Parameters of red blood cell aggregation as correlates of the inflammatory state

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INTRODUCTION

Parameters of red blood cell aggregation as correlates of the inflammatory state. Am J Physiol Heart Circ Physiol 280: H1982–H1988, 2001.—To identify clinically relevant parameters of red blood cell (RBC) aggregation, we examined correlations of aggregation parameters with C-reactive protein and fibrinogen in unstable angina (UA), acute myocardial infarction (AMI), and bacterial infection (BI). Aggregation parameters were derived from the distribution of RBC population into aggregate sizes (cells per aggregate) and changing of the distribution by flow-derived shear stress. Increased aggregation was observed in the following order: UA, AMI, and BI. The best correlation was obtained by integration of large aggregate fraction as a function of shear stress. To differentiate plasmatic from cellular factors in RBC aggregation, we determined the aggregation in the presence and absence of plasma and formulated a “plasma factor” (PF) ranging from 0 to 1. In AMI the enhanced aggregation was entirely due to PF (PF = 1), whereas in UA and BI it was due to both plasmatic and cellular factors (0 ≤ PF ≤ 1). It is proposed that clinically relevant parameters of RBC aggregation should express both RBC aggregate size distribution and aggregate resistance to disaggregation and distinguish between plasmatic and cellular factors.

Acute-phase response; aggregate size distribution; shear stress

Red blood cells (RBC) in the presence of plasma proteins, most importantly fibrinogen, may aggregate to form rouleaux formations (35). The extent of RBC aggregation is determined by opposing forces: the repulsive force between the negatively charged cells, the cell-to-cell adhesion induced by plasma proteins, and the disaggregating shear force generated by blood flow (12, 15, 28, 36). RBC aggregation is thus dependent both on plasma (extrinsic) factors and on cellular (intrinsic) factors. Normally, the blood flow is sufficient for dispersion of RBC aggregates, which is essential for normal tissue perfusion. However, in low-flow states and other pathological conditions, increased RBC aggregation may contribute to circulatory disorders and, particularly in the microcirculation, to the occlusion of microvessels (12). It is assumed that this process is dependent on both the size of RBC aggregates and the cohesive forces within aggregates, expressed by the shear stress required to disperse them. RBC aggregation is increased in various conditions associated with an inflammatory response (40), including stable ischemic heart disease (6, 26, 31), acute myocardial infarction (11, 16, 24), and bacterial sepsis (2, 4).

Evaluation of RBC aggregation is currently available to the clinician practices only indirectly through the erythrocyte sedimentation rate (ESR) (21). ESR correlates poorly with RBC aggregation because of the confounding effects of hematocrit, plasma albumin level, temperature, and hemodilution by anticoagulant. Furthermore, ESR does not differentiate between the RBC tendency to aggregate because of cellular factors (aggregability) and the effect of plasma factors (fibrinogen and albumin levels) on actual aggregation (12, 13). RBC aggregation can be determined more accurately by commercially available systems, the MYRENNE rheometer (30) and the laser-assisted optical rotation cell analyzer (LORCA) (17), which employ light transmission through RBC suspension or light scattering to obtain indexes of RBC aggregation, expressed mainly as the average aggregate size at a certain shear stress. In addition, a cell flow properties analyzer (CFA) has been developed in the laboratory of Yedgar for monitoring RBC aggregation by direct visualization of the aggregation process under controllable shear stress in a narrow-gap (30 μm) flow chamber (8). The CFA analyzes the aggregate size distribution, namely the percentage of the RBC population in each aggregate size (expressed as the number of cells per aggregate), as a function of shear stress. This can provide various measures of RBC aggregation, such as the average, median, or any percentile of RBC population at any aggregate size; the shear stress required to disperse the aggregates; the aggregation kinetics; and the aggregate morphology (aggregate shape parameter) (10).

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These parameters and their combinations may provide a comprehensive characterization of RBC aggregation.

The present study was undertaken to identify and formulate parameters of RBC aggregation and aggregability that correlate with inflammatory indexes in cardiovascular and infectious conditions associated with an inflammatory response. For this purpose, we used the analysis of RBC dynamic organization provided by the CFA (8, 10).

**MATERIALS AND METHODS**

**Patients**

All patients and healthy (control) subjects gave their informed consent for participation, and the study protocol was reviewed and authorized by the institutional review board. Three groups of patients were studied.

Unstable angina. Unstable angina (UA) patients were those with ischemic heart disease and chest pain at rest who were hospitalized with or without acute changes in the electrocardiogram but had no evidence of myocardial infarction. UA patients suffered from chest pain of cardiac origin that was either a new onset (within the last 2 mo before admission), accelerated (increasing in severity, frequency, or duration), or pain at rest (7). We did not enroll patients with postmyocardial infarction angina. Electrocardiogram (ECG) changes suggestive of ischemia were not a requirement.

Acute myocardial infarction. Acute myocardial infarction (AMI) patients were those with an established AMI who were hospitalized in the intensive coronary care unit. To avoid the possible influence of thrombolytic therapy on the aggregation profile of the RBC (35), we did not enroll patients during the first 24 h of their stay in the intensive coronary care unit. The myocardial infarction (MI) patients met two out of three World Health Organization (WHO) criteria: ischemic-type chest discomfort, ECG changes (ST elevation in 2 contiguous leads or new left bundle branch block; LBBB), and elevation in serum levels of cardiac markers (CK MB or cardiac troponin) (39).

Acute bacterial infection. Acute bacterial infection (BI) patients had a fever >38.4°C and one of the following: 1) chest X-ray findings consistent with lobar pneumonia, 2) leukocyturia and positive urine cultures indicating urinary tract infection, 3) clinical findings consistent with soft tissue infection (cellulitis, abscess, and infected pressure sores), or 4) leukocytosis and positive blood cultures indicating septicaemia. We excluded from all groups patients receiving glucocorticoids. The BI patients met the ACCP/SCCM definition of systemic inflammatory response (two of the following four criteria): fever >38°C, tachypnea >20 breaths/min or pCO₂ <32, heart rate >90 beats/min, white count µL >12,000 or <4,000 or >10% bands (5). The BI group was not intended to be homogenous but rather to represent a group of hospitalized patients with large “burden of inflammation.” All patients were febrile and exhibited the typical tachycardia, tachypnea, and/or leukocytosis and thus met ACCP/SCCM criteria for the systemic inflammatory response syndrome (SIRS). Healthy hospital employees served as controls.

**Biochemical Assays**

Blood was drawn from all patients within 3 days of admission. As mentioned, patients with AMI did not have blood drawn during the first 24 h of hospital stay. C-reactive protein (CRP) concentration was determined by laser nephelometry and specific anti-human CRP antibodies (34). Plasma fibrinogen concentration was determined by the method of Claus (see Ref. 29).

**Preparation of RBC Suspension for Determination of RBC Aggregability**

Samples of venous blood were drawn from the antecubital vein and collected into EDTA containing Vacutainers. The RBC were isolated by centrifugation (2,000 rpm for 10 min), washed with PBS, pH 7.4, and resuspended to the desired hematocrit in either the autologous plasma or PBS supplemented with 1% bovine serum albumin (Sigma; St. Louis, MO) and 0.5% dextran-T500 (Pharmacia Biotech; Uppsala, Sweden) to induce aggregation.

**Determination of RBC Aggregation**

RBC aggregation was measured in either the autologous plasma or PBS (pH = 7.4) supplemented with 1% albumin, in which the aggregation was induced by the addition of dextran-T500 to a final concentration of 0.5%. Dextran has been used extensively for studying RBC aggregation (8, 10), and we have previously found that 0.5% dextran-500 induces the formation of RBC aggregates similar in size and shape to those formed in plasma (9). All aggregation measurements were conducted within 6 h after venipuncture.

The aggregability of the RBC was studied using the CFA previously described (8, 10). Briefly, RBC suspension in either autologous plasma or dextran solution is prepared at 6% hematocrit. The suspension is then introduced into the flow chamber connected to a pump exerting laminar flow and to a pressure transducer that enables the control of shear stress during the experiment. The RBC dynamic organization (aggregation/disaggregation) in the flow chamber is directly visualized and recorded through a microscope connected to a charge-coupled device (CCD) video camera that transmits the RBC images to a computer, providing parameters of RBC aggregation.

In evaluation of RBC aggregation, it is important to take into account both the aggregation extent (e.g., aggregate size, the fraction of aggregated RBC) and the strength of intercellular interaction as expressed by the aggregate resistance to disaggregation by flow. As noted above, the CFA provides the aggregate size distribution from which various parameters of the aggregation can be derived. In the present study, to identify the parameters that best correlate with the inflammatory indexes, we first examined the correlation of the inflammatory indexes with the commonly used measures of RBC aggregation, mainly the average aggregate size (AAS) and the disaggregating shear stress (8), and compared them with newly defined parameters formulated by manipulation of the measures provided by the CFA.

Overall, the following parameters were examined for their correlation with inflammatory indexes.

**AAS.** Because experimental models of RBC aggregation have found peak AAS to be achieved at a shear stress of 0.1–0.25 dyn/cm², we defined the AAS at a shear stress of 0.15 dyn/cm² as the peak aggregate size (25).

Small, medium, and large aggregate fraction. We measured the distribution of the RBC population into aggregate size ranges, i.e., the erythrocyte fraction (in %) in small, medium, or large aggregates (SAF, MAF, and LAF, respectively), referring to size ranges of 1–4 RBC/aggregate, 5–32 RBC/aggregate, and 33 or more RBC/aggregate. These ranges were chosen because aggregates of up to 4 RBC are usually in the form of linear rouleaux, aggregates of 5–32 RBC will include branched rouleaux, and larger aggregates will start forming rouleaux networks (3, 32).
These calculations ranged from 0.15 to 4.00 dyn/cm², at which shear stress was monitored at 0.15, 0.5, 1.0, 2.0, and 4.0 dyn/cm². SAF, small aggregate fraction, was singly dispersed by shear stress, and aggregation was monitored for AAS, SAF, MAF, SAF, small aggregate fraction, i.e., the distribution of the RBC population into aggregate size range, namely, the erythrocyte fraction (%) in small aggregates (from single RBC up to 4 RBC in aggregate).

Shear stress. The shear stress required to obtain 50% of RBC population in the small range of aggregate size is represented by \( \tau_{S50} \).

Area under the curve. The area under the curve (AUC) of the plot of an aggregation parameter as a function of shear stress is shown in Fig. 1. This was done for AAS, SAF, MAF, SAF, small aggregate fraction, and defined a PF as follows:

\[
PF = \frac{\Delta A_{g_{FP}} - \Delta A_{g_{Dex}}}{\Delta A_{g_{FP}}}
\]

where \( \Delta A_g = A_{g_{PA}} - A_{g_{C}} \), i.e., the difference in aggregation between the tested (“pathological”) RBC (Ag_{PA}) and that of the control (Ag_{C}) normal RBC; FP is full plasma; and Dex is dextran solution. Hence, \( \Delta A_{g_{Dex}} = |A_{g_{PA}} - A_{g_{C}}| \) in dextran solution, and \( \Delta A_{g_{FP}} = |A_{g_{PA}} - A_{g_{C}}| \) in the autologous patient plasma. Obviously, \( \Delta A_{g_{FP}} \geq \Delta A_{g_{Dex}} \), because the determination of aggregation in full plasma includes both cellular and plasmatic factors. Accordingly, \( 0 \leq PF \leq 1 \). When PF = 0, there is no plasma contribution to the aggregation, meaning that the altered aggregation is all due to changes in cellular factors. When PF = 1, the altered aggregation is all due to plasmatic factors.

Statistical Analysis

Differences between aggregation parameters in different patient groups were evaluated using the two-tailed Student's t-test. A P value of <0.05 was considered statistically significant. The correlation between the various variables was assessed by Pearson's two-tailed correlation coefficient. Calculations were performed using the SPSS software package.

RESULTS

Forty patients in four diagnostic categories were included: 18 patients in the control group (11 men and 7 women, mean age 32 ± 11 yr, range 18–49 yr), 11 patients with UA (8 men and 3 women, aged 61 ± 17 yr, range 42–84 yr), 9 patients with AMI (7 men and 2 women, aged 58 ± 13 yr, range 45–85 yr), and 11 patients with BI (8 men and 3 women, aged 76 ± 10 yr, range 49–85 yr). Of the patients with UA, nine were treated with full-dose heparin and aspirin. Of the patients with AMI, eight received full-dose heparin and aspirin, and seven patients were treated with thrombolysis.

Markers of the Acute-Phase Response

Significant differences were noted in the acute-phase reactants CRP and fibrinogen between the control and patient groups. As shown in Table 1, both were elevated in the patient groups, particularly in BI. However, the disease state is especially manifested in the level of CRP, which is at the background level in the control group (in the range of experimental error) but is elevated severalfold in the coronary syndromes (AMI and UA) and ~30-fold in BI. This makes the CRP level a prominent marker of the inflammatory state.

Indexes of RBC Aggregation

Table 2 depicts the RBC aggregation parameters, measured in the autologous plasma for the controls and the three patient groups. The difference in AAS, the common measure of RBC aggregation, was significant but not dramatic. In comparison, a very significant difference was observed in the value of \( \tau_{S50} \) between the control and the patient groups.

However, as previously discussed (9), when considering the possible contribution of RBC aggregation to the formation of thrombi, one should consider the possible contribution of RBC aggregation to the formation of thrombi.
The pathological RBC aggregation is further manifested by the integrative measures [integral of LAF as a function of shear stress (AUCL)] and complementarily integral of SAF as a function of shear stress (AUCS), particularly in BI, where AUCS is ~50-fold higher than in the control and ~5-fold higher than in the coronary syndromes.

**Correlation of RBC Aggregation Indexes with Markers of the Acute-Phase Response**

As noted in the introduction, our main interest in the present study was to examine the relation of RBC aggregation parameters to inflammatory states. For this purpose, we examined the correlation of the aggregation parameters (described in MATERIALS AND METHODS) with the levels of the acute-phase reactants described in Table 1, regardless of the corresponding disease states. As shown in Table 3, no significant correlation was observed between the acute-phase response (APR) and AAS, whereas excellent correlation was obtained with the aggregation parameters that expressed the distribution into aggregate size ranges. The correlation with the inflammatory markers was further improved when examined against the aggregation parameters that integrate the change in the size distribution with aggregation strength, such as AUCs, which depicts the integral of the dependence of the SAF on the shear stress. When considering the occlusive potential of RBC aggregation, the important fraction is obviously that of the large aggregates (LAF).

**Table 3. Correlation between RBC aggregation parameters and acute-phase reactants**

<table>
<thead>
<tr>
<th>Aggregation Parameter</th>
<th>CRP</th>
<th>Fibrinogen</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>AAS</td>
<td>0.14</td>
<td>NS</td>
</tr>
<tr>
<td>$\tau_{SS0}$</td>
<td>0.35</td>
<td>NS</td>
</tr>
<tr>
<td>SAF</td>
<td>$-0.41$</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MAF</td>
<td>$-0.61$</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>LAF</td>
<td>0.61</td>
<td>0.001</td>
</tr>
<tr>
<td>AUCs</td>
<td>$-0.70$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AUCt</td>
<td>0.87</td>
<td>&lt;0.001</td>
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</table>

NS, not significant. *Because in RBC of control healthy subjects the amount of large aggregates (as defined in the present study) is negligible (see Tables 2 and 4), the calculation of LAF and AUCt here includes only the values obtained with the patient (excluding control) RBC.
Experimental models and clinical studies demonstrate the potential of RBC aggregation to hinder blood flow through the microcirculation in sepsis and septic shock (2, 22). The formation of “sludge” blood under these conditions is assumed to cause tissue hypoxia and acidosis, and it interferes with the ability of normal host defenses and administered antibiotics to reach sequestered bacteria, thus facilitating bacterial multiplication (19). A pathogenic role for RBC aggregation has been postulated in acute coronary syndromes. These studies examined blood viscosity, not RBC aggregation. Furthermore, we mention the importance of RBC aggregation in determining blood viscosity. The size of myocardial infarction after coronary arterial ligations has been shown to increase with increasing blood viscosity (38). Patients with increased blood viscosity after myocardial infarction are generally more likely to develop complications such as cardiogenic shock and heart failure (24).

The assessment of the potential contribution of RBC aggregation to microcirculatory disorders depends to a great extent on the parameters used for its characterization, and might depend on the method used for this purpose. It is thus important to identify and formulate the clinically relevant parameters as performed in the present study. The pathogenic potential of RBC aggregation within the microcirculation is dependent on the size of aggregates formed and the cohesive forces within the aggregate, i.e., the resistance of aggregates to shear-induced disaggregation. To identify clinically relevant measures of RBC aggregation, we employed the monitoring of RBC dynamic organization provided by the CFA to derive diverse aggregation parameters and examined their correlation with the inflammatory markers. Such parameters are pertinent to the diagnosis of patients at risk because of tissue damage by increased RBC aggregability as well as for monitoring disease activity and response to treatment.

The data presented in this study show that the AAS at a certain shear stress, the commonly used parameter of RBC aggregation, is not satisfactory: AAS was increased in both ischemic heart disease and BI compared with healthy subjects, but it did not correlate with the level of CRP.

Similarly, $T_{SS0}$, the parameter expressing the resistance of the RBC aggregates to disaggregation by the flow-induced shear stress, was clearly elevated in pathological states and correlated well with the fibrinogen.

Table 4. CRP and corresponding $AUC_L$ values for individual participants

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UA</th>
<th>AMI</th>
<th>BI</th>
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<tr>
<td>CRP</td>
<td>$AUC_L$</td>
<td>CRP</td>
<td>$AUC_L$</td>
<td>CRP</td>
</tr>
<tr>
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<td>0.5</td>
<td>0.184</td>
<td>5.2</td>
</tr>
<tr>
<td>0.7</td>
<td>0.000</td>
<td>0.5</td>
<td>0.160</td>
<td>9.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.035</td>
<td>0.6</td>
<td>0.189</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>4.0</td>
<td>0.253</td>
<td>3.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>0.5</td>
<td>0.138</td>
<td>13.7</td>
</tr>
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<td>3.4</td>
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<td>0.289</td>
<td>2.7</td>
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<td>0.000</td>
<td>4.6</td>
<td>0.156</td>
<td>0.9</td>
</tr>
<tr>
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<td>0.132</td>
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</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>1.2</td>
<td>0.217</td>
<td>6.7</td>
</tr>
<tr>
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<td>0.000</td>
<td>2.7</td>
<td>0.204</td>
<td>8.5</td>
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<td>0.5</td>
<td>0.000</td>
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</tbody>
</table>

CRP and corresponding $AUC_L$ values for individual participants in control and 3 patient groups. For UA, $r = 0.60$ and $P < 0.05$. For AMI, $r = 0.80$ and $P < 0.01$. For BI, $r = 0.77$ and $P < 0.01$.

UA and BI, both cellular and plasmatic factors contribute to the elevated aggregation (PF < 1). This is despite the fact that in AMI, the increase in fibrinogen (considered the most potent aggregation inducer in plasma) is smaller than in BI. It thus seems that, contrary to expectations, the contribution of plasma to the elevated aggregation is not only due to the elevation of fibrinogen and that other PFs take part in this phenomenon.

**DISCUSSION**

In the present study, we conducted a comparative analysis of various parameters expressing the aggregability and aggregation of RBC in three patient populations and healthy subjects, and examined their correlation with markers of inflammation and APR. An inflammatory response, evidenced by increased levels of CRP, has previously been shown in patients with acute coronary syndromes, and elevated levels of CRP correlated with a graver prognosis (27). The significant effect of BI and sepsis on the inflammatory response has been previously documented (18, 23, 33). The laboratory indexes of the APR in the patient groups studied here indicate the existence of an inflammatory gradient, with values greater than normal in patients with acute coronary disease and further enhanced in BI.

RBC aggregation, as noted above, is a major determinant of blood viscosity, particularly under conditions of low shear stress, and therefore affects flow dynamics mainly within the microcirculation, where such conditions prevail (37). The vessels most likely to be affected by RBC aggregation are the postcapillary venules, where vessel caliber is large enough and shear stress low enough to allow aggregation (whereas flow in capillaries is more dependent on RBC deformability).
oxygen level but not with CRP. This might suggest that CRP, the major index of the inflammatory state, is irrelevant to RBC aggregation, but the contrary is found with the more comprehensive parameters formulated in this study. As shown in Table 3, SAF and LAF, the parameters that expressed the aggregate size distribution of the RBC population, exhibited a clear correlation with both fibrinogen and CRP levels.

Moreover, the correlation between the inflammatory indexes and RBC aggregation was further improved and practically reached the same level of significance for CRP and for fibrinogen when both the aggregation strength and the aggregate size distribution were considered. This is clearly demonstrated by the correlation coefficients and the significance values obtained between the integrative parameters AUCL and AUCS (Table 3), which express the change in the percentage of the RBC population at the indicated size range as a function of shear stress.

Of special interest is the finding that the RBC aggregation parameters formulated in this study correlated with the inflammatory indexes CRP and fibrinogen, regardless of the specific pathological conditions and despite the variability of symptoms among the patients. Although this does not necessarily imply a cause-and-effect relationship, it further strengthens the correlation between the RBC aggregability and inflammatory parameters.

These findings strongly suggest that clinically relevant parameter(s) of RBC aggregation should express both the distribution of the RBC population into aggregate size ranges and the resistance to shear-induced disaggregation. It should be noted that, intuitively, these two properties are expected to be changed in the same direction. However, in previous studies with RBC of thalassemic patients, we found that it is not necessarily so: RBC of both thalassemia major (TM) and intermedia (TI) patients exhibited enhanced aggregation, but whereas the shear stress required to disperse TM-RBC was markedly higher than normal, TI-RBC were dispersed at the normal shear stress (9, 20). Therefore, projection from the change in one parameter onto the other is not valid, and it is important to examine them independently.

As discussed in the introduction, the actual RBC aggregation in the blood is determined by both cellular and plasmatic factors. The distinction between these factors, i.e., determining RBC aggregability (in standard solution) in addition to the actual aggregation (in plasma), provides information as to the source of the altered aggregation in the pathological states and thus may indicate the kind of desired treatment. The PFs presented in Table 5 show that in BI and UA, both cellular and plasmatic factors contribute to the enhanced aggregation (PF < 1), whereas in AMI, the enhanced aggregation is predominantly due to plasmatic factors (PF = 1). This criteria can assist in defining therapeutic strategies. It is noteworthy that UA and BI are known to be associated with oxidative stress, which is well known to alter RBC membrane (1, 14), and antioxidants have been proposed for the amelioration of RBC rheological properties aggregation.

Increased RBC aggregation has been previously observed in inflammatory states (40), including both BI (4) and acute coronary syndromes (11, 16), and has been attributed to the rise of plasma fibrinogen, which is considered the most potent inducer of RBC aggregation. However, the present study seems to disagree with this notion. The CRP level in BI was markedly higher than in the other diseases (Table 1), concomitant with RBC aggregation (Table 2), whereas the fibrinogen level in BI exceeded that in the other diseases to a much lesser extent (Table 1). This suggests that in addition to fibrinogen, another plasmatic factor(s), possibly CRP, might make a considerable contribution to the enhanced RBC aggregation in inflammatory states.

In conclusion, we propose that clinically relevant parameters of RBC aggregation should (1) provide measures of both the aggregation extent, as expressed by the aggregate size distribution, and the strength of the intercellular interaction, expressed by the aggregate resistance to disaggregation by the flow-induced shear stress; and (2) differentiate between the contribution of plasmatic and cellular factors responsible for the altered aggregation by comparing the actual aggregation (in the autologous plasma) and the aggregability of the isolated RBC (in standard solution) to characterize the pathological alteration and indicate therapeutic objectives.

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