Rheological properties of the blood influencing selectin-mediated adhesion of flowing leukocytes

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Submitted 17 May 2002; accepted in final form 27 February 2003

Abbitt, Katherine B., and Gerard B. Nash. Rheological properties of the blood influencing selectin-mediated adhesion of flowing leukocytes. Am J Physiol Heart Circ Physiol 285: H229–H240, 2003. First published March 6, 2003; 10.1152/ajpheart.00408.2002.—We studied how the rheological properties of blood influence capture and rolling adhesion of leukocytes as well as their margination in the bloodstream. When citrated, fluorescently labeled blood was perfused through glass capillaries coated with P-selectin, leukocytes formed numerous rolling attachments. The number of attached leukocytes increased as the hematocrit was increased between 10% and 30% and was essentially constant from 30% to 50%. In EDTA-treated blood, adhesion was absent, and the flux of margined cells varied little with increasing hematocrit. However, the velocity of margined leukocytes increased monotonically, whereas the volumetric flow rate was constant, implying that the flow velocity profile became blunted and wall shear rate increased. Thus increasing hematocrit promoted attachment for a given total flow rate, without increasing margination, even though wall shear rate and blood viscosity increased. Blood was diluted to 20% hematocrit with plasma, 40-kDa dextran (to reduce red cell aggregation), or 500-kDa dextran (to enhance aggregation). Increasing aggregation correlated with increasing leukocyte adhesion and with more slow-moving leukocytes near the wall. Thus flowing erythrocytes promote leukocyte adhesion, either by causing margination of leukocytes or by initiating and stabilizing attachments that follow.

ADHESION OF FLOWING LEUKOCYTES to the vessel wall requires expression of endothelial receptors capable of capturing fast-moving cells but is also dependent on the local conditions of flow and the rheological properties of blood. Whereas the molecular mechanisms of leukocyte adhesion have been well characterized (54), the physical constraints on capture from flowing blood are less well defined (59). Leukocytes must become marginated toward the vessel wall to collide with endothelial cells, in a process that depends on the behavior of red blood cells as well as flow dynamics (e.g., Ref. 22). Specialized fast-acting bonds can then be made, typically through receptors of the selectin family. These allow capture and support a rolling form of adhesion during which the bonds are continually broken and new ones made. The slow-moving leukocytes can receive stimuli from the endothelium, which activate their integrin receptors and enable immobilization and subsequent migration. Initial formation of adhesive bonds is limited by the velocity of the flowing leukocytes, whereas the lifetime of the formed bonds depends on the force exerted by the blood flowing over the attached leukocytes (e.g., Refs. 11 and 23). Thus, in general, the movement of leukocytes to the vessel wall and subsequent efficiency of attachment both depend on the physical characteristics of the blood flow.

The physical basis of the margination stage has been well defined. In vitro and in vivo studies have indicated that margination requires the presence of red blood cells and is promoted by their aggregation, which is itself enhanced by a reduction of the flow shear rate (6, 21, 41, 42, 58). Leukocytes were increasingly distributed toward the wall in blood flowing in glass capillaries as wall shear rate or stress decreased (21, 41). If red blood cell aggregation was abolished by replacement of plasma with saline, redistribution was markedly reduced (21, 41), although changes in hematocrit had relatively little effect (21). In narrow and converging microvessels, red blood cells appear to push slow-moving leukocytes to the wall as they flow past them (9, 51).

The effects of the rheological properties of blood on leukocyte attachment, as opposed to margination, are uncertain. Indeed, how closely margination and attachment correlate is unclear, because the two processes have rarely been studied together. Movement of leukocytes to the wall will be a limiting factor, but rate of attachment will also depend on the forces acting on cells and their velocity when they arrive. In vitro adhesion assays have had limited usefulness in studying these phenomena because of their use of purified subpopulations of leukocytes. However, Munn et al. (38) did show that adhesion of flowing, isolated lymphocytes to endothelial cells was promoted by the addition of red blood cells. Systematic variation in blood rheology is difficult in vivo, but in observing adhesion in the mesenteric microcirculation of the rat, Firrel and Lipowsky (16) found that a natural variation in hematocrit between venules had little effect on the number of

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rolling leukocytes. Recent studies in a similar preparation manipulated the degree of red blood cell aggregation in blood by adding dextrans (43). Increasing aggregation was associated with an increasing flux of rolling leukocytes at a low shear rate, a response equated to enhancement of margination. However, fluxes of free-flowing, marginated cells and rolling adherent cells were not separately measured.

To address the above-mentioned uncertainties, we investigated how systematic changes in interrelated hematological and rheological variables (hematocrit, red blood cell aggregation, and viscosity) would influence the number and behavior of leukocytes adhering to a model vessel wall coated with P-selectin. To interpret the results and evaluate the contribution of margination, it was necessary also to measure the number, flux, and velocity of free-flowing leukocytes near the “vessel” wall under identical conditions but with a nonadherent coating. Data on the effects of rheological parameters on the velocities of free-flowing leukocytes near the wall, rather than on overall flux, are lacking from the literature. Experiments were carried out using a whole blood perfusion system (1), adapted from an in vitro adhesion assay using small glass capillaries (10, 13, 47). In vitro studies of leukocyte adhesion using flowing whole blood have been reported recently (1, 26, 37, 48). However, none of these studies defined how the physical properties of blood modified adhesion or margination.

**METHODS**

**Collection of blood and fluorescent labeling of leukocytes.** Venous blood from healthy adult volunteers was collected into citrate-phosphate-dextrose-adenine (CPDA; concentrate diluted 1:80 in blood to give final concentrations of 20 mM D-glucose, 11 mM trisodium citrate, 2 mM sodium acid phosphate, 2 mM citric acid-H₂O, and 0.2 mM adenine; pH 7.4) for adhesion studies or EDTA (1.5 mg/ml) for studies of margination. Citrate chelates Ca²⁺ incompletely and leaves an adequate concentration for leukocyte binding to P-selectin (1). Experiments were completed within 2.5 h of withdrawal, and the order of studies was rotated to account for changes in adhesion with storage (1). Leukocytes were fluorescently labeled using rhodamine 6G (200 g/ml, Sigma; Poole, UK) in citrated blood or acridine orange (100 µg/ml, Sigma) in EDTA-treated blood.

**Measurement and manipulation of hematological and rheological parameters.** For each blood sample, the total leukocyte count and a two-part differential count (“small” lymphocytes vs. “large” neutrophils plus monocytes) were determined using a Coulter counter (Coulter Multisizer II, Coulter Electronics; Luton, UK). Hematocrit was measured by the microhematocrit method after centrifugation of blood at 15,000 g for 5 min. The viscosity of blood at 37°C was determined at shear rates between 20 and 128 s⁻¹ using a Contraves LS-30 Couette viscometer (Contraves AG; Zurich, Switzerland). The aggregation of red blood cells was characterized at 37°C using an erythrogrammometer (Sefam; Van-doeuvre-les-Nancy, France). This device is an adaptation of a Couette viscometer that assesses red cell aggregation based on the intensity of light backscattered by blood (15). The blood is sheared at a high rate to disrupt aggregation and then brought to a stop abruptly to allow aggregation of the red blood cells to commence. The intensity of the backscattered light is measured as a function of time; as aggregation proceeds, the intensity decreases. The parameter used to characterize the aggregation of blood in this study was S₁₀: a measure of the reduction in intensity of backscattered light and hence the degree of aggregation achieved by the red blood cells 10 s after shear was stopped.

The hematocrit of the blood was manipulated between 10% and 50% by adding autologous plasma or red blood cells to the blood. Plasma was removed from blood centrifuged for 10 min at 1,000 g, the buffy coat was discarded, and red blood cells were taken from the center of the pellet. With the use of this method, leukocytes were not subjected to centrifugation, but leukocyte count varied with hematocrit. In some experiments, to avoid a reduction in the leukocyte count, the blood was centrifuged for 10 min at 1,000 g, and the plasma and the buffy coat were removed to a fresh tube. Red blood cells from the pellet were added to achieve a hematocrit of 10%. For comparison, blood at 40% hematocrit was centrifuged and then remixed.

We varied red blood cell aggregation, blood was diluted to 20% hematocrit by the addition of either 1) low-molecular-mass dextran (40-kDa dextran (Dx40), Sigma) at a concentration of 3.25% (wt/vol) in PBS (GIBCO-BRL); 2) autologous plasma; or 3) high-molecular-mass dextran [500-kDa dextran (Dx500), Sigma] at a concentration of 1.6% (wt/vol) in PBS. The dextran solutions were chosen to have a viscosity of 1.2 mPa·s, close to the average for plasma for the donors tested here. Thus, when either was mixed with blood, the suspending phase viscosity was minimally modified. A hematocrit of 20% was chosen to be comparable to the value found in microvessels in vivo (34, 36). The addition of Dx40 dilutes fibrinogen in plasma and decreases red blood cell aggregation. Dx500 increases red blood cell aggregation, with maximum at −1% (wt/vol) (40), the final concentration used here.

As a marker for potential activation, the surface expression of CD11b on neutrophils was analyzed for samples of blood diluted to 20% hematocrit with plasma, Dx40, or Dx500. CD11b was quantitated by immunofluorescence labeling with R-phycocerythrin-conjugated anti-CD11b (R0841, DAKO; High Wycombe, UK) and flow cytometry, as previously described (8). The two dextrans were also compared for any effects on adhesive behavior of isolated neutrophils. Neutrophils were isolated as described previously (10) and diluted to 10⁶ neutrophils/ml in PBS containing 5% autologous plasma and either 2% (wt/vol) Dx40 or 1% (wt/vol) Dx500 (same final concentrations as in blood, see above). The neutrophils were perfused over immobilized P-selectin, and adhesion was quantified (see Flow-based adhesion assay). Samples were also tested without added dextran, but these had lower suspending phase viscosity (buffer viscosity = 0.7 mPa·s vs. −1.0 mPa·s for each of the dextran solutions diluted in PBS) and therefore experienced lower shear stress.

**Flow-based adhesion assay.** Microslides (glass capillary tubes, 5 cm long, with a rectangular cross section of 3 x 0.3 mm) were coated with purified P-selectin (10 µg/ml recombinant human protein produced from stably transfected Chinese hamster ovary cells and lacking the transmembrane and cytoplasmic domains, R&D Systems; Abingdon, UK) and blocked with albumin (1% BSA, fraction IV, Sigma) or coated with albumin alone, as described previously (1). Previously, we (1) tested a range of coating concentrations of P-selectin (1–10 µg/ml) for use with inflow shears with whole blood and found that a concentration of 10 µg/ml supported stable rolling attachments in sufficient numbers for reliable analysis. Rolling velocities obtained were comparable to those seen in the microcirculation of animals exposed to inflammatory
stimuli, indicating that the model was physiologically relevant (1). Coated microslides were connected to a flow system (10), which allowed blood or PBS-BSA to be perfused through them at a chosen volumetric flow rate at 37°C. The microslides were mounted on a fluorescence videomicroscope (Olympus IMT-2 inverted microscope, Olympus Optical, London, UK), which could be tilted on its back on a specially constructed table. Blood was perfused for 4 min. Events at the surface of the microslide were videorecorded for 15 s in each of eight fields observed between 2 and 4 min of inflow. Blood was perfused vertically upward, unless stated otherwise, to avoid artifacts associated with blood sedimentation. Previous studies have indicated that levels of leukocyte adhesion to P-selectin were similar for whole blood perfused vertically upward or horizontally (1), but this might not be the case in diluted blood or when aggregation was enhanced. In experiments with isolated neutrophils, observations were made using phase-contrast microscopy, and microslides were horizontal. In the vertical orientation, adhesion of isolated neutrophils is negligible, demonstrating that sedimentation is essential for adhesive contact in these suspensions (1).

Results of adhesion. Videorecordings were digitized and analyzed off-line using the Optimas 6.2 computer program (Media Cybernetics; Silver Spring, MD). In the stop frame, adherent circular cells were easily distinguishable from nonadherent cells, which were visible as fluorescent streaks with a velocity of >300 μm/s (see RESULTS). The adherent cells were either stationary or rolling (i.e., tumbling continually over the surface) at a velocity far below the free-flow velocity. To quantify the percentage of adherent cells that were stationary or rolling, and the rolling velocity, a sequence of 20 video images was digitized over 5 s. The images were superimposed using a logical “OR” function, and the distance moved by adherent cells was measured. Stationary cells were defined as those with a velocity of <1 μm/s. The number of adherent cells (rolling plus stationary) was counted in a single field of known dimensions at the start of each of the eight 15-s recordings and converted to adherent cells per millimeter squared. The values were averaged over the 2 min and divided by the number of leukocytes that had been perfused by the midpoint, to normalize adhesion for the leukocyte count of the donor (i.e., number of adherent cells/mm² per 10⁶ cells perfused). Adherent cells could be categorized as either small, relatively faint cells or large, brighter cells. The separate counts were normalized by the number of small or large cells counted in the blood by the Coulter counter. The smaller adherent cells were considered to be lymphocytes, whereas the larger cells were judged to be mainly neutrophils, based on previous observations of the nuclear morphology of fixed adherent cells and ability to respond to formyl peptide (1).

Characterization of margination. When viewing a microslide en face, we could not analyze the density and velocity profile of cells throughout its depth. However, we could analyze the density, velocity, and flux of marginated cells flowing near the wall, which represent the cells of interest when interpreting adhesive behavior. First, all cells visible as fluorescent streaks were counted on at least 12 occasions in at least 6 video fields during a 1-min videorecording. The average number was divided by the leukocyte count, to give a normalized number density of cells close to the wall at any time (i.e., number of cells/μm² of 10⁶ leukocytes in the blood). The velocity of each cell was measured from the distance moved between consecutive video frames. We (53) have previously measured velocities up to 6 mm/s using this method. The depth of sharp focus for the lens used was approximately ±7 μm, but all cells were blurred because of their motion, and so restriction of analysis to those in sharp focus (and thus within 14 μm of the wall) was not possible. Some cells were fainter than others, and thus probably further from the surface, but again no objective choice of cells to include in analysis was possible on this basis. Thus we constructed frequency distributions of cell velocities and used the shape of these distributions, and theoretical predictions of absolute velocity at given distances from the wall, to predict which cells were indeed close to the wall (see Prediction of flow parameters in microslides and effects of the non-Newtonian nature of blood). From these distributions, the flux of “marginated” leukocytes crossing a line across the microslide was determined. This was done by multiplying the number of cells in each velocity interval (interval width typically 100 μm/s) by the midvelocity of that interval and summing over the intervals. The result was expressed as a percentage of the known total flux of leukocytes through the microslide. This method could be used to calculate the flux of cells up to a chosen velocity (e.g., the modal velocity). These fluxes characterize the leukocytes near the wall on one side of the microslide (nearest the microscope objective lens). The fluxes on the opposite side were presumably the same (based on arguments of symmetry) when the microslides were oriented vertically.

Prediction of flow parameters in microslides and effects of the non-Newtonian nature of blood. For fully developed, laminar flow of a Newtonian fluid (i.e., with constant viscosity with varying shear rate) passing between infinite parallel plates, there is a parabolic velocity profile between the plates. For a microslide of finite width, the wall shear rate (γw) is given by

$$\gamma_w = 6Q/(w \cdot h^2)$$  \hspace{1cm} (1)

where Q is the volumetric flow rate, w is the microslide internal width, h is the internal depth, and w >> h. The wall shear stress (τw) = γw·η, where η is the fluid viscosity. The velocity of undisturbed flow at any distance x from the wall (x = h/2) is given by

$$v(x) = \gamma_w \cdot x(1 - x/h)$$  \hspace{1cm} (2)

Near the wall (x << h), this reduces to

$$v(x) \sim \gamma_w \cdot x$$  \hspace{1cm} (3)

Equation 2 can be used to predict the velocity of cells with their centroid at any distance from the wall (x, which cannot be less than their radius). Equation 3 can be used to predict the velocity of a cell grazing the wall, with x given the value of the cell radius (−μm). In fact, the presence of a cell disturbs the flow field near the wall, a problem that has been treated by Goldman et al. (20). Their solution for the velocity of a spherical particle flowing in an otherwise linear velocity gradient near a flat wall depends critically on the grazing distance between the particle and the wall, although as this distance increases, the velocity quickly approaches that predicted by Eq. 3. It has been pointed out that if the distance chosen is equal to 1 μm (the scale of a microvillus on the surface of a leukocyte), then the theoretically predicted velocity is very close to the velocity measured for isolated leukocytes flowing along the wall of a rectangular flow channel, and this velocity is only ~10% slower than the value predicted using Eq. 3 (57). The use of Eqs. 2 and 3 is thus adequate to interpret observed cell velocities in parabolic flows. It may also be noted that because microslides have a finite width (w = 10-100 μm), we avoided edge effects by restricting all observations to a series of videomicroscope fields along the centerline. These fields had
a width of ~300 μm, so that we only observed the central 10% of the microslide.

However, in experiments with blood, the situation is complicated because the fluid is particulate and has a non-Newtonian nature (with viscosity decreasing as shear rate increases). First, there is a tendency for the flow profile to be blunted (i.e., plug flow; see, e.g., Ref. 22). The degree of blunting is likely to depend on the hematocrit of the blood (e.g., see RESULTS). For a given volumetric flow rate, plug flow will cause the wall shear rate and cell velocity near the wall to be greater than predicted. Conversely, experimentally observed changes in the velocity of cells near a wall for a given volumetric flow rate can be useful in allowing quantification of changes in the wall shear rate. This has been done experimentally by Tangelder et al. (56), who measured the velocities of fluorescent platelets across the diameter of arterioles and concluded that the wall shear rate was about twice the value that would have been obtained for parabolic flow.

There remains the problem of estimating wall shear stress. Exclusion of red blood cells from the layer closest to the wall, and the tendency of red blood cell aggregates to migrate toward the vessel wall to be lower than the bulk average. This means that the effective viscosity near the wall will be lower than that measured for evenly mixed blood, which in any case would vary with local shear rate. There is no exact mathematical formulation for the problem of predicting wall shear stress in blood flowing through tubes, and one cannot simply multiply the predicted wall shear rate by the blood viscosity measured in a bulk viscometer. However, there is a simple approach to estimating the proportional changes in wall shear stress in microslides with increasing hematocrit. We note, first, that the "apparent viscosity," which can be measured in tubes, is essentially a measure of the pressure drop required to generate a given flow rate for a given tube diameter. It is thus directly proportional to the wall shear stress, based on simple consideration of balance of forces. Second, previous work has shown that the variation in apparent viscosity (measured in tubes with a diameter of the order of microslide dimensions) with increasing hematocrit closely parallels the variation in viscosity measured using a bulk viscometer with increasing hematocrit (45, 49). Finally, variations in blood viscosity with hematocrit, measured in the Couette viscometer, will give an estimate of the proportional changes in wall shear stress when the same blood is perfused through the microslides, even though absolute values cannot be determined accurately. On the basis of our measurements of bulk blood viscosity at 35 s⁻¹ (i.e., average shear rate in the microslides) as a function of varying hematocrit, this approach predicts that the wall shear stress in microslides should increase in the ratios of 1.0, 1.4, 2.0, 3.1, and 4.3 for hematocrits of 10%, 20%, 30%, 40%, and 50% (based on averages from measurements on blood from 6 donors).

The experimental flow rate was chosen to give $\gamma_w = 70$ s⁻¹ (assuming parabolic flow) for several reasons. Previously, we (1) compared the adhesive behavior of leukocytes attaching to P-selectin from flowing whole blood in the range of 35–280 s⁻¹. At 70 s⁻¹, stable rolling adhesion was detected at a level that was reproducible and easily measurable, but the level decreased rapidly if shear rate was increased. Because the level of adhesion was sensitive to changing shear rate around 70 s⁻¹, it should also be sensitive to manipulations of blood rheology that modify shear rate or stress near the wall. Moreover, the range of shear rates across the microslide (0 s⁻¹ in the center to 70 s⁻¹ at the wall) is similar to the range over which red blood cell aggregation occurs. Above this range, aggregates are dispersed (12, 52). Thus, again, blood rheological behavior should be sensitive to manipulations of red blood cell aggregability under the flow conditions chosen. Because attachment and rolling adhesion were observable previously at double or one-half the current shear rate, we also avoided the possibility that we were approaching any shear threshold for leukocyte capture (30). Finally, the observed white blood cell velocities (~1 mm/s; see RESULTS) are within the range typically found in postcapillary venules, and the approximate value for wall shear stress ($\tau_w \sim 0.4$ Pa in whole blood, based on bulk viscosity measured at an average shear rate of 35 s⁻¹) is comparable to values estimated in the same way for venules in vivo (2–5, 14, 17, 24, 25, 27, 28, 32, 46).

**Statistical analysis.** Effects of treatments were tested using ANOVA. Comparison of individual treatments was made by paired t-test when appropriate.

**RESULTS**

**Characteristics of adhesion and margination of leukocytes in whole blood.** When blood (hematocrit ~40%) was anticoagulated with CPDA and perfused through microslides coated with P-selectin at a wall shear rate of 70 s⁻¹, numerous adherent cells were observed. The great majority of adherent leukocytes (>90%) were rolling continuously. Large cells (mainly neutrophils) bound more efficiently than small cells (mainly lymphocytes) when adhesion was corrected for the number of each type of leukocyte in the blood (e.g., Fig. 2). The velocity of rolling averaged 4 μm/s. The small cells rolled more rapidly than the large cells (data not shown), as previously reported (1). This is consistent with our previous observation that lymphocytes adhere less efficiently and roll more rapidly on P-selectin than neutrophils (29).

To independently characterize the margination of leukocytes, EDTA-anticoagulated blood was perfused through albumin-coated microslides. Rapidly moving fluorescent leukocytes were visible near the wall, but no adhesion was observed. The frequency distribution of the cell velocities was strongly positively skewed (modal value = 650 μm/s, mean = 1,100 μm/s) with a sharp lower cutoff at ~500 μm/s (Fig. 1, data for 40% hematocrit and wall shear rate of 70 s⁻¹). Cells cannot come closer to the wall than one cell radius (defining a lower limit for velocity) but will be visible up to some greater distance (defining an upper limit for velocity). The number of leukocytes clearly visible at any instant was quantified, and, from the number density in each interval of the frequency distribution of velocity, flux up to any particular velocity could be calculated. For example, at 40% hematocrit, the total flux of cells visible from one side of the microslide represented ~3% of the perfused leukocytes.

**Effects of variation in hematocrit on adhesion of leukocytes.** Hematocrit was varied between 10% and 50% by adding autologous plasma or red blood cells to native blood. Variation in the number of adherent leukocytes (corrected for blood count) with hematocrit is shown in Fig. 2 for CPDA-treated blood perfused over P-selectin. Total leukocyte adhesion increased with increasing hematocrit up to 30% but was essen-
initially constant for hematocrits between 30% and 50%. When adhesion of large and small cells was analyzed separately, the large cells followed the above trend, but there was no evident variation in adhesion of small cells with hematocrit (Fig. 2). It is notable that increasing hematocrit had no consistent effect on the velocity of rolling adherent leukocytes (Fig. 3).

We estimate that over the hematocrit range tested, wall shear stress should have increased steadily and be approximately fourfold higher at 50% hematocrit than at 10% for a constant volumetric flow rate (see Methods). The patterns of increasing adhesion and steady rolling velocity in the face of increasing shear stress appear to suggest some stabilizing effect of increasing hematocrit. When the video recordings were examined, it appeared that the adherent cells rolled more steadily at higher hematocrit, in that there were fewer events where cells rolled for a period and then detached or skipped over the surface. To quantify any stabilizing effect on rolling, we calculated the coefficient of variation for rolling velocity measured in 12 consecutive periods of 0.4 s for individual cells at 10% and 40% hematocrit. As previously described for leukocytes rolling on selectins (19), there was considerable temporal variation in velocity for each cell. However, the coefficient of variation at 40% hematocrit (41/±10063%) was significantly lower than at 10% hematocrit (49/±10063%) (means ± SE for 12 cells from 3 experiments, P < 0.05 by Student’s t-test). We also checked the coefficient of variation between cells within a sample (rather than with time). There was great intercellular variation (e.g., within-sample coefficient of variation averaged 108% at 40% hematocrit), but there was no consistent effect of hematocrit on this variation (data not shown). This variation presumably described inherent variability in the leukocyte population.

A higher absolute number of leukocytes in blood at higher hematocrit might increase the efficiency of adhesion if the leukocytes, which adhered first, assisted the capture of subsequent cells (i.e., through "secondary adhesion") (7, 37). Thus we also compared blood at 10% and 40% hematocrit with similar leukocyte count. The lower hematocrit was achieved by removing red blood cells from centrifuged blood and then remixing it. The ratio of adhesion at 10% to 40% was 0.65/±0.12 (mean ± SE from 4 comparisons), which is slightly lower than the ratio obtained when hematocrit was adjusted by dilution (data in Fig. 2). Thus the reduction in corrected binding as the hematocrit was lowered did not appear to arise from a reduction in the absolute leukocyte count.

**Effects of variation in hematocrit on margination of leukocytes.** When the distributions of cell velocities were examined under nonadhesive conditions, it was clear that velocities increased with increasing hematocrit (Fig. 4). All distributions were positively skewed, but the lower velocity cutoff and initial peak were right shifted at higher hematocrit. Because the total volumetric flow rate was constant, higher velocities of cells

**Fig. 1.** Frequency distribution of the velocities of leukocytes flowing near the wall of microslides in whole blood anticoagulated with EDTA. Data are from 352 cells measured in 3 experiments, each with a different donor, with an average hematocrit (Hct) of 41%.

**Fig. 2.** Effects of changes in Hct on the number of leukocytes adherent to P-selectin in blood anticoagulated with CPDA. Data are compared for all cells (•), large cells (○), and small cells (△). ANOVA showed significant variation in adhesion in the ranges between 10% and 30% Hct, for all cells or for large cells (P < 0.05 in each case) but not for the ranges between 30% and 50% Hct. Data are means ± SE from 3 to 6 experiments.

**Fig. 3.** Effects of changes in Hct on the rolling velocity of leukocytes adherent to P-selectin in blood anticoagulated with CPDA. Data are shown for 5 separate experiments. Each symbol represents a different donor. ANOVA showed no significant variation in the rolling velocity with Hct.
near the wall indicate that the velocity profile became increasingly blunted with increasing hematocrit and that the wall shear rate increased steadily. For blood at 10% hematocrit, the minimum cell velocity (H9262 m/s) was similar to the value expected for a leukocyte with a 4-\mu m radius flowing adjacent to the wall at H70 s H11002.1. This suggests that there was relatively little deviation from parabolic flow at the lowest hematocrit. Also, at a hematocrit of 10%, the modal velocity was 430 \mu m/s, and the 95th percentile was 1,800 \mu m/s. Assuming parabolic flow, these velocities would occur 6 and 30 \mu m from the wall. The former thus represents cells flowing near the wall, whereas the latter gives an estimate of the maximum depth at which leukocytes were clearly visible. The number of visible cells up to this depth in uniformly mixed blood would be 30 cells/mm² per 10⁶ cells in blood. Directly counting the number of leukocytes visible near the wall, the latter gives an estimate of the maximum depth at which leukocytes were clearly visible. The number of visible cells up to this depth in uniformly mixed blood would be 30 cells/mm² per 10⁶ cells in blood. Directly counting the number of leukocytes visible near the wall at 10% hematocrit yielded an average value of 44 cells/mm² per 10⁶ cells in blood (Fig. 5A). It appears, therefore, that even at a low hematocrit, aggregation of red blood cells was sufficient to marginate leukocytes toward the wall, without disturbing the flow profile detectably.

To relate the characteristics of margination to adhesive phenomena, it is necessary to consider the velocity of leukocytes (which will influence their probability of attaching to an adhesive substrate), the number density near the wall (which shows whether more cells are near the wall than predicted for an evenly mixed suspension), and the flux of marginated cells (which quantifies total delivery to the region of the wall). The number density of all visible marginated cells (corrected for changes in leukocyte count) did not vary significantly with hematocrit, although there was a tendency toward higher numbers at the lower hematocrits (Fig. 5A). As noted above, the velocity of the marginated cells increased steadily with hematocrit; for instance, the ratio of modal velocity at 50% hematocrit to modal velocity at 10% was ~1.8 (Fig. 5B).

Whereas mean or median velocity also increased monotonically with hematocrit, the modal velocity is the most relevant because it represents the slower cells nearest to the wall. These are the cells potentially able to become attached. The positive “tail” of the distribution may be a true representation of a falling off in density of faster moving cells further from the wall, indicating that there is high density of cells traveling with slow velocity near the wall. However, it may
represent a tailing off in the visibility of cells further from the wall.

When calculating the flux of marginated cells at the different hematocrits, it was important to take possible changes in depth of visibility into account. We only included cells up to the modal velocity, which were clearly visible at all hematocrits. The flux of cells up to the modal velocity was not consistently affected by changes in hematocrit (Fig. 5C). Moreover, if the number density of cells was reanalyzed only to include cells up to the modal velocity, there was still no significant variation with hematocrit. Before any conclusion can be drawn, it must be ascertained that there was an equal volume of analysis at each hematocrit (i.e., that analysis consistently included only those near the wall). If, for instance, cells at greater depth were detected and included in the analysis of flux at lower hematocrit than at higher hematocrit, there would be a systematic overestimate of flux at the lower hematocrit. This could hide any increase in the true flux of cells near the wall at higher hematocrit. Underestimate of flux at higher hematocrit could also arise if cells could not be adequately detected near the wall. The latter cannot apply, as evidently we could detect many cells at higher velocity and higher depth than those included in the analysis. The first problem was also avoided, because analysis up to the modal velocity at the lowest hematocrit only included cells up to ~6 μm from the wall (see above). This is little more than the cell radius. In other words, the velocities measured set an upper limit on the depth at which velocity (or the volume of analysis). This is so small that cells out of the immediate region of the wall were not included even at the lowest hematocrit. Thus our analysis up to the modal velocity can reasonably be said to be unbiased in detecting cells close to the wall irrespective of hematocrit. We conclude that the flux of marginated cells close to the wall was similar at all hematocrits, but the marginated cells flowed faster at a higher hematocrit.

**Effects of variation in red blood cell aggregation on adhesion of leukocytes.** Aggregation of red blood cells was varied by diluting blood to 20% hematocrit with Dx40 (to reduce aggregation), plasma, or Dx500 (to enhance aggregation). When the diluted blood was perfused over P-selectin, the number of leukocytes adhering varied in the order of Dx500 > plasma > Dx40 (Fig. 6A). Also, on a sample-by-sample basis, adhesion correlated with the aggregation index $S_{10}$ (Fig. 6B). The trend was evident for large or small adherent leukocytes when they were analyzed separately (data not shown). Figure 6B also shows that there was no overlap between values for $S_{10}$ measured for the different treatments. Microscopic observation of the diluted blood showed an almost complete absence of aggregates when Dx40 was added.

In a previous study (1) of unmodified whole blood, adhesion was the same for horizontal versus vertical flow. However, this might not hold true for low hematocrit or enhanced aggregation, because red blood cell sedimentation would be more rapid. We thus compared adhesion for blood diluted to 20% hematocrit with plasma or Dx500, perfused vertically or horizontally (Fig. 7). In the vertical orientation, adhesion was greater with Dx500 than with plasma, as before. In the horizontal orientation, adhesion was nearly identical for the two diluents. Thus sedimentation in the horizontal orientation nullified the increase in adhesion caused by increased aggregation.

To test for direct effects of dextrans on the leukocytes, we compared the adhesion of isolated neutrophils to P-selectin, with Dx40 or Dx500 added. The numbers of cells bound for Dx40 (131 ± 31 cells/mm² per 10⁶ cells perfused) or Dx500 (151 ± 8 cells/mm² per 10⁶ cells perfused) were nearly identical. Both dextrans gave lower adhesion than PBS alone (259 ± 26 cells/mm² per 10⁶ cells perfused, mean ± SE, $n = 3$ for all treatments). Because the addition of either 2% (wt/vol) Dx40 or 1% (wt/vol) Dx500 to PBS elevated the suspending phase viscosity by ~40%, this reduction of
adhesion may have been due to the increased wall shear stress. In comparison, if we tested the adhesion of neutrophils in PBS alone but at a 40% increased flow rate and hence shear stress, then adhesion decreased by 33 ± 2% (mean ± SE from 4 experiments), slightly less than the ~45% reduction with dextrans. Thus, whereas the differential effects of Dx40 and Dx500 in blood cannot be attributed to direct effects on neutrophils, dextran might modify adhesion slightly. We checked the level of surface expression of the integrin CD11b, as a marker of neutrophil activation in blood. Expression with added Dx40 or Dx500 was 1.05 ± 0.15 or 1.07 ± 0.15, respectively (values expressed relative to the plasma control, means ± SE from 3 experiments), indicating no significant effect of the dextrans.

Effects of variation in red blood cell aggregation on margination of leukocytes. When aggregation was manipulated in EDTA-treated blood, the number of cells observed flowing near the capillary wall was greater when aggregation was enhanced, but inhibition of aggregation had little effect compared with plasma (Fig. 8A). The frequency distributions of velocities (Fig. 9) showed that the lower cutoffs in velocity and the modal values varied little with modification of aggregation, although the positive skew became slightly more pronounced with decreasing aggregation. Consequently, the modal velocity of the cells was constant (Fig. 8B). The flux of visible leukocytes up to the modal velocity was greater after the dilution of blood with Dx500 than with plasma or Dx40 (Fig. 8C). Flux for plasma and Dx40 did not significantly differ. Thus variation in aggregation did not modify the velocity of cells nearest the wall, but the addition of Dx500 increased the flux of these cells.

DISCUSSION

We report here, for the first time, how systematic variations in hematocrit and red blood cell aggregation in human blood modify selectin-mediated adhesion of flowing leukocytes in an in vitro model. To help explain the observed changes in adhesive behavior, the number, velocity distribution, and flux of leukocytes flowing near the model “vessel” wall were independently quan-
measured. Detailed, independent observations of adhesion and margination of leukocytes have not been made previously in a single model. Here, they were made possible by the choice of surface coatings (P-selectin or albumin) and anticoagulants, which enabled unequivocal distinction and characterization of cells that were rolling adherent or free flowing. The results show that for a given volumetric flow rate, changes in the rheological characteristics of the blood modify the number and velocity of leukocytes traveling close to the wall and influence how efficiently they adhere. However, changes in the characteristics of margination do not explain all the changes in adhesive behavior. Specifically, when the hematocrit was increased between 10% and 50%, the efficiency of adhesion increased up to ~30% hematocrit and was constant thereafter. These trends did not correlate well with an essentially constant flux of marginated leukocytes whose velocity increased monotonically with hematocrit. On the other hand, increasing red blood cell aggregation at constant hematocrit correlated with increasing efficiency of leukocyte adhesion, which could be explained largely by an increasing flux of slow-flowing leukocytes near the wall.

Establishment of an increased concentration of leukocytes in the peripheral zone of the bloodstream (i.e., margination) promotes adhesion. Subsequent attachment is predicted to increase with increasing flux of leukocytes near a vessel wall (i.e., product of number density and velocity), as this will increase the frequency of collisions between receptors and ligands. However, increasing velocity (at any given flux) will tend to reduce the efficiency of attachment as the time for bond formation (and hence probability of capture) reduces. Thereafter, the outcome of bond formation depends on the fluid shear stress, or force applied to captured cells (11, 23). Thus, to understand how the physical properties of the blood modified leukocyte adhesion, it was necessary also to carry out analysis of the number density, velocity, and flux of cells near the walls of nonadhesive microslides. For the adhesion studies, we used P-selectin as a physiologically relevant receptor, able to capture fast-moving leukocytes (velocity ~300–600 μm/s for leukocytes close to the wall in this study) and support stable, rolling adhesion. We have shown previously that the number of leukocyte attachments from native whole blood to P-selectin is highly sensitive to shear rate and shear stress around the range used here (1). Thus one might expect changes in blood rheology to modify adhesion even at a constant volumetric flow, through effects on the 1) movement of leukocytes to the wall, 2) flow velocity in that region, and 3) blood viscosity and hence shear forces.

Increase in the hematocrit of the blood did cause a marked right shift in the velocity distribution of leukocytes near the wall. This can be explained by progressive blunting of the “ideal” parabolic flow profile, so that wall shear rate increased (22, 56). Tangelder et al. (56) estimated that the wall shear rate in arterioles was about double that expected for parabolic flow. This was based on measured velocity profiles of fluorescent platelets flowing in 20- to 30-μm arterioles of the rabbit mesentery. Here, in rather larger “vessels,” the velocity of cells near the wall at 50% hematocrit was nearly double the velocity at 10% hematocrit. The lower limit of cell velocity observed in the latter case suggested that flow was close to parabolic at 10% hematocrit. However, the flux of marginated leukocytes was not significantly affected by hematocrit. Nevertheless, adhesion increased up to 30% hematocrit and was then essentially constant. This is puzzling at first sight, because increasing velocity of marginated cells at constant flux would be expected to cause a monotonic decrease in the efficiency of attachment. Moreover, although it is not possible to exactly define the viscosity near the wall, increasing hematocrit is predicted to increase the wall shear stress markedly for a given volumetric flow rate. Hence, the shear stress applied to cells and force applied to formed bonds will increase. It appears there must be other mechanism(s) by which increasing concentration of red blood cells promotes adhesion in the face of hemodynamic trends that should decrease it.

Munn et al. (38) previously suggested that collision of leukocytes with red blood cells would add a dispersive force normal to the wall that would promote contact by leukocytes and stabilize their adhesion. They found that red blood cells at a hematocrit as low as 5% promoted adhesion when added to isolated lymphocytes perfused over endothelial cells vertically upward. Because we (1) have previously found that adhesion was essentially undetectable for isolated neutrophils flowed vertically over P-selectin, the current studies indicate that a hematocrit of 10% is sufficient to increase adhesion to ~50% of its maximal level (Fig. 4). Above 30% hematocrit, we found that the level of adhesion stayed nearly constant. Examining the balance of pro- and antiadhesive physical factors, the increasing normal forces applied by red blood cells that promote attachment appear to outweigh the inhibitory effects of increasing velocity and stress up to ~30%
hematocrit. Thereafter, the effects of increasing normal forces and increasing local flow velocity may have been balanced. The findings that rolling velocity was essentially constant over the whole range of hematocrit and that fluctuations in rolling velocity with time were significantly reduced at higher hematocrit support the concept that an increasing red blood cell concentration continuously stabilized attachments once they were made. Otherwise, the increase in shear stress alone would be expected to increase rolling velocity (31, 33, 35, 44). Increasing deformation of the leukocytes may also have contributed to stabilization of rolling attachments (14, 16).

In fact, the effect of shear stress (independent of shear rate) on adhesion from flow remains poorly defined. When shear stress was increased at a constant shear rate (by increasing fluid viscosity), the frequency of capture of isolated neutrophils on a low concentration of P-selectin did decrease (11). Rinker et al. (50) found that increasing shear stress at a constant shear rate was associated with a more frequent capture of a monocyte cell line on cytokine-treated endothelial cells. Interestingly, they (50) also found that increasing shear stress at a constant shear rate converted intermittent attachments to more stable rolling ones. It seems that the increasing application of force to adherent cells, either through an increasing concentration of red blood cells or fluid viscosity, can effectively stabilize attachments. Nevertheless, increasing shear force appears to decrease initial capture, because here, and previously (39), increasing the suspension viscosity with dextran at a constant shear rate reduced the number of isolated neutrophils that bound to P-selectin.

When we manipulated the red blood cell aggregation by diluting blood with Dx40, autologous plasma, or Dx500, increasing leukocyte adhesion was found to occur in parallel with increasing red blood cell aggregation. Analogous experiments have recently been described where Dx40 or Dx500 was infused into rats, and the flow rates in mesenteric postcapillary venules was also varied (43). Augmentation of aggregation was associated with increased flux of leukocyte rolling in venules, with the maximal effect at low shear rate, close to the wall shear rate used here. In that study, margination and rolling were used essentially as synonyms. However, the two behaviors (fast free flow near the wall versus slower-moving intermittent attachment) are not the same, and margination does not necessarily imply any form of adhesion. The rolling velocities observed in the rat venules were low enough (43) to make it clear that adherent rather than freeflowing cells were measured, and so margination independent of adhesion was not actually quantified.

Nevertheless, the independent measurements of rolling adhesion and margination made here do suggest that the two are linked when aggregation is varied. Modification of red blood cell aggregation had relatively little effect on the flow velocities of leukocytes. The lower cutoff and modal values for cell velocity did not vary with aggregation, suggesting that the wall shear rate and velocity profile were little altered. Elevation of aggregation with Dx500 was associated with a higher number of leukocytes seen instantaneously near the wall. The net result was that “margined” flux was greater when Dx500 was added if only the slower leukocytes (up to the modal velocity) were included in the analysis. Limiting flux up to the modal velocity is reasonable when trying to explain the levels of adhesion, because only leukocytes near the wall (which will be the slowest) can adhere, and faster visible cells were estimated to be up to 30 μm from the wall. Thus the observed increase in adhesion when aggregation was increased with Dx500 can be explained by a greater flux of leukocytes traveling at essentially the same velocity near the wall compared with leukocytes in blood diluted with plasma or Dx40. An increase in leukocyte margination with increased aggregation is in agreement with results of previous in vitro and intravital studies where red blood cell aggregation was promoted (21, 41, 42, 58).

When discussing the relevance of the present study to leukocyte behavior in vivo, the critical, interrelated, experimental factors are the “vessel” size, blood hematocrit, and shear rates and stresses. It is effectively impossible to match all parameters under all conditions, and we sought at least to make wall shear rates (and hence leukocyte cell velocities) and wall shear stresses close to those observed intravitaly (2–5, 14, 17, 24, 25, 27, 28, 32, 46). The microslide dimensions are fixed and larger than postcapillary venules where leukocyte adhesion typically occurs during inflammation. It is difficult to be sure how closely the velocity profile and margination changes seen here will be reproduced in small vessels, although flow blunting and higher than predicted wall shear rate were observed for blood flowing through small arterioles in rabbits (56). Leukocyte adhesion may also occur in larger vessels, for instance, during development of atherosclerosis, so that studies of rheological constraints on attachment are also relevant to these processes.

Hematocrit varies between microvessels (−20–30%) and large vessels (40–45%) due to the Fahreus effect (for a review, see Ref. 18), and our first series of experiments defined the effects of hematocrit on adhesion for this range. The cross section of the microslides (300 μm × 3 mm) is such that there should be little difference between their “tube hematocrit” and the sample feed hematocrit (18). We checked this by suddenly stopping the flow in microslides perfused with blood at 40% or 20% hematocrit and compared the red blood cell count in the feed reservoir and in the microslides (isolated by cutting the connecting tubes at each end). Counts in the microslide and reservoir differed on average only by 2% (2 experiments at each hematocrit), indicating that any Fahreus effect (additional reduction in hematocrit) in the microslides was within the experimental error. For studies of aggregation, we used a 20% hematocrit comparable to microvessels, even though our model represented an intermediate-
sized vessel. Because, again, not all parameters could be matched, we considered that it was better to mimic the blood rheology as far as possible, because we could not control the vessel size. Use of this hematocrit also gave us the practical advantage of simply diluting the blood with dextrans of viscosity equal to plasma. In this way, leukocytes were not disturbed by centrifugation to allow fluid replacement for maintenance of hematocrit, and the suspending phase viscosity was not altered.

With the caveats outlined above regarding the imperfect ability to match in vivo conditions with in vitro models, the present study still sheds light on critical factors affecting leukocyte adhesion, which are relevant to inflammatory and immune responses. Increases in the plasma level of fibrinogen, and hence red blood cell aggregation, occur during the acute phase response associated with acute inflammation and a range of chronic diseases (55). Hematocrit is generally lower in microvessels than in the systemic circulation, and the results presented here indicate that low hematocrits are adequate for promoting contact and adhesion of leukocytes. In addition to increased red blood cell aggregation during inflammation, fluid loss through increased capillary permeability could lead to a local increase in hematocrit. Both factors might promote leukocyte attachment to the vessel wall. Adhesion is ultimately limited by the efficiency with which cells are brought into contact with the wall. Margination, where leukocytes exist at a higher density near the wall than in the center of the vessel, appears to be driven by red blood cell aggregation (21, 41). Even at 10% hematocrit, we observed more leukocytes than expected near the wall, and at 20% hematocrit, increasing the red blood cell aggregation tendency caused a further elevation in the flux of margined leukocytes. Thus aggregation appears to promote margination even at low hematocrits. It should also be noted that collisions with red blood cells will accelerate the diffusion of leukocytes in blood. While this diffusion might be isotropic initially, it assists in repopulating regions near the vessel wall that become depleted of leukocytes after they adhere to the surface. Thus, even in nonaggregating suspensions, red blood cells promote leukocyte adhesion. For instance, leukocytes in blood diluted with Dx40 in the present study, or lymphocytes with washed red blood cells added (38), adhered much more efficiently than isolated leukocytes in studies using vertically orientated tubes. Thus red blood cells critically influence the adhesion of leukocytes in the circulation by facilitating diffusion through collisions, by causing margination when aggregated, by modifying the flow velocity and shear stress near the wall, and apparently by applying normal forces that promote attachment even in the face of increasing shear stress.

This work was supported by Wellcome Trust Grant 050340.

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