Hematocrit and red cell size are important factors for the transport of blood platelets toward subendothelium in flowing blood. We report that red cell deformability also demonstrated. 

Cholesterol loading and treatment with diamide increased rigidity and increased T. In vitro perfusion experiments in an annular perfusion system with everted human umbilical arteries were performed with perfusates to which such treated red blood cells were added to investigate their influence on platelet adherence to artery subendothelium. Platelet adherence was well correlated with red cell rigidity, with increased adherence at increased rigidity and vice versa. A change in T of 0.10 corresponded to a change in platelet adherence of approximately 50%. These effects were more pronounced at a wall shear rate of 1,800 s⁻¹ than at 300 s⁻¹.

MATERIALS AND METHODS

Perfusion Experiments

Fresh blood from healthy donors was drawn in 1/9 vol of 110 mmol/L trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation for ten minutes at 190 g (20 °C). To 1 vol of PRP, 1 vol of a Krebs-Ringer solution (4 mmol/L KCl, 107 mmol/L NaCl, 2 mmol/L Na₂SO₄, and 20 mmol/L NaHCO₃, pH 5.0), containing 19 mmol/L citrate (plasma concentration) and 27 mmol/L glucose, was added, which gave a final pH of 6.1. Platelets were pelleted (ten minutes, 500 g, 20 °C), resuspended, and washed once in Krebs-Ringer solution at pH 6.1. In the latter suspension (10³ platelets/mL) ¹¹¹In-labeling and aspirin treatment of the platelets was carried out simultaneously. To 9 mL of the platelet suspension, 100 μL ¹¹¹In-oxine solution (100 μCi) (Byk-Mallinckrodt, Petten, The Netherlands), and to enhance the labeling efficiency, 45 μL of oxine (British Drug House, Pool, UK; 6.5 μg/mL dissolved in 95% ethanol) was added. One hundred microliters of 0.1 mmol/L aspirin solution was added (10 μmol/L final concentration) and the suspension was incubated for 15 minutes at 37 °C. The aspirin treatment was done to prevent thrombus formation. Free radiolabel and aspirin were removed by two washes (ten minutes, 500 g, 20 °C) with Krebs-Ringer solution at pH 6.1. The platelets were resuspended in the remaining platelet-poor plasma to a final concentration of 190,000 platelets/μL plasma. Red blood cells were washed three times in isotonic saline by centrifugation (3,000 g, ten minutes, 20 °C). Perfusates (15 mL per experiment) were reconstituted by mixing the reconstituted platelet-rich plasma with the washed red cells to a hematocrit of 0.4 prior to each perfusion experiment. Perfusion experiments with the annular perfusion chamber of Baumgartner were performed by recirculation of 15 mL perfusate in a steady flow system. Arteries were isolated from the human umbilical cord (obtained immediately after birth), stored in buffer (0.2 mol/L TRIS, pH 7.4), and used within 24 hours, after a one-hour treatment with 0.1 mmol/L aspirin in order to inhibit prostacyclin production by the vessel wall.

Perfusion experiments were performed at vessel wall shear rates of 300 s⁻¹ and 1,800 s⁻¹ for three or five minutes at 37 °C. Perfusates were prewarmed for five minutes at 37 °C prior to perfusion.

To obtain a vessel wall shear rate of 300 s⁻¹, a perfusion chamber with a diameter of 6.1 mm was used (effective annular width 1.05 mm) at a flow rate of 35 mL/min. To obtain a vessel wall shear rate
of 1,800 s⁻¹, a perfusion chamber with a diameter of 5.0 mm (effective annular width 0.60 mm) and a flow rate of 100 mL/min was used. Platelet adherence was determined by ⁶¹¹In counting of the artery segments in a gamma counter (Trigamma 600, Baird Atomic Inc, Bedford, Mass). As a control, morphometric evaluation was performed by light microscopy (Leitz Wetzlar dialux 20 EB, E. Leitz GmbH, Wetzlar, FRG) on several segments. Fixation, embedding, and sectioning of the vessel segments were performed according to Baumgartner. Staining was according to Huber et al.¹⁰

**Determination of Red Blood Cell Deformability**

Blood viscosity is mainly determined by the red blood cells, while plasma viscosity and other blood cells are of minor importance. Blood viscosity increases rapidly with increasing hematocrit, but due to red cell deformability, a red blood cell suspension always remains fluid up to 100% packing. Under low shear conditions (< 10 s⁻¹), red cell aggregation is responsible for non-newtonian behavior, causing a shear rate-dependent viscosity, or more properly, apparent viscosity. This apparent viscosity decreases with increasing shear rate, due to the break up of red cell aggregates.¹¹ At shear rates above 100 s⁻¹, no red cell aggregates are present, resulting in a constant apparent viscosity and newtonian behavior. Under newtonian flow conditions, blood viscosity is predominantly determined by the hematocrit, but red cell deformability also plays a role. The more deformable the red cells are, the less blood viscosity will increase with increasing hematocrit.¹²

An empirical relation concerning the viscosity of blood and red cell suspension under newtonian flow conditions was described by Dintenfass:¹³

\[ \mu_r = \mu_0 (1 - TH^{0.6})^{0.25} \]

where \( \mu_r \) is the viscosity of blood or red cell suspension (mPas); \( \mu_0 \) is the viscosity of the suspending medium; \( H \) is the hematocrit (vol/vol), and \( T \) is the so-called “Taylor factor,” a dimensionless parameter, related to red cell deformability. Increasing values of \( T \) indicate decreased red cell deformability (increased rigidity). The \( T \) value, estimated by measurement of viscosity and hematocrit of a red cell suspension, can thus be used as a parameter for red cell deformability. This relationship between blood viscosity and hematocrit is valid for red cells with mean cell volumes (MCVs) between 85 and 100 µm³. For smaller or larger RBCs, the power factor of -2.5 changes. A second prerequisite is that blood should have completely newtonian behavior. Red cell aggregation should be absent. This was attained by performing the viscosimetric studies with RBCs suspended in buffer, in the absence of fibrinogen, and at the highest shear rate possible of 130 s⁻¹.

The viscosity measurements were performed with a Contraves viscosimeter (Contraves Low Shear 30; Contraves A.G., Zurich). The principle of the method is based on the fundamental equation:

\[ \mu = \tau / \gamma \]

where \( \mu \) is the viscosity (mPas), \( \tau \) is the shear stress (dyne/cm²), and \( \gamma \) is the shear rate (s⁻¹). A cylindrical bob is placed in a cylindrical cup with larger diameter, leaving a concentric cylindrical slit of uniform width. The slit is filled with the red cell suspension. During operation, the outer cylindrical cup rotates with known angular velocity, while the central bob is kept in fixed position by means of a torque mechanism. Under this condition, a so-called “Couette-flow” is built up in the cylindrical slit. In such a flow, a linear flow field exists with constant shear rate. The shear rate is proportional to the angular velocity of the outer cup and inversely proportional to the width of the cylindrical slit, while the shear stress is proportional to the torque that is necessary to keep the central bob in fixed position. Exact values for shear rate and shear stress are obtained by calibration. From shear rate and shear stress the viscosity of the sample can be calculated according to equation 2.

Differences in viscosity caused by differences in red cell deformability become more pronounced at higher hematocrits. This was visualized by Dintenfass, in theoretical plots of relative viscosity

\[ (\mu_{relative} - \mu_r) / \mu_r \]

for several different \( T \) values.¹³ T value estimation from viscosity measurements was more sensitive at high hematocrit (> 0.60), while at still higher hematocrit (> 0.80), the flow is unstable. For these reasons, we used suspensions of red cells in isoton saline (1 mL) at a uniform shear rate of 130 s⁻¹ at hematocrit of 0.60 to 0.80 (37 °C). T value can be calculated from equation 1 in a rearranged form, using the relative viscosity and the hematocrit of the suspension:

\[ T = (1 - (\mu_r / \mu)_{observation}) / H \]

The hematocrit was determined with a Coulter counter (model S, Coulter Electronics, Harpenden, England). The \( T \) value determined by this method was used as a parameter for red cell deformability. It could be determined with a variation coefficient of 2%.

**Manipulation of Red Cell Deformability**

Changes in red cell deformability were obtained by treatment of normal red blood cells from healthy donors with various drugs or by cholesterol enrichment. An increase of red cell deformability was achieved by treatment with isoxsuprine (Duphar B.V., Amsterdam) and chlorpromazine (Sigma Chemical Company, St Louis). The incubation with these drugs was for one hour at 37 °C in phosphate-buffered saline (PBS) at a hematocrit of 0.2, at a concentration of 0.5 mmol/L isoxsuprine or 0.5 mmol/L chlorpromazine.¹⁴ Treatment with diamide (Calbiochem, San Diego) and enrichment with cholesterol (British Drug House, Pool, England) decreased deformability. Diamide treatment (0.5 mmol/L), according to Fischer et al.,¹⁵ was for one hour at 37 °C in a medium consisting of 90 mmol/L KCl, 45 mmol/L NaCl, 10 mmol/L Na₂HPO₄/NaH₂PO₄, and 47 mmol/L sucrose, pH 8.0.

Cholesterol enrichment was performed by incubation of red cells overnight at 20 °C in a cholesterol enriching medium according to Shinitzky.¹⁶ Cholesterol enrichment was determined by measuring the cholesterol-phospholipid ratio before and after treatment. Chemical analysis of cholesterol (CHOD-PAP Boehringer Cat. No. 23691, Boehringer, Mannheim, FRG) and phospholipid were done on a total lipid extract of the red cell membrane according to Bligh and Dyer.¹⁸

**RESULTS**

**Effect of Chemical Agents on Deformability**

Normal untreated red cells had \( T \) values in the range of 0.91 ± 0.02 (mean ± SD), obtained from blood samples of 40 healthy donors. Treatment with isoxsuprine, chlorpromazine, or diamide changed red cell deformability, as demonstrated by a change in \( T \) value. The dependence on concentration during incubation on change in \( T \) value is plotted in Fig 1. Figure 1 is the result of the treatment of red cells from one donor and is representative for the results obtained with the red cells of all donors. The absolute changes in \( T \) value, however, differed from case to case, possibly
depending on the initial value of $T$. Nevertheless, isoxsuprine and chlorpromazine treatment consistently yielded $T$ values < 0.90, indicating an increased deformability, while diamide treatment yielded $T$ values > 0.95, indicating a decreased deformability. The incubation procedure used for the perfusion experiments did not affect MCV and red cell shape and caused no damage to the cells. Diamide at or above 5 mmol/L caused an increase at the MCV up to 110 μl, and chlorpromazine at or above 5 mmol/L caused hemolysis.

Microscopic control red cells showed no shape changes upon incubation under these conditions, except for with isoxsuprine at a concentration of 5 mmol/L, where a significant number of cup-shaped red cells was observed (~50%). However, at the concentrations applied in actual treatment procedure (0.5 mmol/L), cup-shaped red cells were only rarely observed (<1%). Cholesterol enrichment also decreased red cell deformability, with $T$ values > 1.0.

The relationship between cholesterol enrichment and change in $T$ values is presented in Table 1. Cholesterol incorporation occurred up to 24 hours (250% of initial value) of incubation, with a parallel increase in $T$ value. Incubation longer than 24 hours (up to 36 hours) gave no further changes, but cell damage, mainly due to spherocytosis, was observed. To avoid cell damage, a standard incubation time was chosen (16 hours), thereby accepting nonoptimal cholesterol incorporation. Also, a large variation of the cholesterol incorporation was observed, possibly due to a biologic variety of the incubation medium.

**Perfusion Experiments With Treated Red Cells**

Treated or untreated red blood cells were compared in perfusion experiments with respect to the changes in platelet adherence that they caused.

The results were obtained from several separate sets of perfusion experiments. In each set of perfusion experiments, 1 unit whole donor blood (500 mL) was processed for perfusion, as described, to yield 20 to 30 perfusates of 15 mL. One sample of the red cells was treated with one of the chemicals or enriched in cholesterol, while a parallel sample was incubated under identical conditions, but without active substance. Thus, each separate set of perfusions consisted of a number of perfusions with treated red cells or an equal number of perfusions with untreated control cells, while platelets and plasma were identical. Difference in platelet adherence obtained with treated red cells as compared to untreated red cells was expressed as a percent difference, taking the untreated control red cells as 100%. Changes in deformability were expressed as a difference in $T$ value between treated and untreated red cells ($\Delta T$). The adherent platelet numbers obtained with normal untreated red cells were 30 to 40 × 10^5 platelets/cm² after three-minute perfusion and 50 to 60 × 10^5 platelets/cm² after five-minute perfusion, which is similar to what has been found in previous studies.5-8 In Fig 2, changes in platelet adherence were plotted against changes in $T$ values obtained from a number of separate sets of perfusion experiments with isoxsuprine-, chlorpromazine-, and diamide-treated red cells at wall shear rates of 300 s⁻¹ (Fig 2a) and 1,800 s⁻¹ (Fig 2b). The results showed an evident association between red cell deformability and platelet adherence. Increase of red cell deformability by isoxsuprine or chlorpromazine treatment (negative $\Delta T$) causes a decrease of platelet adherence, while decreased red cell deformability by diamide treatment caused an increase in platelet adherence. The results of perfusion experiments with cholesterol-enriched red cells are also presented in Fig 2. Cholesterol enrichment also decreased red cell deformability, causing an increase in platelet adherence.
Red blood cells play a dominant role in the process of platelet transport to the vessel wall and the subsequent platelet adherence. In a recent study, we suggested that red cells enhance platelet diffusivity by the so-called “turbulent mixing effect,” according to Keller and Wang and Keller. Red blood cells rotating in the shear field would cause a mixing effect in their surroundings, thereby enhancing diffusivity of blood platelets. With a concentration gradient of platelets between the bulk of the flow and the vessel wall, enhanced platelet diffusivity results in enhanced transport and subsequent adherence of blood platelets. Particles, in this case red blood cells, suspended in a flowing fluid, disturb the flow lines and give resistance to the flow, resulting in an increased apparent viscosity. Rigid particles are not affected by shear forces, but red blood cells are deformed, minimizing the flow disturbance, and as a consequence, resulting in a lowered apparent viscosity. This explains the well-known phenomenon of decreasing apparent blood viscosity with increasing shear rate. In this process, which is called “tank treading,” the internal fluid of the red cell adapts itself to the external flow while the flexible membrane rotates around the cell interior. According to Chien, the rotating disc-shaped red blood cell has a “swept-out volume,” the size of which depends on the deformability of the cell. The more a cell is deformed, the smaller is its swept-out volume. Combining this with the turbulent mixing concept of Keller, it is evident that the mixing effect is diminished with increasing red cell deformability, which results in a decreased platelet diffusivity, causing decreased platelet adherence. Red cell deformability increased with increasing shear rate, so the effect is expected to be more pronounced at a shear rate of 1,800 s⁻¹ than at 300 s⁻¹.

The results of our experiments are in good agreement with these theoretical considerations. Changes in platelet adherence related to changes in deformability, as expressed in ΔT values, were more pronounced at high shear rate (1,800 s⁻¹) than at low shear rate (300 s⁻¹). This indicates that the more deformable red cells are less effective than the more rigid red cells. The effects were best seen in parallel studies in which changes in deformability were related to changes in platelet adherence.

Hardened red cells may have a large trapped volume after packing than the more deformable red cells. Thus, perfusates with hardened red cells, when composed by mixing PRP and packed cells, may have lower hematocrit as a consequence. To estimate the size of this error, we performed studies in which the trapped
volume was measured with \[^{14}\text{C}]\text{sucrose}. Trapped volumes for untreated cells and cells with increased deformability after treatment with isoxsuprine or chlorpromazine was about 2% to 3%. The more rigid red cells, obtained by diamide treatment and cholesterol loading, exhibit only slightly higher trapped volumes of about 5% to 6%. The actual hematocrit in the perfusates composed of these red cells will thus be lower (0.37 or 0.38 instead of 0.40). Due to this lower hematocrit, the enhanced platelet adherence caused by the more rigid red cells is slightly underestimated. The difference is small (5% to 10% less increased platelet adherence) compared to the total effect of 50% to 100% increased platelet adherence caused by the hardened cells.

Whether these studies have therapeutic relevance remains to be determined. Isoxsuprine was originally introduced as a vasodilator, but more recently has been ascribed the putative beneficial effect of improving the microcirculation due to decreased viscosity.23–25 Preliminary data of a clinical study with isoxsuprine showed that prolonged in vivo administration of isoxsuprine enhanced red cell deformability to a similar extent as in vitro incubation.

The clinical relevance of the chlorpromazine data is less clear. Whether in vivo administration of chlorpromazine will cause changes in red cell deformability is unknown, but concentrations required to change red cell deformability in vitro suggest that this is possible. Increase of platelet adherence caused by cholesterol-enriched red cells may have importance in cases of hypercholesterolemia, provided that excess cholesterol is incorporated into the red cells.26–27 In normal individuals, plasma cholesterol levels range from 6 to 7 mmol/L, while patients with hypercholesterolemia can have plasma levels up to 20 mmol/L. Pilot study on red cells from patients with hypercholesterolemia, having plasma cholesterol levels of 10 to 15 mmol/L, showed that these red cells contained no extra cholesterol and had no decreased deformability.

From our results we conclude that red blood cell deformability plays a role in platelet adherence. The influence of red cell deformability in the physiologic range on platelet adherence is less pronounced than the influence of hematocrit and red cell size, as reported in previous studies. Red cell deformability, however, may have a greater clinical importance, because it can be changed by treatment.

ACKNOWLEDGMENT

We thank Dr A. B. T. J. Boink and Dr A. J. H. Maas (Department of Cardiovascular Surgery, University Hospital Utrecht) for offering facilities for viscosity measurement and performing cholesterol determinations.

REFERENCES

22. Wang NH, Keller KH: Solute transport induced by erythro-


Red blood cell deformability influences platelets--vessel wall interaction in flowing blood

PA Aarts, RM Heethaar and JJ Sixma