Visualization of CD2 Interaction with LFA-3 and Determination of the Two-Dimensional Dissociation Constant for Adhesion Receptors in a Contact Area

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Abstract. Many adhesion receptors have high three-dimensional dissociation constants (Kd) for counter-receptors compared to the Kd's of receptors for soluble extracellular ligands such as cytokines and hormones. Interaction of the T lymphocyte adhesion receptor CD2 with its counter-receptor, LFA-3, has a high solution-phase Kd (16 μM at 37°C), yet the CD2/LFA-3 interaction serves as an effective adhesion mechanism. We have studied the interaction of CD2 with LFA-3 in the contact area between Jurkat T lymphoblasts and planar phospholipid bilayers containing purified, fluorescently labeled LFA-3. Redistribution and lateral mobility of LFA-3 were measured in contact areas as functions of the initial LFA-3 surface density and of time after contact of the cells with the bilayers. LFA-3 accumulated at sites of contact with a half-time of ~15 min, consistent with the previously determined kinetics of adhesion strengthening. The two-dimensional Kd for the CD2/LFA-3 interaction was 21 molecules/μm², which is lower than the surface densities of CD2 on T cells and LFA-3 on most target or stimulator cells. Thus, formation of CD2/LFA-3 complexes should be highly favored in physiological interactions. Comparison of the two-dimensional (membrane-bound) and three-dimensional (solution-phase) Kd's suggest that cell-cell contact favors CD2/LFA-3 interaction to a greater extent than that predicted by the three-dimensional Kd and the intermembrane distance at the site of contact. LFA-3 molecules in the contact site were capable of lateral diffusion in the plane of the phospholipid bilayer and did not appear to be irreversibly trapped in the contact area, consistent with a rapid off-rate. These data provide insights into the function of low affinity interactions in adhesion.

MULTICELLULAR organisms use cell contact and adhesion for both structural integrity and short range communication. In the immune system, adhesion of T cells to antigen presenting cells (APCs) is mediated by antigen receptors and adhesion receptors. The T cell antigen receptor and the accessory molecules CD4 and CD8 (cluster of differentiation antigens 4 and 8) bind directly to antigenic peptide: major histocompatibility complex (MHC) protein adducts (15, 24, 31). Parallel interactions involving CD2 with lymphocyte function-associated antigen 3 (LFA-3) and LFA-1 with ICAMs 1, 2, and 3 are required for stable cell–cell adhesion (14, 18, 40). CD2 and LFA-3 are members of the immunoglobulin superfamily (IgSF), each possessing two Fg-like domains, that bind to each other through an interaction involving their NH2-terminal Ig-like domains (1, 20, 32, 39). If the mode of interaction between CD2 and LFA-3 is similar to that employed by other adhesion molecules of the IgSF, then the CD2/LFA-3 interaction may serve as a prototype for adhesive interactions involved in neural cell adhesion, cell migration in development and tumor cell metastasis.

The CD2/LFA-3 interaction was the first heterophilic adhesion mechanism to be fully reconstituted (17, 37, 41). It was also the first heterophilic mechanism for which the solution-phase interaction was rigorously analyzed. The dissociation constant (Kd) and off-rate for the interaction...
between purified soluble forms of CD2 and LFA-3 are 1.6 \times 10^{-5} \text{ M} and greater than 4 \text{ s}^{-1}, respectively (45). It has been suggested that the high $K_d$ and fast off-rate of the CD2/LFA-3 interaction allows for cell–cell dissociation, which is essential for the migration of long-lived T lymphocytes (44).

Before the description of heterophilic adhesion mechanisms such as that involving CD2 and LFA-3, the three part interaction among cellular Fc receptors, hapten-specific antibody, and hapten-coated surfaces or liposomes provided an important model for molecular studies of adhesion. A classic study by McCloskey and Poo (26) showed that the interaction of a cell bearing fluorescently labeled anti-DNP antibody-IgFc receptor complexes with a liposome incorporating DNP-labeled phospholipids efficiently drove accumulation of antibody-Fc receptor complexes into the area of contact between the cell and the liposome. This “trapping” effect correlated with adhesion strengthening. Receptor accumulation did not require an active contribution from the cell, suggesting that it was driven by the extracellular binding interaction ($K_d = 10^{-8} \text{ M}$) between antibody and DNP. Although this study did demonstrate the importance of adhesion molecule trapping in contact areas as a general mechanism, it did not determine an upper limit for the $K_d$ of an interaction that could be mediated by this trapping or syn-capping effect. This is an important question, since the CD2/LFA-3 interaction functions in adhesion with a solution-phase $K_d$ of 16 \text{ M}.

Other studies showing redistribution of adhesion molecules to contact areas have not excluded (or have specifically invoked) interactions with the cytoskeleton as a contributing or necessary process in adhesion receptor accumulation (10, 11, 21, 22, 28, 30, 48). Therefore, such studies do not directly address the role of the $K_d$ of adhesion receptor interactions in receptor redistribution.

We have previously shown that a glycosylphosphatidylinositol anchored isoform of LFA-3 (GPI-LFA-3) is more potent than a transmembrane isoform in mediating adhesion of CD2+ T lymphoblasts to glass-supported planar bilayers reconstituted with LFA-3 (9). This difference in potency is related to the observation that transmembrane proteins are laterally immobile (9, 27), whereas phospholipids (9, 27) and GPI-anchored proteins (9) are laterally mobile, in glass-supported planar bilayers. One likely mechanism for the effect of lateral diffusion on adhesion involves an increase in the rate of productive collisions between laterally mobile LFA-3 and CD2. A second likely mechanism involves trapping of laterally mobile LFA-3 in the contact area, which increases the effective LFA-3 density. In the present study we have labeled purified GPI-LFA-3 with FITC, incorporated FITC-LFA-3 into glass-supported planar bilayers, and characterized changes in the surface distribution and diffusion coefficient of LFA-3 induced by interaction with CD2+ T lymphoblasts. We find that LFA-3 accumulates in contact areas with T lymphoblasts. Equilibrium binding analysis shows that the two-dimensional $K_d$ for the CD2/LFA-3 interaction is low relative to the normal surface densities of CD2 and LFA-3 in biological membranes, suggesting that binding approaches saturation in biological contact areas. Comparison between two- and three-dimensional $K_d$ for the CD2/LFA-3 interaction suggests that CD2 and LFA-3 are under topological constraints such as those described by Bell et al. (4) as a “confinement region”, or a thin subregion of volume within the contact area in which adhesion molecule binding sites are localized. Finally, we detect lateral diffusion of “bound” LFA-3 in the contact area, consistent with a rapid off-rate for the CD2/LFA-3 binding interaction.

**Materials and Methods**

**Monoclonal Antibodies and Cell Lines**

The monoclonal antibodies TS2/9 (anti-LFA-3) and TS2/18 (anti-CD2) were originally described in Sanchez-Madrid et al. (34). The Jurkat T lymphoma cell line was maintained in RPMI 1640 containing 10% fetal bovine serum, 5 mM glutamine and 50 \mu g/ml gentamicin. Jurkat cells in log phase of growth (6-9 \times 10^{6} \text{cells/ml}) were used for all experiments.

**Purification and Modification of LFA-3**

GPI-LFA-3 was affinity-purified from human erythrocytes, as previously described (17). To avoid modification of the CD2 binding surface on critical lyses, LFA-3 was bound to TS2/9-coupled Sepharose CL-4B before conjugation with FITC. Purified LFA-3 was added in a sufficient amount to saturate 1 ml of TS2/9 Sepharose CL-4B (2 mg/ml) in neutralized elution buffer, and the mixture was rotated at 4°C for 4–16 h. The Sepharose was washed extensively at 4°C in 0.1 M NaHCO$_3$/Na$_2$CO$_3$, pH 9.0, 0.05% Triton X-100, and excess buffer was removed. FITC was dissolved in dimethylformamide to a concentration of 80 mM, and the solution was diluted 1:9 in 1 ml of 0.1 M NaHCO$_3$/Na$_2$CO$_3$, pH 9.0, 0.05% Triton X-100 and added immediately to the LFA-3-Sepharose mixture. After 2 h of end over end rotation at room temperature the slurry was centrifuged, the supernatant was removed, a fresh preparation of 8 mM FITC in 0.1 M NaHCO$_3$/Na$_2$CO$_3$, pH 9.0, 0.05% Triton X-100 was added, and the room temperature incubation was repeated. The slurry was transferred to a column and washed with 50 volumes of 25 mM Tris, pH 8.6, 0.15 M NaCl, 1% octyl-\beta-d-glucopyranoside (OG). FITC-modified LFA-3 (FITC-LFA-3) was eluted with 50 mM glycine, pH 3.0, 0.15 M NaCl, 1% OG and collected in tubes containing 1 M Tris, pH 8.6, 1% OG.

**Preparation of Liposomes and Planar Bilayers**

Unilamellar liposomes were prepared by OG dialysis (7,29). Egg phosphatidylcholine (PC) (Avanti Polar Lipids, Inc., Pelham, AL) was dissolved at 0.4 mM in 25 mM Tris, pH 8.0, 0.15 M NaCl, 2% OG, and then mixed with an equal volume of 25 mM Tris, pH 8.0, 0.15 M NaCl, 2% OG containing different amounts of LFA-3 or FITC–LFA-3. Liposome suspensions were formed by dialysis and stored at 4°C under argon to minimize lipid oxidation.

Planar bilayers containing purified LFA-3 or FITC–LFA-3 were formed as previously described (17), with the following modifications. In some experiments, bilayers were formed from a mixture of liposomes containing FITC–LFA-3 and purified LFA-1 (16). A diamond pencil was used to place a shallow scratch in the center of clean coverslips to permit identification of the plane of the bilayer. In a 2:1 tank of 25 mM Hepes, pH 7.4, 147 mM NaCl, 5 mM glucose, 1% bovine serum albumin (HBS), the planar bilayer forming liposomes were transferred to glass slides that had been coated with 10-mm rings of vacuum grease (Dow-Corning, Corning, NY). A Jurkat cell suspension was pipetted within the grease ring just as the coverslip was slowly lowered and pressed into the grease to trap a small number of cells. The incubation was initiated when the system was inverted coverslip down. In some cases, CD2 molecules on Jurkat cells were blocked by preincubating cells with TS2/18 at 100 \mu g/ml for 30 min and washing three times with HBS. Cells were incubated with biotinylated antibodies at room temperature. LFA-3 site densities on planar bilayers were determined by radioimmunoassay, as previously described (9).

**Fluorescence Photobleaching Recovery**

Fluorescence photobleaching recovery (FPR) is used to measure the lateral mobility of fluorescently labeled proteins and lipids in membranes (2). Spot FPR was performed on a Meridian Instruments (Okemos, Michigan) ACAS 570 Interactive Laser Cytometer. Data were analyzed using a nonlinear least squares method (5) to yield both the lateral diffusion coe-
ficient and the fraction of fluorescently labeled molecules that were free
to diffuse on the time scale of the experiment (typically 45-90 s). The
ACAS 570 uses a computer-interfaced scanning stage to generate line
scans through the bleach site that are used to monitor non-bleached re-
gions of the bilayer as an internal control. Bleaching pulses were 30-100
ms in duration using a beam power of 200 µW. Measuring pulses were 4
µs in duration using a beam power of 100-225 µW. The 1/e² diameter of
the laser beam was measured as 1.3 ± 0.07 µm (mean ± SD, n = 6) using
a 40× 1.3 N.A. oil immersion objective. Non-FITC fluorescence was ex-
cluded from the emission detection system by using a 530 ± 15 nm band
pass filter. The fluorescence signal from unlabeled bilayers was less than
10% of that from labeled bilayers, and was subtracted from the total signal.

Image Analysis

Two-dimensional fluorescence images of FITC-LFA-3 distribution in egg
PC planar bilayers were obtained using the Meridian ACAS 570. Most im-
ages were taken as 90 × 90-µm fields using a step size of 0.5 µm. Bright
field images of Jurkat cells on bilayers were used to draw outlines of the
cells (i.e., cell profiles) on the fluorescence images. These outlines were
used to determine projected cell areas and to define areas of the bilayer
not in contact with cells. Projected cell area was defined as the area of a
disk in the horizontal plane through the widest part of the cell. Contact
sites were defined mathematically to include all pixels having fluorescence
values ≥120% of the average pixel intensity outside the cell outlines. The
average autofluorescence of Jurkat cells adherent to unlabeled LFA-3 in
egg PC planar bilayers was subtracted as a background. Average fluores-
cence intensities within areas of cell contact and non-cell contact were
converted to LFA-3 surface densities by setting the average fluorescence
intensity in multiple fields of FITC-LFA-3 equal to the surface density of
LFA-3 measured by iodinated antibody binding. When laser power and
photomultiplier tube gain were held constant, there was a linear relation-
ship between fluorescence intensity (arbitrary units) and LFA-3 site den-
sity determined by radioimmunoassay over the range used in these exper-
iments (data not shown).

Bound receptor densities were determined using conventions for equi-
librium dialysis (43). All data were corrected for background by subtrac-
tion of fluorescence signals from planar bilayers without FITC-LFA-3 or
from cells adherent to bilayers containing unlabeled LFA-3, as appropri-
ate. The density of free LFA-3 in the bilayer (i.e., of LFA-3 not interacting
with cells) was determined from the average LFA-3 density outside the
cell outlines. The density of bound LFA-3 in a contact site was defined as
the average LFA-3 density within the contact site minus the free LFA-3
density in the bilayer. The total number of bound LFA-3 molecules was
determined by integrating the density of bound LFA-3 over the contact
area.

Two-Dimensional Scatchard Analysis

LFA-3 surface densities, determined as described above, were plotted as
bound on the abscissa and bound/free on the ordinate. Linear least
squares analysis was used to determine the slope, which is proportional to
the negative reciprocal of the two-dimensional Kd (mol/µm²). Extrapol-
ation to the abscissa was used to determine the binding at saturation (mol/
µm² of contact area) (35).

Results

Redistribution of LFA-3 into Areas of Planar Bilayers
In Contact with Jurkat T Cells

GPI-LFA-3 was purified from human erythrocytes by immu-
oaffinity chromatography. FITC was conjugated to
LFA-3 bound to a Sepharose-coupled antibody that blocks
interaction with CD2, to protect the CD2-binding site
from modification by fluorophore. FITC–LFA-3 was in-
corporated into phospholipid liposomes which were then

Figure 1. Pseudocolor fluo-
rescence images of FITC-
LFA-3 distribution in glass-
supported planar bilayers.
(a) Uniform distribution
of FITC-LFA-3 (120 mol/
µm²) in a planar bilayer be-
fore interaction with cells.
(b) Distribution of FITC-
LFA-3 (initial density,
1,000 mol/µm²) in a planar
bilayer 30 min after incuba-
tion with Jurkat T cells. (c)
Distribution of FITC-
LFA-3 (initial density, 120
mol/µm²) in a planar bi-
layer 30 min after incuba-
tion with Jurkat T cells. (d)
Distribution of fluores-
cence in a planar bilayer
containing 120 mol/µm² of
FITC-LFA-3 and 500 mol/
µm² of unlabeled LFA-1 30
min after incubation with
TS2/18 pretreated Jurkat T
cells. The projections of at-
tached cells are outlined in
white. The pseudocolor
scale is marked in arbitrary
fluorescence units. Each
image represents 8,100 µm².

Dustin et al. Adhesion Receptor Affinity in a Contact Area
allowed to interact with glass coverslips to form planar phospholipid bilayers on the glass surface. Quantitative fluorescence imaging revealed a uniform distribution of FITC-LFA-3 in the planar bilayers (Fig. 1a).

Upon incubation of planar bilayers with CD2+ Jurkat T cells, FITC-LFA-3 redistributed into areas of contact with cells (Fig. 1b and c, see also Figs. 4 and 5). The following experiment demonstrated that FITC-LFA-3 redistribution required specific interaction with CD2 on T cells. Jurkat cells were allowed to adhere to planar bilayers formed by mixtures of liposomes containing FITC-LFA-3 and purified LFA-1 in the presence of Mg2+. LFA-1 dependent adhesion, mediated by binding of LFA-1 to ICAMs on the Jurkat cells, had no effect on FITC-LFA-3 redistribution into the contact areas. Blocking of CD2 molecules on the Jurkat cells with TS2/18, a mAb known to block interaction of CD2 with LFA-3, prevented redistribution of FITC-LFA-3 into the LFA-1-mediated contact areas (Fig. 1d).

**Kinetics of Contact Area Development**

Contact areas were defined operationally as areas of significantly increased LFA-3 accumulation. Fig. 2 shows the size of contact areas and the average density of bound LFA-3 within contact areas as a function of time after incubation of Jurkat cells with planar bilayers initially presenting 120 LFA-3 mol/μm². The mean size of the contact areas increased rapidly over the first 28 min of incubation and then leveled off at an average size of 98 μm². Likewise, the density of bound LFA-3 in the contact areas increased steadily over the first 31 min before leveling off at an average density of 330 mol/μm². Similar kinetics of contact area expansion were observed with bilayers initially presenting 120 (Fig. 2), 300, and 1,000 LFA-3 mol/μm² (not shown).

**Dependence of Bound LFA-3 Density on Cell Size**

Jurkat cells display size heterogeneity in log phase cultures. The effect of this heterogeneity on FITC-LFA-3 accumulation was assessed. FITC-LFA-3 fluorescence images were analyzed with respect to projected cell area obtained from matched bright-field images. Although larger cells formed larger contact areas within the planar bilayer, the average density of LFA-3 within these contact areas was the same as that in the contact areas formed by smaller cells (Fig. 3).

The spatial distribution of LFA-3 within contact areas was also analyzed. Most cells generated contact areas with a single, central area of concentrated ligand and decreasing ligand density toward the periphery (see Figs. 4 and 5). Peak fluorescence value within a contact area was defined as the greatest fluorescence value held by at least four adjacent pixels (i.e., by an area of at least 1 μm²). The maximum density of bound FITC-LFA-3 was 1,000 mol/μm².

**Lateral Diffusion of LFA-3 in Contact Areas**

Fluorescence photobleaching recovery was used to measure the fraction of FITC-LFA-3 that was free to diffuse in the planar bilayer (i.e., the fractional mobility) and the lateral diffusion coefficient of the mobile fraction (Figs. 4 and 5; Table I). Fig. 4 shows the time course of fluorescence recovery following photobleaching of FITC-LFA-3 in a contact area between a Jurkat T cell and a planar bilayer initially presenting 120 LFA-3 mol/μm². Fig. 5 shows prebleach, 0 s post-bleach and 90 s post-bleach line scans of FITC-LFA-3 fluorescence in contact areas between cells and bilayers initially presenting 120 (A, B), 300 (C, D) and 1,000 (E, F) LFA-3 mol/μm². The pre-bleach line scans vividly display the relationship between initial LFA-3 density in the bilayer and peak LFA-3 accumulation in contact areas. FITC-LFA-3 in planar bilayers reconsti-
Determination of Equilibrium Binding Parameters: Two-Dimensional Scatchard Analysis

The density and total number of bound and free FITC-LFA-3 molecules in contact areas between Jurkat cells and planar bilayers were analyzed by methods that are routinely applied to solution-phase equilibrium dialysis data. In the latter technique, receptors are restricted to a subregion by a semipermeable membrane, and small ligands capable of diffusion through the membrane undergo net concentration on the side of the membrane that contains a specific receptor (43). The adhesion experiment here was modeled as a two-dimensional version of the equilibrium dialysis experiment, in which CD2 and LFA-3 molecules were spatially restricted by the Jurkat cell plasma membrane and the planar bilayer, respectively. Table II summarizes the equilibrium surface density and total number of free FITC-LFA-3 molecules in non-contact regions, and of bound FITC-LFA-3 molecules in regions of cell-bilayer contact, for three initial FITC-LFA-3 densities. It should be noted that the three experimental FITC-LFA-3 surface densities span the physiological range in biological membranes. Data from Table II were transformed into a plot of bound LFA-3/free LFA-3 on the ordinate and bound LFA-3 on the abscissa (35) (Fig. 6). The three points fell on a line with a negative reciprocal slope ($K_d$) of 21 mol/μm$^2$ and an intercept on the abscissa ($B_{max}$) of 430 mol/μm$^2$. This analysis predicts that, since the density of CD2 on a T cell is on the order of 100 mol/μm$^2$, the CD2/LFA-3 interaction in biological membranes will be driven toward saturation upon establishment of cell–cell contact.

Discussion

We have used glass-supported planar phospholipid bilayers reconstituted with defined surface densities of laterally mobile FITC-LFA-3 to quantify the interaction between CD2 and LFA-3 in a model cell–cell contact area. Analysis of LFA-3 surface distribution and lateral mobility in the presence of CD2+ Jurkat T cells reveals time-dependent redistribution of LFA-3 into contact sites and slowing of LFA-3 diffusion. Redistribution of LFA-3 reaches steady-state in 30 min and is maintained for several hours. At low densities of GPI-LFA-3 in planar bilayers adhesion of Jurkat cells increases dramatically between 5 and 20 min of incubation (9), consistent with the time-course of LFA-3 accumulation observed here. At higher GPI–LFA-3 densities adhesion of Jurkat cells is stable at 5 min (9), although redistribution of LFA-3 into contact areas does not reach steady-state until 30 min of incubation, suggesting that formation of a sufficient number of CD2/LFA-3 complexes to stabilize the contacts occurs before the system reaches equilibrium. These results are in qualitative agreement with those of McCloskey and Poo (26), who found that adhesion molecule (antibody–hapten) redistribution is closely associated with adhesion strengthening. We extend the results of McCloskey and Poo (26) on a high-affinity antibody/hapten interaction (solution-phase $K_d = 10^{-8}$ M) to an interaction between adhesion molecules with a much higher solution-phase $K_d$ (1.6 × 10$^{-3}$ M) (44).

Our results indicate that CD2 in Jurkat cell membranes, like LFA-3 in the planar bilayers, also accumulates in con-
Figure 4. Accumulation and lateral mobility of FITC-LFA-3 in a region of contact with a Jurkat T cell. Jurkat cells were allowed to settle on a bilayer initially presenting 120 LFA-3 mol/μm² for 30 min at 24°C. The ACAS 570 was used to monitor fluorescence on a line passing through the center of a cell. The prebleach scan (green) shows FITC-LFA-3 accumulation in the contact area. The vertical line indicates the center of the bleaching spot, which was used to initiate a fluorescence photobleaching recovery experiment. Fluorescence recovery was monitored by line scans at 0 (orange), 1.5, 3, 4.5, 6, 7.5 (red), 9, 15, 21, 27, 33 (blue), 39, 45, 51, 57, 63 (dark red), 69, 75, 81, 87, and 93 (purple) seconds after the bleach. Diffusion coefficient, 1.0 × 10⁻⁹ cm²/s. Fractional mobility, 85%.

Contact areas. There are ~160,000 CD2 molecules on an average Jurkat T cell (33), and ~43,000 are engaged in an average contact area at steady-state. CD2 molecules are uniformly distributed on Jurkat cells in suspension (unpublished observations), yielding an initial CD2 surface density between 100 and 200 mol/μm². The maximum density of bound FITC-LFA-3 within a contact area, which should correspond exactly to the density of bound CD2 within the contact site, is 1,000 mol/μm². Thus, CD2 redistributes by a maximum of 5-10-fold in contact areas. Consistent with this observation, T cell CD2 is found to redistribute into areas of contact with cells expressing high levels of LFA-3 (30).

FITC-LFA-3 in a contact area manifests a reduced lateral diffusion coefficient compared to FITC-LFA-3 outside a contact area. Equilibrium binding of 83% of LFA-3 molecules to CD2 within a contact area causes the diffusion coefficient of LFA-3 to decrease from 5.9 × 10⁻⁹ cm²/s to 1.3 × 10⁻⁹ cm²/s. One explanation for this finding could be that CD2/LFA-3 complexes have a diffusion coefficient similar to that of CD2 in the Jurkat cell membrane (7.2 × 10⁻¹⁰ cm²/s) (23). The suggestion that adhesion receptor/counter-receptor complexes diffuse laterally is intriguing, but may be difficult to establish in this system due to the rapid off-rate (>4 s⁻¹) of the CD2/LFA-3 interaction measured in solution (45). Nearly complete fluorescence recovery is observed even when most of the accumulated FITC-LFA-3 in a contact area is bleached, suggesting that bleached FITC-LFA-3 is replaced by fresh FITC-LFA-3 from the bilayer outside the contact area. This result suggests that transient interactions (19) between CD2 and LFA-3, even in the confines of the contact area, are a more likely explanation for the observed lateral diffusion of LFA-3 than the coupled diffusion of long-lived CD2/LFA-3 complexes.

The binding interaction responsible for the accumulation of LFA-3 in contact areas was analyzed by methods used to analyze interactions between soluble ligands and receptors (35). We obtained a two-dimensional Kd of 21 mol/μm², although the lowest initial LFA-3 density in bilayers was 120 mol/μm². It was not possible to perform equilibrium experiments using planar bilayers initially presenting fewer than 120 mol/μm², because such experiments resulted in surface densities of free FITC-LFA-3 in non-contact regions that were below the fluorescence detection limits of the ACAS 570. Thus, it is not possible to conclude from the Scatchard analysis whether the CD2/LFA-3 interaction exhibits one or more than one class of binding sites. The region of the Scatchard plot sampled here spans the range of physiological LFA-3 surface densities, however, and should be relevant to CD2/LFA-3 interactions in biological membranes. Furthermore, the region of the Scatchard plot near the abscissa typically describes the lowest affinity (highest capacity) sites. Thus, if more than one class of sites is present, the experiments reported here should yield an upper limit for the two-dimensional Kd of the CD2/LFA-3 interaction. Because physiological surface densities of LFA-3 exceed our experimentally de-
Figure 5. Accumulation and lateral mobility of FITC–LFA-3 in regions of contact between Jurkat cells and planar bilayers containing different initial densities of FITC–LFA-3. Jurkat cells were allowed to settle on bilayers initially presenting 120 (A and B), 300 (C and D), or 1,000 (E and F) LFA-3 mol/μm² for 30-120 min at 24°C. Prebleach (green), 0 s post-bleach (blue) and 90 s post-bleach (red) scans are shown to indicate the extents of FITC–LFA-3 accumulation, FITC–LFA-3 bleaching and FITC–LFA-3 fluorescence recovery, respectively. To minimize photobleaching during the fluorescence recovery phase of the experiment, the laser power and photomultiplier tube gain were adjusted for each bilayer to yield an acceptable signal level using minimal monitor beam power. Therefore, fluorescence intensity levels are not directly comparable among the various bilayer samples.

Determined lower limit for the half-saturation point \( (K_0) \) of CD2/LFA-3 binding, the physiological CD2/LFA-3 system appears to operate near saturation once a contact area is formed.

The two-dimensional \( K_d \) measured here should take into account all active and passive contributions of the Jurkat cells to the CD2 affinity for LFA-3. Adhesion is thought to be regulated by a number of cellular processes, such as the association of adhesion receptors with the cytoskeleton and receptor clustering. It is not clear whether these active contributions modulate the two-dimensional \( K_d \), however. To compare the measured two- and three-dimensional \( K_d \) for the CD2/LFA-3 interaction, we make the conservative assumptions that the Ig-like domains of CD2 are conformationally fixed and that the soluble recombinant molecules used by van der Merwe and colleagues to assess the three-dimensional \( K_d \) (45) are identical in unitary interaction to the cell surface CD2 and

<table>
<thead>
<tr>
<th>Sample</th>
<th>Density*</th>
<th>f</th>
<th>D</th>
<th>B/Tot.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC–LFA-3 in egg PC, no cells present</td>
<td>1,000 and 300</td>
<td>72 ± 15</td>
<td>5.9 ± 2.9</td>
<td>—</td>
<td>38</td>
</tr>
<tr>
<td>FITC–LFA-3 in egg PC, Jurkat cells present</td>
<td>1,000</td>
<td>88 ± 10</td>
<td>2.8 ± 1.6</td>
<td>31%</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>84 ± 14</td>
<td>2.3 ± 1.5</td>
<td>63%</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>83 ± 13</td>
<td>1.3 ± 0.9</td>
<td>83%</td>
<td>63</td>
</tr>
</tbody>
</table>

*Initial surface density, FITC–LFA-3 mol/μm².
* Mobility data in the absence of cells were identical for bilayers containing LFA-3 at medium (300 μm⁻²) and high (1,000 μm⁻²) surface densities.
* Mean ± SD.
purified LFA-3 used in this study. We also assume that clustering of CD2 on the T cell does not alter the two-dimensional $K_d$, although such clustering may alter adhesion properties (47). Our analysis therefore considers, as a first approximation, only geometric properties of the contact area.

Bell and colleagues have suggested on theoretical grounds that the binding sites of adhesion molecules in a contact area may be limited to a "confinement region" (3, 4). While this is an intriguing possibility, the concept has not been experimentally tested. The height of the confinement region ($\sigma$) can be calculated from the following relationship (4):

$$\sigma (\mu m) = \left( \frac{K_{d,x,y} (mol/\mu m^2)}{10^{15} \mu m^2/dm^3} \right) + \left( \frac{K_{d,x,y,z} (mol/dm^3) \times N_A (mol/mol)}{1} \right)$$

Table II. Redistribution of FITC–LFA–3 into Regions of Contact between Planar Bilayers and Jurkat T Cells

<table>
<thead>
<tr>
<th>Initial LFA-3 surface density*</th>
<th>Equilibrium surface density of LFA-3 in non-contact regions N</th>
<th>Equilibrium surface density of bound LFA-3 in contact regions</th>
<th>Equilibrium number of bound LFA-3 molecules per cell $\times 10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>$950 \pm 149^f$</td>
<td>$430 \pm 94$</td>
<td>$B + F/4$</td>
</tr>
<tr>
<td>300</td>
<td>$250 \pm 31$</td>
<td>$390 \pm 183$</td>
<td>$2.6$</td>
</tr>
<tr>
<td>120</td>
<td>$68 \pm 15$</td>
<td>$330 \pm 135$</td>
<td>$5.9$</td>
</tr>
</tbody>
</table>

Data include all measurements performed on membranes incubated with cells for at least 1 h (bilayers initially containing LFA-3 at 120 mol/μm² and 1,000 mol/μm²) or at least 30 min (bilayers initially containing 300 LFA-3 mol/μm²). $N$, number of planar bilayers. n, Number of cells.

* FITC–LFA–3 mol/μm².
* Bound LFA-3 mol/μm² in correct areas.
* Accumulation of LFA-3 in contact areas defined as the total (bound plus free) density of LFA-3 in a contact area divided by the density of LFA-3 in areas of membrane not in contact with cells.
* Bound LFA-3 molecules per cell, $\times 10^{-4}$.
* Mean ± SD.

where $K_{d,x,y}$ is the two-dimensional $K_d$, $K_{d,x,y,z}$ is the three-dimensional $K_d$, $N_A$ is Avogadro's number and $1 \times 10^{15} = 1$. Using the two-dimensional $K_d$ of 21 mol/μm² and the three-dimensional $K_d$ of 6 μM (13) (where both $K_d$s are measured at room temperature), the calculated size of the confinement region for the CD2/LFA-3 interaction is 5.8 nm. Our data provide strong support for the confinement region concept. If LFA-3 molecules were separated in the direction normal to the membrane by the same average distance as in the plane of the membrane, then the average separation distance would be 220 nm rather than 5.8 nm. Thus, confinement generates an effective increase in three-dimensional affinity of 220 nm/5.8 nm, or 38-fold. Conversely, the average spacing of molecules at the three-dimensional $K_d$ of 6 μM predicts a two-dimensional $K_d$ of 230 mol/μm². The effective increase in two-dimensional affinity due to confinement is therefore 230 mol/μm²/21 mol/μm², or 11-fold. Note that the 38-fold increase in three-dimensional affinity is related to the 11-fold increase in two-dimensional affinity by the geometric relationship $38^{2/3} = 11$. The decrease in two-dimensional $K_d$, generated by limitation of the CD2/LFA-3 interaction to a confinement region, appears to be important in maximizing binding between CD2 and LFA-3 at physiologic densities on cell surfaces. Since cells with a high surface density of CD2 and LFA-3 express on the order of 100 mol/μm² (9, 25, 33, 37), and surface densities of LFA-3 can range considerably lower than this value (38), loss of the effect of confinement would result, at equilibrium, in a significant reduction in bond formation and a consequent reduction in adhesion strength.

Our observations may also provide a physical basis for the finding that CD2 and LFA-3 are often coexpressed on the same cell, yet do not appear to interact in cis, i.e., in the plane of the same membrane (17,37). The crystal structure of CD2 suggests that bilayers interacting through CD2/LFA-3 bonds are separated by $\sim$15 nm (29,46). The smaller size of the confinement region for the CD2/LFA-3 interaction (i.e., 5.8 nm) could suggest that CD2 and LFA-3 are topologically constrained so that the angle formed between the long axis of either molecule and a line perpendicular to the bilayer is limited to values that allow the binding surfaces to remain in the confinement region. If each molecule is constrained to a confinement region of $\sigma$ (5.8 nm), the long axis of each molecule has length $L$ (7.5 nm for CD2), and the two molecules interact head-to-head through their NH₂-terminal Ig-like domains, then the an-

Figure 6. Scatchard analysis of binding between CD2 in Jurkat cell membranes and FITC-LFA-3 in planar bilayers. Data from Table II were transformed to bound LFA-3 and bound LFA-3/ free LFA-3 and plotted as described in Materials and Methods. $K_d = 21 \pm 4$ mol/μm². $B_{max} = 430 \pm 10$ mol/μm². Errors were determined by using a linear regression program (Regression, version M1.23; Blackwell Scientific Publications, Oxford, UK).
ngle of confinement (θ) can be calculated using the relationship:

\[ \theta = \cos^{-1} \left( \frac{L - (\sigma + 2)}{L} \right) \]

For CD2 and LFA-3 the calculated angle of confinement is ~52°. The mechanism by which the motion of CD2 or LFA-3 would be confined to the cone defined by this angle is unknown; it could, for example, involve interaction of the membrane proximal domain with the bilayer or of the entire molecule with the glycoalkal and neighboring glycoproteins. Of note, the stalk region of six amino acid residues that joins the COOH-terminus of IgSF domain 2 to the transmembrane segment is highly conserved among CD2 molecules in different species, suggesting that this region has an important function (42). The crystal structure of CD2 suggests that a confinement angle >65° would be required for cis interactions to occur (20). Thus, our findings may provide a physical explanation consistent with the observation that cis, non-adhesion promoting, interactions do not compete with trans, adhesion promoting, interactions. Further, CD2 and LFA-3 are homologous (36), are closely linked genetically (50), interact through homologous binding surfaces (46), and are postulated to have evolved from a common ancestor that interacted as a homodimer through cis interactions (49). Prevention of cis interactions of the common ancestor by topological constraints, as manifested in the confinement angle, could have been critical in permitting the evolution of adhesive trans interactions, in analogy to the CD2:CD2 interaction seen in the CD2 crystal structure (20). We note that the calculated confinement distance of 5.8 nm and the predicted membrane separation of 15 nm differ by only 2.6-fold. If either the two-dimensional \( K_d \) measured here or the three-dimensional \( K_d \) measured by Davis et al. (13) is in error by this amount, then the argument for a confinement angle would disappear.

The notion of a confinement region for adhesion receptors has implications for adhesive interactions mediated by receptors of similar or dissimilar size. The CD2/LFA-3 trans interaction occurs naturally along side other adhesion receptor interactions, including those involving LFA-1/ICAM-1 and antigen receptors such as the T cell receptor (TCR) and CD16 (a low-affinity IgG Fc receptor). It has been pointed out (40, 46) that the molecular dimensions of CD2 and LFA-3 (20, 46) are similar to those predicted for the TCR (12) and MHC proteins (6, 8), suggesting that CD2 and LFA-3 may be effective in establishing a contact environment in which the TCR can function optimally. In contrast, LFA-1 and ICAM-1 appear capable of mediating adhesion with a larger intercellular distance than that preferred by the CD2/LFA-3 or TCR/MHC protein interaction. Contact areas involving LFA-1 and ICAM-1 could be physically segregated (i.e., laterally separated) from areas containing CD2/LFA-3 and TCR/MHC proteins, allowing each system to maintain an optimal cell–cell separation distance and confinement region. Adhesion receptors with strikingly different geometries have been described: adhesion may be regulated in some systems by changes in intercellular distance (44).

We have described a novel approach to the molecular analysis of adhesion molecule interactions in a contact area. The results allow calculation of a two-dimensional \( K_d \) that relates directly to the natural function of these molecules in adhesion. Our findings demonstrate that a three-dimensional \( K_d \) must be interpreted in the context of additional topological constraints on adhesion molecules in a membrane environment to determine the relationship between two-dimensional and three-dimensional \( K_d \)s. In fact, both the two- and three-dimensional \( K_d \)s must be known to characterize fully the interaction of adhesion molecules.

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