Determinants of platelet conjugate formation with polymorphonuclear leukocytes or monocytes in whole blood

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Summary

Following preliminary in-vitro experiments, platelet-leukocyte conjugates and their determinants were evaluated in citrated whole blood from 349 subjects (209 women, age 16–92 years) randomly recruited from the general population. Platelet activation by ADP/collagen but not leukocytes stimulation by fMLP or LTB4 resulted in formation of platelet conjugates with PMN or monocytes. In the population study, mixed cell conjugates, platelet P-selectin and leukocyte CD11b were measured by flow cytometry both at baseline and after in-vitro stimulation with ADP/collagen. The latter significantly increased platelet conjugates with either PMN or monocytes, platelet P-selectin and leukocyte CD11b expression. Platelet count significantly correlated with platelet-PMN, platelet-monocyte conjugates and P-selectin both at baseline and upon stimulation. In all conditions, both conjugate levels correlated with each other, when adjusted for gender, age and platelet count. Age correlated with platelet-PMN conjugate numbers in basal and stimulated conditions and with basal P-selectin. ADP/collagen stimulation resulted in higher P-selectin and conjugates values in women. Among risk factors, a significant correlation was found between conjugate and glucose levels. In conclusion, the presence and formation in whole blood from a large population of platelet-leukocyte conjugates reflects primary platelet – but not leukocyte – activation and varies with gender, age, platelet count and blood glucose.

Keywords
Platelet-leukocyte conjugates, platelet P-selectin, cell activation markers, flow cytometry, general population

Introduction

In the framework of an ongoing epidemiological study aimed at characterizing the phenotypes linked to juvenile myocardial infarction, we decided to include the measurement of platelet-leukocyte conjugates.

Cross-talk and binding of leukocytes to platelets have been investigated in various disease states, and platelet-leukocyte conjugates have been proposed as a predictive marker for the cardiovascular disease outcome in several clinical conditions (1–5), but scarce data are available in normal subjects or in large general populations. Information on the capacity of platelets and leukocytes to mutually interact may help better understand the mechanisms of vascular integrity or disruption in inflammatory and thrombotic disease (6–9). At the site of endothelial damage, adhering platelets may indeed attract leukocytes to promote inflammation and atherogenesis (10, 11). After plaque rupture, exposed collagen from the vessel wall, in addition to other mediators, induces platelet aggregation. Platelet-released substances, such as adenosine diphosphate (ADP), stimulate adjacent platelets, further enhancing the process of platelet activation (12, 13). Activated platelets not only may promote further platelet accumulation and fibrin deposition at the site of vascular injury -as was clearly established in the past- but may recruit polymorphonuclear (PMN) and mononuclear leukocytes into an actively forming thrombus (14, 15). On the other hand, leukocytes accumulated in a platelet thrombus can contribute to further platelet activation and deposition (16, 17).

We performed preliminary in-vitro experiments to establish the most appropriate experimental conditions for studying spon-
taneous and induced conjugate formation to be applied to the subsequent population study. Physiological conditions, such as whole blood, temperature of 37°C, and constant stirring to induce a low shear force were selected. For the purpose of conjugate induction, as platelet-leukocyte cross-talk in washed systems and in whole blood are reportedly initiated by either platelet or leukocyte activation (10, 16–19), we initially tested both platelet and leukocyte stimuli.

The purpose of our study was to assess platelet-PMN and platelet-monocyte conjugates in basal conditions and upon in vitro stimulation in a large general population, and to correlate mixed cell conjugates with two markers of platelet or leukocyte activation, namely P-selectin expression on the platelet surface and CD11b on the leukocyte surface, respectively. The possible influence of gender, age and other laboratory and clinical variables was also evaluated.

Materials and methods

Subjects

The first part of this study was performed on healthy volunteers, mainly laboratory personnel (9 women, 4 men, 20–45 years old), who had not taken any medication for at least two weeks.

The second part (population study) was performed on 349 subjects (209 women, 140 men, 16–92 years old), randomly recruited from the general population of the Molise region (Italy), in the frame of a larger epidemiological study. The exclusion criteria were the occurrence of any previous cardio-cerebrovascular event containing 1/10 volume of 3.8% trisodium citrate (Becton Dickinson) and stored at 4°C for about 18 hours (h). Platelet-leukocyte conjugates were also measured by standardized techniques (20).

All subjects were informed that blood samples were obtained for research purposes and their privacy would be protected, and provided written informed consent. The latter as well as the study plan and all the procedures were approved by the Institutional Ethics Committee.

Reagents and antibodies

ADP, n-formyl-methionyl-leucyl-phenylalanine (fMLP) and leukotriene B4 (LTB4) were from Sigma-Aldrich (Milan, Italy), collagen from Mascia Brunelli S.p.A. (Milan, Italy).

Platelet-leukocyte conjugates were identified with a R Phycocerythrin cyanin 5.1 (PC5)-conjugated anti-CD45 monoclonal antibody (MoAb) J33, and a fluorescein isothiocyanate (FITC)-conjugated anti-CD61 MoAb SZ21 (Immunotech, IL, Milan, Italy). CD11b expression on leukocytes (identified by the PC5-conjugated anti-CD45 MoAb) was identified by a Phycocerythrin (PE)-conjugated anti-CD11b MoAb Bear1 (Immunotech). P-selectin and activated GπIIbⅢa expression on platelets were quantified by a FITC-conjugated anti-CD42b MoAb SZ2 (Immunotech) and a PE-conjugated anti-CD62P MoAb AC1.2 (Becton Dickinson, Milan, Italy) and a PE-conjugated anti-CD61 MoAb SZ21 (Immunotech) and a FITC-conjugated anti-GπIIbⅢa complex (PAC-1) MoAb SP-2 (Becton Dickinson), respectively. FITC- and PE-conjugated isotypic MoAbs 679.1Mc7 (Immunotech) were used as negative control for anti-CD61 and anti-CD11b, a PE-conjugated isotypic MoAb X40 (Becton Dickinson) was used as negative control for anti-CD62P and a FITC-conjugated isotypic MoAb G155–228 was used as negative control for PAC-1. All MoAbs were used at saturating concentrations, which were experimentally determined and usually corresponded to the concentrations suggested by the manufacturers.

ThromboFix™ Platelet Stabilizer, IO Test3 lysing solution, PBS buffer (0.01 M Potassium Phosphate, 0.15 M Sodium Chloride, pH = 7.2 ± 0.2) and Flow-Check™ Fluorospheres were from Beckman Coulter (IL, Milan, Italy).

Blood collection and sample preparation

Venous blood was collected on siliconized Vacutainer tubes containing 1/10 volume of 3.8% trisodium citrate (Becton Dickinson). The first 5 ml were used for biochemical analysis (glucose, total cholesterol, LDL, HDL, triglycerides, morphology) while for mixed conjugates and cell activation markers only the following blood was used in order to minimize cell activation due to blood collection.

All samples were processed between 10 and 20 minutes (min) after blood collection, as previously described (21, 22) and lately recommended by Hardling et al. (23). To measure “basal” mixed conjugates, 100 µl of blood were fixed by adding an equal volume of ThromboFix. To measure mixed conjugates formed in vitro upon platelet or leukocyte stimulation, aliquots of 450 µl fresh blood were incubated with the couple of agonists ADP (5 µM final concentration, f.c.) and collagen (2 µg/ml f.c.), with IMLP (1 µM f.c.) or LTB4 (0.15 µM/ f.c) 10 min, at 37°C, under constant stirring at 1,000 rpm, in an aggregometer (Chrono-log Corporation; Mascia Brunelli S.p.A., Milan, Italy). The concentrations of ADP and collagen were selected that induced – in preliminary experiments – a 2- to 4-fold increase of mixed conjugates. The ADP concentration (5 µM f.c.) was within the range of those used by Barnard et al. (24), while the collagen concentration (2 µg/ml f.c.) was similar to that used by Maugeri et al. (22) but 10 times lower than that used by Barnard et al. (24). The time of incubation of whole blood with platelet stimuli was chosen on the basis of previous observations (25). The protocol used was aimed at evaluating platelet-leukocyte conjugates formed in the presence and possibly in competition with platelet-platelet aggregates primarily induced by ADP/collagen challenge; low shear flow conditions were applied, to better mimic the in-vivo condition of leukocytes, mainly contributing to microvascular injury and capillary plugging, following the ischaemia/reperfusion sequence (26). We decided not to prevent platelet aggregation by omitting stirring of the samples or using anti-platelet compounds (25, 27), to more closely mimic the complex cell-cell interactions occurring within the circulation. Reaction was stopped by fixation, and then labelling with the appropriate MoAb for 15 min in the dark, at room temperature (RT). Then, red cells were lysed by adding 10 Test3 lysis solution, as indicated by the manufacturer, and the sample was centrifuged for 10 min x 1,000g, at RT. The supernatant was discarded and samples were re-suspended in 1 ml of phosphate-buffered saline (PBS) buffer and stored at 4°C for about 18 hours (h). Platelet-leu-
Platelet-leukocyte conjugates, platelet P-selectin and leukocyte CD11b expression were measured both at baseline and after platelet or leukocyte stimulation.

For platelet PAC-1 expression, unfixed blood was incubated with MoAb for 15 min, the reaction stopped by addition of cold PBS and the samples were immediately analysed.

**Flow cytometric analysis**
Analyses were performed with a Coulter EPICS XL flow cytometer (Beckman Coulter) that was daily checked by the acquisition of Flow-Check™ Fluospheres. For each measurement 10,000 events were analyzed.

Platelets, including platelet aggregates, were defined by morphological characteristics, using forward light scatter (FS)
versus side light scatter (SS) intensity dot-plot representation (Fig. 1A) and by CD42b positivity. PMN and monocyte populations were defined on the basis of the SS characteristics within the CD45-positive population (Fig. 2A). Data are reported as percentage of fluorescence positive events in platelet, PMN or monocyte populations.

**Statistical analysis**

**Preliminary study**

Statistical analysis was performed by paired t-test, and contrasts were adjusted following the method of Sidak (28). All data (except CD11b expression) were log transformed to normalize their positively skewed distribution. Data are reported as means and standard error of the mean (SEM), and p-values below 0.05 were considered as statistically significant.

**Population study**

Platelet-PMN, platelet-monocyte and P-selectin expression were log transformed to normalize their positively skewed distribution. The influence of age was analysed both as a continuum or after subdividing the population in tertiles. Data are reported as median and 25e-75e percentile, and p-value below 0.05 was chosen as the level of significance.

Univariate and multivariate associations of continuous variables with age and gender were assessed by multivariate analysis of variance (procedure GLM in SAS).

Univariate correlations among continuous variables were calculated using Pearson's correlation coefficient. Multivariate correlations among continuous variables adjusted for gender, age and platelet count were assessed applying multivariate linear regression analysis (Procedure REG in SAS).

A multivariate linear regression analysis with interaction terms among sex or age-tertiles was used to test difference between slopes.

All computations were carried out using the SAS statistical package (Version 8.2 for Windows, SAS Institute Inc., Cary, NC, USA: SAS Institute Inc., 1989).

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**Figure 3:** Effect of ADP/collagen, fMLP, or LTB4 addition to whole blood on mixed platelet-leukocyte conjugate levels and cell activation markers. A) Platelet-PMN and platelet-monocyte conjugates (% CD61-positive leukocytes). B) Platelet P-selectin and PAC-1 expression (% of platelets positive for each antigen). C) CD11b expression on PMN and monocytes (% CD11b-positive leukocytes). Means and sem, n=12, except for LTB4 (n=5-6). p<0.05 (Sidak adjusted p-value) for pairwise comparisons between basal and platelet (ADP/collagen) or leukocyte (fMLP) stimuli. In a subgroup analysis the results obtained with either leukocyte stimulus (fMLP or LTB4) were non statistically different.
Results

Preliminary study

As shown in Figure 3, ADP/collagen stimulation in vitro for 10 min at 37°C under constant stirring induced a significant increase of both mixed platelet-leukocyte conjugates and cell activation markers, namely platelet P-selectin expression and PMN and monocyte CD11b upregulation. Platelet PAC-1 expression was also significantly increased. In particular, platelet/PMN conjugates rose from 3.4 ± 0.4 to 12.0 ± 2.3% (mean ± SEM) and platelet/monocytes from 8.7 ± 2.7 to 21.7 ± 5.1% (mean ± SEM).

Although in basal conditions there appears to be a higher percentage of platelet conjugates with monocytes than with PMN, the relative increase by ADP/collagen stimulation was quite similar (3.5 and 2.5 fold, respectively). In contrast, both fMLP and LTB4 stimulation failed to induce any significant increase of mixed platelet-leukocyte conjugates. While CD11b expression was markedly increased on both PMN and monocytes, platelet activation markers were unaffected by either stimulus (Fig. 3).

The possibility that fMLP or LTB4, directly or through leukocyte activation (7), could down-regulate platelet function, was ruled out by experiments in which whole blood was incubated both with fMLP or LTB4 either shortly before or after stimulation with ADP/collagen; no difference in either mixed conjugate formation or platelet marker expression was found in samples stimulated with ADP/collagen alone or in combination with fMLP or LTB4 (data not shown). On the other hand, CD11b expression on leukocytes was similar when ADP/collagen was used either in the presence or in the absence of fMLP or LTB4 (data not shown).

Population study

Whole blood collected from 349 volunteers within a general population was tested in basal conditions and, on the basis of the preliminary findings reported above, upon in-vitro stimulation with a combination of platelet agonists (ADP/collagen). Mixed platelet-leukocyte conjugates, platelet P-selectin and leukocyte CD11b were measured to monitor platelet and leukocyte activation, respectively.

Demographics, blood cell counts and major cardiovascular risk factors of the population studied are shown in Table 1.

The platelet count was significantly higher in women, and total leukocyte count in men. Systolic and diastolic blood pressure and glucose levels were significantly higher in men. Significantly more men were smokers.

Platelet-PMN and platelet-monocyte conjugates

Table 2 reports values of platelet-PMN and platelet-monocyte conjugates present in basal conditions or formed after in-vitro platelet stimulation. Median basal values of platelet-PMN and platelet-monocyte conjugates were 3.2 and 5.5 %, respectively. ADP/collagen determined a similar significant increase in both platelet-PMN and platelet-monocyte conjugates of about three fold.

A positive significant correlation (p<0.001) was observed between the percentage of basal and stimulated conjugates (r=0.48 and 0.62 for platelet-PMN and platelet-monocytes, respectively) as well as between platelet-PMN and platelet-monocyte conjugates (r=0.66 in basal conditions and r=0.75 after ADP/collagen stimulation). All data had been adjusted for gender, age and

Table 1: Demographics and cardiovascular risk factors of the population studied (n = 349).

<table>
<thead>
<tr>
<th></th>
<th>Total (n = 349)</th>
<th>Women (n = 209)</th>
<th>Men (n = 140)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>45 ± 18</td>
<td>44.4 ± 19</td>
<td>45.1 ± 18</td>
<td>-</td>
</tr>
<tr>
<td>Platelet count (10³/µl)</td>
<td>246.7 ± 60.7</td>
<td>260.0 ± 64.0</td>
<td>226.7 ± 49.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WBC (10³/µl)</td>
<td>6.1 ± 1.5</td>
<td>5.9 ± 1.5</td>
<td>6.4 ± 1.4</td>
<td>0.0012</td>
</tr>
<tr>
<td>PMN count (%)</td>
<td>61.8 ± 7.3</td>
<td>61.6 ± 7.3</td>
<td>61.9 ± 7.3</td>
<td>-</td>
</tr>
<tr>
<td>Monocyte count (%)</td>
<td>5.7 ± 2.0</td>
<td>5.7 ± 1.8</td>
<td>5.1 ± 1.5</td>
<td>-</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130.9 ± 21.3</td>
<td>126.7 ± 21.4</td>
<td>137.0 ± 20.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>77.1 ± 12.0</td>
<td>75.2 ± 11.2</td>
<td>79.9 ± 12.3</td>
<td>0.0002</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>96.0 ± 21.6</td>
<td>91.4 ± 13.1</td>
<td>102.1 ± 29.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>204.4 ± 44.0</td>
<td>203.9 ± 45.9</td>
<td>205.3 ± 41.2</td>
<td>-</td>
</tr>
<tr>
<td>C-reactive protein (µg/dl)</td>
<td>2.3 ± 3.1</td>
<td>2.3 ± 2.88</td>
<td>2.4 ± 3.5</td>
<td>-</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.5 ± 4.8</td>
<td>26.2 ± 5.3</td>
<td>26.9 ± 3.9</td>
<td>-</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>30</td>
<td>23</td>
<td>40</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

*P-values (women vs. men) adjusted for age; means ± SD.

Table 2: Platelet-PMN and platelet-monocyte conjugates in basal conditions and upon platelet stimulation in whole blood from 349 subjects.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>ADP/collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-PMN conjugates</td>
<td>3.2 (2.2–5.2)</td>
<td>9.9 (4.4–22.2)*</td>
</tr>
<tr>
<td>Platelet-monocyte conjugates</td>
<td>5.5 (2.8–10.7)</td>
<td>17.4 (6.9–36.7)*</td>
</tr>
</tbody>
</table>

Data are reported as percentage double fluorescence positive events (CD45/CD61); median and 25-75 percentile (n=349); ADP 5 µM, collagen 2 µg/ml. *p<0.0001 vs basal at multivariate (age, gender and platelet count) analysis.
platelet count. The same correlations did not differ when analysed in women versus men or in different age tertiles.

### Influence of gender and age

Table 3 reports the percentage of mixed platelet-PMN and platelet-monocyte conjugates in basal conditions and after ADP/collagen stimulation, separately for women and men, and in all subjects subdivided in age tertiles of comparable size.

Table 3: Platelet-PMN and platelet-monocyte conjugates in basal conditions and upon platelet stimulation in whole blood from 349 subjects: effect of gender and age.

<table>
<thead>
<tr>
<th>Gender (n = 349)</th>
<th>Age tertiles (n=349)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-PMN conjugates</td>
<td></td>
</tr>
<tr>
<td>Women (n = 209)</td>
<td>Men (n = 140)</td>
</tr>
<tr>
<td>Basal</td>
<td>3.2 (2.2–5.2)</td>
</tr>
<tr>
<td>ADP/collagen</td>
<td>13.4 (4.4–28.6)</td>
</tr>
<tr>
<td>Platelet-monocyte conjugates</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>5.4 (3.0–10.7)</td>
</tr>
<tr>
<td>ADP/collagen</td>
<td>19.9 (7.7–44.1)</td>
</tr>
</tbody>
</table>

Data are reported as percentage double fluorescence positive events (CD45/CD61); median and 25°–75° percentile; ADP 5µM, collagen 2µg/ml. *p<0.001 vs. women. ** p<0.04 vs. age tertile 1. °p=0.03 vs. age tertile 1.

No difference between women and men were found in mixed conjugates measured in basal conditions; in contrast, ADP/collagen stimulation resulted in a significantly (p=0.019) greater amount of platelet-PMN conjugates in women as compared to men (data adjusted for platelet count and age). The plots of platelet-PMN vs platelet-monocytes had a similar slope in women and men both in basal conditions and after ADP/collagen stimulation.

When the whole population was subdivided in age tertiles and data adjusted for platelet count and gender, the oldest tertile had significantly more platelet-PMN conjugates (p=0.04 in basal conditions and p=0.01 after ADP/collagen stimulation), as compared to the youngest one. In addition, a continuous positive correlation with age was observed for platelet-PMN conjugates both at baseline and after ADP/collagen stimulation (r=0.14, p=0.007 and r=0.12, p=0.028, respectively).

By multivariate analysis (data adjusted for gender and age), platelet-PMN and platelet-monocyte conjugates were significantly correlated with platelet count, in both basal conditions and upon stimulation (r values between 0.18 and 0.27, p values between 0.0009 and <0.0001).

### Platelet and leukocyte markers

Table 4 reports data on the expression of platelet or leukocyte activation markers in basal conditions and after ADP/collagen stimulation. Platelet P-selectin, on the one hand, and PMN and monocyte CD11b expression on the other were significantly (p<0.0001) increased after ADP/collagen stimulation upon multivariate analysis (adjusted for gender, age and platelet count).

Table 4: Markers of platelet and leukocyte activation in basal conditions and upon platelet stimulation in whole blood from 349 subjects.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>ADP/collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet P-selectin</td>
<td>2.1 (1.0–3.8)</td>
<td>23.2 (14.1–30.1) *</td>
</tr>
<tr>
<td>PMN CD11b</td>
<td>26.8 (15.4–40.7)</td>
<td>74.5 (57.6–84.3) *</td>
</tr>
<tr>
<td>Monocyte CD11b</td>
<td>39.8 (24.3–55.8)</td>
<td>79.9 (69.3–86.1) *</td>
</tr>
</tbody>
</table>

Data are reported as percentage antigen fluorescence positivity in the appropriate cell population. Median and 25°–75° percentile (n=349). *p<0.0001 vs. basal values at multivariate (gender, age, platelet count) analysis.
Izzi, Pampuch, et al. Platelet-leukocyte conjugates in the general population

Table 5: Markers of platelet and leukocyte activation in basal conditions and upon platelet stimulation in whole blood from 209 women and 140 men.

<table>
<thead>
<tr>
<th></th>
<th>Women (n=209)</th>
<th>Men (n=140)</th>
<th>P-value (women vs. men)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P-selectin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1.9 (1.0–3.8)</td>
<td>2.3 (1.2–3.9)</td>
<td>n.s.</td>
</tr>
<tr>
<td>ADP/collagen</td>
<td>24.0 (17.0–31.8)</td>
<td>20.4 (8.8–27.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Monocyte CD11b</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>41.7 (25.6–59.3)</td>
<td>36.2 (21.0–49.7)</td>
<td>n.s.</td>
</tr>
<tr>
<td>ADP/collagen</td>
<td>82.0 (71.1–88.4)</td>
<td>78.1 (65.2–84.3)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Data are reported as percentage antigen fluorescence positivity in the appropriate cell population. Median and 25%-75% percentile. * data for multivariate analysis adjusted for age and platelet count. n.s. not significant.

Influence of clinical and biochemical cardiovascular risk factors (Table 1)

By univariate analysis, statistical significance was observed between both basal and ADP/collagen stimulated platelet-PMN conjugates on the one side and blood glucose on the other (p=0.032 and p=0.025, respectively) and between basal platelet-PMN conjugates and either systolic (p=0.05) and diastolic (p=0.04) blood pressure, or body mass index (BMI) (p=0.047). These correlations, however, except for that with blood glucose levels, were no more significant when data were adjusted for age, gender and platelet count (data not shown).

Discussion

Cell conjugates induced in vitro by platelet or leukocyte stimulation

In the first part of this study, we observed that in citrated whole blood at 37°C under constant sample stirring, basal platelet conjugates with either PMN or monocytes significantly increased as a consequence of platelet activation (by ADP/collagen), while selective leukocyte activation (by either FMLP or LTB4) did not induce any heterotypic cell interaction. Similarly, markers of platelet activation, such as P-selectin and activated GpIIb/IIa complex, were upregulated by platelet agonists only. In contrast, CD11b, a marker of leukocyte activation, was not only directly upregulated by leukocyte agonists, but also indirectly following platelet activation. Thus, in the experimental conditions used in this study (whole blood, at 37°C, constant sample stirring to induce a low shear force, corresponding to that measured in the microvasculature) (29), platelets, but not leukocytes need to be activated for leukocyte activation to occur and mixed conjugates to be formed. Even more conjugates could possibly have been formed if platelet-platelet aggregation had been prevented (27, 30, 31). In the condition we used, we cannot exclude that occasionally leukocytes are mechanically trapped in between aggregating platelets, but preferred to have a more realistic estimate of conjugate induction within the circulation, where it would likely occur in competition with platelet-platelet interaction.

Previous in-vitro studies on washed cells had elucidated the mechanism(s) by which P-selectin, upon binding its leukocyte counter-receptor PSGL-1, induces a src-kinase-dependent signal in either PMN or monocyte leukocytes, resulting in the activation of the β-integrin CD11b/CD18 adhesive receptor and subsequent platelet-leukocyte firm adhesion (9, 32–34). Both in basal conditions and after ADP/collagen stimulation, more monocytes bound platelets than did PMN, a finding in agreement with previous observations (25, 35). Under the experimental conditions used in this and previous studies (36) studies, the preferential binding of platelets to monocytes rather than to PMN can be explained by a markedly higher density of PSGL-1 on monocytes (36).

We confirmed the essential role of platelet activation and P-selectin expression in mixed conjugate formation in whole blood (17, 19, 22, 24, 25), but could not show any role for primary leukocyte activation, a finding apparently at variance with previous studies in washed systems or in whole blood collected on hirudin as an anticoagulant (29). The ability of proteases, such as cathepsin G, released by activated PMN, to activate in turn platelets, is well documented by in-vitro studies in washed cell systems. In those systems, the platelet-activating effect of proteases could be expressed freely, due to the absence of anti-proteinases in the artificial suspending medium. The addition of anti-proteinases resulted in the loss of platelet activation, unless prior cell-cell adhesion had created a microenvironment in which cathepsin G, released from stimulated PMN, was protected against anti-proteinases (7, 37). Thus, the anti-proteases in stirred blood could prevent cathepsin G-mediated platelet activation.

Li et al. (29) showed that leukocyte activation could to some extent lead to platelet-leukocyte conjugates when physiological calcium concentrations are maintained by using hirudin as anticoagulant. In our whole blood system using citrate as anticoagulant, physiological calcium concentrations abolished any effect of leukocyte activation on platelet P-selectin exposure and conjugate formation. However, also in the presence of hirudin, collagen enhanced platelet-leukocyte conjugate formation more markedly than did FMLP, as if platelet-leukocyte conjugate formation was more dependent on platelet than on leukocyte activation. This difference was explained by the fact that P-selectin, the major adhesion molecule involved in platelet-leukocyte conjugate formation (34), is only expressed on activated platelets, whereas its counterparts PSGL-1 and CD15 are constitutively expressed on leukocytes (29). In reconstituted suspensions of platelets, PMN, and monocytes in citrated plasma, stimulation of monocytes or PMN with FMLP only moderately enhanced the extent of platelet-monocyte but not platelet-PMN conjugate levels. The former heteroaggregates, however, could only be observed for very short (15 seconds) shear exposure times, and dis...
appeared within 120 seconds (25). These findings may explain our observations obtained when whole blood samples were challenged by FMLP under dynamic conditions for a much longer time (10 min). We speculate that the affinity of leukocyte CD11b/CD18 for its platelet counter-receptor decreases with time, as previously suggested for both PMN (38) and monocytes (25).

Measurement of cell conjugates and their determinants in a large population
In the second part of this study, we examined in a general population the determinants of conjugate levels either present immediately after venipuncture, without any agonist addition or further manipulation, or formed following in-vitro platelet activation.

The basal values for platelet-leukocyte conjugates in our study were somewhat lower or within the range of those previously reported in small groups of subjects, studied as controls of cardiovascular (39) or haematological patients (5, 21, 40) or in a pharmacological study (22). Basal platelet P-selectin and leukocyte CD11b expression were also in the range previously reported (41).

In basal conditions, platelet-PMN conjugate and platelet-monocyte conjugate levels correlated with each other; P-selectin expression, however, only correlated with the former. Platelet count, in contrast, correlated with both leukocyte conjugate values. Basal platelet conjugate numbers (with both PMN and monocytes) and the markers of cell activation were not different between women and men, or in different age groups. However, significantly more activated platelet-PMN conjugates were observed in subjects in the oldest age tertile, a finding in agreement with higher, though not significant, basal platelet-monocyte conjugates in the oldest people.

ADP/collagen stimulation provoked an increase in both platelet-PMN and platelet-monocyte conjugates (more than 3 fold – on average – in respect to basal values for both parameters), platelet P-selectin expression (more than 10 fold) and CD11b expression on both PMN and monocytes (almost 2–3 fold).

Platelet-leukocyte conjugate levels after ADP/collagen stimulation correlated with those detected in basal conditions. Highly significant correlations were also observed between conjugate values for platelet-PMN and platelet-monocyte following platelet activation.

A significantly higher percentage of ADP/collagen-induced platelet-leukocyte conjugates was formed in women as compared to men, a finding consistent with a significantly greater platelet P-selectin exposure in women. This difference was apparent after adjustment for platelet count (which was indeed significantly higher in women), suggesting a greater in-vitro sensitivity of women’s platelets to agonists with consequent greater platelet-platelet and platelet-leukocyte conjugate formation. Indeed, greater sensitivity to activation of women platelets by agonists has already been reported (42, 43). Less likely, activated platelets from women might be less sensitive to mutual interaction, being therefore more prone to conjugate with leukocytes than with each other.

Age, too, determined the levels of platelet-PMN conjugates, platelet P-selectin exposure and CD11b on PMN following stimulation of whole blood with ADP/collagen.

An observation of particular clinical interest is that basal and stimulated PMN-platelet conjugate values were positively correlated with blood glucose levels. Blood glucose may influence mixed conjugate levels through greater platelet activation; in fact, although we could not observe any significant correlation between glucose levels and P-selectin expression, hyperglycemia has recently been reported to increase platelet activation in normal subjects (44, 45). The possible relevance for diabetic patients of findings obtained in normal subjects remains to be established. Other common cardiovascular risk factors, such as blood pressure and body mass index (BMI), only correlated with mixed conjugate levels by univariate analysis.

Limitations of this study
Although several physiological conditions were mimicked in our in-vitro experiments, the setting in which platelet-leukocyte interactions were measured differed somewhat from in-vivo conditions. Moreover, the possible role of vascular cells on the interactions with and between blood cells could not be taken into account. Only one stimulus (ADP/collagen) of platelets was used, at fixed concentrations, an experimental condition useful when a large population study has to be performed but less informative than dose-response curves with the agonists to measure EC50 values. On the other hand, under the flow conditions used here, platelet stimuli induced not only platelet activation and P-selectin expression, but most likely platelet aggregate formation as well. The latter could have influenced in a variable way the amount of mixed platelet-leukocyte conjugates measured. Finally, from an epidemiological point of view, several correlations found, although significant, were rather weak (r values lower than 0.5) and are therefore of uncertain patho-physiological significance.

Conclusions
In conclusion, this study – the first one, to our best knowledge, on such a large adult population – shows that the presence in basal unstimulated conditions and the formation upon stimulation of platelet conjugates with leukocytes in whole blood, reflects primary platelet, not leukocyte, activation and may vary with gender, age, platelet count and blood glucose levels. It may provide useful baseline information for future epidemiological studies in normal subjects or patients, complementing the measurements in whole blood – rather than in artificial systems of isolated cells – of platelet-leukocyte interactions and their related activation markers and variables.

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