Influence of erythrocyte aggregation on leukocyte margination in postcapillary venules of rat mesentery

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Pearson, Mark J., and Herbert H. Lipowsky. Influence of erythrocyte aggregation on leukocyte margination in postcapillary venules of rat mesentery. Am J Physiol Heart Circ Physiol 279: H1460–H1471, 2000.—The role of erythrocyte (red blood cell; RBC) aggregation in affecting leukocyte (white blood cell; WBC) margination in postcapillary venules of the mesentery (rat) was explored by direct intravital microscopy. Optical techniques were refined and applied to relate the light-scattering properties of RBCs to obtain a quantitative index of aggregate size ($G$), which, under idealized conditions, represents the number of RBCs per aggregate. WBC margination, defined as the radial migration of WBCs to the venular wall and their subsequent rolling along the endothelium, was measured as the percentage of the potentially maximal WBC volumetric flux within the microvessel lumen ($F_{VBC}^W$). In normal blood, $F_{VBC}^W$ increased exponentially fourfold, and $G$ increased from 1 to 1.15 as wall shear rates ($\dot{\gamma}$) were reduced from a steady-state value of $\sim$600 to <100 s$^{-1}$ by proximal occlusion with a blunt microprobe. Enhancement of aggregation by infusion (iv) of dextran 500 (428 kDa), to attain a systemic concentration of 3 g/100 ml, resulted in a four- and sevenfold increase in G and $F_{VBC}^W$, respectively, as $\dot{\gamma}$ was reduced below 100 s$^{-1}$. Inhibition of RBC aggregation by infusion of dextran 40 (37.5 kDa) caused $F_{VBC}^W$ to fall to one-half of its steady-state level for $\dot{\gamma} < 100$ s$^{-1}$. Thus it appears that the well-known increase of WBC margination with reductions in $\dot{\gamma}$ is strongly dependent on the occurrence of RBC aggregation. Increasing the extent of RBC aggregation during reductions in $\dot{\gamma}$ also increased the firm adhesion of WBCs to the endothelium because of an enhanced probability of contact between leukocytes and the postcapillary venular wall.

erythrocyte sequestration; dextran; Wistar-Furth rats; leukocyte-endothelium adhesion

RECEPTOR-MEDIATED ADHESION of leukocytes (or white blood cells; WBCs) to postcapillary endothelium (EC) is recognized as an essential part of the inflammatory process. It is widely acknowledged that the mechanics and kinematics of WBC-EC interaction may play a significant role in the kinetics of the receptor-ligand bond formation that promotes WBC-EC adhesion. Given that the probability of WBC attachment to the EC is proportional to the frequency at which WBCs strike EC, many studies (1, 4, 8, 9, 20, 21, 30, 35) have examined WBC dynamics during their passage through the capillary network and their subsequent radial migration toward the venular wall (margination).

It has been hypothesized that WBC margination is augmented by the dynamic interaction between WBCs and red blood cells (RBCs) that begins at the exit of the true capillaries. It has been shown by in vitro and in vivo studies (30) that RBCs tend to push the WBC toward the walls of postcapillary venules as blood exits the true capillaries. It has also been hypothesized that the formation of aggregates of RBCs along the venular centerline (where low shear rates promote aggregation) may also enhance the radial migration of WBCs toward the EC as aggregates exclude WBCs from the axial core of RBCs. Vejens (35) observed an increase in WBC margination in vivo when the level of red cell aggregation (RCA) was increased with gelatin or fibrinogen. Further support for this mechanism of margination was provided by Nobis et al. (22) and Goldsmith and Spain (9) by use of small bore glass tubes; they found that RCA enhanced the margination of WBCs and that in the absence of RCA, there was no WBC margination. These findings were supported by in vivo observations of microvascular perfusion with WBC suspensions devoid of RBCs (4), which revealed little or no WBC margination. Additional in vivo assessments of the role of hydrodynamic factors that promote margination have focused on the relative roles of WBC-EC adhesion in venules and hydrodynamic factors. By reversal of the direction of flow within the microvascular network, a significant amount of margination occurred in arterioles, whereas under normograde flow, virtually no arteriolar WBC margination occurred (20). The fact that arteriolar margination with flow reversal occurred to an extent equal to that in venules under normal flow suggests that RBC-WBC interactions may act as a significant impetus for margination and subsequent WBC-EC adhesion.

It is therefore apparent that a quantitative assessment of the effect of RCA on WBC margination may provide valuable insight into the determinants of WBC margination, particularly in light of the potential for enhancing RCA with reductions in wall shear rate ($\dot{\gamma}$).
Influence of Red Cell Aggregation on White Cell Margination

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To date, these processes have been explored only qualitatively because of a lack of suitable instrumentation for providing a direct quantitative measure of the extent of RCA. The present study aims to overcome this deficiency by quantitatively measuring the degree of RCA in postcapillary venules and seeking correlates with the extent of WBC margination and reductions in γ. To this end, in situ methods for evaluating the light-scattering properties of blood in small venules have been applied to calculate an aggregation index, based on the size of RBC aggregates, and to relate this index to the flow of WBCs rolling along the venular wall. The occurrence of RCA was increased by reductions in γ by partial microvessel occlusion with a blunt microprobe, and the extent of RCA was increased further by administration of high-molecular-mass dextran (Dx450; 428 kDa). The results of these manipulations were compared with the effects of administering a low-molecular-mass dextran (Dx40; 37.5 kDa) that is known to act as a disaggregating agent.

METHODS

Animal preparation. Male Sprague-Dawley, Wistar, or Wistar-Furth rats (240–400 g) were anesthetized with pentobarbital sodium (35 mg/kg ip). After tracheostomy, a jugular vein and carotid artery were cannulated with polyethylene tubing for infusions of supplemental anesthetic or removing blood samples, respectively. Systemic arterial pressure was measured with a strain gage pressure transducer (Century Technology model CP-01). The intestinal mesentery was exteriorized via a midline abdominal incision and suffused with warmed (37 ± 1°C) HEPES-buffered (pH 7.4) Ringer solution (4.20 mM HEPES, 0.126 M NaCl, 22.85 mM NaHCO₃, 3.43 mM KCl, and 2.602 mM CaCl₂) with 1% Ringer solution (4.20 mM HEPES, 0.126 M NaCl, 22.85 mM NaHCO₃, 3.43 mM KCl, and 2.602 mM CaCl₂) with 1% polyethylene glycol 8000 (Sigma) previously (17). In brief, light transmitted through a small section of a venule with constant (within 5%) diameter (8). The maximum WBC flux was estimated from the product of systemic WBC concentration ([WBCsys]; no. per mm³, determined by a Coulter Counter) and the venular bulk volumetric flow rate, ignoring the Fahraeus effect for WBCs. The marginal flux at the wall (fwall; cells/mm²) was obtained from video recordings (Panasonic NV 8950) by counting of the number of WBCs that rolled along the EC, past an arbitrary reference point, per minute. The percentage of WBCs that marginated were thus calculated from the expression

\[
F_{\text{WBC}} = \frac{f_{\text{wall}}}{[\text{WBC}_{\text{sys}}] \nu_{\text{mean}} \pi D^2/4} \times 100
\]

where \(V_{\text{mean}}\) is mean blood velocity (in mm/s) and \(D\) is venular diameter (in mm). To enable a comparison of changes in \(F_{\text{WBC}}\) accompanying reductions in γ and minimize its heterogeneity throughout the microvascular network (8), \(F_{\text{WBC}}\) was normalized by dividing by \(F_{\text{WBC}}\) at high values of γ, on the order of 450 s⁻¹, i.e., \(F_{\text{WBC}} = F_{\text{WBC,γ=450}}/F_{\text{WBC,γ=∞}}\).

Preparation of dextran solutions. Dx40 (mol wt 37,500) or Dx500 (mol wt 428,000) solutions were prepared for infusion into the animal from stock solutions made by dissolving 12 g dextran (Sigma Chemicals) in 25 ml of PBS. The dextran solutions were then dialyzed overnight against PBS to remove any impurities and concentrated to ~45% by dialysis against powdered polyethylene glycol 8000 (Sigma).

Spectrophotometric determination of microvessel hematocrit and RCA. Differential spectrophotometry was used to measure the hematocrit (17) and to provide an index of aggregation (14) for RBCs within an individual microvesSEL. To this end, the attenuation of light at two isobestic wavelengths (520 and 546 nm) was measured in real time with the use of two photomultiplier tubes (PMTs), each with the appropriate interference filter placed between it and the light transmitted through the microvesSEL, as described in detail previously (17). In brief, light transmitted through a small blood vessel was projected onto the face of a silicon diode video camera (Dage MTI, model 67). A long working-distance Nikon U20 (×13/0.22 numerical aperture (NA)) objective was used to view vessels in a selected region of the mesentery.

Hemodynamic and blood composition measurements. RBC velocities (\(V_{\text{RBC}}\)) were measured along the vessel centerline by the two-slit photometric technique (36) by online cross correlation of the photometric signatures (IPM, model 102), and mean velocities (\(V_{\text{mean}}\)) were obtained from the well-known empirical relationship \(V_{\text{mean}} = V_{\text{RBC}}/1.6\) (2). Luminal diameters (\(D\)) were measured by the video image shearing method (IPM, model 908) (10). An index of γ was calculated by analogy to that of a Newtonian fluid under conditions of Poiseuille flow, i.e., \(γ = 8V_{\text{mean}}/D\) (15).

Systemic hematocrit and differential WBC counts were measured on blood samples withdrawn from the carotid catheter. Plasma dextran concentration was measured from 0.2-ml blood samples obtained via the carotid artery by use of the anthrone reaction (31). In brief, plasma from the sample was separated by centrifugation, diluted 1:2000 in phosphate-buffered saline (PBS; Sigma), pH 7.4, and added to a 0.2% solution of anthrone in concentrated sulfuric acid. This solution was incubated in a hot-water bath at 95°C for 16 min, after which point the reaction was quenched at 4°C by submersion of the test tube in an ice bath. The absorbance of this solution was measured at 626 nm (Shimadzu UV-160A spectrophotometer) and compared with that prepared from standards of known dextran concentration.

WBC margination was quantified as the percentage (\(F_{\text{WBC}}\)) of the potentially maximum WBC flux flowing within a venule that rolled along the EC lining a 120-μm-long section of a venule with constant (within 5%) diameter (8). The maximum WBC flux was estimated from the product of systemic WBC concentration ([WBCsys]; no. per mm³, determined by a Coulter Counter) and the venular bulk volumetric flow rate, ignoring the Fahraeus effect for WBCs. The marginal flux at the wall (\(f_{\text{wall}}\); cells/mm²) was obtained from video recordings (Panasonic NV 8950) by counting of the number of WBCs that rolled along the EC, past an arbitrary reference point, per minute. The percentage of WBCs that marginated were thus calculated from the expression

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\[
\text{OD}_{\text{total}} = \text{OD}_{\text{abs}} + \text{OD}_{\text{scat}}
\]

where OD is defined in terms of incident (\(I_0\)) and transmitted (\(I\)) light intensities as \(\log(I/I_0)\). For two closely spaced isobestic wavelengths, \(\text{OD}_{\text{scat}}\) is equal at each wavelength, and the differential OD (change in OD (\(\Delta\text{OD}\)) = \(\text{OD}_{\text{abs,546}} - \text{OD}_{\text{abs,520}}\), where \(\text{OD}_{\text{abs,546}}\) and \(\text{OD}_{\text{abs,520}}\) are the absorbance terms at wavelengths of 546 and 520 nm, respectively) is
Fig. 1. Schematic representation of the optical basis for the variation in light scattering due to red blood cell (RBC) aggregation. A: A dispersed suspension of RBCs tends to refract the incident illumination from the substage microscope condenser at an angle greater than the acceptance angle (α) of the microscope objective. B: With RBC aggregation in a suspension of equivalent total hemoglobin concentration in the focal plane, particles with increased surface area and volume refract less light outside the acceptance angle of the objective, and a greater intensity of light is transmitted through the suspension (lower scattering optical density). The effective no. of particles per aggregate (G) was computed on the basis of the theory of large tenuous scattering particles (see Ref. 34) by use of Eq. 6.

Proportional to the product of hemoglobin (Hb) concentration, the difference in molecular extinction coefficients at the two wavelengths, and the pathlength of the microvessel (diameter), thus yielding the relationship

$$\text{Hct}_{\text{micro}} = K \frac{\Delta \text{OD}}{D}$$

where \( \text{Hct}_{\text{micro}} \) is the microvessel hematocrit, \( D \) is the vessel diameter (in μm), and \( K \) is a constant dependent on mean cell Hb concentration and the magnitude of the extinction coefficients at each wavelength (17). In vitro calibration studies, using small bore glass tubes, yielded a value of \( K \) equal to 51.6 when \( \text{Hct}_{\text{micro}} \) was expressed as a percentage (100 × packed cell fraction) (17).

To permit a rapid determination of the magnitude of \( \text{OD}_{\text{scat}} \), \( \text{OD}_{\text{total}} \) was monitored at 520 nm in real time by inputting the PMT voltages (proportional to \( I_{520} \) and \( I_0–520 \)) to an analog log-ratio module (Analogue Devices). Taking advantage of the fact that the molecular extinction coefficients of Hb at 520 and 546 nm differ by a factor of ~2.0, such that \( \text{OD}_{\text{abs},546} \approx 2 \text{OD}_{\text{abs},520} \), and then by applying Eq. 2 at the two isobestic wavelengths, it is easily shown (17) that \( \text{OD}_{\text{scat}} = \text{OD}_{\text{abs},520} - 2 \text{OD}_{\text{abs}} \).

In practice, the relative magnitudes of \( \text{OD}_{\text{abs}} \) and \( \text{OD}_{\text{scat}} \) are dependent on the NA of the microscope objective. As NA is increased, more light is captured by the objective and hence the magnitude of \( \text{OD}_{\text{scat}} \) is reduced. For the NA used here (0.22), \( \text{OD}_{\text{abs}} \) and \( \text{OD}_{\text{scat}} \) comprised ~25 and 75% of \( \text{OD}_{\text{total}} \), respectively. This relatively large contribution of the scattering component permits quantitative estimates of the size of the scattering particle on the basis of the theory of light scattering by “large tenuous scatterers” developed by Twersky (34). As shown therein and validated by in vitro studies of blood flow in small bore glass tubes (16, 17), the scattering component of OD may be expressed as

$$\text{OD}_{\text{scat}} = -\log[10^{-\alpha} + q(1 - 10^{-\alpha})]$$

where \( X = D(\text{Hct}_{\text{micro}} - \text{Hct}_{\text{micro}}^2) \), and \( a \) and \( q \) are defined as

$$a = 2 \frac{b}{\lambda^2} (2\pi)^2(n_i - n_o)^2$$

$$q = 1 - \frac{S}{4(b)\lambda^2(2k)^2 \sin^2(\frac{\alpha}{2})}$$

The parameter \( b \) is the particle thickness averaged over all spatial orientations; \( \lambda \) is the wavelength of incident illumination; \( n_i \) and \( n_o \) are RBC interior and suspending media refractive indexes, respectively; \( V \) is particle volume; \( \alpha/2 \) is the acceptance one-half angle of the photodetector; \( S \) is the particle surface area, and \( k \) is equal to \( 2\pi n_i/\lambda \). In physical terms, \( a \) represents the attenuation of the transmitted beam of light due to scattering at the cell-plasma interface of each particle, and \( q \) represents the ability of the photodetector to accept light obliquely scattered at an angle to the optical axis. As schematized in Fig. 1A for a suspension of dispersed RBCs within the focal plane, only \( I_0 \) refracted by individual particles within the acceptance angle (α) of the photodetector (objective) will contribute to the detected I. Hence, a total OD ensues that is greater than that based on absorption alone for a solution of equal Hb concentration. When RCA occurs, the effective particle size increases, changing its effective surface area and volume as well as reducing the effective number of particles for an equal total Hb concentration in the measuring volume of the focal plane, as illustrated in Fig. 1B. Thus, in its simplest terms, with the onset of aggregation, fewer particles are present to refract light away from the α of the objective, and hence \( \text{OD}_{\text{scat}} \) will tend to decrease relative to \( \text{OD}_{\text{abs}} \). It should be noted that \( \text{OD}_{\text{scat}} \) is a parabolic-like function of the tube hematocrit, \( \text{Hct}_{\text{micro}} \) (16), and as \( \text{Hct}_{\text{micro}} \) approaches 100%, \( \text{OD}_{\text{scat}} \) falls to zero. The net result of RCA is thus to alter the parameters \( a \) and \( q \) as the effective particle diameter, surface area, and volume change.
With a few well-chosen assumptions, one may solve for the
volume of the scattering particle by use of Eq. 4 and the
relationship among \( G, q, S, \) and \( V \) as defined by Eq. 5, given
measurements of \( \text{Hct}_{\text{micro}}, D, \) and \( \text{OD}_{\text{scat}} \). First, if it is
assumed that RCA may result in an average \( V \) composed of
several RBCs, each of volume \( V_{\text{pr}} \), then an index of aggrega-
tion \( (G) \) may be determined from the ratio \( G = V_{\text{pr}}/V_{\text{or}} \) which
reflects the equivalent number of RBCs required to satisfy
Eqs. 4 and 5. Second, if it is assumed that \( b \) represents the
average diameter of either an individual RBC or a spherical
aggregate composed of \( G \) cells, then Eq. 4 may be rearranged
to yield (14)

\[
\text{OD}_{\text{scat}} = -\log[1 - (1 - q_0)G^{-2/3}(1 - 10^{-a_0G^{1/3}})]
\]

(6)

where \( a_0 \) and \( q_0 \) are the values of \( a \) and \( q \), respectively,
corresponding to the nonaggregated monodisperse state
where \( G = 1 \). The values of \( a_0 \) and \( q_0 \) have been determined
previously (16, 17) for RBCs (cat blood) by use of glass tubes,
with RBCs suspended in Ringer solution (no aggregation).
Similar in vitro measurements of nonaggregating rat RBCs
suspended in Ringer solution were obtained here for blood
flow in small bore glass tubes (40–70 \( \mu \)m in diameter), which
gave \( a_0 = 0.116 \) and \( q_0 = 0.114 \). Thus, with measurements of
\( \text{OD}_{\text{scat}}, \text{Hct}_{\text{micro}}, \) and \( D \), the \( G \) was obtained by the numerical
solution (Newton-Raphson method) of Eq. 6. Implicit to this
formulation of \( G \) is the assumption that the precise shape of
the scattering particle, whether it be truly spherical or a
population of rouleaux of many orientations, does not affect
the ability to empirically characterize the scattering cross
section (34) of the suspended particles as a function of pre-
vailing concentrations and \( \gamma \) values.

In vivo application of this procedure for determining \( G \) in
11 venules with \( \gamma > 350 \text{ s}^{-1} \) yielded values of \( G \) equal to 2.2 ±
0.78 (SD) in the absence of dextran and 2.6 ± 0.55 (SD) in the
presence of 3 g/100 ml Dx500, which were significantly
greater than in vitro values of \( G = 1 \) at similar values of
\( \text{Hct}_{\text{micro}} \) and \( \gamma \). This discrepancy appeared to arise because of
the sensitivity of \( q \) to the mismatch in refractive index be-
tween the walls of the glass tubes \( (n = 1.51) \) and the sus-
pending media \( (n = 1.33) \). It was found that the difference
between glass and tissue refractive index affects \( q_0 \), more
than \( a_0 \), which occurred because of a change in the ability to
capture light scattered at an angle to the optical axis. Thus,
to compute in vivo values of \( G \) from Eq. 6, the \( G \) in vitro value
of \( a_0 \) was used, and an average value of \( q_0 \) was computed for
each in vivo experiment as that value resulting in \( G = 1 \) for
a disaggregated state defined by the absence of dextran with
\( \gamma > 350 \text{ s}^{-1} \).

The results from application of this technique to measure
RCA in vitro (12, 16) are shown in Fig. 2 for the flow of rat
RBCs through a 50- \( \mu \)m-diameter glass tube. Homogeneous
(constantly stirred) suspensions of RBCs in Ringer solution,
either with 3% Dx500 or 1% albumin, at a 45% feed hemat-
ocrit were fed into the tube from a pressurized reservoir,
and the mean RBC velocity and \( \gamma \) were varied by manipulation of
the perfusion pressure. In the case of the nonaggregating
Ringer-albumin solution, \( G \) remained invariant with reduc-
tions in \( \gamma \) from 700 to \( \approx 50 \text{ s}^{-1} \). In the presence of Dx500, \( G \)
rose 2.5 times as \( \gamma \) fell from 200 to 30 \text{ s}^{-1}. To demonstrate the
effect of Dx500 concentration on RCA, presented in Fig. 2B is
the variation of \( G \) for concentrations of Dx500 up to 8 g/100
ml at \( \gamma = 50 \text{ s}^{-1} \). The occurrence of a maximum value of \( G \) at
a Dx500 concentration of \( \approx 3 \text{ g/100 ml} \) is consistent with in
vitro findings by use of other techniques, such as direct
counting of the number of RBCs per aggregate (6), viscosity
indexes (6), erythrocyte sedimentation rate (6), or devices
based on optical techniques such as the Myrenne aggregomet-
er (37).

To illustrate the subtle variations in the luminal distribu-
tion of RBCs in nonaggregating and aggregating conditions
in a small venule (58- \( \mu \)m diameter), presented in Fig. 3 are
scenes from video recordings in the absence (Fig. 3A) and
presence (Fig. 3B) of 3 g/100 ml Dx500. Each scene was
recorded by instantaneously stopping the flow with a proximal
microprobe to eliminate motion artifacts and by capturing
the scene before any further occurrence of aggregation.
Before flow cessation, \( \gamma \) was \( \approx 150 \text{ s}^{-1} \). In the absence of
aggregation, where \( G \) was arbitrarily set to equal 1, the RBCs
are seen (Fig. 2A) to be uniformly distributed in the focal
plane. In the presence of Dx500, where \( G \) was \( \approx 2.0 \), the RBCs
can be seen (Fig. 2B) to take on a mottled appearance with
the presence of discrete aggregates.

Quantitation of blood volume and red cell entrapment. To
examine shifts in systemic plasma volume and hematocrit
accompanying the infusion of Dx500, blood volumes were determined by injecting 270 µg of Evans blue dye, made to a 12.5% solution with saline, per kilogram of body weight via the jugular vein. Plasma concentrations of dye were determined from absorbance measurements at 608 nm from arterial samples obtained over a 30-min period. The subsequent exponential decay of plasma dye concentration was extrapolated back to the time of injection to obtain plasma volume (PV_{dye}). This process was repeated after injections of Dx500 to determine the effects of enhanced RCA. To minimize the volume removed from the rat during plasma volume determinations, the supernatant from the three hematocrit tubes (~0.2 ml of blood) used to obtain systemic hematocrit (Hct_{sys}) was collected for absorbance measurements by use of 40-µl microuettes (Starna Cells) to establish PV_{dye} (plasma volume based on dye concentration). We also used Hct_{sys} to estimate plasma volume (PV_{hct}), by ignoring RBC sequestration and by assuming that \( PV_{hct} = 0.08 \times W \times (1 - Hct_{sys}) \), where \( W \) is the rat weight in grams. The difference between \( PV_{dye} \) and \( PV_{hct} \) was taken as a measure of the volume of RBCs sequestered in the circulation.

**Experimental protocols.** To rule out the systemic effects of dextran toxicity, studies were initially conducted to elucidate the effects of dextran infusion on systemic arterial pressure (P_{art}). Dx500 was administered by successive isovolemic exchanges of whole blood with 0.5–1.0 ml of a 40- to 45-g/100 ml stock solution to raise circulating concentrations in 0.5- to 1.0-g/100 ml increments. Up to 10 exchanges, at 15-min intervals, were carried out before Hct_{sys} was measured, and supernatants were stored at −20°C for later measurement of circulating dextran concentrations by the anthrone method (31).

To examine the relationship between RCA and WBC margination, a single postcapillary venule (22–65 µm in diameter) was located, and flow conditions were measured at a position >1 mm downstream of a bifurcation with no inlets or outlets between. A blunted glass microprobe was held in a micromanipulator and positioned immediately downstream of the bifurcation to vary \( \gamma \).

Measurements of optical densities, for computation of Hct_{micro} and the RCA index (G), were made as \( \gamma \) was gradually reduced over a period of up to 70 min before and after dextran infusion. During the reduction in \( \gamma \), video recordings of WBC margination were made over a 3-min period at each \( \gamma \) for subsequent offline measurement of the number of WBCs rolling or sticking to the vessel wall and the average WBC rolling velocity. A sufficient amount of dextran was administered to yield a circulating concentration of ~3 g/100 ml. This concentration of dextran was chosen because it induces maximal levels of RCA (6, 37), and, because dextran precipitates fibrinogen in human blood (27), we found for rat blood that 3 g/100 ml Dx500 precipitated <10% of fibrinogen, which we deemed acceptable.

**RESULTS**

**Systemic effects of dextran infusion.** To establish that the infusion of dextran did not adversely affect systemic hemodynamic parameters, alterations in systemic P_{art} and Hct_{sys} were monitored in response to isovolemic dextran infusion, as shown in Fig. 4. A and B, respectively. Initial studies using Sprague-Dawley rats \((n = 7)\) revealed that within 1 min of the first Dx500 exchange, a 50% drop in P_{art} occurred that failed to rise again in the 15-min interval before the next infusion. This initial Dx500 exchange typically produced a circulating concentration of ~0.8 g/100 ml and a 10% rise in Hct_{sys}, as measured before the second exchange. With subsequent exchanges of Dx500, P_{art} increased and eventually stabilized to a level similar to its initial preexchange value, although Hct_{sys} progressively decreased. This response in P_{art} was likely due to an anaphylactoid reaction (11). Similar changes in P_{art} and Hct_{sys} were found with Wistar Rats \((n = 2)\). In contrast, Wistar-Furth rats revealed no significant change in P_{art} or rise in Hct_{sys}, thus suggesting the absence of an anaphylactoid reaction; hence, this strain of rat was used in the remainder of the studies. However, even with Wistar-Furth rats, Hct_{sys} decreased as a result of fluid shifts and RBC entrapment (32).

The relative shifts in plasma, RBCs, and total blood volume concomitant to Dx500 infusion were investi-
gated by use of the Evans blue dilution protocol. As shown in Fig. 5A, a progressive decline in Hct sys occurred, as was seen in Fig. 4B. Before Dx500 infusion, plasma, RBC, and total blood volumes averaged 9.1 ± 2.3 (SD), 7.4 ± 2.1 (SD), and 15.4 ± 4.4 (SD) ml, respectively. The corresponding drop in circulating RBCs and rise in plasma volume (Fig. 5B) suggest that total blood volume remained fairly constant (dotted line in Fig. 5B). The level of RBC sequestration increased in proportion to Dx500 concentration (Fig. 5C) and reached 25% of the total RBC blood volume at a Dx500 concentration of 3.5 g/100 ml.

Venular hemodynamics in response to Dx500. Presented in Fig. 6 is the time course of Hct micro, V RBC, and γ observed in 11 postcapillary venules in response to a single infusion of Dx500 that resulted in a plasma concentration equal to 3.0 ± 0.5 g/100 ml. During both pre- and postinfusion periods of ~70 min, these parameters remained invariant with time (t-test on regression slope, P > 0.2). However, after administration of Dx500, Hct micro rapidly fell by almost 50%, whereas Hct sys decreased 35%. Although not significant, RBC centerline velocity and the calculated Newtonian γ also decreased by ~30%. A summary of the alterations in hemodynamic parameters after Dx500 infusion is given in Table 1. Before and after Dx500 infusions, venule diameters were not significantly different (paired t-test, P > 0.3). The average resting (nonoccluded venule) centerline RBC velocity decreased significantly after infusion (P < 0.002, paired t-test), and a similar fall in γ occurred in response to Dx500 infusion. After Dx500 infusion, systemic WBC count decreased significantly, whereas differential counts of neutrophils and lymphocytes did not vary significantly from 22.0 ± 2.5 (SD) and 76.9 ± 2.3 (SD)%, respectively, before Dx500 infusion, to 26.1 ± 3.6 (SD) and 73.2 ± 3.4 (SD)%, respectively (paired t-test, P > 0.17).

As shown in Table 1, the 20% reduction in systemic WBC count in response to Dx500 infusion resulted in a 39% reduction in the potential maximum WBC flux.
Influence of red cell aggregation on white cell margination

To further explore the role of RCA on WBC margination, hemodynamic measurements were made in a second series of experiments to investigate the disaggregating effect of Dx40, as shown in Table 1. Under similar levels of systemic and microvascular hematocrits and with a γ similar to that in the Dx500 experiments, a similar 20% reduction in systemic WBC count was found. However, in contrast to the Dx500 experiments, the marginating flux decreased by ~15% (albeit statistically insignificant), and the luminal flux that rolled along the venular wall was unchanged.

To determine whether dextrans adversely affected the WBC population or promoted WBC-EC adhesion, WBC rolling velocity along the EC, the number of adherent WBCs per 100 μm of venule length, and the flux of WBCs rolling along the EC were determined at high (γ > 350 s⁻¹) and low (γ < 250 s⁻¹) γ values (Fig. 7). The high γ (>350 s⁻¹) values corresponded to those found in the normal (resting) flow state, whereas the low γ (<250 s⁻¹) values were induced by a gradual reduction in venular blood flow with a blunted microprobe. After Dx500 infusion, the average WBC rolling velocity decreased significantly by 45% at high and low γ (Fig. 7A). The ratio of WBC rolling velocity (V_{WBC}) to calculated γ (V_{WBC}/γ) was calculated (Fig. 7B) to account for diminished shearing forces acting on the WBC and revealed a significant decline at all γ values in response to Dx500, although a significant reduction after Dx40 infusion was found only at the high γ. The significance of this parameter is explored more fully in the Discussion. After Dx500 infusion, the average number of WBCs adhering to the EC (per 100 μm of venule length) approximately doubled (Fig. 7C) at low and high γ, whereas after the Dx40 infusion, it increased significantly only at high γ. The normalized fractional marginating flux of WBCs (Fig. 7D) significantly increased and decreased at low γ for Dx500 and Dx40, respectively, whereas no significant change was observed at high γ.

To determine whether dextran resulted in WBC activation, the nitro blue tetrazolium test (24) was carried out on blood samples taken at the end of an experiment and on WBCs in freshly drawn Wistar-Furth rat blood samples, which were incubated with 3g/100 ml Dx500 for 1 h. Neither study revealed any sign of WBC activation, nor was there a significant increase in the number of WBCs that showed pseudopod formation after Dx500 infusion as observed at high magnification under Nomarski differential interference contrast microscopy.

Effects of γ reductions on RCA and WBC margination. In vivo measurements of G are shown in Fig. 8. In the absence of Dx500 (Fig. 8, solid line), G remained invariant, with reductions in γ from 600 to 200 s⁻¹ induced with a blunted microprobe (rank sum test, P > 0.1). As γ was reduced further, from 200 to 50 s⁻¹, a small yet significant 20% increase in G was observed compared with values at high values of γ between 400 and 500 s⁻¹ (rank sum test, P < 0.001). After infusion of Dx500 to attain a plasma concentration of 3.0 ± 0.5 (SD) g/100 ml (Fig. 8, dotted line), G increased 40% above control as γ fell to 250 s⁻¹ and then rose significantly by threefold with further reductions in γ to 70 s⁻¹ (rank sum test, P < 0.005). Also, Dx500 resulted in a slight elevation of G for γ > 350 s⁻¹, which may be the result of the reorientation or increased deformation of RBCs caused by the increased plasma-dextran viscosity (see Discussion for details).
Table 1. Hemodynamic parameters in response to infusion of dextran

<table>
<thead>
<tr>
<th>Venule Diameter, μm</th>
<th>(V_{\text{RBC}}), mm/s</th>
<th>1, s(^{-1})</th>
<th>Hct(_{\text{sys}}), %</th>
<th>Hct(_{\text{micro}}), %</th>
<th>WBC(_{\text{sys}}), cells/mm(^2)</th>
<th>Maximal Luminal Flux, cells/min</th>
<th>Marginating Flux, cells/min</th>
<th>Fraction of Luminal WBC Flux, (F_{\text{WBC}})</th>
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<tbody>
<tr>
<td>Preinfusion</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Dx500</td>
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</tr>
<tr>
<td></td>
<td>49.3 ± 10.9</td>
<td>5.1 ± 2.3</td>
<td>527 ± 236</td>
<td>44.0 ± 2.1</td>
<td>37.0 ± 8.9</td>
<td>14,173 ± 5,585</td>
<td>5,521 ± 3,797</td>
<td>19.9 ± 11.5</td>
</tr>
<tr>
<td>Postinfusion</td>
<td>48.4 ± 10.7</td>
<td>3.7 ± 1.4</td>
<td>379 ± 109*</td>
<td>29.3 ± 2.5*</td>
<td>23.2 ± 4.2*</td>
<td>11,428 ± 3,718*</td>
<td>3,391 ± 2,562*</td>
<td>19.4 ± 12.9</td>
</tr>
<tr>
<td>Dx40</td>
<td></td>
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<tr>
<td></td>
<td>36.1 ± 13.4</td>
<td>3.6 ± 2.0</td>
<td>515 ± 285</td>
<td>45.7 ± 1.5</td>
<td>NA†</td>
<td>16,898 ± 5,323</td>
<td>3,100 ± 3,170</td>
<td>26.0 ± 23.8</td>
</tr>
<tr>
<td></td>
<td>36.0 ± 13.5</td>
<td>3.8 ± 1.9</td>
<td>559 ± 283</td>
<td>30.9 ± 1.9*</td>
<td>NA†</td>
<td>13,313 ± 1,747*</td>
<td>2,300 ± 2,081</td>
<td>22.2 ± 22.3</td>
</tr>
</tbody>
</table>

Given are means ± SD of venular diameter, red blood cell (RBC) velocity \(V_{\text{RBC}}\), calculated wall shear rate \(\gamma\), systemic hematocrit \(\text{Hct}_{\text{sys}}\) and white blood cell (WBC) count \((\text{WBC}_{\text{sys}})\), microvessel hematocrit \((\text{Hct}_{\text{micro}})\), and the marginating flux of WBCs observed rolling on the venular wall. Also shown is the calculated maximal flux of WBCs that could be present in the venule, on the basis of prevailing bulk flow rate and leukocrit, and the fraction of this flux that was measured along the venular wall. The normalized margination index \(F_{\text{WBC}}\) represents WBC marginating flux normalized with respect to flux at \(\gamma = 450\) s\(^{-1}\), i.e., \(F_{\text{WBC}} = \frac{\text{WBC}_{\text{sys}}}{\text{WBC}_{\text{sys}} - 450}\). Averages are given for infusion of dextran 500 (Dx500) and dextran 40 (Dx40) that resulted in a systemic concentration of 3.0 ± 0.5 (SD) and 3.2 ± 0.2 (SD) g/100 ml, respectively. *Statistically significant change with dextran infusion \((P < 0.05, t\)-test). †\text{Hct}_{\text{micro}} was not acquired in these experiments (NA, not applicable).

DISCUSSION

Detection of aggregation in vivo. To explore the relationship between RCA and WBC margination in vivo, a new technique was employed for measuring the extent of RCA in microvessels. \(G\) was derived from established principles of the theory of light scattering by a suspension of translucent particles (34). It should be noted, however, that although the present method draws heavily from a firm theoretical foundation, it does rely on a certain level of empiricism. Most notable is the lack of complete applicability of in vitro calibrations to the in vivo environment, as evidenced by the necessity of defining a disaggregated state and value for the parameter \(q\) (defined by Eq. 5 and appearing in Eq. 6 for obtaining \(G\)) at high \(\gamma\) values. Because of differences between the refractive indexes of glass tubes and the vessel wall and its surrounding tissue, the effective light-gathering ability of the microscope optics differs for the two applications. Nonetheless, the
value $G$ appears to provide an index of RCA that is sensitive to the extent of aggregation and its $\dot{\gamma}$ dependence, which under idealized conditions may be interpreted as the number of RBCs per aggregate.

**Physiological effects of dextrans.** Dextrans have been widely used in in vivo and in vitro studies of blood rheology to induce RCA in an attempt to mimic a variety of blood disorders, such as some hyperproteinemias and the low flow state. However, it is well known that dextran infusion into laboratory animals may induce a variety of adverse effects (11). To characterize the potential for such effects in the present study, the effects of dextran on three different strains of rat were investigated to choose the best strain for this study.

Both Sprague-Dawley and Wistar rats developed an anaphylactoid reaction to systemic administration of dextran, as evidenced by a precipitous fall in blood pressure. Even though these rats appeared to adapt to the dextran in their system, as evidenced by systemic blood pressure returning to normal, it could not be assumed that the dextran had no other effects on the circulation. Because no adverse reaction was found in the Wistar-Furth rat, they were selected for this study of dextran-enhanced RCA.

It should be emphasized that although there have been many studies of the aggregation of RBCs from different species, few have examined RCA in rats and none have addressed the variability in RCA among strains of rats routinely used in physiological studies. Baskurt et al. (3) found that RBCs from Swiss-Albino rats aggregated only slightly in native plasma, which agrees with the present findings in Wistar-Furth rats. However, Ohta et al. (23) found that RBCs from Wistar-Albino rats aggregate to a greater degree, similar to the level of RCA for human RBCs. This disparity may also be due, in part, to methodological differences as well as differences in strains of rat.

On isovolemic exchange of concentrated dextran solutions with whole blood, larger decreases in Hct$_{\text{sys}}$ were observed (Fig. 5) than could be attributed to the removal of RBCs from the subject animal during the exchange with cell-free dextran solutions. A series of blood volume measurements were conducted that revealed a steady increase in RBC sequestration with increasing Dx500 concentration, as previously demonstrated by Simchon et al. (32) in studies of regional blood flow in dogs. This loss of RBCs from the circula-
tion may be due to the increased level of RCA that causes rapid RBC settling in regions of low flow and small vessel plugging, or possibly to cell-dextran-EC interactions (33). In addition, alterations in colloid osmotic pressure may have also contributed to a generalized fluid shift from tissue to vascular compartments that resulted in the increased plasma volume and diminished Hctsys.

In vivo levels of RCA. The major goal of the present study has been to elucidate the extent to which RCA influences WBC margination in postcapillary venules. For whole rat blood, where RBCs aggregate only slightly, WBC margination was found to increase exponentially as γ decreased (8). When aggregation was enhanced with Dx500, the exponential increase was twofold greater compared with that in the absence of Dx500 as γ was reduced below 350 s⁻¹ (Fig. 9). When RCA was inhibited with Dx40, WBC margination was greatly reduced, and fairly constant, below 350 s⁻¹ (Fig. 10). These trends were consistent with the measured exponential increase in RCA as γ fell below 350 s⁻¹ (Fig. 8).

Prior to in vivo studies of the effect of vessel hematocrit Hctmicro on WBC margination (8) has suggested that the flux of WBCs at the venular wall is mainly invariant with alterations of Hctmicro at high γ and for 15% ≤ Hctmicro ≤ 45%. As shown herein, significant variations in marginating WBC flux with Hctmicro were found only at the lowest γ values (<100 s⁻¹) and when Hctmicro exceeded 50% at γ > 100 s⁻¹. In vitro studies by Goldsmith and Spain (9) showed that at low γ, with decreasing hematocrit, there was an increasing enrichment of WBCs relative to RBCs, but the enrichment of WBCs themselves did not significantly vary with decreasing hematocrit. Similar results by Nobis et al. (22) in glass tubes support the general conclusion that the enhanced margination of WBCs observed here results primarily from the effect of RCA in contrast to hematocrit variations. Hence the decrease in Hctmicro caused by the presence of dextran may play an insignificant role in the margination process, and thus γ-dependent RCA appears to be the primary determinant of increased WBC margination with flow rate reductions.

The present results also suggest that RCA plays a dominant role in maintaining the marginating WBC flux along venular EC. Although it has been shown by Schmid-Schönbein et al. (30) that as many as 94% of all WBCs that exit the true capillaries make rolling contact with the venular EC, it is apparent that the majority of these WBCs rapidly mix with the RBC stream unless they are excluded from the axial stream by RBC aggregates. Enhancement of the WBC flux in the vicinity of the tube wall near the periphery of the RBC core has been shown by Nobis et al. (22) to occur in vitro with reductions in γ. The twofold increase in rolling WBC flux observed here (Fig. 9) with the presence of the proaggregating agent Dx500 (for γ < 100 s⁻¹) and its almost complete obliteration at all γ values with the presence of the disaggregating agent Dx40 (Fig. 10) clearly support a role for RCA in maintaining the in vivo rolling flux of WBCs. It appears that RCA either serves to maintain the rolling flux from capillary exit to venular EC or promotes the radial migration of WBCs that have been previously entrained by the RBC stream after exit from the capillaries.

In the in vitro studies of Nobis et al. (22), the relative WBC flux rolling along the wall itself was markedly reduced at all γ values, presumably because of the lack of adhesive interactions between WBCs and tube wall. To explore the role of dextran on the adhesive interactions between WBCs and EC, the absolute rolling velocity and marginating flux of WBCs were studied. The nearly twofold increase in WBC flux (compared with control) at low shear in response to the Dx500 (Fig. 9) and the concomitant threefold increase in the level of RCA (Fig. 8) suggest that RCA is a major promoter of WBC margination and subsequent firm adhesion. To explore the effect of dextran on the adhesive interactions between WBCs and EC, the absolute rolling velocities and marginating flux of WBCs were studied. As indicated in Fig. 7A, the significantly lower rolling velocity in the presence of Dx40, compared with control, and its further attenuation with Dx500 are suggestive of an enhanced strength of the adhesive bond between WBCs and EC. Normalization of the Vwbc, by division by γ, to account for alterations in the forces that tend to sweep WBCs from the EC (Fig. 7B) yields a consistent indicator of enhanced adhesive strength, except for the case of low γ values in the presence of Dx40. A similar enhancement of the number of WBCs firmly adhered to the EC also suggests an increased strength of the WBC-EC adhesive bond (Fig. 7C). However, the fact that the rolling flux is largely unaffected at high γ values for either the disaggregating agent (Dx40) or the proaggregating agent (Dx500) and is significantly reduced in the presence of Dx40 and increased with Dx500 at low γ values suggests that the apparent increase in adhesion arises because of a greater degree of margination and a greater probability of contact between WBCs and EC. It is likely that the reductions in Vwbc reflect a shift in the composition of the marginating population of WBCs to include a greater proportion of neutrophils, with specific receptors for ligands on the EC surface, as RCA promotes an increase in the total fraction of all WBCs that maintain their contact with the EC. The data of Fig. 7B (Vwbc/γ) support this hypothesis. It has been shown previously (13) that the average rolling velocity of a population of marginating WBCs is less sensitive to increases in the γ or red blood cell velocity (i.e., strength of the WBC-EC bond) compared with the rolling velocity of an individual WBC, because of the heterogeneity of adhesive and mechanical properties of the circulating WBC population.

The increased amounts of firmly adhered WBCs and decreases in Vwbc could have been caused by a number of additional factors such as tissue changes during the experiment, EC activation, or interference with the normal balance of receptor-ligand interaction at the WBC-EC interface. However, it seems improbable that increased WBC adhesion and decreased WBC flux could be the basis for both Dx500 and Dx40 results.
Two additional factors may preclude a role for dextran-induced EC activation. First, WBC activation would lead to a large systemic loss of WBCs, and, second, WBC activation would lead to an increase in WBC margination at higher $\dot{\gamma}$. Although it may still be argued that Dx500 might enhance WBC-EC interactions and Dx40 might inhibit them, this possibility appears unlikely because margination increases at the point at which RCA increases.

The effect of RCA on the resistance to flow in vivo has also been fraught with controversy. Mchedlishvili et al. (18) found that after infusion of large concentrations of Dx500 into rats, systemic blood pressure increased by $\sim 50\%$, which was attributed to the increased resistance caused by dextran-induced aggregation. In the present study, no such increase in systemic pressure was found after exchange of Dx500 into Wistar-Furth rats, whereas systemic blood pressure initially decreased for Sprague-Dawley and Wistar rats. Cabel et al. (5) measured resistance in the circulation of isolated cat lateral gastrocnemius muscle with 0.5% of dextran 250 present and found venous resistance increased by 30% at high $\dot{\gamma}$ values and decreased by 80% at low $\dot{\gamma}$ values. In vitro studies of RCA in vertically positioned small bore glass tubes (7, 28, 29) showed that the apparent viscosity of nonaggregating RBCs increased threefold as $\gamma$ was reduced below 100 s$^{-1}$. When aggregation was increased to normal levels, by suspending RBCs in plasma, the apparent viscosity changed very little with decreasing $\dot{\gamma}$ in small (<58 $\mu$m) tubes. However, prior in vivo measurements in the low flow state suggest a dramatic rise in apparent viscosity with reductions in $\dot{\gamma}$ (12).

The current findings of a 28% decrease in RBC centerline velocity after Dx500 exchange (Table 1), which agrees with Mchedlishvili et al. (18) and others, suggest that RCA leads to an increase in microvascular resistance. However, these reductions in velocity may arise from the increased viscosity of the suspending medium ($\eta_0$) after infusion of the Dx500. Because Poi-seulle’s law predicts that, in a given vessel and at a given pressure gradient, volume flow rate is inversely proportional to viscosity, the effect of the Dx500 may be estimated from the relationship between bulk viscosity ($\eta$), $\eta_0$, and $\text{Hct}_\text{micro}$. Direct in vivo measurements in unbranched microvessels with blood flow at high $\dot{\gamma}$ values (17) suggest that

$$\eta = a\text{Hct}_\text{micro} + \eta_0$$

(7)

where $a = 0.04$ as determined for cat blood (similar in mechanical properties to rat). With the use of values of $\eta_0 = 1.2$ mPa·s for rat plasma (38) and 2.29 mPa·s for 3.0 g/100 ml Dx500 (M. J. Pearson and M. W. Rampling, unpublished work), an increase in blood viscosity equal to 33% would follow the infusion of Dx500 and 38% reduction in $\text{Hct}_\text{micro}$ noted in Table 1. Thus on the basis of bulk viscometry, RCA would have very little effect on resistance within individual microvessels at physiologically relevant $\dot{\gamma}$ values. However, the disruption of red blood cell aggregates in arteriolar bifurcations and their formation in venous confluences may introduce an additional source of increased resistance to flow attendant to RCA, which remains to be determined by experiments that focus directly on alterations in the resistance to flow.

In summary, it has been shown that RCA greatly enhances WBC margination attendant to a reduction in $\gamma$ below 350 s$^{-1}$, and for $\dot{\gamma}$ values above 350 s$^{-1}$, there is little indication that RCA progresses to the point of affecting WBC margination. Thus the role of RCA may be of prime importance in disorders that manifest a combined reduction in $\dot{\gamma}$ values and increased tendency toward WBC-EC adhesion, such as inflammation, septic shock, and ischemia. In this regard, the present study may also serve to establish a framework for evaluating the contribution of a broad variety of hematological and hemorheological disturbances, such as elevated levels of fibrinogen, the principal promotor of RCA, polycythemia, and abnormal RBC deformability and surface charge. With the availability of the new technique presented here for the measurement of RCA, it is anticipated that greater insights into these pathophysiological disturbances may be gained from future studies by use of the techniques of direct intravital microscopy.

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