

# Effect in Mice of Injection of Viable *Candida albicans* and a Cell-Free Sonic Extract on Circulating Platelets

IAN A. HOLDER AND PAUL NATHAN

Shriners Burns Institute, Cincinnati Unit, and Departments of Surgery, Microbiology, and Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45219

Received for publication 4 October 1972

The ability of intravenously injected *Candida albicans* to cause thrombocytopenia and clotting disorders was tested. Mice injected in this manner showed a decrease in circulating platelets and a shortening of clotting time within hours after challenge. Treatment with heparin changed the death distribution pattern, but did not increase overall long-term survival. A cell-free sonic extract prepared from viable *C. albicans* cells was shown to cause aggregation of platelets in vitro, and injection of the cell-free extract into mice caused a decrease in circulating platelets similar to that observed when viable organisms were injected.

It has been reported that bacterial infections are often associated with thrombocytopenia and coagulation disorders (1-3). Unpublished clinical observations at this institution suggested that such an association might be present in burned patients infected with the yeast *Candida albicans*. However, the evidence was not conclusive because some of the therapeutic agents used to treat these patients could themselves cause thrombocytopenia. The following experiments were designed to determine the ability of *C. albicans* to cause thrombocytopenia or clotting abnormalities, or both, in an experimental mouse model and to produce platelet aggregation in vitro.

## MATERIALS AND METHODS

*C. albicans* (no. 11257) isolated in our laboratory from a fatal septicemia in a patient was used in these studies. The organism, when used for injection, was grown in a liquid synthetic medium of the following composition (per liter):  $K_2HPO_4$ , 1.0 g;  $KH_2PO_4$ , 1.0 g; NaCl, 5 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $(NH_4)_2SO_4$ , 0.1 g; L-asparagine, 1.0 g; and glucose, 20.0 g. Female CF-1 mice weighing 22 to 24 g were used in all experiments. Injections were made intravenously with 0.1 ml of an overnight culture which contained  $1.3 \times 10^7$  organisms.

Prior to injection and at 4 and 6 h postinjection, blood was drawn for various assays. Platelet counts were performed by obtaining 50  $\mu$ liters of blood from the retro-orbital sinus in a heparin-rinsed capillary tube and expelling the blood into an equal volume of ethylenediaminetetraacetate (EDTA) solution (7.5 mg of EDTA/ml). Platelets

were counted in duplicate in a hemocytometer by use of a phase microscope. Blood samples for hematocrits and blood cell counts were collected in heparinized capillary tubes; clotting times were determined with nonheparinized capillary tubes.

In some experiments, heparin (50 units/injection) was given subcutaneously in three separate injections at 30 min prior to challenge with yeast and this dose and route were repeated at 2.5 and 5.5 h after injection of viable cells.

In other experiments, up to 0.5 ml of a cell-free synthetic broth filtrate of an overnight culture of this organism was injected intravenously into mice to determine toxicity of the broth. The cell-free filtrate was prepared by sedimenting by centrifugation the cells contained in an overnight broth culture and passing the supernatant fluid through an 0.45- $\mu$ m membrane.

Additionally, an overnight culture of *C. albicans* 11257 containing  $2.2 \times 10^8$  cells/ml was heat-killed by immersing the culture in a boiling-water bath for 45 min. Sterility was assured by plating 0.1-ml samples on brain heart infusion plates which were then incubated for 24 h at 37 C. The killed cells contained in the culture were sedimented by centrifugation, washed twice with saline, and resuspended in saline at one-tenth the original volume, giving a suspension containing  $2.2 \times 10^9$  organisms per ml. Toxicity was determined by injecting 0.1 ml of this suspension, containing  $2.2 \times 10^8$  dead organisms, intravenously into mice. In some mice, platelet counts, as described previously, were performed prior to injection and 6 h postinjection of heat-killed cells, to determine the effect of nonviable organisms on circulating platelets.

*C. albicans* cell-free sonic extract was prepared

as follows. Plastic petri dishes (150 by 25 mm) were filled with 50 ml of medium described above, and 20 g of agar per liter was added. The solidified agar was overlaid with 20 ml of liquid medium and inoculated with 1 ml of an overnight culture of *C. albicans*. These biphasic plates were incubated at 37 C on a reciprocating shaker adjusted so that the liquid phase moved gently back and forth over the solid phase. The cells were harvested by decanting the liquid phase into sterile centrifuge tubes. The cells adhering to the agar were dislodged with a sterile swab, washed off the surface of the agar with saline, and added to the centrifuge tube. The organisms were sedimented by centrifugation, washed three times in sterile saline, and lyophilized. This biphasic method of growing *C. albicans* gave larger yields of cells than any other method tried. A 100-mg amount of lyophilized cells plus 1 g of glass homogenizing beads in 10 ml of saline were subjected to sonic vibrations at 60% output of a Sonifier Cell Disruptor (Heat Systems Co., Melville, N.Y.) for 10 min. Viable-cell counts before and after sonic treatment indicated that sonic vibration at 40 W output for 10 min reduced the number of organisms from approximately  $5.0 \times 10^8$  to  $2.1 \times 10^4$ /ml. The sonic extract was filtered through a 0.45- $\mu$ m membrane filter (Millipore Corp.), and the sterile filtrate was placed in sterile test tubes in 2-ml portions and frozen at -90 C. Sonic extracts prepared as described above but with *C. albicans* cells which had been heated in a boiling-water bath for 45 min were also prepared.

The effect of these cell-free extracts on aggregation of platelets was tested by mixing 0.1 ml of sonic extract containing  $3.2 \pm 0.2$  mg (dry weight) of material with 0.3 ml of platelet-rich plasma, adding 0.1 ml of 0.025 M CaCl<sub>2</sub> solution, and recording changes in light transmittance by use of an aggregometer (Chrono-Log Corp., Broomall, Pa.) with a Health Servo Recorder EU-20B. In addition, tests were performed with 0.1 ml of a solution of *Escherichia coli* lipopolysaccharide O111:B4 (Difco) which contained 100  $\mu$ g of lipopolysaccharide. The effect of the addition of viable-cell sonic extract to platelet-poor plasma

and the addition of saline to platelet-rich plasma was also tested.

Platelet-rich human plasma was prepared by obtaining 10 ml of whole blood in a plastic tube containing 0.2 ml of 7.5% EDTA. The blood was centrifuged at room temperature for 10 min at  $70 \times g$ , and the platelet-rich plasma was withdrawn from the blood sample with a plastic pipette. Samples containing  $1.5 \times 10^8$  to  $3 \times 10^8$  platelets per ml were used in the assay. A sample of platelet-rich plasma was centrifuged at  $1,000 \times g$  for 10 min at room temperature, to sediment the platelets. The supernatant plasma from this centrifugation was essentially free from platelets and constituted platelet-poor plasma.

The in vivo effect of the viable-cell sonic extracts on platelets was determined by performing platelet counts, as described above, on blood samples from mice which had been given an intravenous injection of 0.1 ml of sonic extract 2 min prior to taking the sample. The 2-min time period was chosen to determine whether the sonic extract had an immediate effect similar in timing to that observed in the aggregometer and to avoid the problem of platelet counting at a later time when a possible hypercoagulable state would interfere with obtaining the sample.

## RESULTS

Four hours after injection of viable *C. albicans*, there was a slight decline in circulating platelets (Table 1). By the sixth hour postinjection, the platelet count was significantly ( $P < 0.001$ ) depressed compared to the control value. Red and white blood cell counts and hematocrits were unchanged over the 6-h period; however, during the time interval required for a depression of circulating platelets, there was a significant ( $P < 0.001$ ) decrease in the time required to form a clot (Table 1). Indeed, the decreased clotting times were noted by the fourth hour postinjection, whereas it was 6 h before a significant decrease in circulating platelets was observed.

The percent mortality at intervals after injection with viable *C. albicans* is indicated in Table 2.

TABLE 1. Effect of injection of viable *C. albicans* on platelets, red and white blood cell counts, hematocrit, and clotting time<sup>a</sup>

| Determination                                     | Normal mice         | Time after injection of <i>C. albicans</i> |                    |
|---|---------------------|--|--------------------|
|   |                     | 4 h  | 6 h                |
| Platelets ( $\times 10^3$ /mm <sup>3</sup> )..... | 120.7 $\pm$ 6 (20)  | 107.2 $\pm$ 6 (20)                         | 65.0 $\pm$ 16 (11) |
| Red blood cells ( $\times 1,000$ ).....           | 1,377 $\pm$ 156 (5) | —  | 1,233 $\pm$ 42 (7) |
| White blood cells ( $\times 50$ ).....            | 118.6 $\pm$ 19 (5)  | —  | 100.1 $\pm$ 13 (9) |
| Hematocrit (%).....                               | 52.1 $\pm$ 1 (25)   | 52.3 $\pm$ 1 (10)                          | 50.3 $\pm$ 1 (9)   |
| Clotting time (s).....                            | 286.2 $\pm$ 7 (25)  | 35.0 $\pm$ 5 (18)                          | 39.3 $\pm$ 6 (9)   |

<sup>a</sup> Results are given  $\pm$  the standard error of the mean with the number of mice tested shown in parentheses. The factor times which each result should be multiplied is given in the first column.

TABLE 2. Death distribution (percent mortality)<sup>a</sup>

| Hour postinfection | Untreated | Heparin-treated |
|--------------------|-----------|-----------------|
| 5                  | 48        | 5               |
| 8                  | 80        | 60              |
| 12                 | 93        | 75              |
| 24                 | 96        | 90              |

<sup>a</sup> The values are based on results with 40 untreated mice and 20 heparin-treated mice.

By the fifth hour postinjection, there was a 48% mortality in the nontreated animals. Mice given heparin demonstrated a 5% mortality. However, by 24 h, there was no apparent difference in survival between the two groups. Clotting times of blood from heparinized mice indicated that the blood remained uncoagulable when tested at 8 h after the treatment.

Mice injected intravenously with 0.5 ml of a cell-free filtrate of the medium in which the *C. albicans* cells were grown showed no evidence of any toxic manifestations over a 10-day period. In addition, in 11 mice injected intravenously with 10<sup>8</sup> heat-killed *C. albicans* cells, there was no mortality or any evidence of toxic reactions over a 10-day period. Platelet counts performed in six of these mice prior to the injections and 6 h postinjection of the heat-killed cells demonstrated that there was no decrease in circulating platelets over this time period.

The effect of cell-free sonic extracts of *C. albicans* on in vitro aggregation of platelets contained in platelet-rich plasma is shown in Fig. 1. There was a rapid increase in transmittance immediately upon introduction of the sonic extract prepared from viable organisms, indicating that the sonic extract did cause platelets to aggregate. A similar, but not as marked, increase in transmittance was observed when sonic extract prepared from heat-killed organisms was added. Large aggregates of platelets were observed through a window on the aggregometer fitted with a magnifying lens which allowed one to see into the glass reaction tube.

No increase in transmittance was observed when saline (0.1 ml) or 0.1 ml of a suspension containing 100 µg of *E. coli* lipopolysaccharide was added to the platelet-rich plasma as a control. No increase in transmittance was observed when sonic extracts prepared from viable organisms were added to platelet-poor plasma. The same phenomena were observed in each of four separate experiments.

The effect of intravenous injection of 0.1 ml of *C. albicans* sonic extract, prepared from viable cells, into mice is shown in Table 3. At 2 min

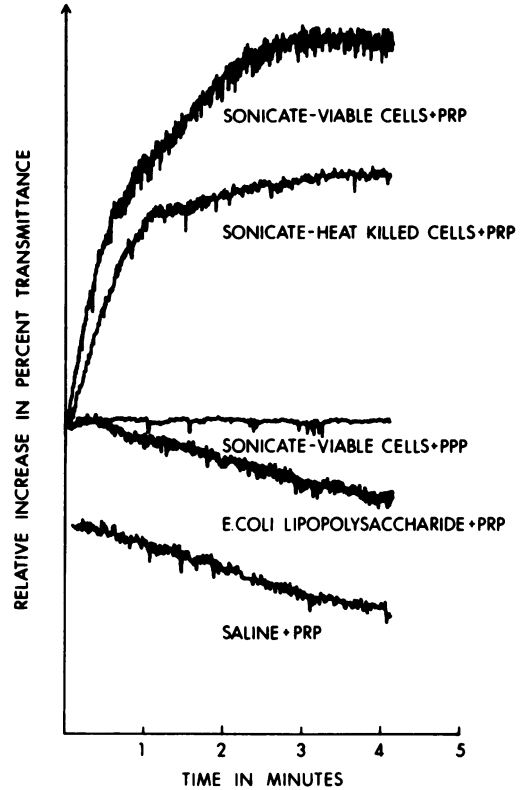


FIG. 1. Typical tracing from an aggregometer study showing the effects of the addition of various preparations to platelet-rich plasma (PRP) and platelet-poor plasma (PPP).

TABLE 3. Effect of intravenous injection of sonic extract prepared from viable *C. albicans* cells on circulating platelets in mice

| Time of test            | No. of mice tested | Thousands of platelets/mm <sup>3</sup> |
|-------------------------|--------------------|--|
| Prior to injection..... | 9                  | 169.8 ± 13.79                          |
| 2 min postinjection.... | 10                 | 116.0 ± 10.95                          |

postinjection, there was a significant ( $P = 0.01$ ) decrease in the number of circulating platelets observed. The results following the injection of sonic extract were similar to those noted after injection of viable organisms.

## DISCUSSION

Clotting defects and depressed levels of circulating platelets have been demonstrated in a wide variety of bacterial septicemias. Corrigan et al. (2, 3) reported changes during infections with many gram-negative and gram-positive

organisms. Although various changes in the clotting mechanisms were encountered, irrespective of the infectious agent, the most frequent abnormality was thrombocytopenia, which occurred in 61% of the patients. Rapaport et al. observed intravascular clotting in *Pseudomonas* septicemia leading to the generalized Schwartzman reaction (9). They attributed this to endotoxin being released from the bacteria. This effect of endotoxin causing thrombocytopenia, platelet aggregation, and clotting disorders is well documented (4, 5).

The data presented here indicate that injection of viable *C. albicans* into mice also causes thrombocytopenia and clotting defects similar to those seen in bacterial infections. The action of endotoxin in the pathogenesis of *C. albicans* infection has been suggested previously (10). Substances having endotoxin-like properties have been isolated by Isenberg et al. (7) and Kobayashi and Friedman (8). However, Kobayashi and Friedman (8) pointed out, "It is tempting to relate pyrogenicity of [their] phenol-extractable material to classical endotoxin, but the facts at hand do not as yet warrant such extrapolation, even though in terms of its behavior it does share some of the characteristics of classical endotoxin." Therefore, although substances with endotoxin-like properties have been associated with *C. albicans*, its possession of a classical gram-negative bacterial endotoxin is still not established. In any case, an extract from disrupted viable *C. albicans* cells did aggregate platelets in vitro and caused thrombocytopenia when injected into mice.

Hasenclever and Mitchell (6) demonstrated that large doses of *C. albicans* have toxic effects when injected into mice. Using a strain which had a high virulence for mice,  $10^5$  cells injected intravenously being an  $LD_{100}$ , they found that injection of  $10^7$  organisms intravenously caused a toxic death in a short period of time. However, no examination of the mechanism(s) of this toxicity was made, nor was any toxicity study done with nonviable cells.

In the studies reported here, the observations of Hasenclever and Mitchell, using viable organisms, are confirmed. However, in our studies, the organisms injected intravenously which caused death in a short period of time were of relatively lower virulence for mice:  $LD_{100}$  of  $1.7 \pm 0.4 \times 10^6$  cells. This may imply that the mechanisms of virulence in *C. albicans* are distinct from those which cause toxicity. Furthermore, the fact that injections of large amounts of cell-free culture filtrate are innocuous suggests that the toxic substances are either not produced in vitro under our growth conditions or are rapidly labile. Injections of extremely large amounts of heat-

killed organisms, which would contain large amounts of endotoxin, also proved to be innocuous; no mortality resulted from these injections, and no effect on circulating platelets was observed. On the other hand, sonic extracts prepared from heat-killed organisms showed some in vitro activity in causing platelets to aggregate, whereas *E. coli* lipopolysaccharide showed no activity in our system.

These observations suggest that endotoxin-like activity is not present in our preparations of heat-killed *C. albicans*. The toxicity observed in our experiments appears to be caused by viable organisms, whereas the product(s) which causes in vitro platelet aggregation can be obtained by sonic disruption of both viable and heat-killed fungi.

Fractionation studies are planned to determine the chemical nature of the substance(s) in the sonic extracts which causes platelet aggregation.

Heparin therapy has been used to treat the coagulation disorders seen in bacterial septicemias in patients. Although heparin inhibited the coagulation defects, it did not appear to increase long-term survival (2). Similarly, the use of heparin did not increase long-term survival in animals injected with viable *C. albicans*. This suggests that in fungal infections, as well as bacterial infections, clotting abnormalities may be part of the pathological process but are not, by themselves, the main cause of death. However, the process(es) by which the clotting abnormalities are caused may be different.

#### LITERATURE CITED

1. Cohen, P., and F. H. Gardner. 1966. Thrombocytopenia as a laboratory sign and complication of gram negative bacteremic infection. *Arch. Intern. Med.* **117**:113-124.
2. Corrigan, J. J., and C. M. Jordan. 1970. Heparin therapy in septicemia with disseminated intravascular coagulation. *N. Engl. J. Med.* **283**:778-782.
3. Corrigan, J. J., R. L. Walker, and N. May. 1968. Changes in the blood coagulation system associated with septicemia. *N. Engl. J. Med.* **279**:851-856.
4. Des Prez, R. M., H. I. Horowitz, and E. W. Hook. 1961. Effects of bacterial endotoxin on rabbit platelets. I. Platelet aggregation and release of platelet factors *in vitro*. *J. Exp. Med.* **114**:857-874.
5. Hardaway, R. M., and D. Johnson. 1963. Clotting mechanism in endotoxin shock. *Arch. Intern. Med.* **112**:775-782.
6. Hasenclever, H. F., and W. O. Mitchell. 1962. Production in mice of tolerance to the toxic manifestations of *Candida albicans*. *J. Bacteriol.* **84**:402-409.
7. Isenberg, H. D., J. Allerhand, and J. I. Berkman. 1963. An endotoxin-like fraction extracted from the cells of *Candida albicans*. *Nature (London)* **197**:515-517.
8. Kobayashi, G. S., and L. Friedman. 1964. Char-

- acterization of the pyrogenicity of *Candida albicans*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*. *J. Bacteriol.* **88**:660-666.
9. Rapaport, S. W., D. Tatter, N. Coeur-Barron, and P. F. Hjort. 1964. *Pseudomonas* septicemia with intravascular clotting leading to the generalized Schwartzman reaction. *N. Engl. J. Med.* **271**: 80-84.
10. Salvin, S. B. 1952. Endotoxin in pathogenic fungi. *J. Immunol.* **69**:89-99.