Premature Red Blood Cells Have Decreased Aggregation and Enhanced Aggregability

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Abstract: Preterm infants are highly susceptible to ischemic damage. This damage is most obvious in the brain, retina, and gastrointestinal tract. Studies focusing on the rheological properties of premature red blood cells (pRBCs) have consistently shown minimal or no RBC aggregation. Previously, measurements of pRBC aggregation kinetics indicated that specific plasma properties are responsible for the decreased RBC aggregation observed in the neonates, but that their specific RBC properties do not affect it. However, the strength of interaction in the pRBC aggregates as a function of medium composition has not been tested. In our previous research, we described clinically relevant parameters, that is, the aggregate resistance to disaggregation by flow. With the help of a cell flow property analyzer (CFA), we can monitor RBC aggregation by direct visualization of its dynamics during flow. We used the CFA to examine pRBC (from 9 premature babies) in the natural plasma and in PBS buffer supplemented with dextran (500 kDa) to distinguish between RBC intrinsic-cellular and plasma factors. pRBCs suspended in the native plasma showed minimal or no aggregation in comparison to normal adult RBC. When we transferred pRBCs from the same sample to the dextran solution, enhanced resistance to disaggregation by flow was apparent.

Key words: red blood cell aggregation, aggregability, preterm infants, plasma factor.

Preterm infants are highly susceptible to ischemic damage. This damage is most obvious in the brain, retina, and gastrointestinal tract. Although they lack the adult compensatory and protective mechanisms for all shifts in blood pressure, hypoxemia, acidosis, and peroxidation, they still exhibit significant resilience to low oxygen states. Much study has been focused on the circulatory and rheological properties of neonate and preterm babies to explain this phenomenon. Studies focused on red blood cell (RBC) flow properties of premature babies have consistently shown decreased aggregation [1, 2], increased deformability [3–5], and therefore low blood viscosity [6–8]. In contrast, impaired flow in narrow vessels (with diameters of less than the critical value of 3.3 µm), because of the large size of neonatal RBCs, has been observed [9]. In parallel, several investigators observed decreased plasma viscosity resulting from a low concentration of plasma proteins in neonatal blood [6–8]. The role of protein plasma concentration in newborn blood flow properties has been intensively discussed in the literature [6–8, 10]. Studies focusing on ischemic damage, such as intraventricular hemorrhage, showed that factors changing the plasmatic composition, such as sepsis, may increase the risk for ischemic damage, though steroid treatment may decrease it [11]. Linderkamp et al. [12] showed that the velocity of premature red blood cell (pRBC) aggregation increased drastically when the suspension medium had been changed from autologous plasma to buffer supplemented with 1% Dextran 500. Similar results have been observed by Meiselman et al. [13, 14] for Dextran 70. Thus the authors have postulated that plasma properties are responsible for the decrease of pRBC aggregation velocity.

It is well known that the intercellular interactions of RBCs are defined by their behavior under she stress and thus these interactions should be determined as a function of shear stress. Yet in many studies, RBC flow properties have been determined in single or limited shear stress, or even in statics [12–14], and their clinical relevance could not be clearly documented, mainly because of the unavailability of suitable instrumentation for identifying the appropriate parameter(s). However, this has limited physiological/clinical relevance, since the dependence on shear stress expressed the strength of intercellular interaction, which is an important hemodynamic determinant. Therefore de-
terminating the dependence of RBC flow properties on shear stress applied to the cells, as proposed here, is essential for the evaluation of their potential contribution to blood flow and microcirculatory disorders. The importance of studying RBC rheology as a function of shear stress was further demonstrated in our recent study [15], in which we searched for clinically relevant parameters of the aggregability of RBCs from patients with inflammatory conditions by correlating aggregation parameters with acute phase reactants. The best correlation was obtained with the RBC parameter that integrated the aggregate size distribution and its dependence on shear stress. The results presented in that study [15] demonstrate that the clinically relevant parameter(s) of RBC intercellular interaction should express the extent of interacting cells and the interaction strength (resistance to flow). These findings motivated us to determine the strength of pRBC interactions and to test the plasma factor in this process.

MATERIALS AND METHODS

Patients. Blood was drawn within 24 h of birth from 9 consecutive premature babies. They were born at a median gestational age of 27 weeks (average 28.75, range 27–33) with a median birth weight of 887.5 g (average 1,019.25 g, range 630–1,970 g). Five babies were ventilated, and all were in an incubator temperature of 37°–38°C with a body temperature of 37°C. Median oxygen pressure was 21.

Preparation of samples. Blood from healthy adult volunteers was drawn from the antecubital vein. Blood samples from babies and volunteers were collected in vacutainers containing EDTA (ethylene diamine tetra acetate). Consent for the use of samples for research was obtained. To differentiate the related effects of plasma and cells on RBC aggregation, we needed to compare RBC aggregation in plasma with a plasma-free standardized medium. We have previously reported [16] that 0.5% of dextran-500 induces the formation of RBC aggregates similar in size and shape to those formed in plasma. We therefore used dextran-500 as a standard aggregating solution [15–23], which eliminates plasma factors, but not RBC factors. The RBCs were isolated by centrifugation (2,000 rpm for 10 min) and washed with phosphate-buffered saline (PBS), pH 7.4, then resuspended to the desired hematocrit (6%) in either the autologous plasma or the PBS supplemented with 1% bovine albumin (Sigma, St. Louis, MO) and 0.5% dextran-500 (Pharmacia Biotech; Uppsala, Sweden) to induce aggregation.

Measurements of RBC aggregation. All aggregation measurements were conducted within 6 h of venipuncture. RBC aggregability was studied using a cell-flow properties analyzer (CFA) as previously described [15, 16, 19–23]. Briefly, the RBC suspension (in plasma or in dextran buffer) is introduced into a narrow-gap (30 µm) flow chamber, which is connected to a pump producing laminar flow and a pressure transducer for monitoring of shear stress during the experiment. The wall shear stress (τ) is calculated from pressure (P) at the entrance to the flow chamber, measured by a pressure-measuring station (Power Lab 2/20, AD Instruments). This is done according to the equation τ = P · h/2 · z, where z and h are the chamber length and gap, respectively. The RBC dynamic organization (aggregation/disaggregation) in the flow chamber is directly visualized and recorded through a microscope connected to a video camera (TM6EX, Pulnix, USA), which transmits the RBC images to a frame grabber (FlashBus, Integral Technologies Inc., USA) in the computer. Images are then analyzed by original image analysis software to provide the parameters of RBC aggregation. We previously found this index to faithfully represent clinically relevant aggregation [15].

Fig. 1. A sample graph demonstrating the derivation of indexes of RBC aggregation by the CFA. AAS is plotted as a function of shear stress (A) or as function of 1/τ (B). The integral (area under the curve: AUC) and ASI (aggregate strength index) are representative of the aggregates’ resistance to shear-induced dispersion.
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**Average aggregate size (AAS):** Because experimental models of RBC aggregation have found peak AAS to be achieved at a shear stress (τ) of 0.01–0.025 Pa, we defined the AAS at τ = 0.015 Pa as the peak aggregate size.

**Small and large aggregate fraction (SAF and LAF):** We measured the distribution of the RBC population into aggregate size ranges, i.e., the erythrocyte fraction (in %) in small (1–4 RBC per aggregate) or large aggregates (33 or more RBC per aggregate). For details, see [15, 20, 21].

**Area under the curve (see Fig. 1A):** The area under the curve (AUC) of the plot of an aggregation parameter as a function of τ was done for AAS and LAF. As shown in [15], AUC expresses the resistance of RBC aggregates to disaggregation by flow.

**Aggregate strength index [18]:** To obtain an appropriate measure of RBC aggregation, we plotted the AAS as a function of 1/τ (Fig. 1B) and took the slope of this line as an aggregate strength index (ASI).

**Statistical analysis.** Values are presented as averages with standard deviation. The differences between groups in frequencies of ordinal variables were calculated with the chi-squared test. The differences in RBC aggregation between the two groups were calculated with the Student’s t-test. The correlations between continuous variables were analyzed with Pearson’s bivariate correlation.

All statistical tests were two-sided. The P values were considered significant when less than 0.05. Statistical calculations were performed with the software package (SPSS Inc., Chicago, Illinois).

**RESULTS**

pRBC suspended in native plasma showed low aggregation in comparison to normal adult RBC (Fig. 2, A and B). Thus in the suspension of normal adult RBC in the autologous plasma (at shear stress 0.015 Pa), we observed large aggregates (LAF = 22.0 ± 1.5), but in contrast, at the same flow large aggregates are not present in the suspension of pRBC. Also, the interaction of cell–cell in aggregates of pRBC is weak, as described by the low value of AUCLAF, AUCAAS, and ASI (Table 1).

pRBC suspended in dextran solution (Fig. 2C) showed a normal value of AAS (Table 1). In dextran solution, pRBC formed large aggregates, and the percentage of RBC in large aggregates is significantly higher for samples drawn from premature babies in comparison to those from normal adults (see Table 1). In parallel, we observed enhanced cell–cell interaction strength in pRBC aggregates in comparison to red cells from normal adults. Thus the values of all integrative parameters (AUCLAP, AUCAAS, and ASI)
DISCUSSION

Vascular flow and RBC properties are of importance in clinical practice, especially in the premature. Diseases prevalent in this population, such as intraventricular hemorrhage, retinopathy of prematurity, and necrotizing enterocolitis, may have intimate connections with blood flow capabilities and lower functional skin capillary density [24]. The strength of RBC aggregates is crucial in determining the blood flow, since rouleaux formations vastly decrease the flow. Therefore much attention has been given in the past to the study of aggregation in the preterm babies. These studies uniformly found minimal or non-aggregation of pRBC when suspended in the native plasma [1, 2]. It may be postulated that this lack of aggregation may be crucial to defend the preterm baby from ischemic or hypoxic damage. According to our knowledge, only in one publication has the role of plasma and cellular factors in pRBC aggregation kinetics been analyzed [12]. Some studies have suggested the notion that the RBC may be partly responsible for the lack of aggregation [21]. The Yedgar laboratories in the Faculty of Medicine at the Hebrew University in Jerusalem have developed the CFA in which RBC aggregation can be directly appraised. This method has been used previously to assess RBC aggregation in various adult diseases [15, 16, 19–23]. For a characterization of strength of cell–cell interaction in the RBC aggregate, the number of clinically relevant parameters has been described [15]. Moreover, we have previously focused on an evaluation of plasma factors [15], particularly the role of fibrinogen [20, 21], in the formation of strong RBC aggregates, which are stable under the flow. Suspending the RBC in dextran eliminates the plasma factors and focuses on the aggregability (i.e., the aggregation potential) of the RBC itself.

We have shown that although there was no aggregation of pRBC in the premature plasma, the pRBC aggregability in dextran solution was greater than for adult red cells. This result is in contradiction to Linderkamp et al. [12] and Whittingstall and Meiselman [14], who observed no difference between full-term neonatal RBC and the aggregability of adult red cells in PBS medium supplemented with 1% dextran-500 [12] or much lower aggregability of full-term neonatal RBC when suspended in 3% dextran-70 solution [13, 14]. Since Linderkamp et al. [12] and Whittingstall and Meiselman [14] characterized RBC aggregability by aggregation kinetic parameters, and we did it by characterizing the strength of cell–cell interaction in the aggregate, these results cannot be accurately compared because the physical phenomena (aggregation kinetics and disaggregation under the flow) discussed are dissimilar.

We believe that the enhanced strength of pRBC–pRBC interaction in comparison to adult cells may be related to the large contact area between two interacted cells because of the big surface area and/or the high deformability [25] of pRBC.

We conclude, in agreement with Linderkamp et al. [12], that a lack of RBC aggregation in premature babies results from plasmatic factors and not from RBC aggregation potential.

Financial support of this work by the U.S.–Israel Binational Science Foundation, No 201203 (to S. Yedgar and G. Barshtein), by the Israel Ministry of Health No 8230 (to S. Yedgar), by the National Blood Foundation–AABB (to S. Yedgar and G. Barshtein), and by the Walter and Greta Stiel Chair for Heart Studies (to S. Yedgar). The authors thank Mrs. D. Fredman for technical assistance.

Table 1. Aggregation in plasma.

|                     | Normal adult |          | Premature baby |          |                     |          |
|---------------------|--------------|----------|----------------|----------|---------------------|----------|
|                     | Average      | St Dv    | Average        | St Dv    |                     |          |
| AAS                 | 15.35        | 1.50     | 3.81           | 0.80     | P < 0.0001          |          |
| LAF                 | 22.0         | 1.50     | 0              | 0        | P < 0.0001          |          |
| AUC_AAS             | 19.60        | 0.70     | 11.30          | 1.10     | P < 0.0001          |          |
| AUC_LAF             | 7.60         | 0.80     | 0              | 0        | P < 0.0001          |          |
| ASI                 | 34.5         | 3.5      | 4.3            | 0.7      | P < 0.0001          |          |
|                     | 8.60         | 0.50     | 10.06          | 1.70     | NS                  |          |
|                     | 3.20         | 1.20     | 24.60          | 7.00     | P < 0.000001        |          |
|                     | 13.60        | 0.50     | 16.50          | 1.60     | P < 0.0005          |          |
|                     | 1.30         | 0.50     | 4.30           | 1.20     | P < 0.000001        |          |
|                     | 15.3         | 1.6      | 21.3           | 2.3      | P < 0.01            |          |

AAS, average aggregate size; LAF, large aggregation fraction; NS, not significant; AUC, area under the curve (AAS [sheer stress]); (LAF [sheer stress]); ASI, slope of the linear regression for plotting AAS as a function of 1/τ.
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