Molecular Dynamics of the Transition from L-Selectin- to β_2-Integrin-Dependent Neutrophil Adhesion under Defined Hydrodynamic Shear

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ABSTRACT Homotypic adhesion of neutrophils stimulated with chemoattractant is analogous to capture on vascular endothelium in that both processes depend on L-selectin and β_2-integrin adhesion receptors. Under hydrodynamic shear, cell adhesion requires that receptors bind sufficient ligand over the duration of intercellular contact to withstand hydrodynamic stresses. Using cone-plate viscometry to apply a uniform linear shear field to suspensions of neutrophils, we conducted a detailed examination of the effect of shear rate and shear stress on the kinetics of cell aggregation. A collisional analysis based on Smoluchowski's flocculation theory was employed to fit the kinetics of aggregation with an adhesion efficiency. Adhesion efficiency increased with shear rate from ~20% at 100 s^{-1} to ~80% at 400 s^{-1}. The increase in adhesion efficiency with shear was dependent on L-selectin, and peak efficiency was maintained over a relatively narrow range of shear rates (400–800 s^{-1}) and shear stresses (4–7 dyn/cm^2). When L-selectin was blocked with antibody, β_2-integrin (CD11a, b) supported adhesion at low shear rates (< 400 s^{-1}). The binding kinetics of selectin and integrin appear to be optimized to function within discrete ranges of shear rate and stress, providing an intrinsic mechanism for the transition from neutrophil tethering to stable adhesion.

INTRODUCTION

The functional significance of many leukocyte adhesive processes is best understood in the context of a cascade of events wherein an initial adhesive mechanism facilitates a subsequent one. An example of this phenomenon occurs with neutrophils forming stable adhesion under physiological shear rates found in postcapillary venules (150–1600 s^{-1}) (Atherton and Born, 1973; von Andrian et al., 1992; Lawrence and Springer, 1991). Members of the selectin family of adhesion molecules (CD62) exhibit sufficiently rapid binding kinetics to allow unactivated neutrophils in the flow stream to interact with endothelial cells and begin rolling at markedly reduced velocities (von Andrian et al., 1992, 1993; Abbassi et al., 1993; Jones et al., 1993). In contrast, members of the β_2 (CD18) integrin family expressed on the neutrophil surface cannot mediate tethering and rolling at these shear rates (Smith et al., 1991; Lawrence and Springer, 1991). Current data support at least two different explanations. The first is that CD18 integrins are in a low-avidity conformation on unstimulated neutrophils (Cai and Wright, 1995; Andis et al., 1993; Diamond and Springer, 1993). The second is that, even in the presence of stimulation, integrin binding kinetics may preclude the formation of adhesive bonds at high shear rates and corresponding brief intercellular contact durations. As the adhesive bonds formed by selectins are insufficient to mediate sustained adhesion (Bargatze et al., 1994; Lawrence and Springer, 1991), neither mechanism alone allows for localization of neutrophils to sites of inflammation at physiological shear rates. In the current paradigm, selectin-mediated rolling reduces the velocity of the cell and may facilitate chemokine activation of β_2-integrins (CD11a,b), which then bind to ICAM-1 and ICAM-2 on the endothelial surface (Huber et al., 1991; Zimmerman et al., 1992; Springer, 1994; Butcher, 1991).

During an inflammatory episode, blood vessels dilate and the shear rate may be reduced by as much as 70% (Kubes et al., 1990; Gaboury and Kubes, 1994). Under these conditions, the mechanisms leading to firm adhesion of neutrophils to the endothelial wall may be very different from those found at normal shear regimes (Gaboury and Kubes, 1994). In particular, L-selectin (CD62L) has recently been shown to mediate neutrophil tethering and rolling only above a threshold level of shear (~90 s^{-1}) both in vitro on a CD34 substrate and in vivo (Finger et al., 1996). On the other hand, CD18 was found to mediate neutrophil capture on endothelial monolayers at shear rates of less than 70 s^{-1} in vitro (Lawrence et al., 1990; Lawrence and Springer, 1991; Smith et al., 1991) and below 200 s^{-1} in mesenteric postcapillary venules (Gaboury and Kubes, 1994). These data support a mechanism by which β_2-integrin binds to its counterstructure on the endothelium below the threshold shear that promotes binding through L-selectin, provided the duration of cell contact is sufficient. The mechanisms that underlie the cooperative process of adhesion through both L-selectin and β_2-integrin over a range of physiological shears remain to be determined.

We have been investigating adhesion between neutrophils (homotypic) in an effort to define the molecular and physical requirements of stable adhesion under defined hydrodynamic shear (Simon et al., 1990, 1992, 1993). Past

Received for publication 28 May 1996 and in final form 23 September 1996.

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0006-3495/96/12/3488/13 $2.00

investigations examined homotypic neutrophil adhesion after chemotactic stimulation in suspension mixed by a magnetic stir bar (Simon et al., 1992). These studies demonstrated that both L-selectin and Mac-1 were required, and it appeared that each of these adhesion molecules bound to distinct ligands on interacting cells (Simon et al., 1993). Much like the case of neutrophil/endothelial adhesion, in homotypic adhesion, neither L-selectin nor β_2-integrin alone is sufficient to allow stable adherence at physiological shear (Simon et al., 1993). Although we have found that cross-linking of L-selectin does enhance the adhesive function of Mac-1 (Simon et al., 1995), a potentially more important role for L-selectin in this experimental model is mediation of the primary adhesion in a two-step cascade necessary at physiological shear rates and stresses.

In the present study, we investigated homotypic neutrophil adhesion using cone-plate viscometry and flow cytometry. We chose cone-plate viscometry as it enables the application of precise and uniform shear rates (Hellums, 1994). Previous studies of homotypic neutrophil adhesion used mixing systems in which the shear field was nonuniform, allowing only estimates of the average shear rate (Simon et al., 1990). Accurate control of the shear rate enabled us to formulate a model of cell adhesion based on Smoluchowski's theory of particle flocculation in a linear shear field (Neelamegham et al., submitted for publication). This analysis was used to predict the collision frequency, adhesion efficiency, and average intercellular contact duration over a wide range of shear rates. Neutrophil adhesion was found to be independent of L-selectin at relatively low shear rates ($\leq 100\ s^{-1}$) and stresses (≤ 1 dyn/cm^2). Remarkably, in cells expressing both selectin and integrin, the adhesion efficiency increased up to a threshold level of shear rate and shear stress. This phenomenon was not observed when L-selectin was blocked with monoclonal antibody (mAb) or cleaved with chymotrypsin. It appears that L-selectin is critical for cells to achieve optimal adhesion efficiency at relatively high levels of shear stress and short intercellular encounter durations.

MATERIALS AND METHODS

Reagents

Anti-L-selectin mAb DREG-200 (IgG1) was a gift of Dr. T. K. Kishimoto, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT. Anti-L-selectin mAbs LAM1-3 (IgG1) and LAM1-14 (IgG1) were obtained from Cell Genesis, Foster City, CA. Anti-CD11b mAb 60.1 (IgG1) was kindly provided as a F(ab')$_2$ fragment by Dr. J. Rushcie, Repligen Corp., Cambridge, MA. Anti-CD11a mAb R3.1 (IgG1) was a gift of Dr. Robert Rothlein (Boehringer-Ingelheim Pharmaceuticals). Anti-CD18 mAb IB4 (IgG2a) was a gift of Dr. J. D. Chambers, Salk Institute, La Jolla, CA. Commercial mAb to L-selectin (Leu 8-FITC) was purchased from Becton Dickinson Immunocytometry, San Jose, CA. Anti-CD18 mAb MHM 23-PE was purchased from DakoPatts, Glostrup, Denmark, and anti-FcγRII mAb (IV.3 Fab) was purchased from Mederex, Annandale, NJ. Fab fragments were produced by digestion with papain and purified by passage over a protein-A-Sepharose column using ImmunoPure Fab preparation kits from Pierce, Rockford, IL. The proteolytic enzyme *O*-sialoglycoprotein endopeptidase (OSGE), which specifically cleaves proteins that are *O*-glycosylated on serine and threonine residues, was purchased from Accurate Chemical and Scientific Corp., Westbury, NY. Formyl-methionyl-leucyl-phenylalanine (fMLP), chymotrypsin, and Ficoll were purchased from Sigma Chemical Co., St. Louis, MO. The fluorescent nuclear dye LDS-751 was purchased from Molecular Probes, Eugene, OR.

Neutrophils

Human blood was collected from healthy volunteers by venipuncture into a sterile syringe containing 10 U of heparin/ml of blood. Neutrophils were isolated using a one-step Ficoll-Hypaque density gradient (Mono-Poly resolving medium, Flow Laboratories, McLean VA) as previously described (Simon et al., 1995) and kept at 4°C in a Ca^{2+}-free HEPES buffer (containing 110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, and 30 mM HEPES, pH 7.4). Neutrophil viability was >95% as assessed by trypan blue exclusion. Neutrophil suspensions (10^6 cells/ml) were incubated for 2 min at 37°C in buffer containing 1.5 mM Ca^{2+} before each experimental run. Samples were then stimulated with 1 μM fMLP and sheared in a cone-plate viscometer maintained at 37°C. Aliquots of 30 μl were taken at 10- to 30-s intervals for up to 7 min after stimulation and fixed in 200 μl of cold 2% glutaraldehyde.

Cone-plate viscometry

Neutrophil suspensions were sheared in a cone-plate viscometer (Ferranti Electric, Commack, NY), which consists of a stationary plate beneath a rotating cone. The design of the viscometer enables a uniform shear rate to be applied to the entire sample (Hellums, 1994). The shear rate (G) is independent of distance from the cone center and is given by (Tees et al., 1993)

$$G = \frac{\omega}{\tan \theta}, \quad (1)$$

where ω is the angular velocity of the cone and θ is the cone angle. A cone with an angle of 1° was used, and the gap between the cone and plate ranged from <10 μm in the center to 610 μm at the outside edge. At a defined shear rate, the shear stress (τ) for a Newtonian fluid is $\tau = \mu G$, where μ is the fluid viscosity. The viscosity of the buffer was measured at ~0.75 cp at 37°C in a Brookfield (Stoughton, MA.) cone-plate viscometer.

Buffer viscosity was increased by adding Ficoll (400,000 mol. wt.; Sigma), a neutral hydrophilic polymer of sucrose that is commonly used in density gradients for cell separation. Ficoll dissolved easily in buffer and did not significantly increase the osmolarity, nor did it appear to penetrate the cell membrane as observed by flow cytometry and light microscopy. We suspended neutrophils in 6% (w/v) Ficoll solutions and found no effect on L-selectin or β_2-integrin expression or their modulation in response to fMLP stimulation. The viscosity of the 6% Ficoll buffer was measured to be 1.7 cp at 37°C.

Flow cytometric detection of homotypic neutrophil adhesion

A FACScan flow cytometer (Becton Dickinson) was used to analyze the particle distributions of fixed-cell suspensions. The neutrophil population was confirmed by gating on its characteristic forward scatter versus side scatter. Singlet neutrophils and aggregates were resolved using autofluorescence derived from glutaraldehyde fixation (Rochon and Frojmovic, 1991), and aggregates were quantitated as integral multiples of the singlet fluorescence channel (Simon et al., 1990).

Inhibition of adhesion with mAb or protease pretreatment

mAbs were preincubated for 15 min at room temperature. Whole antibody to L-selectin was found to cause sustained adherence of stimulated neu

trophils, so Fab and F(ab')$_2$ fragments were used for inhibition studies. Anti-L-selectin DREG-200 Fab (30 μg/ml), anti-L-selectin Lam1-3 (30 μg/ml), and anti-CD11b/CD18 60.1 F(ab')$_2$ (30 μg/ml) were used at concentrations determined to inhibit neutrophil aggregation. The mAb IV.3 Fab (20 μg/ml) to the FcγRII receptor on the neutrophil was used as a positive control. The proteolytic enzyme chymotrypsin was used to cleave L-selectin from the neutrophil surface. Chymotrypsin was preincubated (1 U/10^6 cells) at room temperature for 20 min in buffer without Ca^{2+}, washed, and resuspended in buffer containing 1.5 mM Ca^{2+}. Flow cytometry was used to determine that L-selectin was shed to background levels using this procedure. The proteolytic enzyme OSGE was used to cleave *O*-linked glycosylated peptides from the neutrophil surface. OSGE was preincubated (30 μl of reconstituted OSGE to 2.5×10^6 cells in 250 μl of buffer) for 30 min at 37°C in Ca^{2+}-free buffer. Cells were then washed and resuspended in buffer containing 1.5 mM Ca^{2+}. Protease-treated cells were equilibrated for 2 min at 37°C before each experiment.

Quantitation of receptor expression

To measure receptor expression, sheared neutrophils were fixed in 0.25% paraformaldehyde and labeled with fluorescent mAbs. Samples were labeled for 25 min at 4°C with either Leu-8 fluorescein isothiocyanate (FITC) to L-selectin (10 μg/ml) or MHM-23 phycoerythrin (PE) to CD18 (10 μg/ml). Excess label was removed by centrifugation, and cells were resuspended in 4°C buffer for analysis on the flow cytometer. Receptor expression was quantitated on the green fluorescence channel (FL1) for L-selectin (Leu-8 FITC) and on the red fluorescence channel (FL2) for β_2-integrin (MHM-23 PE). Analysis of cellular events was performed using FACScan analysis software (Becton Dickinson). Receptor number was computed by comparing the binding of anti-CD18 and anti-CD62L to cells with binding to Simply Cellular beads (Simon et al., 1992). These beads have defined numbers of goat anti-mouse sites, and a linear relation was found between mean fluorescence intensity and the number of sites. This relation was used to estimate the initial number of CD18 and CD62L sites on the neutrophil.

ANALYSIS

The following section describes the mathematics used to quantitate the efficiency of aggregation. We outline a model to estimate the rate of recruitment of singlets and multiplets (up to sextuplets) over the first 30 s of the aggregation kinetics.

Calculation of percentage aggregation

The particle distributions of neutrophil aggregates were determined using histograms of fluorescence intensity as described previously (Simon et al., 1990). The extent of homotypic adhesion (F_S = fraction of singlets recruited) was determined by dividing the number of cells in aggregates by the total number of neutrophils detected:

$$F_s = \frac{2D + 3T + 4Q + 5P + 6Sx}{S + 2D + 3T + 4Q + 5P + 6Sx} \qquad (2)$$

where the neutrophil aggregate sizes are given by S = singlets, D = doublets, T = triplets, Q = quartets, P = pentuplets, and Sx = sextuplets and larger unresolved aggregates. Particles larger than sextuplets typically constituted <20% of the total aggregates formed.

Modeling the efficiency of neutrophil adhesion

After chemotactic stimulation, singlet neutrophils rapidly coalesce into aggregates. We fit the kinetics of aggregate formation with a mathematical model to quantitate the absolute rate and extent of neutrophil adhesion over a range of shear rates. The model is based on a theory that describes the interaction of spherical particles mixed in a linear shear field as formulated by Smoluchowski (Smoluchowski, 1917; Simon et al., 1990; Chandrasekhar, 1943). In the current study, we modeled the aggregation kinetics over the first 30 s after chemotactic stimulation. In separate studies, we have determined that adhesion efficiency decreased with time after fMLP stimulation (Neelamegham et al., submitted for publication). However, this decrease is gradual over the initial 30 s (<20% of the initial value) over which the data was modeled. This interval was composed of at least two sample points for all aggregate species measured.

Neutrophil adhesion was modeled as a two-step process. First, cells exposed to the linear shear field of a cone and plate viscometer collide. The collision frequency was dependent on physical parameters of the system, i.e., the radius and concentration of the interacting particles and the applied shear rate (Chandrasekhar, 1943). Second, the adhesion efficiency (E) was defined as the fraction of intercellular collisions that result in firm adhesion. Efficiency as computed was solely assumed to be a function of the intrinsic biological properties of the cell that determine its adhesivity: the number and affinity of adhesive receptors expressed on the cell surface. The efficiency was computed by fitting the kinetics of aggregation from doublets to sextuplets and larger aggregates as quantitated by flow cytometry. Adhesion efficiency was determined to be independent of cell concentration (See Appendix for details).

Estimating the average intercellular contact duration

The defined hydrodynamics of the cone-plate viscometer enabled estimation of the average intercellular contact duration ($t_{contact}$) required by L-selectin and β_2-integrin to mediate cell capture. Although the contact duration is large for head-on collisions, glancing collisions where cells just touch at their radii have much shorter contact durations. We applied an analysis based on the kinematics of rotation of a transiently formed doublet in a linear shear and integrated over the range of collision geometries sufficient for adhesion (Goldsmith et al., 1995). The average intercellular contact duration for the transiently formed doublet exposed to shear rate G was computed to be $\sim 2.6/G$ (See Appendix for details).

RESULTS

The adhesive properties of neutrophils interacting in shear flow were measured with a combination of cone-plate viscometry and flow cytometry. This strategy enabled the

application of precise shear rates to cell suspensions at physiological neutrophil concentrations (10^6 to 5×10^6 cells/ml). Samples were fixed in situ and aggregate distributions and molecular expression levels representative of the entire neutrophil population were obtained over the kinetics of chemotactic stimulation.

Neutrophil adherence determined by flow cytometry

Autofluorescence due to glutaraldehyde fixation enabled the detection of singlets and neutrophil aggregates. Samples were removed by pipette aspiration into fixative at defined time points before and after application of stimulation and shear. In previous studies, we confirmed that the process of sample removal and fixation did not cause aggregate breakup by demonstrating that aggregate distributions were equivalent between live cells injected over time into the cytometer and those fixed in the test tube (Simon et al., 1990; Rochon and Frojmovic, 1991). In the current studies, we confirmed that aggregate distributions were equivalent between samples removed by pipette into fixative and those fixed directly on the plate during shear at comparable times. Fewer than 2% of neutrophils were detected in aggregates in response to shear stress alone (Fig. 1 *a*). Homotypic neutrophil adherence was observed within seconds of stimulation with 1 μM fMLP. At a shear rate of 100 s^{-1}, aggregates were chiefly composed of doublets and triplets (Fig. 1 *b*), whereas at 800 s^{-1}, neutrophils were recruited into aggregates up to sextuplets and larger (Fig. 1 *c*). Formation of larger aggregates at the higher shear rates was consistent with the dependence of encounter frequency on shear rate as predicted by flocculation theory (see Eq. A1 in Appendix). The fraction of singlets recruited into aggregates was computed by gating on each aggregate size and normalizing by the total number of neutrophils detected (Eq. 2).

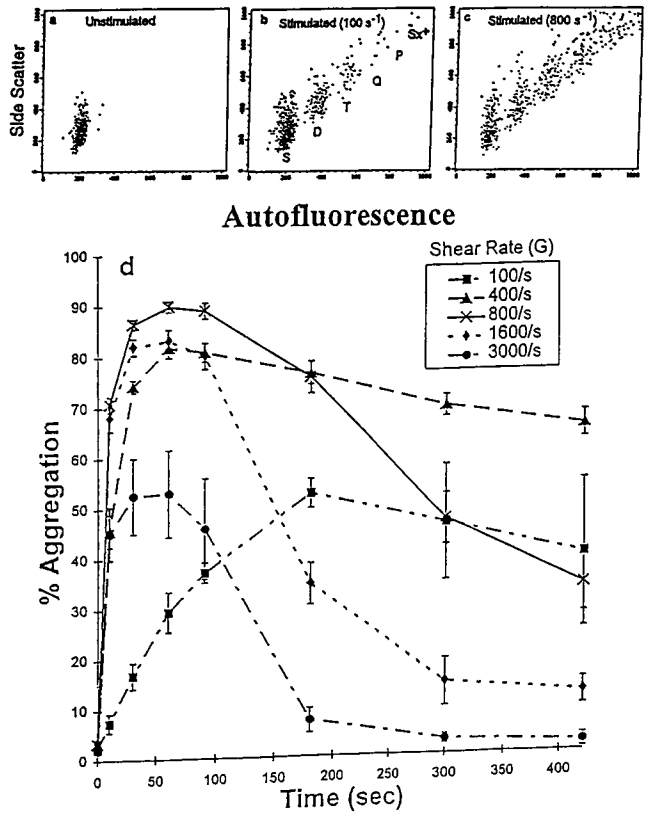

FIGURE 1 Flow cytometric detection and kinetics of neutrophil adhesion. Isolated neutrophils were incubated in 37°C buffer for 3 min, stimulated with 1 μM fMLP, and exposed to shear in a cone-plate viscometer. Samples were taken at various times and fixed with 2% glutaraldehyde. Two-parameter dot-plots of side scatter and glutaraldehyde-induced autofluorescence are shown for (*a*) neutrophils before stimulation, (*b*) fMLP-stimulated cells subjected to $G = 100\ s^{-1}$ for 1 min, (*c*) fMLP-stimulated cells subjected to $G = 800\ s^{-1}$ for 1 min. Particle sizes ranging from singlets to sextuplets were resolved. (*d*) Kinetics of homotypic neutrophil adhesion at shear rates from 100 to 3000 s^{-1}. The percentage of neutrophils recruited into aggregates, mean ± SEM, $n \geq 10$ was plotted, except at $G = 3000\ s^{-1}$ where the mean of three experiments was plotted.

Kinetics of homotypic neutrophil adherence over a range of shear rates

Isolated neutrophils were shear mixed in a cone-plate viscometer and stimulated with 1 μM fMLP. Experiments were performed over a range of shear rates from 100 to 3000 s^{-1}. Stimulation induced adhesion within 10 s, the earliest time point that could be measured with this technique (Fig. 1 *d*). The aggregation rate was highest during the first 30 s and the extent of aggregation was highest at a shear rate of 800 s^{-1}, at which more than 90% of neutrophils were recruited into aggregates. The aggregation rate increased from a minimum at 100 s^{-1} to a maximum at a shear of 800 s^{-1}. However, at shear rates above 800 s^{-1}, the extent of aggregation decreased with increasing shear. At the time point of maximal aggregation, a steady-state plateau phase was observed over a period of 1–2 min for all shear rates. This was confirmed by stimulating cells for 30 s and then diluting aggregates formed (1:50 in buffer containing 1 μM fMLP). Under these conditions, encounter frequency was decreased by a factor of ~2500, thereby limiting intercellular collisions and new aggregate formation. Aggregates remained stable for up to 2 min after dilution with less than 10% dissociation back to singlets (data not shown). At each time point, the aggregate distribution obtained by pipette aspiration was identical to that obtained by glutaraldehyde fixation directly on the plate (data not shown).

We have previously reported that homotypic neutrophil adherence is mediated by molecular recognition of L-selectin and β_2-integrin for distinct counterstructures (Simon et al., 1993). We wished to determine whether the level of the applied shear rate could modulate receptor expression and, in turn, adhesive function. The affect of a relatively low (300 s^{-1}) and high (2000 s^{-1}) shear rate on the kinetics of CD62L and CD18 expression was examined by flow cytometric analysis of the binding of fluorescently conjugated

mAb to sheared cell suspensions. On resting cells, CD62L is maximally expressed (~70,000 sites/cell), whereas CD18 is expressed at a low level (~40,000 sites/cell) (Simon et al., 1992). Within seconds of stimulation with 1 μM fMLP, CD62L was shed from the cell surface and CD18 was rapidly upregulated (Fig. 2). During the first 2 min after stimulation, ~70% of the initial CD62L was shed, and by 7 min, expression was down to background levels. Shear had no significant effect on the rate or extent of L-selectin shedding. During the first 2 min after stimulation, when the aggregation rate was highest, CD18 was upregulated ~5-fold independent of the applied shear rate. After 7 min, the expression of CD18 was significantly higher at 2000 s^{-1} (~14-fold upregulation) than at 300 s^{-1} (~10-fold upregulation) ($p < .05$). Incubating cells at 37°C with shear in the absence of stimulation caused <10% CD62L shedding and CD18 upregulation over the 7-min time course (data not shown). This level of adhesion receptor modulation with incubation alone indicated that the neutrophils remained unactivated in the absence of stimulation.

Blocking of L-selectin and β_2-integrin with mAbs over the time course of aggregation

Three distinct phases of aggregation are evident after neutrophil activation: a rapid phase of singlet recruitment, a plateau phase of aggregate stability, and a disaggregation phase (Simon et al., 1990; Rochon and Frojmovic, 1991). To determine the relative requirements for L-selectin and β_2-integrin in the early phases of singlet recruitment and aggregate stability, blocking mAbs were rapidly added at 10 and 30 s after stimulation. Based on the binding characteristics of anti-CD62L (DREG-200 Fab) and anti-CD18 (IB4) (K_d ~16 nM and ~7.5 nM, respectively), we estimated that ~90% of the available receptors were bound with blocking mAb within 30 s of addition of a saturating concentration of mAb (Simon et al., 1992). At a shear rate of 1200 s^{-1}, addition of anti-CD18 10 s after stimulation prevented further aggregation and caused premature disaggregation of formed aggregates. In contrast, addition of anti-CD62L after 10 s blocked further aggregation but did not cause premature disaggregation (Fig. 3). When anti-CD18 was added 30 s after stimulation, it again caused premature disaggregation, whereas addition of anti-CD62L at this time did not decrease the extent of aggregation (data not shown). The differences in the fraction of aggregates at the time point of disaggregation (t = 120 s) for samples blocked with mAb compared with untreated samples were statistically significant ($p < 0.05$). This led us to hypothesize that L-selectin tethering leads to β_2-integrin-mediated stable adhesion in the first seconds of aggregate formation and that β_2-integrin is required for sustaining formed aggregates.

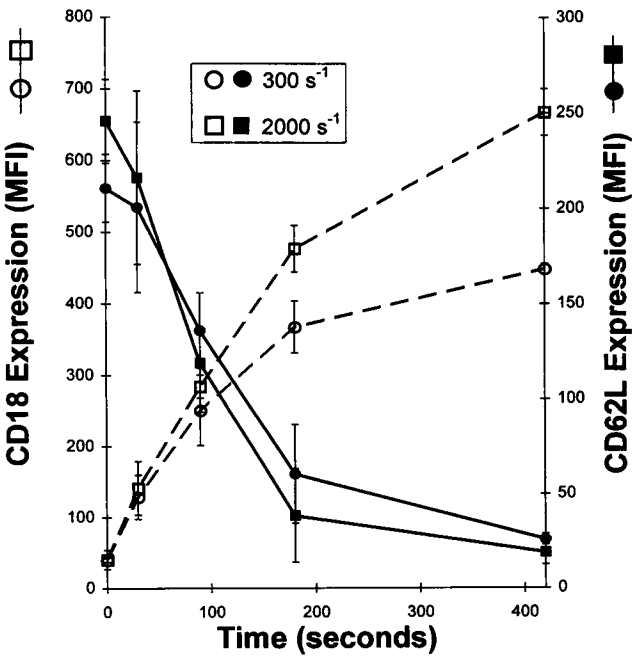

FIGURE 2 Kinetics of the surface expression of CD62L and CD18 at low and high shear. fMLP-stimulated cell suspensions were exposed to G = 300 s^{-1} (●, ○) or G = 2000 s^{-1} (■, □), and aliquots were taken at indicated time points. Cells were fixed with 0.25% paraformaldehyde and labeled with MHM 23-PE to CD18 or Leu-8-FITC to CD62L. The kinetics of anti-L-selectin (●, ■) and anti-CD18 (○, □) binding are plotted as mean fluorescence intensity ± SEM for two experiments.

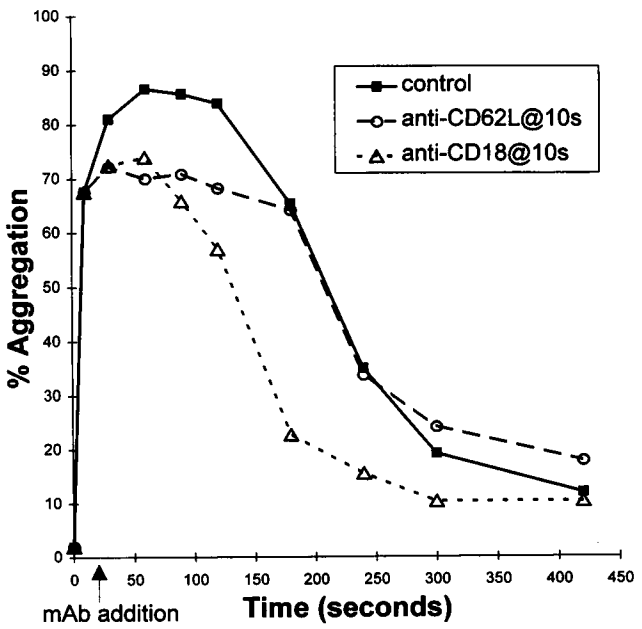

FIGURE 3 Addition of anti-CD62L or anti-CD18 mAbs after fMLP stimulation. Neutrophils (10^6 cells/ml) were stimulated with 1 μM fMLP and exposed to G = 1200 s^{-1}. After 10 s, suprasaturating concentrations of DREG-200 Fab (50 μg/ml) to CD62L or IB4 (50 μg/ml) to CD18 were added. The kinetics of aggregate formation are plotted for control, anti-CD62L, and anti-CD18. Samples were taken at indicated time points and fixed with 2% glutaraldehyde. Shown is a plot representative of four separate experiments.

Dependence of neutrophil adhesion on L-selectin and β_2-integrin at different shear rates

We have previously demonstrated that preincubation with mAbs to L-selectin or β_2-integrin will block homotypic neutrophil adherence (Simon et al., 1992, 1993). Previous studies of aggregation were performed in a test tube vigorously mixed with a small stir bar. Although this technology served well to determine the molecular requirements of homotypic aggregation, it did not provide a quantitative measurement of avidity in a defined shear field. Although the average shear rate estimated for stir-bar aggregometry was low ($\sim 30\ s^{-1}$) (Simon et al., 1990), we demonstrate here that the kinetics and extent of neutrophil aggregation and disaggregation were comparable to a shear rate of 3000 s^{-1} in the cone-plate viscometer. This large discrepancy ($\sim$100-fold) between shear rate and aggregation behavior suggests that the hydrodynamics in the stir-bar system are unpredictable and uncharacteristic of laminar flow conditions. This prompted us to examine the dependence of adhesion efficiency on L-selectin and β_2-integrin at shear rates from 100 to 3000 s^{-1} in the cone-plate viscometer.

Neutrophils were preincubated with saturating concentrations of either anti-CD11a (R3.1 Fab), anti-CD11b (60.1 $F(ab')_2$), anti-CD18 (IB4), or anti-CD62L (DREG-200 Fab, LAM1-3 Fab, or LAM1-14 Fab) and then stimulated with 1 μM fMLP and exposed to a prescribed shear rate (Fig. 4). At a shear rate of 100 s^{-1}, anti-CD11b alone caused a decrease of $\sim$50% in the rate and extent of aggregation and anti-CD11a alone caused a decrease of $\sim$40%. The combination of blocking the CD11a and CD11b subunits of CD18 inhibited adhesion completely (Fig. 4 *a*). At a higher shear rate of 400 s^{-1}, a different pattern of inhibition was observed (Fig. 4 *b*). Blocking CD11a with a Fab fragment of R3.1 did not significantly decrease aggregation (<10%). Blocking with anti-CD11b again resulted in $\sim$50% inhibition of adhesion. Addition of mAbs to both the CD11a and CD11b subunits of CD18 resulted in total inhibition of adhesion (Fig. 4 *b*), equivalent to that of blocking with anti-CD18 (IB4) (data not shown). In contrast to the inhibition seen with anti-β_2-integrin at low shear, blocking L-selectin did not change the rate of aggregation from the control level at 100 s^{-1} (Fig. 4 *c*). Three well established blocking antibodies of L-selectin adhesion, DREG-200 Fab, DREG-56 Fab (data not shown), and LAM1-3 Fab (Simon et al., 1993; Lawrence et al., 1994), preincubated alone or in combination (data not shown) did not inhibit aggregation. However, at a shear rate of 400 s^{-1}, preincubation with mAbs that bind to the lectin-like domain of L-selectin,

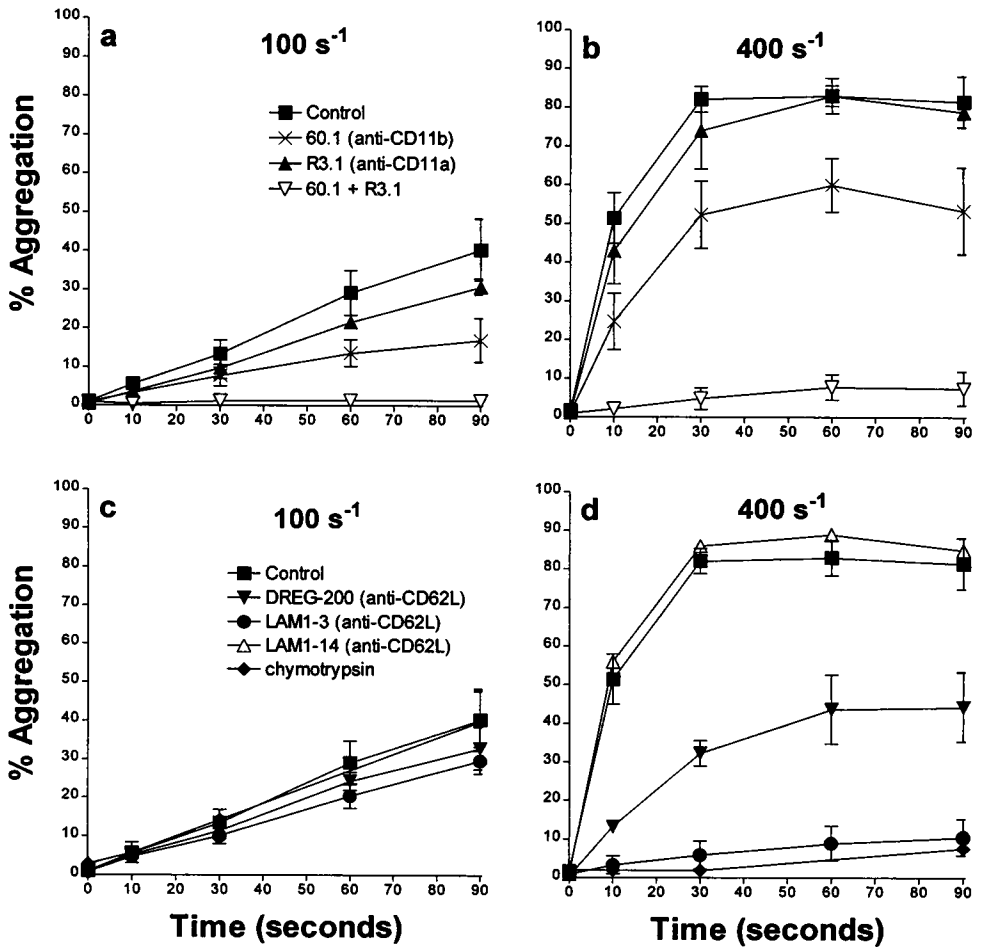

FIGURE 4 Inhibition of neutrophil adhesion by preincubation with anti-CD62L or anti-CD11a, b/CD18 mAbs. Neutrophil suspensions (10^6 cells/ml) were pretreated with DREG-200 Fab (30 μg/ml), LAM1-3 Fab (30 μg/ml), or LAM1-14 Fab (30 μg/ml) to CD62L, 60.1 $F(ab')_2$ (30 μg/ml) to CD11b/CD18, or R3.1 Fab (30 μg/ml) to CD11a/CD18 for 15 min at 25°C. Alternatively, neutrophil suspensions were incubated with chymotrypsin (1 U/10^6 cells) for 15 min at 25°C to shed L-selectin. Cells were then stimulated with 1 μM fMLP and exposed to prescribed shear rates in a cone-plate viscometer. Adhesion kinetics are plotted for untreated, anti-CD11a, anti-CD11b, and both mAbs at shear rates of (*a*) $G = 100\ s^{-1}$ and (*b*) $G = 400\ s^{-1}$. Adhesion kinetics are also plotted for untreated and anti-CD62L and chymotrypsin-treated neutrophils at shear rates of (*c*) $G = 100\ s^{-1}$ and (*d*) $G = 400\ s^{-1}$. The percentage of neutrophils recruited into aggregates is plotted as mean ± SD for n ≥ 3 experiments. The symbols used in *a* apply to *b*, and the symbols in *c* apply also to *d*.

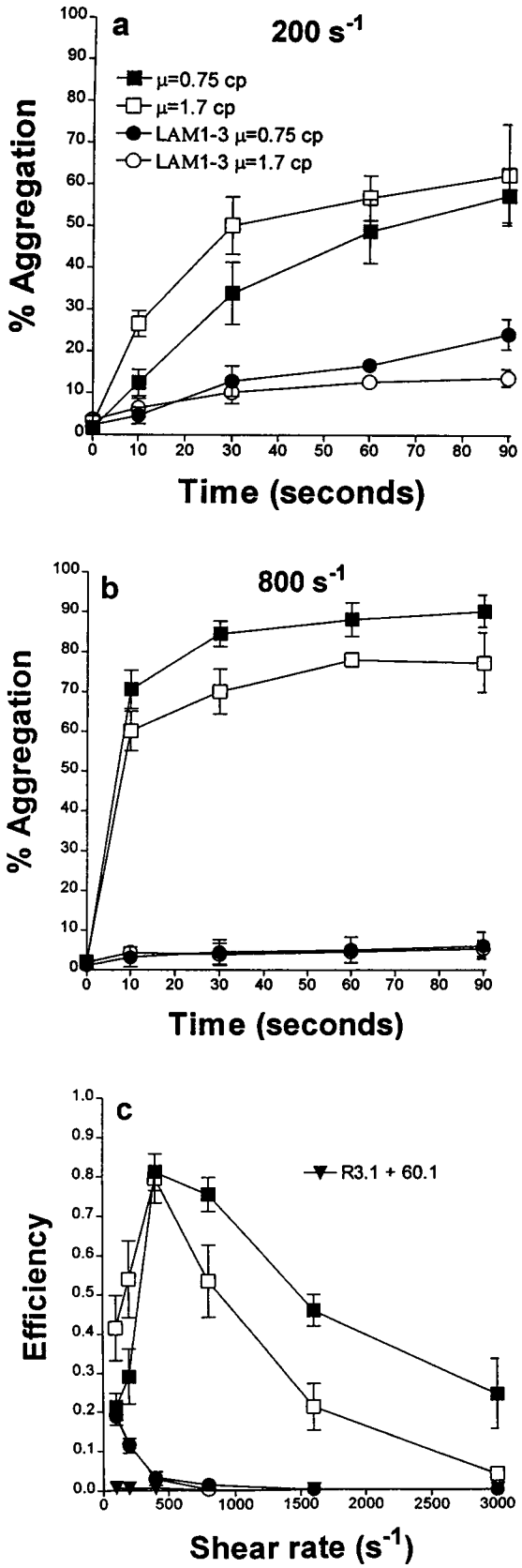

FIGURE 5 The effect of shear stress on neutrophil aggregation kinetics and adhesion efficiency. Isolated neutrophils (10^6 cells/ml) were incubated in 37°C buffer at a low (0.75 cp) or high (1.7 cp) viscosity, exposed to shear in a cone-plate viscometer, and stimulated with 1 μM fMLP.

DREG-200 or LAM1-3(Kishimoto et al., 1990; Lawrence et al., 1994), blocked 70 and 95% of adhesion, respectively (Fig. 4 *d*). Inhibition was dependent on specifically blocking the lectin-like domain as a high-affinity mAb that maps to the short consensus repeats of L-selectin (LAM1-14) did not inhibit adhesion (Fig. 4 *d*). A control mAb, IV.3 Fab (20 μg/ml), which binds the FcγRII receptor on the neutrophil did not affect aggregation at any shear (data not shown).

To confirm the results obtained with mAb blocking of L-selectin, we treated neutrophils with chymotrypsin to remove L-selectin or OSGE to remove the *O*-linked carbohydrate ligand of L-selectin (Bennett et al., 1995). Cells were treated with chymotrypsin at a concentration of 1 U/10^6 cells at room temperature for 15 min. Under these conditions, L-selectin was cleaved to undetectable levels, whereas CD11b/CD18 function was retained as previously shown (Simon et al., 1993; Jutila et al., 1991). The aggregation kinetics were assessed for protease-treated samples at shear rates of 100 s^{-1} (Fig. 4 *c*) and 400 s^{-1} (Fig. 4 *d*). At low shear rates, there was no significant difference in the rate or extent of aggregate formation between control and enzyme treatment. At a shear rate of 400 s^{-1}, removing L-selectin with chymotrypsin or cleaving *O*-linked peptides with OSGE (data not shown) resulted in almost complete inhibition of aggregation. The pattern of inhibition in the presence of protease was equivalent to that observed with blocking mAbs to L-selectin. These results confirmed that β_2-integrin alone could support aggregate formation at low shear rates (<400 s^{-1}) and that both Mac-1 and LFA-1 contribute to homotypic neutrophil aggregation.

Effects of shear stress on selectin- and integrin-mediated adhesion

The effect of increased shear stress on neutrophil adhesion was examined (Fig. 5). Ficoll was used to increase the viscosity of the suspension media, thereby increasing shear stress at a constant shear rate. At 37°C, the normal buffer had a viscosity of 0.75 cp, and addition of 6% Ficoll increased the buffer viscosity to 1.7 cp. The presence of Ficoll had no apparent affect on L-selectin or β_2-integrin expression or modulation in response to fMLP stimulation (data not shown). Increasing the shear stress at a constant

(*a*) Aggregation kinetics of untreated and anti-CD62L-treated neutrophils at G = 200 s^{-1}, at shear stresses of 1.5 dyn/cm^2 and 3.4 dyn/cm^2. (*b*) Aggregation kinetics at G = 800 s^{-1}, at shear stresses of 6 dyn/cm^2 and 13.6 dyn/cm^2 for untreated and anti-CD62L-treated cells. (*c*) Adhesion efficiency was determined at each shear rate from the aggregation kinetics over the first 30 s of fMLP stimulation, as described in Analysis. Adhesion efficiency is plotted for untreated neutrophils in two buffer viscosities, anti-CD62L, and anti-CD11a,b. The adhesion efficiency was determined from the aggregate distributions of each experiment and is plotted as mean ± SD for n ≥ 3 experiments. The extent of aggregation was significantly less than control ($p < 0.05$) for anti-CD11a,b/CD18-treated cells at all shear rates. Anti-CD62L inhibited aggregation significantly only at shear rates ≥200 s^{-1}. The same symbols are valid for all panels.

shear rate had a marked affect on neutrophil adhesion. At shear rates of 100 s^{-1} (data not shown) or 200 s^{-1} (Fig. 5 *a*), doubling the viscosity from 0.75 to 1.7 cp increased the initial rate of aggregation by ~40%. In contrast, at shear rates of 800 s^{-1} (Fig. 5 *b*) and higher (data not shown), increasing the shear stress caused a significant decrease in aggregation. The effect of increased shear stress on L-selectin-independent adhesion was also assessed. Blocking L-selectin with LAM1-3 Fab inhibited the increase in adhesion observed when shear stress was doubled at 100 and 200 s^{-1} (Fig. 5 *a*).

It was apparent from the aggregation kinetics obtained at various shear rates (Fig. 1 *d*) that the rate of aggregation increased with shear rate up to an optimal level before decreasing at shear rates of >800 s^{-1}. A useful measure of the rate at which neutrophils are recruited into aggregates is the aggregation efficiency (see Eq. A2). The efficiency was defined as the probability that a cell-cell collision results in formation of a stable aggregate. It may be used to quantitate the average adhesivity of cells over time. We computed the aggregation efficiency over a range of shear rates for untreated cells and for cells preincubated with anti-CD62L or anti-CD11a,b. The efficiency was calculated from the aggregation kinetics over the initial 30 s as the molecular recognition events that lead to adhesion occur on this time scale. In the control buffer (μ = 0.75 cp), 20–30% of collisions resulted in aggregate formation at shear rates of 100–200 s^{-1} (Fig. 5 *c*). The efficiency more than doubled to ~80% at shear rates from 400–800 s^{-1} and decreased sharply to ~45% at 1600 s^{-1} and ~25% at 3000 s^{-1}. Optimal efficiency required the expression of both L-selectin and β_2-integrin. Blocking with mAb to β_2-integrin inhibited aggregation completely at all of the shear rates tested. L-selectin alone did not support the formation of aggregates that were sustained long enough to be measured by this method. Anti-CD11b decreased efficiency by ≥50% over the full range of shear rates studied (data not shown), whereas anti-CD11a blocked significantly only at shear rates of <400 s^{-1}. At shear rates of ≥200 s^{-1}, mAb to L-selectin significantly decreased adhesion efficiency. However, anti-CD62L reduced adhesion to baseline levels only at shear rates of >400 s^{-1} (corresponding to average contact durations of <0.6 ms), indicating an absolute requirement on selectin tethering at these contact intervals. The adhesion efficiency was unchanged by anti-CD62L at shear rates of ≤100 s^{-1}, corresponding to average contact durations of ≥25 ms.

A plot of adhesion efficiency versus shear rate for the two buffer viscosities provided a direct comparison of the effects of shear rate and shear stress on control and L-selectin-independent neutrophil adhesion (Fig. 5 *c*). Adhesion efficiency increased ~2-fold at the higher viscosity for shear rates of <400 s^{-1}. A peak efficiency of ~80% was observed at 400 s^{-1}, which was equivalent at both viscosities. At shear rates of 800 s^{-1} or higher, increasing the viscosity at the same shear rate significantly reduced the adhesion efficiency. In the absence of L-selectin, adhesion efficiency decreased with shear rate, and increasing shear stress at the same shear rate had no significant effect (Fig. 5).

To illustrate the dependence of adhesion efficiency on shear stress, the data were replotted in Fig. 6. The adhesion efficiency increased with shear stress between 0.75 and ~7 dyn/cm^2. At shear stresses above 7 dyn/cm^2 or shear rates above 400 s^{-1}, the efficiency steadily decreased. The adhesion efficiency of cells in which L-selectin was blocked with LAM1-3 Fab was equal to that of untreated cells at a shear stress of 0.75 dyn/cm^2. At shear stresses ≥1.5 dyn/cm^2, blocking mAb to L-selectin significantly inhibited adhesion efficiency.

DISCUSSION

At sites of inflammation, neutrophils are recruited by a sequential process that involves initial cell contact and rolling on the vessel wall, followed by firm adhesion and transmigration (Springer, 1994; Butcher, 1991). Neutrophils rolling on or firmly adherent to the vessel wall may also recruit additional neutrophils through capture from the free stream (Bargatze et al., 1994; T. K. Kishimoto, personal communication). Therefore, the key questions regarding neutrophil adhesion apply to both homotypic adhesion and margination to the vasculature. What are the relative contributions of the selectin and integrin molecules to the efficiency of cell adhesion under hydrodynamic shear? How does the rate of bond formation and dissociation compare to

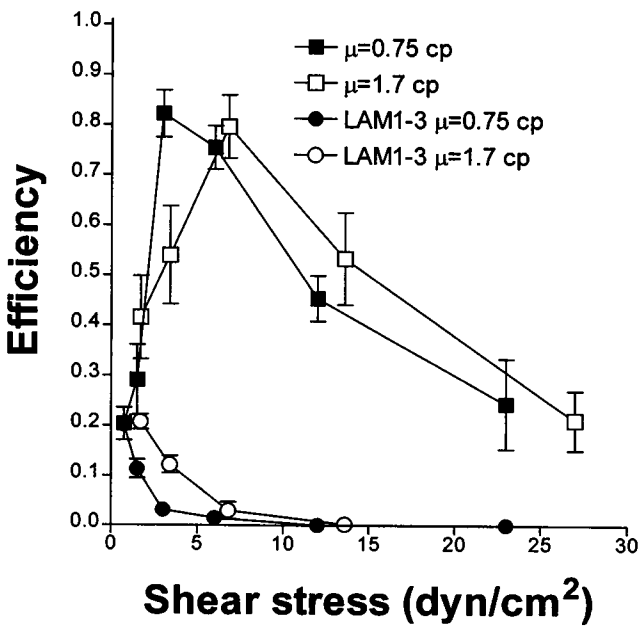

FIGURE 6 The effect of shear rate and shear stress on adhesion efficiency. Neutrophils were suspended in buffers with viscosities of either 0.75 cp or 1.7 cp, stimulated with 1 μM fMLP, and exposed to prescribed shear rates. Efficiency was plotted as a function of the shear stress for viscosities of 0.75 cp and 1.7 cp. The adhesion efficiency is plotted as mean ± SD for n ≥ 3 experiments

the duration over which cells are in contact and exposed to tensile and compressive stresses in a shear field?

In the current study, we employed cone-plate viscometry in combination with flow cytometry to quantitate the kinetics of adhesion between neutrophils in response to chemotactic stimulation. Cone-plate viscometry enabled the application of a linear shear field. It permitted the application of precise shear rates and stresses to neutrophil suspensions, and it allowed us to estimate the collision frequency and average intercellular contact duration. When coupled with a model of cell adhesion in a linear shear field, we were able to predict the adhesion efficiency over a range of physiologically relevant shear rates from 50 to 3000 s^{-1} and stresses from 1 to 30 dyn/cm^2. Adhesion efficiency was found to be invariant with respect to the cell concentrations used and was primarily a function of the intrinsic properties of the receptors (Neelamegham et al., submitted for publication). Neutrophils were found to aggregate with an optimal adhesion efficiency of ~80% over a narrow range of shear rates between 400 and 800 s^{-1} at which both selectin and integrin were necessary. The factors that mediate an increase in adhesion efficiency up to the peak value and effect a decrease in efficiency at shear rates of >800 s^{-1} and stresses of >7 dynes/cm^2 will be addressed.

Receptor expression and adhesion efficiency

Neutrophils adhere within seconds after chemotactic stimulation when sheared in suspension (Simon et al., 1990, 1992). This reversible adhesion requires the expression of L-selectin and β_2-integrin, respectively, on colliding cells (Simon et al., 1993). The L-selectin counterstructure appears to be an *O*-linked sialylated glycoprotein. Removal of these glycoproteins by an endopeptidase has been shown to inhibit neutrophil aggregation (Bennett et al., 1995). L-selectin and β_2-integrin represent two parts in what appears to be a four-component recognition process. The *O*-linked glycosylated ligand is proposed to be the third component, whereas the fourth component, the ligand bound by β_2-integrin, remains unknown. Several lines of evidence suggest that L-selectin functions to transiently tether neutrophils during aggregation. First, neutrophils that are fixed with glutaraldehyde retain their ability to serve as adhesive targets for live cells, which bind via L-selectin and activated β_2-integrin (Kuypers et al., 1990; Bennett et al., 1995). Second, data from at least two laboratories have demonstrated that neutrophils can be captured from the free stream and roll on a monolayer of adherent neutrophils in an L-selectin-dependent manner (Finger et al., 1996; Bargatze et al., 1994).

The molecular dynamics of homotypic neutrophil adhesion appear to be analogous to neutrophil adhesion on the vascular endothelium. We suggest that neutrophil aggregation occurs through transient interactions via L-selectin to an *O*-linked glycoprotein ligand enabling firm adhesion through activated β_2-integrin. The initial expression of relatively high selectin and low integrin levels was sufficient for optimal adhesion efficiency during the first 30 s after stimulation (Fig. 2). Although L-selectin appears to be essential for transient adhesion, activated β_2-integrin plays a critical role in stabilizing the adhesive interaction. This was evident in the experiments in which mAbs were added to either L-selectin or β_2-integrin within 10 s of fMLP activation; a marked decrease in the extent of aggregation was observed (Fig. 3). In contrast, with the addition of anti-L-selectin blocking mAb at 30 s, a time point at which most aggregate formation is complete, the extent of aggregation was unaltered. This provided evidence that selectin was not critical to sustain formed aggregates. Maintenance of newly formed aggregates was attributed to CD18, as addition of anti-β_2-integrin mAb at 10 or 30 s caused the early breakup of aggregates.

Effects of shear stress and shear rate on adhesion efficiency

Adhesion efficiency was found to vary as a function of both shear rate and shear stress applied in the cone-plate viscometer. At both the highest (3000 s^{-1}) and lowest (100 s^{-1}) shear rates applied, ~50% of singlets were recruited into aggregates over the first 90 s of stimulation. Over a range of shear rates between 400 and 1600 s^{-1}, greater than 80% of neutrophils were recruited into aggregates. However, peak efficiency was observed only over a relatively narrow range of shear rates from 400 to 800 s^{-1}.

The relative contributions of the selectin and integrin to adhesion efficiency were assessed by preblocking sites with mAbs to either molecule or by removing L-selectin or its *O*-glycosylated ligand with proteases. We discovered that β_2-integrin alone could support adhesion at shear rates of less than 400 s^{-1}. In this range of shear rates, blocking with anti-CD11b inhibited ~50% of aggregation. The CD11a subunit of β_2-integrin accounted for the other 30–50% of the adhesion (Fig. 4 *a*). The combination of anti-CD11a and anti-CD11b completely inhibited aggregation at all of the shear rates tested, equivalent to blocking with anti-CD18. At the highest shear rate (3000 s^{-1}), anti-CD11b alone completely inhibited aggregation whereas anti-CD11a alone had no significant effect (data not shown). Previous studies examining the molecular requirements for neutrophil aggregation have largely been performed in stir-bar assays, which appear to produce high, nonuniform shear fields. The aggregation kinetics of neutrophil suspensions mixed with the stir bar were equivalent to a shear rate of 3000 s^{-1} in the cone-plate viscometer, where the contribution of LFA-1 was not evident. Although LFA-1 (CD11a) has been previously shown to be involved in lymphocyte aggregation in static adhesion assays (Rothlein and Springer, 1986), its contribution to the homotypic aggregation of neutrophils is a novel observation.

The adhesion observed at shear rates of less than 400 s^{-1} in the presence of blocking mAb to L-selectin or protease

was completely abolished by mAbs to CD18. The efficiency of this β_2-integrin-dependent adhesion decreased in direct proportion to the increase in shear rate for both the low and high viscosity buffers (Fig. 5 *c*). An increase in shear stress at shear rates of $<400\ s^{-1}$ did not affect adhesion efficiency for β_2-integrin-dependent adhesion. Taken together, the data indicate that the decrease in intercellular contact duration as a result of increased shear rate was the primary factor that limited β_2-integrin-dependent neutrophil aggregation.

Both L-selectin and its *O*-glycosylated counterstructure appeared to be critical to optimize efficiency at shear rates of $>100\ s^{-1}$, which corresponds to average intercellular contact durations shorter than ~25 ms. Blocking with anti-L-selectin LAM1-3 or cleaving L-selectin with chymotrypsin significantly inhibited aggregation at shear rates of $\geq 200\ s^{-1}$ and completely inhibited adhesion at shear rates of $>400\ s^{-1}$ and contact durations of <6 ms. In comparison, less effective inhibition was found with DREG-200 and no inhibition was observed with LAM1-14. We propose that L-selectin binds rapidly via its lectin-like domain to *O*-sialylated counterstructures, thereby increasing the duration of the cell collision and the number of integrin bonds that support sustained adhesion.

Neutrophils expressing both L-selectin and β_2-integrin exhibited an increase in adhesion efficiency as shear stress was increased. Suspending neutrophils in the higher viscosity media boosted efficiency by ~2-fold at shear rates of 100 and $200\ s^{-1}$ (Fig. 5 *c*). The increase in shear stress as opposed to shear rate appeared to be the primary factor increasing efficiency up to the peak level. Maximal efficiency was observed at the same shear rate ($400\ s^{-1}$) but over a range of shear stress from 3–7 dyn/cm^2 (Fig. 5 *c*). Above the threshold shear rate, efficiency decreased proportionally with an increase in either shear rate or stress as indicated in Figs. 5 *c* and 6.

Role of selectins in adhesion efficiency

The process of cell capture and rolling has been extensively studied in parallel-plate flow chambers on substrates of human umbilical endothelial cells. Initial attachment and cell rolling is dependent on the selectin family of adhesion receptors, including E-selectin (Abbassi et al., 1993; Kaplanski et al., 1993) and P-selectin (Lawrence et al., 1995) induced on vascular endothelial cells and constitutively expressed L-selectin on leukocytes (Spertini et al., 1991; Smith et al., 1991). Cell rolling mediated by selectins has also been demonstrated on substrates of reconstituted selectin ligands including peripheral node adressin (Lawrence et al., 1995) and glycolipids bearing s-Le^x and s-Le^a (Alon et al., 1995a). The molecular dynamics that support the transient tethering and rolling of cells are thought to require the binding of relatively few selectin molecules to their carbohydrate ligands. These bonds apparently have high tensile strength and a rapid molecular on and off rate (Alon et al., 1995b). The efficiency with which selectins mediate the attachment and subsequent rolling of leukocytes appears to be sensitive to the applied shear. As shear flow was increased in a parallel-plate flow chamber, the efficiency of cell capture and rolling on substrates of P- and E-selectin increased (M. B. Lawrence, personal communication). This effect was also found for neutrophil rolling on L-selectin ligand (CD34) or on a monolayer of neutrophils (Finger et al., 1996). A minimal shear of ~0.5 dyn/cm^2 was required for cell capture, and the number of rolling cells steadily increased to a maximum at a shear of 1 dyn/cm^2 (Finger et al., 1996). In our studies, we also demonstrated that L-selectin mediated an increased adhesion efficiency up to an optimal level that was dependent on the applied shear. The underlying mechanism for the increase in efficiency with shear stress may be a function of the increased cell deformation and effective membrane contact area induced by a greater force of impact. For example, L-selectin is strategically positioned for binding to adjacent ligand as it is preferentially expressed on microvilli (Picker et al., 1991). The surface density of selectin may be manyfold higher than that of the integrin, which is not solely expressed on the microvilli (A. Burns, personal communication). Increased compression of the membranes would effectively increase the number of adhesive bonds, particularly integrins as they must make contact with ligand in the membrane between microvilli. Another consequence of an increased intercellular compressive force would be to overcome the electrostatic repulsive forces due to negatively charged molecules in the membrane glycocalyx.

The interplay of intercellular contact duration and shear stress in modulating adhesion efficiency

The dynamics of cell interactions in a linear shear field are governed by the shear rate and the geometry of cell interaction (see Analysis). For the case of cell collisions in free suspension, the average contact duration is $\sim 2.6\ G^{-1}$, whereas it is ~25-fold less ($\sim 0.1\ G^{-1}$) (Bongrand et al., 1988) for interactions with a planar monolayer. During this interval, cells are exposed to hydrodynamic shear stresses that involve both compressive and tensile force components acting at the adhesive interface between cell membranes. These forces vary with time and are distributed in a complex manner at the interface as the cells rotate and change position in the shear field (Tha et al., 1986). Nonetheless, a first approximation of the duration of contact between cells and the nature of the forces acting on the bonds may shed light on the relation between the kinetics of bond formation, bond lifetime, and efficiency of adhesion.

In the case of homotypic neutrophil adhesion, a newly formed doublet tumbling in a shear field will have a period of rotation of $\sim 5\pi G^{-1}$ (Tees et al., 1993). The adjacent cell membranes will experience a cycle of compression over approximately one-fourth of the total rotation, and the number of selectin bonds would rapidly build up. Over a range of shear rates between 100 and $400\ s^{-1}$, at which adhesion

efficiency increased, the average contact duration is >6 ms, an interval apparently sufficient for optimal selectin bond formation. The estimated molecular on rate for selectins is on the order of 1×10^7 s^{-1} (Alon et al., 1995a,b), high enough to provide ample binding to take place even at the highest shear rate applied (3000 s^{-1}). However, efficiency decreased to ~25% at the highest shear rates, indicating that the tensile forces and the decreasing contact duration (<1.0 ms) may limit the number of integrin and selectin bonds that actually form. The lifetime of L-selectin is reported to be ~0.15 s (Finger et al., 1996). This interval is more than sufficient to support β_2-integrin binding, which has an association rate of $\sim 10^4$ M^{-1} s^{-1} (Cai et al., 1995). The duration of aggregation is on the order of minutes and is more closely correlated with the lifetime of the integrin bond.

A review of studies of cell capture via L-selectin indicate that the efficiency of cell capture to a planar substrate decreased as a function of increased shear rate (Finger et al., 1996; Jones et al., 1993; Lawrence and Springer, 1993). Finger et al. (1996) reported that the peak in cell recruitment of L-selectin-mediated adhesion both in vitro and in vivo occurred over a range of shear stress between ~1.0 and 10.0 dynes/cm^2. In comparison, we found homotypic adhesion to be most efficient over a range of shear stress from 3 to 7 dynes/cm^2. However, a narrower range of shear rates and corresponding contact durations supported optimal adhesion. Apparently, it is the brief contact duration that primarily limits the adhesion efficiency at shear rates above ~400 s^{-1} both in vitro and in vivo (Finger et al., 1996).

CONCLUSION

Neutrophil capture and rolling on endothelial cells is partially mediated by L-selectin (Smith et al., 1991; Abbassi et al., 1993; von Andrian et al., 1992). This event is both necessary and sufficient for β_2-integrin-dependent firm adhesion at normal physiological shear rates (von Andrian et al., 1991; Ley et al., 1991). β_2-Integrin alone is sufficient to mediate cell capture only under conditions of low shear flow (Smith et al., 1991; Lawrence et al., 1990; Gaboury and Kubes, 1994). We demonstrate here that CD11a and CD11b can mediate adhesion at shear rates of less than 400 s^{-1}. β_2-Integrins may require considerably more time for binding than L-selectin; an average intercellular contact duration of 25 ms was necessary for cell adhesion in the absence of L-selectin. L-selectin markedly enhanced the efficiency of homotypic adhesion at shear rates of greater than 100 s^{-1} and was absolutely required at shear rates of greater than ~400 s^{-1} (contact durations of <6 ms). As L-selectin can tether unactivated neutrophils, it may serve to provide sufficient time and cell contact for β_2-integrin to engage in sufficient numbers after cell activation. The presence of ligands for both L-selectin and β_2-integrin on the endothelium should markedly optimize adhesion efficiency over a wide range of shear rates. These results suggest that the interplay between adhesion molecule expression and the hydrodynamic conditions may provide a novel mechanism of selectivity for neutrophil localization at appropriate sites on vascular endothelium.

We thank Dr. James Smolen for his insightful review of the manuscript, Dr. Micah Dembo for early guidance in this project, Dr. T. K. Kishimoto for his gift of reagents, and Michelle Swarthout and Carol McGary for expert secretarial assistance.

This work was supported by National Institutes of Health grants AI23521, 5P50NS23327, AI31652, and HL42550 and a Whitaker Foundation grant.

APPENDIX

Computation of adhesion efficiency

The uniform gradient in the velocity streamlines of a linear shear field causes the cells closer to the rotating cone surface to move faster than the cells near the stationary plate, resulting in cell-cell collisions. The intercellular collision frequency per unit volume, f_{ij}, was computed from prescribed experimental conditions: aggregate radius r_i and r_j (cm), shear rate G (s^{-1}), and the concentration c_i and c_j (cells/ml) of aggregates of size i and j, respectively (Smoluchowski, 1917):

$$f_{i,j} = \frac{16}{3}(r_i + r_j)^3 G c_i c_j. \tag{A1}$$

Collisions between the cells result in firm adhesion with some probability. This may be expressed as the adhesion efficiency, E, defined as the ratio of the number of cell collisions that result in firm adhesion (quantitated by flow cytometry) to the estimated number of collisions in suspension. Mathematically, the rate at which an aggregate a_{ij} of size $i + j$ is formed on collision of particles having radius r_i and r_j is given by

$$\frac{da_{ij}}{dt} = f_{i,j} E. \tag{A2}$$

Upon collision, the aggregating species initially collide and adhere to each other at a single point on the cell surface. These transiently formed aggregates are susceptible to breakage due to hydrodynamic shear forces. On the basis of experimental measurements, we described the rate of disaggregation of these transient aggregates in the first 30 s after application of shear by a power law expression (Neelamegham et al., submitted for publication). The rate at which aggregates of size j disaggregate into smaller aggregates of size i and $j - i$ is given by

$$\frac{db_{i,j}}{dt} = \frac{K j^m E}{i} A_j. \tag{A3}$$

The parameters K and m are disaggregation constants determined experimentally at each shear rate. The concentration, A_i, of aggregates of size i over time can be found by solving the following differential equation:

$$\frac{dA_i}{dt} = \frac{1}{2}\sum_{j=1}^{i-1} \frac{da_{j,i-j}}{dt} - \sum_{j=1}^{N-i} \frac{da_{i,j}}{dt} - \frac{1}{2}\sum_{j=1}^{i-1} \frac{db_{i,j}}{dt} + \sum_{j=i+1}^{N} \frac{db_{j,i}}{dt} \qquad \begin{matrix} i = 1, 2, 3 \cdots N \\ j = 2, 3, \cdots N. \end{matrix} \tag{A4}$$

N is the maximal aggregate size used in the simulation and determines the number of simultaneous differential equations solved at each time point. Experimental observations revealed that typically less than 20% of the

aggregates were larger than sextuplets. For continuity, aggregates up to size 15 ($N = 15$) were modeled, and the aggregation kinetics of singlets through sextuplets were fit to the simulation. The first term on the right hand side of Eq. A4 accounts for the formation of aggregates (e.g., a triplet is formed from a singlet and a doublet). The second term accounts for the depletion of aggregates (e.g., the singlet and doublet populations are depleted in triplet formation). The last two terms account for the disaggregation of large aggregates. The third term quantifies the rate at which large aggregates break up (e.g., the rate at which a triplet breaks up into a doublet and singlet). The fourth term accounts for the rate of formation of small aggregates as a result of disadhesion (e.g., a singlet and doublet are formed when a triplet disaggregates).

The adhesion efficiency was optimized to fit the first 30 s of the kinetic data for all aggregate species over a range of shear rates and experimental treatments. The model assumed that the single cells were spherical in geometry, and only single collisions between two particles were considered at any time. The system of differential equations represented by Eq. A4 was solved simultaneously by the Runge-Kutta Fehlberg 4,5 algorithm (Fehlberg, 1970) with a variable time step on a Pentium computer. Detailed justification for the modeling assumptions and model parameter determination will be described in a separate manuscript (Neelamegham et al., submitted for publication).

Average intercellular contact duration

The average contact duration was calculated on the basis of a collisional analysis for the interaction of two equally sized rigid spheres in a linear shear field (Goldsmith et al., 1995). The contact duration depends on the collision angle, ϕ, that the projection of the axis of revolution of the collision doublet makes with the median plane (see Fig. 1, Goldsmith et al., 1995). Upon collision, the spheres move as a rigid dumbbell with angular velocity, $d\phi/dt$, which varies with the collision angle ϕ as previously described (Wakiya, 1971). Based on this analysis, the predicted contact duration of a doublet that collides with an initial angle ϕ has been shown to be (Goldsmith et al., 1995):

$$t_{\text{contact}} = \frac{5}{G}\tan^{-1}\left(\frac{1}{2}\tan\theta\right). \qquad \text{(A5)}$$

We calculated the average contact duration over all possible collision orientations, ranging from head-on collisions where ϕ tends to $\pi/2$, to glancing collisions where ϕ tends to 0. Neutrophils were assumed to be spherical in shape and their surface was discretized into a fine grid. For each point on the grid we calculated the angle of collision and the corresponding contact duration of interaction from Eq. A5. We also calculated the number of collisions at each grid point, as in a linear shear field, the frequency of collision increases linearly as we move to higher streamlines. The average contact duration calculated, while taking into account the collision orientation at each point and weighing it by the frequency of collision at that point, was found to be $\sim 2.6/G$.

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