Kinetics of Linear Rouleaux Formation Studied by Visual Monitoring of Red Cell Dynamic Organization

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ABSTRACT Red blood cells (RBCs) in the presence of plasma proteins or other macromolecules may form aggregates, normally in rouleaux formations, which are dispersed with increasing blood flow. Experimental observations have suggested that the spontaneous aggregation process involves the formation of linear rouleaux (FLR) followed by formation of branched rouleaux networks. Theoretical models for the spontaneous rouleaux formation were formulated, taking into consideration that FLR may involve both "polymerization," i.e., interaction between two single RBCs (e + e) and the addition of a single RBC to the end of an existing rouleau (e + r), as well as "condensation" between two rouleaux by end-to-end addition (r + r). The present study was undertaken to experimentally examine the theoretical models and their assumptions, by visual monitoring of the spontaneous FLR (from singly dispersed RBC) in plasma, in a narrow gap flow chamber. The results validate the theoretical model, showing that FLR involves both polymerization and condensation, and that the kinetic constants for the above three types of intercellular interactions are the same, i.e., $k_{ee} = k_{er} = k_{rr} = k$, and for all tested hematocrits (0.625–6%) $k < 0.13 \pm 0.03\ s^{-1}$.

INTRODUCTION

Red blood cells (RBCs) in the presence of plasma proteins or other macromolecules may form rouleaux aggregates, depending on flow conditions. The extent of aggregation is determined by opposing forces: the aggregation induced by the presence of macromolecules and the disaggregation induced by the negative surface charge and the flow-induced shear stress. RBC aggregation might thus be enhanced in low-flow states or by alterations in RBC membrane composition or plasma components. RBC aggregation plays a major role in blood flow, particularly in small blood vessels, as it contributes to 1) vascular resistance to blood flow, particularly in the postcapillary venules (Johnson et al., 1985), and 2) the formation of sludge blood (Knisely et al., 1947).

A considerable part of the total microvascular resistance is exerted by the venules. The changes in venular resistance with flow are attributed to geometrical factors (vascular hindrance) and the blood rheological factor (e.g., the effective blood viscosity). Blood viscosity is strongly dependent on RBC aggregation and geometrically enhanced with the aggregation, particularly in the postcapillary venules, where the shear stress is low (Alonso et al., 1993). Accordingly, Johnson et al., have shown that at least 50% of postcapillary resistance can be attributed to RBC aggregation (Cabel et al., 1997).

As noted above, RBC aggregation is a major determinant of blood viscosity. Aggregation-induced elevation of blood viscosity reduces blood flow (increased vascular resistance), which further increases aggregation, etc. Thus elevated RBC aggregation might initiate a self- accelerating "vicious cycle" of growing aggregation, leading to the formation of "sludge blood" and reduced tissue perfusion (Knisely et al., 1947). Indeed, enhanced aggregation has been observed with RBC in numerous diseases associated with microcirculatory disorders, such as cardiovascular diseases, diabetes, and trauma, and has been linked to their pathophysiology (Stoltz and Donner, 1987; Chien, 1987; Jones, 1990).

The organization of RBCs in blood vessels is a dynamic process in which RBCs are continuously aggregated and dispersed (disaggregated), depending on the flow conditions and, in pathological states, on possible changes in the RBC membrane composition and plasma components. Therefore, an understanding the mechanism of the aggregation process is essential for understanding blood flow behavior; such a mechanism has long been sought after for study, particularly in the venular system (Das et al., 1997). As discussed below and previously demonstrated (Barshtein et al., 1998), this mechanism can be elucidated by the kinetics of RBC rouleaux formation.

Previous studies of RBC aggregation have suggested that the aggregation process involves three steps, depending mainly on the shear stress: the formation of short linear rouleaux (composed of several RBCs), formation of long linear rouleaux, and formation of branched rouleaux networks, whose occurrence is decreased with increasing shear stress (Shiga et al., 1983). The process of spontaneous formation of linear rouleaux, as the basis for RBC aggregation, has been the focus of theoretical studies, mainly by Ponder (1924) and Samsel and Perelson (1982). Ponder formulated a model for the growth of average aggregate size (AAS), applying the theory of Smoluchowski for collision between particles. Samsel and Perelson (S and P) viewed the aggregation as a polymer formation and applied related

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formulations to RBC intercellular interactions (see details in the Results and Discussion). The formation of RBC aggregates (rouleaux) has been extensively studied experimentally (Shiga et al., 1983). However, no direct study has been performed to experimentally test the theoretical models, except for the study of Kernic et al. (1973), which supported the assumption of Ponder that RBC aggregation starts with a linear increase of the average aggregate size. The present study was undertaken to test proposed models for the spontaneous formation of linear rouleaux (FLR).

MATERIALS AND METHODS

Blood samples (5 ml) were drawn from healthy volunteers (with their consent, according to Helsinki Committee regulation 98290) into EDTA-containing tubes. RBCs were separated by centrifugation (500 g for 10 min), then resuspended in platelet-free plasma (hematocrit from 0.625 up to 6.0%).

The kinetics of FLR was studied by a computerized cell-flow properties analyzer (CFA) developed in our laboratory for monitoring the process of RBC aggregation in a narrow-gap flow chamber under controllable flow conditions (Chen et al., 1994, 1995). This system, in which RBCs are videotaped during the process of aggregation, provides the distribution of the RBC population into aggregate size (number of RBCs per aggregate) for any stage of the aggregation or disaggregation. This provides a unique tool for analyzing the kinetics of rouleaux formation and examination of the proposed theoretical models. The CFA, which is described in detail in previous publications (Chen et al., 1994, 1995), consists of a thermostatted flow chamber into which 1.0 ml of RBC suspension (6%) is injected and subjected to a continuous (nonpulsatile) flow, induced by applying a constant pressure gradient along the flow chamber. The flow chamber, having a narrow gap of 40 μm between two transparent plates, is placed under a microscope (model DMIL; Leica Microscopy Systems Ltd., Heerburgg, Switzerland) connected to a CCD camera (Pulnex America Inc., Sunnyvale, CA). The digitized RBC images are transmitted to a computer for analysis, which provides the aggregate size distribution. The flow chamber, having dimensions of 1 cm width and 20 cm length between the inlet and the outlet, prevents interference of the boundary shearing formed at the edges of the chamber, thus providing a homogeneous two-dimensional Couette flow in the observed field (Chen et al., 1994). As the RBCs in the middle section (center of the chamber) are monitored, the distance from the inlet to the center allows the control of the shearing history and avoids the entrance effect. The wall shear stress, τ, was calculated by the equation $\tau = \Delta Ph/L$, where ΔP is the pressure difference between the two ends of the chamber, h is the chamber gap, and L is the length of the chamber. As noted above, in this study we focused on the spontaneous formation of linear rouleaux from dispersed cells. As previously shown, maximum dispersion of normal RBCs reaching an AAS of ~1.5 may be achieved already at 2 dyne/cm^2 (Chen et al., 1994). In the present study the RBCs were dispersed by a shear stress of 5 dyne/cm^2, and the process of rouleaux formation from singly dispersed cells was recorded from cessation of flow. For analysis of FLR kinetics, RBC images were taken at intervals of 1 s for the first 10 s from the cessation of flow (t_0) and 5–20 s thereafter until the final aggregation state (2–8 min, depending on RBC concentration). Parameters of the Samsel and Perelson model (see below) were calculated from the analysis of the change in distribution of RBC aggregates (number of cells per aggregate) at different stages of the aggregation process.

THEORY

The theory of Smoluchowski (1917) for the kinetics of aggregation of colloids is based on the assumption that each particle is surrounded by a "sphere influence." Initially, the suspension consists only of single spherical particles, which undergo Brownian motion until they collide. Adhesion between particles requires their penetration into each other's sphere, and once this is achieved all collisions have equal probability of a successful interaction (sticking of particles). This theory also assumes that once formed, the aggregates do not dissociate. If a doublet is formed, the resulting particle undergoes Brownian movement with a reduced velocity until it enters the sphere of influence of another single or multiple particle. As aggregation proceeds, the average diffusion constant of the aggregate population decreases.

The model of Smoluchowski for colliding particles was applied by Ponder to the kinetics of RBC aggregation in stasis (1924). In developing the formula for rouleaux formation in vitro, Ponder limited his model to the average aggregate size (AAS), took into consideration that RBCs are too large for Brownian motion, and made the following assumptions:

1. The distribution of collision velocities is the same for single RBCs and rouleaux of all sizes.
2. The available contact area is the same in all three types of possible collisions: between single erythrocytes (e + e), between a single erythrocyte and a rouleau (e + r), and between two rouleaux (r + r).
3. Every collision achieving the required contact area results in cohesion.
4. Rouleaux, once formed, do not break up (irreversible interaction).

With these assumptions, Ponder (1947) formulated the equation for rouleaux formation (from singly dispersed red blood cells) as a function of time:

$$\mathrm{AAS} = (1 + 0.5KtH), \tag{1}$$

where AAS is the average aggregate size (average number of cells per aggregate), K is a constant, t is time, and H is the total number of RBCs (both singly dispersed and in rouleaux) per unit suspension volume (i.e., RBC concentration). Obviously, when all RBCs are singly dispersed, AAS = 1.

Samsel and Perelson (1982) developed a model for RBC aggregation in which the distribution of the RBC population between single cells and aggregates is considered and the aggregation process is treated as polymer formation, which might include "polymerization" or "condensation." Respectively, a linear rouleau containing n RBCs may be formed by the addition of a single erythrocyte to rouleaux with $n - 1$ RBCs (polymerization), or by condensation of two rouleaux into a larger one. Because individual RBCs may have properties different from those of rouleaux with regard to their membrane mechanics and collision rate, three different rate constants should be considered:

k_{ee}, the rate constant for the interaction between two single RBCs (e + e)

k_{er}, the rate constant for the addition of a single RBC to a rouleau (e + r)

k_{rr}, the rate constant for the interaction between two rouleaux (r + r).

The process of linear RF can thus be described by the change in the concentration of single erythrocytes, E, and of rouleaux, R. $E + R$ = the total number of particles per volume. E is not changed by r + r interaction and R is not changed by e + r interaction. Thus, as shown by Samsel and Perelson (1982),

$$dE/dt = -k_{ee}E^2 - k_{er}RE. \quad (2)$$

$$dR/dt = 0.5(k_{ee}E^2 - k_{rr}R^2). \quad (3)$$

With this assumption, and taking into account that $AAS = E_0/(E + R)$ (the number of total RBC divided by the number of single RBC and of aggregates), combining Eqs. 2 and 3 yields the equation (see details in the Appendix)

$$AAS = 1 + 0.5kE_0t. \quad (4)$$

Equation 4 describes FLR as a process of linear growth, with time as formulated by Ponder in Eq. 1 (Ponder, 1947). Based on this analysis, we experimentally examined the S and P model, by determining if RBC aggregation is performed by both "polymerization" and "condensation," and the validity of the assumption that $k_{ee} = k_{er} = k_{rr}$.

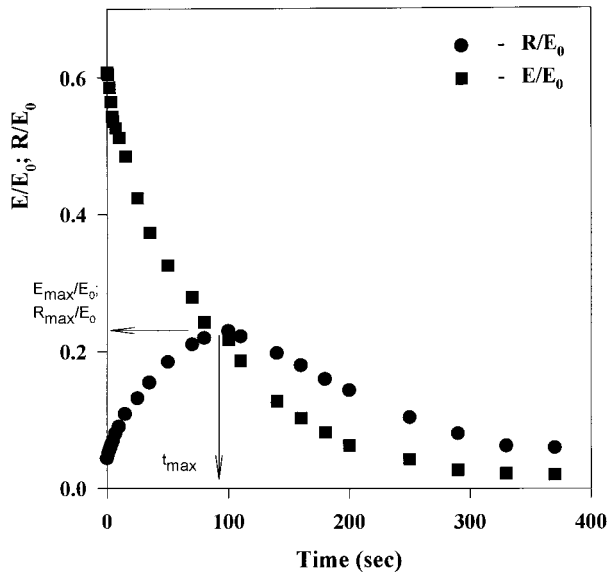

FIGURE 1 The concentration of single RBCs and of rouleaux as a function of the aggregation time. RBCs at the concentration of 2.5% in plasma were singly dispersed by shear stress, and the rouleaux formation was monitored upon cessation of flow ($t = 0$). The values of E and R were determined at each time point with the image analyzer (see Materials and Methods). t_{max} is the time point at which R reaches its maximum value. R_{max} and E_{max} are the values of R and E at $t = t_{max}$. E_0 is the number of total RBCs.

RESULTS AND DISCUSSION

Fig. 1 depicts the RF kinetics in 0.5% RBC suspended in plasma, expressed by the parameters of the S and P model (E and R). This figure shows that the single erythrocyte concentration (E) decreases monotonically, whereas the rouleaux concentration (R) increases up to a maximum value, R_{max}, obtained at t_{max}, after which R decreases to a plateau level. In agreement with the theoretical prediction of Samsel and Perelson (1982), we also found that $R_{max}/E_0 = 0.25$, as shown in Fig. 1. Obviously, if rouleaux are formed only by polymerization (e + e and r + e), no decrease in their number should occur. Thus the decrease in R after t_{max} (Fig. 1) clearly shows that RF includes the condensation mechanism (r + r), i.e., $k_{rr} > 0$, as predicted by S and P. Fig. 1 allows the calculation of the ratio of k_{ee}/k_{rr} and, subsequently, the value of the general kinetic constant (k) as follows: at t_{max} $dR/dt = 0$. Substituting this into Eq. 3 yields the equation

$$k_{ee}/k_{rr} = (R_{max}/E_{max})^2. \quad (5)$$

Using Eq. 5, we derived the experimental values of k_{ee}/k_{rr} for different hematocrits, as depicted in Table 1, and it was found that in all tested cases k_{ee}/k_{rr} is ~1, i.e., $k_{ee} = k_{rr}$.

The initial stage of FLR was found linear up to a critical time t_c, after which deviation from linearity became significant. This is illustrated by Fig. 2, depicting a representative curve of the change of AAS with time. Fig. 3, summarizing all of the experiments, shows that t_c decreases with RBC concentration. This is in agreement with the observation of Kernic et al. (1973), who studied rouleaux formation at low RBC concentration (under slow stirring at 2 s^{-1}) and found that at 4% RBC concentration t_c is ~10 s, which is similar to our observation (Fig. 3). According to the S and P model, linearity in the FLR requires that all of the kinetic constants of the interaction between the RBCs and aggregates be equal. These data thus further confirm the assumption that

TABLE 1 Experimental values of k_{ee}/k_{rr} and k

Sample	RBC (%)	k_{ee}/k_{rr}	k (s^{-1})
I	6	1.05	0.16
II	6	1.1	0.10
III	6	1.0	0.13
IV	6	1.05	0.14
V	5	1.02	0.12
V	2.5	1.05	0.10
V	1.25	1.02	0.11
V	0.625	1.1	0.12

For each blood sample the values of E and R were plotted as a function of time as demonstrated in Fig. 1. The values of k_{ee}/k_{rr} were derived from Eq. 5, using the values of E/R at $t = t_{max}$, i.e., (E_{max}/R_{max}) obtained by the experimental plots. To derive the values of k, for each blood sample AAS was plotted versus time as in Fig. 2, and the values of $d(AAS)/dt$, which is the slope of the linear part of the curve (at $t < t_c$), were substituted in Eq. 4.

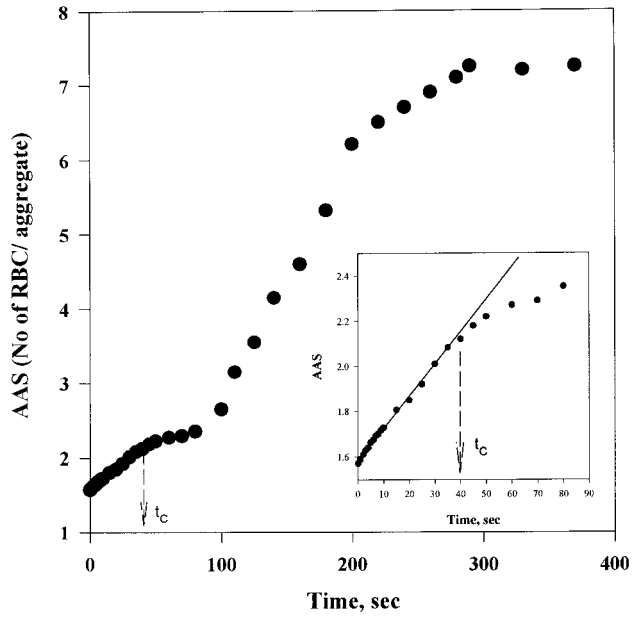

FIGURE 2 A representative curve of RBC average aggregate size (AAS) as a function of time. The same procedure as in the experiment shown in Fig. 1 was performed. t_c is the time point at which the curve starts deviating from linearity.

$k_{ee} = k_{er} = k_{rr} = k$, which is in accord with the model of Ponder (Eq. 1).

Hence only one kinetic constant is required, and this can be derived by application of Eq. 4 to RF at $t < t_c$ (the linear growth phase). The experimental values of k derived in this way (listed in Table 1) show that for all cases k equals $\sim 0.1\ s^{-1}$.

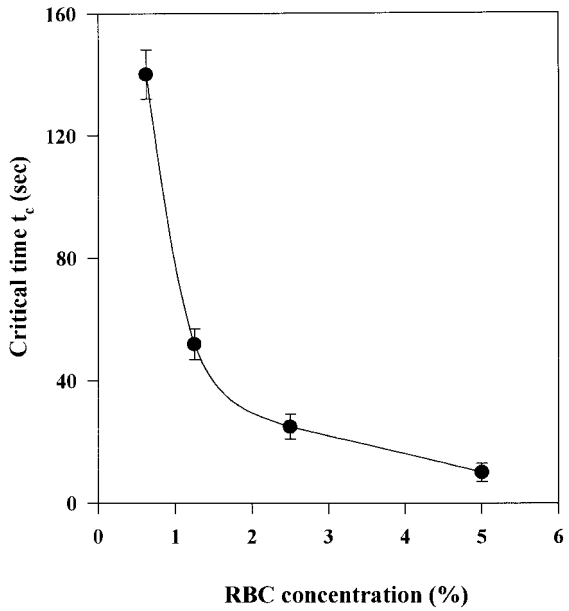

FIGURE 3 Critical time (t_c) as a function of RBC concentration (hematocrit). The same procedure as in the experiment shown in Fig. 1 was performed for each RBC concentration, using blood sample V of Table 1, and the respective t_c values were derived from the curves and plotted versus RBC concentration.

In conclusion, the present study demonstrates that the linear rouleaux growth includes three types of interactions between RBC and their aggregates (e + e, e + r, and r + r), and that the three processes have the same kinetic constants. This supports the mechanism proposed in the model of Samsel and Perelson (1982), in which FLR is performed by both polymerization and condensation of single and aggregated RBCs.

APPENDIX: TRANSFORMATION FROM EQS. 2 AND 3 TO EQ. 4

$$dE/dt = -k_{ee}E^2 - k_{er}RE. \quad (2)$$

$$dR/dt = 0.5(k_{ee}E^2 - k_{rr}R^2). \quad (3)$$

Assuming $k_{ee} = k_{er} = k_{rr} = k$, combining Eqs. 2 and 3 yields

$$dE/dt + dR/dt = -kE^2 - kER + 0.5kE^2 - 0.5kR^2$$

$$= -0.5k(E + R)^2.$$

Since AAS = $E_0/(E + R)$ = the number of total RBCs/the number of single RBCs and aggregates,

$$dE/dt + dR/dt = -0.5kE_0^2/AAS^2. \quad (3a)$$

The equation AAS = $E_0/(E + R)$ can be transformed to $1/AAS = (E + R)/E_0$; differentiation of the latter yields

$$(dE/dt + dR/dt) = E_0 d(1/AAS)/dt. \quad (3b)$$

Combination of Eqs. 3a and 3b yields

$$-0.5kE_0^2/AAS^2 = E_0 d(1/AAS)/dt,$$

which can be reduced to

$$d(AAS)/dt = 0.5E_0k. \quad (3c)$$

Integration of Eq. 3c yields

$$AAS = 0.5kE_0t + AAS_0, \quad (4)$$

where AAS_0 = AAS at $t = 0$.

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