T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1

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Effective interaction between T cells and their targets requires that recognition of specific antigen be coordinated with increased cell–cell adhesion. We show that antigen-receptor cross-linking increases the strength of the adhesion mechanism between lymphocyte function-associated molecule-1 (LFA-1) and intercellular adhesion molecules (ICAMs), with intracellular signals transmitted from the T-cell antigen receptor to the LFA-1 adhesion molecule. The increase in avidity is rapid and transient, providing a dynamic mechanism for antigen-specific regulation of lymphocyte adhesion and de-adhesion.

T-CELL immune recognition requires adhesion receptors as well as the T-cell receptor (TCR), as shown by the ability of monoclonal antibodies directed against these structures to inhibit antigen-specific responses[1]. T-cell adhesion receptors include LFA-1, which binds to its counter receptors ICAM-1 and ICAM-2 (refs 2–4), and CD2, which binds to its counter receptor LFA-3 (ref. 5). LFA-1 is a member of the integrin family, a group of extracellular matrix and cell-adhesion receptors which integrate the extracellular environment with the cytoskeleton[6–8]. The other molecules, ICAM-1, ICAM-2, CD2 and LFA-3 are members of the immunoglobulin superfamily[9]. One unresolved question is how the TCR and adhesion receptors cooperate to balance the competing needs for sensitive antigen recognition and stable cell–cell adhesion. At one extreme, requiring TCR interaction with antigen to contribute the antigen-specific adhesive component, would need a large number of receptor–ligand interactions, lowering the sensitivity of antigen recognition. At the other extreme, strong adhesion of T cells to other cells regardless of antigen expression would lower the efficiency of immune surveillance, as T cells would spend long periods of time in non-productive interactions.

Early studies demonstrated that cytolytic T lymphocytes (CTL) elicited *in vivo* adhered to target cells bearing antigen but not to cells lacking antigen, implying that adhesion was antigen-specific and, by inference, solely due to the TCR[10,11]. With the discovery of adhesion receptors it was initially proposed that these molecules contributed in an additive manner to TCR-specific interactions, so that antigen-specific adhesion could be maintained[12,13], or that they might be involved in an adhesion strengthening step triggered by the TCR[1,14,15]. No evidence for TCR regulation was presented, however, and subsequent studies with CTL lines and clones maintained *in vitro* showed that adhesion was virtually equivalent with both antigen-positive and antigen-negative targets[16,17]. It was proposed that adhesion-molecule interactions preceded TCR binding to antigen, and that the TCR was important only for triggering T-cell effector function[16]; this theory has been widely accepted[18].

Interactions between T lymphocytes and antigen-bearing cells must be reversible. This can be inferred from the existence of circulating T lymphocytes that have previously encountered antigen (memory T lymphocytes) and from studies on CTL. CTL can be observed engaging in repeated cycles of adhesion to target cells, lethal hit delivery and de-adhesion, with a cycle time as short as 15 min[11,14,19]. The mechanism for regulating adhesion and de-adhesion cycles is completely unknown.

We present evidence that LFA-1 function is regulated by phorbol esters and the TCR. Activation of lymphocytes by the TCR rapidly converts LFA-1 to a high-avidity state. TCR engagement thus triggers an adhesion amplification mechanism, allowing antigen-specific adhesion to be driven by a metabolic energy-dependent increase in LFA-1 avidity. The TCR and LFA-1 are coupled by intracellular signalling pathways, as shown by inhibition with dibutyryl cyclic AMP, agents that increase cytosolic cAMP, and the protein kinase inhibitor, staurosporine. The high avidity state of LFA-1 is transient; it peaks 5 to 10 min after TCR stimulation and returns to the low-avidity state by 30 min–2 h, providing a mechanism for de-adhesion.

TABLE 1 Blocking of CD3-stimulated adhesion with monoclonal antibodies

Monoclonal antibody against:	OKT3	OKT3 + anti-IgG2a
	^{51}Cr-labelled resting T-cell binding	
Negative control (X63)	5.0 ± 0.4	56.0 ± 0.1
LFA-1 α (TS1/22)	1.0 ± 0.2	1.0 ± 0.2
LFA-1 β (TS1/18)	0.5 ± 0.1	0.6 ± 0.03
LFA-1 α (TS2/4)	6.8 ± 0.6	57.3 ± 5.5
ICAM-1 (RR1/1)	2.0 ± 0.2	1.5 ± 0.2
CD2 (TS2/18)	5.7 ± 0.3	61.2 ± 5.8

Resting T cells were pretreated with OKT3 (IgG2a; 1:10 culture supernatant) as described in Fig. 1 and the indicated antibody, except RR1/1 which was used to pretreat the plates, for 30 min at 4 °C. All antibodies were washed out. Cells were added to wells with or without 2 μg ml^{-1} horse anti-mouse IgG2a. All antibodies except OKT3 are IgG1 so only the anti-CD3 antibody was cross-linked. ICAM-1 density was 1,000 sites μm^{-2} by ^{125}I-labelled RR1/1 binding. Methods were otherwise identical to Fig. 1. Monoclonal antibodies, as described in Fig. 1. Data shown are representative of two experiments.

Phorbol esters and LFA-1/ICAM-1 adhesion

The first experimental evidence for active regulation of the LFA-1/ICAM-1 adhesion mechanism was the ability of phorbol esters to stimulate LFA-1-and ICAM-1-mediated leukocyte homotypic adhesion[15,20,21]. Adhesion is stimulated rapidly, within one hour, and is not accompanied by any change in cell-surface density of LFA-1 or ICAM-1 (refs 15, 20, 21). It was shown subsequently that LFA-1-mediated adhesion of murine T-cell clones to antigen-negative targets could be enhanced twofold by phorbol 12-myristate-13-acetate (PMA) treatment[22]. Regulation by phorbol esters of LFA-1 avidity, however, could not be distinguished from regulation of ICAM-1 avidity or from a change in some general cellular property such as membrane spreading.

Binding of cells to purified adhesion molecules on inert surfaces is an ideal system to study regulation of adhesion mechanisms. In this situation, only the cell-surface receptor for the purified adhesion molecule is involved in binding, allowing regulation of its avidity (multivalent affinity) to be measured in

isolation from other adhesion mechanisms. The coexistence of ICAMs and LFA-1 on a number of different cell types allowed us to reciprocally assay binding of the same cells to both purified LFA-1 and ICAM-1 on artificial substrates, and so to determine which cell-surface molecule is affected by cellular activation. The hypothesis that PMA stimulates adhesion by increasing the avidity either of cellular LFA-1 or of cellular ICAM-1 predicts that regulation shows sidedness, that is, adhesion to artificial substrates containing purified ICAM-1 or LFA-1 should be differentially affected. Demonstration of sidedness would rule out a general adhesion-promoting effect of phorbol esters, as this should increase adhesion to LFA-1 and ICAM-1 substrates equally. Two distinct LFA-1 counter receptors have been defined, ICAM-1 and ICAM-2. The latter was recently defined by functional complementary DNA cloning[4]. The two immunoglobulin-like domains of ICAM-2 are 35% identical to the two domains closest to the N-terminus of the five immunoglobulin-like domains of ICAM-1. For generality, we have used cells expressing ICAM-1 (JY) as well as those that bind to LFA-1 by an ICAM-1-independent mechanism, which is likely to be through ICAM-2 (SKW3 and resting T cells). Binding of cells to artificial substrates was for six minutes under conditions established to prevent cell-cell aggregation (Fig. 1 legend). Basal binding of JY and SKW3 to ICAM-1 substrates was relatively inefficient over a wide range of ICAM-1 densities (Fig. 1*a*). Adhesion to ICAM-1 was dramatically increased by PMA, however, over a wide range of ICAM-1 densities. By contrast, SKW3 and JY cells adhered to the same extent to LFA-1 substrates in the presence and absence of PMA, although PMA shifted the density of LFA-1 required for half-maximal adhesion by twofold (Fig. 1*b*). Spreading of SKW3 and JY cells on LFA-1 (data not shown) was increased by PMA, which could be the basis of the increased resistance to washing at intermediate LFA-1 densities. Sidedness was even more dramatically illustrated with freshly isolated peripheral blood T lymphocytes. Adhesion to ICAM-1 substrates was almost completely dependent on phorbol ester stimulation (Fig. 1*e*), whereas adhesion to LFA-1 substrates was constitutive and was hardly affected by PMA (Fig. 1*f*). Thus, there is little or no avidity regulation of cell-surface ICAMs and these molecules appear constitutively avid for LFA-1. By contrast, cell-surface LFA-1 is not constitutively avid for ICAM-1, and seems to be converted to a high-avidity state after PMA stimulation.

Temperature effect on LFA-1/ICAM-1 interaction

The LFA-1/ICAM adhesion mechanism is susceptible to inhibition at low temperature (4 °C) in cell-cell binding[14,15] and binding of cells to purified ICAM-1 (ref. 2). This property is relevant

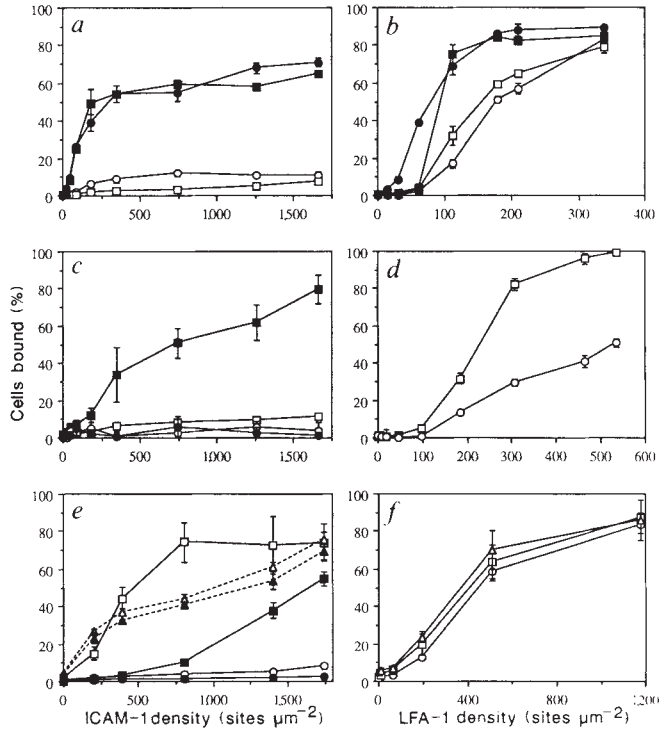

FIG. 1 Effects of activation on binding of cells to purified LFA-1 and ICAM-1. *a*, Binding of JY (circles) and SKW3 (squares) to ICAM-1 adsorbed to plastic without (open) or with (filled) PMA (50 ng ml^{-1}). *b*, same as *a*, binding to LFA-1 adsorbed to plastic. *c*, Binding of JY cells to ICAM-1 at 37 °C (squares) or 4 °C (circles). Cells were treated with PMA (50 ng ml^{-1}) (filled) or with media (open) for 30 min at 37 °C before dispersal and assay. *d*, Binding of JY cells to LFA-1 at 37 °C (squares) or 4 °C (circles). *e*, Binding of resting T cells to ICAM-1; cells pretreated with CD3 monoclonal antibody and then without (open circle) or with (open square) goat anti-mouse IgG, or with PMA (open triangle). Cells treated as above but also pretreated with 2 mM dibutyryl cAMP for 15 min at 24 °C before addition to wells are shown with filled symbols rather than open symbols. *f*, Same symbols as *e*, binding to LFA-1. These data are representative of at least 3 experiments for each group *a–b*; *c–d*, and *e–f*.

METHODS. LFA-1 and ICAM-1 were immunoaffinity purified from JY cell lysates using TS2/4 and RR1/1 sepharose, respectively. ICAM-1 was eluted at pH 12.5 (ref. 2). LFA-1 was eluted at pH 11.5 in the presence of 2 mM $MgCl_2$ (M.L.D. and T.A.S., manuscript in preparation). Purified proteins were at 20–100 μg ml^{-1} in buffered saline with 1% octyglucoside (OG) detergent (+2 mM $MgCl_2$ for LFA-1). Purified LFA-1 and ICAM-1 in 1% OG were adsorbed to polystyrene microtitre plate (Flow, Virginia) wells by addition of 5 μl of the detergent solubilized protein to 45 μl of 25 mM Tris, pH 8.0, 0.15 M NaCl, 2 mM $MgCl_2$ (TSM). After 16 h incubation at 4 °C the plates were incubated for 1 h at room temperature in 1% BSA and TSM, and were then washed with assay media. Site numbers were quantified with ^{125}I-labelled RR1/1 or TS2/4 for ICAM-1 or LFA-1, respectively, at 10 μCi μg^{-1} at a final antibody concentration of 10 μg ml^{-1}. JY was pretreated with TS1/22 (anti-LFA-1 α) for binding to LFA-1, and RR1/1 (anti-ICAM-1) for binding to ICAM-1, and free mAb was completely washed out before the assay. This was the best way to prevent PMA-stimulated JY cell aggregation and did not effect binding to the adsorbed proteins. Similar results were obtained without the antibody pretreatment, but PMA-stimulated cells contained many small aggregates (2–10 cells) especially on ICAM-1 substrates. Other cells gave no homotypic aggregation within the 6-min assay period. Resting T cells were isolated from whole blood by plastic adherence and nylon wool filtration and were 97% CD2$^+$, 93% CD3$^+$ and 96% CD6$^+$. Resting T cells were used within 24 h of drawing blood. T cells were pretreated with anti-CD3 monoclonal antibody (Leu4 ascites at 1:500 or OKT3 culture supernatant at 1:10 as indicated) for 30 min at 4 °C and then washed 3 times at 4 °C. In all assays ^{51}Cr labelled cells (1–2 × 10^4 JY or SKW3, or 5 × 10^4 resting T cells) were added to wells which contained 50 ng ml^{-1} PMA, 2 μg ml^{-1} goat anti-mouse IgG or neither as indicated above and centrifuged at 10 × g for 5 min at 24 °C (or 4 °C for *c*&*d*). In temperature effect experiments similar results were obtained if cells were allowed to settle at 1 × g for 60 min at 4 °C (not shown). Plates were then incubated for 6 min at 37 °C (or 4 °C in *c*&*d*). Unbound cells were removed by washing, and bound cells removed for γ-counting by incubation for 10 min at 37 °C in medium with 10 mM EDTA. Visual inspection of the number of bound cells per well gave the same results as γ-counting. In *a–d*, unbound cells were removed by four complete aspirations of media at assay temperature through an 18 ga. needle as described[49]. In *e* and *f*, unbound cells were removed by flicking media from the plates 8 times with 100 μl added between each wash. Flicking was more effective for thoroughly removing unbound resting T cells which were more difficult to remove due to their small size and it was also quicker, allowing more careful kinetic analysis. Both the aspiration and flicking wash protocols result in higher shear forces than techniques used in earlier studies with cells adhering to ICAM-1 in liposomes or planar membranes[2,49,50]. These differences result in lower percent binding of unstimulated JY cells observed here. Mab used in this study were TS2/4 (native LFA-1 α, IgG1)[51], TS1/18 (native LFA-1 β, IgG1)[51], TS1/22 (LFA-1 α, IgG1)[51], RR1/1 (ICAM-1, IgG1)[21], R6.5 (ICAM-1, IgG2a)[52], CL203 (ICAM-1, IgG1)[53], TS2/9 (LFA-3, IgG1)[51], OKT3 (CD3, IgG2a[54] Ortho Pharmaceuticals, Raritan, New Jersey) and Leu4 (CD3, IgG1)[55] (a gift from R. Evans), 235 (ref. 29) and 38-1 (ref. 25) (CD3 IgMs) were gifts from Drs S. M. Fu and J. Ledbetter, respectively. Anti-CD6 IgM[56] and anti-CD6 IgG2a (JOR-T1, Dr Amador, Stockholm) were obtained from the T-cell panel of the Fourth International Leukocyte Workshop.

to understanding regulation of this adhesion mechanism as it suggests a requirement for membrane fluidity or metabolic energy. As previously demonstrated, binding of JY cells to ICAM-1 was abolished at 4 °C (ref. 2); absence of adhesion was also observed when cells were pretreated with PMA for 30 min at 37 °C before testing adhesion at 4 °C (Fig. 1*c*). Dose-dependent adhesion of JY cells to purified LFA-1 was observed at both 37 °C and 4 °C, although binding at 4 °C was less efficient (Fig. 1*d*). The temperature effect on adhesion to purified ICAM-1 and LFA-1 also shows sidedness, therefore, with binding through cell-surface LFA-1 showing greater sensitivity than adhesion through cell-surface ICAMs.

Definitive evidence for LFA-1/ICAM-1 interaction was obtained by showing that purified LFA-1 protein micelles bind to ICAM-1 on plastic (Fig. 2). Specificity was demonstrated by lack of binding to purified LFA-3, inhibition with EDTA, and inhibition with anti-LFA-1 (TS1/22) and anti-ICAM-1 (RR1/1) monoclonal antibodies, which block cell-cell adhesion. Other antibodies that bind equally well to LFA-1 (TS2/4) or ICAM-1 (CL203) but do not block cell-cell adhesion had no effect. Also, identical results were obtained at 4 °C and 37 °C and thus binding of the purified molecules to each other is not temperature-dependent. Temperature therefore seems to be important for cellular processes that affect the avidity of LFA-1, but not for the function of cell-surface ICAMs, purified LFA-1 or purified ICAM-1.

TCR regulation of LFA-1 avidity

To determine whether regulation of the avidity of LFA-1 was physiologically relevant to antigen-specific T lymphocyte cell-cell interactions, we tested whether it was stimulated by TCR ligation. TCR stimulation triggers phosphatidylinositol turnover and elevates cytoplasmic Ca^{2+} (ref. 23). We studied resting T lymphocytes, rather than cloned T-cell lines, as the latter are already activated by antigen and by maintenance in culture and may be difficult to obtain as a homogeneous resting population. Because resting peripheral blood T cells vary in TCR specificity, bearing different $\alpha\beta$ or $\gamma\delta$ TCR subunits quantitatively associated with the invariant CD3 subunits, an effective way of stimulating them is with a monoclonal antibody against the CD3 component of the TCR complex. Resting T cells were incubated with anti-CD3, washed, and then incubated with a cross-linking anti-IgG, which is required to stimulate hydrolysis of phosphatidyl inositol and Ca^{2+} mobilization[24,25]. Anti-CD3 IgG alone has no effect on T-cell adhesion. Addition of anti-IgG, however, stimulated a significant increase in T-cell adhesion to ICAM-1 substrates, even more than with PMA (Fig. 1*e*). By contrast, there was no effect on adhesion to LFA-1 substrates (Fig. 1*f*). The CD6 antigen, present on T cells in similar amounts as the TCR, was used as a control for nonspecific effects. Cross-linking with an anti-CD6 IgG and anti-IgG did not result in an increase in adhesion (see Fig. 3*a*). The cross-linking anti-IgG could be intact IgG or Fab'_2 (not shown). The TCR-stimulated adhesion to purified ICAM-1 was completely blocked by monoclonal antibodies against LFA-1 α and β subunits and ICAM-1, which have previously been shown to block cell-cell adhesion (Table 1). Antibody TS2/4, which binds to LFA-1 but shows little inhibition of CTL killing[26], and anti-CD2 antibody, however, were not inhibitory.

A remarkable feature of T-lymphocyte interactions with other cells is the rapid progression from strong adhesion to de-adhesion. If the high-avidity state of LFA-1 were reversible, it could provide a mechanism for the unexplained phenomenon of adhesion and de-adhesion cycles. To address this question, we examined the kinetics of TCR-stimulated changes in the LFA-1 avidity state. Remarkably, the high-avidity state of LFA-1 stimulated by TCR cross-linking with anti-CD3 IgG and anti-IgG peaked at 10 min, with complete return to the low-avidity state by 30 min (Fig. 3*a*). By contrast, PMA-stimulated adhesion was maximal at 10 min and remained elevated for at least one hour. Purified T cells show a bimodal expression of LFA-1, as previously reported[27,28], with the two populations differing 2.5-fold in the number of LFA-1 binding sites per cell (Fig. 4). There was no significant change in LFA-1 expression on T cells pretreated with anti-CD3 IgG at 5 or 30 min after addition of anti-IgG. Thus, quantitative changes in LFA-1 surface expression are not involved in regulating either the induced binding at 5 min, or the decreased binding seen at 30 min. Also, addition of PMA to resting T cells that were stimulated by TCR cross-linking for 30 min restored high levels of adhesion within

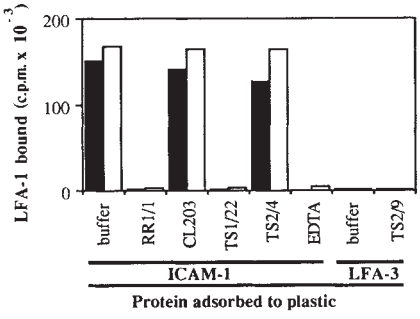

FIG. 2 Binding of LFA-1 protein micelles to ICAM-1 coated plastic. Binding was performed in the presence of the indicated additions at 4 °C (solid bars) or 37 °C (open bars).

METHODS. LFA-1 micelles were prepared by concentrating 2 ml fractions in 1% OG to 50 µl, resuspending in PBS and repeating the concentration step twice to remove residual detergent[57]. Protein concentration could then be determined by Coomassie blue dye binding[57]. LFA-1 micelles were iodinated with Iodogen (Pierce, Rockford, Illinois) to a specific activity of 10 µCi µg^{-1} (ref. 57). ^{125}I-labelled LFA-1 micelles (1 µg ml^{-1}) were incubated in microwells with 50 fmol ICAM-1 per well in 50 µl final volume of Hanks balanced salt solution, 10 mM HEPES, 2 mM $MgCl_2$. LFA-3 (ref. 57) and ICAM-1 were adsorbed to plastic as in Fig. 1. Wells were preincubated with purified antibody at 100 µg ml^{-1} prior to adding LFA-1. After 2 h at 37 °C or 4 °C the wells were washed 6 times with assay media, bound LFA-1 was released with 0.1 M NaOH and subjected to gamma particle counting. Data are representative of two experiments. Monoclonal antibodies, as described in Fig. 1.

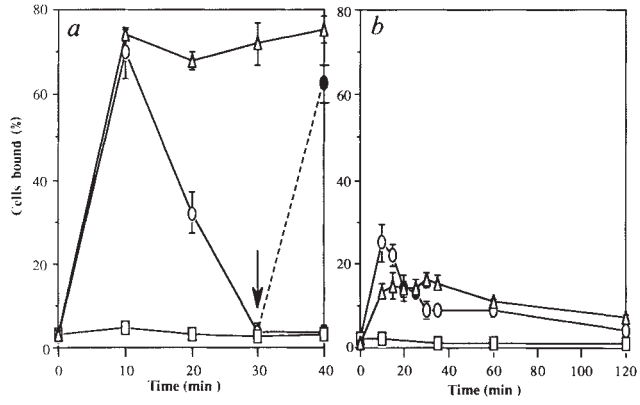

FIG. 3 Kinetics of TCR-stimulated increase in LFA-1 avidity. *a*, Resting T cells were treated with 50 ng ml^{-1} PMA (open triangles) or monoclonal antibody pretreated resting T cells were treated with 1 µg ml^{-1} goat anti-mouse IgG for varying time in suspension before transfer into wells and centrifugation onto an ICAM-1 coated surface. Pretreatment was with 1:500 anti-Leu4/CD3 ascites (open circles) or 1:500 JOR-T1 anti-CD6 ascites (open squares). In one case (arrow) T cells treated with anti-CD3 plus anti-IgG for 30 min at 37 °C were added to wells containing a final concentration of 50 ng ml^{-1} PMA (filled circle). Data is representative of 4 experiments. *b*, as in *a*, except resting T cells were treated with 235 anti-CD3 IgM at 10 µg ml^{-1} (open circles) or 1 µg ml^{-1} (open triangles) or anti-CD6 IgM at 1:500 ascites (open squares). CD6 staining was equivalent to 44% saturation with anti-CD3 monoclonal antibody. These data are representative of two experiments. The indicated time includes the 5 min centrifugation period.

10 min (Fig. 3*a*). This demonstrates that LFA-1 and the adhesion-promoting machinery are still functionally intact after the avidity decrease seen 30 min after TCR cross-linking. In addition, the experimental demonstration of 1.5 cycles of LFA-1 avidity regulation within 40 min indicates that the mechanism is truly capable of cycling, and in a rapid manner. Increased adhesion was also stimulated by anti-CD3 IgM without any need for further cross-linking (Fig. 3*b*), which is consistent with the ability of this anti-CD3 IgM to induce Ca^{2+} flux and phosphatidylinositol turnover in resting T cells[25,29]. The kinetics of the IgM-stimulated adhesion were concentration-dependent. With 10 $\mu g\ ml^{-1}$ anti-CD3 IgM (80% saturation after 30 min at 4 °C, as determined by immunofluorescence flow cytometry), a distinct peak in adhesion to ICAM-1 at 10 min was obtained, which then decreased gradually over 2 h. Absence of an early peak with an even more sustained increase, maximal at 30 min, was obtained with 1 $\mu g\ ml^{-1}$ anti-CD3 IgM (30% saturation after 30 min at 4 °C). No increase in adhesion was seen after addition of anti-CD6 IgM. Stimulation through TCR induces an increase in LFA-1 avidity, therefore, with kinetics dependent upon the degree of cross-linking.

Does the TCR communicate directly with LFA-1, or is it linked to LFA-1 through signalling pathways? Treatment of T cells with the cell-permeable cAMP analogue, dibutyryl cAMP[30–32], before TCR cross-linking or PMA addition, strongly inhibited TCR-stimulated adhesion to ICAM-1 substrates but had no effect on PMA-stimulated adhesion (Fig. 1*e*; Fig. 5). Similar decreases in adhesion triggered by both anti-CD3 IgG plus anti-IgG and anti-CD3 IgM were observed with dibutyryl cAMP (50% of maximal inhibition was achieved at ~1 mM) or the adenylyl cyclase activator forskolin (25 μM) plus the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX) (0.5 mM) (Fig. 5). These agents were used over ranges previously used to inhibit TCR signalling and were clearly not toxic, as PMA-stimulated adhesion was not decreased (Fig. 1*e*, Fig. 5). Butyrate (5 mM), a control for effects of butyrate released by degradation of dibutyryl cAMP, had no effects on unstimulated, or on cPMA- or TCR-stimulated adhesion to ICAM-1 (data not shown). The protein kinase C inhibitor, staurosporine (5 $\mu g\ ml^{-1}$)[33], completely blocked the PMA-stimulated increase in LFA-1 avidity (Fig. 5), but, surprisingly, it only partially blocked TCR-stimulated adhesion. This illustrates either that protein kinase C can feed into the same pathway as the TCR, but is not a direct intermediate in TCR-stimulated signalling, or that the TCR can stimulate some additional mechanisms. Low concentrations of staurosporine (0.01–0.1 ug ml^{-1}) induced a small (2–5 fold) but significant increase in resting T-cell binding to ICAM-1, indicating that staurosporine may inhibit kinases that have an inhibitory effect on LFA-1 avidity (data not shown). The dramatic and opposite effects of PMA and cAMP, which activate protein kinases C and A, respectively, suggest that LFA-1 avidity is regulated by intracellular signalling pathways. These results support the notion that intracellular second messengers connect the TCR and LFA-1, and the concept that LFA-1 can transduce signals from the cytoplasm to the extracellular environment, an example of 'inside-out signalling'.

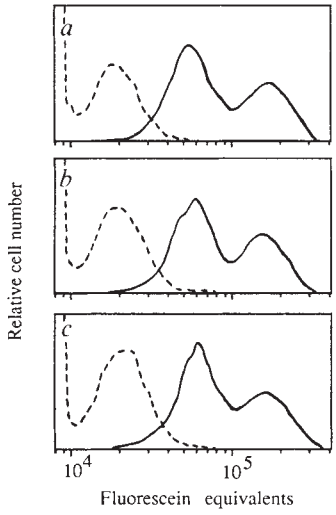

FIG. 4 Expression of LFA-1 on TCR-stimulated resting T cells. Resting T cells were pretreated with OKT3 culture supernatant at 1:10 dilution (~80% saturation) for 30 min at 4 °C and washed. Anti-IgG2a (1 $\mu g\ ml^{-1}$) was either not added (*a*) or added for 5 min (*b*) or 30 min (*c*) at 37 °C before rapid cooling to 0 °C by addition of ice-cold media containing 0.025% NaN_3. Cells were then stained with fluorescein isothiocyanate (FITC) labelled LFA-1 monoclonal antibody (TS1/22) and analysed by immunofluorescence flow cytometry (FITC TS1/22 mAB, solid line; FITC control IgG1, dashed line). Only the relevant portion of the 3 log scale is shown.

TCR regulation of cell–cell adhesion

In interactions between cloned CTL lines and B-lymphoblastoid cell line (BLCL) target cells, both the CD2/LFA-3 and LFA-1/ICAM-1 pathways play a major part in adhesion[16,17]. We examined the effect of TCR cross-linking on the LFA-1/ICAM and CD2/LFA-3 dependent components of conjugate formation between resting T cells and BLCL (Fig. 6*a*, *b*). The BLCL used expressed high levels of ICAM-1 and LFA-3, but were LFA-1^-. We found that unstimulated fresh peripheral blood T cells showed much lower levels of conjugate formation (2–8%) (Fig. 6) than those previously published for cloned CTL (40–80%)[17]. The efficiency of conjugate formation was dramatically increased by TCR cross-linking and the increase in adhesion was completely blocked by anti-LFA-1 monoclonal antibody (Fig. 6*a*). A portion (10–25%) of the basal and stimulated adhesion was blocked by anti-LFA-3 antibody. The TCR-stimulated increase in T-cell adhesion seems to be due mainly to an increase in LFA-1 avidity, with no, or only a minor, contribution from CD2. TCR stimulation of LFA-1 avidity in cell–cell adhesion was transient and was blocked by preincubation of T cells with dibutyryl cAMP (Fig. 6*b*). Thus, the transient increase in LFA-1 avidity stimulated by the TCR is manifested both in binding to purified ICAM-1 and to ICAM-1^+ cells.

Role of LFA-1 avidity in immune response

Based on these findings, we propose the following model for cooperation between TCR and adhesion molecules to mediate antigen-specific recognition. LFA-1 on unactivated cells, such as resting T lymphocytes, is in a low avidity state which may be equivalent to the inactive state of LFA-1 on cells depleted of ATP with sodium azide and 2-deoxyglucose[1,15]. There may be some interaction between LFA-1 in the low avidity state and ICAM-1, but not enough to stabilize cell adhesion. In the absence of antigen, then, the equilibrium governing adherence of T lymphocytes to other cells favours free, mobile T lymphocytes, leading to efficient immune surveillance. On contact with cells bearing specific antigen, TCR ligation generates intracellular signals which lead to energy-dependent conversion of LFA-1 to a high-avidity state and LFA-1/ICAM-dependent adhesion is favoured. Antigen specificity is maintained because the input of energy to convert LFA-1 to the high-avidity stage, whether this energy is used to fuel protein phosphorylation, LFA-1 redistribution or some other mechanism, is controlled by the TCR. Cellular energy expended in converting LFA-1 to a high avidity state drives the adherence-nonadherence equilibrium towards stable adherence, and is analogous to the use of ATP to favour an otherwise energetically unfavourable reaction in intermediary metabolism. LFA-1 is an adhesion servomotor operated by the TCR. As TCR binding to peptide MHC does not have to stabilize cell-cell adhesion but instead triggers an adhesion amplification mechanism, this provides a mechanism for greatly increasing the sensitivity of T cells by lowering the number of TCR-ligand interactions required for antigen

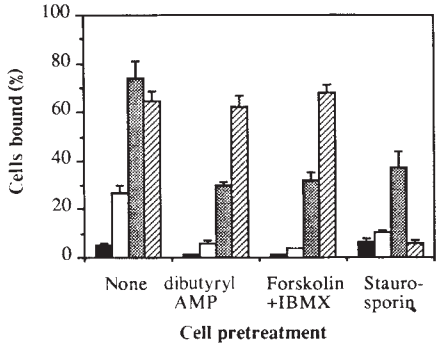

FIG. 5 Effect on LFA-1 avidity increase with pharmacologic agents that act on intracellular cAMP levels or protein kinases. Resting T cells were pretreated with the indicated agents for 15 min at 24 °C before stimulation and assayed for binding to ICAM-1 (1,600 sites μm^{-2}) as in Fig. 1. T cells were unstimulated (solid) or stimulated with anti-CD3 IgM (10 $\mu g\ ml^{-1}$) (open), anti-Leu4/CD3 IgG plus anti-IgG (1:500 Leu4 then 1 $\mu g\ ml^{-1}$ anti-IgG) (shaded), or PMA (50 $ng\ ml^{-1}$) (diagonal stripes). Dibutyryl cAMP (Calbiochem) was dissolved in media and was used at 2 mM. Forskolin (Calbiochem), IBMX (Sigma) and staurosporine (Boehringer) were dissolved in dimethyl sulphoxide (DMSO) and used at 25 μM (0.15% DMSO), 0.5 mM (0.25% DMSO) and 5 $\mu g\ ml^{-1}$ (0.5% DMSO), respectively. DMSO at 0.6% had no effect on adhesion to ICAM-1 (data not shown). Data are representative of two experiments directly comparing these conditions and preliminary experiments to define optimal drug concentrations.

recognition. This view of adhesion strengthening is consistent with the recent observation in murine T-cell clones that LFA-1 and talin, a cytoskeletal protein which co-localizes with a number of integrins at sites of adhesion, redistribute to sites of interaction with antigen-bearing B cells, but not antigen-negative B cells[34]. It is intriguing that redistribution of LFA-1 and talin has been shown to be highly sensitive to low antigen concentrations, as would be expected of adhesion amplification.

The transience of the TCR-stimulated increase in LFA-1 avidity provides a mechanism for regulating the adhesion–de-adhesion cycle. We propose that the TCR triggers a cascade of protein phosphorylation or second messenger production such that an early step in the cascade leads to an increase in LFA-1 avidity and a later step lowers it. The kinetics of the LFA-1 avidity increase triggered by anti-CD3 IgG plus anti-IgG are in good agreement with the kinetics of CTL interaction with target cells. Highly active CTL bind to targets rapidly (0.2–2 min) and can deliver the lethal hit and disengage from the target within an additional 6 min[11,19], but this may be slower for some CTL–target combinations and immunization conditions[19]. It is of interest, therefore, that we have found that the kinetics of avidity regulation are influenced by the number of TCR engaged and the degree of cross-linking. Antigen density may, consequently, influence both the strength and kinetics of adhesion, presumably by affecting the kinetics of the signalling cascade. Duration of adhesion may also be influenced by the level of ICAM expression and whether ICAM-1 or ICAM-2 is the ligand. It is important to remember that as ICAM-1 is inducible by cytokines[1,35], T-cell stimulation could lead to induction of ICAM-1 on antigen-presenting cells, and secondarily alter the kinetics of T-cell interactions.

Although the mechanism of the regulation of LFA-1 avidity is unclear, a change in the conformation of the ICAM binding site or redistribution in the membrane seem most likely; either is compatible with the ability of a non-blocking anti-LFA-1 monoclonal antibody to stimulate LFA-1-dependent homotypic adhesion[36]. Another integrin, gpIIbIIIa, undergoes a well-documented increase in affinity. On unactivated platelets, gpIIbIIIa does not bind fibrinogen but upon activation binds soluble fibrinogen with a dissociation constant, K_d, of 29–45 μM (ref. 37), and no transience is reported. Neutrophil aggregation and binding to endothelium stimulated by chemoattractants is transient[38,39]; there is some evidence that the leukocyte integrin Mac-1 contributes to this[38,40], but estimates vary[40,41]. These phenomena are complicated by chemoattractant-stimulated increases in Mac-1 surface expression, the ability of multiple neutrophil receptors to bind both to endothelial cells and to fragments of C3, rapid shedding from the surface on neutrophil activation of another putative endothelium receptor, Mel-14 (ref. 42), and the generality of the effects on other neutrophil surface receptors that are not integrins[43]. Avidity increases for Mac-1, but not gpIIbIIIa, have been correlated with clustering of receptors in the plane of the membrane[44,45]; surprisingly, redistribution of Mac-1 and increased binding to iC3b are stimulated by phorbol esters but not by a chemoattractant[44], whereas neutrophil aggregation and binding to endothelium are stimulated by both[38,40]. The transience of the increase in avidity of neutrophil Mac-1/CR3 and of the non-integrin CR1 (refs 40, 43), by contrast to the stability of the increase in avidity of LFA-1, stimulated by PMA, suggests that separate mechanisms are involved.

We have defined a mechanism by which lymphocyte adhesion to other cells can be dynamically regulated. The type of inside-out signalling described here in which signals from the cytosol are transduced across the membrane to generate changes in extracellular functions such as adhesion could be of general importance in cell and developmental biology. In the model system studied here, TCR on the entire surface of the cell is ligated, whereas in physiological interactions TCR would be engaged only in the area of cell–cell contact and it would be possible that avidity enhancement would apply only to LFA-1 molecules in or recruited to this area of the cell. Integrins are thought to be important in cell migration as well as adhesion[7]. Spatial gradients of adhesion-molecule avidity on the cell surface could be generated by the same mechanisms as the temporal

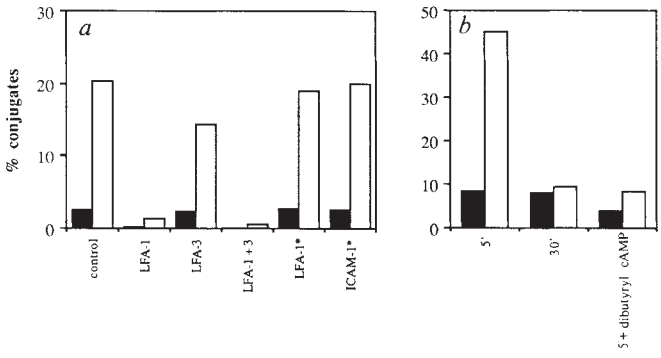

FIG. 6 Regulation of cell–cell adhesion by the TCR. Resting T cells and the genetically LFA-1$^-$ BLCL BBN from patient 1 (ref. 5) were labelled with sulphofluorescein diacetate and hydroethidine, respectively, and conjugates were enumerated by fluorescence flow cytometry[17]. *a*, The resting T cells and BLCL were allowed to co-sediment for 1 h at 4 °C without (solid bars) or with (open bars) OKT3 anti-CD3 monoclonal antibody pretreatment and anti-mouse IgG2a and in the presence of the indicated antibody at 50 $\mu g\ ml^{-1}$. Binding, non-function blocking antibodies are asterisked. LFA-1, LFA-3, LFA-1* and ICAM-1* were TS1/22, TS2/9, TS2/4 and CL203, respectively, and are IgG1, so only OKT3 was crosslinked, as described in Table 1. The pellet was incubated at 37 °C for 5 min and analysed. *b*, Resting T cells were pretreated with OKT3 anti-monoclonal antibody and incubated without or with anti-IgG for 5 or 30 min as indicated (5′ or 30′) and then combined with BLCL and centrifuged at 20g for 5 min at 24 °C, incubated for 5 min at 37 °C and analysed. T cells were pretreated without or with 1 mM dibutyryl cAMP for 15 min at 24 °C before incubation with anti-IgG. METHODS. Cell purification was carried out as in Fig. 1. Labelling of cells was done as described[17]. Resting T cells were combined with BLCL at a 1:2 ratio (5×10^6 cells ml^{-1} total) in 50 μl of assay media. Before analysis the cell pellets were dispersed by adding 0.4 ml balanced salt solution with 1 mM $MgCl_2$ and vortexing for 5 s. Similar results were obtained with a more gentle resuspension.

gradient described here. In models of active cell translocation, it is generally appreciated that a mechanism for de-adhesion is required at the trailing edge of the cell[46,47]. A gradient of integrin avidity from high at the leading edge of a cell to low at the trailing edge could provide a mechanism for de-adhesion at the trailing edge and differential adhesiveness at the leading and trailing edges could drive cell migration. This would be analogous to haptotaxis, the ability of gradients of substrate adhesiveness to promote directed migration of cells[48]. □

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The Oct-1 homoeodomain directs formation of a multiprotein–DNA complex with the HSV transactivator VP16

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The herpes simplex virus transactivator VP16 participates in the formation of a multiprotein–DNA complex with the ubiquitous octamer-motif-binding factor Oct-1. Complex formation is dependent on specific amino acids in the Oct-1 homoeodomain which are in positions analogous to positive control mutations in helix 2 of the λ phage repressor helix-turn-helix motif, indicating that this structure is an ancient target for protein–protein interactions mediating transcriptional control.

PROTEIN–PROTEIN interactions are fundamental to transcriptional regulation in eukaryotic cells. Such interactions are involved both in formation of a pre-initiation complex between RNA polymerase II and general transcription factors[1,2], and in contacts between sequence-specific DNA-binding transcription factors and the promoter-proximal complex (reviewed in ref. 3). Nevertheless, despite the purification of many transcription factors, specific protein–protein interactions remain largely uncharacterized.

In prokaryotes, however, at least one important interaction between a sequence-specific DNA-binding transcription factor, the phage λ repressor, and RNA polymerase has been well characterized. The λ repressor can positively activate transcription from the promoter P_{RM}, which is the promoter of the gene for λ repressor itself[4]. Amino-acid substitutions in λ repressor that abolish positive regulation of transcription from P_{RM}, but which do not affect either operator binding or negative regulation, have been termed positive-control mutations or *pc* mutations. The *pc* mutations map to a solvent-exposed patch on the surface of the DNA-binding domain of the protein which probably directly contacts the RNA polymerase bound to the promoter[5,6].

In eukaryotes, viral transactivator proteins that do not bind to DNA directly but alter patterns of gene expression, provide model systems to study protein–protein interactions. One of these, the herpes simplex virus (HSV) transactivator VP16, also called Vmw65, VF65 and α-TIF, can form a multiprotein–DNA complex with one or more cellular factors, which include the ubiquitous octamer-motif-binding protein Oct-1 (also called