T lymphocytes isolated from human peripheral blood express β1 (VLA) and LFA-1 integrins, but strong binding to integrin ligands occurs only after the delivery of an activation stimulus to the T cell. To gain further insight into activation-dependent regulation of integrin function, we have analyzed integrin activity on three different T-leukemic cell lines: Jurkat, CEM, and H9. This analysis shows important mechanistic differences in integrin regulation. First, phorbol ester treatment results in increased β1 integrin-dependent adhesion of both Jurkat and CEM cells to fibronectin, but decreased adhesion of H9 cells. Second, certain activation stimuli that upregulate β1 integrin activity in peripheral T cells are nonfunctional in these T-cell lines. Third, analysis of a panel of Jurkat mutants lacking surface expression of CD2 and/or CD3 shows that CD2-mediated upregulation of β1 integrin activity is dependent on expression of CD3, whereas CD28-mediated upregulation is not dependent on either CD2 or CD3 expression. Fourth, all T-cell lines tested show an inability to adhere to purified ICAM-1 via LFA-1. The selective alterations in integrin regulation in these cell lines relative to peripheral blood T cells provide important insights into the intracellular processes involved in integrin activation.

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phore upregulate β1 integrin activity in the less phenotypically mature CEM T-cell line. In H9 T cells, ligation of CD3 increases β1 integrin-mediated adhesion, whereas PMA activation inhibits adhesion. In addition, by using a series of Jurkat mutants deficient in expression of CD2 and/or CD3, we show that (1) CD2-mediated β1 integrin activation is dependent on coexpression of CD3; (2) CD3-mediated β1 integrin activation does not require CD2 coexpression; and (3) activation of β1 integrin function through CD28 is independent of CD2 or CD3 coexpression. Furthermore, we show that each of the three T-cell lines examined is deficient in activation-induced LFA-1 adhesion to purified ICAM-1.

MATERIALS AND METHODS

Cells. The T-cell lines CEM and Jurkat E6-1 were obtained from American Type Culture Collection (ATCC; Rockville, MD). H9 (a derivative of HUT78) was obtained from Dr Paula Kavathas (Yale University, New Haven, CT). J65-6A is a subclone of the Jurkat T-cell line E6-1 produced in our lab. All cell lines were maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (GBCO/BRL, Grand Island, NY), L-glutamine, and penicillin/streptomycin. These cell lines were maintained at a cell density of 3 to 5 × 10^6 cells/mL at least 3 days before use in adhesion assays or flow cytometry. Resting peripheral CD4+ T cells were isolated by negative magnetic immunoselection as previously described.1,5,11 Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from either leukapheresis packs or buffy coats by Ficoll-Hypaque density-gradient centrifugation. Resting CD4+ T cells were subsequently isolated from PBMCs using Advanced Magnetics Particles (Advanced Magnetics, Inc, Cambridge, MA) and a cocktail of MoAbs consisting of 1VA12 (anti-HLA class II), FMC63 (anti-CD19), 3G8 (anti-CD6), NIH11b-I (anti-CD11b), 6D33 (anti-CD14), B9.8.4 or 51.1 (both anti-CD8), and 10F7 (anti-glycophorin). The T-cell populations were typically greater than 97% CD3+CD4+ as assessed by flow cytometric analysis.

Isolation of CD2- and CD3- Jurkat mutants. Mutants of the Jurkat cell line deficient in expression of CD3 and/or CD2 were isolated by selection for cells that failed to show increased adhesion to FN after MoAb cross-linking of CD3. Briefly, J65-6A cells were γ-irradiated with 300 rad of γ-rays and split into 30 separate culture flasks. After 5 days of growth to allow for appearance of mutants of different phenotypes, mutant cells were enriched by multiple rounds of panning for nonadherent cells on FN-coated tissue culture dishes. For each selection, 60-mm Petri dishes were coated with 60 μg of FN and incubated overnight at 4°C. The following day, FN was removed and phosphate-buffered saline (PBS)/2.5% bovine serum albumin (BSA) added and incubated for 2 to 3 hours at 37°C. Dishes were then washed with PBS before addition of cells. Cells were first preincubated with saturating amounts of the CD3-specific MoAb OKT3 for 30 minutes at 4°C. Unbound MoAb was removed by two washes with PBS/0.5% human serum albumin (HSA), and then the cells were added to FN-coated dishes in PBS/HSA medium containing 1 μg/mL goat antimouse Ig (GAMlg). Plates were incubated for 30 minutes at 37°C and the nonadherent cells removed by aspirating off the supernatant. The nonadherent cells were recultured until sufficient numbers of cells were available for another selection. After 5 to 6 rounds of selection, flasks containing large numbers of nonadherent cells were identified and screened for expression of CD3 and CD2 by flow cytometry. Three classes of mutants were isolated after cloning by limiting dilution, as defined by CD2 and CD3 expression: CD2+CD3- (J65-6A-D1A), CD2-CD3+ (J65-6A-D2Q), and CD2-CD3- (J65-6A-D1E). Because the isolation of mutants involved an initial selection for cells deficient in adherence to FN, all mutants deficient in CD2 or CD3 expression were tested for their ability to adhere to FN in the presence of PMA, and only those with functional FN receptors were analyzed further for this report.

Antibodies and other reagents. The following MoAbs were used as purified IgG: anti-LFA-1 α chain MoAb TS1/22 (ATCC), anti-α5 MoAb MAB16.14 and anti-β1 MoAb MAB13.11 (both provided by Dr K. Yamada, National Institutes of Health [NIH], Bethesda, MD). The following MoAbs were used as dilutions of ascites fluid: anti-CD28 MoAb 9.3 (J. Ledbetter, Bristol Myers Squibb, Seattle, WA), anti-CD2 MoAbs 95.5-49.16 and 9-1 (Dr B. Dupont, Sloan Kettering, New York, NY), anti-α3 MoAb P1BS and anti-α5 MoAb P1D6 (Telfos Pharmaceuticals, San Diego, CA), anti-α4 MoAb L25.17.27 (Dr P. Estes, Becton Dickinson, Mountain View, CA), and antilymphocytic MoAb 10F7 (ATCC). The following MoAbs were used as dilutions of culture supernatant: anti-CD3 MoAb OKT3 and anti-CD7 MoAb 3A1 (both ATCC), anti-α4 MoAb NIH49d.18 (Drs S. Shaw and Y. Tanaka, NIH), anti-α6 MoAb GoH3.19 (Dr A. Sonnenberg, CLB, Amsterdam, The Netherlands). The anti-β1 chain MoAb B-D15 was purchased from Biosource International (Camarillo, CA). Fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG and goat antirat IgG were obtained from Fisher Biotech (Pittsburgh, PA).

FN from pooled human plasma was purchased from the New York Blood Center (New York, NY). The phorbol ester PMA (Sigma Chemical Co, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) before use. The Ca2+ ionophore A23187 (Sigma) was dissolved in ethanol before use. ICAM-1 was purified by affinity chromatography using the anti-ICAM-1 MoAb 84H10 from the Hodgkin’s lymphoma cell line L245 as previously described.20 The kinase inhibitors staurosporine (Boehringer Mannheim, Indianapolis, IN) and GF 109203X (Calbiochem, La Jolla, CA) were dissolved in DMSO at 1 and 3 mM/mL, respectively.

Adhesion assays. Adhesion assays were performed as described.5 Briefly, 96-well microtiter plates (Costar [Cambridge, MA] #3596 for FN, Nunc Immunoplates [Naperville, IL] for ICAM-1) were incubated overnight at 4°C with the indicated concentrations of FN or ICAM-1 (diluted in PBS with Ca2+ and Mg2+), and unbound binding sites subsequently blocked with PBS/2.5% BSA. Each well contained 40,000 3H-labeled Jurkat, CEM, or H9 T cells, or 50,000 3H-labeled peripheral blood CD4 T cells in a final volume of 0.1 mL PBS/0.5% HSA. For A23187 activation, cells were added to wells containing 1 μg/mL A23187. For PMA activation, cells were added to wells containing 10 ng/mL PMA (Sigma). For CD2 activation, T cells were added to wells containing 10 μg/mL 95.5-49 and a 1:1,000 dilution of MoAb 9-1. For CD3, CD7, and CD28 activation, cells were incubated with saturating amounts of antibody for 30 minutes at 4°C, washed twice, and added to wells containing 0.5 to 1 μg/mL goat antimouse Ig (Organon Teknika, Malvern, PA). After 1 hour of settling at 4°C, plates were rapidly warmed to 37°C for 10 minutes, nonadherent cells were washed off, and the percentage of bound cells was determined by lysing the well contents with detergent and counting γ-emissions. All data are expressed as the mean percent of cells binding from three replicate wells ± SEM.

In all MoAb blocking experiments, MoAbs were added to wells at 10 μg/mL (for purified IgG), 1:500 dilution (for ascites fluid), or 1:5 dilution (for culture supernatants). The MoAbs used in the blocking studies previously were reported by our lab and others to specifically inhibit β1 integrin-mediated adhesion to FN via the α4 chain (L25), the α5 chain (MAB16), or the common β1 chain (MAB13).5,10,12,2 The LFA-1 α chain-specific MoAb TS1/22 has been previously reported to specifically inhibit LFA-1 binding to

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ICAM-1 is completely inhibited in the adhesion of PMA-activated CD4+ T cells to ICAM-1 used in this study (not shown). For assays using kinase inhibitors, cells were added to wells containing 2.5 μM staurosporine or 3 μM GF 109203X with or without 10 ng/ml PMA or goat antinouse Ig. The concentrations of inhibitors used were determined beforehand to be optimal for inhibiting adhesion to FN without inducing cell death and without nonspecific effects on adhesion by DMSO, as determined from DMSO controls.

In assays with altered divalent cation usage, the plates with bound integrin ligands were washed five times with Ca++/Mg++-free PBS. 3Cr-labeled T cells were washed twice with excess Ca++/Mg++-free PBS before the addition of cells and the appropriate cation-containing buffers to the plates. Each well contained 50,000 T cells in a final volume of 0.1 mL PBS/0.5% HSA (extensively dialyzed against Ca++/Mg++-free PBS) supplemented with the indicated concentrations of either MgCl₂ and EGTA, or MnCl₂. For PMA stimulation, cells were added to wells containing 10 ng/ml PMA. After 1 hour of settling at 4°C, plates were rapidly warmed to 37°C for the indicated time period, and the nonadherent cells removed by washing the plates five times with PBS containing 0.5 mMol/L Mg++. Flow cytometry. Single-color flow cytometric analysis was performed essentially as described. Cells were stained by sequential incubations with saturating amounts of test MoAb and FITC-conjugated goat antinouse IgG or goat antirat IgG. Cells were analyzed on a FACScan (Becton Dickinson).

**RESULTS**

To determine whether T-cell lines could serve as in vitro models for peripheral blood T-cell integrin function, we first analyzed the Jurkat, CEM, and H9 T-cell lines for expression of these integrins by flow cytometry. Jurkat is an acute lymphoblastic leukemic (ALL) T-cell line that is characterized as being phenotypically mature (stage III), and thus closely resembles resting peripheral blood T cells. CEM was also derived from a patient with ALL, but this cell line is less phenotypically mature (stage I/II) than Jurkat. The H9 T-cell line was derived from HUT 78, a cutaneous T-cell lymphoma derived from a patient with Sezary syndrome. The parental HUT 78 line from which H9 was derived is characterized as being phenotypically mature and expresses several surface activation markers, suggesting that this cell line is constitutively activated.

Figure 1 shows that each of the three cell lines expresses low but detectable levels of α6, higher levels of α4 and α5, and correspondingly high levels of expression of the β1 chain that is associated with each of the α chains. α3 expression is higher on all three cell lines than is typically seen with peripheral T cells, with the highest expression observed on H9 cells. These cell lines also express high levels of LFA-1 (Fig 1), but no detectable levels of another β2 integrin, Mac-1 (data not shown). Therefore, each of these T-cell lines is equipped with the same set of integrin receptors as peripheral blood CD4+ T cells.

Strong adhesion of peripheral T cells to β1 integrin ligands is induced by the ligation of a number of T-cell surface molecules, including CD2, CD3, CD7, and CD28. We therefore examined the various T-cell lines for the presence or absence of these regulators of integrin function. Flow cytometric analysis shows that Jurkat T cells express high levels of CD2, CD3, CD7, and CD28. CEM cells express CD7 but lack significant expression of CD2, CD3, or CD28, whereas H9 cells express CD3 but lack expression of CD2, CD7, and CD28 (Fig 2).

Resting peripheral blood T cells do not bind well to integrin ligands without prior stimulation induced by ligation of surface regulatory molecules or by the addition of pharmacologic activators such as phorbol esters or calcium ionophore. T-cell lines, on the other hand, are transformed cells that could be considered to be in a constant state of activation. To determine whether the integrins on these transformed cells were in a constitutive high-avidity state, we tested the capacity of Jurkat, CEM, and H9 cells to adhere to immobilized integrin ligands. As shown in Fig 3A, both unstimulated Jurkat and H9 bind only minimally to the α4β1 and α5β1 ligand FN at concentrations below 1 μg/ml, whereas CEM bind well at FN concentrations as low as 0.03 μg/ml. Furthermore, the adhesion of both Jurkat and CEM is enhanced by stimulation of the cells for 10 minutes with the phorbol ester PMA. In contrast, the adhesion of H9 cells to FN actually decreases in the presence of PMA. The inverse correlation between PMA concentration and H9 adherence to FN is consistent over a broad range of PMA con-
three cell lines show differences in their use of α4 or α5 for FN binding. The level of inhibition of FN adhesion to FN was roughly equivalent when the cells were pretreated with either the α4-specific MoAb L25 or the α5-specific MoAb MAB16, whereas adhesion by Jurkat was more strongly inhibited by the α4-specific MoAb and H9 was more strongly inhibited by the α5-specific MoAb. Adhesion to FN by all T-cell lines was completely inhibited by a combination of these α4- and α5-specific MoAbs, indicating that no other α chains participate in FN adhesion by these cells. β2 integrins do not appear to be involved in the adhesion of these T-cell lines because the LFA-1α-specific MoAb TS1/22 had no effect on FN binding and these cell lines do not express the β2 integrin Mac-1.

Because the FN-binding capacity of the three T-cell lines tested could be augmented by PMA (Jurkat) or A23187 (H9) or both (CEM), we sought to determine whether integrin activation in T-cell lines could be induced by the ligation of specific T-cell surface molecules that upregulate β1 integrin activity on peripheral T cells. These include the antigen-specific CD3/TCR complex, as well as the CD2, CD7, and CD28 cell surface antigens. The data in Fig 5 show that CD3 cross-linking induces increased adhesion of Jurkat cells to FN. Interestingly, H9 T-cell adhesion to FN was also augmented by CD3 cross-linking, even though PMA treatment reduces binding to FN. CEM adhesion to FN was unaffected by CD3 cross-linking, because CD3 is not expressed on these cells.

The CD2 antigen was found to be expressed only on Jurkat cells. Consequently, treatment of Jurkat, CEM, or H9 cells with mitogenic pairs of CD2-specific MoAbs only resulted in increased adhesion of Jurkat cells to FN. Similarly, the CD28 antigen is expressed at high levels only on Jurkat cells and MoAb cross-linking of CD28 also only results in increased adhesion of Jurkat cells to FN. These results show that Jurkat T cells express intact CD3, CD2, and CD28 receptors with regard to their ability to upregulate β1 integrin activity on appropriate stimulation. In contrast, even though CD7 is strongly expressed on Jurkat and CEM cells, MoAb cross-linking of CD7 fails to result in augmented adhesion to FN. These results suggest that CD7 signaling is functionally defective in these T-cell lines, and that CD7-mediated upregulation of β1 integrin activity is mechanistically distinct from CD3, CD2, and CD28 upregulation of integrin-mediated adhesion.

The kinetics of activation-dependent upregulation of β1 integrin activity differ depending on the initial activating stimulus used. Whereas PMA treatment results in a rapid and prolonged increase in peripheral blood T-cell adhesion, CD3 cross-linking results in a rapid but transient upregulation. The increase in adhesive strength peaks 10 to 20 minutes after the initiation of activation by anti-CD3 cross-linking, and then declines to baseline by 30 to 60 minutes. When examined at various time points after activation by CD3 cross-linking, the kinetics of adhesion to FN were found to be different for each of the three T-cell lines tested (Fig 6). After CD3 cross-linking, Jurkat T cells follow a pattern similar to that of resting T cells, with adhesion peaking at 10 minutes, followed by a steady decline in adhesiveness.
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Fig 3. Differential activation-dependent adhesion of human T-cell lines to FN. (A) Adhesion of Jurkat, CEM, and H9 T cells to FN (applied at the indicated concentrations) with (■) or without (□) stimulation with 10 ng/mL PMA for 10 minutes at 37°C. (B) Adhesion of Jurkat, CEM, and H9 T cells to 1 μg/well FN after activation for 10 minutes at 37°C under the following conditions: no stimulation (■), 1 μg/mL A23187 (□), or 10 ng/mL PMA (▲). Data are expressed as the mean percent of cells binding from three replicate wells with bars representing standard error of the mean.

reaching baseline levels by 80 minutes. PMA-activated Jurkat cells show a rapid but prolonged increase in binding compared with CD3-mediated upregulation. Unactivated Jurkat T cells show no increase in adhesiveness over 80 minutes. As described above (Fig 3), unstimulated CEM T cells have a characteristically high level of basal binding to FN that is augmented by PMA, but not by CD3 cross-linking. The PMA-induced adhesion appears not to be transient, with levels of binding remaining consistently elevated throughout the 80-minute time course (Fig 6). The kinetic

Fig 4. Adhesion of human T-cell lines to FN is blocked by antibodies specific for α4β1 and α5β1 integrins. The adhesion of Jurkat, CEM, and H9 T cells to FN applied at 1 μg/well with (■) or without (□) stimulation with 10 ng/mL PMA for 10 minutes at 37°C was assessed in the presence of the indicated antibodies. The MoAbs used for this analysis were LFA-1 α chain-specific MoAb TS1/22, α4-specific MoAb L25, α5-specific MoAb MAB18, and β1-specific MoAb MAB13. Data are expressed as the mean percent of cells binding from three replicate wells with bars representing standard error of the mean.
Profile of H9 adhesion to FN is distinct from that of either Jurkat or CEM. The most striking difference is that the level of adhesion of unstimulated (or PMA-treated) H9 cells continually increases with time at 37°C. PMA, which is inhibitory to H9 adhesion at early time points (see also Figs 3, 4, and 5), has little or no inhibitory activity at later time points. CD3 cross-linking augments H9 adhesion to FN at all times examined, but this stimulation of adhesion peaks at 20 to 40 minutes, much later than Jurkat or peripheral blood T cells, and only begins to decline by 80 minutes. In fact, as with Jurkat, at 80 minutes, the levels of adhesion for both unstimulated and CD3-stimulated H9 cells are nearly equivalent. However, for H9 cells this is caused primarily by the constant increase in adhesiveness of the unstimulated cells, and not by a dramatic decrease in adhesion of the stimulated cells.

Because Jurkat cells are similar to peripheral blood T cells in their activation-dependent integrin regulation through PMA, CD2, CD3, and CD28 stimulation, we used this T-cell line to ascertain whether the various integrin regulatory molecules are interdependent, or whether activation through one receptor is independent of the presence of other integrin regulatory molecules. Thus, we produced a series of Jurkat mutants deficient in expression of CD2 and/or CD3, but with normal levels of CD28 and the relevant integrin molecules (Fig 7).

For each mutant phenotype (CD2-CD3\(^{+}\), CD2-CD3\(^{-}\), and CD2-CD3\(^{\beta}\)), two independently generated mutant clones were produced and tested in adhesion assays. Because the results for similar mutants were equivalent, only the results for one of each mutant phenotype are reported in Fig 8. Mutants deficient in only CD2 expression (CD2\(^{\beta}\)CD3\(^{+}\)) retain normal integrin regulation through CD3, CD28, and PMA stimulation. The kinetics of their adhesion and subsequent deadhesion are also identical to those of the wild-type Jurkat (Table I). Mutants deficient in only CD3 expression (CD2\(^{\beta}\)CD3\(^{-}\)) lose the ability to activate integrin function through CD2, even though these mutants express high levels of CD2 on their surface. However, the loss of CD3 expression does not prevent integrin activation via CD28 ligation or PMA stimulation, showing that the \(\beta\)1 integrins expressed on these mutants are functional. A deficiency in expression of both CD2 and CD3 (CD2\(^{\beta}\)CD3\(^{-}\)) also has no effect on integrin regulation by CD28 or PMA.

Activation via CD2 or CD3 results in activation of pro-
tein kinase C (PKC) and other intracellular kinases. PKC-mediated phosphorylation events may play a pivotal role in the activation-dependent regulation of integrin function because (1) PMA, which directly activates PKC, dramatically augments integrin adhesiveness; and (2) anti-CD3-mediated upregulation of integrin function can be inhibited by PKC inhibitors. Because our analyses showed significant differences between anti-CD3-induced and PMA-induced integrin regulation in the overall adhesion of H9 T cells to FN (Figs 3, 4, and 5) and in the kinetics of Jurkat adhesion to FN (Fig 6), we used inhibitors of PKC to determine the relationship between PKC activation and PMA- or anti-CD3-induced integrin activation. We used two different PKC inhibitors: staurosporine, a potent but relatively nonspecific inhibitor of PKC, and GF 109203X (bis-indolylmaleimide), a recently described PKC inhibitor that is more specific than staurosporine for PKC inhibition.

As shown in Fig 9, staurosporine and GF 109203X have differential effects on the FN adhesion of the three T-cell

### Table 1. Kinetics of Adhesion to FN by CD2+CD3+ and CD2−CD3+ Jurkat T Cells

<table>
<thead>
<tr>
<th>Time at 37°C (min)</th>
<th>% FN Binding by CD2+CD3+</th>
<th>% FN Binding by CD2−CD3+</th>
<th>Jurkat T Cells</th>
<th>Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+CD3 Ligation</td>
<td>+PMA</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>49</td>
<td>30</td>
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<td>80</td>
<td>12</td>
<td>59</td>
<td>3</td>
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Data are expressed as the mean percentage of cells binding from three replicate wells. Standard error was less than 3% for all conditions tested.

* Adhesion assays were conducted as described in Fig. 6 legend.
The activation of integrin-mediated adhesion, presumably by altering the conformation of the integrin ligand-binding domain, can be summarized as follows: (1) Stauroporine completely inhibits H9 adhesion and dramatically inhibits CEM adhesion to FN in the basal state and under all activation conditions examined; (2) it slightly augments the basal level of Jurkat adhesion to FN; and (3) it has only a modest inhibitory effect on the adhesion of peripheral T cells to ICAM-1. This suggests that the LFA-1 molecule expressed in Jurkat cells can be made functional under the appropriate divalent cation conditions. However, these same divalent cation conditions result in only a slight increase in the adhesion of CEM and H9 cells to ICAM-1, even though both CEM and H9 cells express higher levels of LFA-1 than do Jurkat cells (Fig 1).

**DISCUSSION**

We have analyzed three human T-cell lines, Jurkat, CEM, and H9 to compare their activation-dependent regulation of integrin function with that which we have previously reported for human peripheral blood T cells. Table 2 summarizes our findings. Of the three T-cell lines examined, the mature (stage III) Jurkat T cells are the most similar to peripheral blood T cells. First, they have similar patterns of surface expression of integrins (including LFA-1, a5b1, a4b1, a5b1, and a6b1) and integrin regulators (CD2, CD3, CD7, and CD28). Second, adhesion of Jurkat to FN is rapidly and dramatically enhanced over levels of unstimulated cells after stimulation of Jurkat with PMA, or MoAb ligation of CD2, CD3, or CD28. Third, the enhanced adhesion after CD3 cross-linking is transient, with levels of adhesion decreasing to baseline by 60 to 80 minutes of stimulation. Fourth, both α4β1 and α5β1 integrins participate in Jurkat adhesion to FN. Despite these similarities, Jurkat T cells differ from peripheral blood T cells in several important ways. First, Jurkat T-cell β1 integrins are not activated by the calcium ionophore A23187 or by cross-linking cell surface CD7. Second, the adhesion of unstimulated Jurkat to integrin ligands is higher than is normally reported for peripheral blood T cells, although this level is relatively low in comparison to the other T-cell lines examined. Third, although they express high levels of LFA-1, Jurkat T cells do not bind to purified ICAM-1 under normal conditions.
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Fig 10. Jurkat, CEM, and H9 T cells bind poorly to ICAM-1, even after PMA stimulation. Adhesion of CD4+ T cells, Jurkat, CEM, and H9 cells to ICAM-1 (left panel) and FN (right panel) was assessed either in the absence (○) or presence (●) of 10 ng/ml PMA. Cells were warmed to 37°C for 10 minutes before nonadherent cells were washed off. Data are expressed as the mean percent of cells binding from three replicate wells with bars representing standard error of the mean. FN was applied at 0.3 μg/well and ICAM-1 at 20 ng/well.

Although little is known about the biologic relevance of CD7 in vivo, cross-linking CD7 in vitro has been reported to induce intracellular calcium mobilization in T cells.36 Because both A23187 and CD7 ligation increase intracellular calcium levels and augment integrin-mediated adhesion by peripheral T cells, it is possible that the failure of CD7 ligation on Jurkat T cells to upregulate integrin function is caused by a defective CD7-mediated calcium response in these cells. In fact, we have found that, whereas ligation of CD2, CD3, or CD28 results in a measurable calcium flux in Jurkat cells, ligation of CD7 does not (not shown). However, A23187 does induce a strong calcium flux in Jurkat cells and still fails to enhance integrin-dependent adhesion. Thus, it is unlikely that integrin regulation in T cells is a direct consequence of a calcium flux, but is likely to be dependent on other intracellular messengers not yet identified. However, these findings do suggest that integrin activation can be induced by multiple, discrete intracellular biochemical mechanisms, and that inactivation of one integrin activator, such as CD7, does not necessarily preclude integrin activation through others, such as CD3.

To better understand the interplay between the various cell surface regulators of integrin function, we produced a series of Jurkat mutants deficient in surface expression of CD2 and/or CD3, but retaining expression of CD28 and all relevant integrins (Fig 7). Analysis of the activation-dependent regulation of FN adhesion in these mutants suggests that activation of Jurkat through CD3 ligation is not dependent on CD2 expression, whereas activation through CD2 ligation appears to be dependent on the coexpression of CD3. In these mutants, integrin activation through CD28 ligation is independent of the expression of either CD2 or CD3, as is integrin activation through PMA. The finding that integrin activation through CD2 is dependent on the coexpression of CD3 are consistent with reports indicating that phosphatidylinositol (PI) hydrolysis, calcium mobilization, and induction of interleukin-2 (IL-2) gene transcription by CD2 ligation are dependent on the expression of CD3.37,38 That CD3-mediated integrin activation is independent of CD2 expression is also consistent with data indicating that PI hydrolysis, calcium mobilization, and IL-2 gene induction by CD3 ligation are unaffected by a lack of CD2 coexpression.39 Our data also suggest that the activation of integrin activity through CD28 is independent of CD2 or CD3. Such independence for other aspects of CD28-mediated T-cell activation has been previously suggested based on the cyclosporin A-resistance of CD28-mediated costimulation of T-cell proliferation.40,41

In contrast to Jurkat, regulation of integrin function by the CEM and H9 T-cell lines differs dramatically from peripheral T cells. Although CEM T cells express sufficient
quantities of all relevant T-cell integrins, they express little or no CD2, CD3, or CD28, and therefore antibodies specific for these integrin regulators are ineffective at augmenting CEM integrin function. CEM T cells express very high levels of CD7, but as with Jurkat, ligation of CD7 has no effect on integrin-mediated adhesion. Although no cell surface regulators of integrin function are effective in CEM T cells, both PMA and A23187 increase the level of CEM adhesion to FN over the level of adhesion by unstimulated cells, indicating that the intracellular machinery for integrin regulation is intact, even if the extracellular machinery is missing or inoperative. The lack of certain T-cell surface glycoproteins in CEM T cells is likely to be a consequence of the early thymocyte developmental stage at which these cells became transformed. Perhaps the most striking difference between CEM and peripheral blood T cells is in the level of adhesion of unstimulated cells; whereas normal unstimulated peripheral blood T-cell adhesion to FN rarely exceeds 5% to 10%, unstimulated CEM T-cell adhesion is commonly 40% to 60%. The striking contrast between the very low levels of constitutive peripheral blood T-cell adhesiveness to FN and that of CEM T cells suggests that the integrins expressed on unstimulated CEM cells are already in an activated or partially activated state. It is interesting to note that the high level of binding of unstimulated CEM cells parallels the strong binding of immature thymocytes to FN.

The most unusual T-cell line of the three tested is H9. Like the other two cell lines, it has a pattern of integrin expression similar to that of peripheral blood T cells, but of the integrin regulators examined, H9 expresses only CD3. MoAb cross-linking of CD3 increases H9 adhesion to FN, although the peak of adhesion occurs at a later time point (20 minutes) than is seen with Jurkat T cells or peripheral T cells (10 minutes). In further contrast to Jurkat, CEM, or peripheral blood T cells, unstimulated H9 T cells increase in adhesiveness to FN over time, reaching levels of adhesiveness by 80 minutes at 37°C that approach those of their CD3-stimulated counterparts. The most surprising and unexpected result was found in the response of H9 T cells to PMA; whereas H9 increases adhesion to FN with CD3 stimulation and in the presence of A23187, the addition of PMA actually inhibits H9 adhesion to FN at early time points. As with CEM, the differences in integrin regulation between H9 and Jurkat or peripheral T cells may reflect inherent differentiation stage-specific changes in integrin function. H9 T cells express a panel of activation antigens consistent with a lymphoblast phenotype, whereas the majority of peripheral blood T cells are quiescent resting cells. We are currently analyzing peripheral blood T-cell blasts to determine if their integrin regulation resembles those reported here for H9 cells.

The discordance in the effects of PMA and CD3 cross-linking on H9 T-cell adhesion to FN is particularly striking and suggests that the ability of CD3 cross-linking to increase β1 integrin function in this cell line is not caused solely by activation of PKC. The use of inhibitors of protein kinases in Fig 9 has allowed us to make some tentative conclusions regarding the relationship between PKC activation and integrin regulation in all three T-cell lines. First, the basal levels of FN adhesion by the T-cell lines are only partially susceptible to PKC inhibition with the PKC-specific inhibitor GF 109203X, suggesting that other factors are responsible for the maintenance of this state. The additional inhibitory effects of staurosporine on the basal adhesion of H9 and CEM suggest the involvement of other kinase species. The slight staurosporine-mediated augmentation of Jurkat cell adhesion to FN is reminiscent of a previous report indicating that staurosporine can augment LFA-1-dependent binding of B cells to ICAM-1. The investigators proposed a model in which a staurosporine acts to inhibit a kinase necessary for maintaining integrins in a low avidity state. Because PMA stimulation results in a decrease in H9 adhesion to FN, we expected that inhibiting PKC would result in increased levels of H9 adhesion. Therefore, we were surprised to see just the opposite effect: GF 109203X could not reverse the inhibitory effects of PMA and staurosporine treatment actually further decreased the level of H9 adhesion. However, GF 109203X can nullify the CD3-dependent adhesion augmentation in H9 T cells. Together, these results suggest that PKC is probably involved in the response of H9 cells to CD3 cross-linking, but that a staurosporine-sensitive kinase other than PKC is required to maintain H9 adhesion to FN. We are currently using biochemical approaches to identify this kinase. Because the H9 parental line HUT 78 lacks expression of the β isoform of PKC, it is intriguing to speculate that the unusual inhibitory effects of PMA on H9 adhesion and the unusual kinetics of activation-dependent adhesion of this cell line might be caused by a lack of expression of a specific PKC isoform or other non-PKC kinase. Minimally, we can conclude that

### Table 2. Differential T-Cell Line Adhesiveness to FN in Response to Stimulators of Integrin Activation

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CD4+ CEM</th>
<th>H9</th>
<th>JURKAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A23187</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Anti-CD7</td>
<td>+</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>+</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Anti-CD2</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Anti-CD28</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Abbreviations:** +, increased adhesion relative to unstimulated cells; -, decreased adhesion relative to unstimulated cells; 0, no increase in adhesion relative to unstimulated cells; NA, not applicable because of lack of expression of specific receptor.
the activation-dependent regulation of T-cell integrins is more complex than a recent proposal suggesting that integrin activation is a PKC-mediated event, and that integrin de-adhesion is a consequence of a phosphatase-mediated reversal of the phosphorylation event. 46

Although Jurkat, CEM, and H9 show activation-dependent regulation of β1 integrin function, and despite the fact that these cell lines express high levels of LFA-1, each of these cell lines fails to bind to purified ICAM-1, even after stimulation with PMA. This is consistent with other lines of evidence implicating defective LFA-1 receptors on Jurkat cells. 49 Divalent cation treatment can restore strong adhesion of Jurkat cells to ICAM-1, suggesting that LFA-1 on Jurkat cells may normally be "locked" in an inactive conformation that prevents it from functioning, even after appropriate activation. Even though CEM and H9 cells express higher levels of LFA-1 than do Jurkat cells, Mg²⁺/EGTA and Mn²⁺ do not increase adhesion of CEM and H9 cells to ICAM-1 to the levels seen with Jurkat cells. This suggests potential differences in the LFA-1 defect in these cell lines, which is currently under investigation. Although the pathophysiological significance of the LFA-1 defects in these tumor lines remains to be defined, changes in integrin expression have been proposed to be important to both tumorigenesis and tumor metastasis. 47-49 Our results suggest that alterations in integrin receptor function, in addition to alterations in levels of expression, may also be relevant to the development of cancer.

In conclusion, our results indicate important mechanistic differences in the regulation of integrin activity in Jurkat, CEM, and H9 T cells. These results are critical in several general respects. First, this analysis suggests important differences in the mechanisms by which various integrin regulatory molecules upregulate β1 integrin activity. For example, CD2- and CD3-mediated activation appear to use common signaling pathways, whereas CD28 cross-linking appears to use a somewhat distinct pathway for upregulating integrin function (Table 2). CD7 cross-linking may also be distinct, because CD7 cross-linking fails to upregulate β1 integrin activity in CD7⁺ cells that respond appropriately to other activation signals. Second, further analysis of these cell lines, and aspects of β1 integrin regulation unique to each, is likely to continue to yield insights into this critical adhesion regulatory event. Finally, our results suggest that alterations in the regulation of β1 and LFA-1 integrin activity in transformed cells may be relevant to our further understanding of the role of integrins in the development of cancer.

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