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RHEOLOGICAL CHARACTERISTICS OF DESIALYLATED ERYTHROCYTES IN RELATION TO FIBRINOGEN-INDUCED AGGREGATION

NOBUJI MAEDA, KAZUHIKO IMAIZUMI, MISUZU SEKIYA and TAKESHI SHIGA

Department of Physiology, School of Medicine, Ehime University, Shigenobu, Onsen-gun, Ehime, 791-02 (Japan)

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The effect of fibrinogen and sialic acid content of erythrocytes on the aggregation of erythrocytes was quantitatively examined by using a rheoscope combined with a television image analyzer and a computer. (1) The electrophoretic mobility of erythrocytes was proportional to the sialic acid content of erythrocytes (the surface potential of erythrocytes could be expressed by the sialic acid content). (2) The aggregation of erythrocytes was accelerated by increasing fibrinogen concentration in the medium (due to the increased bridging force among erythrocytes) or by decreasing the sialic acid content (due to the reduction of the electrostatic repulsive force among erythrocytes). (3) An empirical equation expressing the velocity of aggregate formation (v, in $\mu m^2/min$) by the concentration of fibrinogen (F, in g/dl) and the sialic acid content (S, in μmol/ml red blood cells), $\log v = -0.065\ F^{-1.2}S + 2.2\ F^{0.35}$, was deduced. (4) The contribution of the bridging force of fibrinogen to the erythrocyte aggregation was much greater than that of the electrostatic repulsive force produced by sialic acid on the surface of erythrocytes.

Introduction

Erythrocytes in blood sometimes aggregate at low shear rates but disintegrate at high shear rates. The accelerated aggregation of erythrocytes increases the low shear viscosity and induces the sludging in the capillary. Many factors participate in the aggregation of erythrocytes: (i) the physical conditions (shear rate), (ii) the properties of erythrocytes (concentration, deformability, surface charge, shape) and (iii) the properties of plasma proteins (especially high molecular weight proteins such as fibrinogen, immunoglobulins, etc. [1,2]. The aggregation of erythrocytes in blood results from bridging between adjacent cells by plasma proteins, but the negative surface charge of erythrocytes is very important for the repulsive force against the aggregation.

The negative surface charge of erythrocytes mainly originates from sialic acid present in the sugar moiety of membrane-bound glycoproteins [3]. The role of sialic acid in determining the life-span of circulating erythrocytes has frequently been discussed, and the short survival of desialylated erythrocytes in the circulation has been shown [4]. Further, the increased aggregability of acquired sialic acid-deficient erythrocytes and the resultant increase in blood viscosity have been shown [5].

In this report, the effect of the negative surface charge of erythrocytes and fibrinogen on the aggregation of erythrocytes was quantitatively examined (while maintaining constant the other factors influencing the aggregation). An empirical equation for the relationship of the aggregation rate, sialic acid content and fibrinogen concentration is deduced. The results provide valuable evidence that the negative surface charge of erythrocytes may play a significant role in the aggregation

of erythrocytes, but macromolecules such as fibrinogen are essential and powerful mediators inducing the aggregation.

Materials and Methods

Erythrocytes and plasma. Fresh blood was obtained from a healthy adult male (red cell type A^+), and heparinized (10 units heparin/ml blood were used). Blood was centrifuged at 3000 rpm for 5 min at 4°C. Plasma was collected and recentrifuged at 15000 rpm for 15 min at 4°C to remove platelets. Erythrocytes were washed twice with isotonic phosphate-buffered saline (42.6 mM Na_2HPO_4/7.4 mM NaH_2PO_4/90 mM NaCl/5 mM KCl/5 mM D-glucose, pH 7.4). Erythrocytes were used within 4 h after collection.

Protein preparations. Human serum albumin (fatty acid-free, Fraction V) was purchased from Miles Lab. Inc. After dissolving in isotonic phosphate-buffered saline, a tiny amount of charcoal was removed by centrifugation at 15000 rpm for 15 min at 4°C. Human fibrinogen was purchased from AB Kabi, Stockholm, (Grade L; 90% clottable) and was used after passing through lysine-Sepharose 4B column (purchased from Pharmacia Fine Chemicals; 1.0 × 3.0 cm column equilibrated with isotonic phosphate-buffered saline, pH 7.4) to remove contaminated plasminogen and/or plasmin [6]. Neuraminidase (sialidase; from *Clostridium perfringens*; activity, 0.86 unit/mg protein, using *N*-acetylneuramin-lactose) was purchased from Sigma Chem. Co.

The concentration of fibrinogen was determined spectrophotometrically by using an extinction coefficient of 15.1 [7] for 1% solution at 280 nm (1 cm). The preparation showed a single band in cellulose acetate electrophoresis, and the sedimentation coefficient ($s_{20,w}$) was 7.82 S and the schlieren pattern showed a single and symmetrical peak.

Desialylation and the determination of sialic acid content. Washed erythrocytes (hematocrit, 20%) were incubated with neuraminidase (10–50 mU/ml) in isotonic phosphate-buffered saline at 37°C with gentle shaking for various times. After incubation, the mixture was cooled and the erythrocytes were washed with cold isotonic phosphate-buffered saline twice.

In order to determine the content of sialic acid in erythrocytes, ghosts were prepared from a known amount of erythrocytes by hypotonic hemolysis according to Dodge et al. [8]. The ghost preparation was incubated in 0.1 N H_2SO_4 at 80°C for 60 min to release sialic acid bound to membrane proteins, and then the thiobarbituric acid method [9] was adopted for the determination of sialic acid.

Measurement of electrophoretic mobility of erythrocytes. The electrophoretic mobility of erythrocytes with various contents of sialic acid in the cell surface was measured by free-flow electrophoresis (C. Desaga, GmbH; model FF 48) according to the method of Hannig [10]. The triethanolamine-acetate buffer system of Hannig and Zeiller [11] was used with a small modification.

10 mM triethanolamine-acetate buffer containing 0.26 M sucrose and 5 mM D-glucose (pH 7.4, 300 mOsm) was used for the separation buffer, and 100 mM triethanolamine-acetate buffer (pH 7.4) for the electrode buffer. The electrophoresis was performed at 4°C under a voltage gradient of 800 V for the migration distance of 7 cm (the electric current in this condition was 45 mA). The residence time [12] of the sample in the electric field was 127 s. The electrophoretic mobility was expressed by $(\mu m \cdot s^{-1})/(V \cdot cm^{-1})$.

Rheological measurements. (1) Viscometry: a cone-plate viscometer (Tokyo Keiki Co., model E, mounted 0.8° cone) was used at 37°C. The erythrocyte concentration was adjusted on the basis of the hematocrit. The hematocrit was determiend by the microcapillary centrifugation technique, without correction for the buffer volume trapped among cells. (2) Measurement of 'rouleau' (one-dimensional aggregate) formation: the apparatus combined a rheoscope [13] (composed of a transparent cone-plate viscometer and an inverted microscope [14]) with a TV image analyzer (Luzex 450, Toyo Ink Co.), and a computer (Hewlett Packerd, HP-85) was used for the measurement of the velocity of rouleau formation, as reported previously [2,15].

The washed erythrocytes were resuspended in a medium composed of 70% autologous plasma and 30% isotonic phosphate-buffered saline (pH 7.4) or in an artificial medium composed of human serum albumin and fibrinogen in various concentrations

(pH 7.4). The final hematocrit was adjusted to 0.26%. The mixture was immediately applied to the rheoscope, and the rouleau formation was observed at a shear rate of 7.5 s^{-1} at 25 °C. The number of particles (i.e., erythrocyte, one-dimensional aggregate (rouleau) and three-dimensional aggregate, as shown in Fig. 4) and the total area occupied by particles in a limited area of TV image (actual frame size was 190 × 130 μm) were consecutively encoded by the analyzer at an interval of approx. 1.3 s, and transferred to the computer. An estimate of the velocity of rouleau formation was represented by the increment of area occupied by one particle (v, μm^2/min).

Morphological and hematological examination. The morphology of erythrocytes was examined by the scanning electron microscope, aa described previously [16]. The number of erythrocytes was counted by a microcellcounter (Sysmex, model CC-110). The hemoglobin concentration was determined by the cyanmethemoglobin method [17].

Results

Desialylation of erythrocytes and the electrophoretic behavior

The time course of desialylation of erythrocytes is shown in Fig. 1. Sialic acid on the surface of erythrocytes was easily removed by neuraminidase, but 10–15% of total sialic acid was fairly resistant to hydrolysis in this experimental condition.

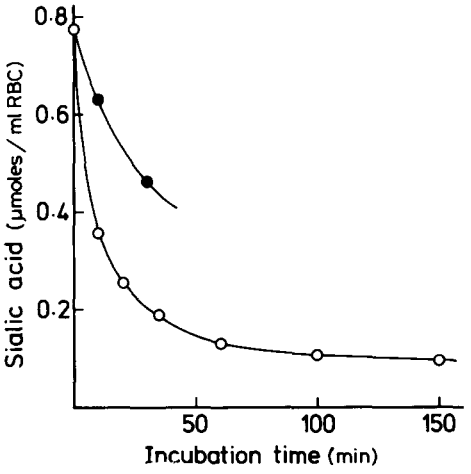

Fig. 1. Time course of desialylation of erythrocytes by neuraminidase. Treatment with neuraminidase of 10 (●) and 50 (○) mU/ml, in isotonic phosphate-buffered saline (pH 7.4) at 37 °C. RBC, red blood cells.

The electrophoretic mobility of the desialylated erythrocytes was examined by free-flow electrophoresis. The electrophoretic patterns of erythrocytes with different contents of sialic acid are shown in Fig. 2. The peak was single and almost symmetrical, but those with lower contents of sialic acid were slightly tailed.

A linear relationship between the electrophoretic mobility and the content of sialic acid was obtained, as shown in Fig. 3. It was clear that erythrocytes were effectively fractionated by the negative charge difference due to sialic acid content on the surface of erythrocytes.

Effect of desialylation on the suspension viscosity of erythrocytes

The suspension viscosity of erythrocytes in isotonic phosphate-buffered saline (hematocrit, 33%) was not affected at shear rates of 18.1–752 s^{-1} by the degree of desialylation, within experimental

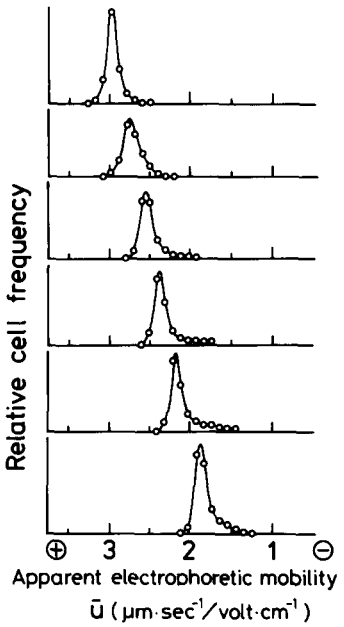

Fig. 2. Free-flow electrophoretic patterns of desialylated erythrocytes. Sialic acid content from top to bottom: 0.754, 0.699, 0.584, 0.484, 0.398 and 0.222 μmol/ml red blood cells, respectively. Details for the electrophorpetic conditions in the text.

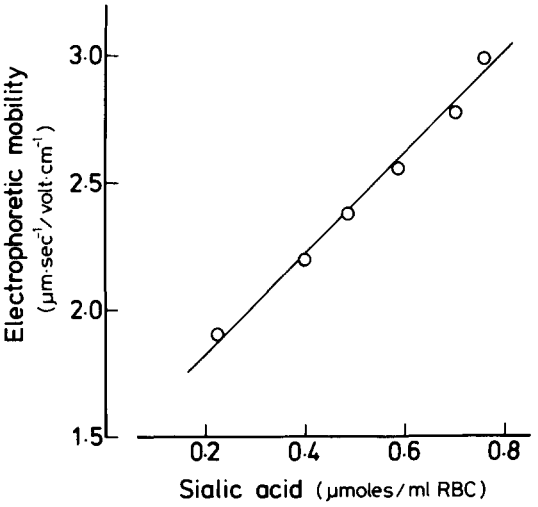

Fig. 3. Relationship between the electrophoretic mobility and the content of sialic acid. The electrophoretic mobility is expressed by the peak position in Fig. 2. RBC, red blood cells.

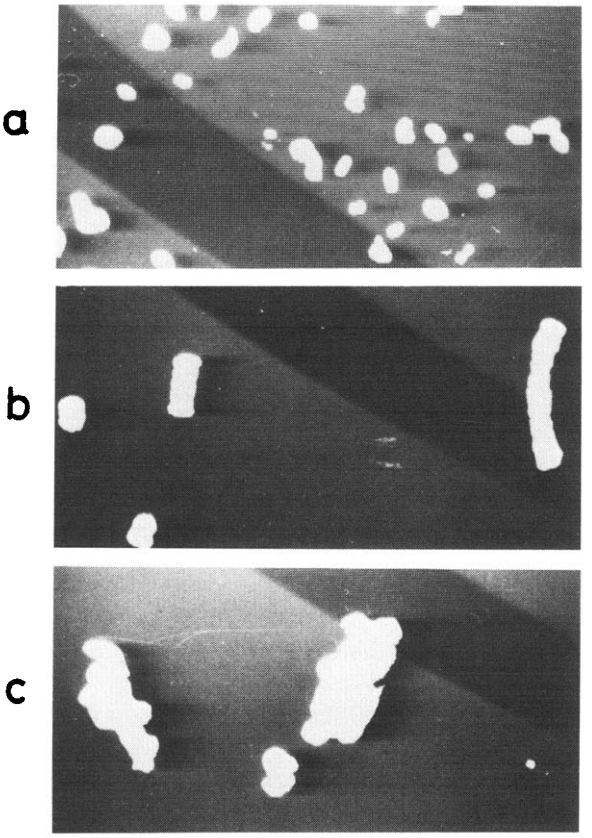

Fig. 4. Representative time course of aggregate formation (after processing with TV image analyzer). (a) First stage, dispersed state of individual cells and formation of some one-dimensional short rouleaux; (b) second stage, formation of one-dimensional long rouleaux; (c) third stage, formation of three-dimensional aggregates.

error. In this connection, the red cell shape, the mean corpuscular volume and the mean corpuscular hemoglobin concentration (which were important factors influencing red cell rheology) were not altered by desialylation. The absence of an increase in the suspension viscosity at higher shear rates suggests that the deformability of erythrocytes may not be influenced by the desialylation.

Effect of desialylation on the aggregation of erythrocytes

The aggregation of desialylated erythrocytes was measured in the autologous plasma and in the artificial medium by the low-shear rheoscope combined with the TV image analyzer. A representative pattern of gradual aggregate formation is shown in Fig. 4. The velocity of the rouleau (one-dimensional aggregate) formation was estimated by the increasing velocity of area per particle in progressing from the first stage to the second stage.

(1) Aggregation in the autologous plasma. The aggregate formation of desialylated erythrocytes was measured in medium composed of 70% autologous plasma and 30% isotonic (phosphate-buffered saline (pH 7.4). In this condition, no three-dimensional aggregate was formed. The relationship between the velocity of rouleau formation (v) and the sialic acid content is shown in Fig. 5.

As the sialic acid content of erythrocytes decreased, the aggregation of erythrocytes was accelerated, i.e., the interaction among erythrocytes in the presence of plasma proteins increased. The semilogarithmic plot of the velocity of rouleau formation against the sialic acid content gave a straight line, as shown in the inset in Fig. 5. An empirical relation between the velocity of rouleau formation and the sialic acid content is obtained: $\log v = a \cdot S + b$, where S is sialic acid content, a and b are constants.

(2) Aggregation in the artificial medium. In order to clarify the effect of the individual plasma proteins, especially fibrinogen, and the sialic acid

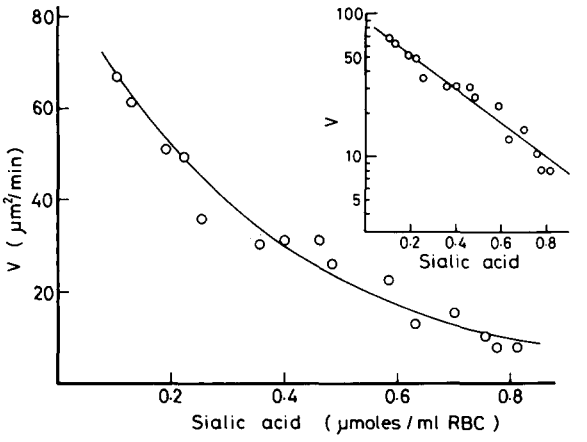

Fig. 5. Effect of desialylation on the rouleau formation of erythrocytes. Measured in 70% autologous plasma + 30% isotonic phosphate-buffered saline (pH 7.4). v, the increasing velocity of area per particle due to rouleau formation. The inset is the semilogarithmic plot, RBC, red blood cells.

content on the aggregation of erythrocytes, the artificial medium (basically composed of 0.3 g/dl fibrinogen and 5 g/dl albumin in isotonic phosphate-buffered saline, pH 7.4) was used. The influence of fibrinogen and sialic acid content on the aggregation of erythrocytes is shown in Fig. 6.

As the concentration of fibrinogen in the medium increased, the velocity of rouleau formation increased in all erythrocyte preparations with different contents of sialic acid. Furthermore, as the sialic acid content in the surface of erythrocytes was decreased by the treatment with neuraminidase, the velocity of rouleau formation increased. It is especially noteworthy that the aggregation of erythrocytes never occurred in the absence of fibrinogen, independently of the sialic acid content. The relationship between the velocity of rouleau formation (v) and the sialic acid content (S) is shown in Fig. 7. Linear relations were also obtained between log v and S in all fibrinogen concentrations, as observed in autologous plasma (Fig. 5). The phenomena are represented by the equation, $\log v = \alpha \cdot S + \beta$, where α (slope) and β (intercept) are dependent on the fibrinogen concentration in the medium (the equation will be discussed in detail in the Discussion).

The deviations of fibrinogen concentration and sialic acid content from the physiological conditions influence the aggregation of erythrocytes in the following manner, as roughly estimated from Figs. 6 and 7: a 10% increase in fibrinogen concentration from 0.3 g/dl accelerates the velocity of aggregation 18% (Fig. 6), whereas a 10% decrease

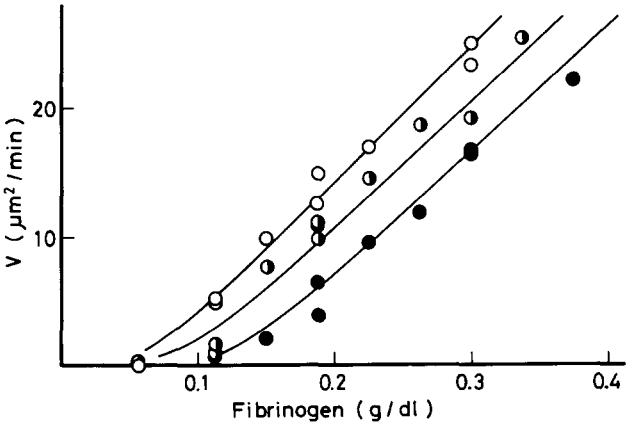

Fig. 6. Effect of fibrinogen and sialic acid content on the rouleau formation of erythrocytes. Measured in 5 g/dl albumin in isotonic phosphate-buffered saline at 25 °C. Two different experiments are summarized in the figure. Sialic acid content: ○, 0.17–0.20; ◑, 0.52–0.56; ●, 0.76–0.81 μmol/ml red blood cells.

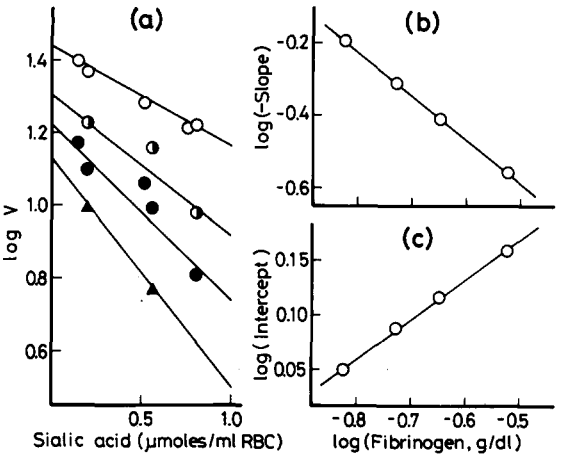

Fig. 7. Effect of sialic acid content on the rouleau formation of erythrocytes. (a) Relationship between the velocity of rouleau formation and sialic acid content. Measured in the artificial medium as shown in Fig. 6. Fibrinogen concentrations were 0.300 (○), 0.225 (◑), 0.187 (●) and 0.150 (▲) g/dl. The straight lines are calculated according to the empirical equation, $\log v = -0.065F^{-1.2}S + 2.2F^{0.35}$ (see Discussion). (b) Dependence of the slope in (a) on fibrinogen concentration. (c) Dependence of the intercept in (a) on fibrinogen concentration (see Discussion).

of sialic acid content from 0.8 μmol/ml red blood cells accelerates the velocity of aggregation only 6% (Fig. 7). Therefore, it must be concluded that the change in fibrinogen concentration has more effect on the aggregation of erythrocytes, compared with the change in the electrostatic repulsive force due to the surface negative charge of erythrocytes.

Discussion

The aggregation of erythrocytes is very important for affecting blood viscosity at low shear rates, and further relates to the sludging in the capillary. A TV image analyzer combined with a low-shear rheoscope was properly adopted for measuring the velocity of aggregate formation.

Factors influencing the aggregation of erythrocytes

Various factors affecting erythrocyte aggregation have been studied in detail by Chien and Jan [18], using carbohydrate macromolecules (dextran) with various molecular weights and changing the negative charge of erythrocyte surface with neuraminidase. They have proposed that the net aggregation force (F_a) is determined by the macromolecular bridging force (F_b), the electrostatic repulsive force (F_e), the mechanical shearing force (F_s), and the membrane bending force (F_m) with the relation $F_a = F_b - F_e - F_s - F_m$. Recently, the importance of red cell shape to aggregation has been pointed out [2]. The erythrocyte concentration, another specific factor influencing the velocity of aggregation in the present method [2], was maintained at 0.26% in hematocrit. In the present experiment, the quantitative relationship between the macromolecular bridging force of fibrinogen and the electrostatic repulsive force of sialic acid was clarified by fixing the other factors, as follows.

(i) Macromolecular bridging force. Plasma is very heterogeneous in protein composition. Aggregation in medium composed of 70% autologous plasma and 30% isotonic phosphate-buffered saline was accelerated by desialylation. Among plasma proteins, fibrinogen and immunoglobulin are very effective for bridging of erythrocyte surfaces. As is well known, fibrinogen increases the viscosity of erythrocyte suspensions in the low shear rates [19,20] due to the accelerated aggregation of erythrocytes [2,19–22]. Recently, the effect of immunoglobulin on the aggregation of erythrocytes and the dependence on the molecular weight of immunoglobulin have been reported [23]. The present results also show that fibrinogen increased the aggregation of erythrocytes markedly (Fig. 6). Although the heterogeneity of α chain of fibrinogen in molecular weight [24–26] and of β and γ chains in sialic acid content [24,27,28] has been reported, the same fibrinogen preparation, which was homogeneous in electrophoretic mobility in cellulose acetate membrane (pH 8.8) and in the Schlieren pattern of analytical centrifuge, has been used in our experiment.

(ii) Electrostatic repulsive force. The negative charge of the erythrocyte surface produces an electrostatic repulsive force among erythrocytes [18,29]. It was clearly recognized that the electrophoretic mobility of erythrocytes paralleled the sialic acid content (Fig. 3), as shown previously [30]. Thus, about 90% total sialic acid (which is easily removed by neuraminidase) behaves as effective negative charge on erythrocytes at pH 7.4. We have no evidence as to whether the rest of the sialic acid is effective electrophoretically but is not hydrolyzed by the enzyme as it may be buried in the protein moiety.

(iii) Mechanical shear force. The aggregate formation of erythrocytes is shear-dependent. Thus, the aggregate formed in the presence of fibrinogen disintegrated on application of the high shear rate. The shear rate in this experiment was maintained at 7.5 s^{-1}, because of the experimental precision in encoding the area by the image analyzer.

(iv) Membrane binding force. The deformability of erythrocytes is closely related to the membrane bending. We have shown that the aggregation of erythrocytes is extremely suppressed by cross-linking spectrin with diamide, due to the decreased deformability [31]. The desialylation procedure did not affect the deformability, because the high shear viscosity unchanged.

(v) Shape factor. The red cell shape is very important for the side-to-side or end-to-side attachment between erythrocytes and between erythrocyte and neighboring rouleau, as described previously (echinocytosis or spherocytosis decreases the aggregation, remarkably) [2,15]. The scanning electron microscopic observation of de-

sialylated erythrocytes showed no alteration of their morphology.

Quantitative consideration of the macromolecular attraction and electrostatic repulsion among erythrocytes

In the present study, the quantitative relation between the macromolecular bridging force of fibrinogen and the electrostatic repulsive force of the surface negative charge of erythrocytes was studied in artifical medium (isotonic 50 mM sodium phosphate-buffered saline containing 5 g/dl albumin) without alteration of other factors.

The electrostatic repulsive force is a function of the surface potential of erythrocytes, which is represented by the zeta potential (ζ) [18]. The zeta potential is calculated with the use of the Helmholtz-Smolchowski equation, $\zeta = 360\pi \cdot \eta \cdot u/D$, where η is the viscosity, u is the electrophoretic mobility and D is the dielectric constant of the fluid medium. In the present experiment, the zeta potential can be estimated by the electrophoretic mobility of erythrocytes, as η and D are fairly constant due to the lower concentration of fibrinogen. Further, the electrophoretic mobility was proportional to the sialic acid content of erythrocytes in the range examined here. Thus, the surface potential, which produces the electrostatic repulsive force, is proportional to the sialic acid content.

To evaluate the relative contribution of the attractive force of fibrinogen and the repulsive force of surface negative charge of sialic acid on the aggregation of erythrocytes, an empirical equation was deduced from the relationship of the velocity of rouleau formation (v, in $\mu m^2/min$), the fibrinogen concentration (F, in g/dl) and the sialic acid content (S, in μmol/ml red blood cells) in Fig. 7a, as follows.

First, the relation between $\log v$ and S could be approximately expressed by the linear equation as already described (Fig. 7a),

$$\log v = \alpha \cdot S + \beta$$

where α and β are dependent on fibrinogen concentration (both the correlation coefficients were more than 0.93).

Second, the slope (α) and the intercept (β) were fitted to the power curve of fibrinogen concentration. As shown in Fig. 7b and c, the plots of log(−slope) and log(intercept) against log(fibrinogen concentration) gave straight lines (both the correlation coefficients were more than 0.98). Thus, the following equations were deduced:

$$\log(-\alpha) = m \cdot \log F + \log n$$

$$\log(\beta) = m' \cdot \log F + \log n'$$

The constants, m, n, m' and n', which were calculated by the least-squares method, were −1.2, 0.065, 0.35 and 2.2, respectively.

Conclusively, an empirical equation

$$\log v = -0.065F^{-1.2}S + 2.2F^{0.35}$$

was obtained. The calculated lines for various concentrations of fibrinogen are shown in Fig. 7a. It was clear that the empirical equation fitted well to the experimental points (in the range $v > 5$ $\mu m^2/min$ in Fig. 6). It was clear that the attractive force of fibrinogen is more powerful than the repulsive force of the surface negative charge of erythrocytes. Based on a model of cell electrophoresis of Levine et al. [32], the contribution of the electrostatic surface potential to the repulsive force among erythrocytes will become much smaller.

Effectiveness of fibrinogen in the aggregation of erythrocytes

The effect of macromolecules on erythrocyte aggregation has been frequently studied by various methods [1]. In these macromolecules, the effect of high molecular weight dextrans on erythrocyte aggregation has been systematically examined [18,29,33]. In spite of the methodological differences (e.g., shear rate, hematocrit, etc.), the effect of various dextrans on erythrocyte aggregation using our method (data not shown here) agreed well with those observed by Chien and Jan [18]. The good agreement on the degree of erythrocyte aggregation has also been observed for microscopic observation, determination of erythrocyte sedimentation rate and measurement of low-shear viscosity [33].

Compared with Dextran T-500, fibrinogen in

the same molar concentration was more effective in erythrocyte aggregation. It could be roughly estimated that fibrinogen is 10-times as effective as dextran of the same molecular weight (if available), probably due to the presence of charged groups in the fibrinogen molecule and to the molecular structure. However, it was noticed that the shape of rouleaux or aggregates induced by dextran was extremely different from those induced by fibrinogen. It has been observed that polylysine is an extreme effector for aggregation of erythrocytes [34]. Therefore, the molecular weight, the conformation of the macromolecule (e.g. the molecular shape, the molecular flexibility, etc.) and the amount of positively or negatively charged groups in the molecule should be taken into consideration with regard to the aggregation of erythrocytes.

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