Mechanical Anchoring Strength of L-Selectin, β_2 Integrins, and CD45 to Neutrophil Cytoskeleton and Membrane

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ABSTRACT The strength of anchoring of transmembrane receptors to cytoskeleton and membrane is important in cell adhesion and cell migration. With micropipette suction, we applied pulling forces to human neutrophils adhering to latex beads that were coated with antibodies to CD62L (L-selectin), CD18 (β_2 integrins), or CD45. In each case, the adhesion frequency between the neutrophil and bead was low, and our Monte Carlo simulation indicates that only a single bond was probably involved in every adhesion event. When the adhesion between the neutrophil and bead was ruptured, it was very likely that receptors were extracted from neutrophil surfaces. We found that it took 1–2 s to extract an L-selectin at a force range of 25–45 pN, 1–4 s to extract a β_2 integrin at a force range of 60–130 pN, and 1–11 s to extract a CD45 at a force range of 35–85 pN. Our results strongly support the conclusion that, during neutrophil rolling, L-selectin is unbound from its ligand when the adhesion between neutrophils and endothelium is ruptured.

INTRODUCTION

Anchoring strength of transmembrane receptors is of fundamental interest in cell adhesion and cell migration, where extraction of receptors could be involved. Quickly extracting a receptor from a cell surface or rupturing a bond usually requires a force of tens of piconewtons, which can be measured with an atomic force microscope (Binnig et al., 1986), laser tweezers (Ashkin, 1992), or a biointerface probe (Evans et al., 1995). In his groundbreaking work, Bell (1978) predicted that a force of ~100 pN would be needed to extract an integral glycoprotein from a lipid bilayer. However, a force of only 10–20 pN was detected when a glycophorin A was extracted at a speed of 0.4 μm/s from a red cell membrane (Evans et al., 1991). Bell (1978) also hypothesized that the logarithm of the lifetime of a receptor-ligand or antibody-antigen bond will decrease linearly with the pulling force applied to separate the bond. In like manner, to extract a receptor from a cell surface may require a time period that is force dependent. Here we report the correlation between the time and the force needed to extract an L-selectin (CD62L), a β_2-integrin (CD18), or a CD45 receptor from a human neutrophil surface with a newly developed technique, which is based upon a micropipette manipulation system (Shao and Hochmuth, 1996).

In response to an inflammatory stimulus, human neutrophils move toward, attach to, and roll on endothelium before their firm arrest and migration out of blood vessels to sites of injury or infection. To mediate neutrophils' rolling and firm arrest, L-selectin and three β_2-integrins (lymphocyte function-related antigen 1 or LFA-1, macrophage 1 or MAC-1, and gp150,95) bind to their ligands on endothelium at different strengths (Springer, 1995). L-selectin, which consists of an NH_2-terminal lectin-like domain, an epidermal growth factor-like domain, two consensus repeats, a transmembrane domain, and a cytoplasmic tail, is an 80-kDa molecule constitutively expressed on resting human neutrophils (McEver, 1994). For L-selectin, the ligand on endothelium is sialylated carbohydrate structures related to sialyl $Lewis^X$. The neutrophil-endothelium linkage established by L-selectin can be broken by blood flow through bond dissociation or molecular extraction on either surface. However, new linkages are always formed before old linkages are broken, thus enabling neutrophils to roll on endothelium (Puri et al., 1997). β_2-Integrins, also constitutively expressed on neutrophils, are heterodimers with their α- and β-subunits noncovalently linked together. To mediate the firm arrest of neutrophils, β_2-integrins have to bind to their ligands on endothelium more strongly than L-selectin and have a stronger anchor to neutrophil cytoskeleton and membrane. Like other transmembrane receptors, L-selectin and β_2-integrins are embedded in neutrophil membranes via hydrophobic interactions. In addition, the cytoplasmic tail of L-selectin is linked to the cytoskeleton through α-actinin (Pavalko et al., 1995), whereas the β-chains of β_2-integrins can be bridged to the cytoskeleton through α-actinin or filamin (Pavalko and LaRoche, 1993; Sharma et al., 1995). Knowledge of the overall anchoring strength of L-selectin and β_2-integrins will help us better understand their distinctive roles in neutrophil adhesion.

CD45, also called T200 or leukocyte common antigen, is a family of high-molecular-weight transmembrane glycoproteins expressed in various isoforms on leukocytes. At least four isoforms have been identified, with molecular masses of 180 kDa (CD45RO, or gp180), 190 kDa, 205 kDa, and 220 kDa (CD45RA). On human neutrophils, CD45RO is the main isoform expressed (Lacal et al., 1988; Caldwell et al., 1991). The cytoplasmic tail of CD45, which is heavily involved in the signal transduction through its protein tyrosine phosphatase activity, can be linked to the cytoskele-

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ton through ankyrin (Moffat et al., 1996), fodrin, or spectrin (for fodrin, the in vitro association constant $K_a = 9.1 \times 10^8$ M^{-1}; for spectrin, $K_a = 3.1 \times 10^8$ M^{-1}) (Bourguignon et al., 1985; Lokeshwar and Bourguignon, 1992).

In our study, a human neutrophil was allowed to touch an antibody-coated bead for a short period of time, after which a pulling force of tens of piconewtons was applied to the neutrophil with micropipette suction (Shao and Hochmuth, 1996). Although the rate of bond formation between the neutrophil and bead (0.96–1.90 s^{-1}) was slightly larger than the rate of bond formation for mouse anti-rabbit-IgG-coated beads and rabbit-IgG-coated surfaces (~0.5 s^{-1}) (Pierres et al., 1995), single bond adhesion events were still dominant in our study, as indicated by the infrequency of adhesion (Chesla et al., 1998; Piper et al., 1998) and our Monte Carlo simulation (Appendix A). We found that receptors were extracted from neutrophil membranes when neutrophils were separated from beads. L-selectin was not anchored to neutrophil surfaces as strongly as β_2-integrins, whereas CD45 was anchored more strongly than β_2-integrins at lower forces but more weakly than β_2-integrins at larger forces. Strong anchorage of β_2-integrins ensures strong linkage of neutrophils to their substrates. The fast off-rate of the bond between L-selectin and its ligand (Puri et al., 1997, 1998) implies that L-selectin is unlikely to be extracted from neutrophil membranes during neutrophil rolling.

MATERIALS AND METHODS

Neutrophil and bead

With a density gradient method, human neutrophils were isolated from venous or finger prick blood of healthy donors. Antibody-coated beads, which were usually injected into the experimental chamber 30 min before the adhesion assay, were produced by incubating latex beads (2×10^5/ml; covalently linked with Fc specific goat anti-mouse IgG; Sigma, St. Louis, MO) with mouse anti-human monoclonal antibodies: anti-CD62L (10 μg/ml; SK11 clone; IgG2, κ), anti-CD18 (10 μg/ml; L130 clone; IgG1, κ; Becton-Dickinson, San Jose, CA), or anti-CD45 (2 μg/ml; BRA-55 clone; IgG1, κ; Sigma). For detailed neutrophil and bead preparation procedures, see Shao and Hochmuth (1996).

Cytochalasin D treatment

A 10 mM stock solution was obtained by dissolving cytochalasin D (Sigma) in dimethyl sulfoxide. Before use, some stock solution was diluted to 5 μM with endotoxin-free Hanks' balanced salt solution (HBSS) (Sigma) that was buffered with 25 mM HEPES. Then 300 μl of human autologous plasma and 300 μl of 5 μM cytochalasin D in HBSS were mixed together. Neutrophils in 50% plasma (diluted with HBSS) and antibody-coated beads in phosphate-buffered saline (PBS) were added to the mixture. The total volume was brought to ~750 μl. About half an hour after the addition, the adhesion assay was performed.

Adhesion assay

As shown in Fig. 1 *a*, a pulling force can be applied to a human neutrophil that has been in contact with an antibody-coated bead for ~0.1 s. If the neutrophil and bead did not adhere to each other, the neutrophil would move away freely; if they did adhere to each other, a tensile force (F) would develop between them and a retarded motion of the cell would be observed. The adherent cell would eventually break loose under the pulling force and resume its free motion. The duration between the moment when the adherent cell is pulled and the moment when the cell resumes its free motion is defined as the *adhesion lifetime*. For every neutrophil-bead pair, this procedure is repeated ~100 times unless described otherwise.

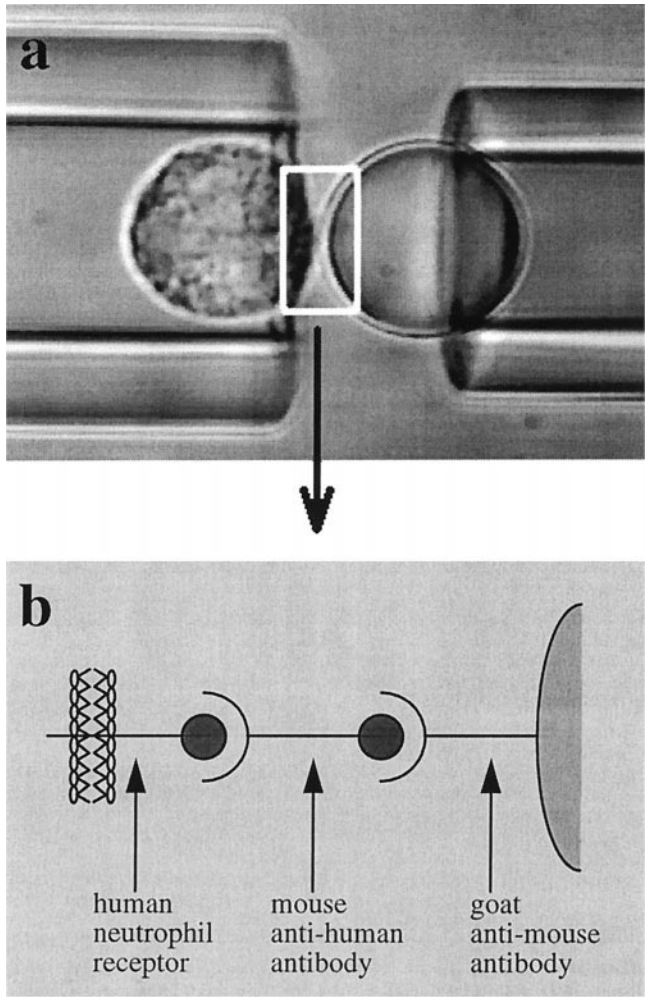

FIGURE 1 A microscopic view of the adhesion assay. (*a*) A human neutrophil is placed in a micropipette whose diameter (~9.5 μm) is almost the same as that of the neutrophil. A latex bead (~10 μm in diameter), coated with antibodies to human receptors (CD62L, CD18, or CD45), is positioned at the opening of the pipette containing the neutrophil. A precise suction pressure (Δp) is applied to the neutrophil with a micropipette manipulation system (Shao and Hochmuth, 1996). A larger positive pressure can be superimposed on the suction pressure so that the neutrophil moves toward the bead and touches it. Then the positive pressure is removed and only the suction pressure remains, so that a suction force is imposed on the neutrophil. (*b*) A diagram of the molecular linkage when a neutrophil adheres to an antibody-coated bead.

Force calculation

Adhered neutrophils either moved at a smaller and smaller velocity to a stationary position or moved at a constant velocity, in which case tethers or long membrane cylinders were formed (Shao and Hochmuth, 1996; Shao et al., 1998). For both cases, the pulling force (F) was calculated as

$$F = \Delta p \cdot \pi R_p^2 (1 - U/U_f), \quad (1)$$

where Δp is the suction pressure, R_p is the radius of the pipette that contains the cell, U is the velocity of the cell, and U_f is the velocity of the nonadherent cell under the same pressure. For neutrophils moving more and more slowly to a fixed position, the average velocity during the adhesion lifetime was used for U. It can clearly be seen that the force will be zero when the cell moves as a neutrally buoyant body ($U = U_f$), and that the force will be just the pressure times the cross-sectional area of the pipette when the cell is stationary ($U = 0$). For a detailed discussion of this formula, see Shao and Hochmuth (1996).

Data acquisition

The adhesion lifetime was measured field by field from a videotape on which the whole experimental process was recorded at a speed of 60 fields/s. After the adhesion lifetimes were measured for one suction pressure, they were plotted in a histogram. Outliers, presumably caused by multiple bonds, were identified and excluded from consideration with a method described by Devore (1995). The distance was measured with video calipers (Vista Electronics, La Mesa, CA). Video calipers were calibrated before every analysis by use of an image of a reticule (10 μm between parallel lines), which was recorded after every experiment with the same setup.

Flow cytometry

Goat anti-mouse-antibody-coated beads in PBS (5×10^5/ml) were incubated with mouse monoclonal anti-human CD45 antibodies labeled with fluorescein isothiocyanate (1 μg/ml) (Sigma). The same incubation condition and wash procedure as those used for the beads in the adhesion assay were applied. The beads were stored at 4°C in PBS until their fluorescence intensities were measured with a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

RESULTS

Four control experiments were done: one with Sigma beads that were washed twice with PBS (the bare beads), one with Sigma beads that were incubated with purified mouse IgG2a, κ (Sigma), one with Sigma beads that were incubated with purified general type mouse IgG (Sigma), and one with anti-CD45-coated beads while anti-CD45 was present in the chamber ($\sim$5 μg/ml). For the first case, two adhesion events occurred in 414 trials with three pairs of neutrophil and bead, corresponding to an adhesion frequency of 0.5%; for the second case, one adhesion event occurred in 408 trials with four pairs of neutrophil and bead, corresponding to an adhesion frequency of 0.25%; for the third case, seven adhesion events occurred in 380 trials with three pairs of neutrophils and bead, corresponding to an adhesion frequency of 1.8%. All three of these frequencies are substantially lower than the adhesion frequencies observed when beads were coated with specific antibodies to human receptors. For the last case, the adhesion frequency decreased from 11% to 5%.

For every antibody type and every suction pressure, each cell-bead pair was used for $\sim$100 touches. Fig. 2 shows the record of the number of adhesion events (n) and the number of trials (N) for a particular cell-bead pair in the experiment with neutrophils and anti-CD62L-coated beads under a suction pressure of 0.75 pN/μm^2. In all of the experiments for other cell-bead pairs and antibody types, the relations between n and N are similar to this one. Because cell-bead pairs were often changed during the experiments, it is assumed that n will depend linearly upon N. Therefore, the adhesion frequency can be obtained by dividing the total number of adhesion events (n_T) by the total number of trials (N_T). The adhesion frequencies observed in our adhesion experiments were generally low (6–24%), which indicates that the adhesion was mediated by only a single bond between cell and bead. Intuitively, low adhesion frequency means that the contact time at each touch is short enough for cell and bead not to form more than one bond. With a binomial stochastic model, it can be predicted that cell-cell adhesion will probably be mediated by only a single bond if the adhesion frequency is below 25% (Chesla et al., 1998; Piper et al., 1998). With a Monte Carlo simulation (Mahama and Linderman, 1994, 1995), we studied cell-bead interactions by immobilizing antibodies on a virtual bead surface and distributing freely diffusing receptors on a virtual cell surface (Appendix A) (Shao, 1997). We found that adhesion between cell and bead will be dominated by single bond events whenever the combination of the three parameters (the concentration of receptors, the concentration of antibodies, and the diffusion coefficient of receptors) yields an adhesion frequency below 25%. However, even at very low adhesion frequencies, multiple bonds cannot be avoided, although their numbers are very small. Fig. 3 shows a typical histogram of the lifetime measured from the adhesion between a neutrophil and an anti-CD62L-coated bead under a pressure of 0.75 pN/μm^2. Some long lifetimes, probably caused by multiple bonds, lie away from most of the data. All histograms at other pressures and for other antibodies look alike.

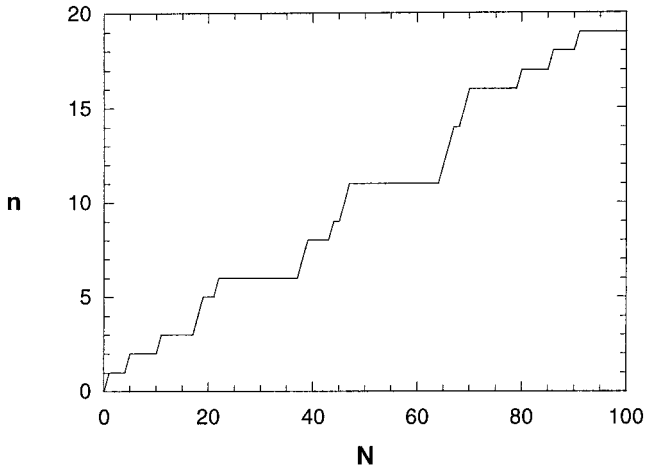

FIGURE 2 The number of adhesion events (n) versus the number of trials (N) in an experiment with a neutrophil and an anti-CD62L-coated bead. The average force applied on the cell is 34 pN. For this particular cell-bead pair, a linear fit gives an average adhesion frequency of 21%.

Adhesion between neutrophils and anti-CD62L-coated beads

At three different suction pressures (0.5, 0.75, and 1 pN/μm^2), the adhesion between neutrophils and anti-CD62L-

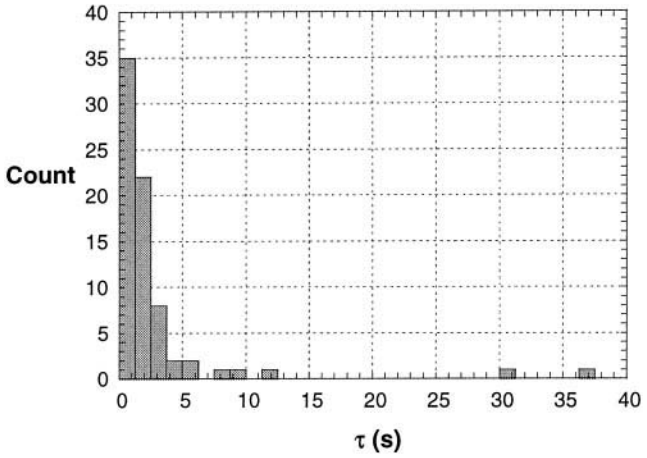

FIGURE 3 The histogram of the adhesion lifetime (τ) between neutrophils and anti-CD62L-coated beads at a force of ~34 pN.

coated latex beads occurred at frequencies of 10% (n_T = 76), 24% (n_T = 74), and 23% (n_T = 57), respectively. The lifetimes were averaged after the outliers were removed. The correlation between the adhesion lifetime and the force is shown in Fig. 4.

Adhesion experiments were also done with latex beads (~2 × 10^5/ml) incubated at three different concentrations of anti-CD62L: 10, 0.1, and 0.001 μg/ml. The adhesion lifetimes between neutrophils and these beads were measured under a suction pressure of 1 pN/μm^2. The adhesion happened at frequencies of 10% (n_T = 55), 6% (n_T = 33), and 11% (n_T = 57). This lack of dose response in the adhesion frequency is probably caused by the variation in antibody concentrations on the bead surfaces, as only a few pairs of cell and bead were used in the experiments at each incubation concentration. The lifetimes measured from these three experiments are comparable to the results shown in Fig. 4.

Adhesion between neutrophils and anti-CD18-coated beads

Because LFA-1, MAC-1, and gp150,95 all have a β_2-chain, the antibody to CD18 can bind to any of these three receptors on the neutrophil surface. Under six different suction pressures (1.25, 1.5, 1.75, 2, 3, and 4 pN/μm^2), the adhesion between neutrophils and anti-CD18-coated beads occurred at frequencies of 17% (n_T = 61), 18% (n_T = 30), 17% (n_T = 27), 14% (n_T = 57), 23% (n_T = 44), and 16% (n_T = 26), respectively. The correlation between the adhesion lifetime and the force is shown in Fig. 5 (*circles*). Statistical analysis shows that the slope of the fitted line in Fig. 5 has a significant difference from the one in Fig. 4 ($p < 0.05$), but the intercept does not ($p > 0.2$) (Zar, 1996). Also shown in Fig. 5 (*squares*) are the measurements for the adhesion lifetime between anti-CD18-coated beads and neutrophils treated with 2 μM cytochalasin D. The cytochalasin D experiments were done at pressures of 1.5, 2, and 2.5 pN/μm^2. The adhesion happened at frequencies of 25% (n_T = 64), 23% (n_T = 40), and 20% (n_T = 40), respectively.

Adhesion between neutrophils and anti-CD45-coated beads

Under five different pressures (0.5, 0.75, 1, 1.5, and 2 pN/μm^2), the adhesion occurred at frequencies of 7% (n_T = 8), 6% (n_T = 18), 10% (n_T = 12), 11% (n_T = 26), and 9% (n_T = 22), respectively. Four more data points were measured from another work (Shao et al., 1998). In those experiments, the adhesion frequencies are 11% at 0.625 pN/μm^2 (n_T = 26), 10% at 0.75 pN/μm^2 (n_T = 32), 11% at 0.875 pN/μm^2 (n_T = 27), and 11% at 1 pN/μm^2 (n_T = 21).

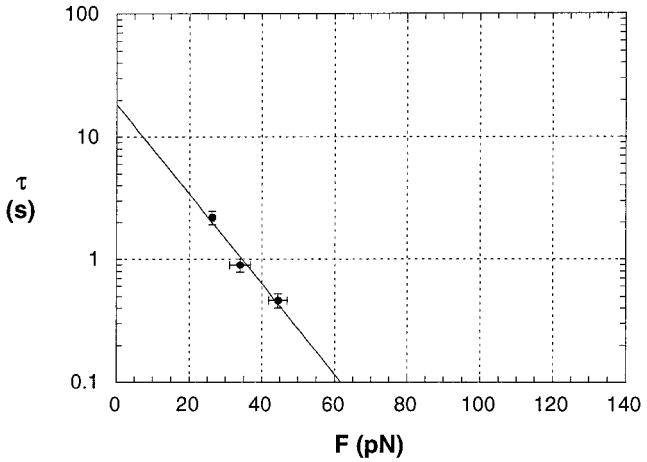

FIGURE 4 The correlation between the pulling force (F) and the adhesion lifetime (τ) for neutrophils and anti-CD62L-coated beads. The equation that fits through the filled circles is $\tau = 18e^{-0.085F}$. For convenience of comparison, Figs. 4, 5, and 6 are plotted on the same scale. All error bars in Figs. 4, 5, and 6 show the standard errors of the mean.

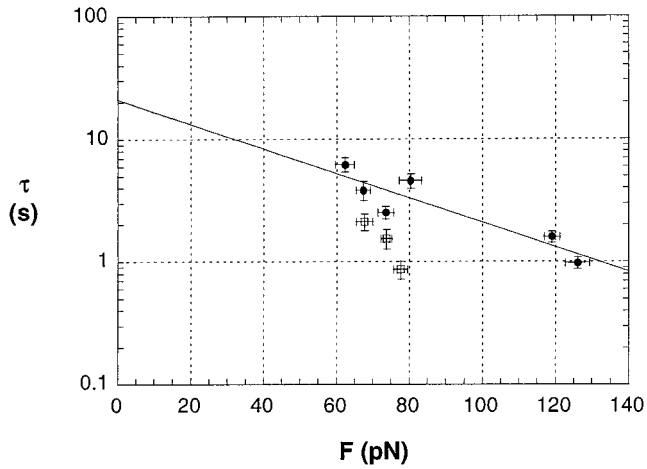

FIGURE 5 The correlation between the pulling force (F) and the adhesion lifetime (τ) for neutrophils (●) or cytochalasin D-treated neutrophils (□) and anti-CD18-coated beads. The equation that fits through the circles is $\tau = 21e^{-0.023F}$.

The correlation between the adhesion lifetime and the force is shown in Fig. 6 (*circles*). Statistical analysis shows that the slope of the fitted line in Fig. 6 has a significant difference from the one in Fig. 5 ($p < 0.01$), and so does the intercept ($p < 0.001$). However, the slope of the fitted line in Fig. 6 is not significantly different from the one in Fig. 4 ($p > 0.2$), although the intercept does ($p < 0.001$) (Zar, 1996). Also shown in Fig. 6 (*squares*) are the measurements for the adhesion lifetime between anti-CD45-coated beads and neutrophils treated with 2 μM cytochalasin D. Here, the cytochalasin D experiments were done at pressures of 0.25, 0.5, 0.75, and 1 pN/μm^2. The adhesion occurred at frequencies of 10% ($n_T = 7$), 13% ($n_T = 37$), 11% ($n_T = 22$), and 28% ($n_T = 15$), respectively.

Receptors were very likely to be extracted from neutrophil surfaces

The termination of adhesion between cell and bead could be caused by any one of the following three mechanisms (Fig. 1 *b*): extraction of receptors, dissociation of the bond between receptor and mouse antibody, and dissociation of the bond between mouse antibody and goat antibody. The goat anti-mouse antibody is covalently bound to the bead surface, so it is very unlikely that this antibody is uprooted. Because the three types of beads have the same goat anti-mouse antibodies and the Fc portions of the three mouse anti-human antibodies (either mouse IgG1 or IgG2) are 90–95% homologous (Kuby, 1997), it is assumed that all of the bonds between mouse antibody and goat antibody have similar average binding strengths. If the bond between mouse antibody and goat antibody were to break in the experiments with anti-CD62L (or in the experiments with anti-CD45), we would not expect to see longer adhesion lifetimes at ~40 pN in the experiments with anti-CD45 (or at ~80 pN in the experiments with anti-CD18) (Figs. 4–6).

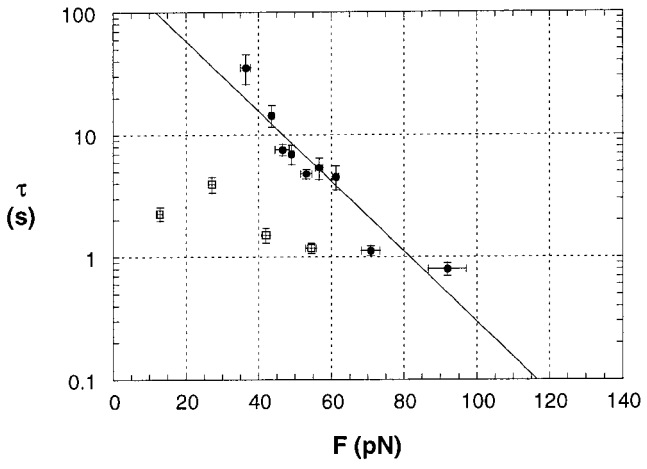

FIGURE 6 The correlation between the pulling force (F) and the adhesion lifetime (τ) for neutrophils (●) or cytochalasin D-treated neutrophils (□) and anti-CD45-coated beads. The equation that fits through the circles is $\tau = 220e^{-0.066F}$.

If the bond between mouse antibody and goat antibody were to break in the experiments with anti-CD18 and the lifetime of this bond relates linearly to the pulling force, we would not expect to see longer adhesion lifetimes at ~40 pN in the experiments with anti-CD45 (Figs. 5 and 6). Therefore, the results shown in Figs. 4–6 suggest that the bond between mouse antibody and goat antibody did not break for anti-CD62L, anti-CD18, or anti-CD45. It is very likely that only two failure mechanisms remain: extraction of receptors and dissociation of the bond between receptor and mouse antibody.

To determine the location of failure, the adhesion between neutrophils and anti-CD62L-coated beads was studied with a single bead touched hundreds of times by different neutrophils under a pressure of 1 pN/μm^2. As can be seen in Fig. 7 *a*, the adhesion frequency decreased steadily from 23% (a linear fit to the first 100 points) to 8% (a linear fit to the last 100 points; $p < 0.001$) in 1212 trials. This is an indication of a gradual depletion of the antibody-binding sites on the bead surface (Chesla et al., 1998). The same correlation between n and N was observed when another anti-CD62L-coated bead was touched 989 times by nine neutrophils. In this case, the adhesion frequency decreased from 19% to 13% ($p < 0.001$). This frequency decrease was not observed when different pairs of neutrophil and bead were used in the adhesion lifetime measurement, so the decrease in the adhesion frequency was not likely to have been caused by the dissociation of mouse antibodies from the bead surface. Therefore, our data strongly suggest that the bond between receptor and mouse antibody did not break in the case of anti-CD62L, because the adhesion frequency (dn/dN) would have been a constant if this bond had broken. In other words, L-selectin was extracted. With the theory presented in Appendix B, this slow decay of adhesion frequency can be described by (Eq. B9)

$$n = 2N_{ab}^0[1 - \exp(-k_f' N_{rc}^0 N)], \quad (2)$$

where N_{ab}^0 is the initial number of antibodies in the apparent contact area (~1 μm^2), N_{rc}^0 is the initial number of receptors in the contact area, and k_f' is a constant proportional to the forward reaction rate constant. Fitting the experimental data in Fig. 7 *a* with Eq. 2 gives us $N_{ab}^0 = 220$ and $k_f' N_{rc}^0 = 4.9 \times 10^{-4}$ (correlation coefficient = 0.99937).

After a single anti-CD18-coated bead was touched 1397 times by 10 neutrophils under a suction pressure of 3 pN/μm^2, the adhesion frequency decreased from 46% to 20% ($p < 0.001$) (Fig. 7 *b*). This experiment was done with a different batch of beads from Sigma, and the initial adhesion frequency was too high for Eq. 2 to be used. Nevertheless, the data in Fig. 7 *b* strongly suggest that β_2-integrins were extracted when neutrophils separated from anti-CD18-coated beads, because no adhesion frequency decrease was observed when different pairs of neutrophil and bead were used. This conclusion was also supported by the fact that the adhesion lifetime was dramatically reduced when neutrophils were treated with 2 μM cytochalasin D (Fig. 5).

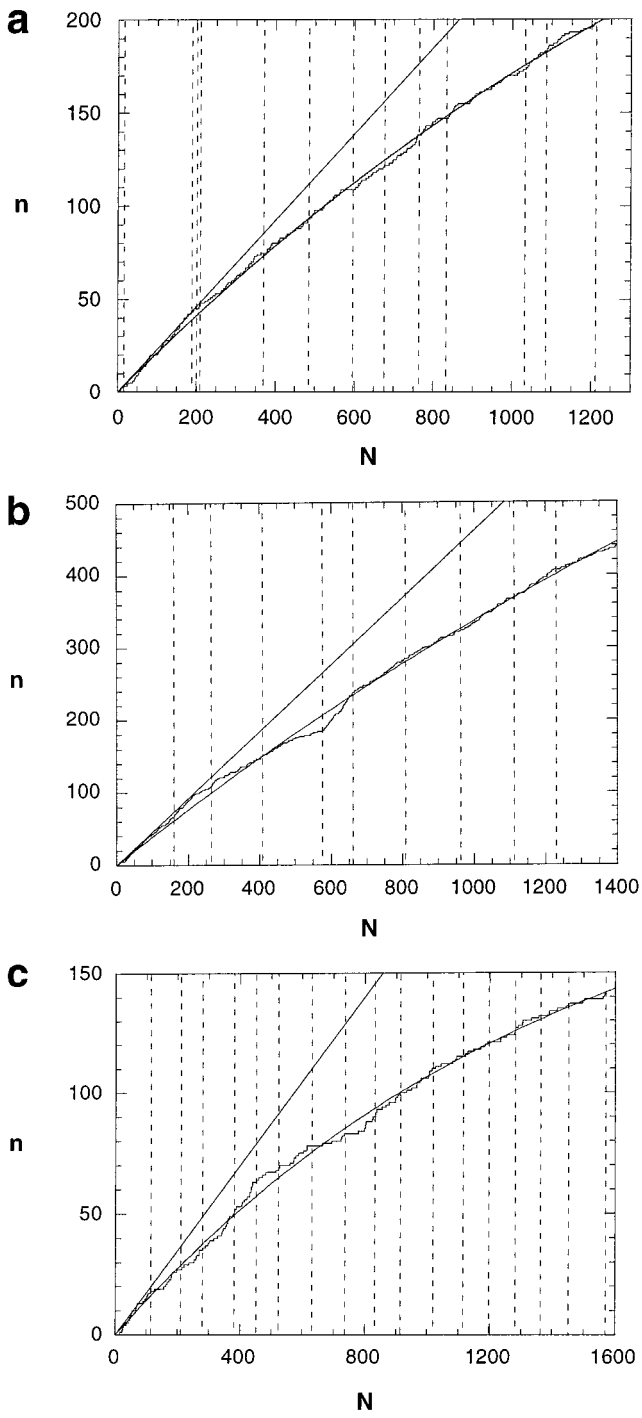

FIGURE 7 The decrease in adhesion frequency when one antibody-coated bead was touched by several neutrophils. Data from different cells are separated by dashed lines. Curves fitted with Eq. 2 are shown together with the experimental measurements. A linear fit to the first 100 trials is also shown as a straight line, the slope of which represents the initial frequency. (*a*) The decrease in adhesion frequency when one anti-CD62L-coated bead was touched by 13 neutrophils. (*b*) The decrease in adhesion frequency when one anti-18-coated bead was touched by 10 neutrophils. (*c*) The decrease in adhesion frequency when one anti-CD45-coated bead was touched by 17 neutrophils.

After an anti-CD45-coated bead endured numerous touches of neutrophils under a suction pressure of 1.5 pN/μm^2, the adhesion frequency decreased from 18% to 3% ($p < 0.001$) in 1571 touches by 17 neutrophils (Fig. 7 *c*). However, the adhesion frequency did not decrease when different pairs of neutrophil and bead were used during the adhesion lifetime measurement. Thus the frequency decrease was not caused by the dissociation of mouse antibodies from the bead surface. To further illustrate this, we analyzed the stability of the mouse antibody concentration on the bead surface with flow cytometry and found that the antibody concentration changed very slowly when the bead storage time changed from 40 min to ~14 days (Fig. 8). So CD45 was probably extracted from neutrophil surfaces in our adhesion experiments, as supported by the decreased adhesion lifetimes when neutrophils were treated with cytochalasin D (Fig. 6). Fitting the data in Fig. 7 *c* with Eq. 2 gives us $N_{ab}^0 = 107$, with $k_f' N_{rc}^0 = 6.9 \times 10^{-4}$ (the correlation coefficient = 0.99831).

DISCUSSION

Receptors (CD62L, CD18, or CD45) were very likely to be extracted when adhered neutrophils were separated from antibody-coated beads. Other interpretations of the data shown in Figs. 4–6 are still possible, although not very likely. The adhesion between the neutrophil and bead was enabled by formation of antibody-receptor bonds. The rate of bond formation between a surface and a cell or a bead has been reported with a parallel flow chamber assay (Kaplanski et al., 1993; Pierres et al., 1995). For mouse anti-rabbit-IgG-coated beads and rabbit-IgG-coated surfaces, the rate of bond formation was below 0.5 s^{-1} (Pierres et al., 1995). Here, the rate of bond formation is essentially the adhesion frequency divided by the contact time of every touch. For a

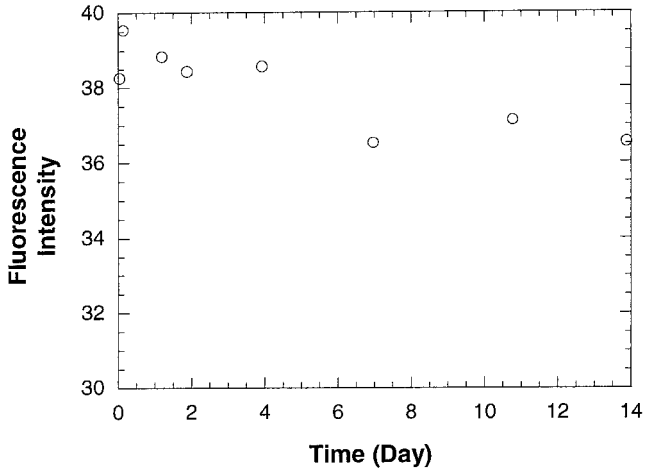

FIGURE 8 The fluorescence intensities of anti-CD45-FITC on bead surfaces at different storage times after incubation. For bare beads, the average fluorescence intensity is 2.47; for beads incubated with a mixture of anti-CD45 (40 μg/ml) and anti-CD45-FITC (1 μg/ml), the average fluorescence intensity is 6.52.

contact duration of 0.1 s, the rates of bond formation are 1.90 ± 0.78 (anti-CD62L), 1.75 ± 0.30 (anti-CD18), and 0.96 ± 0.19 s^{-1} (anti-CD45). If the initial number of antibodies (N_{ab}^0), the initial number of receptors (N_{rc}^0), and the contact area (A_c) are known, the two-dimensional forward reaction rate constant can be calculated (Appendix B). The number of antibodies in the contact area can be calculated by fitting $n = 2N_{ab}^0[1 - \exp(-k_f' N_{rc}^0 N)$ to the curve shown in Fig. 7 *a*. With flow cytometry, the total numbers of L-selectin and β_2-integrins on a neutrophil surface are estimated to be ~65,000 and ~66,000, respectively (Simon et al., 1992). Thus, for a neutrophil whose volume is 299 μm^3 and whose actual surface area is 2.1 times its apparent surface area (Ting-Beall et al., 1993), the average concentration of β_2-integrins is $\sim 145/\mu m^2$. If we assume that a microvillus is cylindrical with a flat circular tip (~0.2 μm in diameter) and L-selectin is uniformly distributed on neutrophil surfaces other than microvillus tips, the average concentration of L-selectin on microvillus tips can be calculated to be $\sim 940/\mu m^2$, because 78% of L-selectin is located on microvilli (Bruehl et al., 1996). For a contact area of five microvillus tips (~0.157 μm^2) (Shao et al., 1998), the two-dimensional forward reaction rate constants are $\sim 0.29 \times 10^{-12}$ cm^2/s for human CD62L and mouse anti-CD62L, and $\sim 1.7 \times 10^{-12}$ cm^2/s for human CD18 and mouse anti-CD18.

When the adhesion experiments were done with neutrophils treated with 2 μM cytochalasin D, the adhesion lifetimes dramatically decreased (Figs. 5 and 6). This suggests that treatment with cytochalasin D makes it easier to extract receptors. Cytochalasin D inhibits actin polymerization by binding to the plus end of actin filaments (Alberts et al., 1994). Thus it alters the actin-rich network of the cytoskeleton, which is the base for anchoring proteins. However, the time scale during which cytochalasin D can completely disrupt the cytoskeleton is still not clear. If the treatment is long enough, one receptor may eventually only bind to just one actin molecule because the binding of other actin molecules to this one has been completely abolished. Then what we are extracting will probably be the whole chain of molecules, i.e., receptor, linkage protein, and actin. In our experiments, almost all neutrophils became irregular in shape 3 h after they were added to the cytochalasin-D-containing experimental chamber. Spherical neutrophils were harder to find as time progressed. Therefore, the time needed to extract receptors from neutrophils treated with cytochalasin D may depend not only upon the force, but also upon the time after neutrophils encounter cytochalasin D.

In the force range we applied, the logarithm of the average adhesion lifetime depends linearly upon the force. Integrins on the cell membrane exhibit both random motion and directed motion (Schmidt et al., 1993). This suggests that they do not bind to the cytoskeleton all the time, so integrins could have been extracted at different states during the adhesion experiments, and so could CD62L and CD45. Note that all of the bonds depicted in Fig. 1 *b* are reversible bindings, i.e., they can dissociate without the need for pulling forces. When the adhesion lifetime is measured, one of those bonds or connections could be already in the process of dissociation. If this happens, lifetimes for bonds other than the failure location deduced earlier would be obtained. Of course, accidental inclusion of multiple bond adhesion events is also possible.

The in vitro association constant between α-actinin and the cytoplasmic tail of L-selectin is 1.8×10^6 M^{-1} (Pavalko et al., 1995). Although both L-selectin and β-chains of β_2-integrins can be anchored to neutrophil cytoskeleton through α-actinin (Pavalko and LaRoche, 1993; Pavalko et al., 1995; Sharma et al., 1995), extracting a β_2-integrin is more difficult than extracting an L-selectin, as shown in Figs. 4 and 5. This could be attributed to the interaction between α-chains of β_2-integrins and the cytoskeleton and to the fact that β_2-integrins can also be linked to the cytoskeleton via filamin (Sharma et al., 1995). Extracting receptors not only breaks the binding between receptor and cytoskeleton, but also breaks the association between receptor and membrane; our results show a similarity to the lifetime of a single bond between rabbit IgG and mouse anti-rabbit IgG under different pulling forces (Pierres et al., 1995). This is probably because specific and nonspecific adhesions are mediated by the same forces, i.e., van der Waals forces, hydrogen bonding forces, and electrostatic forces. Assume that the overall interaction between a receptor and the membrane-cytoskeleton complex can be integrated into a single "bond." Then the effective "bond" interaction length can be calculated from Bell's hypothesis and the fitted lines in Figs. 4–6 (Bell, 1978): 3.4 Å for L-selectin extraction, 0.93 Å for CD18 extraction, and 2.7 Å for CD45 extraction.

The intercept of the fitted line in Fig. 4 is 18 s. Does this mean that an L-selectin will spontaneously jump out of the neutrophil surface in 18 s? We think that the intercept should be interpreted as the upper limit for the lifetime of the connection between L-selectin and the cytoskeleton. The natural off rate for a bond between L-selectin and its ligand is ~8 s^{-1} (Puri et al., 1998). Under any amount of tensile force, the lifetime of such a bond will be less than 0.125 s. Therefore, it is unlikely that L-selectin is extracted when a neutrophil is rolling on endothelium. Instead, the bond between L-selectin and its ligand is probably broken.

APPENDIX A: SINGLE BONDS OR MULTIPLE BONDS? A MONTE CARLO SIMULATION

In this appendix, a Monte Carlo simulation (Mahama and Linderman, 1994; Mahama and Linderman, 1995) is rendered to study whether bonds are formed and how many bonds are formed each time a neutrophil touches an antibody-coated bead. When a neutrophil makes contact with an antibody-coated bead, they can adhere to each other if antibodies bind to receptors on the neutrophil surface. The number of antibody-receptor bonds formed in this process depends on the diffusion coefficient of receptors (D), the concentration of receptors (C_{rc}), the concentration of antibodies (C_{ab}), the contact time (Δt_c), the contact area (A_c), the on rate for a complex to form a bond (k_{on}), and the reverse reaction rate constant. Although the antibodies remain stationary on the bead surface, the recep-

tors undergo a perpetual Brownian motion on the surface of neutrophils. Close contact between the two surfaces still allows the receptors to "walk" around in the contact region (Dustin et al., 1996). (This was also shown by our own observation that the adhesion frequency increased when the contact time between neutrophil and bead increased.) After a neutrophil touches a bead surface, its receptors diffuse into and out of the contact area as if they are looking for the antibodies on the bead surface. Once a receptor encounters an antibody and a complex is formed, the probability that this complex will become a bond can be calculated with (Hammer and Apte, 1992)

$$P_{\text{complex}\rightarrow\text{bond}} = 1 - \exp(-k_{\text{on}}\Delta t), \qquad \text{(A1)}$$

where Δt is the average lifetime of a complex. Δt can be calculated as the characteristic time for a receptor to diffuse from one location to another one, and k_{on} is related to the rotational and orientational diffusion.

Because of the richness of neutrophil microvilli, it is very likely that the contact points between the neutrophil and bead are tips of microvilli. Because the overall apparent contact area is around 1 μm^2 (estimated from video micrographs such as the one shown in Fig. 1 *a*), it is assumed that five microvilli are in contact with the bead when a neutrophil touches an antibody-coated bead (Shao et al., 1998). It is also assumed that bond formation on each microvillus tip is independent of that on the others, as the contact time is only ~0.1 s, so one touch is simulated as the bead surface touched five times by one microvillus. A plain square is selected as the simulation region on the neutrophil and on the bead. A circular region at the center of the square is designated as the contact area, the diameter of which is equal to the diameter of a microvillus (D_{tip}). Triangular grids with a lattice spacing of 5 nm (d) are used in both simulation regions. A number of antibodies are randomly immobilized on the bead surface, whereas some receptors are randomly distributed on the neutrophil surface. Receptors are subjected to a Brownian motion and actually serve as antigens. No antibodies or receptors can occupy the same location on their respective surfaces. After receptors and antibodies are placed on the grid, the number of bonds before any motion of receptors is counted by checking if any receptor has the index of an antibody binding site location that is inside the contact area and if a random number is less than the probability calculated with Eq. A1. Then, at each Monte Carlo time step, a receptor is chosen randomly and a random adjacent location is selected to move to. If the new location has been occupied by either a free receptor or a bound receptor, then the move is rejected. If the new location has been out of the boundary, periodic conditions are imposed, and this receptor will appear at the other side of the simulation square. If the new location is empty, the current location is unoccupied and the receptor moves to the new location. If there is a free antibody binding site in the new location and the new location is inside the contact area, a complex is formed, and whether this complex can form a bond is determined by the probability calculated with Eq. A1.

The adhesion frequency is defined as

$$f = \frac{\mathrm{d}n}{\mathrm{d}N}, \qquad \text{(A2)}$$

where n is the number of adhesion events after N trials. If C_{rc} and C_{ab} are kept constant for every touch, there will be a linear correlation between n and N. The adhesion frequency will be simply $n_{\text{T}}/N_{\text{T}}$, where n_{T} is the total number of adhesion events and N_{T} is the total number of trials.

In the following simulation, different combinations of C_{rc}, C_{ab}, and D will be used while the other four parameters are kept as constants: $N_{\text{T}} = 100$, $\Delta t_{\text{c}} = 0.1$ s, $k_{\text{on}} = 50$ s^{-1}, and $D_{\text{tip}} = 100$ nm. For each set of parameters, 10 simulations are run. The simulation results for $C_{\text{rc}} = 200/\mu\text{m}^2$, $D = 1000$ nm^2/s (or 10^{-11} cm^2/s), and some different values of C_{ab} are shown in Fig. 9. It is not a surprise that the adhesion frequency increases as C_{ab} increases. Trials can go seven times without any adhesion events, although the total adhesion frequency is as high as 53%. Trials can also have two consecutive adhesion events, even when the total adhesion frequency is as low as 5%. Similar behavior was observed in the neutrophil adhesion experiment. When $C_{\text{ab}} = 50/\mu\text{m}^2$, there is one simulation where the total number of adhesion events is 20, yet the total number of multiple bond adhesion events is zero in 100 trials. This shows that low adhesion frequency cannot guarantee single bonds and that high adhesion frequency cannot guarantee multiple bonds. Therefore, there is always a chance that multiple bonds will appear in the neutrophil adhesion experiment. However, for low frequency adhesions, the probability of forming multiple bonds is indeed very small. For the adhesion lifetime measurement, these multiple bond events should give larger values for the lifetime, which will lie away from the lifetime for single bond events. It should also be noted that even at an adhesion frequency as high as 50%, more than half of the adhesion events are still single bond events. So adhesion lifetime experiments can still be performed, even when the adhesion frequency is high, as long as the difference between the lifetime of a single bond and the lifetime of a multiple bond is larger than the resolution of the technique.

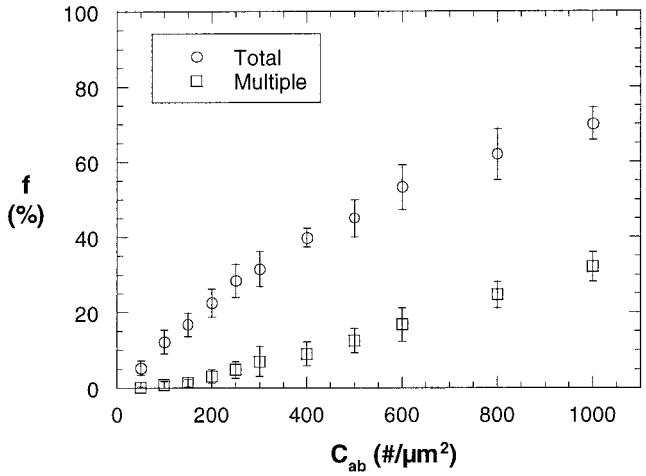

FIGURE 9 Dependence of the total adhesion frequency (○) and the multiple bond adhesion frequency (□) upon the number of antibodies (C_{ab}) when $C_{\text{rc}} = 200/\mu\text{m}^2$ and $D = 1000$ nm^2/s ($N_{\text{T}} = 100$, $\Delta t = 0.1$ s and $D_{\text{tip}} = 100$ nm). The adhesion frequency (f) is averaged from 10 simulations (100 trials in every simulation); error bars show the standard deviation.

More details about the simulation method can be found in Shao (1997).

APPENDIX B: SURFACE ADHESION KINETICS WHEN ONE BEAD IS TOUCHED BY NUMEROUS NEUTROPHILS

When one antibody-coated bead is repeatedly touched by different neutrophils for hundreds of times, the rate of bond formation can be described with the following forward reaction equation:

$$\frac{\mathrm{d}(n/A_{\text{c}})}{\mathrm{d}t} = k_{\text{f}}\frac{N_{\text{rc}}}{A_{\text{c}}}\frac{2N_{\text{ab}}}{A'_{\text{c}}}, \qquad \text{(B1)}$$

where t is the time, n is the number of bonds being formed, A_{c} is the total contact area between the neutrophil microvilli and bead surface, A'_{c} is the apparent contact area between the neutrophil and bead (~1 μm^2), k_{f} is the forward reaction rate constant, N_{rc} is the number of receptors in A_{c} at time t, and N_{ab} is the number of antibodies in A'_{c} at time t. Note that every antibody has two binding sites, and we assume that they are both functional in our adhesion experiments.

For low frequency adhesions that involve only single bond adhesion events, n will be equal to the number of the accumulated adhesion events, and Eq. B1 can be rewritten as

$$\frac{\mathrm{d}n}{\mathrm{d}N} = 2k'_{\text{f}}N_{\text{rc}}N_{\text{ab}}, \qquad \text{(B2)}$$

where N is the number of touches and

$$k_f' = \frac{k_f \cdot dt/dN}{A_c'} = \frac{k_f \cdot \Delta t_c/1}{A_c'}, \quad \text{(B3)}$$

where Δt_c is the contact time of every touch between cell and bead. Because a new neutrophil is used for about every 100 touches, it is assumed that the number of receptors in the contact area is a constant, i.e.,

$$N_{rc} = N_{rc}^0. \quad \text{(B4)}$$

Assume that a bound receptor will dissociate from the antibody in N_c touches after its binding; then

$$N_c = \text{the smallest integer} \geq \frac{\Delta t_b}{\Delta t_N + \Delta t_c}, \quad \text{(B5)}$$

where Δt_b is the lifetime of the bond between receptor and mouse antibody and Δt_N is the elapsed time between every two adjacent touches. Therefore, if we assume that the unbound receptor does not bind back to any of the antibodies in the contact area, the number of antibody binding sites at any N will be

$$2N_{ab} = 2N_{ab}^0 - n[N] + n[N - N_c], \quad \text{(B6)}$$

where N_{ab}^0 is the initial number of antibodies in A_c' and $n[N - N_c] = 0$ when $N \leq N_c$. Here the square brackets denote functions, i.e., $n[N - N_c]$ means that n is a function of $N - N_c$. From Eqs. B2, B4, and B6, we have

$$\frac{dn}{dN} = k_f' N_{rc}^0 (2N_{ab}^0 - n[N] + n[N - N_c]). \quad \text{(B7)}$$

The initial condition is $n[N] = 0$ when $N = 0$. This equation can be solved progressively on the following separate intervals: $(0, N_c]$, $(N_c, 2N_c]$, $[2N_c, 3N_c]$, . . . , although the expression of the solution will become increasingly complicated. It can be predicted that the adhesion frequency will start at $2k_f' N_{rc}^0 N_{ab}^0$, then decrease to a constant where the binding and the unbinding in the contact area reach an equilibrium state. The constant, $2k_f' N_{rc}^0 N_{ab}^0/(1 + k_f' N_{rc}^0 N_c)$, can be solved by assuming that $n[N]$ has the form of a linear function. We also numerically solved Eq. B7 and found that $dn/dN \to 2k_f' N_{rc}^0 N_{ab}^0/(1 + k_f' N_{rc}^0 N_c)$ as $N \to \infty$ for 30 groups of $k_f' N_{rc}^0$, N_{ab}^0, and N_c.

When the unbinding is infinitely fast ($N_c = 0$),

$$n = 2k_f' N_{rc}^0 N_{ab}^0 N, \quad \text{(B8)}$$

and the adhesion frequency will remain a constant.

When the unbinding is infinitely slow ($N_c \to \infty$),

$$n = 2N_{ab}^0[1 - \exp(-k_f' N_{rc}^0 N)] \quad \text{(B9)}$$

and the adhesion frequency will tend to zero.

Fig. 10 illustrates how N_c will affect the relation between n and N for $N_{ab}^0 = 220$ and $k_f' N_{rc}^0 = 4.9 \times 10^{-4}$. It can be seen that the adhesion frequency will be the same when N is less than 100, regardless of the value of N_c.

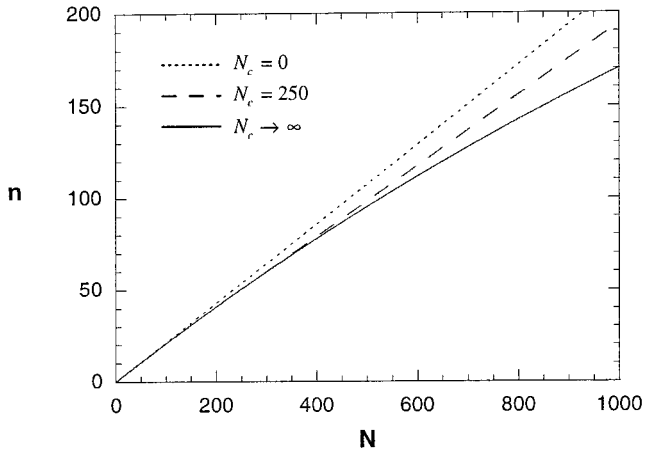

FIGURE 10 The effect of N_c (unbinding of bound receptors from bead surfaces) on the dependence of n upon N. When N_c is equal to 250 or other nonzero integers, the adhesion frequency first decreases according to $dn/dN = 2k_f' N_{rc}^0 N_{ab}^0(\exp - k_f' N_{rc}^0 N)$ and then approaches a constant.

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