Adhesion-activating Phorbol Ester Increases the Mobility of Leukocyte Integrin LFA-1 in Cultured Lymphocytes

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Abstract

Lymphocytes activate adhesion to intracellular adhesion molecule 1 (ICAM-1) via leukocyte function-associated antigen 1 (LFA-1), their major β_2 integrin, in response to PMA (phorbol 12-myristate 13-acetate) without an increase in the number of receptors expressed. The molecular details of the mechanism are unknown. To determine the effect of PMA activation on LFA-1 movement within the plasma membrane, we used the single particle tracking technique to measure the diffusion rate of LFA-1 molecules on EBV-transformed B cells before and after PMA activation. Diffusion of LFA-1 on unactivated cells was restricted compared to CR1 (CD35), another transmembrane protein of equivalent size. PMA caused a 10-fold increase in the diffusion rate of LFA-1 without any effect on CD35. The increased LFA-1 motion induced by PMA was random, not directed, indicating that it was due to a release of constraints rather than the application of forces. The diffusion rates of LFA-1 are consistent with cytoskeletal attachment before and free diffusion after PMA. Cytochalasin D led to an equivalent increase in mobility and, at low doses, stimulated adhesion. We propose that the release of LFA-1 from cytoskeletal constraints is an important early step in activation of adhesion, implying that the nonadhesive state of LFA-1 is actively maintained by the lymphocyte cytoskeleton. (*J. Clin. Invest.* 1996. 97:2139–2144.) Key words: cytoskeleton • cell membrane • diffusion • microscopy • microspheres

Introduction

Control of adhesion is physiologically important to leukocytes (1–3). These cells must patrol the body in a nonadherent state in the blood and lymph, and then migrate through tissues as adherent cells, making rapid transitions between adherent and nonadherent states. One way lymphocytes modulate interactions is by regulating their avidity for intracellular adhesion molecule 1 (ICAM-1)[1] substrates via leukocyte function-associated antigen 1 (LFA) (4–7). Increased LFA-1–mediated adhesion requires cell metabolism, and is not simply the result of increased expression of LFA-1 (4, 7). Possible molecular mechanisms for this avidity increase fall into two categories: those based on an increase in affinity of individual receptors for ligand and those based on rearrangement or altered cytoskeletal association of receptors (8, 9).

Since a major function of integrins is to link extracellular contacts to the cell's cytoskeleton (1–3), we hypothesized that cytoskeletal associations might change as cell adhesion is activated. We therefore examined the behavior of LFA-1 before and after activation with phorbol ester in real time on living cells, using single particle tracking (SPT) as an assay. In the SPT assay, small (40–100 nm) gold particles are bound to membrane proteins via antibodies or other specific ligands (10–13). The movement of these specifically bound particles in the plane of the membrane can be visualized by video-enhanced differential interference contrast microscopy on living cells (14). Computer analysis of each video frame can determine the location of the centroid of the particle with nanometer-level accuracy (13, 15). This results in a particle track, as illustrated in Fig. 1. Analysis of these particle tracks can distinguish between diffusing proteins and those restricted by cytoskeletal interactions based on their degree of Brownian motion (12). SPT has advantages over fluorescence recovery after photobleaching (FRAP), the other major technique for measuring diffusion: SPT can track both random and directed motion and can measure diffusion rates over much longer time courses.

We found that LFA-1 movement in the plasma membrane was quite restricted in resting MP cells (an EBV-transformed B cell line). Calculated diffusion rates were much lower than those of a control lymphocyte membrane protein, CR1 (CD35), a transmembrane protein of equivalent size. There was a 10-fold increase in Brownian motion of LFA-1 after PMA activation of the lymphocytes. This occurred without a general change in membrane fluidity, since the behavior of CD35 was not affected. The increase is consistent with release of LFA-1 from cytoskeletal constraints and was equivalent to the effect of cytochalasin D. Furthermore, cytochalasin D treatment was able to increase adhesion of lymphoblasts to ICAM-1–coated substrates nearly as effectively as PMA.

These data suggest a model for regulation of LFA-1 avidity in lymphocytes. We propose that in resting lymphocytes adhe-

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1. *Abbreviations used in this paper:* ICAM-1, intracellular adhesion molecule 1; LFA-1, leukocyte function-associated antigen 1; SPT, single particle tracking.

sion is inhibited by cytoskeletal constraint on LFA-1 as follows: At the moment of cell–cell contact, it is unlikely that the location of a receptor on one cell will exactly match that of a ligand on the other. Therefore firm adhesion is unlikely to be established in an unactivated cell, where the receptors are relatively immobile. After activation, the LFA-1 is free to move in the membrane at a 10-fold greater rate. This allows receptor to find ligand, effectively activating cell adhesion.

Methods

Cell preparation

Coverslips (22 × 22 mm, No. 0; Thomas Scientific, Swedesboro, NJ) were incubated at room temperature with a solution 1 μg/ml poly-L-lysine (Sigma Chemical Co., St. Louis, MO) in PBS for 30 min. They were then washed four times with PBS and once with stage medium (Hank's Balanced Salt Solution, Gibco BRL, Grand Island, NY) supplemented with 2 mM $CaCl_2$ and 4 mM $MgCl_2$. MP cells (an EBV-transformed B cell line from a normal donor) were cultured in RPMI with 10% FBS. For experiments, cells were washed twice with PBS and then incubated in Neuraminidase solution (0.1 U Neuraminidase X [Sigma Chemical Co.] in 0.13 M NaCl, 0.05 M Na Acetate, pH 6.5) at 37°C for 1–2 h to dissolve excess glycocalyx and facilitate access of antibody-coated beads to integrins. Cells were then transferred to stage medium and allowed to settle onto coverslips at 37°C for 30 min. This resulted in well spread cells with thin lamellae that were relatively stationary and free of organelles. Experiments were then performed at room temperature.

Single particle tracking

Method. Procedures for labeling and tracking LFA-1 were adapted from methods developed for tracking concavalin A–coated beads (10, 11). Lymphocytes were immobilized on poly-L-lysine–coated coverslips in a viewing chamber through which solutions could be perfused. Approximately 100-nm gold beads were coated with IB4, a mAb specific for β_2 integrins (16). These were added to stage medium and allowed to settle onto cells. The binding of these beads was specific: while IB4 beads began to bind to cells within a few minutes, beads coated with an irrelevant antibody did not bind, even after > 1 h. Moreover, IB4-coated beads did not bind to EBV-transformed B cells which lacked LFA-1 (from a patient with Leukocyte Adhesion Deficiency). Beads of this size bind single or small clusters of integrins, and the motion of the beads reflects the motion of the protein in the membrane (10, 11). Beads were observed by video-enhanced DIC microscopy, and videotapes of their motion were recorded. Bead (and thus receptor) motions were later analyzed by computer algorithms developed for this purpose (15). The location of the bead was determined in each video frame, i.e., 30 times a s, for 15 s (starting with attachment of the bead). By calculating a centroid, bead locations could be determined with nanometer-level precision. This resulted in a "particle track" of the bead's motion. Only beads which were observed to settle out of the medium and attach to cells were analyzed.

Analysis. Particle tracks were analyzed mathematically to separate the random and directed components of motion. The motion of a randomly diffusing particle is described by the equation:

$$\text{m.s.d.}_{\text{random}} = 4Dt \tag{1}$$

where m.s.d. is mean square displacement and D is the diffusion coefficient. If there is also a directed component to the particle's motion, that component is described by:

$$d = vt \tag{2}$$

where d = displacement, v = velocity, and t = time. Squaring both sides,

$$d^2 = (vt)^2 = \text{m.s.d.}_{\text{directed}} \tag{3}$$

Combining equations 1 and 3 results in an equation to describe motion with both random and directed components:

$$\text{m.s.d.}_{\text{total}} = 4Dt + (vt)^2 \tag{4}$$

Note that this is a quadratic equation. By fitting this equation to the data, D, a measure of the thermal motion, can be extracted (i.e., D = the first order coefficient divided by 4).

Analysis of random vs. directed motion. The probability that a particle in random motion will diffuse to a point between x and x + dx in time t is described by the equation

$$P(x)\,dx = 1/(4\pi Dt)^{1/2} \exp(-x^2/4Dt)\,dx \tag{5}$$

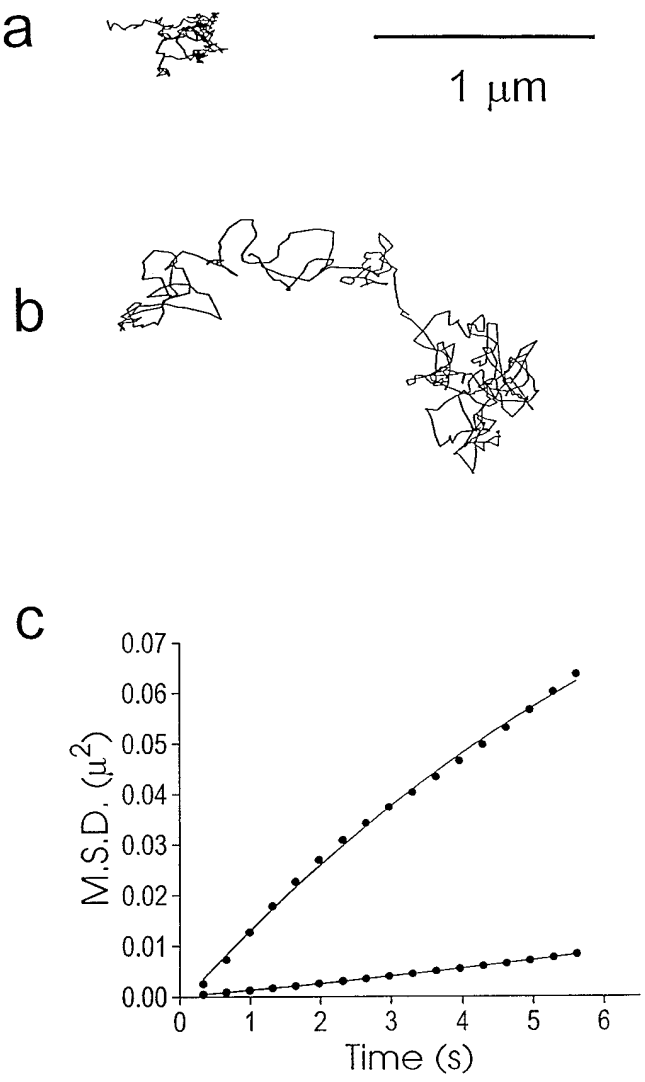

Figure 1. Particle tracks of gold-tagged LFA-1 generated by computer analysis of video sequences. In each case, the particle is tracked in 450 video frames (15 s), and a line is drawn connecting these 450 locations. These tracks are analyzed to generate a diffusion coefficient, D, as a quantitative measure of their thermal motion (explained in detail in Methods). (*a*) A particle with $D = 2.8 \times 10^{-11}$ cm^2/s. This is an example of what we will call "constrained motion." (*b*) A particle with $D = 3.7 \times 10^{-10}$ cm^2/s. This is an example of free diffusion. (*c*) *M.S.D.* (mean square displacement) plots generated from the particle tracks in *a* and *b* and fit with quadratic equations. Diffusion coefficients are derived from these curves as described in the "Analysis" section of Methods.

where D is the diffusion coefficient. The probability that a particle could traverse distance x or greater by executing a random walk (i.e., without a directed component of motion) is obtained by integrating this equation from x to infinity. Thus, to determine whether the motion of a given particle was random or directed, a value for D from each track was determined. Using D and the particle locations at t = 0 and t = 15 seconds, the probability that this movement occurred by random motion was determined. Tracks generating a $P < 0.05$ were considered to have a significant component of directed motion.

Adhesion assays. ICAM-1 was purified from JBL cells (a gift of T.A. Springer, Center for Blood Research, Boston, MA) by immunoaffinity chromatography on M174 agarose with elution at pH 3.0 (17). ICAM-1 was adsorbed to plastic by detergent dilution, and the plates were blocked with 5% nonfat dry milk (7, 18). The ICAM-1 site density was determined by binding of iodinated RR1/1 (a gift of R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). MP cells (10^6/ml) were labeled with calcein AM (Molecular Probes, Eugene, OR) at 2 μg/ml for 10 min at 37°C in RPMI 1640 2% BSA (adhesion media). TS1/22 (a gift of T.A. Springer, Center for Blood Research, Boston, MA), an mAb specific for the α_L subunit of LFA-1, was used in some cases to block specific adhesion. The cells were pretreated as indicated and 10^5 cells were added to each well to give a final vol of 100 μl. The plates were centrifuged for 5 min at 10 *g* (200 rpm), read in a Cytofluor 1300 microplate fluorimeter (Millipore Corp., Dedham, MA) (pre-FL) and incubated for 10 min at 37°C by flotation in a 37°C water bath. The plates were washed four times with room temperature assay media and the fluorescence reanalyzed (post-FL). The percent adhesion was calculated as:

$$\%\ cells\ bound = [\,(post\text{-}FL - media\text{-}FL)\,/\,(pre\text{-}Fl - media\text{-}FL)\,] \times 100. \quad (6)$$

Results and Discussion

To investigate the relationship between avidity change and cytoskeletal association of LFA-1, we tracked LFA-1 movements on the lamellae of MP cells before and after perfusing PMA in a concentration (50 ng/ml) that activates both adhesion to ICAM-1 substrates (7, 19) and LFA-1/ICAM-1–mediated homotypic aggregation of B cells (4). Approximately 100-nm diameter colloidal gold beads coated with IB4 mAb against the β_2 subunit of LFA-1 were allowed to settle onto cells, and their motion was tracked with video-enhanced DIC microscopy and analyzed by computer (10, 15) (Fig. 1).

SPT analysis can separate random from directed components of particle motion, and a diffusion coefficient (D) can be assigned to the random component of motion (10–12) (see "Analysis" section of Methods). We used D simply as a measure of the degree of thermal motion of the LFA-1 molecules in the membrane. This random motion increased 10-fold after PMA, from $2.3\pm0.5 \times 10^{-11}$ cm^2/s before PMA to $2.9\pm0.4 \times$

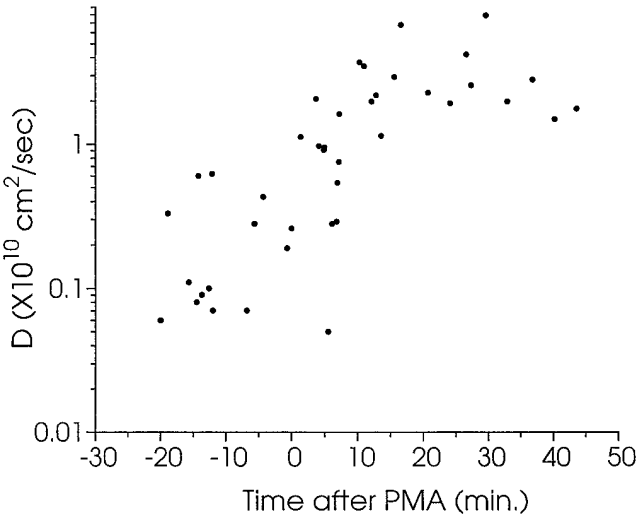

Figure 2. PMA (50 ng/ml) perfused through the viewing chamber results in an increase in the Brownian motion of β_2 integrins on the dorsal surface of the lamella. The diffusion coefficient D, a measure of the average Brownian component of motion, is plotted vs. time after PMA perfusion (note the log scale). Each data point represents an independent measurement. By 10 min after PMA perfusion, the Brownian (thermal) motion of the β_2 integrins on the dorsal surface of the lamella has increased more than an order of magnitude ($2.3\pm0.5 \times 10^{-11}$ cm^2/s before PMA vs. $2.9\pm0.4 \times 10^{-10}$ cm^2/s 10 min after PMA).

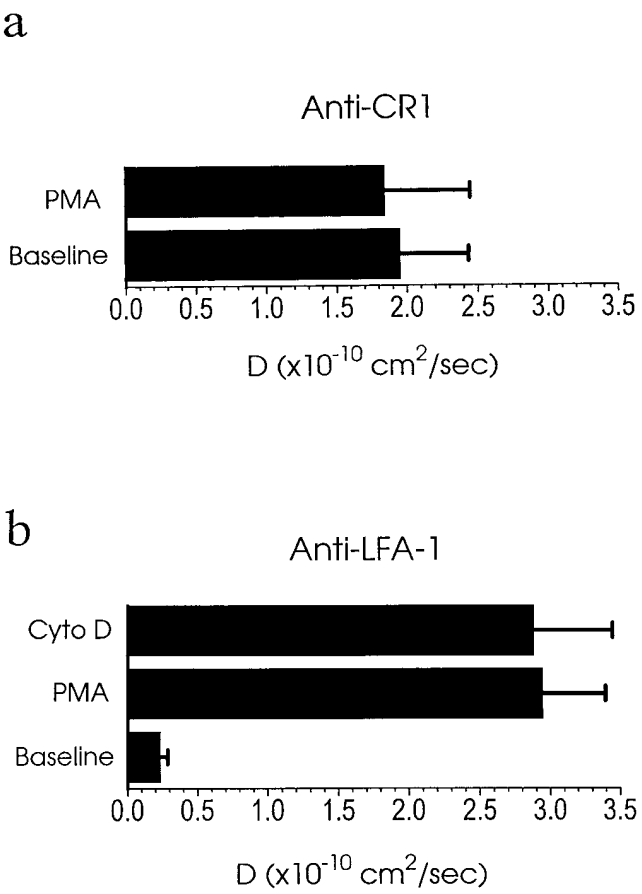

Figure 3. PMA causes a change in mobility consistent with a change in cytoskeletal associations, not a global change in membrane properties. (*a*) The random component of motion of Complement Receptor 1 (*CR1*), another transmembrane protein with a cytoplasmic tail, is relatively unconstrained and does not change with PMA. 10 measurements were made before PMA treatment (50 ng/ml) and 9 after. Plotted are mean diffusion coefficient (D) ±SEM. The mean diffusion coefficient before PMA treatment is not significantly different from that 10 min after PMA. Thus the increase in lateral mobility of LFA-1 in response to PMA is not likely to be a global effect on membrane fluidity. (*b*) Cytochalasin D (*Cyto D*), which disrupts actin filament organization, increases the lateral mobility of LFA-1 in the lymphocyte membrane to an extent similar to 50 ng/ml PMA. (Mean diffusion coefficient (D) ±SEM, calculated as in *a.* Before PMA, $n = 14$; after PMA, $n = 17$; cytochalasin D treated cells, $n = 20$.)

Table I. Analysis of Randomness of Membrane Protein Motion

	Random	Directed
Restricted ($D < 10^{-10}$ cm^2/s)	19	3
Unrestricted ($D > 10^{-10}$ cm^2/s)	19	1

Using a statistical analysis (11, 28) based on random walk theory (29), we analyzed all particle tracks for anti–LFA-1 coated gold. We calculated the probability that the distance traversed by each particle from the beginning to the end of its particle track could be the result of a random walk, as described in Methods. With a cutoff of $P = 0.05$, the motion of the LFA-1 molecules is mostly random, with few gold particles showing any significant directed component either in the unactivated (restricted) or the activated (unrestricted) state. This indicates that the major effect of PMA is an increase in random motion of receptors, with no significant role for directed motion.

10^{-10} cm^2/s (Fig. 2). The final diffusion rate agrees well with that of diffusing β_1 integrins in fibroblasts measured by fluorescence photobleaching (20).

To test whether the PMA effect on LFA-1 could be explained by a general increase in membrane fluidity (with no specificity for particular membrane proteins), we tracked the motions of CR1 (CD35), another B cell transmembrane protein with a cytoplasmic tail of 43 amino acids, similar in size to the 58 amino acid α_L and 46 amino acid β_2 cytoplasmic tails of LFA-1. Thus CR1 should experience membrane changes similarly to LFA-1. Before PMA treatment, CR1 diffused with a mean D of 2.1×10^{-10} cm^2/s. This D is consistent with a protein diffusing in the membrane, i.e., not constrained by the cytoskeleton. Therefore, CR1 is a good probe for changes in membrane fluidity. After PMA, the mean D of CR1 was 1.8×10^{-10} cm^2/s. This is not a statistically significant change (Fig. 3 *a*). Thus, the PMA effect is not consistent with a global change in membrane properties.

Since LFA-1 is an integrin, and integrins are known to associate with the actin cytoskeleton via various proteins (21–27), we hypothesized that the increased lateral mobility of LFA-1 after PMA resulted from release from cytoskeletal constraints. Indeed, the magnitude of the diffusion coefficients before PMA are consistent with cytoskeletal attachment, while those after PMA are consistent with relatively free diffusion of transmembrane proteins (20, 28–30). We therefore compared lateral mobility of LFA-1 induced by PMA to the effect of cytochalasin D, which disrupts actin filament organization (Fig. 3 *b*). Cytochalasin D resulted in a similar increase in mobility of LFA-1, suggesting that release from cytoskeletal constraints could account for the change in D in both cases.

We next analyzed the particle tracks to determine whether the increased LFA-1 mobility was consistent with purely random motion resulting from diffusion after release from constraints, or if active transport processes contributed. Applying a statistical analysis to distinguish directed from random motion (11, 31) based on random walk theory (32), we determined that the movement of LFA-1 in both the constrained and released state was consistent with entirely random (thermal) motion in the vast majority of cases (Table I). Therefore, the major change in LFA-1 behavior with PMA is a dramatic increase in receptor diffusion, consistent with a release from cytoskeletal constraints.

Recent experiments using integrin chimeras in CHO cells suggest that PMA regulates adhesion without affecting affinity for ligand (33). The increase in lateral mobility of LFA-1 that we observed could explain PMA activation of adhesion. As one cell contacts another, the likelihood that the positions of the receptor on one cell and ligand on the other will match exactly is very small. As the proteins diffuse, however, they will encounter each other and bond formation can occur. Redistribution of LFA-1 to sites of cell–cell contact has been observed in some cases (34–37), though not always (38). Our data suggest that redistribution is a passive (i.e., diffusive) process.

Cytochalasin D is generally thought to be an inhibitor of LFA-1–dependent adhesion (4, 5). Disruption of the actin cytoskeleton certainly has effects on processes potentially involved in adhesion besides increasing the mobility of LFA-1, such as cell spreading and reinforcement of cell-substrate connections. In many cell types, however, some populations of actin filaments are more sensitive to the effects of cytochalasins than others (39–42). We therefore examined the effect of low doses of cytochalasin D, not large enough to cause total disruption of the actin cytoskeleton, for their effect on adhesion of MP cells to ICAM-1. At 500 molecules/μm^2, an ICAM-1 density at which activation is required for efficient adhesion (7), cytochalasin D doses < 3 μg/ml stimulated adhesion of MP cells to ICAM-1 (Fig. 4 *a*). The optimal cytochalasin D dose was between 0.1 and 0.3 μg/ml, leading to a greater than fourfold increase in adhesion. This stimulated adhesion was reversed by adhesion blocking antibodies to LFA-1 (Fig. 4 *a*). Cytochalasin D (0.3 μg/ml) was then compared to PMA for stimulation of adhesion to surfaces coated with varying ICAM-1 densities. Over a wide range of ICAM-1 densities, cytochalasin D was nearly as effective as PMA at stimulating adhesion (Fig. 4 *b*). Furthermore, the effect of cytochalasin D together with PMA was no greater than PMA alone. These results suggest that cytochalasin D activates adhesion by a mechanism similar to that of PMA. The data support the hypothesis that f-actin inhibits adhesion in unstimulated lymphocytes, and increased receptor lateral mobility is a critical event in LFA-1 avidity regulation.

The theoretical basis for diffusion-limited adhesion is not new (43, 44). Briefly, if the strength of adhesion depends on the number of bonds formed and this depends on the number of random encounters of receptor with ligand, the development of strong adhesion will depend on the lateral mobility of the receptor. Experimental evidence has confirmed these theoretical predictions in several models. Anti-DNP IgE in cell-surface Fcε receptors on RBL cells passively rearrange to form contacts with cell-size phospholipid vesicles containing a DNP-lipid hapten. This correlates with a time-dependent increase in specific adhesion (45). Futhermore, development of CD2-mediated adhesion of Jurkat cells to LFA-3 in an artificial lipid bilayer depends on the lateral mobility of the LFA-3 (46). In these models, however, regulation of receptor mobility was not addressed. Our data suggest a mechanism of lymphocyte adhesion activation involving release of receptors from cytoskeletal constraint to activate adhesion. Importantly, this suggests that maintenance of the low avidity state is an active process, requiring actin cytoskeleton. If lymphocyte-specific proteins are required for this cytoskeletal-constraint mechanism to maintain low avidity, this process may explain why transfection of LFA-1 into nonleukocytes results in unregulated LFA-1 mediated adhesion (47).

We propose that the increase in lateral mobility is an important early step in adhesion, accelerating receptor-ligand bond formation. Once ligand is bound, however, establishment of new connections to the cytoskeleton may be required, e.g., for structural support. This second step is likely to be inhibited by high doses of cytochasin D. This hypothesis might be best examined by using SPT to compare wild-type B cells to those expressing various cytoplasmic-tail-truncation mutant LFA-1 molecules.

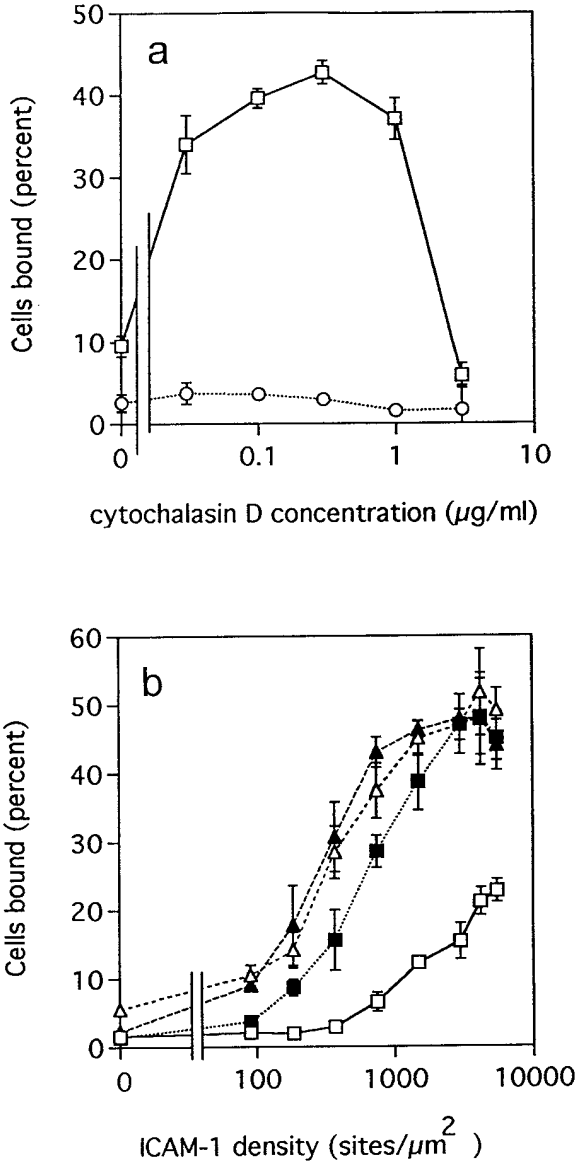

Figure 4. Cytochalasin D stimulates MP cell adhesion to ICAM-1 coated substrates. (*a*) Calcein-labeled MP cells were treated with the indicated concentration of cytochalasin D without (*square*) or with (*circle*) 10 μg/ml TS1/22 antibody for 15 min at 37°C in adhesion media and then added to wells coated with 500 molecules/μm^2 of purified ICAM-1. The plates were centrifuged, analyzed for fluorescence, incubated at 37°C for 10 min, washed four times, and reanalyzed for fluorescence. Pre and post wash fluorescence values were used to calculate cells bound in percent of input±standard deviation of 4 points. Data are representative of two experiments. (*b*) Calcein-labeled MP cells were pretreated without (*open symbols*) or with (*filled symbols*) cytochalasin D at 0.3 μg/ml for 15 min at 37°C in adhesion media and added to wells coated with the indicated densities of ICAM-1 without (*squares*) or with (*triangles*) 50 ng/ml PMA. The plates were processed as in *a.* Data are representative of three experiments.

Acknowledgments

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