Characterization of Binding of Human Fibrinogen to the Surface of Germ-tubes and Mycelium of *Candida albicans*

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The binding of human fibrinogen to germ-tubes and mycelium of *Candida albicans*, forms usually found in infected tissues, was studied *in vitro* by an immunofluorescence assay. Binding was quantified by using ^{125}I -labelled fibrinogen. The degree of binding differed according to the morphological form of the fungus. Binding relative to that of the yeast form was greater for mycelium (12-fold) than for germ-tube (7.7-fold). Pretreatment of yeasts with fragments D and E (terminal degradation products of fibrinogen) before fibrinogen binding showed that fragment D possessed a higher affinity for *C. albicans* than fragment E. Binding of fibrinogen was diminished when *C. albicans* was pretreated with 2-mercaptoethanol alone or in combination with pronase, or pretreated with α -mannosidase or trypsin. Binding was not decreased by pretreatment with pronase alone or chitinase. Inhibition experiments using *C. albicans* dialysed culture filtrate, *C. albicans* mannan, chitin, sugars or amino sugars were done by preabsorbing the fibrinogen with each of the above mentioned compounds. *C. albicans* dialysed culture filtrate inhibited the binding more strongly than *C. albicans* mannan. However, fibrinogen binding to *C. albicans* was not significantly reduced by mannose, several other sugars or chitin. These studies demonstrate the existence of a fibrinogen-binding factor (FBF) strongly associated with the surface of germ-tube and filamentous forms of *C. albicans*, and indicate a possible role for FBF in the pathogenicity of *C. albicans*.

INTRODUCTION

Candida albicans is a dimorphic pathogen. As a commensal, it grows primarily as a yeast (blastospores), but in infected tissues, blastospores and filamentous forms are simultaneously found (Odds, 1979). The mycelium, initiated by germ-tubes, plays a role in the pathogenicity of *C. albicans*, particularly in colonization of the host. Kimura & Pearsall (1980) have shown that the degree of fungal adherence to host epithelial cells is correlated with the formation of germ-tubes.

The interactions of *C. albicans* with serum proteins are of considerable interest, since exposure of *C. albicans* to mammalian serum results in a variety of morphological effects, such as germ-tube induction (Taschdjian *et al.*, 1960; Hasenclever, 1978). Ray & Wuepper (1976) have demonstrated that *C. albicans* activates the alternative complement pathway, resulting in the formation of chemotactic factors, and Kozutsumi *et al.* (1980) isolated a protein from rabbit serum which binds to yeast mannan (MBP). MBP has the properties of a lectin and may have a role in defence against pathogenic yeasts (Bull & Turner, 1984). An analogous binding protein was isolated from human and bovine sera (Kawasaki *et al.*, 1985). A role for coagulation proteins in the pathogenicity of *C. albicans* has been little documented, although envisaged by Maisch & Calderone (1981), who examined adherence of *C. albicans* to a fibrin-platelet matrix formed *in*

Abbreviation: FBF, fibrinogen-binding factor.

vitro, and by Philippidis *et al.* (1971) and Kambayashi *et al.* (1983), who reported that severe *C. albicans* septicaemia can induce a disseminated intravascular coagulation.

For other micro-organisms, the role of coagulation factors and especially of fibrinogen has been studied. Espersen *et al.* (1984) demonstrated the enhancing effect of human fibrinogen on the virulence of *Staphylococcus aureus* in mice. More recently, Chhatwal *et al.* (1985) reported binding of human fibrinogen to groups A, B, C and G streptococci, and demonstrated that fibrinogen binding inhibited fixation of the third component of human complement on the bacterial surface.

In the present study, binding of human fibrinogen to the surface of germ-tubes and mycelium of *C. albicans* was investigated by immunofluorescence and quantified by using ^{125}I -labelled fibrinogen. The effect of various inhibitors and enzymes pretreatment on binding was also studied.

METHODS

Organisms and culture conditions. *Candida albicans* 1066 (serotype A) originally isolated from a case of candida septicaemia was used throughout. Blastospores were grown at 37 °C on Sabouraud glucose agar (Merck) for 24 h. Germ-tubes and mycelium were obtained by incubation of 24-h-old blastospores for various times (1, 2, 4, 8 and 24 h) at 37 °C in Medium 199 (Flow Laboratories).

Immunofluorescence assay. Two sources of fibrinogen were used: (i) purified human fibrinogen, free of factor XIII (Diagnostica stago) and (ii) whole fresh human plasma.

Fungal elements were washed twice in 0.15 M-phosphate-buffered saline (PBS), pH 7.2, containing 5% (w/v) sodium citrate (PBSC) and adjusted to 5×10^6 cells ml^{-1} . Portions (0.1 ml) of suspension were centrifuged at 500 g for 15 min and the pellets were resuspended in 0.2 ml purified fibrinogen solution (0.25 mg ml^{-1} in PBSC) or in 0.2 ml whole fresh plasma. After 30 min incubation at 37 °C, the fungi were washed twice in PBS and resuspended in 50 μl PBSC. One drop (20 μl) was placed on a microscope slide and allowed to dry. Then 20 μl rabbit anti-human fibrinogen serum (Diagnostica stago) at 1/100 dilution in PBSC was added. Slides were placed in a moist chamber, incubated for 30 min at 37 °C and washed three times in PBSC. Fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG (20 μl 1/100 dilution; Biosis) was added. Slides were incubated for 30 min at 37 °C, washed three times in PBSC and mounted in PBS containing 90% (v/v) glycerol. They were observed under a Nikon microscope equipped for epifluorescence.

^{125}I -Labelled fibrinogen binding assay. Human fibrinogen radiolabelled with ^{125}I (specific activity, 137.5 $\mu\text{Ci mg}^{-1}$, 5.09 MBq mg^{-1}) was from Amersham. Fungal cells (24 h blastospores, 2 h germ-tubes and 24 h mycelium) were washed twice in PBSC. The reaction mixture consisted of 0.1 ml 8% (v/v) fungal suspension and 0.1 ml ^{125}I -labelled fibrinogen (500 $\mu\text{g ml}^{-1}$). After incubation for 30 min at room temperature the fungi were sedimented by centrifugation (15 min at 2000 g) and were washed six times in PBSC. Radioactivity in the sediment was measured in a γ -spectrometer (Kontron MDA 312). For determination of surface areas, a blastospore was considered as a microsphere (5 μm diameter), while a germ-tube was considered as a cylinder (3 μm diameter and 15 μm length) plus a microsphere of 5 μm diameter. Mycelium was considered as a cylinder of 5 μm diameter and 250 μm length. For germ-tubes, the surface area used for determinations was the tube surface only. Data presented are mean values from triplicate experiments.

Competitive binding by fibrinogen fragments D and E. Competitive binding to the surface of 24-h-old mycelium was investigated between non-radiolabelled fibrinogen fragments D or E (Diagnostica stago) and ^{125}I -labelled fibrinogen. In the first phase of the assay, mycelial cells (0.1 ml) were incubated with 50 μl fragment D or fragment E (3–50 $\mu\text{g ml}^{-1}$) for 30 min at room temperature. In the second stage, 50 μl ^{125}I -labelled fibrinogen (100 $\mu\text{g ml}^{-1}$) was added. After incubation for 30 min at room temperature, the mycelium was washed six times in PBSC by centrifugation (15 min at 2000 g), and the radioactivity in the sediment was measured.

Binding inhibition assays. Reaction mixtures consisted of 50 μl iodinated (^{125}I) fibrinogen (100 $\mu\text{g ml}^{-1}$) and 50 μl inhibitor at different concentrations. After 30 min at room temperature, 0.1 ml 24-h-old mycelial suspension (8%, v/v) was added and the radioactivity in the sediment was measured after 30 min of contact at room temperature and several washings. The following substances were tested for their possible inhibitory effect. (i) Culture filtrate from *C. albicans* 1066, grown for 48 h in Medium 199, extensively dialysed against distilled water and lyophilized. The dialysed culture filtrate consisted of 30% protein. Protein was measured by the Lowry method using BSA as a standard. (ii) Mannan, prepared from blastospores of *C. albicans* 1066 by the method of Peat *et al.* (1961). (iii) Chitin (from crab shells; Sigma). (iv) Sugars and amino sugars [D(+) -glucose, D(+) -galactose, D(+) -mannose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine; all from Sigma].

Exposure of mycelial surface to 2-mercaptoethanol and enzymes. SH-Reducing treatment. Washed mycelium (1 mg) was suspended in 2 ml 0.15 M-PBS, pH 7.2, containing 0.2 M-2-mercaptoethanol and incubated for 1 h at 37 °C. **Pronase E treatment.** The mycelium (1 mg) was treated for 1 h at 37 °C with 250 μg pronase E (Merck) and 0.2 M-2-mercaptoethanol in 0.15 M-PBS, pH 7.6, (2 ml). Some mycelium was treated with pronase E without the reducing

agent. *Trypsin treatment.* The mycelial suspension (1 mg in 2 ml 0.15 M-sodium phosphate buffer, pH 7.6) was incubated for 1 h at 37 °C with 250 µg trypsin (Merck). *α -Mannosidase treatment.* The mycelium (1 mg) was treated for 1 h at 37 °C with 200 µl (approximately 80 U) α -mannosidase (Sigma) in 2 ml 0.15 M-PBS, pH 6.8. The enzyme was dialysed against distilled water before use. The preparation of enzyme contained less than 0.05% proteases, β -galactosidase, α -galactosidase and β -N-acetylglucosaminidase. *Chitinase treatment.* The mycelium (1 mg) was treated for 1 h at 37 °C with 250 µg-chitinase (Calbiochem) in 2 ml 0.15 M-sodium phosphate buffer, pH 5.5.

For each treatment, control samples using buffer without enzymes were incubated for 1 h at 37 °C. After different treatments, mycelium was carefully washed in 0.15 M-PBS containing 0.6 M-KCl as osmotic stabilizer, and was used in immunofluorescence and in ^{125}I -labelled fibrinogen binding assays.

RESULTS

Binding of fibrinogen to C. albicans

A study of the binding of fibrinogen to *C. albicans* by immunofluorescence indicated that non-budding blastospores showed binding. Of the budding blastospores, produced under budding conditions or under hyphae-inducing conditions, only a few exhibited a strong fluorescence of their emerging bud. Fig. 1(e) shows a fluorescent bud and a fluorescent hypha emerging from a parent yeast under hyphae-inducing conditions. After 1 h (Fig. 1a) or 2 h (Fig. 1b) germ-tubes showed very strong binding on all the surface of the emerging element. The fluorescence exhibited was mostly homogeneous (Fig. 1d) and 8-h-old mycelium (Figs 1e and f) was also very strongly fluorescent. In 3-d-old mycelium (Fig. 1c) cell-wall fluorescence was heterogeneous and diminished compared with newly-formed germ-tubes (Figs 1a and b). A study by immunofluorescence assay of fibrinogen binding with whole fresh plasma gave binding patterns identical to those obtained with pure fibrinogen.

Binding of ^{125}I -labelled fibrinogen confirmed the observations from immunofluorescence microscopy. When the surface area of fungus exposed to fibrinogen was used as the denominator for measurement of binding, the mean value for the number of bound fibrinogen molecules per mm^2 was $34 \times 10^7 \pm 2 \times 10^7$ (SD) for blastospores, $265 \times 10^7 \pm 6 \times 10^7$ (SD) for germ-tubes and $408 \times 10^7 \pm 9 \times 10^7$ (SD) for mycelium. Thus, fibrinogen binding relative to that of blastospores was 12 times greater for mycelium and 7.7 times greater for germ-tubes.

A study of the capacity of mycelium to bind fibrinogen (Fig. 2) showed that, for 8 µl fungal material, 100% binding was obtained from 250 µg radiolabelled fibrinogen ml^{-1} (final concentration). In the following inhibition experiments, the [^{125}I]fibrinogen concentration used was $25 \mu\text{g ml}^{-1}$, corresponding to 33% binding.

Fibrinogen binding after binding of fibrinogen fragments D and E

The binding of fibrinogen fragments on the mycelial surface of *C. albicans* depended on the concentrations used (Fig. 3). The binding of fragments D or E is expressed as the percentage inhibition of radiolabelled fibrinogen binding. For $50 \mu\text{g ml}^{-1}$ of these fragments, inhibition of binding was easily obtained with fragment D (52% inhibition), but only moderately with fragment E (11% inhibition).

Inhibition of fibrinogen binding by yeast extracts and other substances

Only dialysed culture filtrate strongly inhibited the fibrinogen binding. Thus 75% inhibition was observed with 250 µg filtrate (Fig. 4). At the same concentration, the observed inhibition with mannan extracted from *C. albicans* (Fig. 4) was less strong (38%). Slight inhibition was observed with chitin ($\leq 7\%$ inhibition with 250 µg) (Fig. 4). The specificity of the inhibition was tested by examining the effect of other constituents on binding. Mannose, a constituent of mannan, was not inhibitory. All other sugars [D(+)-glucose and D(+)-galactose] and amino sugars [N-acetyl-D-glucosamine and N-acetyl-D-galactosamine] tested did not inhibit fibrinogen binding.

Fig. 1. Stages in the development of germ-tubes and mycelium from blastospores of *C. albicans*, as seen by immunofluorescence microscopy of fibrinogen-treated organisms. For explanation, see text. Bars, 10 μ m.

Effects of 2-mercaptoethanol and pretreatment with enzymes

The binding of fibrinogen to *C. albicans* was quantified after treatment of 24-h-old mycelium with pronase E alone, pronase E and 0.2 M-2-mercaptoethanol, 0.2 M-2-mercaptoethanol alone, trypsin, α -mannosidase or chitinase (Table 1). Pronase E enhanced fibrinogen binding, and chitinase did not reduce binding significantly, but α -mannosidase, trypsin, 2-mercaptoethanol and 2-mercaptoethanol + pronase resulted in a significant loss of fibrinogen-binding activity.

DISCUSSION

The binding of host proteins, particularly of human fibronectin (Skerl *et al.*, 1984), to blastospores of *C. albicans* is considered to play a role in adherence of *C. albicans* to mammalian cells. With human fibrinogen, the present study demonstrated that binding can be obtained with

Fig. 2. Measurement of [^{125}I]fibrinogen binding to mycelium of *C. albicans*. Each point represents a mean of duplicates for one [^{125}I]fibrinogen concentration. Vertical bars represent SD. For conditions of incubation see Methods.

Fig. 3

Fig. 4

Fig. 3. Inhibition of fibrinogen binding to *C. albicans* by preincubation of the mycelium with fragment E (▲) or fragment D (△) of human fibrinogen. Each point represents a mean of triplicate experiments. Vertical bars represent SD. For conditions of incubation see Methods.

Fig. 4. Inhibition of fibrinogen binding to mycelium of *C. albicans* by preabsorbing the fibrinogen with *C. albicans* culture filtrate (●), mannan (○) and chitin (□). Each point represents a mean of triplicate experiments. Vertical bars represent SD. For conditions of preabsorbing see Methods.

filamentous forms (germ-tubes and mycelium). However, we could observe with only a few budding cells the binding of fibrinogen at their buds; no binding was observed with mother cells. The fibrinogen-binding factor (FBF) appeared early during germ-tube formation; it was detected within 30 min and disappeared as the mycelium aged. These observations were probably associated with the ultrastructural changes in the cell wall during germ-tube formation from blastospores of *C. albicans* (Cassone *et al.*, 1973). The binding of fibrinogen to the filamentous forms, usually found in infected tissues (Odds, 1979) and implicated in adherence processes (Kimura & Pearsall, 1980), may suggest a role for FBF in the pathogenicity of *C. albicans*. The FBF could account for adherence of *C. albicans* to the fibrin-platelet matrix formed *in vitro*, as demonstrated by Maisch & Calderone (1980, 1981). In addition, this binding was not inhibited by plasma components. The binding site of fibrinogen is probably located on fragment D

Table 1. *Effect of treatment with 2-mercaptoethanol and enzymes on fibrinogen binding to C. albicans mycelial surface*

Pretreatment*	Percentage of [¹²⁵ I]fibrinogen binding $\pm$ SD†	Relative binding‡
PBSC	44 $\pm$ 5	1.0
Pronase E	56 $\pm$ 7	1.3
2-Mercaptoethanol	13 $\pm$ 2	0.3
Pronase E and 2-mercaptoethanol	16 $\pm$ 2	0.4
Trypsin	24 $\pm$ 3	0.5
α -Mannosidase	18 $\pm$ 2	0.4
Chitinase	38 $\pm$ 4	0.9

* For conditions of treatment with hydrolytic enzymes see Methods.

† Data presented are mean values from triplicate experiments $\pm$ SD. *C. albicans* treated with buffer without enzymes or SH-reducing agent served as the control.

‡ Binding relative to that of mycelium treated with PBSC.

because this has a higher affinity for *C. albicans* than fragment E. Pretreatment of germ-tubes with 2-mercaptoethanol alone or in combination with pronase decreased fibrinogen binding, but the difference between the two pretreatments was not significant (Table 1). It seems possible that 2-mercaptoethanol releases the FBF. These results are comparable with the observations of Davies & Wayman (1975) and Chattaway *et al.* (1976) in which 2-mercaptoethanol was found to release a large amount of cell wall material from *C. albicans* blastospores. These released components may be implicated in concanavalin A agglutination (Cassone *et al.*, 1978; Tronchin *et al.*, 1981). Pretreatment with trypsin also decreased fibrinogen binding. The role of other cell wall components was studied. Pretreatment with chitinase gave no significant decrease in fibrinogen binding, but α -mannosidase released mannose from the cell wall of *C. albicans* and decreased the binding.

Mannan and proteins may be components of FBF. However, purified mannan did not significantly inhibit the binding at low concentration and inhibition was obtained only at high concentration. With dialysed culture filtrate of *C. albicans*, on the other hand, a strong binding inhibition was obtained even at low concentration (Fig. 4). These results confirm that proteins and glycosidic components present on the mycelial surface are involved in fibrinogen binding.

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REFERENCES

- BULL, F. G. & TURNER, N. R. (1984). A serum mannan-binding protein and candidiasis. *Sabouraudia* **22**, 347–350.
- CASSONE, A., SIMONETTI, N. & STRIPPOLI, V. (1973). Ultrastructural changes in the wall during germ-tube formation from blastospores of *Candida albicans*. *Journal of General Microbiology* **77**, 417–426.
- CASSONE, A., MATTIA, E. & BOLDRINI, L. (1978). Agglutination of blastospores of *Candida albicans* by concanavalin A and its relationship with the distribution of mannan polymers and the ultrastructure of the cell wall. *Journal of General Microbiology* **105**, 263–273.
- CHATTAWAY, F. W., SHENOLIKAR, S., O'REILLY, J. & BARLOW, A. J. E. (1976). Changes in the cell surface of the dimorphic forms of *Candida albicans* by treatment with hydrolytic enzymes. *Journal of General Microbiology* **95**, 335–347.
- CHHATWAL, G. S., DUTRA, I. S. & BLOBEL, H. (1985). Fibrinogen binding inhibits the fixation of the third component of human complement on surface of groups A, B, C, and G streptococci. *Microbiology and Immunology* **29**, 973–980.
- DAVIES, R. & WAYMAN, F. J. (1975). The effect of thiols on *Saccharomyces fragilis*. *Antonie van Leeuwenhoek* **41**, 33–58.
- ESPERSEN, F., CLEMMENSEN, I., FRIMODT-MOLLER, N. & JENSEN, K. (1984). Effect of human IgG and fibrinogen on *Staphylococcus aureus* intraperitoneal infection in mice. *Acta pathologica, microbiologica et immunologica scandinavica* **92**, 311–317.
- HASENCLEVER, H. F. (1978). The *in vitro* interactions of *Candida albicans* with non specific serum proteins. *Mycopathologia* **65**, 169–176.
- KAMBAYASHI, J., KOSAKI, G. & SHIMADA, Y. (1983). Disseminated intravascular coagulation associated

- with severe infection in surgical patients: a retrospective and prospective study. *Bibliotheca haematologica* **25**, 49–58.
- KIMURA, L. H. & PEARSALL, N. N. (1980). Relationship between germination of *Candida albicans* and increased adherence to human buccal epithelial cells. *Infection and Immunity* **28**, 464–468.
- KOZUTSUMI, Y., KAWASAKI, T. & YAMASHIMA, I. (1980). Isolation and characterization of a mannan-binding protein from rabbit serum. *Biochemical and Biophysical Research Communications* **95**, 658–664.
- KAWASAKI, N., KAWASAKI, T. & YAMASHIMA, I. (1985). Mannan-binding protein and conglutinin in bovine serum. *Journal of Biochemistry* **98**, 1309–1320.
- MAISCH, P. A. & CALDERONE, R. A. (1980). Adherence of *Candida albicans* to a fibrin-platelet matrix formed *in vitro*. *Infection and Immunity* **27**, 650–656.
- MAISCH, P. A. & CALDERONE, R. A. (1981). Role of surface mannan in the adherence of *Candida albicans* to fibrin-platelet clots formed *in vitro*. *Infection and Immunity* **32**, 92–97.
- ODDS, F. C. (1979). *Candida and Candidosis*. Baltimore: University Park Press.
- PEAT, S., WHELAN, W. J. & EDWARDS, T. E. (1961). Polysaccharides of baker's yeast. IV. Mannan. *Journal of the Chemical Society* **1**, 29–34.
- PHILIPPIDIS, P., NAIMAN, J. L., SIBINGA, M. S. & VALDESDAPNEA, M. A. (1971). Disseminated intravascular coagulation in *Candida albicans* septicemia. *Journal of Pediatrics* **78**, 683–686.
- RAY, T. L. & WUEPPER, D. (1976). Activation of the alternative (properdin) pathway of complement by *Candida albicans* and related species. *Journal of Investigative Dermatology* **67**, 700–703.
- SKERL, K. G., CALDERONE, R. A., SEGAL, E., SREEVALSAN, T. & SCHELD, W. M. (1984). *In vitro* binding of *Candida albicans* yeast cells to human fibronectin. *Canadian Journal of Microbiology* **30**, 221–227.
- TASCHDJIAN, C., BURCHALL, J. & KOZINN, P. (1960). Rapid identification of *Candida albicans* by filamentation on serum and serum substitutes. *American Journal of Diseases of Children* **99**, 212.
- TRONCHIN, G., POULAIN, D., HERBAUT, J. & BIGUET, J. (1981). Cytochemical and ultrastructural studies of *Candida albicans*. II. Evidence for a cell wall coat using concanavalin A. *Journal of Ultrastructure Research* **75**, 50–59.