Letters to the Editor

Measurement of Selectin Tether Bond Lifetimes

Smith and others (Smith et al., 1999) estimated selectin bond lifetimes by measuring the duration of pauses when neutrophils in flow tethered to selectins or their ligands immobilized on the wall of a flow chamber. Smith et al. claim that the use of higher temporal and spatial resolution in their study is responsible for differences with other studies. Indeed, a table comparing their kinetic and mechanical estimates with those from other labs is entitled "Effect of sampling rate and magnification on estimates of selectin bond lifetimes." Smith et al. claim higher spatial resolution because they used a 20× microscopic objective whereas they state that previous investigations used a 10× objective; this is incorrect, because many of the studies cited, and similar studies, have used 20× and 40× objectives (Alon et al., 1997, 1998; Puri et al., 1998; Ramachandran et al., 1999). The differences in temporal resolution were 48 frames per second (fps) compared to 30 fps in earlier studies for P-selectin and E-selectin (Alon et al., 1995, 1997; Kaplanski et al., 1993; Ramachandran et al., 1999) and 240 fps compared to 30 fps in earlier studies for L-selectin and PNAd (Alon et al., 1997, 1998; Puri et al., 1998). It is difficult to understand how a 1.6-fold difference in frame rate in the P- and E-selectin studies could be responsible for 2.6 to 3.7-fold differences in unstressed off rates.

Although Smith et al. state that differences with other studies were "attributable to the higher temporal and spatial resolution analysis," they never demonstrated this by using different frame rates in their own study and determining whether this affected the selectin bond lifetime estimates. Therefore, the differences between the study of Smith et al. and other studies may result from differences in the biological preparations, the hardware and software for image acquisition and calculation of cell pause duration (bond lifetime), or data analysis. We wish to point out two unusual aspects of data analysis in Smith et al.

Dissociation of receptor-ligand bonds follows the kinetics $-dC/dt = k_{off}C$, where C is the concentration of receptor-ligand complexes (or number of bonds). Integration yields $k_{off} = -(\ln C)/t$. In cell tethering experiments, the number of cells that pause with a duration $\geq t$ is used as the measure of the number of receptor-ligand bonds (C) remaining at time t. Therefore, lnC is plotted versus t to yield the slope $(\ln C)/t = -k_{off}$ (Alon et al., 1995). However, Smith et al. plotted not the number of tether bonds remaining with time, but the number of bonds that dissociated in each video frame time interval. Therefore, they plotted not lnC, but $\ln(-\Delta C/\Delta t)$, versus t. Smith et al. stated in the Fig. 4 legend that "The dissociation rate constant, k_{off}, is equal to the negative slope of the corresponding distributions" and thus used these plots to determine k_{off}, as confirmed by Dr. Lawrence (personal communication), and our own curve fits to the data which yield the same values reported by Smith et al. (Fig. 1 *A*, representative dataset for L-selectin at 0.5 dyn/cm^2 from Fig. 4 *C* of Smith et al.). Thus the k_{off} values reported in Smith et al. were calculated with an incorrect equation. When the data of Smith et al. are replotted to allow calculation of k_{off} by the correct equation, different k_{off} values are obtained (Fig. 1 *B*). Furthermore, deviation from a straight line is noted that reveals heterogeneity in dissociation kinetics (Fig. 1 *B*). Similarly curved lines can result when tethers involve multiple bonds (Chen and Springer, 1999), which if true would invalidate the assumption of first order dissociation kinetics.

To obtain estimates of the unstressed k_{off} (k_{off}°) and the bond separation length (σ), Smith et al. plotted k_{off} against the estimated force on the bond, and fit the Bell equation to the data. Smith et al. suggested that the data at 250 pN did not fit well, and used only data from 38 to 125 pN to calculate k_{off}° and σ. They claimed two different regimes of force-enhanced bond dissociation, based on the anomalous

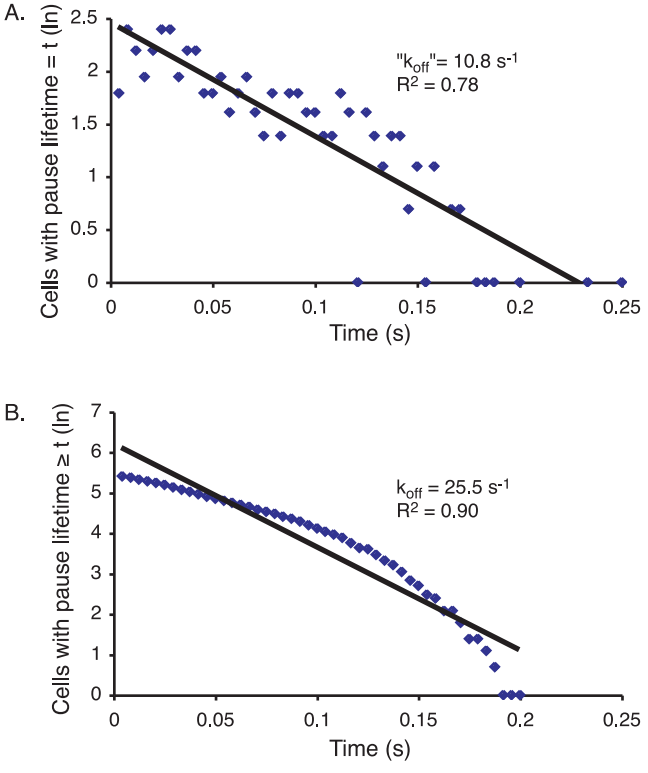

FIGURE 1 Calculations of k_{off} for L-selectin at 0.5 dyn/cm^2 from Fig. 4 *C* of Smith et al. (*A*) Data plotted as in Smith et al., with the number of cells that dissociated in each 1/240 s video frame time period. (*B*) Data replotted to show the number of cells with pauses longer than t, and to allow k_{off} to be correctly calculated from the −slope. Note that if k_{off} was calculated using the first 90% of cells to dissociate (up to 0.14 s), as is commonly done (Alon et al., 1998), the k_{off} would be 16.1 s^{-1}

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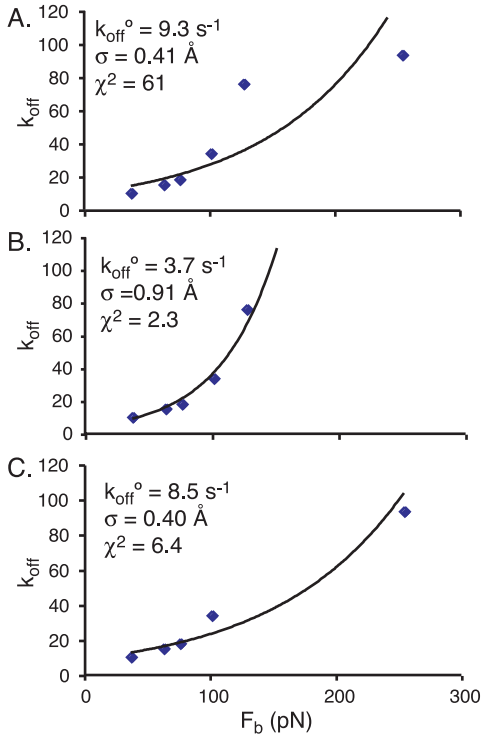

FIGURE 2 Fit of data on L-selectin k_{off} from Fig. 6 *A* of Smith et al. to the Bell equation. (*A*) All six data points. (*B*) Omission of the 250 pN data point. (*C*) Omission of the 125 pN data point. The calculated values for k_{off}°, σ, and χ^2 are shown. The values in *B* differ slightly from those of Smith et al. because the least squares fit to an exponential equation was calculated with Excel; our fit appears to be better because we obtained a χ^2 value of 2.3 for the five data points omitting the 250 pN value, whereas Smith et al. report a χ^2 value of 27.4.

data point at 250 pN. However, multiple data points would be required to support the existence of each regime, whereas the regime at higher force is based on a single point. Other investigators have measured transient tether k_{off} at forces up to 360 pN, and found no anomalies in Bell fits (Alon et al., 1997; Chen and Springer, 2001; Ramachandran et al., 1999). In the footnote to their Table 1, Smith et al. justify omission of the 250 pN data point based on the much lower χ^2 values obtained. We fit the same data to the Bell equation and calculated χ^2 (Fig. 2). Since Smith et al. found the greatest discrepancy between their results and those of other investigators with L-selectin, and also found the largest effect of omitting the point at 250 pN, we focus on this dataset (Fig. 2 *A*). As it must, the χ^2 value is decreased when one of the six data points is omitted; however, χ^2 is decreased almost as much when the 125 pN value is omitted (Fig. 2 *C*) as when the 250 pN value is omitted (Fig. 2B). Furthermore, our χ^2 value of 61 for all six data points is much less than the value of 1624 reported by Smith et al. We can only reproduce χ^2 values this large when we use the curve fit to the data points at 38–125 pN to calculate χ^2 for the data points at 38–250 pN, which represents an incorrect method of data analysis. Therefore, although there is considerable scatter in the data of Smith et al., there is no justification for eliminating any particular data point because it does not fit with the others. It follows that there is no statistical justification for claiming two different regimes of force-enhanced bond dissociation.

When the 250 pN data points are used, the calculated k_{off}° and σ values are much closer to previously reported values. For example, the k_{off}° and σ values for L-selectin reported by Smith et al. were 2.8 s^{-1} and 1.11 Å, but are 9.3 s^{-1} and 0.41 Å when the 250 pN value is included (Fig. 2 *A*), compared to 7.0 s^{-1} and 0.24 Å in a previous report (Alon et al., 1998).

In conclusion, it is incorrect to attribute differences in k_{off}° and σ estimates between Smith et al. and other studies to differences in temporal and spatial resolution. It appears that the differences are due to an incorrect method for calculating k_{off}, the large amount of scatter in the data, and the high sensitivity of the estimates of k_{off}° and σ to omission of a single data point.

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