Role of Erythrocytes in Leukocyte-Endothelial Interactions: Mathematical Model and Experimental Validation

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ABSTRACT The binding of circulating cells to the vascular wall is a central process in inflammation, metastasis, and therapeutic cell delivery. Previous in vitro studies have identified the adhesion molecules on various circulating cells and the endothelium that govern the process under static conditions. Other studies have attempted to simulate in vivo conditions by subjecting adherent cells to shear stress as they interact with the endothelial cells in vitro. These experiments are generally performed with the cells suspended in Newtonian solutions. However, in vivo conditions are more complex because of the non-Newtonian flow of blood, which is a suspension consisting of 20–40% erythrocytes by volume. The forces imparted by the erythrocytes in the flow can contribute to the process of cell adhesion. A number of experimental and theoretical studies have suggested that the rheology of blood can influence the binding of circulating leukocytes by increasing the normal and axial forces on leukocytes or the frequency of their collision with the vessel wall, but there have been no systematic investigations of these phenomena to date. The present study quantifies the contribution of red blood cells (RBCs) in cell capture and adhesion to endothelial monolayers using a combination of mathematical modeling and in vitro studies. Mathematical modeling of the flow experiments suggested a physical mechanism involving RBC-induced leukocyte dispersion and/or increased normal adhesive contact. Flow chamber studies performed with and without RBCs in the suspending medium showed increases in wall collision and binding frequencies, and a decrease in rolling velocity in the presence of erythrocytes. Increased fluid viscosity alone did not influence the binding frequency, and the differences could not be attributed to large near-wall excesses of the lymphocytes. The results indicate that RBCs aid in the transport and initial engagement of lymphocytes to the vascular wall, modifying the existing paradigm for immune cell surveillance of the vascular endothelium by adding the erythrocyte as an essential contributor to this process.

INTRODUCTION

Cell adhesion under static conditions or in laminar flow in saline solution may not accurately mimic in vivo conditions (Melder et al., 1995). Cell capture to the vascular endothelium is a complex process that depends on the forces of adhesion, the fluid dynamics, and the kinetics of adhesion molecule association and dissociation on the cell surfaces (Hammer and Apte, 1992; Melder et al., 1995; Skierczynski et al., 1995). As a cell contacts the surface, the participation of various adhesion molecules is determined by the force of contact, which must overcome surface protein electrostatic repulsive forces to bring the receptor-ligand pair into close proximity, and the time of contact, which must be long enough to allow at least one bond formation, (Alon et al., 1995).

Even though RBCs constitute ~95% of the particles in blood and 30–45% of the total volume (Cokelet, 1987), they have been largely ignored as contributors to the process of leukocyte adhesion. Because erythrocytes may increase both the contact force and contact time, studies performed in the absence of these cells may be inadequate for extrapolating to in vivo conclusions. For example, a receptor-ligand pair that does not extend very far above the glycocalyx (the glycoprotein-rich envelope surrounding the cell) may not be able to engage in RBC-free saline solution, but with the additional forces imparted by RBCs, the cell membranes may come into closer contact, allowing bond formation (Bell et al., 1984). Also, larger contact forces should result in larger contact areas, thereby increasing the number of adhesion molecules available for binding; this would increase the probability of cell arrest. It has also been suggested that the localization of certain adhesion receptors at the tips of microvilli of the lymphocyte facilitates intermembrane contact and molecular association (Melder et al., 1991; Skierczynski et al., 1995). Once initial attachment has occurred, RBC normal forces may flatten the microvilli, allowing engagement of additional receptors that are in the valley, instead of on the microvilli tips.

The presence of RBCs has been shown to change the spatial distribution of latex beads, platelets, and leukocytes in the flow stream, leading to near wall excesses of these species (Eckstein et al., 1988; Goldsmith and Spain, 1984; Nobis et al., 1985). This is another factor that could contribute to leukocyte binding in blood. Finally, frequent collisions between RBCs and the circulating cell in the free stream cause fluctuations of the cell trajectory (Goldsmith and Karino, 1977; Schmid-Schönbein et al., 1980). This process results in dispersion of the white blood cells (WBCs) and a "random walk" that eventually brings the binding cell to the wall (Eckstein and Belgacem, 1991; Eckstein et al., 1988). Without RBCs, this process is neg-

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ligible and binding should be diminished. WBC-WBC collisions have been neglected in this process because of the much larger number of RBCs ($\approx 5 \times 10^9$/ml) compared with WBCs ($\approx 7.4 \times 10^6$/ml) in blood (Cokelet, 1987). Thus the number of WBC-WBC collisions will be negligible compared with RBC-WBC collisions.

Our recent work (Melder et al., 1995) has provided evidence regarding the above-mentioned aspects of RBC-enhancement of leukocyte adhesion. The present study quantifies the role of these processes using both experimental approaches and mathematical modeling.

Blood rheology

The unusual flow characteristics of erythrocyte suspensions have been the subject of much research. In the years 1917–1938, Robin Fåhraeus pioneered the current understanding of the anomalous viscosity and concentration variations measured in vessels of different size (Goldsmith et al., 1989). Because of differences in the velocity of the erythrocytes relative to the plasma, blood hematocrit decreases as flow proceeds through smaller branches of the vasculature; this phenomenon is known as the Fåhraeus effect and is most pronounced in vessels with diameter <0.3 mm. A related effect, that of decreased viscosity in smaller vessels, is known as the Fåhraeus-Lindqvist effect, and can be explained in terms of a marginal plasma layer that contains few erythrocytes. Because the highest shear rates are found near the vessel wall, a plasma-rich layer in this region decreases the total flow resistance through the tube (Chien et al., 1984).

Since the time of Fårhaeus, many investigators have attempted to understand these effects by considering the two-phase properties of the plasma and RBCs as blood flows through a vessel. It has been shown that the aggregation of RBCs at low shear rate results in the movement of RBC rouleaux toward the center of the flow stream, leaving a plasma-rich layer at the wall (Chien et al., 1967; Cokelet and Goldsmith, 1991). The lower shear rates in smaller vessels are therefore accompanied by a decrease in viscosity. At higher flow rates, however, the rouleaux are exposed to high shear and consequently break up. Blood at high shear rate therefore approaches Newtonian behavior. Migration of RBCs and RBC aggregates to the center of flow changes the flow velocity profile across the tube. The profile may no longer be parabolic, but assumes a shape approaching plug flow; i.e., the profile is more blunt, especially in smaller vessels (Chien et al., 1984; Goldsmith, 1967; Goldsmith, 1971; Schmid-Schoenbein and Zweifach, 1975).

In addition to the ability of erythrocytes to aggregate, the mechanical properties of these cells determine the flow characteristics of blood. The flexibility of RBCs allows them to deform in response to shear stress, lowering the viscosity considerably compared with a suspension of rigid spheres or hardened erythrocytes (Chien et al., 1967). This partially explains the reduction in blood viscosity at higher shear rates (Goldsmith, 1967; Schmid-Schoenbein and Zweifach, 1975).

A number of meticulous studies have revealed the flow characteristics of individual erythrocytes and have shown how these properties contribute to the bulk blood rheology. The behavior of a particle suspension in developed Poiseuille flow depends on a number of factors, including shear rate, particle deformability and the diameter of the particle relative to the tube. It has been shown that at low shear rates and in dilute suspensions, rigid spherical particles follow an axial trajectory through a long tube with no radial migration. On the other hand, any deformable particle will be subjected to a force that results in migration of that particle to the center of the flow stream. This was shown experimentally using tracer RBCs (Goldsmith and Marlow, 1979) and has also been treated mathematically for both rigid particles and fluid drops (Cox and Brenner, 1968; Cox and Hsu, 1977; Leal, 1980). The tendency for migration increases with particle size, consistent with the observation that larger rouleaux occupy the central flow region.

Because radial migration also occurs in the absence of RBC aggregation, a plasma-rich layer exists even at higher shear rates (where aggregation is minimal). The extent of this cell-poor zone has been estimated by microscopic examination of blood flow in vertical tubes (Charm et al., 1966), in flow through glass slits (Palmer, 1965), and in vivo (Schmid-Schoenbein and Zweifach, 1975). These studies conclude that the plasma layer is statistical in nature—erythrocytes occasionally cross this zone to contact the tube wall. The width of the layer varies little with shear stress, but decreases as hematocrit increases (Charm et al., 1966), with average values ranging from 2.5 to 12 μm in the physiological range of hematocrit and shear rate. In opposition to the radial force pushing the RBCs to the axis of flow is the dispersion caused by particle collisions. By tracing the paths of individual RBCs in a concentrated suspension of ghost cells, Goldsmith and co-workers showed that erratic radial fluctuations on the order of a cell diameter occur as the tracer cell collides with other cells in the suspension (Goldsmith, 1971; Goldsmith and Marlow, 1979).

Blood rheology and leukocytes

The preceding discussion of erythrocyte rheology is central to the topic of leukocyte interactions with the vessel wall. In considering the flow of leukocytes through a vessel, the local flow environment of blood must be considered. For example, where rouleau formation occurs, the larger RBC aggregates can exclude leukocytes from the axial flow region, forcing them toward the wall, where they are more likely to interact (Nobis et al., 1985). Even without RBC aggregation, simple dispersion due to collisions with RBCs can lead to increased collision frequency with the vessel wall. The dispersion of particles in shear flow has been

treated theoretically (Eckstein and Belgacem, 1991; Phillips et al., 1992) and observed experimentally with platelets in vivo (Tangelder et al., 1985), with latex spheres in glass channels (Eckstein et al., 1988), and with rigid spheres and leukocytes in ghost cell suspensions (Goldsmith and Karino, 1977). Other studies with platelets in blood have shown a correlation between hematocrit and platelet adhesion, presumably because of RBC-mediated enhancement of delivery to the wall (Turitto and Baumgartner, 1975). Because dispersion is related to the momentum transfer between adjacent layers in the shear flow, the "mean free path" of the tracer particles varies with the local shear rate and is largest near the wall. Using three dimensional Laser-Doppler technology, Blackshear and co-workers (1971) measured the velocities of tracer latex spheres in a RBC ghost cell suspension normal to the surface in channel flow. In this system, the tracer particles had normal velocities toward and away from the surface exceeding 25% of the axial centerline velocity. Keller (1971) extended the analysis by estimating actual collision rates between RBCs and the wall, and found that the collision rates increased linearly with Reynolds number, consistent with the hypothesis that higher shear rates result in more dispersion.

In addition to the increased frequency of collision between WBCs and the vessel wall due to dispersion and rouleau-induced segregation, the behavior of WBCs at the wall can be influenced by the erythrocytes in the bulk flow. The presence of a plasma layer at the wall on the order of a cell diameter may decrease the local kinematic force that pushes the WBC along the vessel, because of fewer RBC collisions. This can be advantageous for a cell attempting to bind to the vascular endothelium, as the axial force in this region is lessened. On the other hand, transient collisions with passing erythrocytes may increase the tendency for a leukocyte at the vessel wall to roll along the endothelium. Using a model system, Schmid-Schönbein et al. (1975) estimated the transient forces imparted to a cell at the vessel wall by collisions with passing RBCs. The measured shear stress varied greatly with time, especially at higher flow rates, with mean values considerably greater than those measured only in the suspending medium (without particles) (Schmid-Schönbein et al., 1975). Taken together, the plasma-rich zone and collisions with passing erythrocytes, predominantly from the radial direction, could trap the cell at the vessel wall and at the same time move it along in the axial direction, thereby increasing the vascular surface area sampled by these cells.

The present study examines the mechanical effects of RBCs on leukocytes interacting with the endothelium to identify and quantify the biophysical mechanisms responsible for binding enhancement in the presence of RBCs. Specifically, we address the following questions: 1) To what extent do RBCs in the flow stream affect the cell rolling and binding on endothelial cell monolayers? 2) Can we develop a theoretical framework to describe RBC-enhanced cell adhesion? After presenting the initial observations and impetus for this work, we will describe the mathematical model, its predictions, and the detailed experimental analysis of RBC-enhanced lymphocyte adhesion.

Initial observations and impetus

The enhancement of T-cell adhesion in erythrocyte suspensions has been described previously (Melder et al., 1995). In this study, we showed that cell adhesion in vivo was qualitatively and quantitatively different from cell adhesion in Hanks balanced salt solution (HBSS) in the parallel plate flow chamber. Suspecting that the flow medium might be responsible for the discrepancies seen, we attempted to mimic in vivo conditions by adding RBCs to the flow medium in the in vitro experiments. This produced dramatic increases in binding efficiencies of T cells to TNF-α-activated human umbilical vein endothelial cell (HUVEC) monolayers over a range of wall shear stresses from 1.1 to 4.6 dyn/cm^2. The effect was significant at all shear rates, but was especially pronounced at the high rates, where no cells were able to bind without RBCs, but many cells bound in the medium containing RBCs. The rolling behavior was also affected by the presence of RBCs, as a higher proportion of cells were induced to roll in the RBC-containing samples. The hypothesis that the RBCs contribute forces that result in enhanced lymphocyte-endothelial interactions formed the basis for the present study.

POPULATION BALANCE MODEL OF CELL INTERACTIONS

To mechanistically define the effects of RBCs in the interactions discussed above, we begin with a mathematical model that describes the behavior of circulating cells as they roll and bind to the endothelium. The system is illustrated in Fig. 1. This simple model based on population balances for the free flowing, rolling and bound cells has the advantage of incorporating few adjustable parameters. It allows direct comparison with experimental data, elucidating the most important and sensitive parameters.

In this treatment, the fluid dynamics above the surface have been decoupled from the interactions at the surface. Other analyses have addressed the details of particle migration in particulate shear flow in an attempt to describe the transport of these particles across shear surfaces (Eckstein and Belgacem, 1991; Phillips et al., 1992). These models are useful in understanding the rheology of the system, but require fundamental assumptions concerning the relationship between migration and shear rate to define a constitutive equation. In the present analysis, we assume, as a first approximation, that the rate of collision with the surface (R_h) is constant with time and distance along the monolayer, which is in agreement with our experimental observations over the distances studied. This eliminates Δy from the analysis and considers only cells that are contacting the surface. Because the convective delivery is much greater than the rate of depletion due to interaction, this assumption

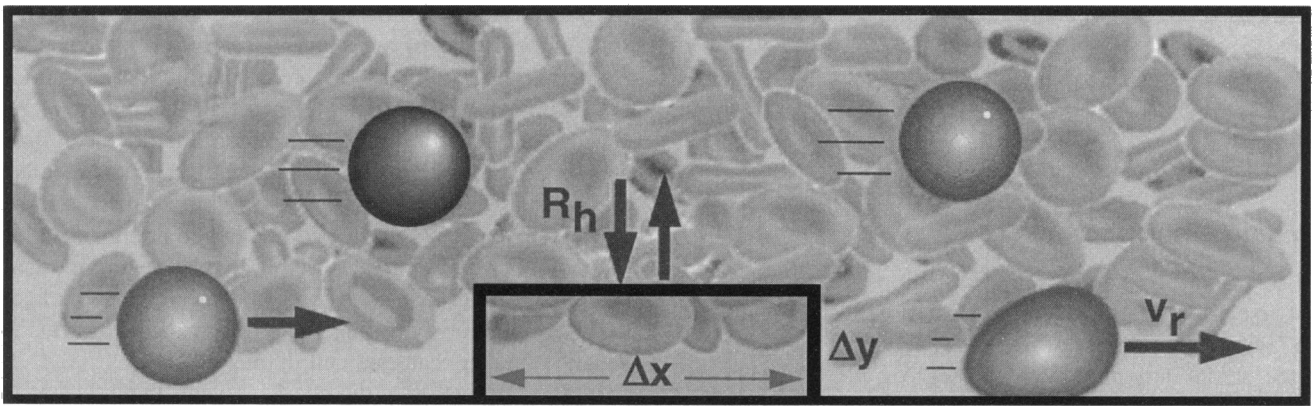

FIGURE 1 Control volume for population balance model (*boxed area*). Fluid flow is from left to right; cells can enter, and leave the volume by rolling on the surface or flowing in suspension. They can also enter or leave from the bulk due to radial fluctuations in trajectory.

is justified. We therefore neglect the depletion of cells in the fluid due to cell interactions at the surface. A population balance on the number of bound cells gives

$$\frac{\partial \sigma_b}{\partial t} = R_{hb} + \sigma_r k_{rb} - \sigma_b(k_{br} + k_{bd}) \quad (1)$$

where σ_b is the bound cell density on the surface, σ_r is the rolling cell density on the surface, t is time, k_{rb} is the roll:bind rate constant, k_{br} is the bind:roll rate constant, k_{bd} is the bind:detach rate constant, R_{hb} is the rate per area at which cells contact the surface and bind without first rolling. The generation terms in this equation are due to newly formed contacts (R_{hb}) and the arrest of rolling cells ($\sigma_r k_{rb}$), whereas the depletion terms are due to detachment ($\sigma_b k_{bd}$) and the inception of rolling of bound cells ($\sigma_b k_{br}$).

Similarly, for rolling cells, the population balance is given by:

$$\frac{\partial \sigma_r}{\partial t} = -v_r \frac{\partial \sigma_r}{\partial x} + R_{hr} + \sigma_b k_{br} - \sigma_r(k_{rd} + k_{rb}) \quad (2)$$

where R_{hr} is the rate at which cells hit the surface and roll, k_{rd} is the roll:detach rate constant, and v_r is the rolling velocity.

This system of coupled equations must be solved with the boundary conditions $\sigma_r = \sigma_b = 0$ $x \leq 0$ (no binding or rolling upstream of the monolayer), and the initial conditions $\sigma_r = \sigma_b = 0$ at $t = 0$.

Parameter values

All of the parameters in the model were estimated from experiments using the flow chamber with T lymphocytes suspended in HBSS or HBSS-RBC mixtures (Melder et al., 1995; Munn et al., 1994, see Materials and Methods). A summary of the parameters with appropriate units and ranges is given in Table 1.

Analytical approximation

Experimental observations indicated that the rolling density reaches a quasi-steady-state value relatively quickly, and that the gradient in the x direction is small within a large spatial range. An approximation for the steady-state rolling density in the region in which the experiments are evaluated can therefore be obtained from Eq. 2 with $\partial\sigma_r/\partial t = \partial\sigma_r/\partial x = 0$, and by noting that $\sigma_b k_{br} \ll R_{hr}$ (the contribution due to the initiation of rolling of bound cells is negligible compared with cells from the free stream hitting and rolling). The rolling density can then be expressed as the ratio of the rate of the initiation of rolling to the total rate of loss due to binding and detachment:

$$\sigma_r = \frac{R_{hr}}{k_{rd} + k_{rb}}. \quad (3)$$

Similarly, the bound density is approximately linear with time in the regime of interest, and does not depend on x. Again, we neglect the contribution of bound cells that detach or begin rolling compared with new collisions, i.e.,

$$\sigma_b(k_{br} + k_{bd}) \ll R_{hb} + \frac{R_{hr}k_{rb}}{k_{rd} + k_{rb}}. \quad (4)$$

TABLE 1 Model parameters

	Definition	Range*	Base value#	Units
k_{rd}	Roll:detach rate constant	0.03–0.12	0.08	s^{-1}
k_{rb}	Roll:bind rate constant	0–0.05	0.025	s^{-1}
k_{br}	Bind:roll rate constant	0–0.004	0.002	s^{-1}
k_{bd}	Bind:detach rate constant	0	0	s^{-1}
R_{hr}	Rate at which flowing cells hit surface and begin to roll	0.01–0.8	0.4	$mm^{-2}s^{-1}$
R_{hb}	Rate at which flowing cells hit surface and bind	0.004–0.13	0.07	$mm^{-2}s^{-1}$
v_r	Rolling velocity	0.01–0.05	0.02	$mm^{-1}s^{-1}$

*Range of values encompasses experiments with and without RBCs. Determinations were performed at a flow rate of 0.08 ml/min (shear rate = 105 s^{-1}).
#Base value refers to the parameter value used in the sensitivity analysis.

With this approximation and substitution of Eq. 3 into Eq. 1 we get

$$\sigma_b = \left(R_{hb} + \frac{R_{hr}k_{rb}}{k_{rd} + k_{rb}}\right)t. \quad (5)$$

Numerical solution

To solve the original system of equations (Equations 1, 2) we first discretize the x coordinate, dividing the length of the layer into a number of regions. This allows replacement of the partial derivative with respect to x with slopes of the function at each x location, thus producing a system of ODEs that is easily solved using the 4th and 5th order Runge-Kutta method. With the discretization, the system of equations is described by a $2n \times 2n$ matrix where n is the number of bins.

Model predictions and sensitivity analysis

Fig. 2 illustrates the sensitivity of the results to the parameters v_r, k_{rd}, R_{hb}, and R_{hr}. Within the ranges of expected values for these parameters (encompassing both conditions with and without RBCs), the model predictions are most sensitive to the rate at which cells initiate adhesion from the bulk stream (Fig. 2, *g* and *h*). The model shows that the rolling velocities affect the results only slightly, because it is the surface density, not the residence time, that governs the behavior of the model (Fig. 2, *a* and *b*). Increasing v_r slightly decreases the rolling and bound densities through the gradient term in Eq. 2. Increasing k_{rd} decreases the steady-state rolling density and the binding rate nonlinearly because of its exponential form; in the region of higher k_{rd}, where we are operating, the model is relatively insensitive to this parameter (Fig. 2, *c* and *d*). Similar results are seen with the other rate constants; they are not as important, in the range of relevant values, as the rate R_{hr}. Because the number of bound cells is also indirectly affected by the number of rolling cells, σ_b is also sensitive to R_{hr}. On the other hand, whereas the bound density is affected by R_{hb} (Fig. 2 *f*), the rolling density is affected very little by variations in this parameter, as bound cells rarely begin rolling (Fig. 2 *e*). As predicted by the analytical approximation (Eq. 3), the rolling density reaches a steady-state value relatively quickly. In addition, although the spatial data have been excluded from the plots for clarity, the gradients in the x direction are small except at the entrance and exit regions of the monolayer.

The results described above suggest that the initial formation interaction is the limiting feature of the process, and that the kinetics of attachment/detachment are less important. R_{hr} is much more important than R_{hb} in this system, mainly because of the arbitrary definition of rolling. Upon contact, every cell translates a finite distance along the chamber before becoming arrested, even if no formed bonds break in the process. This is a simple consequence of contact area formation under fluid shear stress, the extent of which increases with time until arrest or steady-state rolling have been achieved; this process is accompanied by a slight shift of the center of mass. We have chosen to consider as "bound without rolling" only cells that move less than one cell diameter before arresting in the measurement of R_{hb}. Because the majority of cells roll more than one cell diameter before binding, R_{hr} dominates, and scales the results in an approximately linear fashion. Only after a cell is rolling do the rate constants for detachment and binding control the outcome. Furthermore, because of the relative unimportance of R_{hb}, almost all firm adhesion comes from the pool of cells that were previously rolling, even if they rolled only a short distance greater than one cell diameter.

Further inspection of the rate R_{hr} reveals that it is actually composed of 1) the absolute rate of collision with the surface (R_h) and 2) the probability that a collision results in rolling (P_{hr}):

$$R_{hr} = R_h P_{hr} \quad (6)$$

Because of this linear relation, either the rate of collision (governed by cell transport to the vascular wall) or the likelihood that a contacting cell will roll (governed in part by the contact force and contact time) should directly affect the observed rolling and bound densities. Because RBCs can affect both of these processes, we hypothesized a mechanical role for RBCs in leukocyte-endothelial interactions in enhancing R_{hr} through either R_h, P_{hr}, or both.

MATERIALS AND METHODS

Lymphocyte and erythrocyte isolation

Normal human T lymphocytes were isolated from platelet pheresis residue, diluted 1:1 with HBSS, and centrifuged over a Histopaque gradient (Sigma Chemical Co., St. Louis, MO). The lymphocyte layer was depleted of monocytes by incubation with L-phenylalanine methyl ester (PME, E.I. du Pont de Nemours, Wilmington, DE) for 15 min (Leung, 1989). The T lymphocytes were then isolated according to a modified method of antibody-mediated cell depletion using anti-CD19, CD16, and CD33 antibodies (Yaumachi and Bloom, 1993) and using a magnetic depletion technique (Melder et al., 1995). Erythrocytes were obtained from the above procedure as the pellet from the Histopaque gradient. RBCs were washed three times in HBSS before use.

Cell labeling

Lymphocytes were labeled with a cytosolic fluorescent dye to allow easy visualization of the cells against the RBC background. The membrane-permeant form of calcein, calcein acetoxymethyl ester (calcein-AM, Molecular Probes, Portland, OR) is an appropriate dye for this purpose, and is routinely used in our laboratory. This dye was well-retained by the cells, had an extremely strong signal in the 488/530 channel, and caused no detectable change in the adhesion properties of these cells (Weston and Parish, 1990,1992). The cells were loaded with the dye immediately before the flow chamber studies by incubation at 4°C with 1 μM calcein-AM for 30 min.

HUVEC monolayer preparation

For seeding the HUVEC monolayers, clean glass microscope slides were first coated with fibronectin (100 μg/ml). When cell monolayers were required, the excess fibronectin was aspirated and the HUVECs were added at $3 \times 10^5/cm^2$. The monolayers were generally confluent and ready to use

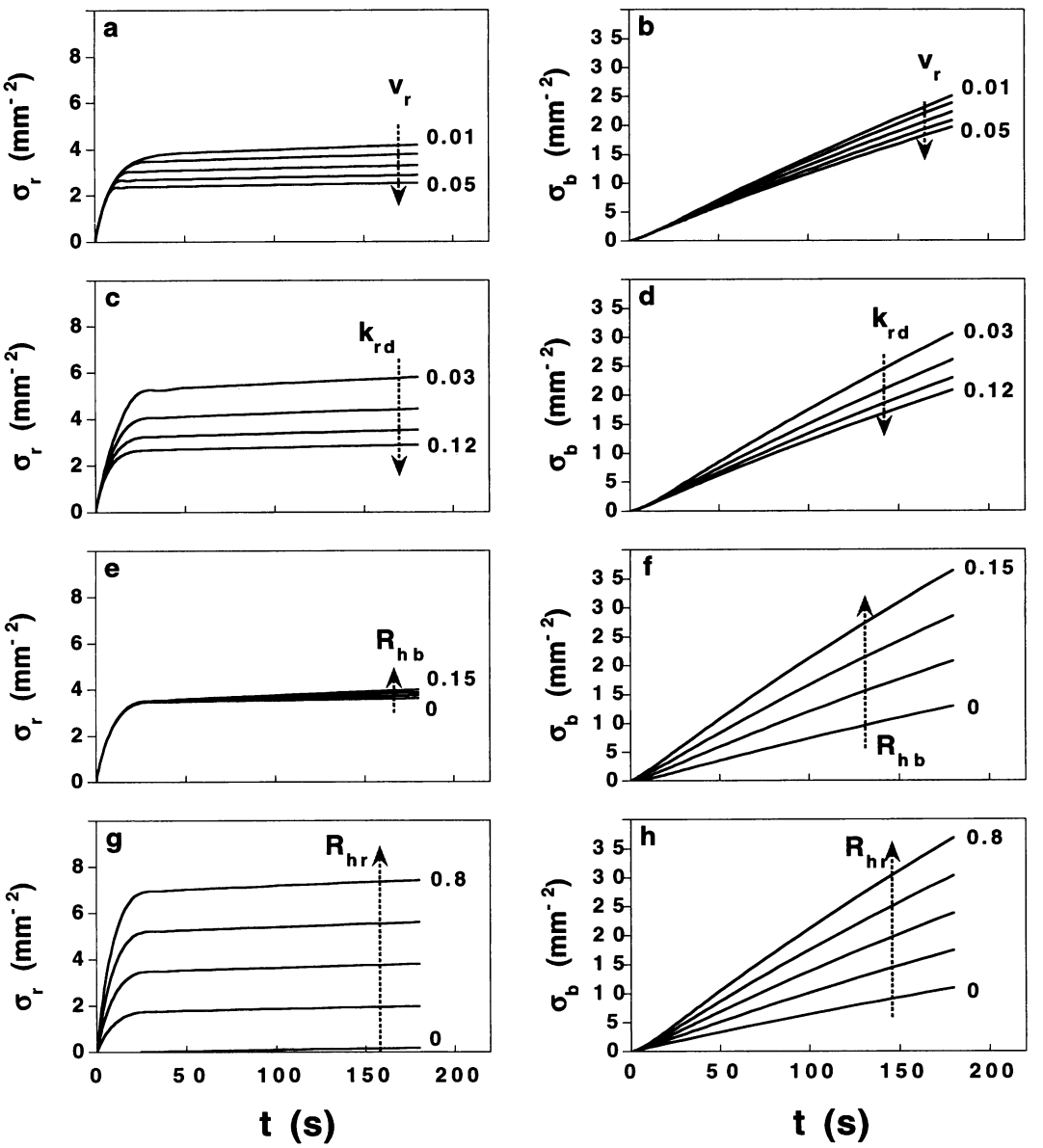

FIGURE 2 Sensitivity of model parameters. Varied parameters for each run are indicated in the plot. The remaining parameters were assigned the base value indicated in Table 1. Each line represents the time course at $x = 800$ μm. The model solution is most sensitive to the rate at which cells collide, and roll on the endothelium, R_{hr}.

within 48 h. In activation experiments, HUVEC monolayers were treated with TNF-α (1 μg/ml).

Flow chamber studies

Adhesion studies used a vertically oriented parallel plate flow chamber. A silastic gasket forms a gap of 78 $\pm$ 4 μm between a plexiglass block and microscope slide when the apparatus is assembled and held together with vacuum. The chamber width (w) is 1.25 cm, and the length is 5.5 cm (Melder et al., 1995; Munn et al., 1994).

In adhesion experiments, cells suspended at 5×10^6/ml were perfused over HUVEC monolayers in the parallel plate flow chamber with and without RBCs. A computer-driven variable speed syringe pump (Harvard Apparatus, South Natick, MA) pulled cells suspended from a reservoir through the chamber. An IBM PC controlled the operation of the pump. Ten fields were sampled at each flow rate and the images recorded on a video cassette. Experiments were performed using a microscope with a vertical stage, so the cell solution flowed vertically through the chamber. This configuration eliminates cell sedimentation toward the surface, allowing examination of rheological effects in the absence of gravitational effects. All adhesion data are expressed in terms of shear rate to avoid complications of estimating the local shear force on a cell at the wall. Although the shear stress at the wall can be calculated from pressure drop measurements, the actual force on a cell rolling or adhered to the wall may be much higher than this force would indicate, and will be transient in time because of stochastic RBC collisions. All data are therefore presented on the basis of equivalent shear rates, assuming parabolic flow.

Flow rates presented in these studies ranged from 0.04 to 0.16 ml/min. The corresponding range of the maximum fluid velocities (assuming parabolic flow) was 1.0 to 4.1 mm/s, and the average fluid velocities ranged from 0.68 to 2.7 mm/s, respectively. The wall shear

rates calculated as $S_w = 3/2 \times Q/b^2w$ (with b the chamber half-height) were 53 to 210 s^{-1}.

Rolling, collision, and trajectory studies

The determinations of rolling velocities were performed on TNF-α (5 h) activated HUVEC monolayers using methods similar to those used in the binding experiments (Melder et al., 1995; Munn et al., 1994). Computer-assisted image analysis located the x, y coordinates of a given cell in consecutive video frames using standard segmenting and particle analysis techniques and the National Institutes of Health-supported Image 1.58 software (available via anonymous ftp at zippy.nimh.nih.gov). Cell coordinate data were then manipulated to give the cell velocity in each frame. For these studies, a cell was defined as rolling if the instantaneous velocity fell below the theoretical free stream velocity of a cell at a distance r (one cell radius) above the surface, and above the minimum velocity for rolling, determined by the noise in the image analysis process. The calculation of the maximum velocity makes use of the wall effects described by Brenner (Brenner, 1961), and the minimum velocity for rolling was set at 20 μm/s. Experiments including RBCs were performed using the vertical stage microscope; those without RBCs were performed using a horizontally oriented stage because of the rarity of interaction in vertical flow without RBCs present. For rolling velocities, images were digitized at 0.067 second intervals (15 frames/s). In the trajectory and collision frequency analyses, videotapes were analyzed at temporal resolution of 30 frames per second to give individual cell velocities in each frame.

Determination of model parameters

The rate constants for the model were determined by frame-by-frame analysis of videotape from experiments with T lymphocytes interacting with TNF-α-activated HUVEC monolayers with or without RBCs present in the HBSS flow medium. The rate constant for rolling cell detachment, k_{rd}, for example, was calculated as the number of rolling cells that detached per unit time normalized to the total number of rolling cells in the same field. The rates of initiation of rolling, R_{hr}, and immediate binding, R_{hb}, were determined by recording the outcome of individual cell collisions (roll, bind, or detach); these were tallied for each category and normalized with respect to time.

RESULTS AND DISCUSSION

The goal of the experimental study was to characterize the firm adhesion and rolling of the lymphocytes to quantify the effects of blood rheology in these processes. The following description of these experiments performed in the presence and absence of RBCs includes 1) a comparison of model parameters and predictions with experimental findings, 2) quantification of the concentration of lymphocytes near the wall under dynamic conditions, 3) a titration of RBC hematocrit as it affects firm adhesion, 4) assessment of the influence of RBCs on rolling velocities and cell trajectories, and 5) estimation of the collision frequencies between the lymphocytes and the monolayer surface. Also included are results from control experiments that show the effect is indeed due to mechanical interactions in the RBC flow, and not due to biochemical activation or viscosity differences.

Enhancement of lymphocyte-endothelial interactions: comparison with the model

The mathematical model suggested that RBCs enhance either the dispersion of the T lymphocytes, or the normal force of interaction, or both. The model parameters and predictions corresponding to the experiments in 0 and 32% hematocrit (Hct) RBCs are shown in Table 2. There were insignificant changes in k_{rb}, k_{rd} and k_{br}. The major changes appeared in R_{hb} and R_{hr}, which increased by an order of magnitude. Also shown in Table 2 are the calculated binding rates and rolling densities at 2 min for the model and experiment. Fig. 3 gives the model predictions for bound and rolling densities for the parameter sets in Table 2, reproducing the dramatic enhancement of interaction seen in experiments.

Assessment of lymphocyte concentration near the wall

We performed further experiments to verify that RBCs increase the rate of onset of rolling, R_{hr}. We first addressed the possibility that the RBCs were affecting the distribution of T lymphocytes in the free stream, thereby enhancing collision frequencies. Previous studies have shown that platelets or latex beads of diameter >2 μm will accumulate near the wall in shear flow because of dispersive interac-

TABLE 2 Model predictions and comparison with experiment

	0% Hematocrit		32% Hematocrit		
	Experimental mean (sd)	Model value	Experimental mean (sd)	Model value	Units
k_{rd}	0.06 (0.03)	0.063	0.08 (0.04)	0.08	s^{-1}
k_{rb}	0.02 (0.03)	0.023	0.02 (0.01)	0.02	s^{-1}
k_{br}	0.002 (0.002)	0.002	0.002 (0.001)	0.00	s^{-1}
k_{bd}	0	0	0	0	s^{-1}
R_{hr}	0.05 (0.04)	0.05	0.5 (0.3)	0.5	$mm^{-2}s^{-1}$
R_{hb}	0.009 (0.005)	0.009	0.08 (0.05)	0.08	$mm^{-2}s^{-1}$
Binding rate*	0.012 (0.02)	0.018	0.15 (0.12)	0.17	$mm^{-2}s^{-1}$
Rolling density#	0.38 (1.2)	0.5	5.7 (5.5)	4.3	mm^{-2}

*Binding rate is the slope of the bound density curve at $t = 0$.
#Rolling density is the value at 150 s after initiation.

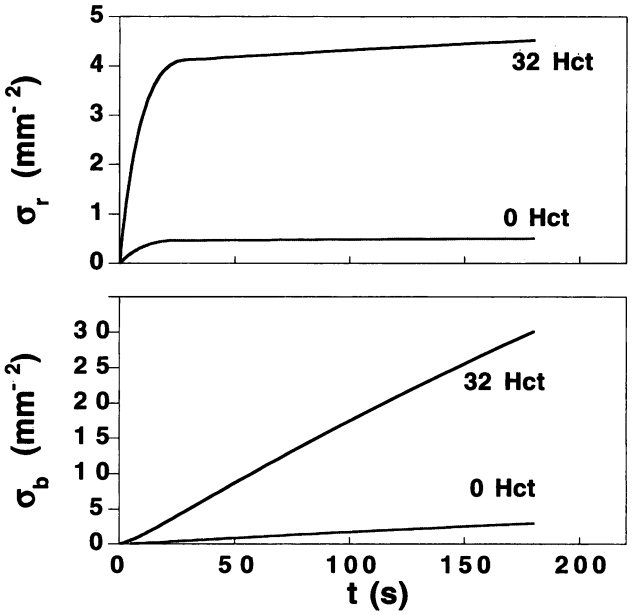

FIGURE 3 Model predictions of bound, and rolling cell density in 0, and 32% Hct RBCs. These plots mimic the behavior seen in vitro. Parameter values were measured in vitro in the parallel plate flow chamber, and are listed in Table 2.

tions with RBCs (Eckstein and Belgacem, 1991; Eckstein et al., 1988; Goldsmith and Karino, 1977). A larger concentration of T lymphocytes near the wall would inevitably cause a higher surface collision rate and increased interaction. However, we found no evidence for higher near-wall concentrations in the presence of RBCs.

Fig. 4 compares the concentration of labeled T lymphocytes near the wall (<13 μm from the surface) in the presence and absence of RBCs. No hematocrit or flow conditions produced near-wall concentrations that were significantly greater than those in RBC-free HBSS. This observation does not directly contradict previous reports, however, because the experimental conditions differed. The previous studies cited used RBCs suspended in dextran-containing saline or plasma with latex beads or platelets. Thus, the mechanical properties and dimensions of the particle of interest are not the same. Also, it is possible that in the presence of dextran or plasma, the ensemble behavior of the RBCs changes, as these media allow aggregation of erythrocytes. Erythrocyte aggregation should be absent in HBSS.

Indeed, Goldsmith and Spain found that in whole blood, leukocytes accumulated at the periphery near the wall in tube flow. In washed blood, on the other hand, they saw no accumulation near the wall, but instead, an average migration of the white cells to the axis of flow (Goldsmith and Spain, 1984). They attributed this difference to the aggregation of RBCs in whole blood, and the lack thereof in washed blood.

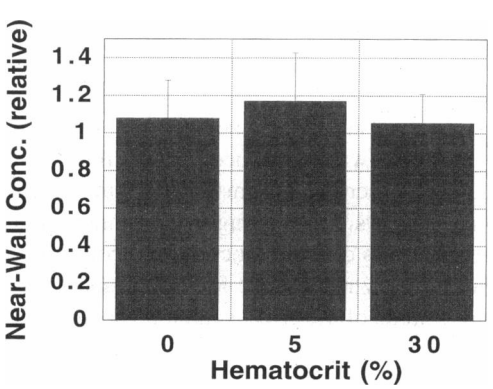

FIGURE 4 Normalized cell concentrations near the surface as a function of hematocrit. Flow rate in all cases was 0.08 ml/min (wall shear rate = 105 s^{-1}). T lymphocytes at 5 × 10^6/ml were perfused through the flow chamber in HBSS with 0, 5, or 30% Hct RBCs, and the number of cells per field within 13 μm of the surface was determined using epifluorescence microscopy, and image analysis of the videotaped experiments. The near wall concentration is calculated as the concentration within this distance normalized to the bulk cell concentration. There were no significant differences in near wall concentration ($p > 0.05$) between the samples with and without RBCs.

Effect of hematocrit

If the hypothesis that physical interactions between the RBCs and the adherent species are enhancing the binding interactions is correct, then the interactions should be affected by the concentration of RBCs in the flow medium. Fig. 5 demonstrates this relationship with T lymphocytes binding to TNF-α-activated HUVEC monolayers suspended in various concentrations of RBCs. It is interesting that the bound density rises with hematocrit but then plateaus at 30% RBCs. Perhaps at the higher concentrations a shrinking of the RBC-poor zone described by other groups (Goldsmith, 1967; Goldsmith and Spain, 1984; Nobis et al., 1985; Schmid-Schoenbein and

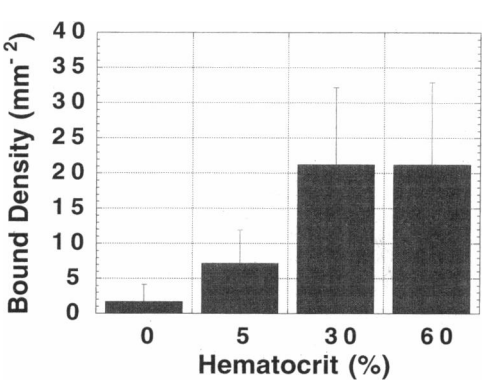

FIGURE 5 The effect of hematocrit on cell binding. T lymphocytes were suspended in HBSS with various concentrations of RBCs, and perfused over TNF-α-activated monolayers at 0.1 ml/min (wall shear rate = 132 s^{-1}). The bound cell density after 10 min of flow is plotted. Error bars represent the standard deviation from the mean of 10 measurements. Enhancement is approximately linear up to a hematocrit of 30%, at which point it levels off.

Zweifach, 1975) increases the number of collisions at the wall, canceling any positive effects of the RBCs on adhesion. In this scenario, increased axial (tangential) forces imparted by the RBC would compete with the larger normal forces that dominate at lower hematocrits where a substantial RBC-poor zone exists.

Rolling velocities

If the RBCs are providing additional forces normal, and possibly tangential, to the surface as a T lymphocyte interacts with the HUVEC monolayer, then the behavior of rolling cells should also be influenced by these forces. Larger tangential forces imparted by passing RBCs might increase the rolling velocity transiently. On the other hand, larger normal forces caused by the increase in torque as a red cell "squeezes" past the rolling cell may increase the contact area and the number of adhesive bonds, and thus slow the rolling. To investigate these possibilities, we measured the velocities of rolling cells at 1/15 s intervals at various hematocrits and flow rates. The data are summarized in Fig. 6 as cumulative frequency histograms. The velocities responded to shear rate as expected-higher shear resulted in higher rolling velocities. Surprisingly, though, the rolling velocities decreased slightly as more RBCs were added. This result suggests that the normal force contributed by passing RBCs is more important than the axial forces in influencing rolling interactions.

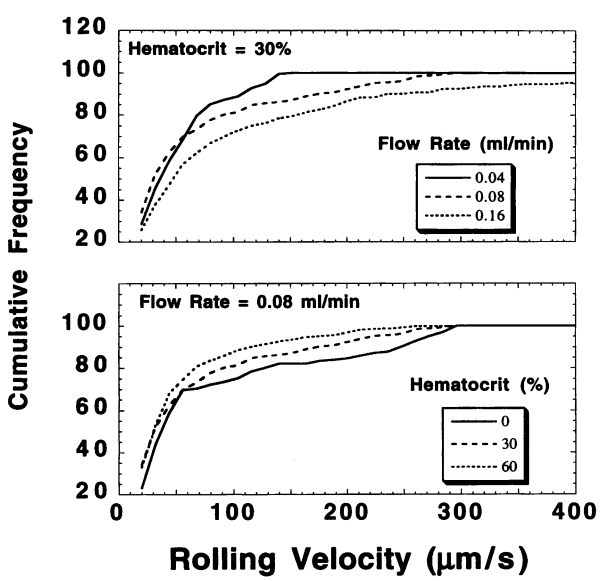

FIGURE 6 The velocities of individual cells were determined at 1/15 second intervals as they rolled on TNF-α activated HUVEC monolayers. Panel *a* shows the cumulative frequency histogram for a hematocrit of 30% for three different flow rates, and panel b shows the dependence of velocity on hematocrit at a constant flow rate. Either increasing the flow rate or lowering the hematocrit results in velocity shifts to higher values.

Cell trajectory analysis

Further evidence for RBC-induced collision enhancement can be gathered by tracking the velocities of individual cells as they pass through the flow chamber and interact with the endothelium. Figs. 7 and 8 show the velocity profiles of six typical cells in the absence (Fig. 7) and presence (Fig. 8) of RBCs. At left in these figures are the velocities as a function of distance in the chamber, and at right are the corresponding time profiles. A reproducible and characteristic feature of these plots is that RBCs affect the maximum velocities before interaction. Inspection of Figs. 7 and 8 reveals that the peak velocities are consistently higher in the presence of RBCs. Without RBCs, detached cells barely exceed the fluid velocity at a distance of one cell radius above the surface (Fig. 7). With the addition of RBCs, however, detached cells can achieve velocities more than double that at a distance of one cell radius (corresponding to the fluid velocity at a distance of 2 cell radii above the surface), and then rapidly reattach a short distance downstream. This is further evidence that RBC-T cell collisions result in lateral motion of the T cells toward the wall, thus facilitating the exit of these cells from the bulk stream and increasing their collision frequency with the surface.

It could be argued, however, that higher fluid velocity near the wall in the presence of RBCs is responsible for the more dramatic accelerations and decelerations in this case. Blunting of the parabolic profile due to RBC migration to the axial flow should decrease the velocity at the center and increase it near the wall. However, our experimental determinations of the free stream velocities of fluorescently labeled T lymphocytes show very small increases in velocity (<5%) due to blunting of the velocity profile, and this effect should be minimal.

Collision frequencies

Additional experiments attempted to directly quantify the collision frequency between fluorescently labeled T lymphocytes and the monolayer surface. Frame-by-frame analysis of videotaped experiments was performed to determine collision frequencies, which increased dramatically with the addition of RBCs. Fig. 9 shows the resulting data at three different flow rates. Although it is clear that increases in collision frequency are at least partially responsible for the enhancement of binding, the magnitude of the effect cannot be deduced from these data. Because the sampling rate in these determinations was 1/30 of a second, any collision that occurred more rapidly than this limit would not be detected. Indeed, in experiments with nonactivated HUVEC monolayers very few collisions were detected (data not shown), indicating that many events of short duration are occurring, and most of these are not detectable using video rate sampling. Only when sufficient adhesion between cells and surface occurs do the interactions last long enough to be measured. The data in Fig. 9 indicate, therefore, that the *detectable* collisions increase in number in the presence of RBCs, implying that either the absolute number of

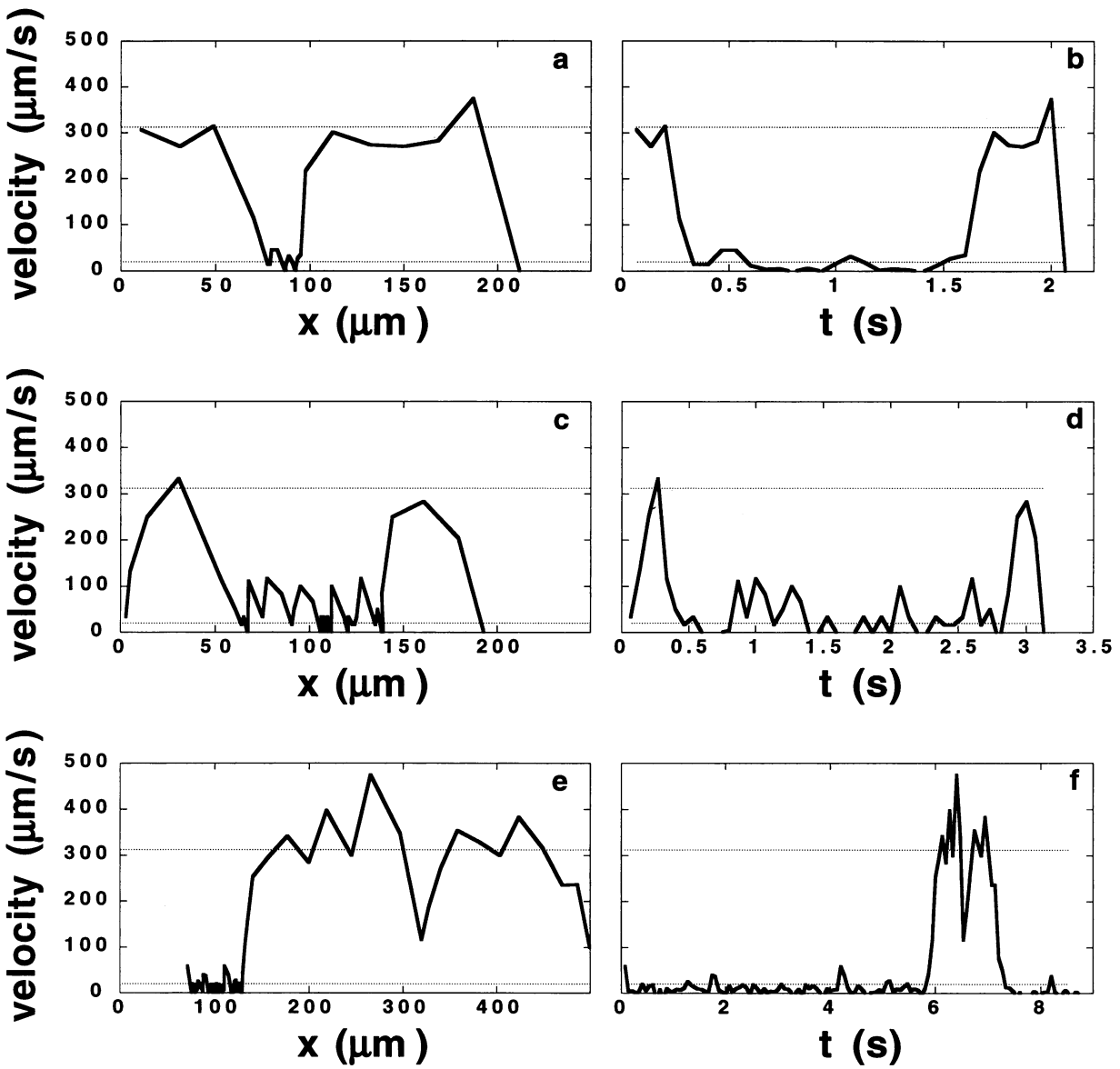

FIGURE 7 Velocities, and trajectories of three cells through the flow chamber at 0% hematocrit. Each cell was tracked at 1/30 second intervals to determine the velocity as a function of distance traveled (*left*), and time (*right*). Also shown are the minimum velocity for rolling (*lower dotted line*), and the fluid velocity at a distance of 1 cell radius from the surface (*upper dotted line*). Flow rate in these experiments was 0.08 ml/min.

collisions increase, or the efficiency of collision increases, or both. This is further evidence for an increase in R_{hr}, but cannot be used to distinguish between the effects of R_h and P_{hr} in Equation 6.

Controls for mechanical interactions

We have shown that the presence of RBCs in the suspending medium enhances lymphocyte interactions with the endothelium in vitro. The enhancement appears to require the physical presence of the RBCs, as pretreatment of the HUVEC cells with an RBC solution does not produce increases in the binding of cells suspended in saline that are subsequently passed over the monolayer-HUVEC monolayers mounted in the flow chamber and superfused with a 32% Hct suspension for 10 min at 0.1 ml/min did not subsequently capture more cells than a control layer pretreated with HBSS alone (data not shown).

It may be proposed that the difference in adhesion is simply due to the increase in viscosity upon the addition of RBCs. Increased viscosity would lead to increased shear stress, but also increased torque on the adhering cell; this could result in larger normal binding forces. But our results show that the differences in viscosity between blood and saline are not responsible for the enhanced binding. Cells suspended in a dextran-HBSS solution (viscosity = 7 cP) bind with the same frequency as those suspended in HBSS (viscosity = 0.89 cP, data not shown).

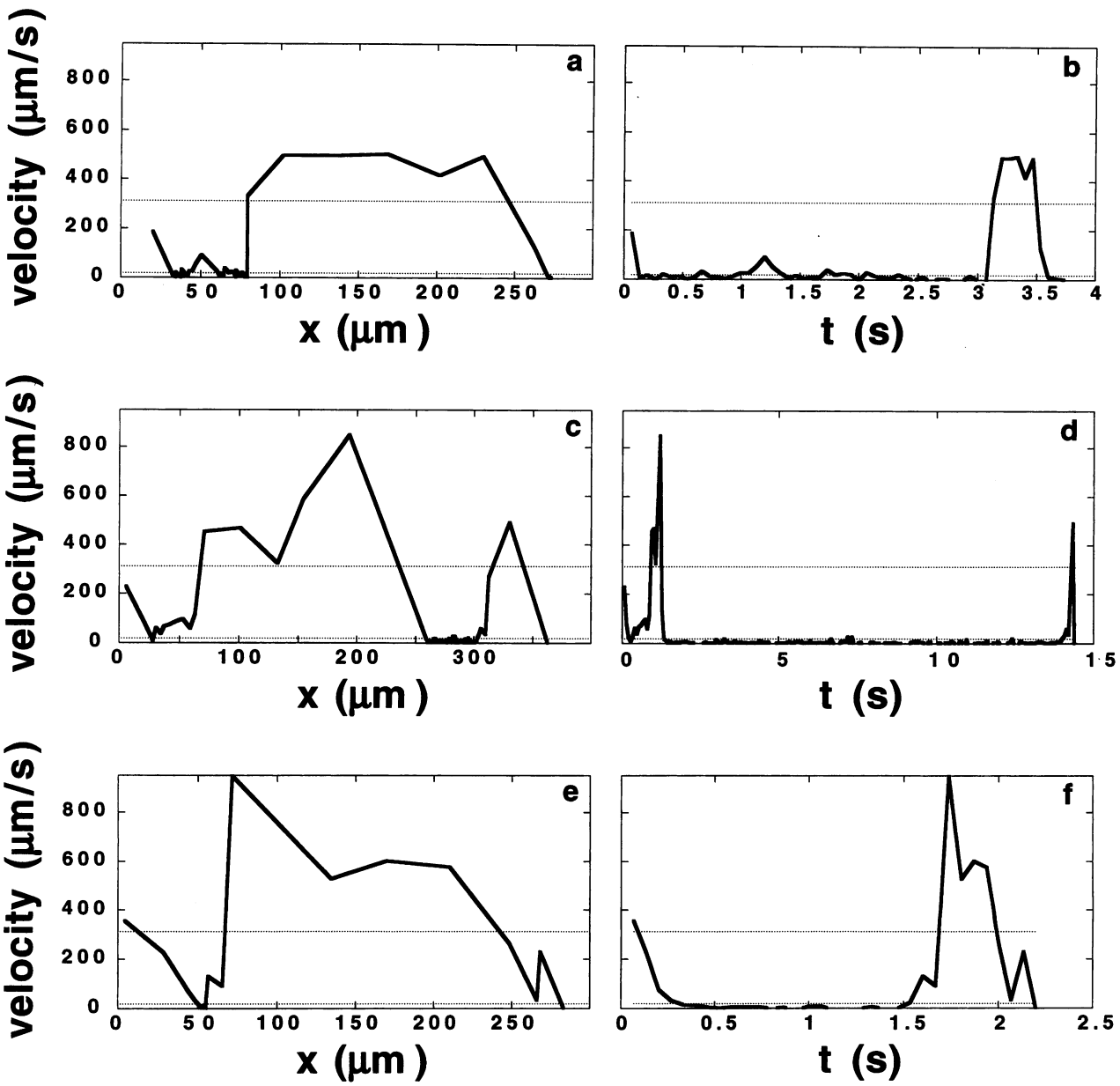

FIGURE 8 Velocities, and trajectories of three cells through the flow chamber at 30% hematocrit. The cell was tracked at 1/30 second intervals to determine the velocity as a function of distance traveled (*left*), and time (*right*). Also shown are the minimum velocity for rolling (*lower dotted line*), and the fluid velocity at a distance of one cell radius from the surface (*upper dotted line*). The maximum velocity achieved before attachment and after detachment is much higher with RBCs present (compare with Fig. 7). Flow rate in these experiments was 0.08 ml/min.

CONCLUSIONS AND IMPLICATIONS

Comparison between the model and data confirmed that the major contribution of RBCs to the adhesion of lymphocytes is in the initial formation of adhesive contact. From the experimental results, we can conclude that the effect is not due to increased concentrations of lymphocytes near the surface in the presence of RBCs, and that viscosity effects and biochemical activation are not important in this phenomenon. Enhancement of adhesion increased with hematocrit up to a value of 30% Hct. Finally, the analyses of rolling velocities provide evidence that the RBCs are contributing additional forces that influence the behavior of lymphocytes in the vicinity of the wall.

The main question that is still unresolved concerns the relative contributions of R_h and P_{hr} in determining R_{hr}: that is, how important is the absolute collision rate, R_h, and how important is the probability of rolling, given that a collision occurred, P_{hr}? Our method for estimating cell contact with the surface probably underestimates the collision frequency between passing cells and the wall. Using the normal velocity near the wall measured by Blackshear et al. (1971), we can estimate the collision frequency that should occur

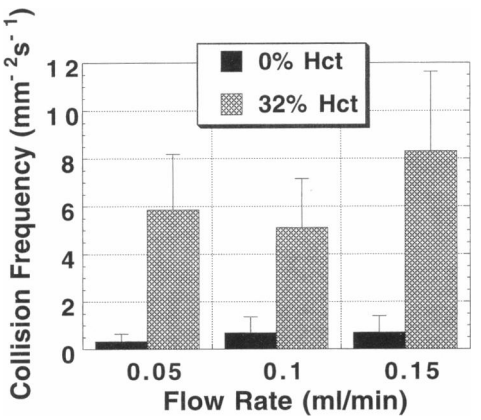

FIGURE 9 Collision frequencies between passing T lymphocytes, and the monolayer surface in the parallel plate flow chamber. Data are limited to collisions that lasted at least 1/30 second. The higher collision frequencies in the presence of RBCs may be responsible for the enhancement of binding seen in this case. The indicated flow rates correspond to wall shear rates of 66, 131, and 197 s^{-1}.

under these conditions. Assuming that half of the cells are moving toward the wall, and half are moving away at a velocity of 10% of the centerline velocity, and that the collision frequency can be approximated by the velocity-averaged flux near the wall, then the calculated collision rate is ~640 mm^{-2} s^{-1}. Given that this is a very rough approximation, it is still a remarkable result, as our observed values were on the order of 6 mm^{-2} s^{-1}. If this calculation is reasonably correct, then the vast majority of collisions between circulating cells and the vascular wall occur on time scales less than 1/30 of a second, and result in no perceptible interaction. Further, because this calculated rate of collision, R_h is two orders of magnitude higher than the value estimated, the efficiency of rolling upon collision, P_{hr}, must decrease proportionally for R_{hr} to maintain its correct value (Eq. 6), and the efficiency of the collisions is therefore extremely small. Only direct measurements of P_{hr} and R_h with high frequency detection systems will resolve this question.

In summary, lymphocyte adhesion to the vascular endothelium is greatly affected by the particulate nature of blood. We have shown that RBCs increase the frequency of adhesion, the rolling behavior and the rate of successful collision with the surface. Although we cannot, at this time, differentiate the relative importance of the RBC normal force from that of increased lymphocyte dispersion, our data suggest that both are important. A possible means by which to distinguish these effects would be through measurements of contact areas and forces, as well as precise determinations of contact frequency.

The present study may modify the existing paradigm for immune cell surveillance of the vascular endothelium, adding the RBC as an essential contributor to this process. Circulating leukocytes must first contact the wall before attaching and extravasating, and are driven by the RBCs to sample a larger fraction of the vascular area. At the same time, the RBCs may promote otherwise unstable rolling interactions. In addition, the results described call for the re-examination of previous in vitro studies performed in the absence of erythrocytes that have quantified the adhesion of leukocyte populations and metastatic cancer cells with endothelial monolayers. Although these studies are qualitatively informative, extrapolation of results to in vivo conditions is not possible without first quantitatively evaluating the contribution of RBCs.

APPENDIX 1

Observations based on the cell trajectory analysis

Upon examining ~250 cell trajectories such as presented in Figs. 7 and 8, a few general observations are noteworthy: 1) The distance that a cell requires to decelerate from the free stream velocity as it contacts the monolayer and either binds or begins rolling is 30–60 μm, independent of hematocrit or shear rate in the range tested. This is apparent by inspection of the decreases in velocity in the left panels of Figs. 7 and 8. The independence of shear rate and cell velocity on the deceleration process suggests that molecular kinetics govern the process. 2) The peaks in the velocity profiles in the left hand panels are asymmetric, whereas those at right are symmetric. The asymmetry in the distance-velocity plots indicates that although the adhering cell requires 2–3 cell diameters to achieve initial capture (decelerate from the free stream velocity), the detachment of these cells requires a much shorter distance (<20 μm). In contrast, the *time* required for a cell to initiate adhesion is comparable to the *time* for detachment. In addition, these times do not appear to vary with flow rate or RBC concentration, suggesting that molecular kinetics have exclusive control over the processes. 3) There are two characteristic length scales involved in the interactions. One is a short length that is consistently on the order of a cell circumference, with the cell maintaining contact with the surface (see for example, Fig. 7, *c* and *d*). This behavior is presumably due to the stochastic rolling caused by nonuniform distribution of adhesion molecules on the lymphocyte surface described by Alon et al. (1995). The other length scale is much longer and more variable, and is characterized by the detachment-reattachment of interacting cells. Previous work has shown that adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin) is heterogeneous on TNF-α-activated monolayers, and that these expression levels correlate with firm adhesion (Munn et al., 1995). It is possible that this surface heterogeneity could be responsible for the detachment of a cell as it rolls into an area of low receptor density. However, topological aberrations in the surface or transient interactions with passing red cells leading to fluid dynamic perturbations (Barbee and Davies, 1995) cannot be ruled out in this process.

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