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Perioperative Variation in Phagocytic Activity against *Candida albicans* Measured by a Flow-Cytometric Assay in Cardiovascular-Surgery Patients

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Candidiasis is an opportunistic fungal infection that frequently occurs following modifications of host defenses. Major surgery can be responsible for such alterations, and therefore it increases the risk of fungal infection. The purpose of this study was to evaluate the perioperative impairment of leukocyte function in patients after cardiovascular surgery by measuring the phagocytic activity against *Candida albicans* by a flow-cytometric method. The average postsurgical decrease in phagocytosis in our patients was 11.4%. By univariate analysis, three factors, all related to antibiotic therapy, were significantly associated with an important decrease in phagocytosis: the use of antimicrobial therapy before surgery, the number of different antibiotics taken, and the length of antibiotic treatment. The results of our study showed that the use of antibiotics in patients undergoing cardiovascular surgery alters the normal phagocytic activity of the host immune system against *C. albicans* and that flow cytometry is a rapid and simple technique that helps in early identification of patients at high risk for *Candida* infections. The mechanisms by which surgery and antibiotics decrease phagocytosis remain to be elucidated.

Candidiasis is a well-known disease that occurs mainly in cancer patients and in those with other hematologic malignancies. Its incidence has been observed to be increasing in nonneutropenic surgical patients. In the past decade, the number of cases of severe *Candida* infection has increased dramatically. According to data from the National Nosocomial Infections Surveillance System, the increase in the rate of *Candida* septicemia was between 219 and 487%, and *Candida albicans* accounted for 76% of these infections (1). During this period, cardiac-surgery patients had the second-highest nosocomial fungal infection rate (3). Diagnosis of candidemia is difficult to establish; it usually relies on a high index of suspicion, since blood cultures are often negative and serodiagnostic methods lack specificity and sensitivity (9, 14). Host defenses play an important role in susceptibility to *Candida* infections (5), particularly nonspecific immunity involving the polymorphonuclear leukocytes (PMNL). Several microscopic methods have been developed for the quantification of leukocyte functions, but the lack of reproducibility and accuracy has prevented their use on a routine clinical basis (2, 4). In previous studies, flow cytometry has been shown to be a more rapid and simpler method of evaluating the phagocytic activity of the PMNL than microscopic techniques (8, 13). The objective of this study was to assess the effect of cardiac surgery on the phagocytosis by PMNL of *C. albicans* by use of a flow-cytometric assay.

MATERIALS AND METHODS

Patients and blood samples. Between March and November 1995, 81 patients scheduled for cardiovascular surgery at the Montreal Heart Institute were enrolled in the study. Heparinized blood was drawn from each patient into a sterile 7-ml Vacutainer tube (12 by 75 mm) (Becton Dickinson, Rutherford, N.J.) prior to the surgery and either 6 days postoperation or on discharge. Blood samples were kept at room temperature until the phagocytosis assay was performed (≤4 h). The study protocol was approved by the Institute's Medical Ethics Committee, and written informed consent was obtained from all participating patients.

Labelling of *C. albicans* with FITC. Cultures of *C. albicans* ATCC 44374 were grown overnight at 37°C in Sabouraud dextrose broth with agitation (200 to 250 rpm). The cells were then washed two times in phosphate-buffered saline (PBS; pH 7.3), resuspended in carbonate buffer (0.5 mol liter^{-1}; pH 9.5), and counted with a hemocytometer before being labelled. For the staining, 1 μl of fluorescein isothiocyanate (FITC; 0.005% [wt/vol]; Sigma, St. Louis, Mo.) prepared in carbonate buffer was added for every 10^6 yeast cells, and the cells were then incubated for 2 h at 4°C. The cells were then washed three times with PBS and stored in PBS at 4°C in small aliquots (500 μl). The fluorescence of the labelled cells (>95% stained) was verified by flow cytometry at regular intervals, and labelling was performed again whenever necessary. Figure 1A shows the flow cytometry results for unlabelled *C. albicans*, whereas Fig. 1B displays a histogram of the fluorescence emitted by yeast cells after FITC staining.

Phagocytosis assay. The assay comprised four samples. Heparinized whole blood (75 μl) was added to each sample. The first and second samples served as a negative control and an anti-CD14–phycoerythrin (PE) conjugated monoclonal antibody staining control, respectively. The next two samples received labelled *C. albicans* in a 3:1 ratio of leukocytes to yeast cells. Leukocytes were enumerated with a Coulter Counter ST (Coulter, Miami, Fla.) and manually recounted with a hemocytometer (1:10 dilution in Turk's solution). Labelled yeast cells were also enumerated with a hemocytometer. The counts of leukocytes and labelled *C. albicans* were then adjusted to the appropriate ratio (3:1). The first three tubes were incubated at 37°C in a shaking water bath for 90 min, while the fourth sample was put on ice. Thereafter, 3 μl of anti-CD14–PE (MY4) (Coulter) was added to each sample except for the first one, and all the tubes were left on ice for 30 min. The samples were then lysed with 2 ml of FACS lysing solution (Becton Dickinson, San Jose, Calif.) for 10 min, washed three times in PBS, and fixed with 2% paraformaldehyde (500 μl).

Flow cytometry. The analysis was performed on a FACStar flow cytometer (Becton Dickinson, San Jose, Calif.) with computer-assisted evaluation of data (Consort 30/Lysis II). For each sample, a total of 10,000 events were analyzed. Green fluorescence from FITC (FL1) was collected through a 530-nm-band-pass filter, and red fluorescence from PE (FL2) was collected through a 585-nm-band-pass filter. Leukocyte subpopulations were differentiated by forward scatter (FSC; a function of cell size) and side scatter (SSC; a function of cell granularity). A two-color contour plot analysis was used to establish the percentage of *Candida* cells phagocytized based on the uptake of labelled *C. albicans* by the targeted leukocytes. An example of data from a flow-cytometric analysis is shown in Fig. 2. Figure 2A shows the distribution of different blood cells according to their FSC and SSC properties (lymphocytes [R1], monocytes [R2], and granulocytes [R3]). R2 and R3 gates were then set to select monocytes and granulocytes, respectively (Fig. 2B), thus excluding from analysis lymphocytes and FITC-labelled yeast cells that had not been phagocytized. Figure 2C illustrates a three-dimensional contour plot of anti-CD14-labelled granulocytes (weak fluorescence [CD14w]) and monocytes (strong fluorescence [CD14s]). The use of anti-CD14–PE in our assay (in addition to FSC and SSC gating) ensures that

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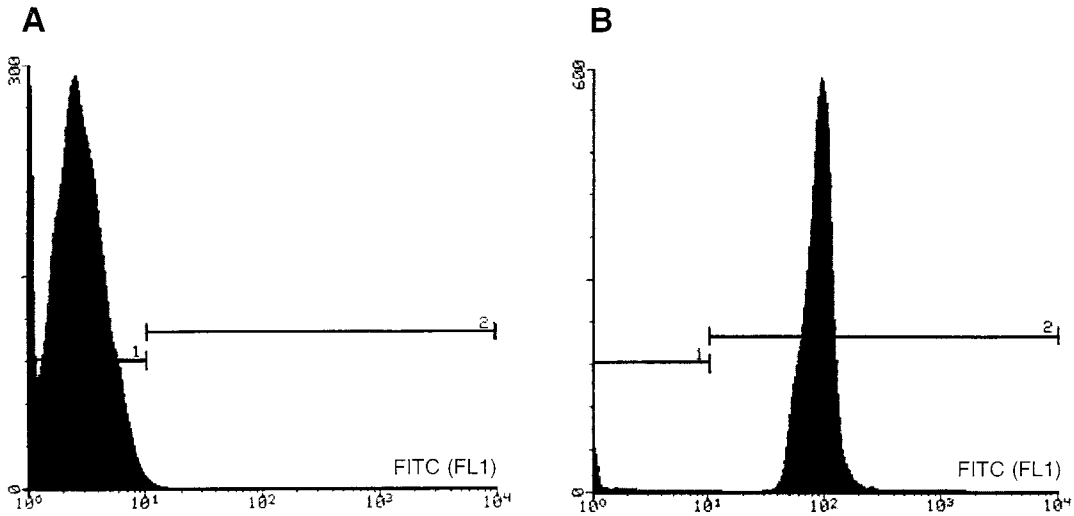

FIG. 1. Labelling of *C. albicans* with 0.005% FITC as assessed by flow cytometry. (A) Histogram of unlabelled yeast cells. (B) Histogram of FITC fluorescence emitted by *C. albicans* after the staining.

measurements are made on phagocytes (granulocytes and monocytes) only. Anti-CD14-labelled cells that have been involved in phagocytosis shift to the right and display double fluorescence (FITC and PE fluorescence) resulting from the ingestion of FITC-labelled *C. albicans* (Fig. 2D). Figure 2E shows the result of an analysis of the fourth sample, which had been incubated on ice as a control for differentiating between phagocytized and adherent yeast particles; phagocytes displaying dual fluorescence at 4°C reflect superficial adherence of labelled yeast cells. Therefore, the absolute percentage of phagocytized *Candida* cells was determined by subtracting the value (FITC- and PE-positive phagocytes) obtained at 4°C (Fig. 2E) from the one obtained at 37°C (Fig. 2D). The variation in phagocytosis observed between the pre- and postoperative blood samples was calculated as [(percent phagocytosis postsurgery − percent phagocytosis presurgery)/(percent phagocytosis presurgery)] × 100.

Statistical analysis. Univariate testing was performed by Pearson correlation analysis, the Student *t* test, and one-way analysis of variance. Variables which were tested by univariate analysis were age, sex of the patient, body mass index, diabetes, transfer from another hospital, length of hospitalization before surgery, use of antimicrobial therapy before surgery, duration of antibiotic therapy, number of antibiotics, length of stay in the surgical intensive care unit, duration of use of a central intravascular catheter and other medical device, number of blood transfusions, and reoperation. A *P* value of less than 0.05 was considered significant.

RESULTS

Table 1 shows that in our patient group there were 61 males and 20 females with a median age of 61.7 (range, 31 to 78 years). Sixty-three patients had coronary artery bypass grafting (CABG) (77.8%), 11 had valve surgeries (VS) (13.6%), 6 had both CABG and VS (7.4%), and 1 had another vascular procedure (1.2%). A total of 62 patients (76.5%) exhibited a decrease in phagocytosis after surgery, whereas an increase in postoperative phagocytosis was observed in 19 patients (23.5%). The mean percentage (± the standard deviation) of PMNL exhibiting phagocytic activity prior to surgery was 23.5% ± 6.5%, while the value obtained postoperatively (average number of days postoperation, 5.86 ± 0.41) was 19.9% ± 4.8%; thus, the difference was 3.6% ± 5.9% ($P = 0.0001$). Using the formula previously described, the postsurgical phagocytosis percentage represents an average decrease of 11.4% compared to the presurgery percentage of phagocytosis. A statistical analysis of the relationship between the epidemiological factors described above and the perioperative variation in phagocytosis was carried out (Tables 2 and 3). The results showed that three factors, all related to antimicrobial therapy, were significantly associated with an important decrease in phagocytosis in our patients. The data showed that patients who were treated with antimicrobial agent (s) before surgery had a twofold decrease in phagocytic activity against *C. albicans* (23.4% versus 10.9%; $P = 0.0007$). Decrease in phagocytic activity were also observed in those who took more than one antibiotic (22.3% versus 8.6%; $P = 0.021$) and in those who were on antibiotic therapy for 3 days or more (21.3% versus 7.8%; $P = 0.014$) (Table 2).

DISCUSSION

The use of broad-spectrum antibiotics has been identified as an independent predictor of serious *Candida* infections (18). The mechanisms underlying the association between antibiotics and the risk of fungal infection have not been completely clarified, although it is known that antibiotics can suppress the normal microbial flora. Previous studies have demonstrated that antibiotic-treated patients exhibit an increased frequency of yeast carriage (11), but few reports have mentioned the effect of such treatment on the host immune system (7). In this study, we have shown that there is a decrease in the phagocytic activity of PMNLs against *C. albicans* in cardiovascular-surgery patients, especially in those receiving antibiotic therapy. Pallister and Warnock (12) found that phagocytosis of *C. albicans* by human PMNL was impaired when patients were treated with a combination of antimicrobial and antineoplastic drugs at therapeutic concentrations. Furthermore, trimethoprim and sulfamethoxazole, individually or in combination, were found to suppress in vitro the respiratory burst of the PMNL after phagocytosis of *C. albicans* (16). It was demonstrated by Morán et al. (10) that vancomycin, at therapeutic doses, had a negative effect on chemotaxis of neutrophils. Chemotaxis is the first step required before ingestion of particles by phagocytic cells occurs, and its inhibition leads to alteration of the normal host defense mechanisms. These results suggest that different chemotherapeutic molecules act as modulators of the immune system. Another investigation found an association between decreased levels of immunoglobulin G receptor on the surface of the PMNL and a decrease in candidacidal activity in induced abdominal sepsis (17). Moreover, protein-calorie malnutrition was related to an inhibition of phagocytosis of *C. albicans* (15), suggesting a role for nutritional factors in

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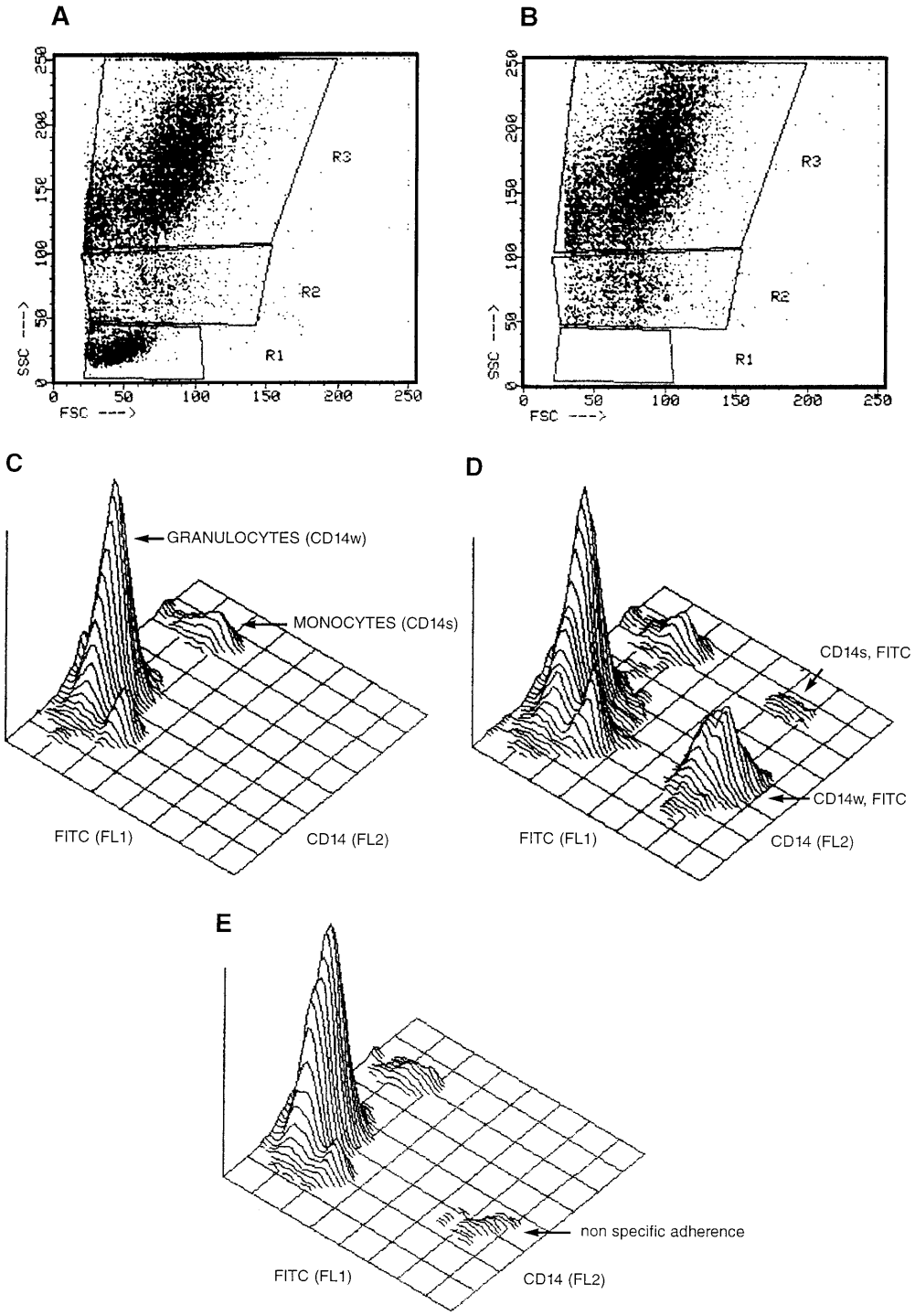

FIG. 2. Flow-cytometric analysis of the phagocytosis assay: (A) Dot plot representing the distribution of leukocytes according to FSC and SSC. R1, lymphocytes; R2, monocytes; R3, granulocytes. (B) As in panel A, except that a gate was set to eliminate lymphocytes (R1) from the analysis. (C) Three-dimensional contour plot of unstimulated granulocytes and monocytes, obtained after labelling of cells with anti-CD14–PE. The conjugated monoclonal antibody is weakly fluorescent with granulocytes (CD14w) and strongly fluorescent with monocytes (CD14s). (D and E) Three-dimensional contour plot of phagocytes after incubation with FITC-labelled *C. albicans* (ratio, 3:1) at 37°C (D) and as a control sample at 4°C (E). Phagocytosis was assessed as the difference between the total number of FITC- and PE-positive phagocytes (granulocytes and monocytes) at 37°C and that at 4°C, the latter representing nonspecific adherence of labelled yeast particles to phagocytes.

host defenses. However, these last two findings were achieved with an animal model; therefore, human studies would be necessary to ascertain their effect on the human immune system. The functional impairment of the PMNL in our study cannot be explained solely by the use of antimicrobial therapy, since a decrease of 8.6% is also noted in patients not treated with antibiotics (excluding prophylaxis). It has been recognized that surgery is an important risk

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TABLE 1. Characteristics of the study population

Characteristic	Value[a]
Sex (M/F)[b]	61/20
Age (years)	61.7 ± 10.5
Weight (kg)	76.1 ± 12.9
Type of surgery to which subjected	
CABG	63 (77.8)
VS	11 (13.6)
CABG plus VS	6 (7.4)
Other vascular procedure	1 (1.2)

[a] Values are as follows: for sex, total numbers; for age and weight, means ± standard deviations; and for surgery types, totals (with percentages of study population in parentheses).
[b] M, male; F, female.

factor for *Candida* infections in nonneutropenic patients (6). Advances in cardiovascular surgery that result in more highly invasive procedures could have a negative impact on the host's immune system.

In conclusion, the results of this study show that cardiovascular surgery in itself and the use of antibiotics in these patients influence the host immune system by altering the normal phagocytic activity of the PMNL against *C. albicans* soon after surgical procedures. The mechanisms of this relationship remain to be elucidated. The simplicity and rapidity of the flow cytometry technique in evaluating the functional activity of the PMNL has again been demonstrated in this work. This assay can be used in clinical settings to assess the state of immunodeficiency after surgery and to identify patients at risk for developing *Candida* infections.

TABLE 2. Epidemiological factors versus perioperative variation in phagocytic activity of PMNL in 81 patients

Factor	% Perioperative variation[a]	*P*
Antibiotic therapy before surgery		0.0007[b]
No (n = 76)	−10.9 ± 25.8	
Yes (n = 5)	−23.4 ± 4.5	
No. of antibiotics		0.021[b]
1 (n = 63)	−8.6 ± 25.6	
>1 (n = 18)	−22.3 ± 20.9	
Duration of antibiotic therapy (days)		0.014[b]
<3 (n = 58)	−7.8 ± 26.2	
≥3 (n = 23)	−21.3 ± 19.7	
Sex of patient		0.53
Male (n = 61)	−10.6 ± 26.7	
Female (n = 20)	−14.7 ± 20.1	
Transfer from another hospital		0.73
No (n = 57)	−10.9 ± 25.0	
Yes (n = 24)	−13.1 ± 25.9	
Reoperation		0.82
No (n = 64)	−11.9 ± 26.1	
Yes (n = 17)	−10.4 ± 22.3	
Diabetes		0.96
No (n = 68)	−11.6 ± 26.1	
Yes (n = 13)	−11.9 ± 20.7	
Length of hospitalization prior to surgery (days)		0.57[c]
1 (n = 24)	−9.9 ± 26.2	
2 (n = 24)	−16.3 ± 26.1	
>2 (n = 33)	−9.5 ± 24.0	

[a] Data are expressed as means ± standard deviations.
[b] Based on one-tailed *t* test.
[c] Based on one-way analysis of variance.

TABLE 3. Correlations between epidemiological factors and perioperative variation in phagocytic activity of PMNL in 81 patients

Factor	r[a]	*P*
Age (years)	−0.0032	0.98
Body mass index (kg/cm^2)	0.25	0.023
Length of stay in surgical intensive care unit (days)	−0.070	0.53
Duration of use of central intravascular catheter or other medical device (days)	0.073	0.52
No. of blood transfusions	−0.086	0.45

[a] Pearson correlation coefficients.

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