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Cooperativity between Selectins and β_2-Integrins Define Neutrophil Capture and Stable Adhesion in Shear Flow

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Abstract—A cooperative, sequential process of molecular recognition governs leukocyte capture, rolling, and arrest on inflamed endothelium. Flowing neutrophils are captured *via* heterotypic adhesive interactions mediated by endothelial E-selectin, whereas homotypic interactions between neutrophils are mediated by L-selectin. To elucidate how each selectin facilitates the transition to CD18-mediated stable adhesion, E-selectin and L-selectin were expressed at defined site density in a murine pre-B-cell line. Direct observation of two-body collisions revealed that 30% of neutrophil interactions with E-selectin transfectants formed doublets at low shear rate $G = 14\ s^{-1}$ whereas a threshold shear rate $14\ s^{-1} \leq G \leq 110\ s^{-1}$ was necessary for L-selectin adhesion. Adhesion *via* L-selectin resisted rupture at high shear stress, while E-selectin tethered doublets remained intact longer once formed. Moreover, higher expression of L-selectin (1100 sites/μm^2) than that of E-selectin (220 sites/μm^2) was required for comparable heterotypic adhesion efficiency. With a threefold rise in active CD18 upregulated on chemotactically stimulated neutrophils, homotypic adhesion efficiency increased 10-fold compared to less than 5-fold for heterotypic adhesion to selectin transfectants. Co-expression of E-selectin and ICAM-1 boosted adhesion efficiency threefold more than either receptor alone over the range of active CD18 expression. These data are the first to quantify adhesion efficiency mediated by selectin tethering and conformational activation of β_2-integrin in neutrophils in shear flow.

Keywords—Selectin, Integrin, Viscometry, Cell adhesion, efficiency, Shear stress.

INTRODUCTION

Neutrophil capture from the blood stream and recruitment to sites of inflammation is a cooperative and sequential process of molecular recognition.[5,18,23,28,46] E- and P-selectin upregulated on activated endothelium initiate leukocyte capture and facilitate rolling by binding sialylated ligands, including L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) expressed on neutrophils.[7,18,51] E-selectin and L-selectin exhibit distinct binding kinetics and bond mechanics, which are reflected in differences in the dynamics of neutrophil adhesion supported by each.[39,54] E-selectin functions to capture leukocytes from the blood stream,.[20,31] while recent data suggests L-selectin primarily functions in capture through neutrophil–neutrophil homotypic interactions.[9,35,37,45] Whereas selectins support initial cell capture from the blood stream through transient adhesive interactions, neutrophil arrest and transmigration requires intracellular signaling and activation of β_2-integrins (CD18). Supporting the transition from rolling to arrest is ICAM-1, which is upregulated on inflamed endothelium and serves as a ligand for arrest through β_2-integrins.[25,42]

Neutrophils transported along adjacent streamlines in laminar shear collide and form doublets under compressive stress with a capture efficiency (ε_c) that can be directly observed in a transparent cone-plate rheoscope. Transient neutrophil doublets rotate through periods of tensile stress, even in the absence of chemotactic stimulation and CD18 bond formation, provided that L-selectin engages PSGL-1 between neutrophils and shear is maintained.[11] Upon stimulation with chemotactic peptide, doublets and larger aggregates are stabilized by formation of CD11a/CD18 and CD11b/CD18 bonds.[11,15,35] Adhesion efficiency modeled from the kinetics of neutrophil aggregation in shear mixed suspensions, therefore, represents an ensemble of the probability of initial capture (ε_c) and of transition to stable adhesion (ε).[34] Previous studies suggest that optimum adhesion efficiency requires sequential engagement of selectins and integrins.[49,50] Current evidence indicates that adhesion efficiency mediated by selectins and integrins varies with shear rate (G) and shear stress (S), and by the level of neutrophil stimulation.[11,15,34] However, a comparison of adhesion mediated by L- and E-selectin as a function of expression of active CD18 has not been published.

In this study, we applied two-body collision theory[44] which takes into account biophysical parameters of cell radii, cell concentration, and fluid shear rate to predict

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the kinetics of neutrophil homotypic and heterotypic aggregation with selectin transfectants in sheared cell suspensions. Aggregates were sampled over the time course of chemotactic stimulation and shear and their size and composition measured by two-color fluorescence flow cytometry. Adhesion efficiency (ε) was estimated from the ratio of aggregate formation rate to collision frequency as previously described.[15,16,32–34] The objective of this study was to examine neutrophil capture mediated by E-selectin or L-selectin in order to define cooperativity between selectin-mediated capture and integrin-dependent stable adhesion. Two types of experiments were employed in these studies. Direct observations of shear-induced collisions and selectin-dependent capture of unstimulated neutrophils were videotaped in a transparent counterrotating cone and plate rheoscope. Neutrophil stable aggregation was measured by employing two-color fluorescence flow cytometry of cell suspensions sheared in a cone-plate viscometer. These data are the first to quantify capture and adhesion efficiency in terms of active CD18 and selectin site density and provide insight into the molecular basis of efficient transition to firm adhesion after selectin-mediated capture.

MATERIALS AND METHODS

Reagents

Formaldehyde solution was purchased from Fisher Scientific (Pittsburgh, PA). Formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC) conjugated anti-E-selectin antibody BBA-21 was from R&D Systems (Minneapolis, MN). Phycoerythrin- (PE) labeled antibody MHM23 to CD18 and FITC-labeled antibodies to CD45 and ICAM-1 (CD54) were from Caltag Laboratories (Burlingame, CA). FITC- and PE-labeled anti-L-selectin antibody Leu-8 were from Becton Dickenson (San Jose, CA). The Rhodamine-PE conjugate of monoclonal antibody 2LPM19c was purchased from DAKO (Carpinteria, CA) for detection of CD11b/CD18 (Mac-1) expression. Protein Design Labs (Mountain View, CA) generously provided the murine anti-E-selectin mAb EP5C7 (denoted MuEP5C7, IgG_2). R15/7 antibody to β_2-integrin was generously provided by Dr Robert Rothlein at Boehringer-Ingelheim Pharmaceuticals (Ridgefield, CT). Antibody 327C (kindly provided by ICOS Corp., Bothell, WA) binds CD18 at the I domain and reports on a neoepitope expressed on the active binding site[27] and was labeled with Alexa-488 (Molecular Probes, Eugene, OR) following the manufacturer's protocol. In all experiments antibodies were used at saturating concentrations, as determined by flow cytometry using anti-CD45-FITC, 2LPM19c-FITC, MHM23-PE, and anti-CD54-FITC at 5 μg/ml, 327C at 10 μg/ml, and BBA-21-FITC, Leu-8-FITC and -PE, MuEP5C7, at 20 μg/ml.

Isolation of Human Neutrophils

Whole blood was obtained from healthy adult individuals by venipuncture using sterile syringes containing heparin (10 units/ml of blood, Elkins-Sinn, Inc., Cherry Hill, NJ) approved human subjects protocol (University of California, Davis Protocol Identification 993120). Neutrophils were isolated from whole blood by using a one-step density gradient (PMN, Robbins Scientific Corp., Sunnyvale, CA) following the manufacturer's protocol. Following isolation, neutrophils were washed once with modified HEPES buffer (10 mM KCl; 110 mM NaCl; 10 mM glucose; 1 mM $MgCl_2$; and 30 mM HEPES, pH 7.4). The neutrophils were resuspended at $\sim 2.5 \times 10^7$ cells/ml and maintained at room temperature in a calcium-free HEPES buffer. Neutrophils remained inactivated at room temperature for up to $\sim$4 h after separation based on the criteria that incubation for 20 min at 37°C yielded less than 10% upregulation of CD11b/CD18. Neutrophil viability was assessed by trypan blue exclusion and they were deemed to be >98% viable.

For the experiments in the rheoscope, neutrophils were isolated from whole blood by using a two-step centrifugation procedure as previously described,[11] and suspended in Ca^{++}, Mg^{++}-free Tyrodes, containing human serum albumin, and kept on ice until used. Approval for drawing blood from human subjects into citrate was obtained from the Research Ethics Committee of the McGill University Health Center, Montreal General Hospital (Protocol Identification REC-95006).

Tissue Culture and Preparation of 300.19 Cells

300.19 cells (murine pre-B lymphocytes), which grow as a suspension in culture, were stably transfected with human E-selectin, L-selectin, or both E-selectin and ICAM-1 cDNA using a modified SRa vector containing a neomycin resistance marker.[19] 300.19 cells were maintained in culture as described previously.[15]

Adhesion Molecule Expression on 300.19 Cells and Neutrophils

FITC conjugates of monoclonal antibodies BBA-21 against E-selectin, Leu-8 against L-selectin, and anti-CD54 against ICAM-1 were used to assess the surface expression by each 300.19 cell line, as well as unstimulated neutrophils. Quantum Simply Cellular calibration bead sets (Bangs Laboratories Inc., Fishers, IN) containing four defined site densities of goat–anti-mouse antibody ranging from $\sim$10,000 to 200,000 sites/bead were used to generate standard curves of relative binding sites/cell versus mean fluorescence intensity for each antibody as described previously.[15] Neutrophils or 300.19 cells at 5×10^6/ml in modified HEPES buffer were incubated with antibodies to the respective adhesion molecule for 30 min at room

temperature, and site densities were computed assuming average cell diameters of 300.19–9 μm and PMN ~7.5 μm.

Videomicroscopic Observations

As previously described,[11] two-body collisions between neutrophils and 300.19E cells in Couette flow were observed and videotaped in a transparent counterrotating cone and plate rheoscope (model MR-1, Myrenne Instruments, Fremont, CA) with a nominal cone angle of 2°. The rheoscope was mounted on a Zeiss Axiovert inverted microscope (Carl Zeiss Ltd., Montreal, QC, Canada). Equal concentrations of neutrophils and 300.19 cells (8×10^6/ml) were suspended in Tyrodes buffer with either 5 or 10% Ficoll added to increase the suspending phase viscosity to 2.7 or 5.5 mPa s at 22°C. Aliquots of the neutrophil suspension on ice and the 300.19 cells at room temperature were mixed and added to buffered Ficoll containing Mg^{++} and Ca^{++}, gently mixed and allowed to warm to room temperature before pipetting 30 μl onto the plate of the rheoscope

Two-Body Collisions. At shear rates of 110 and 220 s^{-1}, cell trajectories and collisions were recorded over 5 s at framing rates of 250 and 500 s^{-1} with a high-speed digital camera (KODAK Motion Corder Analyzer SR-500c, Roper Scientific MASD, San Diego, CA). At low $G = 14$ s^{-1}, recordings were made over 22 s at a framing rate of 60 s^{-1}. Sequences were captured on a computer and collisions analyzed using NIH Image 1.62. Transient doublets were those in which the cells separated while rotating in the quadrant of the orbit under tensile force. Nonseparating doublets were those in which the cells, tethered through selectin bonds, remained attached under tensile force, and rotated past the orientation in which they were aligned with the flow. The time averaged capture efficiency, ε_c, was obtained directly by dividing the number of nonseparating collision doublets by the total number of two-body collisions analyzed. Such nonseparating doublets were followed until they broke up or disappeared from view. The measured doublet lifetime, τ_{meas} (lifetime of adhesion), corresponded to the period between first apparent contact and separation (breakup). The mean values, $\langle\tau_{meas}\rangle$, were compared with those predicted for doublets of rigid spheres, $\langle\tau_{theor}\rangle$ assuming rectilinear approach of the colliding spheres rotating as a rigid dumbbell with an equivalent ellipsoidal axis ratio, r_e^D, given by

$$\tau_{theoretical} = \frac{\pi\left(r_e^D + 1/r_e^D\right)}{G\left(r_e^D + 1\right)} \quad (1)$$

For equal-sized spheres,[3] $r_e^D = 2.0$ and $\langle\tau_{theor}\rangle = 5\pi/6G = 2.62/G$. Values of r_e^D for rotating rigid dumbbells of unequal diameter have been tabulated previously[1] For PMN:300.19 cells $b_1/b_2 = 0.83$, $r_e^D = 1.84$, and $\langle\tau_{theor}\rangle = 2.64/G$.

Rates and Extent of Aggregation. The rates of formation for nonseparating doublets, triplets, and higher order heterotypic and homotypic multiplets were videotaped over 22.5 s at 60 frames/s or 12 s at 125 frames/s, at lower magnification to obtain more cells in the field of view. All experiments were conducted at 22 ± 1°C.

Determination of Active CD18 Expression on Neutrophils

Total CD18 site density was determined by equilibrating suspensions of neutrophils (10^6 cells/ml) for 2 min at 37°C, before fMLP stimulation. Neutrophils were activated over a dose range of fMLP (1 nM–1 μm) and incubated for 7 min at 37°C. Samples were then kept on ice and stained with FITC-labeled mAb to CD18 (MHM23) for 30 min. To measure the site density of active CD18, neutrophils were preincubated with saturating concentration of 327C, an antibody that only binds to the ligand binding open conformation of CD18. After equilibration, the prescribed dose of fMLP was added to neutrophil suspensions, and samples were incubated at 37°C and then fixed in 1% cold paraformaldehyde. After 15-min fixation, samples were washed to remove excess antibody and analyzed by flow cytometry. Mean fluorescence intensities were computed from Quantum Simply Cellular bead standards to determine site numbers, and site density was calculated assuming neutrophil diameter of 7.5 μm.

Cone and Plate Viscometry Applied to Study Neutrophil Adhesion Assays

The kinetics of neutrophil homotypic and heterotypic aggregation was studied at 37°C in a Haake VT550 cone-plate viscometer as previously described.[16] The neutrophils and 300.19 cells were mixed and introduced into the viscometer at final concentrations of 10^6 CD45-FITC (10 μg/ml) labeled neutrophils/ml and between 3×10^5 and 5×10^6 LDS-751 (0.3 μg/ml) labeled 300.19 cells/ml addition of fMLP stimulation between 1 nM and 1 μM upon initiation of shear. At preselected times, samples were removed by pipette and fixed for flow cytometric analysis as described previously.[16] The extent of neutrophil heterotypic aggregation reached a maximum of ~50% singlet recruitment at $G = 600$ s^{-1} where shear stress, $S \sim 0.6$ N/m^2, and this level of shear was chosen for the remaining experiments[15] It should be noted that the above value of S is the same as that in the cone-plate rheoscope experiments carried out at $G = 110$ s^{-1} in 10% Ficoll.

Adhesion Efficiency

The *probability* with which colliding cells adhere and form stable aggregates is termed the adhesion efficiency[34] and expressed as

$$\text{Adhesion efficiency} = \frac{\text{Rate of stable aggregate formation}}{\text{Frequency of intercellular collisions}}.$$

The numerator is derived from flow cytometric quantitation of heterotypic aggregation kinetics up to the peak extent of

aggregation at 30 s of shear and chemotactic stimulation. The denominator predicts the number of collisions per unit time from a model based on Smoluchowski's two-body collision theory[44] and assumes rectilinear approach of the cells as rigid spheres, as described previously.[33,50] For particles of unequal size, as those that are formed during heterotypic aggregation of neutrophils and 300.19 cells, the collision frequency, C, between particles assumed to be spheres of radii b_1 and b_2 at concentrations N_1 and N_2 is

$$C = \varepsilon \frac{4}{3} N_1 N_2 G (b_1 + b_2)^3 \qquad (2)$$

In the following experiments, neutrophils ($b_1 = 3.75\mu$m) were sheared with 300.19 cells ($b_2 = 4.5\mu$m). The two-body collision theory model accurately predicted both homotypic and heterotypic aggregation kinetics in neutrophil-300.19 cell suspensions.

Statistical Analysis

Data are presented as mean values, and error bars indicate standard error of the mean. One-way analysis of variance was used for multiple comparisons and *t* tests for comparisons between two groups. Posttests were performed by Newman–Keuls method. $P < 0.05$ was considered significant.

RESULTS

Expression of Adhesion Molecules on 300.19 Cells

Human cDNA encoding expression of L-selectin, E-selectin, and ICAM-1 was transfected into a murine pre-B cell line (300.19) that grows in tissue culture as a single-cell suspension. Since the 300.19 cells do not produce the glycoprotein selectin ligand sialyl lewis-x they do not adhere to each other, but serve as cellular targets expressing molecules recognized by human neutrophils in sheared, mixed cell suspension. Thus, in heterotypic aggregation selectins on 300.19 cell lines engage in one-way adhesion to ligands on neutrophils, whereas in homotypic aggregation neutrophils engage in two-way molecular bridging with selectins and integrins on opposing cells binding their respective ligands. Three sets of L-selectin clones (300.19L) were isolated that expressed a range of site densities from a low level of ~70,000 sites/cell up to a maximum of ~280,000 sites/cell. 300.19L_{Med} expressed at ~200,000 sites/cell corresponding to the site density on circulating leukocytes (Table 1). Two stable clones of E-selectin 300.19 cells were expanded; 300.19E_{High} expressed ~60,000 sites/cell commensurate with the receptor density upregulated on inflamed endothelium and 300.19E_{Low} expressed ~40,000 sites/cell. A third clone denoted 300.19E/I was cotransfected with cDNA encoding E-selectin and ICAM-1. 300.19E/I expressed E-selectin at ~34,000 sites/cell and ICAM-1 at ~750,000 sites/cell (Table 1).

Unstimulated Neutrophils: Direct Observation of Neutrophil–300.19 Adhesion in the Rheoscope

Shearing cell suspensions in a cone-plate rheosc ope enabled the visualization of two-body collisions between neutrophils and 300.19 cells and comparison of E-selectin versus L-selectin-mediated capture and tethering. These experiments were performed with 300.19 cells expressing high selectin site density (Table 1) and in the absence of chemotactic stimulus to observe selectin function alone. Cells collide and form doublets that are subjected to normal forces acting along the doublet axis. These are alternately

TABLE 1. Expression levels of selectins on 300.19 cells. The number of selectin sites expressed per 300.19 cell was determined for E-selectin (antibody BBA21-FITC) and L-selectin (antibody Leu-8-FITC) and are presented as *M* ± SE. 300.19 cells were incubated with saturating concentrations of labeled antibody at 37°C for 30 min, washed, and analyzed by flow cytometry. Site numbers were determined by comparing mean fluorescence intensities of antibody bound to cells with Quantum Simply Cellular bead standards as described in Materials and Methods. Representative adhesion efficiencies were determined at $G = 600\ s^{-1}$ with 2.5 nM fMLP stimulation for equal concentrations of neutrophils and 300.19 cells (10^6/ml).

Cell type	Expression (sites/cell)	Selectin density (sites/μm^2)	Representative adhesion efficiency, ε (%)
PMN	120,000	680	4.0
300.19L_{Low}	73,300 ± 8200	288	1.2
300.19L_{Med}	205,400 ± 20,200	807	2.1
300.19L_{High}	284,100 ± 3800	1114	2.6
300.19E_{Low}	41,300 ± 3400	162	2.2
300.19E_{High}	57,750 ± 2200	226	2.5
300.19E/ICAM-1	34,400 ± 2700	135	5.7

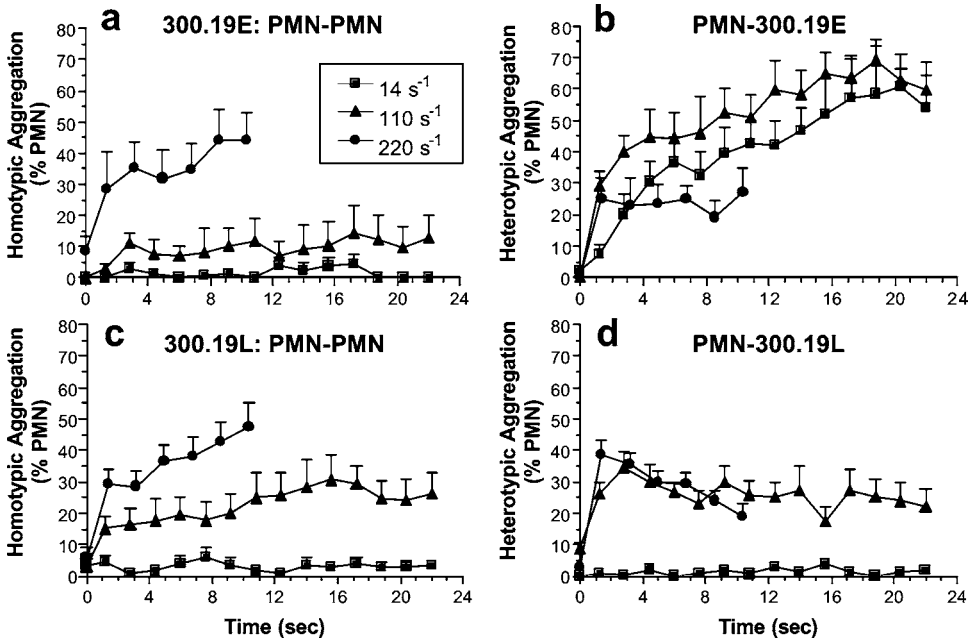

FIGURE 1. Rheoscope measurement of the kinetics of selectin-mediated aggregation. Aggregation of unstimulated neutrophils sheared with 300.19E (a and b) and 300.19L cells (c and d) at equal concentrations of 8 × 10^6/ml was observed at G = 14, 110 and 220 s^{-1} corresponding to S = 0.077, 0.604, and 1.21 N/m^2 in a 10% Ficoll buffer at 22°C. Heterotypic and homotypic aggregation were recorded over the first 20 s after initiation of shear in the rheoscope and computed as a percentage of neutrophils in aggregates. Beginning at the instant of application of shear, frames were chosen at suitable intervals of time and the number of singlets and aggregates were counted. Data are presented as $M \pm$ SE from n = 5–10 separate experiments.

compressive and tensile in successive quadrants of each half rotational orbit in the shear field.[11,15] Adhesion bonds formed during homotypic or heterotypic two-body collisions must survive while rotating under tensile force to form permanent doublets. Indeed, such doublets were observed over multiple rotations in the rheoscope. The kinetics of selectin-dependent heterotypic neutrophil aggregation in 10% Ficoll over a range of shear rate is shown in Fig. 1. Selectin bonds exhibited sufficient lifetime and strength to sustain aggregates over many rotations, even in the absence of chemotactic stimulation and CD18 bond formation. A striking difference in the dynamics of aggregation of unstimulated neutrophils with 300.19L versus 300.19E is evident in Figs. 1a and 1b. Homotypic aggregation required a threshold shear rate to elicit L-selectin-dependent aggregation. In contrast, heterotypic aggregation with 300.19E occurred even at the lowest shear of 14 s^{-1}, with neutrophil recruitment into aggregates increasing steadily up to 60% after 20 s. 300.19L sheared at 14 s^{-1} exhibited almost no aggregation (Figs. 1c and 1d). At a shear rate of 110 s^{-1}, 300.19E aggregation rose to ~65% after 20 s, compared to 35% for 300.19L aggregation which peaked at ~3 s of shear, then slowly decreased. At the highest shear rate of 220 s^{-1} aggregation with 300.19E decreased to ~24%, remaining steady beyond 10 s, compared to 38% for 300.19L, again decreasing after 3 s. The more rapid and stable capture of neutrophils by 300.19E versus 300.19L at G < 220 s^{-1} correlated with a pronounced difference in the relative distribution of neutrophils in homotypic and heterotypic aggregates. Thus, for 300.19E, less than 5% of neutrophils were taken up in homotypic aggregates at 14 s^{-1} and only ~10% at 110 s^{-1}, as compared to ~ 65% in heterotypic aggregates (Figs. 1a and 1b). In contrast, homotypic aggregation of neutrophils sheared with 300.19L continuously increased with time of shear (Figs. 1c and 1d). Finally, adhesion to 300.19L cells required continuous application of shear, as disaggregation rapidly ensued upon reduction of the shear rate, similar to that previously described in neutrophil homotypic aggregation.[11] In contrast, neutrophil aggregates with 300.19E remained intact and even increased in size and number when the shear rate was reduced from 220 s^{-1} to 14 s^{-1} (data not shown). The level of sustained 300.19E aggregation is attributed to the longer lifetime of E-selectin tethered doublets which remained intact over multiple rotational orbits. Treatment of neutrophil-300.19E aggregation with an anti-E-selectin decreased aggregation to background levels (data not shown). At 14 s^{-1} two factors may influence the number of homotypic aggregates available. L-selectin exhibits a shear threshold required for tethering that limits its formation of both neutrophil-300.19L and neutrophil homotypic aggregation. This is consistent with the low number of homotypic in both 300.19E and 300.19L at 14 s^{-1}.

TABLE 2. Heterotypic doublets: Two-body collision lifetimes. *n* = Number of captured (nonseparating) doublets (*n*)* are those observed to break up before leaving rheoscope field of view. Aggregation of unstimulated neutrophils with 300.19E or 300.19L at equal concentrations of 8 × 10^6/ml was observed at *G* = 14, 110, and 220 s^{-1} corresponding to *S* = 0.077, 0.604, and 1.21 N/m^2 in a 10% Ficoll buffer. The duration of each heterotypic doublet interaction was recorded, and the mean doublet lifetimes, $\langle\tau_{meas}\rangle$ (±SE), were normalized by the mean theoretical collision lifetime, $\langle\tau_{theor}\rangle$ for noninteracting rigid spheres as described in Materials and Methods. Captured doublets were defined as those in which cells remained attached while rotating past the orientation in which they were aligned with the flow and under tensile force.

	Shear rate s^{-1}	Neutrophil—300.19L		Neutrophil—300.19E	
		$\langle\tau_{meas}\rangle/\langle\tau_{theor}\rangle$	*n*	$\langle\tau_{meas}\rangle/\langle\tau_{theor}\rangle$	*n*
All collisions	14	1.5 ± 0.1	124	3.4 ± 0.4	219
Captured doublets	14	4.4 ± 0.3	3(0)*	8.7 ± 1.0	65(7)*
All collisions	110	7.1 ± 2.6	89	6.8 ± 1.2	104
Captured doublets	110	17.5 ± 6.2	30(21)*	16.5 ± 2.6	39(1)*
All collisions	220	11.9 ± 2.9	70	6.5 ± 1.6	82
Captured doublets	220	33.2 ± 7.3	22(13)*	22.2 ± 5.3	20(0)*

Capture Efficiency and Doublet Lifetimes of Selectin-Bound Transient Aggregates

Significant differences between E- and L-selectin in mediating heterotypic adhesion of unstimulated neutrophils were also reflected in the capture efficiency (ε_c) and relative mean doublet lifetime (τ_{meas}/τ_{theor}) as a function of increasing shear rate and shear stress (Table 2, Fig. 2a). Neutrophil suspensions sheared in 10% Ficoll at 14 s^{-1} exhibited 10-fold higher ε_c for 300.19E than for 300.19L. In contrast, at a higher shear rate of 110 s^{-1} and stress of 0.60 N/m^2, the values of ε_c were similar for both E-selectin at 37.5% and L-selectin at 33.7%. However, increasing shear stress to 1.21 N/m^2 at 220 s^{-1} led to an ~35% decrease in ε_c for 300.19E, while ε_c remained constant for 300.19L. Capture efficiency *via* E-selectin also decreased 73% with a doubling of shear rate from 110 to 220 s^{-1} at a constant shear stress of 0.60 N/m^2 (Fig. 2a). These data show that above a threshold in shear rate (~14 s^{-1}), capture *via* L-selectin was more resistant to increased shear rate as compared to E-selectin and may reflect its higher molecular association rate.[38]

To further characterize tethering of neutrophils *via* L- versus E-selectin, the lifetimes of neutrophil-300.19 cell interactions were observed directly in the absence of chemotactic stimulus in the rheoscope. Doublet lifetime was recorded following heterotypic collisions until a doublet either separated or moved out of the rheoscope's field of view. Doublet formation between neutrophils and 300.19L or 300.19E at 110 s^{-1} occurred with similar efficiencies of ~35% and the permanent doublets exhibited a mean lifetime of $\langle\tau_{meas}\rangle$ (~17 × $\langle\tau_{theor}\rangle$) ~0.4 s (Fig. 2b; Table 2). However, as many as 70% of neutrophil-300.19L doublets were observed to separate before moving out of the rheoscope field of view. In contrast, neutrophil-300.19E doublets remained intact within the visual field indicating a significantly longer lifetime for E-selectin tethered doublets.

Detection and Kinetics of Homotypic and Heterotypic Aggregation Stimulated by Chemotactic Peptide

To acquire snapshots of the extent of aggregation, aliquots of neutrophil-300.19 suspensions were collected and rapidly fixed over the time course of shear and stimulation in the cone-plate viscometer. Heterotypic and homotypic aggregates remained stable during analysis in the flow cytometer as illustrated by the histograms of Fig. 3 showing two-color fluorescence. Aggregation was most rapid following addition of chemotactic stimulus and reached a peak within 30 s. The relative distribution of neutrophils in either homotypic or heterotypic aggregates was a function of their concentration relative to the 300.19 cells, the dose of fMLP stimulus, and the magnitude of the shear rate. Following maximum aggregation, disaggregation ensued at a rate dependent on the particular 300.19 selectin engaged.

The kinetics of neutrophil recruitment into stable aggregates with $300.19E_{High}$ and $300.19L_{Med}$ are presented in Fig. 4, which shows ~45% of neutrophils recruited into aggregates in response to fMLP stimulation. Neutrophils were recruited into homotypic and heterotypic aggregates most rapidly over the initial 30 s of shear and stimulation, forming aggregates containing up to 5 neutrophils, with less than 5% forming larger aggregates. The extent of neutrophil aggregation with E- and L-selectin were comparable at 2.5 nM fMLP stimulation, when sheared at 600 s^{-1}, 0.6 N/m^2, and a 1:3 ratio of neutrophils (10^6/ml) to 300.19 (3 × 10^6/ml). Neither 300.19E nor 300.19L formed homotypic aggregates with other 300.19 cells. Virtually all heterotypic aggregates consisted of a single 300.19 bound to one or more neutrophils (Fig. 3). Under these shear conditions, neutrophil recruitment was up to twice as fast through

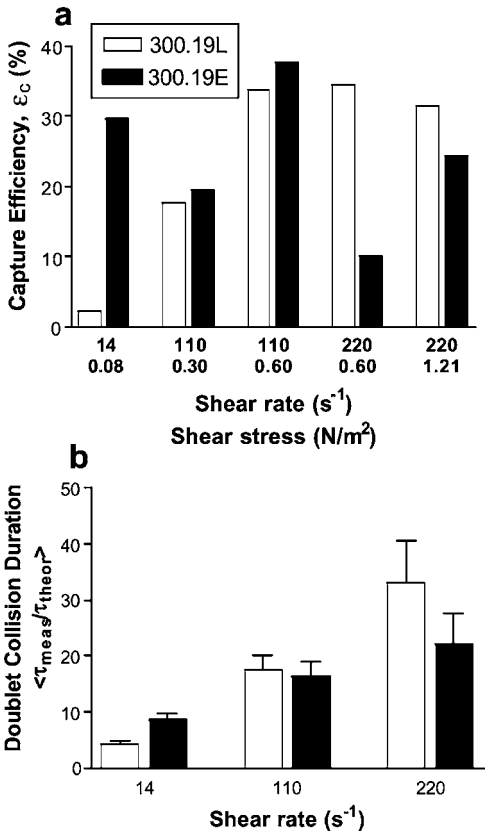

FIGURE 2. Selectin capture efficiency measured in the cone-plate rheoscope. Heterotypic collisions between neutrophils and 300.19E or 300.19L, as in Fig. 1, were observed in the rheoscope in the absence of chemotactic stimulus. (a) Shear dependence of two-body collision capture efficiency, ε_c, defined as the fraction of all two-body neutrophil-300.19 collisions in which the doublets remained intact as they rotated through the quadrant in which tensile normal stress acts to rupture the selectin bonds. Observations were made at shear rates of 14, 110, and 220 s^{-1}, and in 5 and 10% Ficoll buffer, η = 2.7 and 5.5 mPa s, respectively. (b) Shear rate dependence of mean relative doublet lifetime, $\langle\tau_{meas}\rangle/\langle\tau_{theor}\rangle$, where $\langle\tau_{theor}\rangle = 2.64/G$, measured in 10% Ficoll. Bars are ± SE for n = 124, 89, and 70 for G = 14, 110, and 220 s^{-1}, respectively.

E-selectin as through L-selectin (i.e., 20% stable doublets with 300.19E at 12 s, compared to only 10% for 300.19L). A comparable fraction of neutrophils in shear engaged in aggregation, as $300.19L_{Med}$ supported 42% neutrophil recruitment (31% heterotypic versus 11% homotypic) and $300.19E_{High}$ supported 48% recruitment (41% heterotypic versus 7% homotypic). Thus, distribution of neutrophils in homotypic versus heterotypic aggregates was a function of the particular selectin and the availability of single neutrophils.

Although the majority of heterotypic aggregates in both 300.19E and 300.19L were doublets, L-selectin supported more aggregates containing three or more neutrophils. At maximum aggregation, ~55% of neutrophil-300.19L were triplet or larger aggregates than ~35% for 300.19E. In addition to differences in the forward rate of aggregation, neutrophil-300.19E aggregates exhibited much slower kinetics of disaggregation. Neutrophil-300.19E aggregates were sustained up to ~5 min, as compared to reversible adhesion at 2 min for 300.19L or homotypic aggregation. These results suggest that E-selectin facilitates neutrophil capture more avidly than L-selectin at a relatively high shear rate of 600 s^{-1}. However, consistent with observations in the rheoscope, L-selectin more avidly supports large aggregate formation.

Modeling the Dynamics of Homotypic and Heterotypic Aggregation

To quantify the rate of aggregate formation as measured by flow cytometry, we applied a model on the basis of two-body collision theory (Eq. 2) as described in the Methods section. Heterotypic and homotypic aggregation kinetics were fit with a time-averaged efficiency of adhesion over the first 30 s of shear. Over this time frame, disaggregation is insignificant, with decreases in the homotypic aggregate fraction observed as 300.19 cells are bound by preexisting aggregates, both experimentally and computationally. The model estimates the collision frequency from input parameters of cell radius, concentration, and shear rate. Efficiency was then estimated by optimizing a best fit of the model predictions to experimental kinetics of singlet depletion and aggregate formation from doublets up to quintuplets. As computed, adhesion efficiency, ε, represents an ensemble probability that selectin mediated capture transitions to integrin-dependent stable aggregation. Using this approach, heterotypic and homotypic efficiency was computed over a range of experimental parameters, including the ratio of neutrophil to 300.19 cell concentration, selectin site density, and dose of fMLP stimulus. In Fig. 5, experimental data are plotted as mean and standard error at each time point, while the model's prediction fit at a given efficiency is shown as solid lines through the data points. Figure 5 confirms that the kinetics of $300.19E_{High}$ and $300.19L_{Low}$ capture by neutrophils stimulated with 2.5 nM fMLP were accurately predicted with adhesion efficiencies of 2.4 and 1%, respectively. These efficiencies are statistically different since the mean values of the discrete data points are at $p < .05$.

Figure 5 also reveals the molecular requirements for heterotypic aggregation. In the absence of chemotactic stimulus and CD18 activation, neutrophils exhibited insignificant heterotypic and homotypic aggregation. Heterotypic aggregation of neutrophils with nontransfected 300.19 parent cells occurred at less than 0.1% efficiency even at 1 μM fMLP stimulation. Aggregation with parent cells was an order of magnitude less than adhesion of 1 nM fMLP stimulated neutrophils to either 300.19 selectin transfectant.

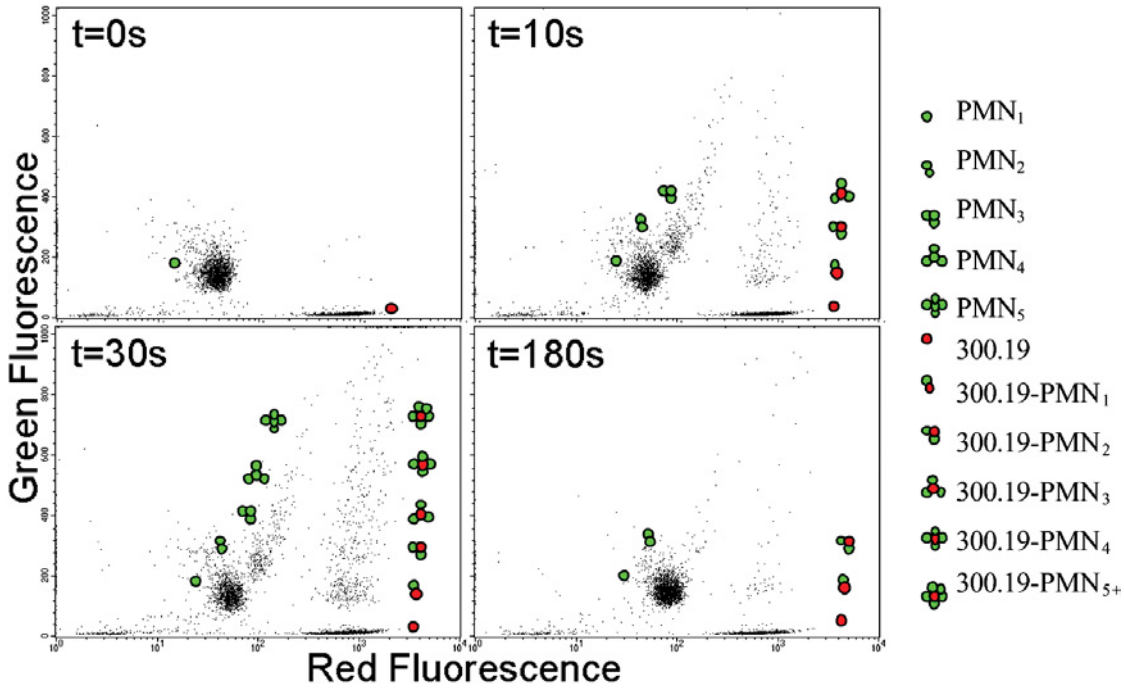

FIGURE 3. Flow cytometric detection of homotypic and heterotypic neutrophil adhesion. Representative dot plots of neutrophil aggregation. Aliquots were removed from the viscometer and fixed at 0, 10, 30, and 180 s after stimulation with fMLP (2.5 nM). Neutrophils (10^6/ml) labeled with anti-CD45-FITC emit green fluorescence, and 300.19 cells (10^6/ml) labeled with the nucleic acid dye LDS751 emit red fluorescence. Neutrophil homotypic aggregates (PMN_i) up to pentuplets emit increasing green fluorescence, while heterotypic aggregates (300.19-PMN_i) emit both red and increasing green fluorescence.

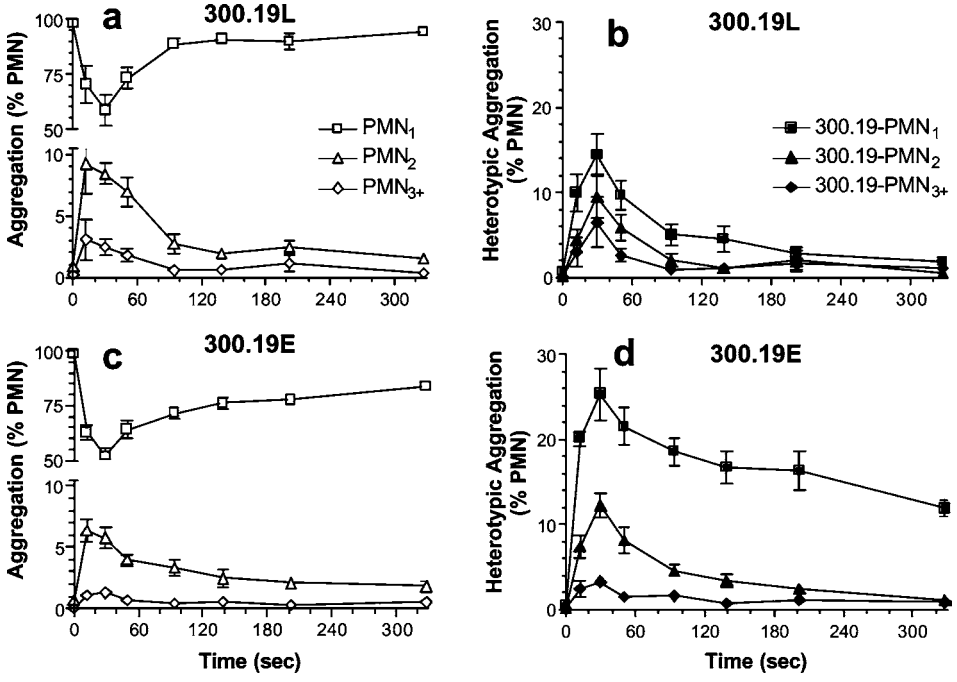

FIGURE 4. Kinetics of homotypic and heterotypic aggregation detected by flow cytometry. Neutrophils- (10^6/ml) labeled fluorescent green and 300.19 cells (3×10^6/ml) fluorescent red were equilibrated at 37°C for 2 min in the cone-plate viscometer. Chemotactic stimulation was added within 1 s of initiation of fluid shear. Samples obtained at prescribed time points fixed in 1.5% cold formaldehyde and read by FACScan flow cytometer. Populations of aggregates quantified on the dotplots as denoted in Fig. 3. Plots of homotypic and heterotypic aggregation at shear rate of 600 s^{-1} and fMLP at 2.5 nM depict the percentage of total neutrophils with 300.19L in (a) homotypic and (b) heterotypic aggregates and 300.19E in (c) homotypic and (d) heterotypic aggregates. Data presented as *M* ±SE from $n = 5$ separate experiments.

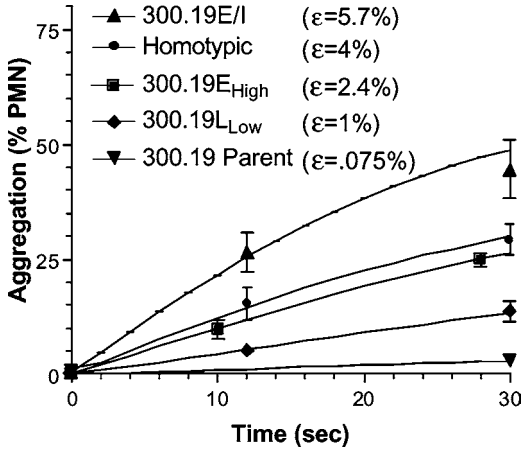

FIGURE 5. Adhesion efficiency and specificity of homotypic and heterotypic aggregation. Kinetics of neutrophil aggregation with 300.19 cells fit with an adhesion efficiency computed from the mathematical model (% in parenthesis). PMN (10^6/ml) alone or with 300.19E$_{High}$ or 300.19L$_{Low}$ (10^6/ml) were sheared at 600 s^{-1} with 2.5 nM fMLP in the cone plate viscometer, or as a control sheared with untransfected 300.19 Parent cells (10^6/ml) and stimulated with 1 μM fMLP. Experimental data are denoted with symbols as mean ±SE from n = 5 separate experiments.

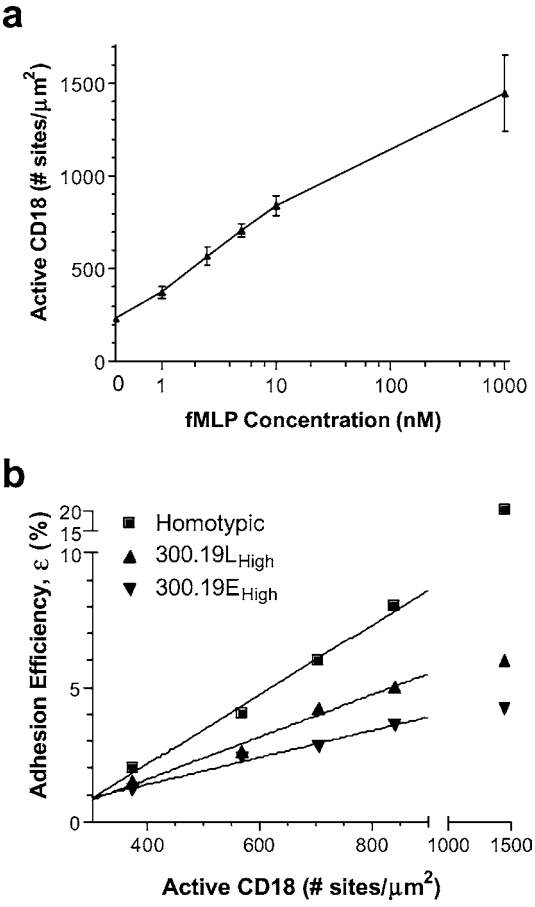

FIGURE 6. Cooperativity of selectin expression and β_2-integrin activation in heterotypic adhesion. (a) Expression of active CD18 was detected by analysis of the binding of Alexa labeled antibody 327C on unstimulated, or neutrophils stimulated for 5 min with fMLP at the dose indicated on the abscissa. Data presented as mean ±SE from n = 4 separate experiments. (b) Adhesion efficiency of neutrophils (10^6/ml) alone or with 300.19E$_{High}$ or 300.19L$_{Low}$ (10^6/ml) is presented over a range of active CD18 site densities as determined by correlation of fMLP concentration to 327C fluorescence in unsheared samples. Samples were sheared at 600 s^{-1} and stimulus added immediately prior to initiation of shear. Adhesion efficiency calculated from initial 30 s of aggregation, and fit to data from n = 3–5 experiments.

Binding of activated CD18 was also necessary for heterotypic adhesion, since blocking CD18 on neutrophils with antibody R15.7 abolished aggregation[15] Blocking mouse ICAM-1, a potential CD18 ligand on 300.19, with antibody YN.1 decreased the rate but not the extent of heterotypic aggregation. In contrast, cotransfecting 300.19 with E-selectin and ICAM-1 boosted adhesion efficiency by approximately twofold. Taken together, the data clearly show that efficient capture requires both selectin tethering and CD18 recognition of human or murine ICAM-1 expressed on 300.19.

Adhesion Efficiency of Neutrophils Increases with CD18 Activation

Expression of activated CD18 has previously been shown to be a sensitive indicator of neutrophil activation and increased adhesive capacity[26] The number of active CD18 receptors upregulated on neutrophils over a dose range of fMLP stimulation was measured with fluorescently conjugated mAb 327C, which reports on the high-affinity ligand binding state of the I domain (Fig. 6a). Neutrophils that were freshly isolated from venous blood expressed a baseline level of ~250 active CD18 sites/μm^2. Stimulation with fMLP from 1 nM up to 1 μM increased active CD18 by fivefold to a peak level of ~1500 sites/μm^2 (Fig. 6a). Expression of active CD18 increased most rapidly (i.e., fourfold) between 0 and 10 nM and increased an additional 70% at a saturating dose of 1.0 μM fMLP. Measurement of total CD18 sites with antibody MHM23 that recognizes a common epitope on constitutive and active CD18 allowed determination of the fraction of total CD18 sites in the activated state. Active CD18 increased from ~20% of total CD18 on unstimulated neutrophils to ~50% after stimulation with a saturating dose of 1 μM fMLP.

We next examined the relationship between CD18 activation and the efficiency of neutrophil adhesion to 300.19L$_{High}$ and 300.19E$_{High}$. Homotypic adhesion efficiency increased in direct proportion to fMLP activation rising by 10-fold over a 5-fold increase in site density of active CD18 (Fig. 6b). Heterotypic adhesion with 300.19E and 300.19L also increased in proportion to the site density of active CD18, but at a slower rate than homotypic aggregation. The rate of increase in percent efficiency ($\Delta\varepsilon$) with active CD18 site density was 13×10^{-3} for homotypic, 8

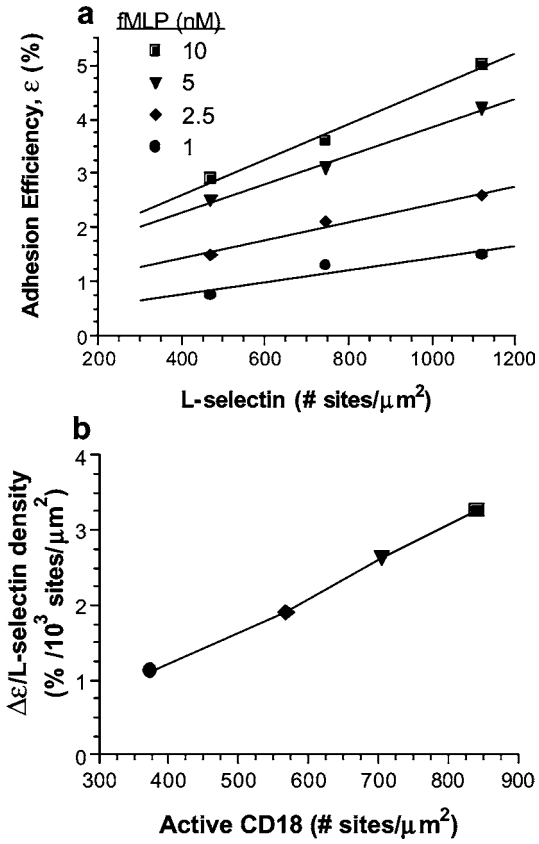

FIGURE 7. Dependence of heterotypic adhesion efficiency on L-selectin site density and CD18. (a) Efficiency of heterotypic adhesion as a function of L-selectin site density over a range of fMLP stimulus. Heterotypic aggregation assays performed with 10^6 PMN/ml and 10^6 300.19 cells/ml at a shear rate of 600 s^{-1}. Selectin site density was varied by selecting 300.19 clones expressing low, medium, and high levels of L-selectin. (b) The slope of efficiency ($\Delta\varepsilon$) vs. L-selectin site density curves in (a) were calculated at each fMLP-induced active CD18 site density. Adhesion efficiency was calculated from the initial 30 s of aggregation and fit to data from n = 3–5 experiments.

$\times 10^{-3}$ for 300.19L_{High}, and 5×10^{-3} for 300.19 in units of $\Delta\varepsilon(\%)$ site/μm^2 active CD18 site density. However, E-selectin is more efficient on a per-receptor basis than L-selectin since at nearly equivalent selectin site density on 300.19L_{Low} and 300.19E_{High}, E-selectin adhered 1.5-fold more efficiently than L-selectin (Fig. 5). These data also reveal greater cooperativity between L-selectin and active CD18 in mediating homotypic adhesion than in heterotypic adhesion through either E- or L-selectin. This occurs despite a twofold higher expression of L-selectin on 300.19L_{High} compared to neutrophils.

Cooperativity of L-Selectin Expression and CD18 Activation in Heterotypic Adhesion

We next examined the relationship between adhesion efficiency and site density of L-selectin on 300.19, over a range of fMLP-induced active CD18 surface expression (Fig. 7a). Heterotypic adhesion efficiency increased more rapidly with the rise in active CD18 site density than with L-selectin site density. As fMLP stimulation was increased from 1 to 10 nM, aggregation efficiency with 300.19L_{High} ($\sim$1100 sites/μm^2) increased fourfold while 300.19L_{Low} and 300.19L_{Med} exhibited smaller increases over this range. Synergy in adhesion *via* L-selectin and active CD18 sites resulted in a sixfold boost in efficiency when L-selectin and active CD18 density were both increased to $\sim$225% of initial site density. Increasing the density of L-selectin alone provided no more than a onefold increase in efficiency, while boosting active CD18 alone increased efficiency less than fourfold. Plotting the slope of adhesion efficiency over the range of L-selectin site density revealed the cooperativity between L-selectin tethering and firm adhesion over the range of fMLP stimulated active CD18 (Fig. 7b).

Receptor Cooperativity in Adhesion to E-Selectin and ICAM-1 Cotransfectants

We next assessed mechanisms of adhesion through E-selectin and ICAM-1 coexpressed on 300.19 cells and the influence of fMLP stimulation on adhesion efficiency (Fig. 8). Human cDNA encoding E-selectin and ICAM-1 was transfected into 300.19 to produce a cell line expressing both vascular adhesion molecules (300.19E/I). ICAM-1 was expressed at $\sim$3000 sites/μm^2, a level at least twice the

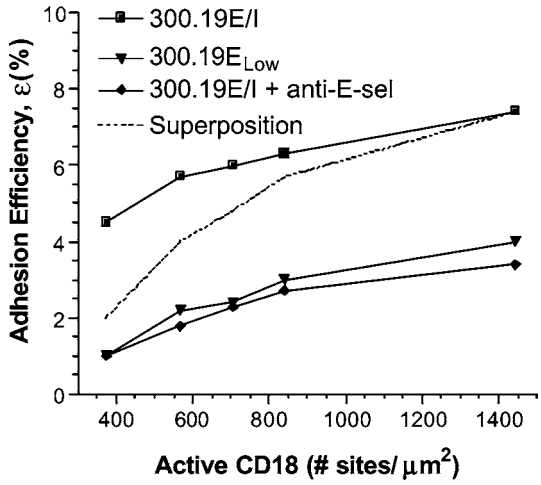

FIGURE 8. Adhesion efficiency enhanced by E-selectin and ICAM-1 binding synergy. Efficiency of aggregation for 300.19 coexpressing E-selectin and ICAM-1 (300.19E/I) compared to recruitment through E-selectin or ICAM-1 alone at comparable selectin site densities. PMN (10^6/ml) and 300.19 (10^6/ml) were sheared at 600 s^{-1} over a range of induced active CD18 site density from 375 to 1450 sites/μm^2. E-selectin was blocked on 300.19E/I by preincubation with MuEP5C7 antibody. Dashed line shows the sum of efficiencies for 300.19E_{Low} and 300.19E/I + anti-E-selectin. Adhesion efficiency calculated from the initial 30 s of aggregation and fit to data from n = 3–5 experiments.

maximum expression of active CD18 on fMLP stimulated neutrophils. Expression of E-selectin on 300.19E/I and 300.19E$_{Low}$ was comparable at ~135 sites/μm^2 (Table 1). Neutrophil aggregation with 300.19E/I in response to 1 nM fMLP stimulation (i.e., 350 active CD18/μm^2) was threefold greater than recruitment through E-selectin or ICAM-1 alone. Over the range of active CD18, heterotypic aggregation through coexpressed E-selectin and ICAM-1 was at least twice as efficient as recruitment through E-selectin or ICAM-1 alone. Adhesion to 300.19E/I also exceeded the efficiency of homotypic aggregation, but only at the lowest active CD18 site density of 350 sites/μm^2. Blocking adhesion through E-selectin with MuEP5C7 allowed assessment of heterotypic adhesion *via* ICAM-1 alone. Under these conditions (i.e., 300.19E/I + anti-E-sel), heterotypic adhesion *via* ICAM-1 was consistently lower than 300.19E or 300.19L and was comparable to that previously reported for 300.19 transfected with ICAM-1[16] Cooperativity between E-selectin and ICAM-1 was more pronounced at low levels of CD18 activation, as indicated by the dashed line in Fig. 8. We depicted the efficiency predicted by superposition (dashed line; Fig. 8) of E-selectin and ICAM-1 by summing the curves of 300.19E$_{Low}$ and 300.19E/I + anti-E-sel. Only at the highest dose of 1 μM fMLP did adhesion mediated by E-selectin and ICAM-1 separately add up to the maximum efficiency (i.e., $\varepsilon = 7.4\%$) afforded by coexpression of these receptors on 300.19E/I.

DISCUSSION

In this study, a murine 300.19 pre-B cell line was transfected with human L-selectin, E-selectin, and ICAM-1. Adhesive interactions with human neutrophils in sheared suspension were studied over a range of concentration of chemotactic stimulus and selectin site density to investigate the molecular basis of human neutrophil recruitment. Heterotypic aggregation featured receptors and ligands sufficient for neutrophil capture and arrest on the endothelium including CD18, L-selectin, PSGL-1, E-selectin, and ICAM-1, or its homolog on murine 300.19 cells. The data revealed intrinsic differences in adhesion supported by L-selectin and E-selectin, as well as cooperativity between selectin and integrin receptors in the transition to stable adhesion. We report the first data on how neutrophil adhesion efficiency is scaled by the site density of selectins and activated CD18.

A Mathematical Model of Aggregation Reveals Dynamics of Capture through E- and L-Selectin

Neutrophil aggregation kinetics, for both homotypic and heterotypic aggregates, were modeled with two-body collision theory. Adhesion efficiency, ε, was computed as the ratio of the measured rate of aggregation to collision frequency. As such ε represents a superposition of capture efficiency, ε_c,[11] and the efficiency of transition to firm adhesion. This is an empirical model that accounts for prescribed or measured geometric and hydrodynamic parameters such as cell radius, concentration, aggregate geometry, and shear rate. We presented data that quantify how adhesion efficiency increased with the density of active CD18, expression of ICAM-1, and the site density of selectins. As applied in these experiments, this model provided a consistent quantitative indicator of the adhesive ability of a well-characterized population of cells in sheared suspensions.

Other computational models have attempted to simulate and thereby define how synergy between selectins and integrins promotes leukocyte recruitment.[4,6,21,48] Bhatia *et al.* recently published a model that applies adhesive dynamics modeling to simulate leukocyte rolling and arrest *via* selectin and integrin binding. As described, the adhesive dynamics model is a stochastic simulation that for prescribed values of mechanical and kinetic properties of selectins and β_2-integrins predicts whether a leukocyte will roll or arrest under shear. In these simulations, synergy was predicted between selectin and integrin receptor-ligand interactions, but at much lower site densities than reported in this study. At $G = 100$ s^{-1} they predicted that as few as 3 sites/μm^2 of each receptor resulted in arrest, and at $G = 600$ s^{-1} cell arrest occurred at ICAM-1 and selectin site densities as low as 10/μm^2 and 50/μm^2, respectively[4] These site densities are an order of magnitude lower than *in vivo* expression on endothelial cells.[8] and the minimum site density observed here for efficient neutrophil recruitment in sheared cell suspensions. This discrepancy may be a function of their choice of parameters in applying the adhesive dynamics model including the reactive compliance and the intrinsic on-rate for integrin-ICAM-1 interactions.

Neutrophil Stable Adhesion Is Dependent on Selectin and CD18

Homotypic neutrophil adhesion is supported by at least six molecular interactions, involving L-selectin, PSGL-1, CD11a/CD18, CD11b/CD18, ICAM-3, and an unknown ligand bound by CD11b/CD18.[35] Thus, neutrophil–neutrophil adhesion differs from recruitment to inflamed endothelium in that each neutrophil presents a complementary set of receptors and ligands. This two-way receptor-ligand interaction serves to increase adhesion efficiency as compared to heterotypic adhesion involving a neutrophil and transfectant or endothelial cell. This is indicated by previous reports that show efficiency decreases by 50% when one set of selectin or CD18 receptors are blocked by antibodies.[12,13,35,41] In the current studies, murine 300.19E cells acted as an analog for endothelial cells, previously shown to preferentially bind L-selectin and PSGL-1 on neutrophils.[15] They also expressed a ligand for CD18 since pretreating neutrophils with anti-CD18 blocked adhesion. A murine homolog of human ICAM-1 appeared to serve

as a CD18 ligand, as antibody YN.1 to mouse-ICAM-1 bound 300.19 and partially inhibited heterotypic aggregation. However, murine ICAM-1 by itself, without coexpression of E- or L-selectin, was not sufficient for aggregation with human neutrophils even at maximal fMLP stimulation ($\sim$1500 active CD18/μm^2). Likewise, expression of selectin expression alone was insufficient for stable heterotypic aggregation, as neutrophils required chemotactic stimulation to aggregate with 300.19 selectin transfectants. In fact, upregulation of active CD18 sites was up to five times more potent in boosting adhesion efficiency on a per-receptor basis than either E- or L-selectin. While clustering CD18 receptors also contribute to the avidity of neutrophil adhesion, upregulation of active CD18 provided a sensitive measure of neutrophil activation, which increased in direct proportion to adhesion efficiency.

Comparison of E-Selectin and L-Selectin in Neutrophil Adhesion Efficiency

L-selectin forms a two-way molecular bridge in binding to PSGL-1 between adjacent neutrophils as observed *in vitro* and during secondary capture of neutrophils from the blood stream by those rolling along the venule wall.[45,50] This adhesive interaction afforded two- to fourfold higher adhesion efficiencies in homotypic versus heterotypic adhesion to E- or L-selectin. Moreover, L-selectin bonds in heterotypic interactions formed more efficiently as shear rate increased as compared to E-selectin. Direct observation of two-body collisions in the cone-plate rheoscope revealed that 300.19L capture efficiency remained over $\sim$30% at shear rates up to 220 s^{-1} and over a threefold increase in shear stress up to 1.2 N/m^2. In contrast, 300.19E capture efficiency decreased by >30% with a doubling of the shear rate (110–220 s^{-1}) at constant shear stress. In contrast, efficiency *via* E-selectin doubled with a doubling of shear stress at both 110 and 220 s^{-1}. Additional evidence of L-selectin shear resistance was the formation of larger stable heterotypic aggregates with 300.19L than 300.19E in the cone-plate viscometer, as well as the rheoscope. Consistent with previous reports,[2,14] adhesion *via* L-selectin required application of a threshold level of shear (i.e., $G > 14$ s^{-1} and $S > 0.08$ N/m^2), whereas 300.19E exhibited a 30% capture efficiency at $G = 14$ s^{-1}. E-selectin in this system did not exhibit a shear threshold for adhesion, consistent with a previous report that indicated a fourfold lower shear stress required for rolling adhesion on E-selectin versus L-selectin.[22] Taken together, the data suggest that L-selectin can form bonds more efficiently than E-selectin at higher rates of tensile force loading.[11] Consistent with recent published data,[40] the lifetimes of L- and E-selectin bonds can actually increase as applied tensile force increases, as observed in Fig. 2b.

In the rheoscope, the rapid rates of neutrophil capture in the first 3–5 s were similar for 300.19E and 300.19L. The 70% decrease in E-selectin capture efficiency as G increased from 110 to 220 s^{-1} at constant $S = 0.6$ N/m^2 suggests a slower association rate for E-selectin versus L-selectin with increased shear rate (i.e., decreased collision duration). This corroborates recent reports that E-selectin binds to sLe^x tetrasaccharide with an $\sim$20% lower k_{on} than L-selectin.[38] and that E-selectin binding to ESL-1 (E-selectin Ligand-1).[52] is $\sim$30% slower than L-selectin to GlyCAM-1 (Glycosylation-dependent cell-adhesion molecule-1).[36] Following the rapid phase of adhesion neutrophils sheared with 300.19E continued to form heterotypic aggregates, while neutrophils bound to 300.19L spontaneously dissociated. Previous observations of homotypic aggregation at $G = 110$ s^{-1} in the rheoscope[11] indicated that the lifetime was 378 $\pm$ 35 ms for neutrophil homotypic captured doublets, close to the value measured here for 300.19L heterotypic doublets. L-selectin also differed in its ability to support homotypic versus heterotypic doublets, since 21% of all neutrophil homotypic doublets broke up within the rheoscope field of view, far fewer than 70% of 300.19L-neutrophil doublets that dissociated in this study. This data indicates a hierarchy in doublet lifetimes, with neutrophil-300.19E doublets lasting longest, followed by neutrophil homotypic and then neutrophil-300.19L.

E-selectin supports rolling on inflamed endothelium and facilitates the transition to firm adhesion of neutrophils in the circulation[30,43] L-selectin and PSGL-1 express sLe^x ligands bound by E-selectin in tethering neutrophils to endothelial cells,[53] whereas L-selectin predominantly binds PSGL-1 on neutrophils. At comparable selectin site densities, neutrophil recruitment through E-selectin on 300.19 cells was up to fourfold more efficient than L-selectin[15] However, adhesion efficiency (ε) was equivalent for L-selectin expressed at fivefold higher site density than for E-selectin (Table 1). L-selectin appears to support effective neutrophil adhesion by allowing bond formation over a higher range of shear rate and shear stress than does E-selectin. Data also suggests that the high efficiency of 300.19L$_{High}$ at high shear rates and stresses was due to more efficient capture and was not a function of prolonged doublet lifetimes, as with 300.19E$_{High}$. Considering the primary role of E-selectin in neutrophil rolling and L-selectin in secondary capture of neutrophils from the blood stream, the long lifetime of E-selectin and the rapid bond formation and shear resistance of L-selectin are well-adapted to these distinct functions in neutrophil recruitment.

Synergy between Selectin and Integrin in Efficient Neutrophil Adhesion

Cooperativity between selectin tethering and integrin arrest has recently been reported for leukocyte recruitment in the microcirculation of the mouse, with ICAM-1 expression boosting the number of rolling cells and slowing the observed rolling velocities[17,47] Rolling *via* selectins occurs

in the absence of integrin engagement, but stable adhesion is not supported by selectin binding alone, even at low shear stress (~0.1 N/m^2). Likewise, CD18 engagement supports capture of leukocytes in the absence of selectin expression, but only at low shear rates (i.e., $G < 200$ s^{-1}).[10,15] We report that adhesion efficiency increased with both increasing active CD18 and selectin site density. Homotypic adhesion efficiency increased 10-fold over a 4-fold increase in CD18 site density. We previously reported that homotypic adhesion efficiency decreased in proportion to shedding of L-selectin from 500 sites/μm^2 to zero efficiency at undetectable levels of L-selectin expression.[29] Neutrophil adhesion to 300.19E and 300.19L increased with increased active CD18 and selectin site density. Synergy was most prevalent at low stimulus dose and expression of active CD18, with coexpression of E-selectin with ICAM-1 providing threefold higher efficiency than E-selectin or ICAM-1 expressed alone. 300.19E/I even provided twice the efficiency of homotypic aggregation despite the two-way molecular bridging available to support homotypic aggregates.

These experiments were carried out at prescribed values of fluid shear rate, shear stress, and receptor expression, and demonstrate that adhesion efficiency and the relative distribution of neutrophils participating in homotypic *versus* heterotypic aggregates is regulated by receptor site density and the extent of chemotactic stimulation. Over a narrow dose range of fMLP from 1 to 10 nM, neutrophils exhibited a sixfold increase in active CD18 that correlated with a 1- to 10-fold increase in adhesion efficiency as a function of the selectin site density. It is well established that membrane expression of selectins, selectin-ligands, CD18, and ICAM-1 are all regulated by the extent of leukocyte or endothelial stimulation.[24] Here, we quantified the relation between the efficiency of neutrophil capture and stable adhesion as a function of receptor expression thereby providing a basis for analysis and modeling of neutrophil recruitment during inflammation.

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