness. Such differences have been demonstrated after intraesophageal challenge in mice with cytarabine-damaged gastrointestinal mucosa; *C. tropicalis* invaded the blood in 69% of mice while the figure for *C. albicans* was only 23% (33). Once the organisms enter the bloodstream and adhere to NBTE, other factors may influence the development of endocarditis. These may include the ability to evade host macrophage ingestion and killing, an enhanced growth rate within the vegetation, the further deposition of fibrin and platelets, or other factors. These properties deserve further study to improve our understanding of the pathogenesis of endocarditis.

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