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Cytometry Part B (Clinical Cytometry) 72B:215–222 (2007)

Brief Observation

Granulocyte– and Monocyte–Platelet Adhesion Index in Coronary and Peripheral Blood After Extracorporeal Circulation and Reperfusion

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Background: Neutrophil-granulocyte and mononuclear-cell functional changes occur during cardiopulmonary bypass and cardiovascular surgery. In particular, leukocyte–platelet interaction, leading to generation of heterotypic coaggregates, represents an amplification mechanism of the local inflammatory response and tissue damage.

Methods: Samples of 20 patients were drawn from venous coronary sinus before cardioplegic arrest and immediately after reperfusion, as well as from peripheral blood at 5 and 24 h postoperatively. The granulocyte and monocyte surface expression of CD162, CD15s, CD18, and CD11b were quantified by flow cytometry at the different times. Parallel variations of circulating leukocyte–platelet conjugates (percentages) and a derived (cell number-normalized) leukocyte–platelet adhesion index were measured using a combination of antibodies against CD45, CD14, and CD41a. The evaluation of platelet functional state was carried out using antibodies against CD62P (P-selectin) and PAC-1.

Results: Monocyte and granulocyte cell number increased markedly in coronary blood at reperfusion and in peripheral blood postoperatively when compared with measurements done before cardioplegia. A very different course characterized the changes of the leukocyte–platelet adhesion index with respect to the variations of circulating leukocyte–platelet coaggregates (percentages). Leukocyte molecules expression showed no significant variations for CD15s on both the leukocyte subsets, while a significant up-modulation for CD162 was observed on monocytes at 24 h after extracorporeal circulation ($P = 0.0002$), and for CD11b on granulocytes at 5 h postoperatively ($P = 0.033$). A loss of CD162 expression was observed in coronary blood at reperfusion ($P = 0.0038$) on granulocytes, associated to a down-modulation of CD18 ($P = 0.0033$) and CD11b ($P = 0.0184$) in peripheral blood at 24 h postoperatively. No significant up-regulation of platelet activatory molecules expression was found at coronary reperfusion, as well as postoperatively in the peripheral blood, when compared with the before-cardioplegia derived data.

Conclusions: The over time variations of a normalized leukocyte–platelet adhesion index seem to reflect the cumulative leukocyte–platelet functional interaction more accurately than the parallel measurements of cellular conjugates. The absence of platelet activation suggests that the leukocyte membrane modifications play a main role in controlling the formation and stability of heterotypic leukocyte–platelet coaggregates after cardiac surgery with extracorporeal circulation. © 2006 International Society for Analytical Cytology

Key terms: granulocyte; monocyte; platelet; coronary blood; cardiopulmonary bypass; flow cytometry

The generation of leukocyte–platelet coaggregates, occurring after coronary ischemia and reperfusion, contributes to amplification of the local inflammation and tissue damage by up-regulating leukocyte integrin expression and their adhesion to endothelium (1–3).

In a previous paper, we used some flow cytometric procedures to calculate a normalized monocyte–platelet adhesion index in coronary blood samples, drawn before and after cardioplegia/reperfusion from patients under-

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Received 3 July 2006; Revision 12 April 2006; Accepted 26 September 2006

Published online 28 November 2006 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.b.20157

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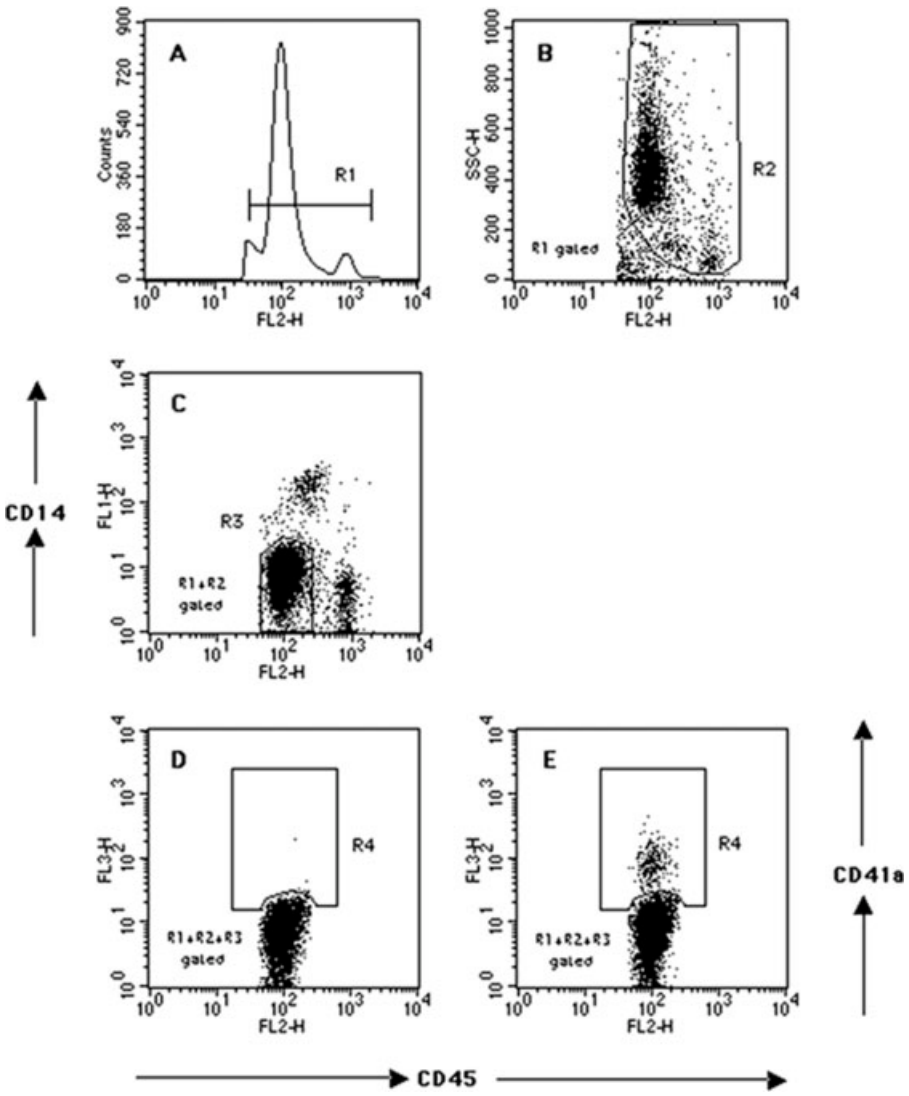

FIG. 1. Measurement of granulocyte–platelet aggregates in whole blood sample. Flow cytometry data collection is triggered using the CD45 fluorescence signal (FL2) and $CD45^{+}$ leukocytes are initially gated by region R1 (**A**). These events are then discriminated on the basis of their side scatter characteristics (R2; **B**), and granulocytes are identified as $CD45^{low}$ and $CD14^{low}$ events (R3; **C**). A final gate (R1+R2+R3) is used to derive two dual-parameter dot plots of FL3 versus FL2, representing the percentage of positivity for the CD41a platelet-specific marker on granulocyte population (R4; **E**) versus the corresponding isotype control (R4; **D**).

going cardiac surgery with cardiopulmonary bypass (CPB) (4). In the present study, we studied both intra-operative coronary and peripheral blood samples at 5 and 24 h postoperatively in a larger number of patients. The aim of this study has been to compare, over time, the monocyte/granulocyte–platelet adhesion index variations, the corresponding changes of circulating leukocyte–platelet coaggregates (percentages), and the surface modulation of main receptors involved in leukocyte–platelet coaggregation.

MATERIALS AND METHODS

We studied 20 Caucasian patients (range, 43–85 years; with a male/female ratio of 9/11) undergoing operation for aortic valve replacement that was isolated or associated with coronary artery bypass graft surgery. All surgical procedures were performed at CPB and all patients received similar medication and intra-operative and postoperative care. We evaluated sinus coronary blood samples drawn before cardioplegia and immediately after reperfusion, as well as 5 and 24 h postoperative peripheral blood specimens.

The study protocol was approved by the local ethical committee, and informed consent was obtained from each subject before enrollment.

Instrument calibration, sample collection and preparation for flow cytometry analysis, leukocyte and platelet count, as well as measurement of monocyte–platelet coaggregates or monocyte surface markers expression, were carried out as previously described (4). The granulocyte population was gated only morphologically (bivariate plot of forward vs. side scatter) and 20,000 events at least were analyzed for each sample. The percentages of granulocyte–platelet coaggregates were quantified accordingly to a CD45/CD14-based gating sequence (5,6), as reported in Figure 1. The accuracy of the flow cytometric quantification of cellular conjugates was determined, both for granulocytes and monocytes, by 10 repeated measurements. We obtained coefficients of variation of 10.4% for monocyte–platelet conjugates and 4.0% for granulocyte–platelet con-

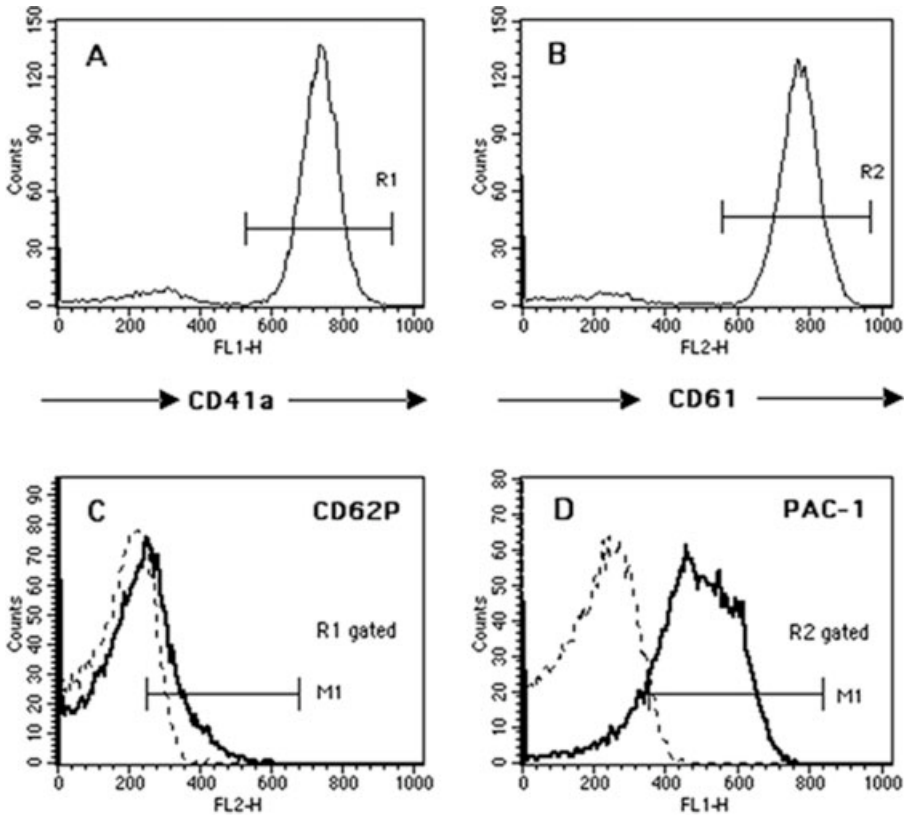

FIG. 2. Analysis of platelet activation in whole blood sample. The platelets, previously clustered by a dual-parameter dot plots of side versus forward scatter, both collected in four-decade log scales, are subsequently identified on the basis of their bright CD41a (FL1; **A**) and CD61 (FL2; **B**) fluorescence intensities. The overlay histogram function (**C**, **D**) was used to quantify the percentage of positive platelet for each activation marker (bold histograms) and their median fluorescence intensity (linear scale) with respect to the isotype controls (dotted histograms).

jugates. Surface expression of platelet activation markers (CD62P and PAC-1) was quantified using the overlay histogram function, as described in Figure 2.

Mean values of measurements and their standard errors of the mean (mean ± SEM) were calculated with StatView 5.0 software (SAS Institute, Cary, NC). The potential existence of statistically significant differences between groups was explored with Fisher's test for repeated measurements. $P < 0.05$ was considered statistically significant.

RESULTS

Leukocyte cell number increased significantly at the moment of coronary reperfusion and in postoperative peripheral blood samples, when compared with measurements done in coronary blood before cardioplegia (Table 1).

To study the functional cell interaction between circulating phagocytes (monocytes and granulocytes) and platelets, in coronary and in peripheral blood coaggregates, we calculated their adhesion index according to the following formula, previously reported (4):

$$\% = [\text{Adhesion index}] \times \frac{\text{Number of platelets}/\mu 1}{\text{Number of leukocytes}/\mu 1} \times \frac{1}{T}$$

where % is the flow cytometric percentage of circulating granulocytes and monocytes forming conjugates with platelets, and T represents the time of coronary ischemia (or cardioplegia) associated with extracorporeal circulation. In our previous paper, a normalization for the T value was carried out on after-cardioplegia coronary blood samples in order to attenuate the significant trapping effect of extracorporeal apparatus on calculation of monocyte–platelet conjugates (4). In the present work, performed on a larger number of patients, we did not observe significant inverse correlation between the variation of flow cytometric percentages of monocyte/granulocyte–platelet

Table 1
Postreperfusion and Postoperative Variations of Leukocyte and Platelet Number (Cells/μl) in Coronary (CB) and in Peripheral Blood (PB) After CPB

	CB		PB	
Cell type	Before cardioplegia	After cardioplegia	5 h postoperatively	24 h postoperatively
Platelets	113111 ± 8339	125000 ± 9080 (P = N.S.)	110200 ± 12383 (P = N.S.)	123750 ± 11125 (P = N.S.)
Monocytes	157 ± 20	217 ± 26 ($P = 0.05$)	614 ± 117 ($P = 0.015$)	986 ± 94 ($P < 0.0001$)
Granulocytes	1794 ± 171	6568 ± 664 ($P < 0.0001$)	10298 ± 1060 ($P = 0.0007$)	10647 ± 1205 ($P < 0.0001$)

CPB, cardiopulmonary bypass; N.S., not significant; $P < 0.05$ was considered statistically significant.

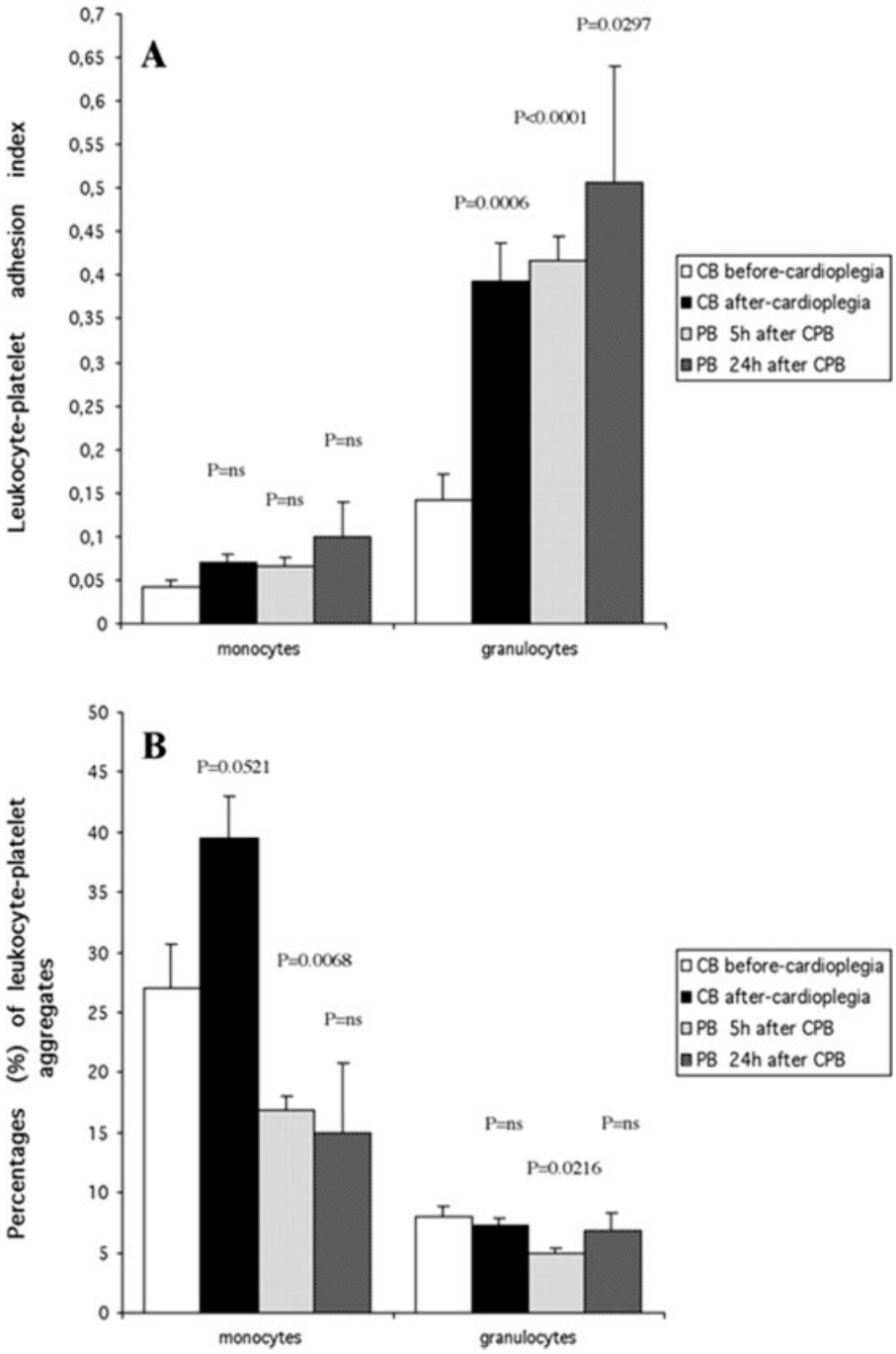

FIG. 3. Representation of monocyte and granulocyte capacity to coaggregate with platelets in coronary vein blood (CB) before and after cardioplegia, as well as in peripheral blood (PB) at 5 and 24 h after cardiopulmonary bypass (CPB). The variations of a normalized leukocyte–platelet adhesion index (**A**) are compared with the corresponding changes of percentages of leukocyte–platelet conjugates determined by flow cytometry (**B**). Statistically significant differences ($P < 0.05$) were detected when compared with measurements done in coronary blood before cardioplegia. Data are expressed as mean ± SEM.

conjugates, determined before and after cardioplegia, and the corresponding times of coronary ischemia. Therefore, no *T* values normalization was applied to the after-cardioplegia coronary blood samples. Obviously, no normalization for the time of cardioplegia was used to derive both monocyte- and granulocyte-platelet adhesion index in peripheral blood sample, at 5 and 24 h postoperatively. As shown in Figure 3, a completely different course characterizes the changes of the leukocyte-platelet adhesion index when compared with the corresponding percentages of circulating leukocyte-platelet coaggregates.

The expression of pro-adhesive receptors CD162 and CD15s showed no significant variations on coronary monocytes at reperfusion (after cardioplegia) when compared with measurements done before cardioplegia (Fig. 4). On the other hand, the coronary granulocytes exhibited a significant loss of CD162 expression at reperfusion (Fig. 4A). Interestingly, when compared with the before-cardioplegia condition, our data indicated a progressive and significant increase of CD162 levels on peripheral blood monocytes after CPB (Fig. 4A). Taking into account the molecules involved in platelet–leukocyte interaction or known to

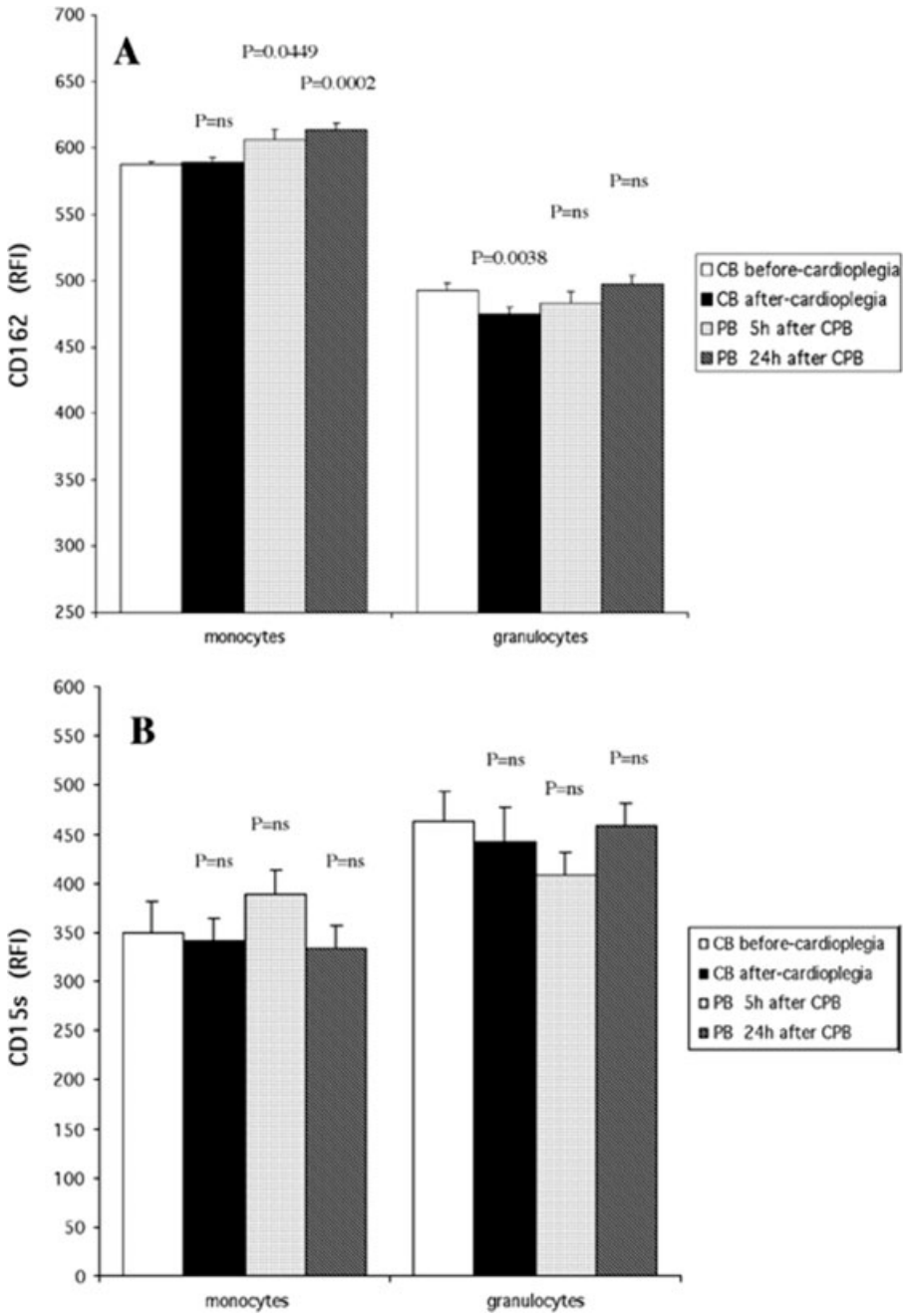

FIG. 4. Comparative analysis of monocyte and granulocyte surface expression (relative fluorescence intensity, RFI) (linear scale) of the pro-adhesive receptors CD162 (**A**) and CD15s (**B**). The evaluations have been performed in coronary vein blood (CB) before and after cardioplegia, as well as in peripheral blood (PB) at 5 and 24 h after cardiopulmonary bypass (CPB). Statistically significant differences ($P < 0.05$) were detected when compared with measurements done in coronary blood before cardioplegia. Data are expressed as mean ± SEM.

stabilize leukocyte–platelet coaggregates, our data showed a progressive increase of granulocyte CD11b expression in coronary blood at reperfusion and in peripheral blood at 5 h after CPB, followed by its clear loss at 24 h postoperatively (Fig. 5B). Moreover, we observed a significant loss of CD18 expression on peripheral blood monocytes and granulocytes at 5 and 24 h after CPB, respectively, compared with before-cardioplegia samples (Fig. 5A). Finally, when compared with the before-cardioplegia condition, our data indicated a decrease of the platelet activation marker PAC-1, especially at 5 h after CPB. Also the expression of the P-selectin molecule (CD62P), involved in the formation of leukocyte–platelet coaggregates, exhibited a slightly but significant decrease at reperfusion and in peripheral blood at 5 h postoperatively (Table 2).

DISCUSSION

The heterotypic leukocyte–platelet functional interaction is an important prognostic factor in clinical conditions characterized by vascular injury and microangiopathy (2,7). Flow cytometric procedures, applied to the evaluation of whole blood samples, may represent an essential tool in investigating this cellular cross talk (8).

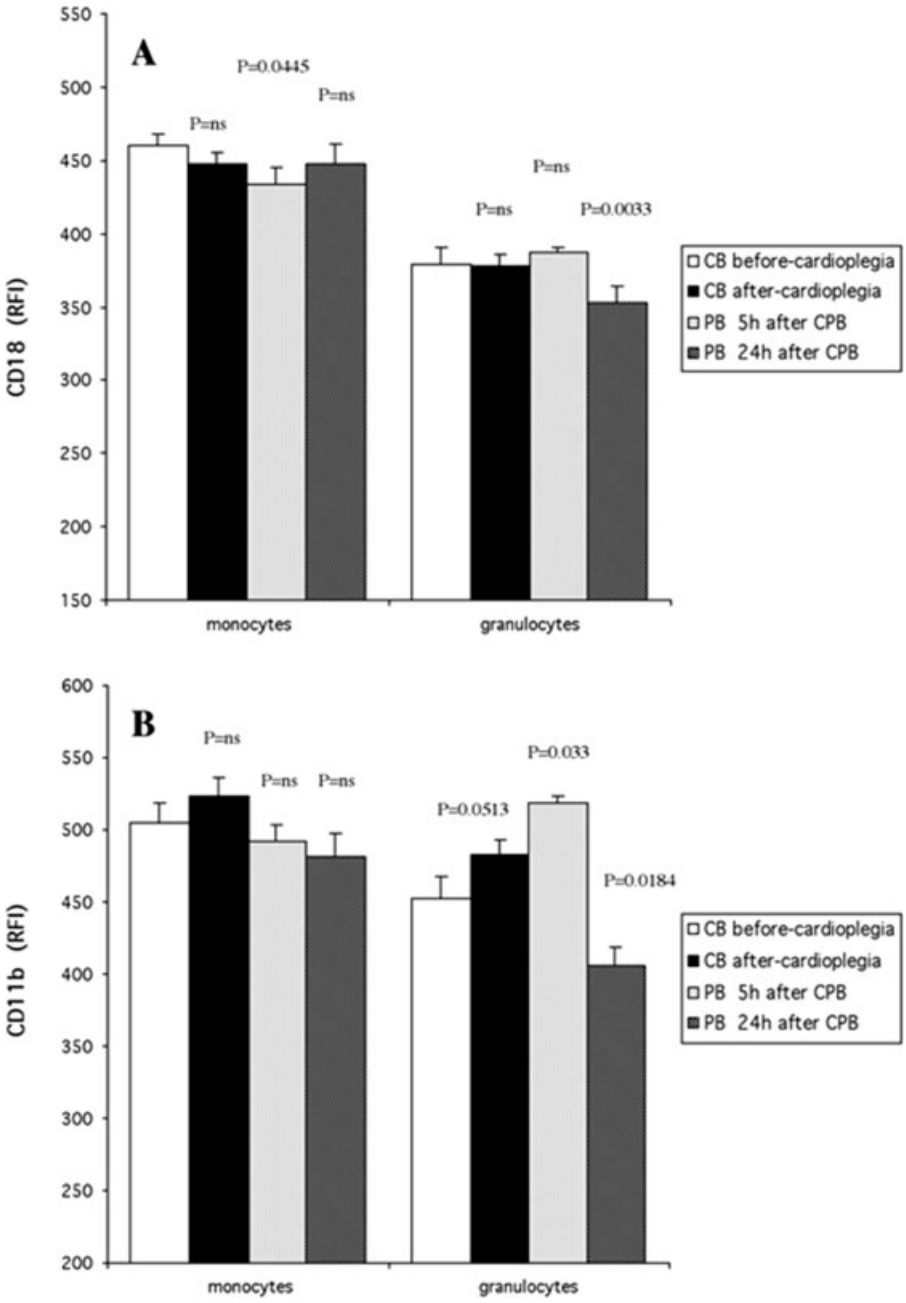

FIG. 5. Comparative analysis of monocyte and granulocyte surface expression (relative fluorescence intensity, RFI) (linear scale) of the integrin molecules CD18 (**A**) and CD11b (**B**). The evaluations have been performed in coronary vein blood (CB) before and after cardioplegia, as well as in peripheral blood (PB) at 5 and 24 h after cardiopulmonary bypass (CPB). Statistically significant differences ($P < 0.05$) were detected when compared with measurements done in coronary blood before cardioplegia. Data are expressed as mean ± SEM.

So far, the level of the leukocyte–platelet functional interaction has been quantified in terms of percentages of heterotypic aggregates (9,10). In the present study, we propose the calculation of a normalized leukocyte–platelet adhesion index as a parameter able to reflect more accurately the cell functional changes underlying leukocyte–platelet coaggregation. We assume that, at the association–dissociation equilibrium, the percentages of circulating leukocytes forming aggregates with platelets (as determined by flow cytometry) depends not only on the pro-adhesive condition of interacting cell types (adhesion index), but also on their probability to reach a membrane contact. Obviously, we can assume that this probability increases with a growing platelet concentration and, on the contrary, it decreases with a rising level of leukocytes. Therefore, in clinical conditions characterized by rapid and dramatic numerical changes of circulating leukocyte subsets, such as cardiac surgery with extracorporeal circulation, the derivation of this normalized adhesion index is essential to know the real pro-adhesive interaction between leukocytes and platelets.

In a previous paper, we calculated the normalized monocyte–platelet adhesion index in venous coronary sinus before cardioplegic arrest and after reperfusion (4). In this study, we included the evaluation of peripheral blood samples at 5 and 24 h postoperatively. Moreover,

Table 2
Postreperfusion and Postoperative Variations of Platelet Activation Markers (CD62P and PAC-1) in Coronary (CB) and in Peripheral Blood (PB) After CPB

	CB		PB	
	Before cardioplegia	After cardioplegia	5 h postoperatively	24 h postoperatively
RFI of $CD62P^+$	150.70 ± 7.05	141.23 ± 5.83 ($P = 0.0338$)	127.80 ± 8.98 ($P = 0.0742$)	147.33 ± 4.30 ($P =$ N.S.)
RFI of PAC-1^+	189.76 ± 19.11	187.17 ± 16.66 ($P =$ N.S.)	77.56 ± 2.63 ($P = 0.022$)	172.59 ± 8.84 ($P =$ N.S.)

CPB, cardiopulmonary bypass; N.S., not significant; RFI, relative fluorescence intensity; $P < 0.05$ was considered statistically significant.

we extended the study to granulocytes, and we compared, over time, the monocyte/granulocyte–platelet adhesion index variations, the traditional flow cytometric determination of aggregates (as percentages), and the parallel changes of main receptors involved in coaggregation.

A completely different behavior characterizes the over time changes of the leukocyte–platelet adhesion indexes and the corresponding variations of circulating leukocyte–platelet coaggregates (Fig. 3). In particular, the increase in the granulocyte–platelet adhesion index in coronary blood samples at reperfusion, as well as in peripheral blood postoperatively, seems to be completely masked by evaluating the percentages of conjugates, when compared to samples before cardioplegia. Also for monocytes, taking into account a slight concordance at the moment of coronary reperfusion, the comparison with the before-cardioplegia derived data shows an opposite postoperative course between leukocyte–platelet adhesion indexes and percentages of leukocyte–platelet coaggregates (Fig. 3).

The quantitative evaluation (relative fluorescence intensity) of leukocyte surface expression of main receptors involved in leukocyte–platelet interaction, such as the P-selectin glycoprotein ligand-1 (CD162) and the sialyl $Lewis^x$ antigen (CD15s), shows no significant changes for CD15s, both on monocytes and granulocytes, when compared with before-cardioplegia samples (Fig. 4B). On the other hand, the decrease in the granulocyte CD162 expression at reperfusion is followed by its return to the before-cardioplegia level, while the monocyte CD162 expression increases in the 5 and 24 h postoperative peripheral blood samples (Fig. 4A). The expression of the integrin complex CD11b/CD18, known to stabilize the leukocyte–platelet adhesive interaction (11,12), does not exhibit significant over time modifications on monocytes, when compared with the before-cardioplegia level, while a progressive increase of CD11b expression is observed on granulocytes at reperfusion and at 5 h postoperatively. Interestingly, the decrease of CD18 and CD11b granulocyte surface expression, observed at 24 h postoperatively, is associated with a corresponding reduced statistical significance and a largest experimental variability in the adhesion index determination, when compared with previous measurements (Fig. 3A). This probably reflects a higher instability of pro-adhesive forces involved in granulocyte–platelet coaggregation.

Finally, the lack of platelet activation, observed in coronary blood at reperfusion, as well as in peripheral blood postoperatively (Table 2), indicates that the leukocyte functional modifications are the main controllers of leukocyte–platelet coaggregation after cardiac surgery with extracorporeal circulation.

Obviously, we cannot exclude that other leukocyte and platelet membrane modifications, not involving the expression of pro-adhesive receptors, may influence the adhesion index determination (13).

Taken together, our data suggest that the flow cytometry-derived calculation of a normalized leukocyte–platelet adhesion index is more informative on the real functional interaction between circulating leukocytes and platelets, than the simple percentage determination of cellular conjugates. Moreover, taking into account the role of the leukocyte–platelet cross talk in the activation of innate immune response (14), the determination of this functional adhesion index might find application in clinical studies evaluating the modulation of inflammatory response following the use of innovative devices in cardiac surgery with CPB. In fact, preliminary results obtained by our group indicate that the use of a novel technique, based on a minimal extracorporeal circulation (MECC®) system, seems to reduce the monocyte–platelet coaggregation at the end of extracorporeal circulation (15).

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the personnel of the "G. Pasquinucci" Hospital intensive care unit and laboratory department.

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