Increased Strength of Erythrocyte Aggregates in Blood of Patients with Inflammatory Bowel Disease

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**Background:** Increased strength of red blood cell (RBC) aggregates are present during the acute inflammatory response and contribute to erythrocyte aggregation and may lead to microvascular dysfunction. Inflammatory bowel diseases (IBDs) are characterized by damage to the bowel wall. This damage may be at least partially attributed to microvascular ischemia caused by enhanced erythrocyte aggregation. The aim of this study was to evaluate the strength of RBC aggregates in the blood of patients with IBD.  

**Methods:** The strengths of RBC aggregates were characterized by integrative RBC aggregation parameters, determined by measuring of RBC aggregation as a function of shear stress. The results are represented as the area under the curve (AUC) of aggregate size plotted against shear stress. For each patient, dynamic aggregation and disaggregation of RBC were recorded and analyzed according to the RBC aggregate size distribution at the different shear stresses. Aggregation indices were correlated with disease activity and inflammatory biomarkers.  

**Results:** We examined 53 IBD patients and 63 controls. IBD patients had significantly elevated concentrations of inflammation-sensitive proteins and aggregation parameters. The strength of large aggregates, represented by AUC for large fraction aggregates, among patients (15.2 ± 18.6) was double that of controls (7 ± 10.9) (P = 0.006). The strength of large aggregates correlated with disease activity (r = 0.340; P < 0.001) with concentration of fibrinogen (r = 0.575; P < 0.001) and with concentration of high sensitivity C-reactive protein (r = 0.386; P < 0.001).  

**Conclusions:** The strength of RBC aggregates is increased in patients with IBD and correlates with the intensity of the acute phase response. This could contribute to bowel damage in these diseases. (Inflamm Bowel Dis 2009;00:000–000)  

**Key Words:** erythrocyte aggregation, acute phase response

There are multiple lines of evidence to suggest a role for erythrocyte aggregation (EA) in the etiopathogenesis of microcirculatory slow flow,1–9 tissue hypoxia,10,11 endothelial dysfunction,12 and reduced capillary density.13 A detrimental microcirculatory rheological milieu might exist in patients suffering from inflammatory bowel disease (IBD) and a potential role of a compromised microcirculatory function has been suggested in the past in both patients and animal models of IBD.14–16 This may be responsible at least partially for tissue damage and necrosis present in the inflamed bowel wall.  

Several studies in the past have suggested the presence of increased EA in the peripheral blood of IBD patients.17,18 We further explored this phenomenon by using a system that enables us to measure the strength of red blood cell (RBC) aggregates. In this research, we examined the role of cellular and plasmatic factors in aggregation of RBC from IBD patients. A potential therapeutic intervention to reduce the aggregation might therefore be of interest.19–24

**MATERIALS AND METHODS**

**Study Design and Patient Selection**

We included IBD patients who were in routine follow-up at the IBD Center, Department of Gastroenterology and Liver Diseases, Tel Aviv Sourasky Medical Center (Tel Aviv, Israel). Excluded were individuals who had a recent (≤3 months) episode of infection and/or those with a history of malignancy. Disease activity of IBD was determined by using the Crohn’s Disease Activity Index (CDAI) scoring system for CD25 and that of the Mayo Clinic for patients with ulcerative colitis (UC).26 The control group included healthy volunteers, members of the medical staff, who had no underlying process of infection/inflammation, did not take any steroid or nonsteroidal antiinflammatory medication, and did not have any overt malignancy.

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Analytical Procedures

Inflammation-sensitive Biomarkers and Biochemical Analysis

Venous blood was drawn from the antecubital vein following an overnight fast. White blood cell count (WBCC) and differential were performed using the Coulter STKS (Beckman Coulter, Nyon, Switzerland) electronic analyzer, erythrocyte sedimentation rate (ESR) by the method of Westergren\textsuperscript{27} and the concentration of fibrinogen was determined using the method of Clauss\textsuperscript{28} on a Sysmex 6000 (Sysmex, Hyaga, Japan) analyzer. High-sensitivity C-reactive protein (hs-CRP) was determined using a Behring BN II Nephelometer (DADE Behring, Marburg, Germany) according to the method of Rifai et al.\textsuperscript{29}

RBC Aggregation

1. Preparation of RBC suspension for determination of RBC aggregability. Samples of venous blood were collected into EDTA-containing vacutainers. RBC were isolated by centrifugation (2000 rpm for 10 min) and washed with phosphate-buffered saline (PBS), pH 7.4.

2. Determination of RBC aggregation. All aggregation measurements were conducted within 2 hours of venipuncture. RBC aggregation was studied using a computerized cell flow-properties analyzer (CFA) previously described.\textsuperscript{30} Briefly, RBC suspension in either autologous plasma or dextran solution was prepared at 6% hematocrit. The suspension was then introduced into a narrow-gap (40 \(\mu\)m) flow-chamber, which was connected to a pump exerting laminar flow and a pressure transducer that monitors shear stress during the experiment. The RBC dynamic organization (aggregation/disaggregation) in the flow-chamber was directly visualized and recorded through a microscope connected to a charge-coupled device video camera, which transmits the RBC images to a computer. Images were then analyzed by image analysis software to provide parameters of RBC aggregation. We used the area under the curve of average aggregate size (AUC\textsubscript{AAS}) plotted against the exerted shear stress to determine RBC aggregation. AUC expresses both RBC aggregate size and the shear stress required for aggregate dispersion. We previously found this index to faithfully represent clinically relevant aggregation in various disease states.\textsuperscript{30,31} Additionally, the number of RBCs in large (more than 33 RBC per aggregate) aggregates was plotted against shear stress. The strength of large aggregates was respectively expressed by AUC determined for large aggregates fraction (AUC\textsubscript{LAF}).

3. Determination of plasma contribution to RBC aggregation. In order to differentiate the effects of IBD on RBC aggregation mediated through alteration in plasma properties from the intrinsic properties of RBC, it is necessary to compare RBC aggregation in plasma to aggregation in a plasma-free standardized medium. Dextrans have been used extensively for studying RBC aggregation.\textsuperscript{32,33} We have previously reported that 0.5% of dextran-500 induces the formation of RBC aggregates similar in size and shape to those formed in plasma.\textsuperscript{34} We therefore used dextran-500 as a standard aggregating solution, which eliminates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD</th>
<th>UC</th>
<th>Controls</th>
<th>(P) Value</th>
<th>Significance Between</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>34</td>
<td>18</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13 (38%)</td>
<td>10 (56%)</td>
<td>41 (65%)</td>
<td>0.011</td>
<td>CD - controls</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.9 ± 12</td>
<td>46.7 ± 12.1</td>
<td>41.8 ± 14</td>
<td>0.012</td>
<td>CD - UC</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>23.3 ± 4</td>
<td>23.2 ± 2.9</td>
<td>23.7 ± 3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Current smokers</td>
<td>3 (9%)</td>
<td>1 (6%)</td>
<td>16 (34%)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>186 ± 44</td>
<td>219 ± 40</td>
<td>206 ± 46</td>
<td>0.033</td>
<td>CD - UC</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>100 ± 34</td>
<td>132 ± 32</td>
<td>121 ± 38</td>
<td>0.005</td>
<td>CD - UC</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>56 ± 17</td>
<td>60 ± 18</td>
<td>62 ± 16</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>148 ± 88</td>
<td>135 ± 81</td>
<td>113 ± 69</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; CD, Crohn’s disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; UC, ulcerative colitis.

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plasma factors but not RBC ones.\textsuperscript{35} If RBC aggregation is reduced when examined in autologous plasma but unchanged when examined in dextran-500, the reduction in aggregation can be attributed to altered plasma composition, whereas RBC aggregation that is reduced to the same extent in both plasma and dextran-500 can be attributed to altered RBC membrane properties. In order to quantify the contribution of plasma factors to the actual aggregation, we defined a plasma factor (PF) as follows:

$$PF = (ΔAg_{FP} - ΔAg_{Dex})/ΔAg_{FP}$$

where $ΔAg = Ag_{pa} - Ag_{C}$, ie, the difference in aggregation between the tested ("pathological") RBC ($Ag_{pa}$) to that of the control (C), normal RBC. Hence, $ΔAg_{Dex} = (Ag_{pa} - Ag_{C})$ in dextran solution, and $ΔAg_{FP} = (Ag_{pa} - Ag_{C})$ in the autologous patient plasma (FP = full plasma). Obviously, $ΔAg_{FP} ≥ ΔAg_{Dex}$, since the determination of aggregation in full plasma includes both cellular and plasma factors. Accordingly, $0 ≤ PF ≤ 1$. 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.

**Ethical Considerations**

The protocol was approved by the local ethics committee. Written informed consent was obtained from patients and control participants before inclusion.

**Statistics**

All continuous variables were summarized and displayed as mean ± standard deviation (SD) for each patient group, and all the categorical variables were summarized and displayed as number and percentage of participants in each group. The hs-CRP has nonnormal distribution; thus, we used a logarithmic transformation, and all the results expressed as hs-CRP are back-transformed geometric means and SD. For all continuous variables, an independent-samples $t$-test was performed to compare the various parameters between IBD and control groups. For all categorical variables, the chi-square test was used to determine the difference between groups.

**Sample Size Calculation**

In accordance with previous articles,\textsuperscript{36} in order to find a difference of 30\% between AUC\textsubscript{AAS} in controls compared to patients, with a type I error of 0.05 and a power of 0.9, the size of the population should have been at least 28 participants in each group.

In order to detect possible confounders we used a linear regression model that included the following variables: gender, age, smoking status, body mass index (BMI), use of medications (steroids, 5-ASA, cytotoxic medications), triglycerides level, and cholesterol level. None of these variables were statistically significant. Despite that fact, we used Pearson correlation for confounding variables in order to adjust for possible confounders, such as age, BMI, cholesterol level, and triglycerides level.

Finally, in order to assess the correlation of RBC aggregation with disease activity, patients and controls were classified into 5 categories, according to disease activity level,\textsuperscript{26,37} as presented in Table 1.

**TABLE 3. Medications in the Study Group**

<table>
<thead>
<tr>
<th>Medication</th>
<th>CD</th>
<th>UC</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-ASA</td>
<td>24</td>
<td>11</td>
<td>—</td>
</tr>
<tr>
<td>Steroids</td>
<td>6</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Cytotoxic drugs\textsuperscript{a}</td>
<td>11</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>Untreated patients</td>
<td>3</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>Statins</td>
<td>—</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Antihypertensive drugs\textsuperscript{b}</td>
<td>1</td>
<td>3</td>
<td>—</td>
</tr>
</tbody>
</table>

\textsuperscript{a}6-MP, azathioprine, methotrexate.

\textsuperscript{b}ACE inhibitors, beta blockers, calcium channel blockers, diuretics.

**TABLE 4. Inflammation-sensitive Biomarkers Among IBD Patients and Healthy Controls**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients</th>
<th>Controls</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCC (x10\textsuperscript{3}/μL)</td>
<td>7222 ± 2460</td>
<td>6470 ± 1460</td>
<td>0.057</td>
</tr>
<tr>
<td>Neutrophils (x10\textsuperscript{3}/μL)</td>
<td>4559 ± 1841</td>
<td>3727 ± 1104</td>
<td>0.005</td>
</tr>
<tr>
<td>Platelets (x10\textsuperscript{3}/μL)</td>
<td>301450 ± 86000</td>
<td>248770 ± 51200</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>23.4 ± 17.5</td>
<td>11.7 ± 8.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>324 ± 83</td>
<td>267 ± 48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>4.4 ± 5.3</td>
<td>1.34 ± 2.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

WBCC, white blood cell count; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein.

**TABLE 5. Inflammation-sensitive Markers in CD and UC Patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD N=34</th>
<th>UC N=18</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCC (x10\textsuperscript{3}/μL)</td>
<td>7182 ± 2200</td>
<td>7077 ± 2850</td>
<td>NS</td>
</tr>
<tr>
<td>Neutrophils (x10\textsuperscript{3}/μL)</td>
<td>4683 ± 1783</td>
<td>4324 ± 1979</td>
<td>NS</td>
</tr>
<tr>
<td>Platelets (x10\textsuperscript{3}/μL)</td>
<td>307880 ± 84000</td>
<td>292720 ± 92000</td>
<td>NS</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>25.1 ± 19.1</td>
<td>21.2 ± 14.4</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>327 ± 89</td>
<td>323 ± 71</td>
<td>NS</td>
</tr>
<tr>
<td>Hs-CRP (mg/l)</td>
<td>5.4 ± 5.8</td>
<td>3.1 ± 4.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

WBCC, white blood cell count; ESR, erythrocyte sedimentation rate; hs-CRP, high-sensitivity C-reactive protein; NS, not significant.
The SPSS statistical package was used to perform all statistical evaluation (SPSS, Chicago, IL).

RESULTS

A total of 53 IBD patients (34 CD, 18 UC, and 1 indeterminate colitis) as well as 63 healthy individuals who served as controls participated in this work. Details regarding age, gender, as well as BMI and smoking habits in IBD patients and in the controls are reported in Table 2 and medication intake is reported in Table 3. Of note, there was no difference between patients and controls regarding age, BMI, or cholesterol profile, while a slight increment in tri-
glyceride concentration was noted in the patients as opposed to the controls (Table 2).

The values of inflammation-sensitive biomarkers of both patients and controls are reported in Table 4. As expected, a heightened inflammatory response is seen in the patient group. No significant difference was noted between CD and UC patients (Table 5).

When RBC aggregation was determined in plasma, a clear increment was noted in patients as opposed to controls (Fig. 1, 2). Interestingly, this increment is mainly due to large aggregates (Table 6). There was no statistically significant difference between aggregation indices of CD and UC patients (data not shown), although in CD aggregation was slightly stronger.

The aggregation increment was especially pronounced in patients with enhanced disease activity. In fact, we found a significant correlation between disease activity and aggregation indices: \( r = 0.388 \) \((P < 0.001)\) for the \( \text{AUC}_{\text{AAS}} \) and \( r = 0.34 \) \((P = 0.001)\) for the \( \text{AUC}_{\text{LAF}} \) (Table 7). Importantly, aggregation indices were controlled for cholesterol and triglycerides levels.

Finally, we tested the aggregation in dextran solution in the absence of plasma and found no difference between patients and controls (Fig. 1; Table 8).

In order to quantify the contribution of plasma factors to the actual aggregation we calculated the plasma factor (PF), as described in Materials and Methods. The value of PF calculated for \( \text{AUC}_{\text{AAS}} \) was 0.85, and of \( \text{AUC}_{\text{LAF}} \) was 0.9, suggesting that RBC aggregation of IBD patients is related to plasma factors and not to the intrinsic properties of the cell membranes.

**DISCUSSION**

This is the first study to document disease activity-related elevation of RBC aggregates strength of IBD patients (Table 6). This phenomenon is plasma-dependent and correlates significantly with the intensity of the acute phase response as evaluated by different inflammation-sensitive biomarkers.

IBD exacerbation is associated with an acute phase

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### TABLE 7. Correlations Between RBC Aggregation, Disease Activity and Inflammation-sensitive Biomarkers Among the Whole Cohort in Plasma, Adjusted to Cholesterol and Triglycerides Levels

<table>
<thead>
<tr>
<th></th>
<th>( \text{AUC}_{\text{AAS}} )</th>
<th>( \text{AUC}_{\text{LAF}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR</td>
<td>0.497 (&lt;0.001)</td>
<td>0.468 (&lt;0.001)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.500 (&lt;0.001)</td>
<td>0.575 (&lt;0.001)</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>0.371 (&lt;0.001)</td>
<td>0.386 (&lt;0.001)</td>
</tr>
<tr>
<td>Disease activity</td>
<td>0.388 (&lt;0.001)</td>
<td>0.340 (0.001)</td>
</tr>
</tbody>
</table>

The value in the table is the correlation coefficient and in parentheses is the statistical significance of the correlation.

### TABLE 8. Strength of RBC Aggregates in IBD Patients and Controls Analyzed in Dextran Solution

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{AUC}_{\text{AAS}} )</td>
<td>15.1 ± 4.7</td>
<td>15.8 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>( \text{AUC}_{\text{LAF}} )</td>
<td>2.3 ± 4.8</td>
<td>3.1 ± 6.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

\( \text{AUC}_{\text{AAS}}, \) area under the curve of average aggregate size; \( \text{AUC}_{\text{LAF}}, \) area under the curve determination of large fraction aggregates; NS, not significant.
response and hyperfibrinogenemia. Fibrinogen is a major determinant of EA \(^{38}\) and is associated with hyperviscosity as well.\(^{39}–^{41}\) A vicious cycle in which hyperviscosity and EA augment each other may be detrimental in terms of microcirculatory flow, especially in the presence of low perfusion pressures.\(^{13}\) The resulting tissue ischemia could perpetrate the damage that has been created in the tissues by the inflammation per se. Although we could not show a cause and effect relation between the EA intensity and that IB activity, the significant correlation that was demonstrated between them does support such a possibility. A confounding factor could be the difference between cholesterol levels among patients and controls in our cohort. Although of minor effect,\(^{42}\) both cholesterol and triglycerides\(^{43}\) could have an effect on RBC aggregation. In order to exclude such a possibility, the presented correlates (Table 7) were controlled for cholesterol and triglycerides levels.

In a previous study we documented the existence of increased EA in the peripheral venous blood of IBD patients.\(^{17}\) We further explored this phenomenon and showed that it is plasma-dependent. In fact, in the absence of plasma, no correlation was noted between the aggregability of the cells and disease activity. In the experiments without plasma, we used dextran, a known aggregating agent for RBCs.\(^{44}\) However, despite these known aggregating properties, we found no difference between patients and controls, suggesting that the cellular properties that are essential for aggregation are not altered in IBD patients.

The present documentation of plasma dependency regarding EA suggests that this is the main mechanism of elevation of aggregates strength in patients with IBD. Multiple acute phase proteins have been suggested as candidates that increase these forces and include fibrinogen, haptoglobin, ceruloplasmin, and immunoglobulins.\(^{34,37,45–49}\) However, fibrinogen is probably responsible for most of these interactions.\(^{39}\) This information is relevant for studies that are aimed to reduce viscosity and improve the patient’s rheological profile.\(^{24}\)

Despite demonstrating these phenomena in the peripheral blood, we assume that similar changes in rheological properties exist in the intestinal microvasculature. Thus, significant ischemia and tissue damage may occur and contribute to augmentation of the inflammatory response and to the microvascular dysfunction of the gut wall in IBD patients. Finally, it should be noted that some of the patients were on antiinflammatory treatment during the research. This treatment had no effect on the intrinsic aggregability properties of the cells, as shown in the studies that were performed in the presence of dextran. A theoretical possibility exists that they have affected plasma-dependent aggregation but this could not be clarified in the present study and remains a certain limitation of our present work. Yet the significant correlation that was found between the aggregation and disease activity does suggest that the use of antiinflammatory therapy did not abolish our main finding, namely, the plasma-dependent increased inter-erythrocytic forces.

Our findings contribute to the understanding of the microvascular dysfunction in IBD patients. Together with endothelial dysfunction it provides a better explanation to microcirculatory disturbances that may eventually lead to hypoxia, gut wall damage, and compensatory angiogenesis.\(^{50,51}\)

We conclude that increased plasma-dependent elevation of RBC aggregates strength exists in patients with active IBD. The strength of aggregates correlates with the concentrations of several inflammation-sensitive biomarkers, suggesting a cause-and-effect relationship between the intensity of the inflammatory response and the aggregation of cells. Due to the relevance to microcirculatory flow and dysfunction, these observations might pave the way for potential therapeutic implications in patients with IBD.

ACKNOWLEDGMENT

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REFERENCES


