

## Adherence of Platelets to *Candida* Species In Vivo

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**The in vivo interactions of platelets with *Candida* species yeast cells were investigated in a murine model. Mice were injected intravenously via the lateral caudal vein, and blood drawn by periorbital puncture was collected in phosphate-buffered saline–formaldehyde to avoid in vitro platelet activation. The study of the clearance of blastoconidia of *Candida albicans* and *Candida glabrata* showed that these cells disappeared quickly from the bloodstream. Microscopic observation of blood samples, stained by Calcofluor white or May Grunwald Giemsa, demonstrated the rapid attachment of platelets to fungal elements of all the *Candida* spp. tested. The attachment of murine platelets to *C. albicans* cells, observed by scanning electron microscopy, revealed morphological changes. The platelets lost their discoid shape, generated pseudopodia, and flattened against the yeast cells. The reversibility of platelet binding to *C. albicans* by chelating agents suggests a cation-dependent link. In contrast, the fixation of *C. glabrata* and *Candida tropicalis* was not modified by chelating agents. The mechanisms involved in the in vivo adherence of platelets to *Candida* cells may therefore differ according to the species of *Candida*.**

Disseminated *Candida* infections involve the translocation of *Candida* cells through the blood circulation. In the bloodstream, the fungus may interact with various (soluble) proteins such as fibrinogen (3), fibronectin (16, 28), complement, and other plasma components (8, 9); with the vascular endothelium (27); and with glycoproteins of the subendothelial extracellular matrix (26) such as collagen and laminin (4, 17). It may also interact with blood cells such as neutrophils, which are usually considered to be the primary effective cells against *Candida* yeast (6). Platelets, known to be critical mediators of homeostasis and blood coagulation, have recently been implicated in the metastatic processes of tumor cells (2, 12, 30) and in the pathogenesis of infectious diseases (34).

Several in vitro studies have suggested that platelets interact with *Candida* cells. Maish and Calderone demonstrated the adherence of *Candida albicans* to fibrin-platelet clots (18, 19). The same group showed that cell wall fragments of *C. albicans* were able to aggregate platelets (29). Previously, we reported that resting platelets adhere to *C. albicans* germ tubes and that this attachment is associated with morphological changes (24, 25). Klotz et al. studied the adherence of *C. albicans* to the endothelium and to the subendothelial extracellular matrix and demonstrated that the interaction between yeast and platelets occurred after platelet aggregation (14, 15). However, although these authors detected platelet aggregation with yeast cytosol, they were unable to produce platelet aggregation with viable whole yeast. A recent study by Willcox et al. (30) concludes that *C. albicans*, unlike all other species, is unable to aggregate platelets. This study also demonstrated the ability of platelets to kill some *Candida* spp. but not *C. albicans*. However, Yeaman et al. showed that platelets are activated by *Candida* species to secrete platelet microbicidal peptides and

that these peptides are active against the *Candida* organisms (32, 33, 35).

In vivo interactions between *Candida* species and platelets have been only partially studied. Holder and Nathan (11) observed that injection of sonic cell extract of *Candida* into mice produced platelet aggregation. In a rabbit model, Calderone et al. (5) studied endocarditis elicited by traumatic destruction of aortic valves and demonstrated the close apposition of *Candida* cells and platelets in the lesion. Demonstration of *Candida* in blood smears from patients has been reported, and Kates et al. (13) showed platelets in close association with yeast. Platelet anti-*Candida* activity has been described and has been shown to play a role in the severity of experimental *C. albicans* endocarditis (33).

Although platelets are considered a factor in the pathogenesis of candidiasis, it is unclear whether these cells promote or limit the progression of the disease. We describe an in vivo procedure for the study of the adherence of mouse platelets to *Candida* cells. The amount of platelet binding to *Candida* species was measured by fluorescence microscopy after coloration by Calcofluor white. The results obtained demonstrate an interaction between platelets and all *Candida* species. In addition, the fixation of platelets to the circulating blastoconidia of *C. albicans* in vivo was confirmed by scanning electron microscopy (SEM), which showed that the attachment to the platelets was associated with morphological changes.

### MATERIALS AND METHODS

**Organisms and culture conditions.** *C. albicans* 1066 (ATCC 66369), originally isolated from a case of septicemia, was used throughout this work. Two clinical isolates of *C. albicans* and isolates of *Candida* spp. *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, and *C. guilliermondii* were obtained from the Mycological Laboratory of the Faculty of Medicine, Angers, France. All strains were maintained by subculture on Sabouraud dextrose agar slants (Merck, Darmstadt, Germany) at 37°C for 24 h twice a month.

For assay purposes, blastoconidia were inoculated at a concentration of  $2 \times 10^6$  per ml in medium YNB broth (1.7% yeast nitrogen base [Fisher Scientific]) containing 2% glucose and 5% ammonium sulfate (YNB-G-SA) for 24 h at 37°C, pH 4.5. In some experiments, germ tubes were obtained by incubation of blastoconidia for 3 h in YNB-G-SA at 37°C and pH 7. Cells were harvested by centrifugation (10 min, 500  $\times$  g), washed three times in 0.15 M NaCl, and finally

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resuspended in this buffer at the required concentration after a hemacytometer count.

**Mice.** Female, outbred Swiss mice, 6 to 8 weeks old, weighing 18 to 20 g (Centre d'Élevage Déprés-France) were maintained under conventional conditions before experimentation. Mice were injected intravenously via the lateral vein, and blood was drawn by periorbital puncture immediately after the mice were killed by cervical dislocation. Blastococonidia of all the *Candida* spp. studied were suspended in 0.1 ml of 0.15 M NaCl for inoculation.

**Clearance of *C. albicans* and *C. glabrata* blastococonidia from the bloodstream.** One hundred microliters of *C. albicans* or *C. glabrata* blastococonidia suspensions ( $2 \times 10^7$  cells) was injected into the lateral tail veins of mice. One, 3, and 15 min after injection, the mice were bled by periorbital puncture, and 20  $\mu$ l of the blood was immediately mixed with 250  $\mu$ l of distilled water. After 5 min the erythrocytes were lysed, and the blastococonidia were labeled by mixing 1 volume of 0.1 mM fluorescein brightener (Calcofluor white [Sigma, St. Louis, Mo.]) in phosphate-buffered saline (PBS; 0.15 M, pH 7.2) with 5 volumes of sample. The enumeration of the blastococonidia was realized by a direct hemacytometer count under fluorescence microscopy using a Nikon incident-light fluorescence microscope with an excited filter with peak transmission at 365 nm and a barrier filter that transmitted light at wavelengths  $>420$  nm.

**Interactions of *Candida* spp. cells with platelets in vivo.** For the adherence assays, the mice were injected intravenously via the lateral tail vein with  $5 \times 10^7$  to  $7 \times 10^7$  *Candida* spp. blastococonidia or *C. albicans* germ tubes. After 3 min, blood was drawn and 20  $\mu$ l of blood was immediately mixed in a plastic tube with 1 ml of PBS or PBS containing 3.3 mM formaldehyde to prevent in vitro platelet activation. In experiments to study the role of divalent cations, blood was collected in PBS containing 10 mM EDTA or 10 mM EGTA or 0.13 M sodium citrate. For negative controls, 20  $\mu$ l of blood from an uninfected mouse was mixed with all the above buffers supplemented with  $5 \times 10^5$  blastococonidia. The blood samples were incubated in the buffers for 1 to 60 min prior to examination by photonic fluorescence microscopy following yeast labeling with Calcofluor white as described above.

In some experiments, mice were bled 1, 3, and 15 min after inoculation and the blood samples were treated with Calcofluor white or the May Grunwald Giemsa stain (MGG [Ral, Paris, France]) before examination by photonic microscopy.

For the SEM study of platelet attachment to *C. albicans* ATCC 66369 cells,  $5 \times 10^7$  blastococonidia were inoculated. After 15 min, 500 to 600  $\mu$ l of blood was collected in 25 ml of PBS and passed immediately through a filter (10- $\mu$ m pore size; MSI, Westboro, Mass.) to remove the erythrocytes. Then the filter was washed once again with 25 ml of PBS and set in a sterile tube containing 1 ml of 0.15 M NaCl. Cells were removed by gentle agitation, the filter was discarded, and the preparation containing the holding cells was fixed in 2.5% glutaraldehyde, postfixed in 2% OsO<sub>4</sub>, dried at the critical point, coated with gold-palladium, and examined with a JEOL JSM 35 microscope as described by Miegville and Morin (21).

**Statistical analyses.** For each experiment, at least three mice were inoculated and each bloodletting was divided into three tubes. Statistical significance was determined by using the paired *t* test. All comparisons were two sided, and a *P* value of  $<0.05$  was considered significant.

**RESULTS**

**Clearance of *C. albicans* ATCC 66369 and *C. glabrata* blastococonidia from the bloodstream.** Blastococonidia ( $2 \times 10^7$ ) were inoculated into the mice by intravenous injection. One, 3, and 15 min after inoculation, blastococonidia were numbered after staining by Calcofluor white. Figure 1 indicates that blastococonidia of *C. albicans* disappeared more quickly from the bloodstream than did *C. glabrata* cells. To illustrate, 1 min after

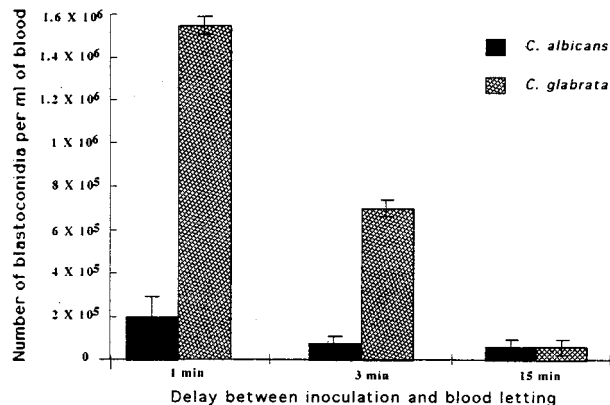


FIG. 1. Comparison between levels of *C. albicans* ATCC 66369 and *C. glabrata* blastococonidium clearance from the bloodstream after intravenous injection of  $2 \times 10^7$  cells into the lateral tail veins of mice. Cells recovered by periorbital puncture were counted 1, 3, and 15 min after the inoculation. Data are means  $\pm$  standard deviations of three mice, with triplicate samples for each.

inoculation, with the blood volume of mice being approximately 3 ml, the percentages of remaining circulating blastococonidia of *C. albicans* and *C. glabrata* were estimated at 3 and 22.5%, respectively, of the blastococonidia initially injected ( $P < 0.001$ ). Three minutes after inoculation, the decrease of circulating yeast cells became more pronounced, leading to the disappearance of 98 and 89% of the blastococonidia initially injected for *C. albicans* and *C. glabrata*, respectively ( $P < 0.01$ ).

However, for a longer period (15 min), the observed difference between the concentrations of blastococonidia in blood for *C. albicans* and *C. glabrata* was not significant ( $P > 0.1$ ).

As it was difficult to bleed several mice precisely 1 min after injection and as the number of blastococonidia per milliliter of blood was very low 15 min after injection, 3 min was chosen as the time to bleed mice after injection for further studies.

**Choice of the blood buffer for the studies of interaction between platelets and blastococonidia.** As the time between the taking and the observation of blood may vary, the composition of the buffer and the time of incubation of blood in this buffer, ensuring no modification of the in vivo interaction of platelets with yeast, were determined for *C. albicans* ATCC 66369 and *C. glabrata* (Table 1).

Blood samples recovered 3 min after inoculation showed a high percentage of yeast cells associated with platelets whatever the time of incubation, the buffer composition (PBS or PBS-formaldehyde), and the *Candida* species tested (87 to 93%;  $P > 0.1$ ) (Table 2).

TABLE 1. Effect of incubation time and buffer composition for blood collection on *C. albicans* ATCC 66369 and *C. glabrata* platelet fixation<sup>a</sup>

<i>Candida</i> spp.	Blood collection buffer	% of yeast cells bound to platelets in blood from:					
		Inoculated mice at:			Uninfected mice <sup>b</sup> at:		
		<1 min	5 min	30 min	<1 min	5 min	30 min
<i>C. albicans</i>	PBS	92.0 $\pm$ 0.6	90.0 $\pm$ 0.4	88.0 $\pm$ 0.4	3.0 $\pm$ 0.7	5.0 $\pm$ 0.8	47.4 $\pm$ 0.8
	PBS-For	96.2 $\pm$ 0.3	95.0 $\pm$ 0.2	87.0 $\pm$ 1.8	1.2 $\pm$ 0.3	1.8 $\pm$ 0.8	0.8 $\pm$ 0.2
<i>C. glabrata</i>	PBS	98.0 $\pm$ 0.3	98.2 $\pm$ 0.6	93.0 $\pm$ 0.4	0.2 $\pm$ 0.1	0.5 $\pm$ 0.1	66.2 $\pm$ 0.9
	PBS-For	98.7 $\pm$ 0.8	97.5 $\pm$ 0.6	95.8 $\pm$ 0.4	0.2 $\pm$ 0.1	0.4 $\pm$ 0.2	0.2 $\pm$ 0.1

<sup>a</sup> Mice were inoculated as described in Materials and Methods, and blood recovered by periorbital puncture was incubated for 1, 5, and 15 min in 0.15 M PBS (pH 7.2) or PBS-3.3 mM formaldehyde (PBS-For) prior to examination. Data are means  $\pm$  standard deviations of three observations of six independent experiments.

<sup>b</sup> Negative controls were performed by incubating blood from uninfected mice under the same conditions as those for blood from infected mice and with the same buffers supplemented with *Candida* cells.

TABLE 2. Interaction between *Candida* spp. and platelets<sup>a</sup>

<i>Candida</i> spp.	% of yeast cells bound to platelets in blood from:	
	Inoculated mice	Uninfected mice
<i>C. albicans</i>	95.0 ± 0.2	1.8 ± 0.8
<i>C. albicans</i> isolate 1	90.2 ± 1.1	1.2 ± 0.3
<i>C. albicans</i> isolate 2	92.6 ± 0.8	1.7 ± 0.4
<i>C. glabrata</i>	99.8 ± 0.2	2.5 ± 0.9
<i>C. guilliermondii</i>	98.6 ± 1.3	0
<i>C. tropicalis</i>	98.7 ± 0.4	0
<i>C. kefyr</i>	96.4 ± 0.1	0
<i>C. parapsilosis</i>	99.9 ± 0.1	0

<sup>a</sup> Mice were inoculated as described in Materials and Methods, and blood recovered by periorbital puncture and diluted in PBS-3.3 mM formaldehyde. Negative controls were performed as described for Table 1. Data are means ± standard deviations of three observations of six independent experiments.

When the blood from an uninfected mouse was collected in PBS supplemented with blastoconidia, immediate or delayed (5 min) observations showed a low percentage of yeast bound to platelets: 3 to 5% for *C. albicans* and 0.2 to 0.5% for *C. glabrata*. However, after 30 min of incubation the percentage of yeast associated with platelets was considerably higher: 47.4% for *C. albicans* and 66.2% for *C. glabrata*. In contrast, when the blood was diluted in PBS-formaldehyde, no modification of the initial binding occurred during the 30-min incubation period. These results were proof of in vitro fixation of platelets to blastoconidia when blood was collected in PBS. To avoid this, PBS-3.3 mM formaldehyde was chosen as the blood buffer and the time of incubation was 1 to 30 min for further studies.

**Interaction between *C. albicans* and platelets: results of photonic microscopy.** When blood from mice inoculated with blastoconidia or germ tubes of *C. albicans* ATCC 66369 was observed in the presence of Calcofluor white with a UV microscope, it was very easy to locate yeast cells (Fig. 2A, C, E, and G). Platelets rapidly attached to germ tubes (Fig. 2F) or blastoconidia (Fig. 2B, D, and H): 1 to 3 min after inoculation yeast cells fixed one or two platelets or formed microaggregates (Fig. 2A to F) with platelets. Fifteen to 30 min after inoculation, only a few blastoconidia (Fig. 2G and H) or germ tubes (data not shown) were observed, usually associated with large aggregates of platelets. Quantitative studies showed that whatever the strain of *C. albicans* used (ATCC 66369 or clinical isolates), the proportion of blastoconidia bound to platelets was high (90 to 95%) (Table 2). The attachment of platelets to fungal elements was confirmed when blood smears stained by MGG were examined by photonic microscopy (Fig. 3A and B). Platelets were seen in close association with blastoconidia (Fig. 3A) or germ tubes (data not shown). However the distribution of the yeast on the blood smear was heterogeneous: free yeast cells or yeast cells with a crown of platelets were observed in the center of the smear, whereas yeast cells associated with large aggregates of platelets were observed at the front edge and sides of the smears.

**Interactions between *C. albicans* and platelets: results of SEM.** The binding of platelets to blastoconidia or germ tubes of *C. albicans* ATCC 66369 was observed 15 min after inoculation. The results of SEM (Fig. 4) show yeast cells in aggregates of platelets (Fig. 4C) and platelets attached to fungal elements (Fig. 4A, B, and D). After fixation on the surface on the fungus, the platelets lose their discoid shape, producing spikes or pseudopodia (Fig. 4A and B). Sometimes the plate-

lets flatten and spread over the blastoconidia (Fig. 4A) or germ tubes (Fig. 4D).

**Interactions between *Candida* spp. and platelets.** The interactions between mouse platelets and several common species of *Candida* were studied in vivo by photonic microscopy. The results show that the platelets are able to bind to blastoconidia of all strains (Table 2). As we observed for *C. albicans* in blood smears or after staining with Calcofluor white, the platelets were seen in close association with circulating blastoconidia. To illustrate, Fig. 3C and D show about eight platelets attached to a blastoconidium of *C. parapsilosis* and its bud after staining with Calcofluor white and Fig. 3B shows two blastoconidia of *C. krusei* associated with large aggregates of platelets at the edge of a blood smear.

**Role of divalent cations in the interaction between platelet and yeast.** When blood from mice inoculated with *C. albicans* ATCC 66369 was collected in PBS-EDTA or in PBS-sodium citrate, 30 min after incubation in these buffers, there was a small but significant decline ( $P < 0.02$ ) in the percentages of blastoconidia bound to the platelets (PBS-EDTA, 64.5% ± 3.1%; PBS-citrate, 59.2% ± 2.7%) compared to the percentages observed after 5 min of incubation (PBS-EDTA, 92% ± 3.2%; PBS-citrate, 85.8% ± 3.1%). After 30 min of incubation in PBS-EGTA the percentage of blastoconidia bound to platelets was greatly depressed (20.2% ± 1.3%;  $P < 0.001$ ). Similarly, the percentages of *C. albicans* bound to platelets decreased sharply after 60 min (PBS-EDTA, 44.3% ± 3.5%;  $P < 0.001$ ; PBS-citrate, 42.3% ± 1.9%;  $P < 0.001$ ).

For *C. glabrata* and *C. tropicalis*, whatever the buffer used or the time of incubation, no statistically significant differences in the reduction of the proportion of yeast cells binding to platelets were observed. To illustrate, the percentages of *C. tropicalis* bound to platelets after incubation with EDTA were 96% ± 0.8% after 5 min and 90.8% ± 1.7% after 60 min.

## DISCUSSION

In the bloodstream, platelets circulate in the form of disk-shaped cell fragments that do not normally interact with the vascular endothelial lining. When the endothelial lining is damaged or the blood vessel is injured, platelets adhere to subendothelial components such as collagen and microfibrils. This adherence elicits the secondary activation of the platelets for the generation of a hemostatic plug. In vitro, the addition of collagen or ADP to blood or platelet-rich plasma (PRP) induces the activation and aggregation of platelets. To aggregate to each other, the platelets in the bloodstream or in PRP must be activated and express fibrinogen receptors.

Apart from the fact that platelets have a vital function in hemostats, they may have a role in the pathogenesis of tumoral metastases (20) and in the dissemination of bacteria (7, 10) or parasites (1, 23, 36). There is circumstantial evidence that platelets may interact with *Candida* (15, 24, 25, 29). Although numerous studies have investigated the ability of yeast cells and yeast cell components to aggregate platelets in PRP (22, 29, 30), little is known concerning the adherence of platelets to yeast cells, which could be a critical initial event in inducing the activation and aggregation of platelets.

In the present study, we have used a murine model to study the abilities of different species of *Candida* to bind platelets in vivo. The data presented here indicate that after injection of blastoconidia into mice the rate of clearance of *C. albicans* from the bloodstream was high, leading to the disappearance of 97% of the inoculated cells within the first minute of incubation. In contrast, the rate of clearance observed with *C. glabrata* was eightfold lower.

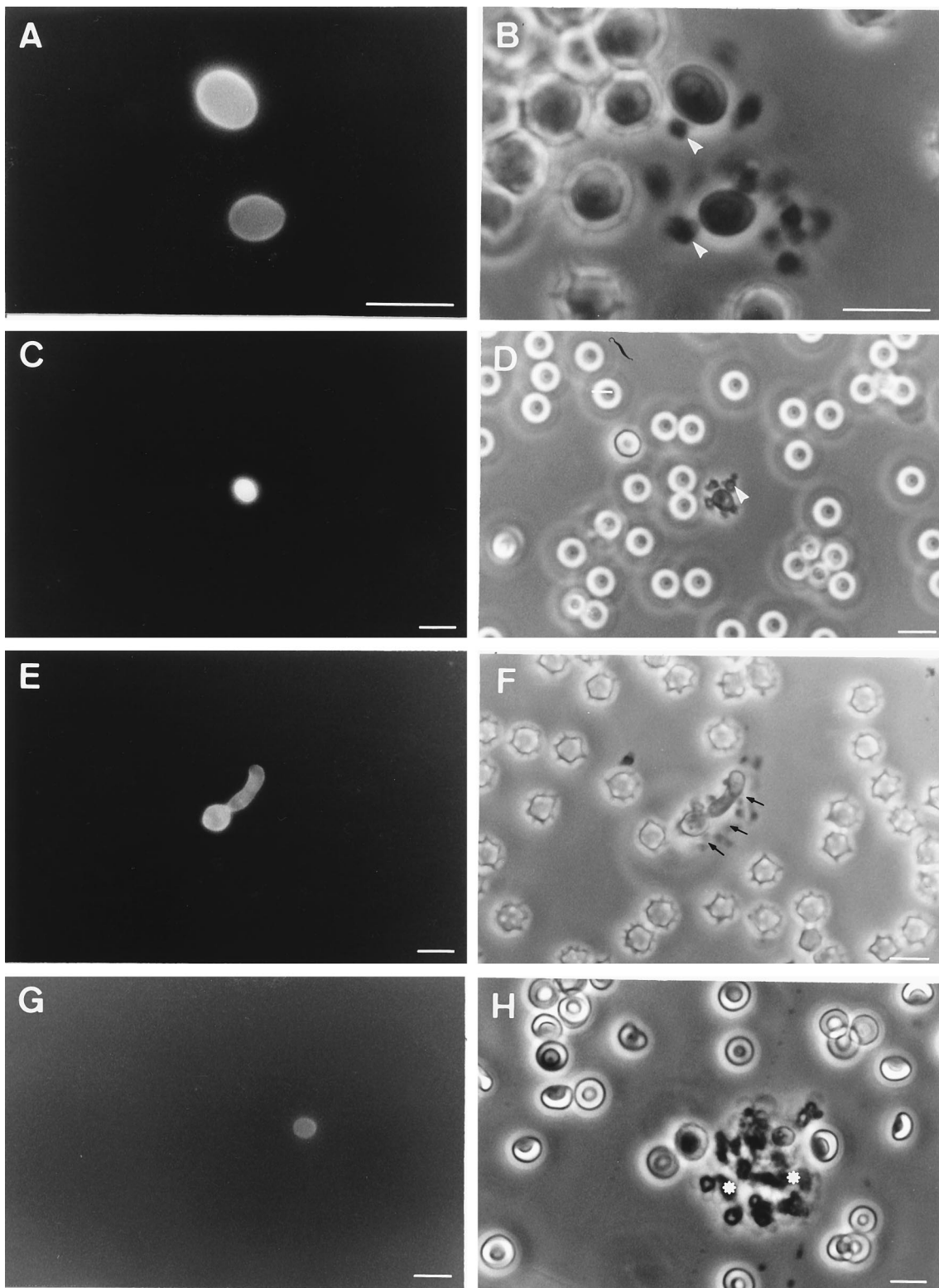


FIG. 2. Immunofluorescence (A, C, E, and G) and phase-contrast (B, D, F, and H) photographs of platelet adherence to *C. albicans* ATCC 66369 blastoconidia (A to D, G, and H) and germ tubes (E and F). Blood samples were recovered 3 (A to F) or 15 min (G and H) after inoculation of the mice, and yeast cells were visualized by Calcofluor white. Note that platelets (arrowheads) adhered to blastoconidia. Note the aggregates of platelets (small arrows) in close association with germ tubes or with blastoconidia (\*). Bars, 10  $\mu$ m.

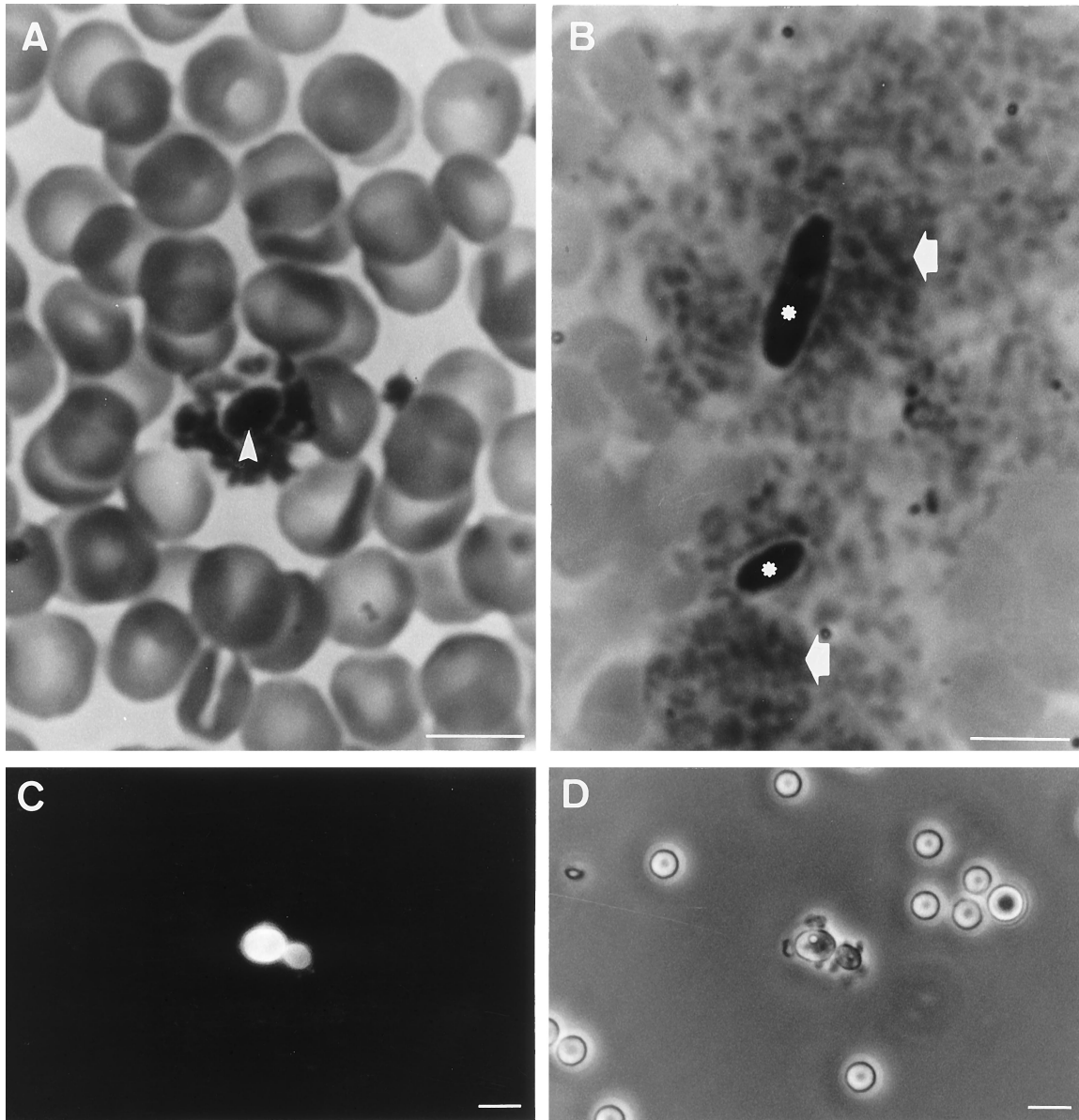


FIG. 3. In vivo studies of the interactions between *C. albicans* ATCC 66369 (A) and *C. krusei* (B) and platelets. Blood smears were stained with MGG. Immunofluorescence (C) and phase-contrast (D) photographs of platelet adherence to *C. parapsilosis* blastoconidia are shown. Note blastoconidia of *C. albicans* (arrowhead) with a crown of platelets. Note blastoconidia of *C. krusei* (\*) associated with big aggregates of platelets (arrows). Bars, 10  $\mu$ m.

Microscopic observation of blood samples stained by Calcofluor white or MGG showed rapid binding of platelets to blastoconidia of all *Candida* spp. The attachment of murine platelets onto *C. albicans* cells observed by SEM was associated with morphological changes. Platelets lost their discoid shape, generated spikes or pseudopodia, and flattened against the yeast cells. Some fungal elements were trapped in platelet aggregates. These findings suggest an activation of the platelets. Our observations support earlier results obtained by an in vitro procedure for the study of the interaction between washed resting human platelets and *C. albicans* germ tubes (24, 25). These results have been confirmed by Yeaman et al. in a flow cytometric analysis of *C. albicans* adherence to platelets in vitro, which indicated that these organisms bind directly to platelets in the absence of plasma (31). Our present results may be compared to those reported by Klotz et al., who

showed that blastoconidia of *C. albicans* bound firmly to the surfaces of platelets aggregated by ADP whereas inactivated platelets did not adhere to yeast cells (15).

The reversibility of platelet binding to *C. albicans* blastoconidia by EDTA, EGTA, and sodium citrate suggests a cation-dependent link. In contrast these chelating agents did not modify the attachment of platelets to blastoconidia of other *Candida* species, suggesting that the mechanisms involved in the in vivo interaction of platelets with *Candida* cells may differ according to the species of *Candida*.

There is some discrepancy between our results and those of Klotz et al., who concluded that whole *C. albicans* cells are unable to aggregate platelets in vitro (15).

Other species of *Candida* have been shown to be able to cause platelet aggregation. It has been suggested that *C. albicans* does not aggregate platelets and is not killed by anti-

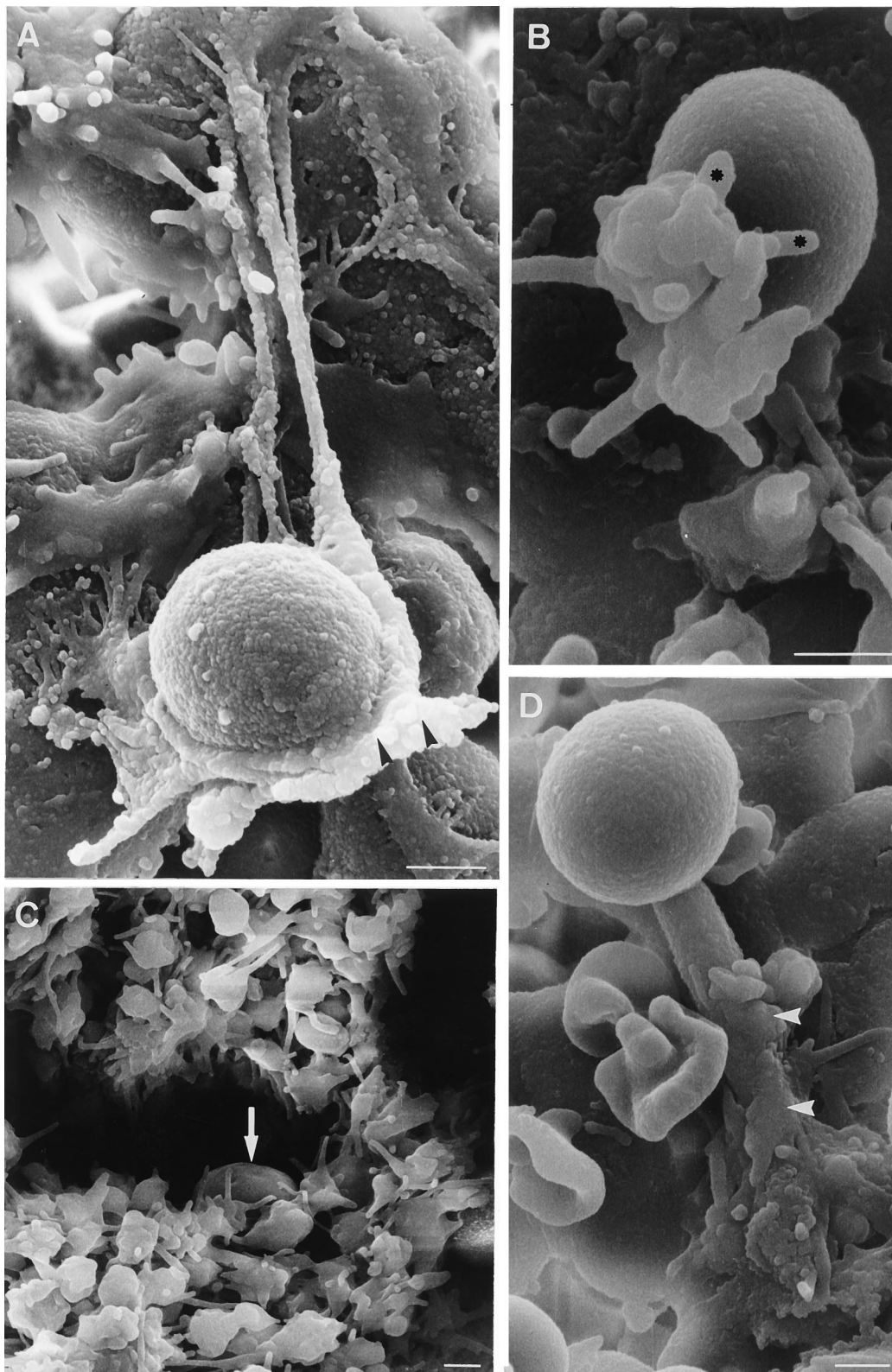


FIG. 4. SEM visualization of platelet adherence to *C. albicans* ATCC 66369. Adherence assays were performed, and platelets that adhered to the fungus were examined by SEM as described in Materials and Methods. Binding of platelets to blastoconidia (A, B, and C) or germ tubes (D) of *C. albicans* was observed 15 min after inoculation. Note that platelets with pseudopods (\*) adhered to blastoconidia (arrow) and that some platelets partially spread on germ tubes (arrowheads). Note the blastoconidium (arrow) associated with a big aggregate of platelets. Bars, 1  $\mu$ m.

crobal proteins released by activated platelets, implying a lack of recognition that may in fact contribute to the survival of this species (30). However, the presence of sodium citrate in the PRP might explain the nonadherence of platelets to *C. albicans*.

In conclusion, attempts to define the role of platelets in disseminated candidiasis have produced conflicting results. Further *in vivo* studies using variant strains should be carried out to determine whether the adherence of platelets to blastoconidia of *C. albicans* or other *Candida* species and the subsequent activation and aggregation of platelets are injurious or beneficial to the host.

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