Selectin- and Integrin-Mediated T-Lymphocyte Rolling and Arrest on TNF-α-Activated Endothelium: Augmentation by Erythrocytes

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ABSTRACT The adhesive and hemodynamic forces that lead to lymphocyte rolling and arrest on activated endothelium and the biophysical role of various adhesion molecules and blood elements in this process are poorly understood. By quantifying their behavior both in vivo and in vitro, we show here that erythrocytes facilitate selectin- and integrin-mediated rolling and binding of T-lymphocytes on tumor necrosis factor α-activated endothelium. The relative contribution of selectins and integrins to this process can be distinguished by using a simple mathematical expression of lymphocyte capture within the range of physiological shear stress. The need for selectin participation in lymphocyte capture increases with shear stress (>1 dyn/cm^2), and both β1 and β2 integrins act in synergy to produce adhesive drag on captured cells. These findings are potentially useful in developing strategies for intervening with T-cells in a variety of normal and pathological responses as well as for the delivery of genetically modified T-cells to their targets in vivo.

INTRODUCTION

When a blood-borne leukocyte comes in contact with the vessel wall in vivo, it may continue to move with the flow stream, may roll on the endothelium, or may attach transiently or permanently to the endothelium and extravasate (Lawrence and Springer, 1991; Ley et al., 1991; von Andrian et al., 1991). The outcome of this interaction depends on the balance between adhesive forces exerted by cellular adhesion molecules (CAMs) and hydrodynamic forces generated by the blood (Hammer and Apte, 1992). Recent in vitro and in vivo studies have focused on the role of two types of CAMs in leukocyte rolling and arrest: selectins (Bevilacqua et al., 1987; Geng et al., 1990; Kansas et al., 1993; Larsen et al., 1989) and integrins (Bevilacqua, 1993; Hakkert et al., 1991; Springer, 1990; Hession et al., 1992). These studies have led to a paradigm in which selectins mediate rolling whereas integrins produce cell arrest (Hammer and Lauffenburger, 1987; Lawrence and Springer, 1991; Ley et al., 1991; von Andrian et al., 1991). The biophysical basis of this paradigm as applied to lymphocytes is not known. Both integrins and selectins have been shown to be important in the recirculation and localization of lymphocytes (Tamatani et al., 1993; Tamatani et al., 1991).

Previous studies of lymphocyte interaction with vascular endothelium have established a probabilistic view of lymphocyte adhesion (Bjerknes et al., 1985) and have shown that lymphocytes may roll on the endothelium under some circumstances (Berg et al., 1993). In addition, some studies (Nobis et al., 1985; Schmid-Schönbein, 1987; Schmid-Schönbein et al., 1980) have suggested that erythrocytes may contribute to the forces influencing leukocyte interactions with the vascular endothelium; however, the extent to which this influences the outcome of these interactions is not clear. Because T-lymphocytes are involved in a wide variety of normal and pathological responses and can be used as vectors for gene therapy, we designed the present in vivo-in vitro study to answer the following questions: 1) Under what conditions do lymphocytes roll and adhere to activated endothelial cells? 2) What is the role of various CAMs alone or in combination in this process? 3) Do erythrocytes (RBCs) play any role in lymphocyte adhesion?

We have selected both in vitro and in vivo methods to investigate this phenomenon. A parallel plate flow cell was employed to examine the kinetics and mechanics of lymphocyte binding to activated endothelial cells in vitro and compared to the interaction of lymphocytes with activated endothelial cells in vivo in the dorsal skin of mice. We report here a quantitative description of the role of various CAMs in lymphocyte adhesion and describe the influence of erythrocytes on this process.

MATERIALS AND METHODS

Preparation of human lymphocytes

Human T-cells obtained from leukapheresis of normal platelet donors were isolated by Ficoll-Hypaque centrifugation followed by depletion of the B-cell, natural killer cell, and monocyte populations using antibodies directed against CD19 (clone J4.119; AMAC, Westbrook, ME), CD16 (B73.1; Becton-Dickinson, San Jose, CA), and CD15 (clone MMA; Becton-Dickenson) at 10 μg antibody/15 $\times$ 10^6 cells, followed by adsorption to a paramagnetic substrate for 30 min (Advanced Magnetics, Cambridge, MA) and removal by a magnetic field. The lymphocytes isolated from this procedure were typically 72% CD4$^+$ and 28% CD8$^+$ cells by flow cytometric analysis. The cells were labeled for fluorescence microscopy with 0.01 mM calcein (Weston and Parish, 1990, 1992) in PBS for 15 min, washed, and resuspended in Hanks' balanced salt solution (HBSS) (with Ca^{2+} and Mg^{2+}) for injection i.v. into SCID mice (100 μl cells in PBS) or used in the in vitro flow chamber with erythrocytes. Cells used for in vitro experiments without RBCs were not labeled.

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In vivo methods

Dorsal skin chamber implantation was performed under anesthesia with ketamine (9 mg/100 g body weight) and xylazine (0.9 mg/100 g body weight) (Leunig et al., 1992). The interaction of blood cells with the vasculature of the dorsal skin was observed through the transparent window of the chamber. Implanted dorsal chambers were activated with tumor necrosis factor α (TNF-α) by direct superfusion into the dorsal window (50 ng in 10 μl PBS; Cetus/Chiron, Emeryville, CA) for 5 h before cell injection. Cells (30–40 million) were injected over a period of 30 s, and the arrival of cells in the vessels of the chamber was monitored by fluorescence video-microscopy and recorded. Antibody blocking studies were done by incubating the cells with 10 μg of antibody for 30 min at 4°C before injection using 1) anti-CD49d (clone HP2/1; AMAC, Inc.), 2) anti-CD18 (clone IB4 obtained from Karl Arfors), 3) anti-L-selectin (clone Dreg 56; AMAC). All antibody blocking studies were limited to 5 min after the injection of cells to avoid shedding of the bound antibody, and observations of each section of vessel were limited to 30 s periods. Blood velocities were determined by the i.v. injection of 1-μm-diameter fluorescent latex spheres (5×10^8 in 50 μl PBS), and the mean of the top three velocities determined from direct measurement of "on screen" images was used to approximate the flow velocity. Relative wall shear stress was approximated by assuming a 32% hematocrit in these vessels with a corresponding viscosity (Chien et al., 1984). The percentage of rolling and binding cells was determined as a fraction of all cells observed in a 200-μm section of vessel for a 30-s period between 2 and 5 min after the injection of cells. A cell that maintained stable arrest for at least 5 s was considered bound.

Flow cell measurements

T-cells isolated from normal human donors were perfused over TNF-α-activated 2° HUVEC monolayers with HBSS in a parallel plate flow cell, as previously described (Munn et al., 1994). The bulk cell concentration was 1×10^6 cells/ml in all in vitro experiments. Each flow experiment was started at high wall shear stress (4.1 dyn/cm^2), then the flow rate was decreased in steps, and the cumulative bound cell density was determined at five fields with the 10× objective at each level of shear stress. The time interval at each flow step was adjusted so that the total number of cells delivered to the surface is constant for each shear stress level (Table 1) (Munn et al., 1994). Antibody blocking of individual and multiple CAMs was used to evaluate their participation in the lymphocyte capture process. Antibodies used in these studies were as follows: a) anti-CD49d (clone HP2/1), b) anti-CD18 (clone IB4), c) anti-L-selectin (clone Dreg 56), d) anti-CD2 (clone S5.2, anti-Leu-5b; Becton-Dickinson, Inc.), e) anti-E selectin (clone H18/7; Becton-Dickinson), f) anti-VCAM-1 (clone 1G11; AMAC), g) anti-ICAM-1 (CD54, clone 84H10; AMAC). Antibody treatment of the lymphocytes was done with 10 μg antibody/15 $\times$ 10^6 cells (antibody excess by staining), and the HUVEC monolayers were treated with 10 μg antibody in 0.5 ml culture medium.

In individual experiments, the number of bound cells per field was determined for five adjacent fields for each level of shear stress, and the mean values were used to calculate the individual binding density. Variation in cell number for adjacent fields was generally less than ±15% of the mean. A minimum of four independent experiments were combined for each treatment to produce a mean and standard deviation for each binding density shown. Rolling velocities were determined from direct measurement of on-screen images over several frames.

Calculation of capture efficiencies

In vitro capture efficiencies were calculated from the binding densities in each treatment group. A capture event was defined as a stable arrest of a cell from the flow stream for at least 5 s. Because the cell flux at the surface of the flow chamber varies with the flow rate (shear stress), the time interval at each flow step was adjusted so that the total number of cells delivered to the surface was constant for each shear stress level (Munn et al., 1994). This normalization permitted us to calculate a capture efficiency defined as the number of cells that bind (N_b) at a given shear stress divided by the total number of cells (N_t) that have passed near the surface ($E = N_b/N_t$). The cumulative binding curves are fit to the equation $N_b/N_t = E_o' e^{-\kappa S}$, where S is the shear stress, and E_o' and κ are adjustable parameters (this equation fit the data well, with R^2 greater than 0.95 in all cases). The value of E_o was then calculated from $E_o = E_o'[1 - e^{-\kappa \Delta S}]$, where ΔS is the difference in shear stress values for successive steps. Thus, $E = E_o e^{-\kappa S}$. This process was performed for each of the antibody treatment and control groups, and the resulting function was plotted. Differences in the resulting parameters, κ and E_o, were compared between various treatment groups with the t-test and Mann-Whitney test.

TABLE 1 Flow parameters for in vitro experiments

Flow rate (ml/min)	Shear stress* (dynes/cm^2)	Time to steady state‡	Total flow time§ (s)
2.0	4.04	104	129
1.5	3.06	90	115
1.0	2.02	92	117
0.8	1.63	91	116
0.6	1.23	93	118
0.4	0.82	98	123
0.2	0.41	118	143

Experiments were started at a high flow rate, then decreased with time according to a protocol designed to deliver equal flux at each flow rate. The initial flow period at 0.2 ml/min was selected so that the delivered flux just reaches steady state before data collection commences. The remaining flow times were calculated (Munn et al., 1994) by equalizing the total number of cells, G_i, passing at each step according to

$$G_i = \frac{Cx}{2}\left(\frac{u_{i-1}}{u_i} + \frac{2vx}{u_i h} + 1\right) + Cu_i\left(1 + \frac{vx}{u_i h}\right)\left(t_{wi} - \frac{x}{u_i}\right)$$

where u_i and u_{i-1} are the current and previous velocities, respectively, and t_{wi} is the time spent at flow rate i (including sampling time).

*Shear stress was calculated assuming a viscosity of 0.0089P.

‡Time required after the change of flow rate to re-establish steady-state flux condition.

§ Observation time + time to steady state.

Flow cell measurements with RBCs

Experiments were conducted in vitro with reconstituted blood [calcein-labeled T-cells and RBCs at 32% hematocrit based upon 40–100 μm vessel size (Chien et al., 1984)] perfused through the parallel plate flow chamber (Munn et al., 1994). Reconstituted blood was prepared by Ficoll-Hypaque gradient separation of RBCs from mononuclear cells followed by repeated washing in PBS. RBCs were resuspended in HBSS or fetal bovine serum, and calcein-AM-labeled lymphocytes were added to a concentration of 1×10^6 cells/ml. T-cells suspended in HBSS or 32% hematocrit in HBSS were perfused through the flow chamber at constant shear stresses of 1.1, 2.3, or 4.6 dyn/cm^2 for 5 min. Ten fields were then sampled and the numbers of rolling and firmly attached cells (adherent for at least 5 s) were counted. The rolling fraction (i.e., the number of rolling cells divided by total cells bound and rolling) and the capture efficiency (i.e., the number of bound cells divided by the number of cells passing the surface during the 5-min flow period) were determined. Rolling fractions shown are the mean ± SD of four independent experiments. For the normalization, the viscosities of the RBC solutions were estimated using the equation of Quemada (Chien et al., 1984) and verified by direct measurement of pressure drop across the flow chamber.

Successive images of rolling cells, both in vitro and in vivo, forming composite photographs, were captured 1 s apart and combined by image

addition, then processed using a despeckling filter and a 5 × 5 gradient convolution.

RESULTS AND DISCUSSION

Lymphocyte rolling and arrest in vivo

The characteristics of in vivo interaction of lymphocytes with TNF-α-activated endothelium were evaluated in the mouse dorsal skin chamber model. We injected calcein-labeled human T-lymphocytes into SCID mice and observed their interaction with vessels of the dorsal skin through the surgically implanted transparent window (Leunig et al., 1992). Five hours before the tail vein injection of fluorescent lymphocytes, the vessels within the chamber were activated with TNF-α. This time period was sufficient to activate endothelial cells and to initiate the capture of passing lymphocytes (Bevilacqua et al., 1989; Yan et al., 1994). Within 30 s the injected cells could be observed entering the arterioles in the skin chamber, and by 1 min they could be observed in both the post-capillary venules and the 50–100-μm-diameter collecting venules. Upon entering these vessels, over 30% of the total cells observed in the collecting venules (approximately 90% of all cells contacting the vessel walls) were observed to roll along the wall of the venules with a velocity of approximately 20 μm/s (Table 2). Rolling was observed over distances of 40–200 μm and was interrupted with jumps of 50–100 μm. Some rolling cells were observed to halt and adhere to the vessel wall; the duration of the adhesion was 5 to 10 s in the large venules and several minutes or longer in the post-capillary venules. The range of wall shear stress in these larger vessels, based on the measurement of the flow velocities, was between 1.2 and 3.0 dyn/cm^2.

We then attempted to reduce rolling and arrest of lymphocytes by anti-CAM antibodies in a manner similar to that of neutrophils (von Andrian et al., 1991). Treatment of the T-lymphocytes with antibodies against LFA-1 (CD18) or VLA-4 (CD49d) produced a significant reduction in the number of cells rolling on and binding to the vessel walls (Table 2). The greatest reduction in rolling and binding cells was seen after blocking by both anti-CD18 and anti-VLA-4. Thus, adhesion mediated by CD18/ICAM-1 and VLA-4/VCAM-1 appears to function in a complementary manner. In addition, blocking the L-selectin on the lymphocytes also resulted in reduced numbers of rolling and binding cells. In contrast, treatment with anti-CD2 antibody did not produce a significant decrease in the number of rolling or adhering cells. Quantification of the velocities of cells that could roll after these treatments indicated that the cells treated with anti-L-selectin antibody had unchanged rolling velocities but those treated with anti-VLA-4 and anti-CD18 rolled at a significantly increased rate (Table 2), indicating that the adhesive drag on these lymphocytes was reduced by blocking the integrins. This further suggested that the reduction in number of rolling cells observed after selectin blocking was due to reduced efficiency of initial lymphocyte capture, but the reduction in rolling cells that followed integrin blocking may be due to the decrease in the adhesive force that maintains cell contact under dynamic conditions.

Lymphocyte behavior in vitro

The mechanism of lymphocyte adhesion may be influenced by the flow environment. Because it is difficult to precisely modulate shear rates in vivo, we employed a parallel plate flow chamber to quantify the rolling and arrest of cells on endothelial monolayers under defined shear stress (Lawrence et al., 1987, 1990; Lawrence and Springer, 1991; Munn et al., 1994). As in our in vivo experiments, the endothelial cells were activated with TNF-α and T-cells were introduced into the flow chamber (Munn et al., 1994). Shear stress was initiated at 4.1 dyn/cm^2 and subsequently reduced in seven steps to 0.4 dyn/cm^2, and the number of bound cells was quantified at each step. Adhesion was defined as stable arrest for at least 5 s. Lymphocyte accumulation was not observed on nonactivated endothelium; however, the arrest of lymphocytes on TNF-α-activated endothelium increased significantly ($p < 0.05$) between 2 and 5 h after stimulation (Fig. 1 *A*).

To test the hypothesis that various CAMs contribute to different degrees to the net adhesive force at different levels of shear stress, we blocked various CAMs with monoclonal antibodies and evaluated the changes in the binding curve of the treated as compared to control cells over a range of physiological shear stresses. In these in vitro experiments, pretreatment of the T-lymphocytes with anti-LFA-1 anti-

TABLE 2 Effect of antibody blocking of L-selectin or integrins on T-cell rolling and binding in vivo

Treatment	% rolling	% binding	Rolling velocity (μm/s)	Shear stress (dyn/cm^2)
Isotype control ($N = 4$, $n = 10$)*	34.1 ± 12.3	8.8 ± 3.5	19.0 ± 16.5	2.9 ± 1.5
L-selectin ($N = 4$, $n = 6$)	12.1 ± 3.9‡	3.0 ± 2.5‡	23.7 ± 13.5	2.0 ± 0.8
VLA4+CD18 ($N = 4$, $n = 6$)	7.1 ± 2.9‡§	1.4 ± 1.3‡	64.4 ± 17.8‡	1.7 ± 0.7
VLA4 ($N = 4$, $n = 7$)	11.8 ± 3.2‡§	2.5 ± 2.2‡	53.7 ± 25.8‡	1.2 ± 0.3
CD18 ($N = 4$, $n = 6$)	18.6 ± 8.8‡§	2.7 ± 1.0‡	47.3 ± 21.7‡	1.7 ± 0.3

*The number of mice and vessels observed are indicated as N and n, respectively. Mean values ± SD are shown for N.
‡Significant difference from control, $p < 0.05$
§Significant difference between single and double antibody groups, $p < 0.05$.

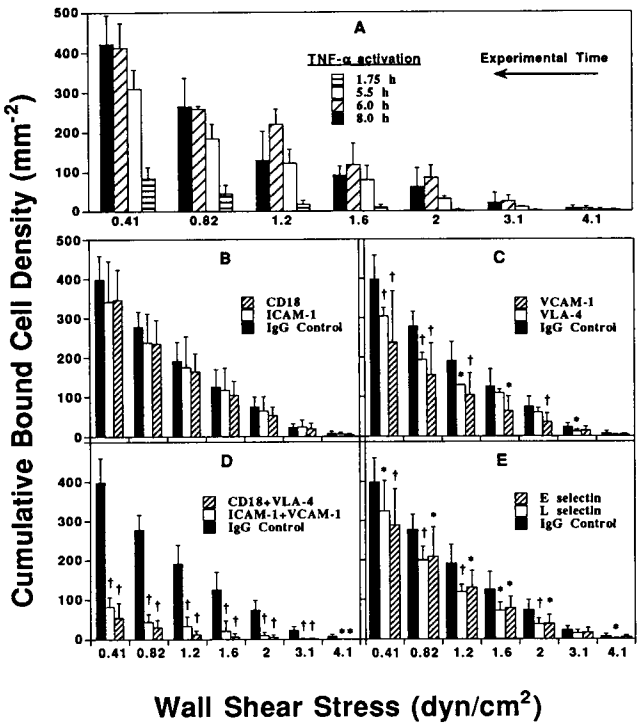

FIGURE 1 In vitro cell capture in the parallel plate flow chamber. A quantitative analysis of the kinetics of lymphocyte adhesion to activated endothelium as well as the contribution of various selectins and integrins to the process of lymphocyte capture on TNF-α-activated endothelium was conducted. Endothelial cell monolayers were activated with TNF-α for various time intervals and installed in the parallel plate flow chamber. T-lymphocytes suspended in the flow solution were perfused through the chamber at different flow rates, thus generating a range of physiological shear stress levels. Flowing cells were observed for stable arrest, defined by adhesion to the endothelium for at least 5 s. Because optimal lymphocyte adhesion was observed between 5.5 and 8 h after endothelial activation (*A*), subsequent mechanistic studies were conducted within this time interval. Expression of ICAM-1, VCAM-1, and E-selectin was strongly indicated by immunofluorescent staining of the monolayers during this time period (not shown). Blocking various adhesion molecules with monoclonal antibodies permitted a dissection of the adhesion process. The symmetry of the blocking effect was examined by using antibodies against either the lymphocyte or the endothelial side of the interactions. (*B–E*) Results of antibody blocking (averaged from four experiments each) on the cumulative bound cell density for TNF-α-activated monolayers. All treatments are compared with irrelevant IgG control antibody averaged from 11 experiments (*solid bars*). Significance is indicated at $p < 0.05$ (*) or $p < 0.01$ (†), and the error bars represent one standard deviation. Treatment with a combination of CD18 + VLA4 or ICAM-1 + VCAM-1 resulted in a dramatic and synergistic reduction in the number of binding cells.

body did not result in a significant reduction of the number of binding cells at 0.4 to 2.0 dyn/cm^2 when compared to lymphocytes treated with an irrelevant control antibody (Fig. 1 *B*). Treating the endothelial cells with anti-ICAM-1 also showed little effect on lymphocyte binding at shear stress levels lower than 2 dyn/cm^2. Blocking of VLA-4 or VCAM-1 produced significant reductions in binding across the range of shear stresses tested (Fig. 1 *C*). However, simultaneously blocking both LFA-1 and VLA-4 or their ligands, ICAM-1 and VCAM-1, produced a dramatic and synergistic reduction in cell binding, significantly less than LFA-1, VLA-4, ICAM-1, or VCAM-1 alone ($p < 0.01$, Fig. 1 *D*). Treatment of the lymphocytes with an antibody directed against CD2 did not produce a significant change in the number of adherent cells in vitro (not shown). To examine the role of selectins, we also used anti-L and E-selectin antibodies (Fig. 1 *E*). These experiments showed significant ($p < 0.05$) reductions in the number of bound cells at shear stresses from 0.4 to 2 dyn/cm^2.

Relative contributions of CAMs

Because the observed number of bound cells in these experiments represents the accumulation of cells at multiple levels of shear stress within a physiological range, the change in bound cell density with changing shear stress is directly related to the binding efficiency as a function of the detachment force generated by the flow. Therefore, to quantify the relative contributions of various CAMs alone or together and to facilitate their comparison, we fit the binding data to the following equation: $E = E_0 e^{-\kappa S}$, where E is the capture efficiency at shear stress S, E_0 is the zero shear efficiency, and κ is the capture coefficient. The parameter κ describes the relative sensitivity of lymphocyte capture to shear stress and must therefore be a function of the reaction rate and bond strength because the force placed on the initial bond is linear with increasing shear stress (Alon et al., 1995). In the case of TNF-α activation of the endothelium, κ is determined by the cooperation of various molecules acting in concert to produce a transient arrest that can lead to stable, long-term adhesion. As the efficiency of capture increases, κ will decrease. In practical terms, this indicates that a high adhesive force at the time of contact will permit relatively little decrease in the capture efficiency with increasing shear stress. Consequently, molecules with a rapid k_f such as P-selectin (1.5×10^7 s^{-1}; Alon et al., 1995) will have a dominant effect at high flow rates, especially if present in high concentration.

E_0 provides a relative index of the efficiency of the adhesion process after initial capture, over a range of shear stress levels, and reflects the stability of the adhesion complex. In this setting, the stable adherent cell utilizes multiple molecular species to maintain adhesion and resist detachment, reflecting ligand-receptor affinity and concentration. Thus, blocking integrin-mediated interactions decreases the net adhesive force and may permit cells to roll via selectin interactions (rolling is not considered to be a stable adhesion event for the purpose of quantification in these studies) or detach. E_0 thus provides a functionally useful means of comparing the relative net adhesive force between different cell populations and activated endothelium.

We found reproducible differences in E_0 and κ for various antibody treatments of lymphocytes or HUVECs (Fig. 2, *A* and *B*). There was no significant reduction in E_0 for anti-VLA-4 or anti-VCAM-1 antibodies alone; however, a significant reduction in E_0 ($p < 0.05$) was obtained with the addition of antibodies to VCAM-1 and ICAM-1 simultaneously, from

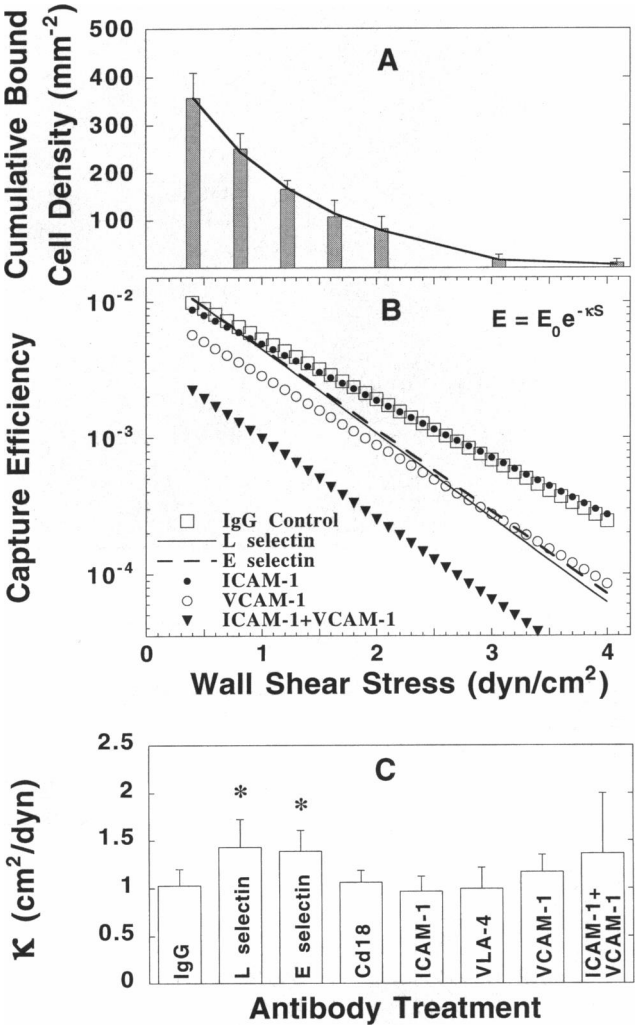

FIGURE 2 Comparison of binding efficiencies. Lymphocyte binding data were fit to a mathematical model to define separate components of the adhesion process. (*A*) Typical cumulative curve from experimental data obtained in Fig. 2. These plots were then fit to a two-parameter exponential equation (see Materials and Methods), in which the slope of the resulting line (κ) reflects the efficiency of lymphocyte capture as a function of the shear stress. Together with the second parameter (E_o), a binding efficiency, E, may be calculated for any given shear stress. (*B*) Comparison of the resultant binding efficiency curves obtained in this manner for various antibody treatments over the range of shear stresses considered. Antibody blocking can alter the efficiency plots by changing the slope of the line, its relative position along the y axis, or both. Blocking either VCAM or a combination of VCAM-1 + ICAM-1 results in a downward shift of the efficiency plot; however, blocking either L- or E-selectin principally changes the slope of the line (i.e., the efficiency of lymphocyte capture at different levels of shear stress). The significant change in the slope (κ) of the capture efficiency plot for the anti-L- and E-selectin treatment indicates that these molecules are more important at the higher shear stresses (*C*). Significant differences ($p < 0.05$) compared to IgG control are marked with an asterisk.

0.015 ± 0.003 (control) to 0.004 ± 0.001 (antibody treatment). In contrast, blocking of L- and E-selectins significantly affected κ (Fig. 2 *C*, $p < 0.05$) but not E_o, indicating that these molecules are more important at higher shear stresses, where κ has the greatest influence. The effect of combined blocking of selectin and $\beta 1$ integrin interactions on the parameter values was inconclusive because of extremely low numbers of arresting cells at shear stress levels over 2 dynes/cm^2. However, the contribution of the selectins to T-lymphocyte adhesion appears to be fundamentally different from that of the integrins, perhaps because of different molecular binding kinetics, as previously suggested (Lawrence and Springer, 1991; Dembo et al., 1988; Jones et al., 1994), and depends on the shear stress.

The in vitro findings provide a quantitative justification of the hypothesis that selectins decelerate passing lymphocytes, allowing the integrins to generate adhesive drag, similar to the model proposed for the neutrophil system (Lawrence and Springer, 1991; Ley et al., 1991; von Andrian et al., 1991). However, our work with lymphocyte adhesion indicates that the VCAM-1/VLA-4 interactions play a central role in the initial contact between T-cells and activated endothelium, which is then reinforced by stabilization of the adhesion by ICAM-1/LFA-1. Such a system would help to explain why anti-ICAM-1/LFA-1 treatment alone was unable to produce a significant reduction in T-cell adhesion in vitro but was still able to produce strong synergy with the anti-VCAM-1/VLA-4 treatment. It is notable, however, that anti-LFA-1 treatment reduced the frequency of cell rolling and binding in vivo, possibly reflecting a difference in the types of endothelium being tested and the amount of ICAM-1 expressed. Selectins appear to fill the role of mediator of the initial interaction, especially at high shear stresses and under conditions of low VCAM-1 and ICAM-1 expression. This is supported by the rapid decrease of in vitro capture efficiency after L- or E-selectin blocking seen at the high shear stresses (reflected by high κ), where selectins may facilitate the subsequent integrin-dependent interactions.

Recent work (Alon et al., 1995) has shown that tethering of neutrophils to P-selectin in vitro is likely to be a quantum event, with a single bond being sufficient to initiate attachment of a cell from the flow in a monotonic system. However, a system containing at least three different adhesion-mediating species of molecules will produce a net adhesive force that is proportional to the relative concentration and reaction rates of each ligand-receptor pair. As a result, the subtraction of the L-selectin contribution to the net force may not be noticeable as a change in rolling velocity (Table 2), but could still produce a significant decrease in the efficiency of the initial capture event. Furthermore, it is unlikely that only L-selectin is involved in the initial capture and rolling events. E- and P-selectin (Luscinskas et al., 1995) can potentially interact with other ligands on the surface of leukocytes, such as PSGL-1 (Moore et al., 1994). Because these molecules are also up-regulated on TNF-α-activated endothelium, they may also contribute to the capture and rolling of passing lymphocytes in this system, as indicated by the in vitro blocking experiments with anti-E selectin.

Differences between in vivo and in vitro observations

Striking differences were seen in the behavior of the T-cells in the flow chamber versus the mouse skin vasculature. Under

our activation protocol in vitro, most T-cells did not spontaneously roll on the monolayer. The attachment of T-lymphocytes to the endothelial monolayers proceeded rapidly after the initial contact. In general, cells contacting the surface arrested in less than one revolution with rare rolling cells, although some cells detached from the monolayer and reattached at some point downstream. Rolling of lymphocytes across the monolayer could be increased, however, if the lymphocytes were allowed to make initial contact under conditions of low shear stress followed by a gradual increase in shear stress. Increasing the shear stress from 2 to 14 dyn/cm^2 in steps of 1 dyn/cm^2 after the arrest of the lymphocytes at low shear stress resulted in approximately 33% loss in bound cells (2.75% cell loss/dyn/cm^2). Once the cells were observed to roll and detach from the monolayer during the first minute of increased shear stress, no further cell detachment could be observed until the shear stress was again increased, suggesting a narrow "window" of permissive conditions for rolling to occur in this setting. Under these conditions, a few cells in each field would roll slowly at velocities ranging from 7.8 ± 3.5 μm/s at 1.8 dyn/cm^2 to 9.9 ± 4.4 μm/s at 14 dyn/cm^2, until they moved out of the field or detached from the monolayer. This is in contrast with the events in vivo, where rolling and frequent transient attachment to the vessel wall were the dominant forms of interaction.

These differences between in vivo and in vitro studies point to several interesting features of lymphocyte-endothelial interaction. Lymphocytes can roll over TNF-α-activated endothelium; however, the process by which this occurs appears to be different in vitro from that in vivo. In vitro rolling required the lymphocytes to establish some level of firm adhesion to the endothelium before the application of shear stress. Without this provision, most cells (96%) would quickly detach from the monolayer without rolling or simply maintain firm adhesion as long as the shear stress was constant. The fact that such behavior was generally not observed in vivo within the vessel sizes observed (Fig. 3 *A*) suggests the involvement of other forces in the vicinity of the vessel wall. We hypothesized that a normal force contribution from the erythrocytes (RBCs) helps to maintain contact between the lymphocytes and the endothelium, as suggested for other leukocytes (Nobis et al., 1985; Schmid-Schönbein, 1987; Schmid-Schönbein et al., 1975, 1980). To test this hypothesis, we prepared platelet-free blood with only the T-lymphocyte populations at a 32% hematocrit (Chien et al., 1984) and tested the effect of the modified flow medium on lymphocyte capture in vitro (Fig. 3, *B* and *C*). The total number of lymphocytes delivered to the monolayer decreased as a consequence of the decreased sedimentation rate of the lymphocytes, which was in agreement with our theoretical predictions (Munn et al., 1994), but the efficiency of cell capture approximately doubled at moderate shear stresses with the addition of erythrocytes. Lymphocyte capture was rare at over 4 dyn/cm^2 without RBCs in the flow medium (Fig. 3 *C*). The addition of RBCs also increased the fraction of rolling cells versus adherent cells, and rolling velocities were comparable to those measured in vivo (Table 2). Because in vitro flow velocities were based on estimates of

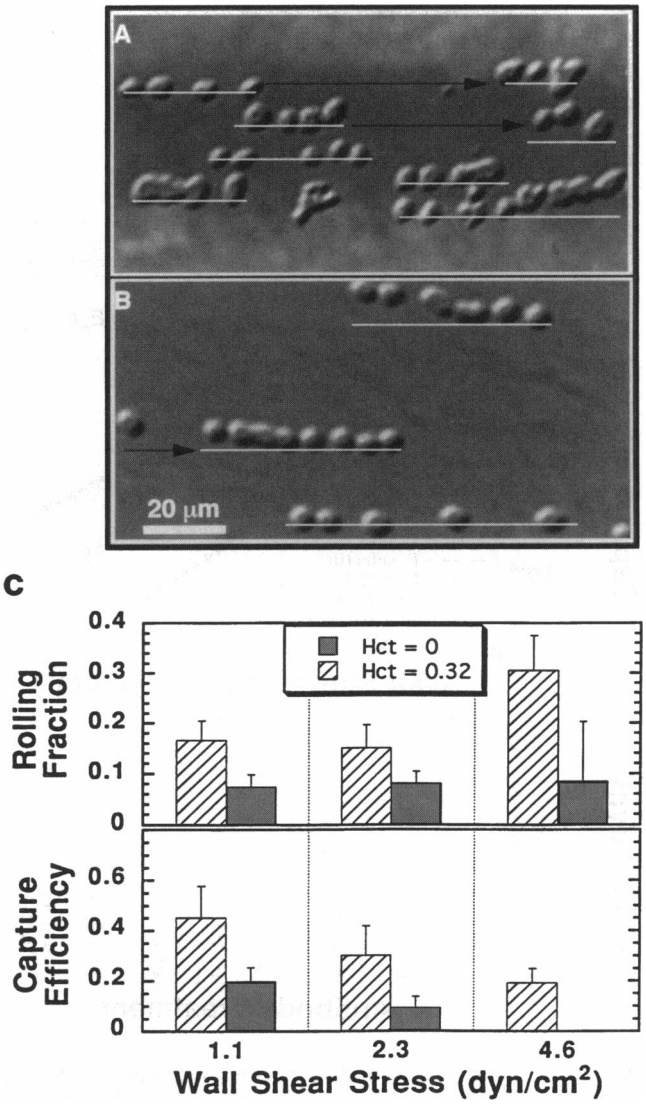

FIGURE 3 Human T-cell rolling in vivo and in vitro in the presence of RBCs. The lack of similarity in the characteristic behavior of lymphocytes on activated endothelium in the parallel plate flow cell and in the venules of the skin prompted an examination of the role of RBCs in the process of lymphocyte rolling, capture, and adhesion. (*A*) Composite image of cells rolling along the vessel wall at 1-s intervals. The images of individual rolling cells are highlighted with a white line, and intermittent jumps are indicated with a black arrow. Variation in the spacing between cell images in the same line reflects changes in rolling velocity. The venule appears as a dark background extending across the field. (*B*) Simulation of this event in vitro after the addition of RBCs to the flow medium at a hematocrit comparable to the observed venules. Approximately 20% of the fluorescently labeled lymphocytes were observed to roll over the endothelial cells. Rolling cells appear as in (*A*) and demonstrate similar velocity variations. (*C*) The increase in the fraction of rolling cells in the presence of RBCs in vitro. Flow rates were adjusted to produce equal shear stresses, with and without RBCs, assuming bulk viscosities of 2.4 cP (1.1 dynes/cm^2), 2.2 cP (2.3 dynes/cm^2) and 1.95 cP (4.6 dynes/cm^2). The efficiency of cell capture approximately doubled at moderate shear stresses with the addition of erythrocytes and could not be observed at all over 4 dyn/cm^2 without RBCs in the flow medium. The addition of RBCs also increased the fraction of rolling cells versus adherent cells, with 15–30% of the lymphocytes observed to roll over the endothelial cells at 32 ± 26 μm/s (2.3 dyn/cm^2), which is comparable to the rolling velocities observed in vivo (Table 2).

bulk viscosity of the flow medium and the cell free layer near the endothelial cells may have a lower viscosity than the bulk estimate, the increase in cell binding might be explained by decreased wall shear stress due to lower flow rates used with the RBC containing suspensions. This is unlikely, however, because experiments performed using equal flow rates for both RBC-containing and RBC-free suspensions produced significantly higher lymphocyte binding in the RBC-supplemented solutions (unpublished observations).

Similar to the behavior in vivo, cells that detached from the monolayer while rolling frequently reattached a short distance downstream, an event that was only rarely observed in RBC-free experiments. Furthermore, the combined effects of anti-LFA-1 and VLA-4 treatment in vivo (Table 2), and in vitro with added RBCs (not shown) did not demonstrate the synergistic effect seen in vitro without RBCs, suggesting that the presence of RBCs in vivo may have masked the effect by promoting reattachment of dislodged lymphocytes. In addition, the in vitro rolling velocities increased to 32 ± 26 μm/s at 2.3 dynes/cm^2, which was not significantly different from the observed in vivo rolling velocities. Thus, these observations suggest that RBCs provide a critical function in maintaining lymphocyte contact with the endothelium and by increasing the frequency of lymphocyte capture.

Two principal mechanisms may be considered with regard to the enhancement effect of RBCs in the flow stream. First is the segregation of RBCs by radial migration in Poiseuille flow, which is associated with RBC deformation (Goldsmith and Spain, 1984; Karnis and Mason, 1966). Because RBCs are considerably more deformable than leukocytes, this effect would not be expected to influence leukocyte distribution directly (Schmid-Schönbein et al., 1981). This could potentially lead to an increased lymphocyte flux in the vicinity of the endothelium, resulting in increased interaction. However, direct observation of the number of passing cells within the flow chamber was unable to demonstrate an increase in the cell flux near the wall. An alternative mechanism is the outward dispersive effect of RBCs produced by cell interactions at physiological hematocrits (Goldsmith and Spain, 1984) during flow in a narrow tube. The random lateral displacements of RBCs may produce repeated collisions with lymphocytes located near the wall and consequently promote increased lymphocyte-wall collisions. If the distribution of adhesion molecules on the wall is heterogeneous, this would result in 1) an increased probability of encountering a favorable binding site and 2) a greater, sustained, normal force upon the rolling cells than would result from adhesion dependent torque alone.

Revised paradigm for lymphocyte-endothelial interaction

The likely sequence of events leading to lymphocyte attachment appears to be initiated by RBCs promoting lymphocyte interaction with the vessel walls, followed by selectin-mediated deceleration (especially at high shear stress), then VLA-4/VCAM-1-mediated adhesion and reinforcement of adhesion by LFA-1/ICAM-1. However, the degree of involvement of any particular CAM in this sequence may be the result of a rate-limiting process of molecular binding (Dembo et al., 1988). Consequently, our findings suggest that CAM involvement in lymphocyte capture is dependent on the shear rate for given ligand concentrations. The effect of the CD18-mediated interactions on lymphocyte rolling may be to generate additional adhesive drag on slowly moving cells. Although CD18 may not directly mediate rolling interactions (Alon et al., 1995), it may be able to augment adhesion once established by more kinetically favored molecules. VLA4-VCAM interactions are likely to function in a similar manner at higher shear rates. Other studies that have examined the heterogeneity of CAM expression on the surface of TNF-α-activated HUVEC cells using a quantitative imaging technique have shown wide variations in the expression of ICAM-1 and VCAM-1 on the surface of activated HUVECs. Localization of captured T-cells from the flow cell experiments coincides with increased levels of expression of these molecules (Munn et al., in press). This supports the concept that increased adhesive drag from β1 and β2 integrins decelerates and immobilizes passing cells and that variations in expression on the monolayer may account for the detailed behavior of the rolling lymphocytes.

The role of RBCs in the process of attachment is manifested in at least three ways. First, the overall efficiency of lymphocyte capture over a range of physiological shear stresses is enhanced. Second, the "permissive window" of conditions for rolling is increased, because a RBC normal force adds to the initial adhesive force (and torque-induced normal force; Schmid-Schönbein et al., 1975) to facilitate lymphocyte contact. Third, the RBCs aid in rapid lymphocyte recapture after detachment from the endothelium. This phenomenon may be of even greater significance in conditions of high arterial shear stress. In vivo observations of arterioles within the dorsal windows used in this study indicated occasional lymphocyte rolling under shear stress of >10 dyn/cm^2 (not shown).

Observations of lymphocyte-endothelial interaction after TNF-α activation in this system represent a maximum response to a high concentration of cytokine at 5 to 6 h after stimulation. Thus, the presence of both high selectin and β1 integrin levels may promote some overlap in adhesive roles at intermediate levels of shear stress, especially in the presence of RBCs, which may not be as significant with other activating agents or for different incubation periods. This potential functional overlap in mediating the initial adhesive contact of lymphocytes with activated endothelium remains to be defined in terms of shear stress binding thresholds. However, earlier in the activation process, before increased integrin expression, the selectins may be more critical to the lymphocyte localization process (Yan et al., 1994).

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