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Detection of Circulating Antigen in Experimental *Candida albicans* Endocarditis by an Enzyme-Linked Immunosorbent Assay

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A double-antibody sandwich enzyme-linked immunosorbent assay was developed for the detection of circulating *Candida albicans* antigen during the course of experimental *C. albicans* endocarditis. The enzyme-linked immunosorbent assay was positive in 75% of rabbits with polyethylene catheter-induced experimental aortic valve *C. albicans* endocarditis but was negative in all controls, including catheterized animals that received intravenous *Candida* or catheterized but uninfected animals, and in rabbits with experimental fungal or bacterial endocarditis of other etiologies. The enzyme-linked immunosorbent assay was much more sensitive than blood culturing or fever determinations in experimental *C. albicans* endocarditis. This assay is more sensitive than currently available serological techniques, is highly specific, and deserves further study in the diagnosis of invasive, disseminated *C. albicans* infections, including endocarditis.

Fungal endocarditis occurs principally in the following three clinical settings: after cardiac surgery, in the intravenous drug addict, and after prolonged intravenous therapy, including hyperalimentation and treatment for bacterial endocarditis (1, 8, 20, 24). This disease is a devastating complication of cardiac surgery and appears to be increasing in frequency. The pathogen varies with the clinical setting, but *Candida albicans* is the most commonly implicated organism in fungal endocarditis (8, 20). Diagnosis is difficult because blood cultures are frequently negative (25) and delays are common. Thus, despite the introduction of newer antifungal agents and an aggressive medical-surgical approach to these infections, the mortality rate remains high and still exceeds 80% (8, 20). Rapid and specific diagnostic tests are critically needed, and numerous serological procedures have been evaluated. Antibodies directed against cell wall or cytoplasmic antigens can be detected by agglutination (4, 16), precipitation (4, 5, 7, 13, 27, 28), countercurrent immunoelectrophoresis (11), and radioimmunoassay (12). All of these tests are variably sensitive and lack specificity, with up to 40% false-positive and -negative rates (4, 27). These antibody detection tests rely on an intact host immune response and usually take weeks to become positive (21, 28). Detection of circulating antigen offers more hope for the early diagnosis of systemic candidal infections. Gas-liquid chromatography (15, 17), countercurrent immunoelectrophoresis (14), hemmagglutination-inhibition (33), radioimmunoassay (19), and enzyme-linked immunosorbent assay (ELISA) (25, 30, 31) have proven highly specific in the detection of circulating antigen in systemic candidiasis in experimental animals and patients.

Due to the relative rarity of *Candida* endocarditis, evaluation of these serological procedures at only one institution is impossible. We employed our well-characterized rabbit model (2, 21) to determine the sensitivity and specificity of an ELISA method for the detection of circulating antigen in experimental *C. albicans* endocarditis.

MATERIALS AND METHODS

Animal models. *C. albicans* endocarditis was induced in New Zealand white rabbits (2 to 3 kg) by modification of previously published methods (6, 21). Sixteen animals were lightly anesthetized with 60 mg of sodium pentobarbital (Barber Veterinary Supply Co., Richmond, Va.), and the right carotid artery was exposed and ligated. A sterile polyethylene catheter (Radio-opaque, Intramedic, PE-90; Clay-Adams, Parsipanny, N.J.) was passed across the aortic valve. After 1 h of catheter-induced trauma (which uniformly results in the deposition of nonbacterial thrombotic endocarditis), 10^6 to 10^7 colony-forming units (CFU) of *C. albicans* were injected through the catheter. The catheter was ligated, and the wound was closed; catheters remained in place for the duration of the experiments. The test organism (serotype A, clone 4) was

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originally isolated from a patient with prosthetic valvular endocarditis and was kindly provided by Richard A. Calderone, Georgetown University, Washington, D.C. Yeast cells were grown for 24 h in Neopeptone broth (Difco Laboratories, Detroit, Mich.), centrifuged (3,000 rpm for 15 min), and washed in 0.9% NaCl twice. A 10^{-2} dilution was used as the inoculum in a volume of 1.0 ml. This procedure uniformly results in the development of *C. albicans* endocarditis.

Several control models were used, as follows. (i) Ten noncatheterized animals were given identical inocula of 10^6 to 10^7 CFU of *C. albicans* intravenously. This procedure failed to produce systemic infection, and all organs (heart, lung, liver, spleen, kidney) were negative by culture and histological study at autopsy (S. A. Harding, J. P. Brody, and D. E. Normansell, Am. J. Clin. Pathol., in press). (ii) Five animals were catheterized as above, but no organisms were administered. (iii) *Candida krusei* endocarditis (organism again provided by R. A. Calderone) was induced by an identical procedure in three animals but required $\geq 10^8$ CFU in the inoculum to produce consistent, uniformly fatal disease. (iv) Experimental *Staphylococcus aureus* and *Streptococcus sanguis* endocarditis was produced in 12 rabbits after catheter-induced aortic valve trauma by methods described previously (22, 23). (v) Sera from 15 rabbits with experimental meningitis due to *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Escherichia coli* after intracisternal inoculation (W. M. Scheld and M. A. Sande, J. Antimicrob. Chemother., in press) were also tested.

Experimental design. Blood was drawn for the ELISA determination (see below) before inoculation of all animals. This procedure was repeated at 24 h after the initiation of the experiments in all groups and every 2 days thereafter. Catheterized urine was removed at these times from three additional animals. In addition, rectal temperatures were recorded, and blood (1.0 ml) was obtained via the central ear artery for culture on tryptic soy agar (Difco) pour plates at these times. Animals were followed until death or sacrificed at 30 days if still alive. The hearts were removed under sterile conditions, and the aortic valve area with vegetations was dissected free, weighed, and homogenized in saline. Serial 10-fold dilutions were made in tryptic soy agar (Difco), and the results were expressed as CFU per gram of tissue. Small kidney sections were prepared similarly.

Anti-*Candida* antibody production. Five recent clinical isolates (blood or sterile body fluid) were obtained through the microbiology laboratory at the University of Virginia Hospital. After 48 h of growth on Sabouraud agar slants (BBL Microbiology Systems, Cockeysville, Md.), cells were harvested by washing with phosphate-buffered saline (PBS) (pH 7.2). The cells were washed with 20 volumes of PBS and checked for purity by subculture on chocolate agar for 48 h at 37°C. The organisms were pooled and heat killed by exposure to 80°C for 1 h. A 1:1 emulsion of a 20% suspension of the organisms with Freund complete adjuvant was injected subcutaneously biweekly in New Zealand white rabbits (2.5 to 3.5 kg). Rabbits with a whole cell agglutinin titer of $\geq$1:640 (17) at 8 to 12 weeks were bled weekly for an additional 8 weeks, and the sera were pooled. Purified immunoglobulin G was prepared by chromatography on diethylaminoethyl cellulose (Whatman DE52), adjusted to 1 mg/ml with PBS, and stored at −70°C until used as coating antibody or horseradish peroxidase-conjugated antibody in the ELISA procedure.

ELISA procedure. A double-antibody sandwich ELISA for the detection of circulating antigen was used in these studies. Anti-*Candida* antibody (200 μl containing 2 μg of immunoglobulin G in PBS) was used to coat polyvinyl chloride microtiter wells (Cooke Engineering Co. [Dynatech Corp.], Alexandria, Va.) for 3 h at 37°C. After three washes with PBS–0.05% Tween 20, a 200-μl heat-inactivated (56°C for 30 min) serum sample was added to each well and incubated for 3 h at 37°C. PBS-Tween washes were repeated. Anti-*Candida* antibody was conjugated to horseradish peroxidase type VI by the method of Nakane and Kawaoi (18). Fluorodinitrobenzene-blocked peroxidase was oxidized with sodium periodate to form aldehyde groups. Peroxidase-aldehyde was bound to free amino groups of immunoglobulin G unidirectionally with high affinities. This peroxidase-labeled immunoglobulin retained both its enzymatic and immunological properties. Working dilutions of unlabeled coating antibody and peroxidase-labeled immunoglobulin G were determined by checkerboard titrations in microtiter wells with known antigen-positive and -negative sera. Peroxidase-labeled immunoglobulin G was then added to each well ($\simeq$0.5 μg of immunoglobulin G per ml in 200 μl) and incubated for 3 h at 37°C or held overnight at 4°C. After washing with PBS-Tween, 200 μl of freshly prepared substrate (5-aminosalicylic acid with 0.05% H_2O_2 [9:1, vol/vol]) was added and incubated for 1 h at 25°C. The reaction was stopped with 50 μl of 3 N NaOH. Positive reactions were easily read visually by the appearance of a dark brown color or spectrophotometrically by absorbance twice known negative controls at 450 nm. Known positive controls (rabbit serum spiked with 10 μg of purified *C. albicans* mannan per ml, kindly provided by J. E. Bennet, NIH, Bethesda, Md.) were included on each plate with known negative controls (PBS and normal rabbit serum). Purified antigens, including extracts of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* (Hollister-Stier Laboratories, Spokane, Wash.), pneumococcal vaccine (Merck Sharp & Dohme, West Point, Pa.), and group A meningococcal vaccine (J. Colabro, University of Rochester, Rochester, N.Y.), failed to yield positive reactions with this procedure.

RESULTS

Clinical and microbiological characteristics of experimental *C. albicans* endocarditis. All 16 animals followed a clinical course similar to that described previously (21). The median duration of survival was 22 days (range, 8 to $\geq$30). Four animals survived to sacrifice at 30 days. All hearts contained grossly visible, friable vegetations weighing up to 0.86 g. Quantitative aortic valve vegetation cultures revealed a mean ± standard deviation *Candida* concentration of $\log_{10}$ 7.4 ± 0.9 CFU/g. All animals with

endocarditis had renal involvement grossly visible as multiple cortical abscesses. Quantitative kidney cultures were all positive with a mean ± standard deviation fungal concentration of $\log_{10}$ 4.4 ± 1.6 CFU/g. Despite such evident pathology, fever and positive blood cultures were unusual. Only 22 out of 146 (17%) temperature recordings were above 39.6°C, the upper limit of normal in the New Zealand white rabbit. In addition, only 1 out of 96 (1%) 1-ml blood cultures obtained was positive for *C. albicans*.

ELISA determination of *Candida* antigen. This ELISA procedure was capable of detecting 1 ng of purified mannan per ml under the conditions described. If the heat inactivation step was omitted, the level of sensitivity decreased to 100 ng of purified mannan per ml. This heat-labile inhibitor in normal rabbit serum has not been characterized further.

A positive ELISA was defined when a serum dilution of ≥1:2 on two sequential samples yielded a readily visible brown-color reaction noted by two independent observers and was confirmed spectrophotometrically. By these criteria, 12 out of 16 animals (75%) with experimental *C. albicans* endocarditis produced a positive ELISA. Urine samples were positive for antigen in all three animals studied. All controls were negative; these included the 10 animals that received 10^6 to 10^7 CFU of *C. albicans* intravenously without prior catheterization, the 5 uninfected catheterized rabbits, animals with experimental *C. krusei* (3 animals), *S. aureus* (6 animals), or *S. sanguis* (6 animals) endocarditis, and animals with experimental meningitis due to *S. pneumoniae* (6 animals), *H. influenzae* (4 animals), or *E. coli* (4 animals).

Rabbits with endocarditis and a positive ELISA revealed titers of 1:4 to 1:8 at 24 h after injection (Fig. 1). Titers then progressively rose and reached a median level of 1:512 by 5 to 7 days (range, 1:256 to 1:1,024). Titers were maintained in this range until the animal died or was sacrificed. Urine titers were generally lower (range, 1:16 to 1:256). A total of 2 out of 10 animals that received 10^6 to 10^7 CFU of *C. albicans* intravenously in the abscence of catheter-induced aortic valve trauma produced a titer of ≤1:2 soon after injection; the other 8 animals produced consistently negative results. These results were considered negative because the titers were all ≤1:2 on only one determination and quantitative heart and kidney cultures were negative at autopsy.

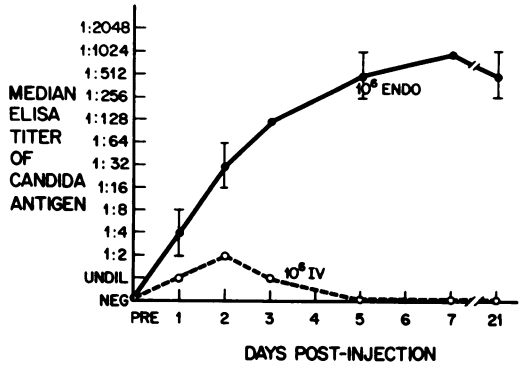

FIG. 1. *Median maximal serum ELISA titer of Candida antigen versus day after injection of 10^6 CFU of C. albicans. Symbols: ●, pattern followed by 12 out of 16 rabbits with endocarditis (ENDO); ○, pattern followed by 2 out of 10 uncatheterized rabbits after intravenous (IV) injection. UNDIL, Undiluted; PRE, preinjection. Bars represent range of ELISA determinations.*

DISCUSSION

This study demonstrates that an ELISA determination of circulating antigen in experimental *C. albicans* endocarditis is feasible, relatively rapid and sensitive, simple, and highly specific. The method was capable of detecting ≤1 ng of purified mannan per ml and was much more sensitive than blood culturing in this experimental model.

The experimental model of endocarditis employed in these studies is similar to the disease in humans and pursues an indolent course. Only 17% of the temperature recordings were positive in the animals used in this study; this figure is lower than those reported for postcardiac surgery *C. albicans* endocarditis in patients, but <50% of the patients were febrile, and another 25% had only transitory fever in one series (24). Other investigations have found fever in virtually all patients (1, 20) with well-established disease.

Methods for the detection of circulating antibody in systemic candidiasis have included such standard techniques as agglutination, immunodiffusion, and countercurrent immunoelectrophoresis (4, 5, 7, 11–13, 16, 27, 28), but all are unsatisfactory due to the following problems: (i) lack of antigen standardization, often employing cell wall or cytoplasmic components or both; (ii) variable sensitivity in the detection of invasive disease (≤40 to 93%, depending on the technique); (iii) poor specificity, with inability to differentiate colonization from systemic infection (up to 40% false-positives in some series); (iv) inability to detect disease early (require 12 days to turn positive in experimental candidiasis [21, 27] and a similar interval in patients), thus precluding rapid institution of specific therapy; (v) dependence on an intact immune response

for formation, often severely attenuated in the immunosuppressed patient, where systemic candidiasis is prevalent; and (vi) persistent positivity for months (or longer). Thus, these tests cannot be used to assess the response to antifungal therapy.

All of these problems could be avoided by the use of an assay for circulating antigen. Miller et al. (17) were the first to report antigen detection in systemic candidiasis. They demonstrated abnormal peaks in serum by gas-liquid chromatography in 6 out of 6 patients with candidemia and 2 out of 4 patients with documented invasive disease; the peaks were absent if superficial colonization alone was present. Similarly, an extremely specific hemagglutination-inhibition assay detected circulating *Candida* mannan in 4 out of 14 patients with systemic candidiasis (33). Recently, a radioimmunoassay capable of detecting 100 ng of *Candida* antigen per ml was positive in 70% of mice with systemic candidiasis (19), and countercurrent immunoelectrophoresis detection of circulating cell wall polysaccharide *Candida* antigen was positive in 13 patients, 8 of whom were proven to have invasive candidiasis (14). Although considerable overlap did occur, gas-liquid chromatography detected ≥ 1 μg of D-arabinitol per ml in 15 out of 20 patients with systemic candidiasis versus 3 out of 28 patients with only superficial colonization (15). These methods deserve further evaluation in the diagnosis of systemic *Candida* infection.

Warren et al. (30) employed an ELISA technique similar to the method used in these experiments to detect circulating antigen in experimental systemic candidiasis in mice and in two rabbits. These earlier experiments did not evaluate nonlethal infection, antigen titer, or pathologic correlations, however. Although the antigen was not identified, later results suggested that the ELISA detected antigens other than mannan (31). In addition, the procedure revealed unacceptable false-positive and -negative rates when evaluated against unknown human sera (32). The methods of antibody production, antibody and sera addition in the ELISA, and enzyme conjugation (alkaline phosphatase) were different from those in the procedures outlined in these experiments. The ELISA reported here is much more sensitive (≤ 1 versus ≤ 250 to 500 ng of purified mannan per ml), requires 20 times less coating antibody, and is equally as specific as the procedure used by Warren and colleagues. Recently, an ELISA-inhibition technique revealed circulating *Candida* antigen in all seven patients with systemic disease (25); however, the method is complicated and less sensitive (≥ 25 ng of mannan per ml), and results are not available for 24 h.

The ELISA, originally described in 1972 (3), is as sensitive and specific as the radioimmunoassay in many situations, but requires nonradioactive materials and inexpensive reagents, and has shown exciting promise in the detection of various infectious diseases (29). The ELISA method reported here detected ≤ 1 ng of purified mannan per ml and was positive for circulating antigen in over 70% of animals with experimental *C. albicans* endocarditis. However, 4 out of 16 animals with endocarditis produced negative results; quantitative aortic valve and kidney cultures were all positive and revealed no differences when compared with the 12 rabbits positive by the ELISA. This may represent a detection of different antigens because a whole-cell immunization scheme was employed, and antibodies directed against different parts of the *Candida* yeast cell or pseudohypha have been described (10, 26). Alternatively, blocking antibody or nonspecific reactions with serum proteins may render the ELISA falsely negative (9). These possibilities require further analysis. The test employed in this study was negative in all controls, demonstrating high specificity. The procedure is easy to perform, inexpensive, requires relatively simple reagents, and can provide rapid results (7 h). The ELISA deserves further study in the detection of circulating antigen in systemic candidiasis, including endocarditis. A similar method revealed 100% sensitivity and specificity in the detection of circulating antigen in experimental systemic candidiasis in immunosuppressed rabbits pretreated with cortisone (Harding et al., in press). The ELISA may be of great value in the diagnosis of *Candida* endocarditis and in following the course after the institution of therapy because circulating antigen, unlike antibody, should decline and eventually disappear with successful intervention.

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