A synergistic effect of albumin and fibrinogen on immunoglobulin-induced red blood cell aggregation

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Immunoglobulin (Ig) preparations are routinely administered to patients suffering from a variety of autoimmune disorders, immune deficiencies, and Guillain-Barre syndrome. Clinical data implicate therapeutically administered intravenous Ig in the precipitation of life-threatening thrombotic events (7, 9, 10, 13, 21, 25, 28, 29, 31, 35). Ig preparations have been shown to induce red blood cell (RBC) aggregation, which may contribute to the increased blood viscosity associated with their use (11, 23, 37). RBC aggregation affects blood viscosity most markedly in areas of low shear stress (<4 dyn/cm²), such as arterial bifurcations. The same sites are also prone to atherosclerosis and thrombosis (18). Increased RBC aggregation and the resulting elevated blood viscosity promote low-flow downstream from atherosclerotic lesions, a condition that favors thrombogenesis (12). Regional low flow resulting from RBC aggregation can therefore set the stage for vascular thrombosis, especially in persons with underlying vascular atherosclerotic disease. Elucidating the mechanism for Ig-induced RBC aggregation could help identify those patients at risk for thrombotic complications.

Aggregation of RBCs is governed by opposing forces: on the one hand, the repulsive electrical force between negatively charged cells and the shear force exerted by blood flow, which together disaggregate RBCs; and, on the other hand, the cohesive force induced by the presence of various plasma proteins that promote the formation of rouleaux structures and larger aggregates (8, 20, 26, 30). The equilibrium between these forces determines the extent of RBC aggregation, which in turn is the major determinant of blood viscosity at low shear rate (2) and thus a key element in hemorheology.

The relative roles of plasma proteins in RBC aggregation are not clear, as disparate findings have been reported. It is believed that fibrinogen, a 340-kDa fibrous hexamer, is the most potent aggregator of RBCs in plasma (26). Some studies (11, 37) have shown that Ig induces RBC aggregation, whereas others have found no effect (16). Data on albumin are even more conflicting. While it is clear that albumin does not directly aggregate RBCs, inconsistent results have been obtained regarding its effect when combined with other plasma proteins. Two studies (14, 38) found an inverse relationship between plasma albumin and RBC aggregation in diabetics. Some investigations described enhancement of fibrinogen-induced RBC aggregation by albumin (17, 22), but one study (14) showed inhibition. Ig-induced RBC aggregation has been inhibited by albumin in one study (17) but enhanced by albumin in another (34). Reinhart and Nagy (24) found that albumin increased the erythrocyte sedimentation rate (2) and thus a key element in hemorheology.

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rate when added to fibrinogen and Ig but inhibited it when added to either alone. However, the sedimentation rate is an imperfect correlate of RBC aggregation; because it is affected by the shape of RBC aggregates, RBC aggregates are not visualized directly, and aggregation is measured under static, no-flow conditions (36).

The present study was undertaken to examine the combined effects of albumin and fibrinogen on Ig-induced RBC aggregation. First, we examined the effect of Ig administered in vivo on RBC aggregation, as a function of plasma albumin and fibrinogen concentrations. The combined effects of albumin and fibrinogen on Ig-induced RBC aggregation were further investigated in vitro, using either human plasma or a suspension containing controlled concentrations of Ig, fibrinogen, and albumin as a medium for RBC aggregation.

MATERIALS AND METHODS

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 at the Sourasky Tel Aviv Medical Center.

Aggregation of RBCs from patients receiving intravenous Ig therapy. To study the effect of intravenous Ig administered in vivo, we recruited patients scheduled for Ig treatment from the Tel Aviv Medical Center hematology and rheumatology clinics. All patients filled out a clinical questionnaire. Venous blood was drawn for determination of RBC aggregation immediately before the infusion of Ig (Omr-IgG-am, Omrix Biopharmaceuticals; Rehovot, Israel) was started and again at its termination. Plasma fibrinogen concentration was determined by the method of Claus (5) using a thrombin reagent (Dade Behring; Newark, DE) according to the manufacturer’s instructions. Plasma albumin was measured by the Bayer Advia 1650 system (Bayer Diagnostics; Tarrytown, NY). Plasma Ig was measured by nephelometry using the BN II system (Dade Behring).

Preparation of RBC suspension for determination of RBC aggregability. Samples of venous blood were drawn from the antecubital vein and collected into EDTA-containing Vacucontainers. The RBCs were isolated by centrifugation (2,000 rpm for 10 min), washed with PBS (pH 7.4), and resuspended at the desired hematocrit in either autologous plasma or PBS supplemented with predetermined concentrations of human fibrinogen (F4883, Sigma; St. Louis, MO), human serum albumin (A3782, Sigma), and Ig, as described below.

Determination of RBC aggregation. All aggregation measurements were conducted immediately after venipuncture. RBC aggregability was studied using a cell-flow properties analyzer as previously described (3). Briefly, RBC suspension was prepared at 5% hematocrit. The suspension was then introduced into a cell-flow properties analyzer, consisting of a narrow-gap (30 μm) flow chamber connected to a pump exerting laminar flow and a pressure transducer that monitored 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 videocamera, which transmitted the RBC images to a computer. Images were then analyzed by image analysis software (a modified version of SigmaScan Pro [SPSS; Chicago, IL]), which computes the average aggregate size (number of RBCs/aggregate) by dividing the total aggregate volume by the volume of a single RBC.

RBC aggregation was monitored in the flow chamber under increasing shear stress and characterized by the area under the curve (AUC) of average aggregate size plotted as a function of the shear stress exerted (AUC_AAS). The wall shear stress taken for this calculation ranged from 0.15 to 4.00 dyn/cm², at which normal RBCs are singly dispersed. The AUC expresses both the extent of RBC aggregation and its dependence on shear stress, thus reflecting the strength of the intercellular interactions. We (1) previously found this index to faithfully represent clinically relevant aggregation in various disease states. The change in AUC after Ig infusion was expressed as the relative increment in RBC aggregation, calculated as follows

\[ 100 \times \frac{(AUC_{after\ Ig}) - (AUC_{before\ Ig})}{(AUC_{before\ Ig})} \]

RBC aggregation after the addition of Ig to plasma in vitro. To study the effect of Ig on RBC aggregation over a wide range of plasma fibrinogen concentrations, we drew blood samples from healthy volunteers and hospitalized individuals with acute coronary syndromes. The latter group is known to have elevated levels of plasma fibrinogen (1).

RBC aggregation was examined in autologous plasma at baseline. Aggregation studies were then repeated after adding Ig in vitro to achieve a final concentration of 25 mg/ml, approximately the concentration resulting in vivo from the administration of Ig at a dose of 0.5 g/kg body wt.

RBC aggregation under controlled concentrations of fibrinogen, Ig, and albumin. To define more precisely the contribution of the major plasma proteins to RBC aggregation, we studied the aggregation of RBCs from healthy volunteers under a range of controlled concentrations of fibrinogen, Ig, and albumin. To establish the dose response, RBC aggregation was examined at concentrations of fibrinogen ranging from 0 to 1,000 mg/dl (increments of 200 mg/dl) in a suspension containing 0 or 25 mg/ml Ig and 0 or 4.5 g/dl albumin and at concentrations of albumin ranging from 0 to 5.5 g/dl (increments of 1 g/dl) in a suspension containing 0 or 25 mg/ml Ig and 250 or 500 mg/dl fibrinogen.

Statistical analysis. Differences in RBC aggregation (AUC_AAS) and plasma protein concentrations before and after Ig administration were calculated with Student’s t-test. Correlations between continuous variables were analyzed with Pearson’s bivariate correlation. All values are expressed as means ± SE. All statistical tests were two-sided. P values were considered significant when <0.05. Statistical calculations were performed with the SPSS software package.

RESULTS

Effect of Ig administered in vivo on RBC aggregation. Thirteen patients (10 men and 3 women) received intravenous Ig treatment for a total of 18 sessions (5 patients were sampled twice). The indication for Ig treatment was chronic lymphocytic leukemia in four patients, non-Hodgkin’s lymphoma in one patient, Hodgkin’s disease in one patient, multiple myeloma in four patients, autoimmune vasculitis in two patients, and common variable immune deficiency in one patient. Ig was given at a mean dose of 0.4 g/kg (range 0.3–0.6 g/kg). Results were analyzed per treatment session rather than per patient.

Baseline values of plasma proteins are shown in Table 1. After Ig treatment, plasma globulin increased from 3.5 ± 0.5 to 4.2 ± 0.5 g/dl (P < 0.001), plasma fibrinogen decreased from 325 ± 26 to 290 ± 19 mg/dl.
Effect of Ig on RBC aggregation in vitro. Plasma was collected from 15 patients hospitalized with an acute coronary syndrome and 4 healthy individuals. Concentrations of plasma proteins are shown in Table 1. Baseline RBC aggregation in human plasma correlated positively with plasma globulin (r = 0.58, P = 0.009) and plasma fibrinogen (r = 0.47, P = 0.04) and negatively with plasma albumin concentrations (r = -0.54, P = 0.01). As with patients receiving Ig in vivo, addition of Ig to human plasma in vitro was not associated with an overall change in mean AUC\textsubscript{AAS}. However, the effect of Ig on RBC aggregation was dependent on the concentrations of fibrinogen and albumin in plasma; the relative increment in RBC aggregation correlated with plasma fibrinogen levels (r = 0.64, P = 0.003). A marked increase in RBC aggregation was noted in plasma samples with fibrinogen concentrations of above 450 mg/dl (Fig. 2). The plasma sample with the highest fibrinogen concentration (600 mg/dl) exhibited a 29-fold increase in AUC\textsubscript{AAS} after the addition of Ig.

Furthermore, the effect of Ig on RBC aggregation in the presence of a high fibrinogen concentration was strongly dependent on the albumin concentration. When plasma fibrinogen was <450 mg/dl, there was an inverse correlation between albumin levels and AUC\textsubscript{AAS} (r = -0.69, P = 0.004; Fig. 3A). However, in the four plasma samples in which the fibrinogen concentration exceeded 450 mg/dl, AUC\textsubscript{AAS} correlated linearly with the plasma albumin concentration (r = 0.99, P = 0.006; Fig. 3B).

RBC aggregation under controlled concentrations of fibrinogen, Ig, and albumin. The response of RBC aggregation to increasing concentrations of albumin (0 to

Table 1. Baseline concentrations of plasma proteins in the various patient groups studied

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Fibrinogen, mg/dl</th>
<th>Ig, g/dl</th>
<th>Albumin, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients receiving intravenous Ig</td>
<td>13</td>
<td>325.8 ± 26.5</td>
<td>3.5 ± 0.5</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Patients with ischemic heart disease</td>
<td>15</td>
<td>380.6 ± 31</td>
<td>2.7 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Control subjects</td>
<td>4</td>
<td>268.9 ± 28.4‡</td>
<td>2.7 ± 0.1</td>
<td>5.2 ± 0.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. *Value smaller than in patients with ischemic heart disease (P = 0.02); †value greater than in patients with ischemic heart disease (P = 0.001) and in patients receiving intravenous Ig (P < 0.001).

(P < 0.01), and plasma albumin decreased from 3.8 ± 0.1 to 3.4 ± 0.1 g/dl (P < 0.001).

Overall, RBC aggregation was not significantly altered after the administration of Ig. The relative change in AUC after Ig infusion ranged from an increase of 78% to a decrease of 43%. The results depicted in Fig. 1 show that the response to Ig differed among patients with low plasma albumin (≤3.5 g/dl) and those with normal to high plasma albumin (>3.5 g/dl). Before Ig infusion, AUC was higher in the low albumin group compared with the normal-high albumin group.

The relative increment in AUC\textsubscript{AAS} after Ig infusion ranged from an increase of 78% to a decrease of 43%. The results depicted in Fig. 2 show that the response to Ig differed among patients with low plasma albumin (≤3.5 g/dl) and those with normal to high plasma albumin (>3.5 g/dl). Before Ig infusion, AUC was higher in the low albumin group compared with the normal-high albumin group.

The relative increment in AUC\textsubscript{AAS} after Ig infusion was 29-fold higher in patients with plasma albumin above 3.5 g/dl (Fig. 2). The plasma sample with the highest fibrinogen concentration (600 mg/dl) exhibited a 29-fold increase in AUC\textsubscript{AAS} after the addition of Ig.

Furthermore, the effect of Ig on RBC aggregation in the presence of a high fibrinogen concentration was strongly dependent on the albumin concentration. When plasma fibrinogen was <450 mg/dl, there was an inverse correlation between albumin levels and AUC\textsubscript{AAS} (r = -0.69, P = 0.004; Fig. 3A). However, in the four plasma samples in which the fibrinogen concentration exceeded 450 mg/dl, AUC\textsubscript{AAS} correlated linearly with the plasma albumin concentration (r = 0.99, P = 0.006; Fig. 3B).

RBC aggregation under controlled concentrations of fibrinogen, Ig, and albumin. The response of RBC aggregation to increasing concentrations of albumin (0 to
5.5 g/dl) was examined in four sets of suspensions, produced by the following combinations: 1) low or high fibrinogen concentrations (250 and 500 mg/dl, respectively); and 2) the presence or absence of Ig at a concentration of 25 mg/ml. In a suspension containing Ig (25 mg/ml) and a high fibrinogen concentration, AUCAAS correlated positively with albumin concentration ($r = 0.94$, $P < 0.01$; Fig. 4). However, in the other three suspensions, there was no effect of increasing albumin concentration on RBC aggregation. At albumin concentrations of 0–3.5 g/dl, AUCAAS did not differ significantly among the four suspensions. At albumin concentrations of 4.5 and 5.5 g/dl, AUCAAS in the suspension containing a high fibrinogen concentration supplemented with Ig was increased twofold over the other three suspensions ($P < 0.001$; Fig. 4).

We also assessed the contribution of fibrinogen to RBC aggregation in four sets of suspensions, produced by the following combinations: 1) the presence or absence of a physiological concentration of albumin (4.5 g/dl); and 2) the presence or absence of Ig at a concentration of 25 mg/ml.

RBC aggregation (log AUCAAS) correlated with fibrinogen concentration in a suspension containing 25 mg/ml Ig and 4.5 g/dl albumin ($r = 0.86$, $P = 0.02$). In contrast, fibrinogen concentration did not affect AUCAAS in albumin-free suspensions (Fig. 5). At fibrinogen concentrations above 400 mg/dl, the absolute value of AUCAAS was significantly greater in a suspension containing both Ig and albumin compared with the other three suspensions ($P < 0.01$; Fig. 5).

**DISCUSSION**

Our results suggest a three-way interaction among the three main plasma proteins involved in RBC aggregation. The observations in vivo imply that infusion of Ig increases RBC aggregation in the presence of an elevated plasma fibrinogen concentration and a normal to high plasma albumin concentration. Both these conditions are required for Ig to augment RBC aggregation, and their effects are synergistic. These findings are supported by the results of the in vitro studies: RBC aggregation in autologous plasma was enhanced after the addition of Ig if the plasma fibrinogen concent-
interactions among various plasma proteins. Our findings in vivo and in vitro confirm a negative correlation between plasma albumin concentration and RBC aggregation, as reported by others (14, 38). However, when studied at high concentrations of both fibrinogen and Ig, albumin takes on an important role as an inducer of RBC aggregation.

Aggregation of RBCs in a suspension increases with increasing molecular size of macromolecules in the suspension (4). Therefore, it is reasonable to speculate that synergistic enhancement of RBC aggregation by plasma proteins is a result of the formation of multi-protein complexes. Being an anionic protein, it is likely that albumin by itself directly disaggregates negatively charged RBCs. Albumin may, however, interact with other plasma proteins, with the effect of enhancing their ability to aggregate RBCs. Fibrinogen and Ig are cationic proteins and thus can bind to albumin. It is possible to construct a plausible model to explain the synergistic effects of all three plasma proteins, based on the following assumptions: 1) albumin can bind to both fibrinogen and Ig simultaneously, using two separate binding sites; and 2) fibrinogen and Ig interact with albumin using binding sites that are distinct from the RBC membrane binding sites of these molecules. If these assumptions hold true, then sufficient concentrations of these plasma proteins could result in the formation of large, positively charged complexes of fibrinogen, Ig, and albumin, which efficiently bridge RBCs and promote the formation of large aggregates.

Several observations support this model. Albumin-IgG complexes are present in normal human plasma (27). Such complexes probably increase in number after the administration of Ig. Albumin-fibrinogen complexes can be produced experimentally, and these complexes have been shown to interact with platelets (32). Both Ig and fibrinogen have been extensively shown to interact with RBCs. Ig infused into healthy subjects coats RBCs without causing hemolysis (19). Fibrinogen binding to RBC membranes has recently been shown to be inhibited by the peptide Arg-Gly-Asp-Ser, suggesting both specific and nonspecific fibrinogen binding to RBCs (15). Thus both fibrinogen and Ig can each bind to albumin and RBC membranes and could theoretically form an intercellular matrix that stabilizes RBC aggregates. The significant decrease in plasma fibrinogen and albumin after intravenous Ig administration, reported also by Madl et al. (16), is consistent with the formation of Ig-albumin-fibrinogen complexes, resulting in reduced levels of free albumin and fibrinogen.

The level of plasma albumin, a so-called “negative acute phase protein,” decreases in acutely ill patients, and albumin levels are inversely related to the risk of death. A recent metaanalysis has shown that the time-honored practice of administering albumin to critically ill patients does not reduce mortality and may in fact increase it (6). In view of our findings, it is tempting to hypothesize that reduction in plasma albumin levels in acutely ill patients is an adaptive response that reduces the viscosity of blood containing high concentrations of RBC-aggregating acute phase proteins such as
fibrinogen and Ig. Increased RBC aggregation may reduce oxygen delivery to tissues by increasing the thickness of the marginal cell-free layer (33), thus reducing the already compromised tissue oxygenation in critical patients.

In clinical practice, fibrinogen and albumin concentrations tend to vary reciprocally, and it is uncommon to find elevated levels of both in the same patient. Madl et al. (16) studied patients with sepsis who received Ig and found no change in RBC aggregation. These patients probably had high concentrations of fibrinogen and low concentrations of albumin, as is typical of acutely ill patients.

Induction of RBC aggregation by therapeutically administered Ig may have important clinical consequences. Thrombotic complications of Ig treatment are increasingly reported and include myocardial infarction (9, 10, 25), ischemic stroke (7, 28, 31), and retinal vein occlusion (21). Provocative administration of Ig to bone marrow transplant recipients was associated with increased mortality in two large registries. Death was attributed to venoocclusive disease of the liver, which could be explained by increased blood viscosity resulting from RBC aggregation (13, 35). Ig is currently contraindicated in children with cyanotic heart disease, who have an elevated hematocrit and blood viscosity, as it was found to promote cyanotic episodes and poor surgical outcomes (29). Knowing what constellation of plasma protein concentrations places a patient at an increased risk of these complications may facilitate the selection of candidates for Ig treatment.

In conclusion, albumin, fibrinogen, and Ig synergistically induce RBC aggregation. Therefore, patients with high plasma concentrations of both albumin and fibrinogen are susceptible to enhanced RBC aggregation after Ig infusion, which may place them at risk for thrombotic complications. Our results should aid in constructing a model of RBC aggregation by plasma proteins that takes into account interactions among the proteins themselves as well as between proteins and RBCs.

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DISCLOSURES

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