Lifetime of the P-selectin–carbohydrate bond and its response to tensile force in hydrodynamic flow

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SELECTINS **tether to the blood vessel wall leukocytes that are flowing in the bloodstream and support subsequent labile rolling interactions as the leukocytes are subjected to hydrodynamic drag forces[1,2]. To support this rolling, selectins have been proposed to have rapid bond association and dissociation rate constants, and special mechanical properties linking tensile forces and bond dissociation[3–6]. We have visualized transient tethering and release of neutrophils in hydrodynamic flow on lipid bilayers containing densities of P-selectin below those required to support rolling. We report here that transient tethers had first-order kinetics and other characteristics suggesting a unimolecular interaction between P-selectin and its glycoprotein ligand (PSGL-1). The unstressed dissociation constant (off rate) was 1 s^{-1}. Hydrodynamic shear stresses of up to 1.1 dyn cm^{-2}, corresponding to a force on the bond of up to 110 pN, increased the off rate only modestly, to 3.5 s^{-1}. The data was adequately matched by a proposed equation[7] relating off rate to the exponential of tensile force on the bond and the bond interaction distance, and gave a bond interaction distance of 0.5Å. This distance is compatible with hydrogen and metal coordination bonds between P-selectin and PSGL-1. Fast on and off rates, together with the high tensile strength of the selectin bond, appear necessary to support rolling at physiological shear stresses.**

We used video microscopy to visualize neutrophil adhesion in shear flow to P-selectin reconstituted in lipid bilayers in a parallel-wall flow chamber. At densities from 400 to 30 sites per μm^2 of P-selectin, neutrophils in hydrodynamic flow tethered to the bilayer and then rolled across it with a jerky motion. During rolling, cells paused for short periods of time, then translated forward for a distance of less than one cell diameter, perhaps reflecting dissociation of one and retention of other selectin–carbohydrate bonds (cell 3, Fig. 1*a*, *b*). At 15 or fewer P-selectin sites per μm^2, the jerkiness increased and periods during which the cells tethered or rolled were separated by periods during which the cell moved forward at the hydrodynamic velocity, the velocity predicted for a non-adherent cell in flow near the wall (Fig. 1*a*, cells 1 and 2)[8,9]. Tethering events were defined as transient when they were separated by at least 50 μm of motion at the hydrodynamic velocity, and when no rolling (<1 μm displacement) occurred while the cell was tethered. At 15 sites per μm^2, 67% of the tethering events were transient, and at 6, 5, 3 and 1 sites per μm^2, all tethering events were transient. The frequency of transient tethering was linearly related to P-selectin site density (Fig. 2). Tethering was highly specific because it was inhibited by pretreatment of the bilayers by monoclonal antibody to P-selectin, pretreatment of neutrophils with *O*-glycoprotease, or addition of EDTA (Fig. 2). This agrees with findings that the ligand for P-selectin on human neutrophils is a sialyl $Lewis^x$-like structure that is displayed by the mucin-like P-selectin glycoprotein ligand (PSGL-1), which is *O*-glycoprotease-sensitive, and that ligand binding requires Ca^{2+} (refs 10–12).

To determine the kinetics for release of tethered neutrophils from P-selectin bilayers (the cellular off rate) the duration of tethering events at a wall shear stress of 0.36 dyn cm^{-2} was measured for P-selectin densities from 1 to 30 sites per μm^2 (Fig. 3*a*). Each set of data from 1 to 15 sites per μm^2 fit a single straight line, demonstrating first-order kinetics. Furthermore, the cellular dissociation rate constant k_{off} was unaffected by P-selectin density from 1 to 15 sites per μm^2 (Fig. 3*a*, *b*). Although there may be heterogeneity in the structure of carbohydrate ligands for P-selectin displayed on PSGL-1[12], our finding of a single dissociation rate constant suggests the ligands are homogenous at least with respect to dissociation rate, and agrees with homogenous affinity[13].

The independence of cellular dissociation rate on P-selectin density at 15 sites per μm^2 and below, the lack of rolling, and the linear rather than higher-order dependence of tethering frequency on selectin density, suggest that a quantal tethering unit has been identified. The data are consistent with the hypothesis that this quantal tethering unit is a monovalent bond between a single PSGL-1 molecule on the neutrophil and a single P-

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selectin molecule on the lipid bilayer; however, PSGL-1[10,11] and P-selectin are dimers[13], raising the feasibility of dimeric interactions. A single bond dissociation model was favoured by a variety of statistical tests, including Pearson's linear correlation coefficient, and examination of the χ^2 deviation by both q (ref. 14) and F-tests[15,16], for P-selectin densities $\leqslant 5$ sites per μm^2. By contrast, a two-bond dissociation model gave better fits for most data sets with P-selectin densities $\geqslant 15$ sites per μm^2, suggesting multivalent tethering.

Tensile force on non-covalent bonds between receptors and ligands is predicted to increase the rate of dissociation[4,7]. The magnitude of this effect, which is particularly relevant for understanding the biophysics of selectin-dependent rolling[5,6], has not previously been measured in any system. The k_{off} was similar at shear stresses of 0.17 and 0.36 dyn cm^{-2}, but was consistently faster at 0.73 dyn cm^{-2} (Figs 3*b* and 4*a*). Examination of a wider range of wall shear stresses revealed that at 1.1 dyn cm^{-2}, the k_{off} increased to 3.5 s^{-1} (Fig. 4*b*). Using Goldman's solution for the hydrodynamic force on a sphere near a wall[8], and approximating the lever arm between the P-selectin bond and the centre of the neutrophil as 4 μm (Fig. 4*c* and legend), give a tensile force of 112 pN on the bond at 1.1 dyn cm^{-2}. Bell[7] proposed that the dissociation rate constant is related to the force on a bond, F_b, by the equation $k_{off}=k^{\circ}_{off}\exp(\sigma F_b/kT)$, where k°_{off} is the dissociation rate in the absence of applied force, σ is the distance over which separation of the receptor and ligand weakens their interaction, k is Boltzmann's constant and T is temperature. A fit of this equation to our data (Fig. 4*b*) yields $k^{\circ}_{off}=0.95\pm0.17\ s^{-1}$ and $\sigma=0.49\pm0.08$Å. Our value of σ is probably accurate to a factor of 2; the main uncertainty derives from the estimate of the lever arm and hence F_b. The σ of 0.5Å is consistent with hydrogen and calcium coordination bonds between the selectin and carbohydrate ligand[17,18]; the variation in length of hydrogen bonds seen in crystal structures is of the same magnitude[19]. By contrast, hydrophobic bonds depend on the exclusion of water molecules at binding interfaces and are estimated to have a σ of the same order as the diameter of a water molecule of 3Å (ref. 20). σ is also linked by deformation of the interface on a stressed molecule to the lateral dimensions of the binding interface and a small σ may be favoured by a smaller recognition surface for selectins[17,18] than for typical hydrophobic protein–protein interactions[21]. The modest coupling between force and dissociation rate that we have found is ideal for maintenance of the rolling adhesion between the leukocyte and the vessel wall, because k_{off} increases only moderately at shear stresses in the physiological range (3.5-fold at 1.1 dyn cm^{-2}). Thus, the P-selectin–carbohydrate bond has high tensile strength. By contrast, with $\sigma=3$Å for hydrophobic bonds that provide the binding energy for most protein–protein interactions, there would be a 1,000-fold increase in k_{off} at 1.1 dyn cm^{-2} according to Bell's expression; such fast dissociation clearly would greatly lower the probability of formation of a second receptor–ligand bond and would prevent rolling interactions.

The average force required to pluck a membrane protein from a cell by extracting its transmembrane domain from the bilayer is estimated to be 20 pN[22,23]. We hypothesize that the cytoplasmic domains of selectins and their counter-receptors, such as PSGL-1, anchor these molecules to the cytoskeleton so that they can resist extraction at forces up to 112 pN; the cytoplasmic domain

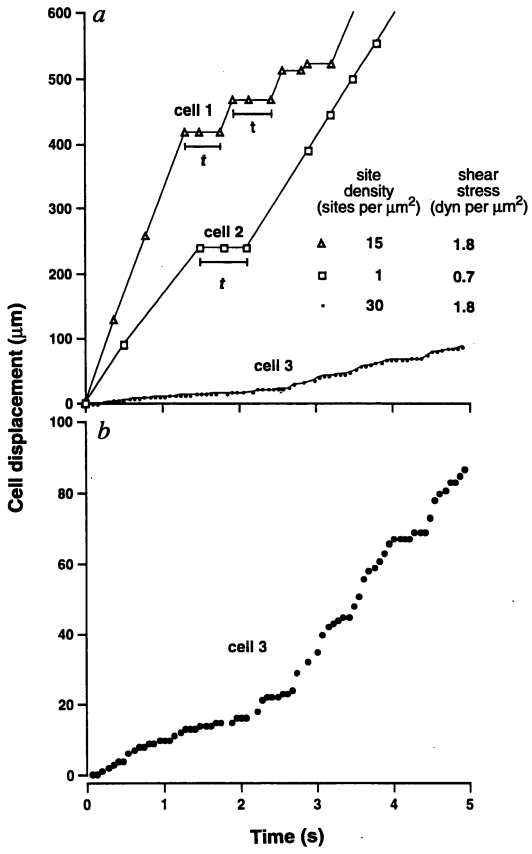

FIG. 1 Displacement in the direction of flow of representative neutrophils interacting with purified P-selectin in the parallel-wall chamber. *a*, Three individual neutrophils. Duration of transient tethers is marked (*t*). *b*, Enlargement of the displacement of cell 3.
METHODS. Purified P-selectin was incorporated in phosphatidylcholine bilayers on glass slides that were assembled in a parallel-wall flow chamber and mounted on an inverted phase-contrast microscope[3]. Selectin site density was determined and neutrophils were prepared and infused as previously described[3,28]. Images from a Nikon plan ×10 objective and a TEC-470 CCD video camera (Optronics, Goleta, CA) that yielded a 0.43 mm^2 field of view 0.76 mm in the direction of flow and 0.57 mm wide were recorded and played back on Hitachi time lapse TLC 2051 or Hi8 recorders. Playback at slow motion or frame by frame with positions of cells measured on transparencies on the monitor, or frame grabbing with a SCION LG-3 on a Macintosh Quadra 650 and analysis with NIH Image 1.55 and a routine for following motion of cells (E. Finger), were used to track neutrophils. Coordinates of cell centres were determined within one pixel accuracy (+/−0.7 μm).

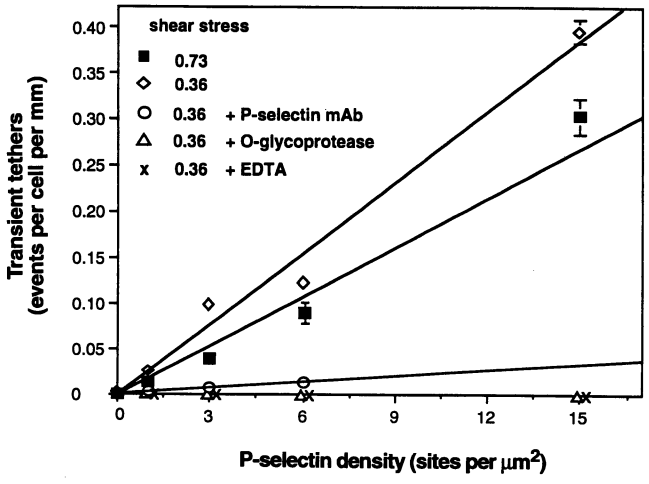

FIG. 2 Frequency of transient tethering of neutrophils is proportional to selectin site density. Using methods described in Fig. 1, the number of transient tethers was counted and divided by the number of cells that flowed across the field that were within the focal plane of the bilayer and by the path length (0.76 mm). For inhibition studies, bilayers were pretreated with 20 μg ml^{-1} G1 mAb to P-selectin at 37 °C[29], and tethering measured in absence of mAb; neutrophils were pretreated with 50 μg ml^{-1} *O*-glycoprotease, a gift from A. Mellors (University of Guelph, Ontario) for 30 min[30]; or 5 mM EDTA was present in the assay.

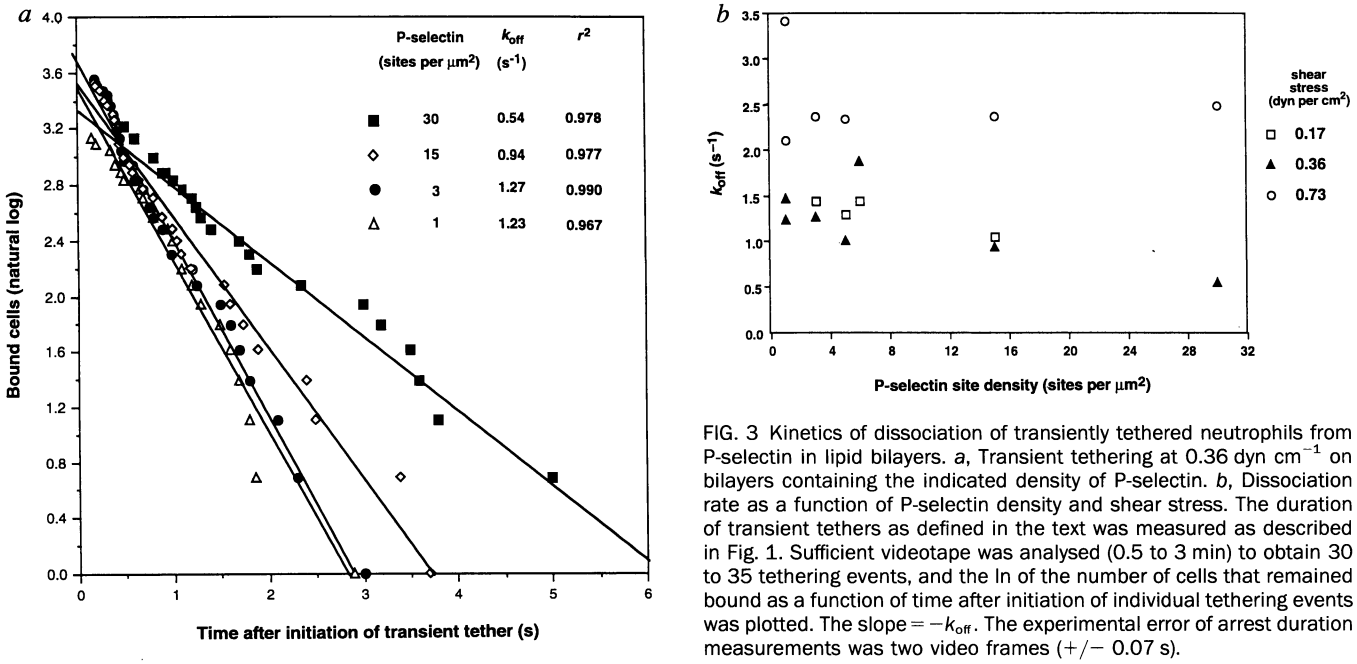

FIG. 3 Kinetics of dissociation of transiently tethered neutrophils from P-selectin in lipid bilayers. *a*, Transient tethering at 0.36 dyn cm^{-1} on bilayers containing the indicated density of P-selectin. *b*, Dissociation rate as a function of P-selectin density and shear stress. The duration of transient tethers as defined in the text was measured as described in Fig. 1. Sufficient videotape was analysed (0.5 to 3 min) to obtain 30 to 35 tethering events, and the ln of the number of cells that remained bound as a function of time after initiation of individual tethering events was plotted. The slope $= -k_{off}$. The experimental error of arrest duration measurements was two video frames (+/− 0.07 s).

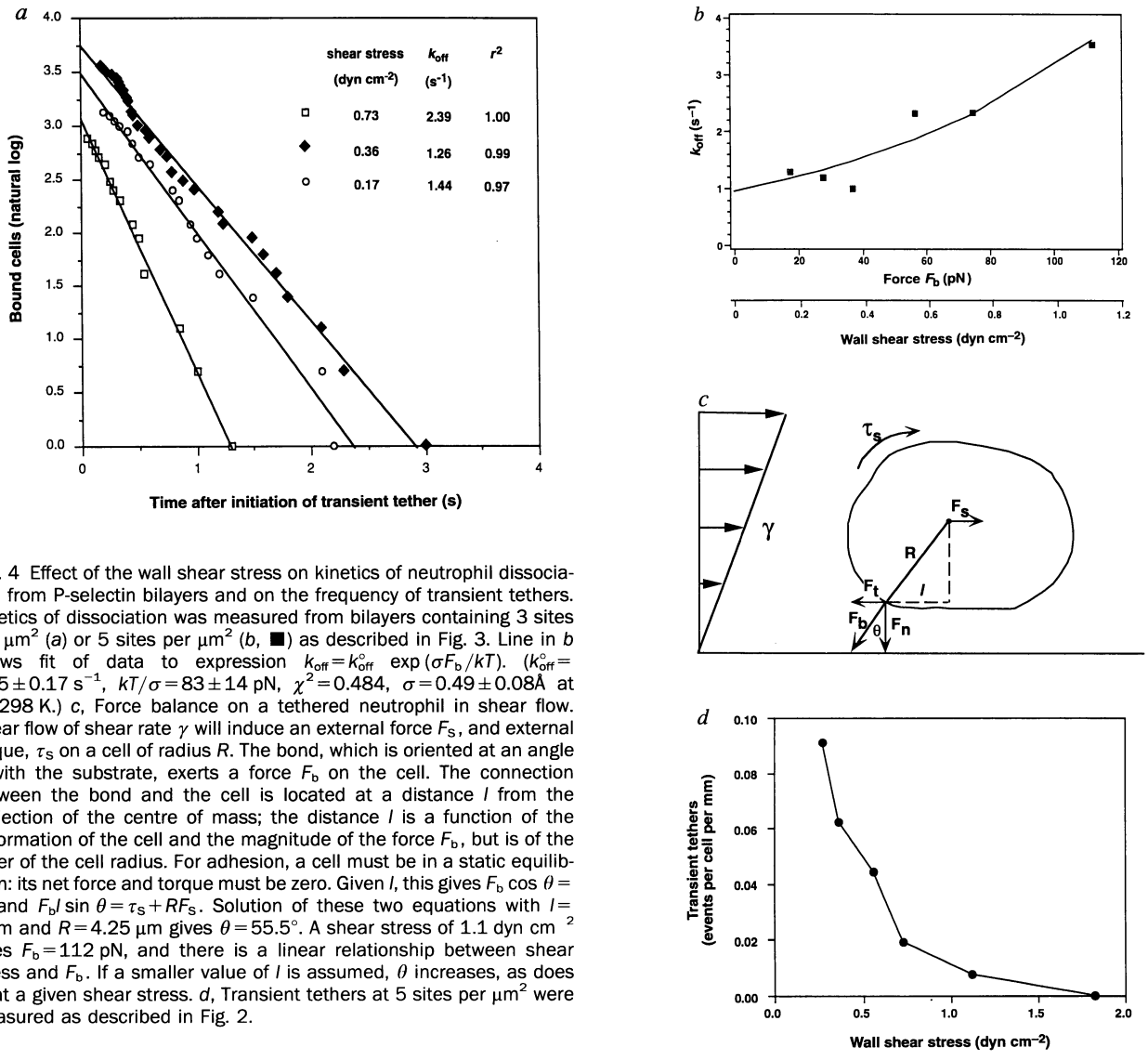

FIG. 4 Effect of the wall shear stress on kinetics of neutrophil dissociation from P-selectin bilayers and on the frequency of transient tethers. Kinetics of dissociation was measured from bilayers containing 3 sites per μm^2 (*a*) or 5 sites per μm^2 (*b*, ■) as described in Fig. 3. Line in *b* shows fit of data to expression $k_{off} = k^{\circ}_{off} \exp(\sigma F_b / kT)$. ($k^{\circ}_{off} = 0.95 \pm 0.17\ s^{-1}$, $kT/\sigma = 83 \pm 14$ pN, $\chi^2 = 0.484$, $\sigma = 0.49 \pm 0.08$Å at $T = 298$ K.) *c*, Force balance on a tethered neutrophil in shear flow. Shear flow of shear rate γ will induce an external force F_S, and external torque, τ_S on a cell of radius *R*. The bond, which is oriented at an angle θ with the substrate, exerts a force F_b on the cell. The connection between the bond and the cell is located at a distance *l* from the projection of the centre of mass; the distance *l* is a function of the deformation of the cell and the magnitude of the force F_b, but is of the order of the cell radius. For adhesion, a cell must be in a static equilibrium: its net force and torque must be zero. Given *l*, this gives $F_b \cos\theta = F_S$ and $F_b l \sin\theta = \tau_S + RF_S$. Solution of these two equations with $l = 4$ μm and $R = 4.25$ μm gives $\theta = 55.5°$. A shear stress of 1.1 dyn cm^{-2} gives $F_b = 112$ pN, and there is a linear relationship between shear stress and F_b. If a smaller value of *l* is assumed, θ increases, as does F_b at a given shear stress. *d*, Transient tethers at 5 sites per μm^2 were measured as described in Fig. 2.

and an intact cytoskeleton are required for rolling on L-selectin[24]. At 1.8 dyn cm^{-2} or 183 pN and above, we did not detect tethering at low P-selectin densities (Fig. 4*d*), which may reflect extraction from the membrane. Extraction does not appear to occur during rolling[3], which at high P-selectin densities occurs up to 35 dyn cm^{-2} (ref. 3), corresponding to a force of 3,500 pN; maintenance of rolling may require that this force be distributed over multiple selectin–ligand bonds.

High on rates and off rates have been proposed to be important for selectin-mediated rolling[3]. Using the $K_a = 1.4 \times 10^7\ M^{-1}$ for binding of truncated, monomeric P-selectin to neutrophils[13] and our measured value of $k_{off}^{\circ} = 0.95\ s^{-1}$, $k_{on} = 1.5 \times 10^7\ s^{-1}$. Both k_{off} and k_{on} are indeed fast compared to other tabulated macromolecular interactions[25]. The CD2–LFA-3 interaction has a 40-fold slower on-rate but 6-fold faster off-rate[26] and does not mediate rolling[27]. However, interaction of a cell-bound monoclonal antibody with dinitrophenol does not mediate rolling despite a k_{on} twofold faster and a k_{off} only threefold slower than P-selectin[9]. We suspect the antibody–dinitrophenol interaction fails to support rolling because it depends on hydrophobic bonds with lower tensile strength, or because of extraction from the membrane. Thus, not only fast on and off rates, but also high tensile strength of the receptor–ligand bond and anchoring of the receptor and ligand to the cytoskeleton may be important for rolling. Although not all adhesion molecules support rolling, other adhesion molecules including the integrins and cadherins bear tensile force. These molecules may be distinguished from adhesion molecules that function primarily in signalling, and from receptors for soluble ligands. Common features may have been selected in the binding sites of selectins, integrins and cadherins, including a requirement for divalent cations, that allow them to resist tensile stress. □

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