INFECTION AND IMMUNITY, Dec. 1981, p. 938-943
0019-9567/81/120938-06$02.00/0

Vol. 34, No. 3

Platelet Interactions with *Candida albicans*

KATHERINE G. SKERL, RICHARD A. CALDERONE,* AND T. SREEVALSAN

Microbiology Department, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C. 20007

Received 8 May 1981/Accepted 20 August 1981

The interaction of human platelets and *Candida albicans* was studied. Platelet-rich plasma was obtained from freshly drawn blood or outdated platelet concentrates. From the platelet-rich plasma, a platelet extract was derived which stimulated germ tube formation by *C. albicans* when incubated with yeast cells at 37°C. The active component(s) was heat stable, trypsin sensitive, and ribonuclease and deoxyribonuclease insensitive, and possessed cationic properties since it readily attached to carboxymethyl-Sephadex. The active component(s) seemed to bind to heparin also, since germ tube-promoting activity was eluted from a heparin-cyanogen bromide-activated Sepharose 4B column. In addition, platelet-derived growth factor (Collaborative Research, Inc.) stimulated germination when incubated with low amounts (0.4% final concentration) of bovine calf serum. The aggregation of platelets, prepared as platelet-rich plasma by *C. albicans* cell wall or alkali-extracted cell wall fractions, was also studied. Aggregation of platelets was observed when cell wall or cell wall fractions were incubated with platelet-poor plasma at 37°C for 20 min and then added to platelet-rich plasma. The component of platelet-poor plasma which promoted aggregation of platelets by *C. albicans* cell wall or alkali-extracted fractions was inactivated at 56°C (30 min) and by cobra venom factor, indicating a role for the alternate complement pathway in the aggregation response.

During the pathogenesis of endocarditis caused by the yeast *Candida albicans*, vegetations occur on the traumatized heart valve, which superficially are composed of fibrin, erythrocytes, and platelets. The *Candida* yeasts are trapped in the fibrin clots between the damaged tissue and these surface components (2). Once *Candida* sp. takes up residence on the damaged valve endocardium, continued evolution of the vegetation seems to be dependent upon the presence and influence of the yeast, as well as upon the aggregation of platelets on the surface. Uncolonized vegetations remain small and eventually heal. Although platelets appear to be very important in the disease process, they appear to exert their influence by the release of some factor(s) since they are not usually observed within the vegetation. In the inner portions of the vegetations, *Candida* sp. cells can be observed within phagocytes or in a free state (2). Many of the yeasts germinate, producing abundant pseudohyphae.

Two important factors appear, therefore, to be involved in the continued development of the vegetation on the heart valve. First, the aggregation of the platelets on the surface of the initial vegetation, which is presumably influenced by some component of the yeast, and second, the germination of the yeasts with pseudohyphal formation, possibly mediated by soluble platelet components since platelets contain several growth-promoting factors for mammalian cells (15-17). Our experiments were designed to determine by in vitro assay whether *C. albicans* could induce the aggregation of human blood platelets and to characterize the role, if any, of platelet components in germ tube formation by *C. albicans*.

MATERIALS AND METHODS

Organisms. *C. albicans* (1-A) was grown in two different media. For platelet aggregation studies, the yeast was cultured in 25 ml of Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) for 18 to 24 h (37°C, 150 rpm), and for experiments on germ tube formation, the yeast was cultured in 25 ml of Phytone-peptone broth (BBL Microbiology Systems) containing 1 mg of glucose per ml for 18 to 24 h (25°C, 150 rpm) (10). *C. krusei* (isolate courtesy of R. D. King) was cultured in a similar manner for experiments involving platelet aggregation. Zymosan (4 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) was boiled in distilled water for 20 min, then washed three times with physiological saline, and suspended in saline.

Quantitation of germ tube formation. Yeast-phase cells of *C. albicans* were harvested, washed 2 times in sterile saline, and adjusted to a concentration

of 1×10^7 cells per 0.1 to 0.2 ml. Yeast cells were incubated with platelet-poor plasma (PPP), platelet extract (PE), or treated platelet extract, and at 0, 30, 45, 60, 90, and 120 min, a sample was obtained, and germ tube production was determined microscopically. Generally, 100 or more cells were counted, and a percent germination was determined. All experiments were repeated, and similar results were obtained. The volume used in each assay was 0.2 to 0.3 ml (yeast cells, 0.1 to 0.2 ml; extract, 0.1 ml).

Extraction of human platelets. Outdated, concentrated platelet suspensions or freshly drawn human blood platelets were used as a source of the extract. Both were equally effective as sources of extract, but because it was more convenient to obtain the outdated suspensions, they were used almost exclusively. The platelets were obtained from the Georgetown University Hospital Blood Bank or from the American Red Cross Blood Service, Washington Region. Platelet suspensions were centrifuged (1,200 rpm, 10 min, 25°C) to remove erythrocytes and leukocytes. The supernatant (platelet-rich plasma [PRP]) was pooled and centrifuged (4,500 rpm, 10 min, 4°C). The supernatant obtained at this step represented PPP. The pellet containing the platelets was washed 5 times with sterile saline or platelet buffer (16), suspended in deionized water, and then hand homogenized for approximately 2 min with a hand-operated Potter-Elvehjem tissue grinder. The temperature during extraction was 4°C (erythrocyte and leukocyte contamination was variable (<1 to 1%). If lysis was complete (as judged byphase-contrast microscopy), the mixture was centrifuged at 4°C (12,062 × *g* for 10 min). The supernatant (PE) was collected and dialyzed against tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.1 M, pH 7.4) overnight. PE could be stored at −20°C with little loss of activity. The protein concentration of the extract was determined by the Lowry procedure (9).

Characterization of PE. To partially characterize its germ tube-inducing activity, 0.5 ml of PE was tested for heat stability (either by boiling at 100°C or incubating at 56°C for 30 min). In addition, a 0.5-ml volume of extract was treated with 10 μg of ribonuclease A (Sigma Chemical Co.), 10 μg of deoxyribonuclease (Worthington Chemical Co.), or 10 to 100 μg of trypsin (Worthington Chemical Co.). The enzymes were added to the extract in a total volume of 10 μl, incubated at 37°C for 30 min, and then tested for activity. Controls (no enzyme) were similarly diluted with saline. Soybean trypsin inhibitor (15 to 150 μg) (Sigma Chemical Co.) was added after trypsin digestion. Enzyme-treated extract was dialyzed overnight (4°C) against Tris-hydrochloride (0.1 M, pH 7.4).

Column chromatography. The cationic property of the germ tube-inducing component was determined by carboxymethyl (CM)-Sephadex column chromatography. Dialyzed PE or PPP (1 ml) was added to a column of CM-Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.; C-50 medium, total bed volume, 15 ml), equilibrated with the Tris buffer (0.1 M, pH 7.4). The column was washed with Tris-hydrochloride buffer, and 5-ml fractions were collected. When protein could no longer be washed out of the column (usually a total of about 60 ml), the bound proteins were eluted with 0.39 M NaCl and collected in 5-ml fractions. Both the Tris-hydrochloride wash and 0.39 M NaCl eluate fractions were pooled, concentrated to about 1 ml with a Diaflo PM-10 membrane (molecular weight cut off, 10,000; Amicon Corp., Lexington, Mass.), and dialyzed overnight (4°C) against Tris-hydrochloride.

PE was also purified by heparin-cyanogen-bromide-activated Sepharose 4B (Pharmacia) column chromatography (7). A 6-ml column bed of Sepharose 4B conjugated with 1,000 U of heparin (Sigma Chemical Co.) was prepared, and 1 to 3 ml of PE was added. The column was washed with 0.01 M sodium bicarbonate containing 0.15 M NaCl (pH 8.0), and antiheparin proteins were eluted with 0.5 M NaCl (pH 8.0). The wash and NaCl eluate were concentrated and dialyzed as described above.

Platelet preparation for aggregation studies. Freshly drawn whole blood was obtained from normal donors. The blood was collected in 4.5-ml Vacutainer tubes (B-D), centrifuged at 1,200 rpm at room temperature for 10 min to obtain a PRP fraction (supernatant), and then used directly in aggregation experiments. PRP was maintained at room temperature for use in experiments.

Reaction between *C. albicans* or zymosan and platelets. *C. albicans* cell wall (or *C. krusei*) was prepared by a modified procedure of Reiss et al. (13). Each organism was grown in 500-ml batches of Sabouraud dextrose broth in 2-liter flasks for 48 h (37°C). The cells were collected by centrifugation, washed three times with deionized water, standardized, and broken with a Braun homogenizer (Bronwill Scientific Inc., Rochester, N.Y.). The amount of breakage was determined by phase microscopy. Wall fractions were pelleted and washed with sodium dodecyl sulfate in sucrose (13). After extensive washing with deionized water, the cell wall was lyophilized.

With *C. albicans*, the lyophilized cell wall was further fractionated according to Reiss et al. (13). An alkali-soluble, alkali-sonicate (obtained by sonicating cell wall that had previously been extracted with alkali), and an alkali-insolublexfraction were obtained. All fractions were standardized for hexose content by the Anthrone test (20).

In most experiments, the cell wall (4 mg [dry wt]/ml) of *C. krusei*, *C. albicans*, or zymosan (4 mg [dry wt]/ml) was incubated with 4 volumes of undiluted PPP at 37°C for 20 min. A 0.1-ml amount of this mixture was then incubated with 0.3 ml of PRP (prewarmed at 37°C), and aggregation followed at 37°C. We used a Chronolog platelet aggregometer (Chronology Corp., Havertown, Pa.) and chart recorder. Fractionated cell wall (alkali soluble or alkali sonicate, 1.75 mg of hexose per 0.5 ml) was conjugated overnight (4°C) with 0.5 ml of packed 0.46-μm latex beads (Sigma Chemical Co.), using a continuous aliquot mixer (Miles Laboratories, Inc., Elkhart, Ind.). The latex cell wall conjugates were each washed two times with phosphate-buffered saline and suspended in 0.5 ml of phosphate-buffered saline. A 1:10 dilution of conjugated beads was made in PPP and incubated at 37°C for 30 min. A volume of 40 μl of PPP-incubated latex beads was incubated with PRP, and aggregation of PRP was followed as previously described. Binding of the frac-

tions to the latex beads was approximately 25% or about 112 μg of hexose per 0.5 ml of packed beads.

RESULTS

Effect of PE on germ tube formation of *C. albicans*. Preliminary experiments indicated that yeast cells began germination as early as 30 min after incubation with PE. However, maximum germination occurred at 90 min postincubation. The active component(s) of PE was heat stable at 56°C (30 min incubation, 90 to 100% germination) and somewhat less stable at 100°C (2 min), trypsin sensitive, and ribonuclease and deoxyribonuclease insensitive (data not shown). Furthermore, the activity of PE as well as PPP when fractionated on CM-Sephadex was associated with the NaCl eluate, indicating the cationic property of the active component(s) (Table 1). The percent germination at various concentrations of PE is also presented in Table 1. The salt eluate of PPP from CM-Sephadex was also stimulatory, whereas noncationic proteins were not stimulatory (Table 1).

Since growth factors for mammalian cells have been isolated from platelets, some of which bind to heparin (12), we attempted to determine whether the active component(s) which promotes germ tube formation had a similar characteristic. Yeast cells, therefore, were incubated with various concentrations of heparin and a constant amount of PE. At concentrations of 1,000 or 2,000 U of heparin per 0.8 ml, germ tube formation was reduced (Fig. 1). Heparin itself, at a concentration of 1,000 U/ml, did not inhibit germination in Phytone-peptone broth, a medium which promotes germination at 37°C.

These results suggested that the active component(s) interacted with heparin. Direct proof for such an interaction was sought by determining whether the active component(s) could bind

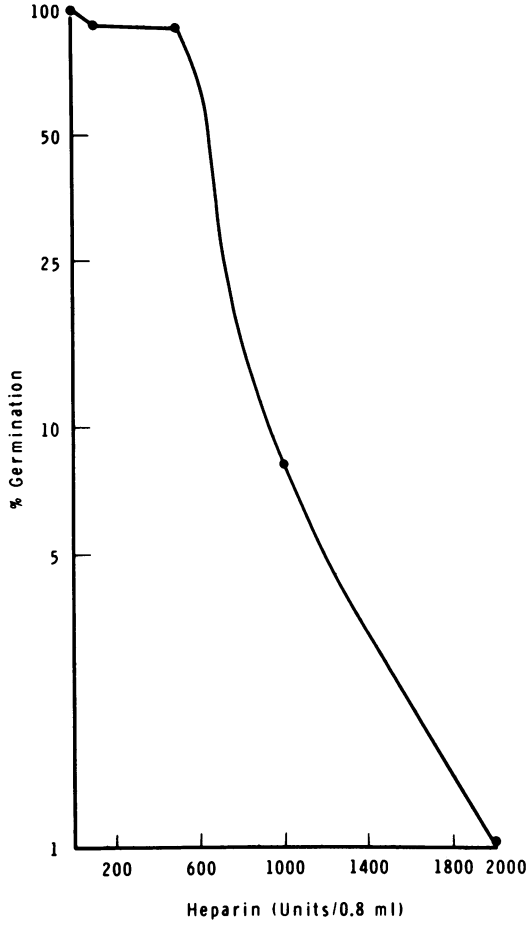

FIG. 1. *Effect of heparin on the induction of germ tube formation by PE. Heparin (0 to 2,000 U per 0.8 ml) was incubated with PE (20 μg of protein), and then yeast cells (1.0 × 10^7/0.2 ml) were added to the mixture. Germ tube formation was determined after 90 min at 37°C.*

TABLE 1. *Effect of PE or PPP on germ tube formation after CM-Sephadex chromatography*

Treatment[a]	Protein (μg)	% Germination
PPP	760	100
PPP after CM-Sephadex	580	0
CM-Sephadex eluate of PPP	12.5	65
PE	130	100
PE after CM-Sephadex	48	0
CM-Sephadex eluate of PE	3.3	23
	7.7	62
	10.2	86
	20.4	98

[a] PE or PPP was chromatographed on CM-Sephadex and eluted with 0.39 M saline. Percent germination was measured at 120 to 150 min of incubation time with 10^7 yeast cells in a total volume of 0.2 ml.

to a heparin-Sepharose 4B column. The heparin-associated proteins, eluted with NaCl, were then tested for germ tube-promoting activity. These data are shown in Table 2. The heparin-Sepharose proteins induced germination at a concentration of 1 and 21 μg/0.1 ml.

Platelet-derived growth factor (Collaborative Research, Inc., Waltham, Mass.), at a concentration of approximately 16 U (12 μg of protein), promoted germination. Yeast cells were incubated with platelet-derived growth factor in 0.4% bovine calf serum (final concentration). Within 1 h, over 95% of the cells had germinated. Yeast cells incubated in only 0.4% bovine calf serum had not germinated. Clumping of the germ tubes prevented quantitation at longer incubation times. Control cells (0.4% bovine calf serum

only) did germinate after longer periods of incubation (39% after 2.5 h), but the percent germination did not increase upon further incubation (data not shown).

Leukocyte contamination of the PRP used for extraction was minimal (<1 to 1%) so that the active component was not derived from leukocytes. In fact, an extract prepared from a leukocyte suspension (100 times more WBC than in PRP) produced only a minimal stimulation when incubated with yeast cells for 2.5 h.

Platelet aggregation. The aggregation of human blood platelets by *C. albicans* cell wall and alkali-extracted fractions is shown in Fig. 2 and 3. When cell wall was preincubated with PPP for 20 min and then exposed to PRP, aggregation of platelets was observed within minutes (Fig. 2). When, however, *C. albicans* cell wall (control) was not preincubated with PPP and then added to PRP, aggregation was not observed for at least 10 min (Fig. 2). *Candida* sp. cell wall alone, incubated in PPP, did not aggregate to any considerable extent (data not shown).

We partially characterized the wall components which promote aggregation, using a fraction obtained by cold-alkali extraction. The alkali-digested cell wall was then further extracted in cold alkali by ultrasonic homogenization. These two fractions (cold alkali soluble, alkali sonicate) and the remaining wall residue (alkali insoluble) were then tested for their ability to promote aggregation. Both the alkali-soluble and the alkali-sonicate fractions were coupled to latex beads, which, in turn, were incubated in PPP. Each of the three fractions promoted aggregation to some extent (Fig. 2, alkali insoluble; Fig. 3, alkali soluble and alkali sonicate). Each of these fractions had been normalized to a hexose concentration as described above, since all fractions are primarily composed of carbohydrate. Latex beads incubated in PPP but with no *Candida* sp. components (control) aggregated platelets to lesser extent (Fig. 3).

TABLE 2. *Effect of PE on germ tube formation after heparin-Sepharose 4B column chromatography*

Treatment[a]	Protein (μg)	% Germination
PE	230	100
Heparin-Sepharose 4B wash	40	0
Heparin-Sepharose eluate	1	21
	21	100

[a] PE column wash or column NaCl eluate was incubated with *C. albicans* yeast cells, and percent germination was measured at 150 min.

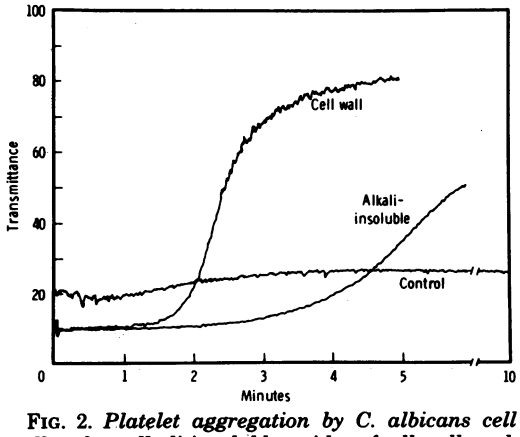

FIG. 2. *Platelet aggregation by C. albicans cell wall and an alkali-insoluble residue of cell wall, each previously incubated in PPP. Control cell wall was not incubated in PPP.*

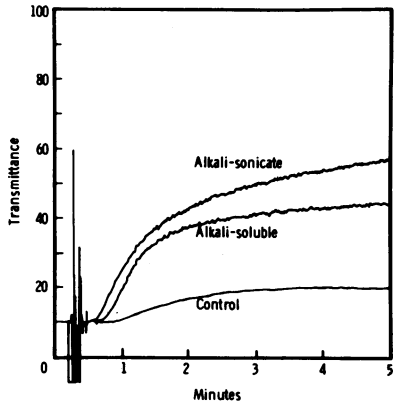

FIG. 3. *Platelet aggregation by alkali-soluble and alkali-sonicated fractions of C. albicans cell wall. The control reaction consisted of latex beads preincubated with PPP and then added to PRP.*

To further characterize the aggregation response, *C. albicans* cell wall was incubated with PPP which had been heated (56°C, 30 min) to inactivate complement. Cell wall incubated with complement-inactivated PPP did not induce an aggregation response, whereas cell wall incubated with PPP (not heat inactivated) promoted aggregation (Fig. 4, cf. A with D). Cobra venom factor was also used to determine the role of the alternate complement pathway in the aggregation response. PPP, pretreated with cobra venom, did not promote an aggregation response by *Candida* sp. cell wall. In addition, PPP, after incubation with cell wall, was not able to promote platelet aggregation by cell wall not previously exposed to PPP (Fig. 4B), whereas the cell wall previously incubated in the same PPP did cause platelet aggregation (Fig. 4C).

As a comparison to *C. albicans*, zymosan and *C. krusei* cell walls were tested for their ability

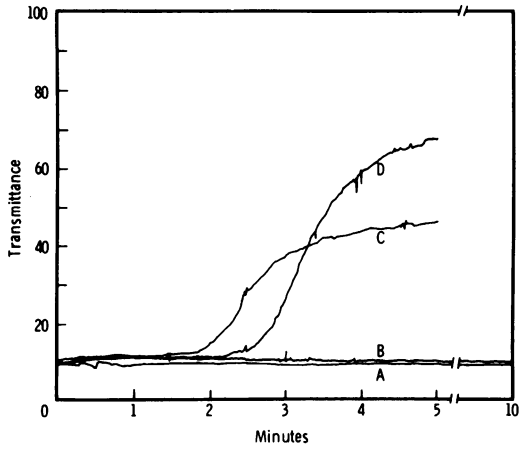

FIG. 4. *Platelet aggregation by C. albicans cell wall. A, PPP was heated at 56°C, 30 min; B, PPP previously absorbed with C. albicans cell wall; C, absorbed cell wall (from B) used to aggregate PRP; D, cell wall preabsorbed in PPP.*

to induce an aggregation response (Fig. 5). Both zymosan and *C. krusei* cell walls, pretreated with PPP, induced an aggregation response similar to that of *C. albicans*.

DISCUSSION

After attachment of *C. albicans* to the traumatized heart valves, two events which seem to be critical to the development of the endocardial vegetations are the aggregation of platelets at the surface of the vegetation and the growth of the yeast within the vegetation. The experiments reported here suggest that *Candida* sp. mediates a platelet aggregation response through a complement-dependent mechanism and that platelets promote the germination of *Candida* sp. Thus, yeast and platelets influence each other in vitro in a manner consistent with in vivo observations.

Platelet aggregation occurred with *Candida* sp. cell wall or wall extracts provided that they were preabsorbed with PPP. The preabsorption was necessary to allow for fixation of complement via the alternate pathway. This observation is based upon the fact that heat-inactivated PPP or cobra venom-treated PPP did not promote aggregation of platelets by cell wall or cell wall components. The alkali extracts (alkali soluble or alkali sonicate) are primarily composed of glucose and mannose polysaccharides and have been described in detail by Reiss et al. (13). *C. krusei*, a comparatively nonpathogenic *Candida* sp., aggregated platelets equally as well as *C. albicans*. Platelet aggregation has been described previously with zymosan (22), as well as with *Histoplasma capsulatum* (5), although with *H. capsulatum*, plasma fibrinogen was necessary for the aggregation response.

Adhesion and aggregation of platelets, in turn, stimulate further platelet adhesion and aggregation (3). Uncolonized vegetations remain small and eventually heal, indicating the role of the yeast in the development of the vegetation. Thus, through a platelet aggregation mechanism, massive vegetations form on the damaged endothelium of the heart valves. In addition, an insoluble, fibrinous material makes up a large component of the vegetation (2, 18). This observation indicates the presence of thrombin, which converts fibriongen to fibrin. Interestingly, the enzyme plasmocoagulase, which can convert prothrombin to thrombin, has been described from a number of fungi, including *C. albicans* (21). This enzyme may play an important role in the development of the vegetation, and additional work is needed to define its role in endocarditis. Disseminated intravascular coagulation is not an uncommon manifestation of disseminated candidiasis (11), and enzymes like plasmocoagulase may be involved in endocarditis and disseminated intravascular coagulation.

Within the vegetations of infected animals, *C. albicans* was observed proliferating as pseudohyphae (2, 18, 19). Platelets, although abundant at the surface of the clot, were rarely observed within the clot, indicating that lysis and release of platelet constitutents had occurred. Platelets contain several low-molecular-weight proteins which are potent mitogens for mammalian cells (15–17). Therefore, it is possible that the proliferation of *Candida* sp. in the vegetation may be induced by platelet components. Our results support this notion since PE was found to stimulate germ tube formation of *C. albicans*. The active component(s) of both PE and PPP was readily eluted from CM-Sephadex by high molar

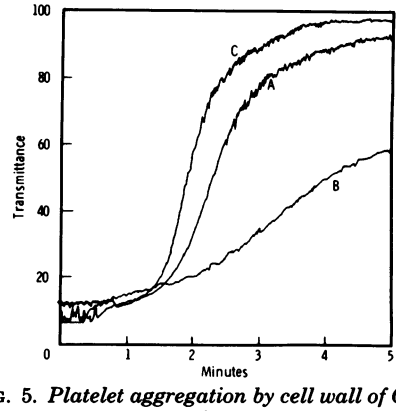

FIG. 5. *Platelet aggregation by cell wall of C. krusei (A), zymosan (B), and C. albicans (C).*

salt, indicating its cationic properties. In comparison, the germ-tube-inducing component of serum (4, 8, 14) has been described as anionic (1). PE was also heat stable and trypsin sensitive, characteristics similar to those of other platelet proteins, such as platelet-derived growth factor, platelet factor 4, and platelet basic protein (12, 17). Platelet factor 4, platelet basic protein and platelet-derived growth factor also bind to heparin, although they differ in their affinity (12). The data presented indicated that yeast cells did not germinate when heparin was incubated with PE before the addition of *Candida* sp. cells. In addition, the active component was eluted from a heparin-Sepharose 4B column. Finally, platelet-derived growth factor proved stimulatory when incubated with bovine calf serum. Whether other platelet factors are equally stimulatory remains to be tested. Recently, nerve growth factor has been shown to promote phase transition in *H. capsulatum* (M. Sacco, B. Maresca, B. V. Kumar, G. Medoff, and G. S. Kobayashi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F24, p. 323).

In vivo, one can imagine a platelet release reaction (or lysis) occurring which promotes the enlargement of the vegetation. The platelet components (and probably other plasma components) could then trigger germ tube production by *C. albicans*. Occurring simultaneously, the aggregation response of platelets would certainly be an important and highly interrelated event which occurs during the pathogenesis of this disease. Platelet aggregation also may play a role in the pathogenesis of bacterial endocarditis (6).

ACKNOWLEDGMENTS

The authors thank Stephanie Coleman for typing the manuscript.

The work was supported by Public Health Service grant 1R23HL21370 from the National Heart, Lung and Blood Institute and by American Heart Association grant 79856.

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