Effect of Pathogenic Yeasts on Human Platelet Aggregation

Teresinha de Jesus Carvalho Neiva and Jairo Ivo dos Santos

Federal University of Santa Catarina, Centre of Health Science, Florianópolis, SC, Brazil

We investigated the effects of *Candida albicans*, *Cryptococcus neoformans* and the respective culture supernatants on human platelet aggregation (PA). Both yeasts were unable to aggregate the platelets directly. On the other hand, cells of these yeasts significantly ($P < 0.01$) inhibited PA at concentrations equal to or higher than 1×10^6 cells/mL for *C. albicans* and equal to or higher than 1×10^5 cells/mL for *C. neoformans*. When the supernatants of one-week broth cultures were added to the activated platelets no inhibition in aggregation was observed. Apparently somatic components of these yeasts, but not their metabolic products, exert an antagonistic effect on the aggregation of human platelets, possibly aiding the fungi in their evasion of the microbicidal defense system during vascular dissemination.

Key Words: *Candida albicans*, *Cryptococcus neoformans*, platelet aggregation.

Platelets are blood components that participate in physiological as well as pathological processes, such as homeostasis, thrombosis, atherosclerosis and infections [1-4]. In platelet aggregation (PA), the platelets interact with each other to form a hemostatic plug or thrombus. This process can be triggered *in vitro* by mixing fresh platelets with agonists, such as collagen or adenosine diphosphate (ADP), which activate the resting platelets [5].

Many fungal species, or their products, can interfere directly in platelet functions. The inhibition of PA, induced by components present in extracts of moulds and mushrooms, is well known [6-10]. In addition, pathogenic fungi, like the yeasts *Candida albicans* and *Cryptococus neoformans*, can cause infections that are particularly severe in immunocompromised individuals [11, 12]. During fungal infection, these patients are under increased risk for fungal proliferation in their blood and further vascular dissemination [13,14].

Received on 15 May 2003; revised 08 August 2003.

Address for correspondence: Dr..Teresinha de Jesus Carvalho Neiva, Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Campus Universitário, Florianópolis, Brazil, 88040-970. Phone: 0xx48 331 9712. Fax: 0xx48 331 9542. E-mail: neiva@ccs.ufsc.br

The Brazilian Journal of Infectious Diseases 2003;7(6):370-374

The mechanisms by which fungal pathogens initiate vascular infections are believed to be complex, and they involve interactions of fungal cells with plasma components, e.g., fibronectin [15], endothelial cells [16] and platelets [17], and avoidance of plasma host defenses, such as complement and phagocytosis. Platelet activation also releases inflammatory mediators, e.g., arachidonic acid metabolites and platelet microbicidal proteins [5,18]. Hence, fungal cells can interact with blood components, including those associated with hemostasis processes.

Therefore, we evaluated the effect of *C. albicans* and *C. neoformans* cells, as well as their culture supernatants on human PA, *in vitro*.

Materials and Methods

Reagents. Collagen and ADP were purchased from Chronolo. Corp., USA, while the remaining reagents were purchased from Sigma (USA).

Fungal preparation. Candida albicans (strain 3153 from the London School of Hygiene and Tropical Medicine Collection) and *C. neoformans* (strain ACL-68, previously isolated from the cerebrospinal fluid of an AIDS patient) were cultured in Sabouraud's dextrose agar during five days at 25°C. Afterwards,

the yeast cells were suspended in sterile saline, counted in a haemocytometer and were diluted to various concentrations of 1 x 10^4 to 1 x 10^7 cells/mL. Supernatants were also obtained from one-week cultures in Sabouraud broth. These cultures yielded approximately 1 x 10^7 cells/mL after one week of incubation. To obtain the supernatant, the broth was centrifuged at 500 g for 5 min, and then the supernatant was collected and centrifuged again at 8,000 g for an additional 5 min. The supernatant was then collected and used for the platelet aggregation test. Sterile Sabouraud's broth was used as a control.

Subjects. The study included 12 healthy volunteers, who gave informed consent before participating in this study.

Isolation of platelets. Human blood platelets were obtained from healthy, drug-free individuals and were collected in tubes containing 3.8% sodium citrate. Platelet-rich plasma was prepared by centrifugation of citrated blood at room temperature for 6 min at 180 *g*. Platelets were adjusted to 2.5 x 10^8 cells/mL with sterile saline.

Measurement of PA. PA was determined by the turbidimetric method [19] using a Net Lab aggregometer. Aliquots of 450 µL of a platelet suspension were transferred into small cuvettes and stirred at a constant speed of 180g at 37ºC. The platelets were pre-incubated for 5 min at 37ºC with different yeast concentrations, or with the supernatants of the cultures, and were stimulated with 6 µM ADP and 2 µg collagen/mL. The extent of aggregation (%) was recorded continually for 5 min after addition of the agonist. To evaluate the ability of *C. albicans and C. neoformans* to aggregate platelets, fungal cells (1 x 10^7 cells/mL) or culture supernatants were mixed with platelets (2.5 x 10^8 cells/mL) and incubated at 37ºC during 10 min.

Statistical analysis. Data were expressed as mean ± SD. The Student's t-test was employed to determine if there were differences between the groups. Differences were considered significant when the probability was $P < 0.05$. The statistical program INSTAT-2 was utilized.

Results and Discussion

We observed that *C. albicans* and *C. neoformans* cells were unable to aggregate platelets directly (Figure 1). Studies on interactions of yeasts and platelets have demonstrated a direct agonistic effect on platelet aggregation. The yeast forms of the dimorphic fungi *Histoplasma capsulatum* can induce platelet aggregation *in vitro* [20]. Likewise, a *Saccharomyces cerevisiae mannan* fraction was found to be lethal when injected intravenously into mice, resembling the effect induced by the administration of platelet-activating factor [21,22]. In addition, somatic fractions of this yeast, which were chemically defined as being composed of phosphocholines, were also capable of activating platelets [23]. Willcox et al. [24] found that human PA is induced by all *Candida* species, except for *C. albicans*. The aggregation of platelets releases microbicidal or microbistatic substances that are active against *Candida spp.* [24,25]. Therefore, the ability of *C. albicans* to inhibit the aggregation of platelets could contribute to its survival in the blood during vascular infections.

High cell counts of both *C. albicans and C. neoformans* were capable of preventing PA in the presence of collagen and ADP (Table 1). The inhibitory effect was always concentration dependant. A significant inhibition was observed after incubation of platelets with 1 x 10^6 cells/mL of *C. albicans*. The maximum inhibitory effect was obtained when 1 x 10^7 cells/mL was used. On the other hand, *C. neoformans* produced a more intense inhibitory effect on PA than *C. albicans*. We found that as low as 1 x 10^5 cells/mL were capable of inhibiting PA (Table 2). This antagonistic effect could also explain the capacity of this fungus to evade the microbicidal mechanisms triggered by PA. Numerous moulds and mushrooms, or their products, can easily inhibit PA *in vitro* [5,6,8,26,27]. In these cases, the antagonistic effects derive from substances, such as proteins, hydrazine derivatives, peptides, nucleosides,

Table 1. Effect of *Candida albicans* on platelet aggregation, stimulated by collagen and ADP. Platelets were pre-incubated with *C. albicans* cells for 5min at 37°C before stimulation. Each value represents the mean ± SD of 12 independent experiments

	Aggregation (%) Collagen- 2 µg/ml	**Aggregation (%) ADP-6 µM**
Control	75.4 ± 7.3	69.8 ± 10.0
1 X 10^5 cells/mL	65.9 ± 8.0	68.4 ± 8.2
1 X 10^6 cells/mL	57.2 ± 2.7*	58.8 ± 6.0*
1 X 10^7 cells/mL	35.0 ± 5.8*	29.8 ± 7.0*

Student's t-test: P < 0.01*.

Table 2. Effect of *Cryptococcus neoformans* on platelet aggregation, stimulated by collagen and ADP. Platelets were pre-incubated with *C. neoformans* cells for 5 min. at 37°C before stimulation. Each value represents the mean ± SD of 12 independent experiments

	Aggregation (%) 2 µg/mL Collagen	**Aggregation (%) 6 µM ADP**
Control	85.7 ± 11.0	73.4 ± 8.4
1 X 10^4 cells/mL	76.2 ± 10.0	72.0 ± 6.1
1 X 10^5 cells/mL	70.0 ± 7.1*	56.0 ± 6.0*
1 X 10^6 cells/mL	58.0 ± 3.2*	49.0 ± 1.4*
1 X 10^7 cells/mL	7.4 ± 3.6*	8.8 ± 2.3*

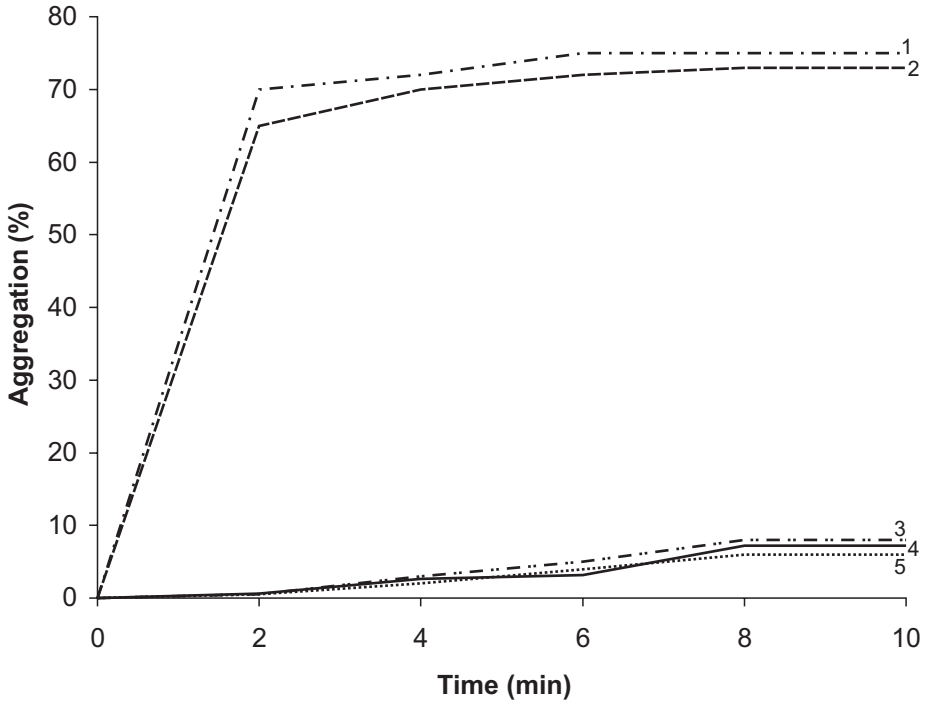
Student's t-test: P < 0.01*.

Table 3. Effect of *Candida albicans* and *Cryptococcus neoformans* culture supernatants on platelet aggregation, stimulated by collagen and ADP. Platelets were pre-incubated with the supernatant of one-week cultures of the yeasts, for 5 min. at 37°C, before stimulation. Each value represents the mean ± SD of 5 independent experiments

	Aggregation (%) 2 µg/ml Collagen	**Aggregation (%) 6 µM ADP**
C. albicans		
Control	66.2 ± 4.4	61.6 ± 10.5
Supernatant (from 1 X 10^7 cells/mL)	64.2 ±3.6	
C. neoformans		
Control	73.8 ± 4.0	57.0 ± 8.8
Supernatant (from 1 X 10^7 cells/mL)	69.2 ± 2.3	

Student's t-test: P > 0.05 (n.s).

Figure 1. Platelet aggregation in the presence of 2 µg/ml-Collagen (1), 6 µM ADP (2), 1 X 10^7cells/mL *Candida albicans* (3), 1 X 10^7 cells/mL of *Cryptococcus neoformans* (4) and platelets plus saline (5).



nucleotides, lecithins, saponins and sterols, which are present in the fungal extracts and can directly inhibit the aggregating agents [28].

Finally, to determine if soluble by-products from yeast cultures have any effect on PA, centrifuged supernatants from one-week cultures containing 1 x 10^7 cells/mL were added to the platelets before being activated by collagen and ADP. No change in the intensity of PA was observed for the supernatants of the two yeasts (Table 3). It is known from *in vivo* studies, that the capsule prevents the platelets from adhering to *C. neoformans* [29]. However, the polysaccharide capsule does not necessarily play a role in the inhibition of PA, since we observed that the supernatant of one-week cultures of *C. neoformans*, which should contain soluble capsular antigens, did not inhibit PA.

In conclusion, unidentified *C. albicans* and *C. neoformans* cell component(s) can directly inhibit platelet activation, playing a role in fungal evasion from host defenses, during vascular dissemination.

References

1. McCrae K.R., Bussel J.B., Mannucci P.M., et al. Platelets: an update on diagnosis and management of thrombocytopenic disorders. Hematology (Am. Soc. Hematol. Educ. Program.) **2001**;1:282-305.
2. Theilmeier G., Michiels C., Spaepen E., et al. Endothelial von Willebrand factor recruits platelets to atherosclerosis-prone sites in response to hypercholesterolemia. Blood **2002**;99:4486-93.
3. Roux W., Pieper C., Cotton M. Thrombocytopoenia as marker for HIV exposure in the neonate. J Trop Pediatr **2001**;47:208-10.
4. Yeaman M.R. The role of platelets in antimicrobial host defense. Clin Infec Dis **1997**;25:951-70.
5. Colman R.W., Hirsh J., Marder J.V., Salzman E.W. Hemostasis and thrombosis. Basic principles and clinical practice. Philadelphia, USA: J.B. Lippincott Company, 3rd edition, **1994**.

6. Ando T., Tsurumi Y., Ohata N., et al. Vinigrol, a novel antihypertensive and platelet aggregation inhibitor agent produced by a fungus, *Virgaria nigra*. I. Taxonomy, fermentation, isolation, physicochemical and biological properties. J Antibiot (Tokyo) **1988**;41:25-30.
7. Chu M., Truumees I., Gunnarsson I., et al. A novel class of platelet activating factor antagonists from *Phoma sp*. J Antibiot (Tokyo) **1993**;46:554-63.
8. Erkel G., Lorenzen K., Anke T., et al. Kuehneromycins A and B, two new biological active compounds from a Tasmanian *Kuehneromyces sp* (*Strophariaceae*, *Basidiomycetes*). Z Naturforsch **1995**;50:1-9.
9. Kagamizono T., Nishino E., Matsumoto K., et al. Bassiatin, a new platelet aggregation inhibitor produced by *Beauveria bassiana* K-717. J Antibiot (Tokyo) **1995**;48:1407-12.
10. Fabian K., Lorenzen K., Anke T., et al. Five new bioactive sesquiterpenes from the fungus *Radulomyces confluens* (Fr.) Christ. Z Naturforsch **1998**;53:939-45.
11. Zuger A., Louie E., Holzman S.R., et al. Cryptococcal disease in patients with the acquired immunodeficiency syndrome. An Int Med **1986**;104:234-40.
12. Walsh T.J., Groll A.H. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. Transpl Infect Dis **1999**;1:247-61.
13. Kobza K., Steenblock V. Demonstration of *Candida* in blood smears. Br Med J **1977**;1:1640-1.
14. Kates M.M., Phair J.B., Yungbluth M., Weil S.L. Demonstration of *Candida* in blood smears. Lab Med **1988**;19:25.
15. Jakab E., Paulsson M., Ascenio F., Ljungh, A. Expression of vitronectin and fibronectin binding by *Candida albicans* yeast cells. APMIS **1993**;101:187-93.
16. Filler S.G., Pfunder A.S., Spellberg J.P., Edwards J.E. *Candida albicans* stimulates cytokine production and leukocyte adhesion molecule expression by endothelial cells. Infec Immun **1996**;64:2609-17.
17. Maisch P.A., Calderone R.A. Role of surface mannan in adherence of *Candida albicans* to fibrin-platelet clots formed *in vitro*. Infect Immun **1981**;32: 92-7.
18. Caccese D., Pratico D., Ghiselli A., et al. Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation: role of arachidonic metabolism. Thromb Haemost **2000**;83:485-90.
19. Neiva T.J.C., Benedetti A.L., Tanaka S.M.C.N., et al. Determination of serum aluminum, platelet aggregation and lipid peroxidation in hemodialyzed patients. Braz J Biol Med Res **2002**;35:345-50.
20. DesPrez R.M., Steckley S., Stroud R.M., Hawiger J. Interaction of *Histoplasma capsulatum* with human platelets. J Infec Dis **1980**;142:32-9.
21. Nagase T., Mikami T., Suzuki S., et al. Lethal effect of neutral mannan fraction of baker's yeast in mice. Microbiol Immunol **1984**; 28:997-1007.
22. Mikami T., Fukushi K., Ishitani M., et al. Induction of platelet activating-factor in mice by intravenous administration of a neutral fraction of baker's yeast mannan. Lipids **1991**;26:1404-7.
23. Nakayama R., Kumagai H., Saito K. Evidence for production of platelet-activating factor by yeast *Saccharomyces cerevisae* cells. Biochim Biophys Acta **1994**;1199:137-42.
24. Willcox M.D., Webb B.C., Thakur A., Harty D.W. Interactions between *Candida* species and platelets. J Med Microbiol **1998**;47:103-10.
25. Robert R., Nail S., Marot-Leblond A., et al. Adherence of platelets to *Candida* species *in vivo*. Infect Immun **2000**;68:570-6.
26. Gentry P.A., Ross M.L., Bondy G.S. Inhibitory effect of trichothecene mycotoxins on bovine platelets stimulated by platelet activating factor. Can J Vet Res **1987**;51:490-4.
27. Pinto A., Cirino G., Meli R., et al. Inhibitory effects of fungi on aggregation of rabbit platelets and rat polymorphonuclear leucocytes *in vitro*. J Ethnopharmacol **1998**;22:91-9.
28. Hammerschmidt D.E. Szechwan purpura, N Engl J Med **1980**;302:1191-3.
29. Nail S., Robert R., Dromer F., et al. Susceptibilities of *Cryptococcus neoformans* strains to platelet biding *In vivo* and to the fungicidal activity of thrombin-induced platelet microbicidal proteins *in vitro*. Infec Immun **2001**;69:1221-5.