Stimulation of Integrin-mediated Adhesion of T Lymphocytes and Monocytes: Two Mechanisms with Divergent Biological Consequences

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Summary

We show that the adhesion of T lymphoid cells to immobilized fibronectin can be increased by two distinct mechanisms. The first is by increasing the affinity of the fibronectin receptor/ligand interaction using the anti-$\beta 1$ integrin monoclonal antibody 8A2. The second is by treating the cells with phorbol 12-myristate 13-acetate (PMA), which alters events that occur after receptor occupancy (e.g., cell spreading) without affecting receptor affinity. The effects of these two mechanisms on adhesion in the presence of physiological concentrations of soluble fibronectin suggest that they have different biological consequences. Under these conditions, the net effect of increasing the affinity of the fibronectin receptors is to decrease cell adhesion, whereas the increase in adhesion induced by PMA is unaffected. This suggests that the high affinity receptors are not primarily available for cell adhesion under these circumstances, and that they have an alternative function. We further show that high affinity binding of soluble fibronectin can be induced by either differentiation of the monocytic cell line THP-1 or by cross-linking the T cell receptor complexes on the T lymphoid cell line HUT-78. The differentiated monocytic cells express two populations of fibronectin receptors: a minority in a high affinity state, and the majority in a low affinity state. Thus they will both continue to adhere in the presence of physiological concentrations of soluble fibronectin and bind significant amounts of soluble fibronectin at the cell surface.

T lymphocytes and monocytes need to adhere to extracellular matrix molecules such as fibronectin, collagen, and laminin during migration into sites of inflammation, and these interactions are mediated by members of the integrin family of adhesion receptors (1, 2). The principal fibronectin receptors expressed by peripheral blood mononuclear cells are $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (3), and both are implicated in the migration of lymphoid cells into sites of inflammation (4, 5) and beneath bone marrow–derived stromal cells in culture (6). $\alpha 5\beta 1$ recognizes the central cell-binding domain of fibronectin (7), whereas $\alpha 4\beta 1$ binds to the alternatively spliced V region (8–12). The minimal sequence identified for binding to this region is leucine–aspartic acid–valine (12, 13). $\alpha 4\beta 1$ also recognizes the cell surface receptor vascular cell adhesion molecule 1 (VCAM-1) (14).

Fibronectin is a prominent adhesive ligand that exists in both soluble and insoluble forms (15). Soluble fibronectin is a disulfide linked dimer, ~440 kD, that is present in all body fluids, including plasma (concentration 450–900 nM), amniotic fluid, bronchoalveolar lavage fluid, synovial fluid,

and pleural effusions (15). Insoluble fibronectin multimers are formed by certain cells in culture, resulting in a fibrillar matrix at the cell surface (16, 17). Integrin $\alpha 5\beta 1$ is required for assembly of this matrix (18–20). A similar fibrillar matrix of fibronectin is in stromal or reticular connective tissue, in basement membranes, around muscle fibers, and in the walls of blood vessels (15).

The affinity of the integrins for soluble ligands is highly regulated. This has been demonstrated in ligand binding studies with $\alpha IIb\beta 3$ (21–24), $\alpha M\beta 2$ (25) and $\alpha 5\beta 1$ (26, 27). The increase in affinity of $\alpha 5\beta 1$ for soluble fibronectin was induced by the anti-$\beta 1$ monoclonal antibody 8A2 (28), which is one of a group of antibodies that increase $\beta 1$-mediated cellular adhesion to immobilized ligands (29–31). Other means of stimulating $\beta 1$-mediated adhesion have also been described, including cell stimulation with phorbol esters or by cross-linking a variety of cell surface receptors (32–38). However, these studies did not directly measure the affinity of the receptor-ligand interaction, and so it is uncertain whether the increase in adhesion is due to changes in affinity or due

to an effect on events that occur following receptor occupancy (e.g., cell spreading). Similar stimuli and monoclonal antibodies also promote $\beta2$-mediated cell aggregation or adhesion to immobilized ligands (39–46).

In this paper we examine how regulation of integrin function impacts on the adhesion of T lymphocytes and monocytes to fibronectin. The adhesion to fibronectin mediated by integrins $\alpha4\beta1$ and $\alpha5\beta1$ could be affected by either altering the receptors' affinity for fibronectin or by altering events that follow ligand binding, and the presence of soluble fibronectin has dramatically different effects on cell adhesion induced by these two mechanisms. In addition, we have studied two physiological mechanisms for inducing high affinity fibronectin binding. The first is the differentiation of monocytic cells, where we find that the number of high affinity fibronectin receptors expressed by the differentiated cells constitutes only a minority of the total number of fibronectin receptors on the cell surface. The second mechanism for inducing high affinity fibronectin binding is by cross-linking the TCR complex on T lymphoid cells.

Materials and Methods

Antibodies and Reagents. Production, characterization, and purification of the murine anti–human $\beta1$ monoclonal antibody 8A2 (IgG1-κ isotype) has been described (27, 28). The concentration of purified IgG was determined by absorbance at 280 nM (1 OD = 0.8 mg/ml) (47). The rat anti–human $\alpha5$ monoclonal antibody Ab 16 (20) was used at a concentration of 20 μg/ml. The murine anti–human $\alpha4$ monoclonal antibody HP2/1 (48) was used as a 1:10 dilution of tissue culture supernatant. The murine anti-CD3 monoclonal antibody cell line OKT3 was purchased from the American Type Culture Collection (ATCC; Rockville, MD) and the antibody used as purified IgG. Phorbol 12-myristate 13-acetate (PMA) and cytochalasin D were purchased from Sigma Chemical Company (St. Louis, MO).

Cells. The T lymphoid cell lines Molt-4 and HUT-78 were purchased from the ATCC, and were maintained in RPMI medium (Biowhittaker Inc., Walkersville, MD) supplemented with 10% FCS (Biowhittaker Inc.), 1% glutamine, and 1% penicillin and 1% streptomycin (both from Sigma Chemical Co.). The monocytic cell line THP-1 was purchased from the ATCC, and maintained in the same medium with the addition of 20 mM 2-ME (Sigma Chemical Co.). Human T lymphocytes were purified from the peripheral blood of normal donors by centrifugation on a Ficoll-Paque gradient (Pharmacia LKB, Piscataway, NJ), panning for monocytes on serum-coated dishes, and passaging over a nylon wool column (49).

Stimulation of Cells. Cells were incubated with the indicated concentrations of PMA or 8A2 for the duration of the assays (30 min). THP-1 cells were differentiated by the addition of 100 nM of PMA to the culture medium for 48 h before the assays. HUT-78 cells were stimulated by cross-linking their TCR with OKT3 IgG (10 μg/ml) for 5 min at 22°C, followed by mixing with the cross-linking antibody (affinity isolated polyclonal goat anti–mouse IgG [1 μg/ml; Sigma Chemical Co.]) for the 30-min duration of the assay.

Flow Cytometry. Cells were washed twice at 4°C with buffer (PBS, pH 7.4, plus 1% FCS and 0.02% sodium azide), resuspended at 10×10^6 cells/ml, and 50 μl incubated with the same volume of primary antibody for 20 min at 4°C. The cells were washed once in excess cold buffer, and incubated with a 1:200 dilution of secondary antibody (FITC-labeled goat anti–mouse IgG or FITC-

labeled goat anti–rat IgG, Tago, Inc., Burlingame, CA) for 20 min at 4°C in the dark. Background fluorescence was determined by incubating an aliquot of cells with secondary antibody alone. After a further wash in excess cold buffer, the cells were resuspended in 500 μl of the same buffer and analyzed immediately on a FACScan® (Becton Dickinson & Co., Mountain View, CA), using the Lysis II program. To determine the effect of the 8A2 antibody on the expression of $\alpha5$ on differentiated THP-1, one aliquot of the cells was also incubated with Ab16 plus 100 nM of 8A2 Fab fragments (27), followed by the FITC-labeled goat anti–rat secondary antibody. The expression with and without 8A2 was analyzed as above, and the result expressed as a mean fluorescence index where the background fluorescence was normalized to a value of 1.

Purification of Human Fibronectin. Fibronectin was isolated from fresh human citrated plasma by affinity chromatography on gelatin-Sepharose (Pharmacia LKB), as previously described (27). The isolated fibronectin yielded a single band on SDS-PAGE under nonreducing conditions, and a closely spaced doublet of 215,000 and 230,000 daltons under reducing conditions, consistent with the reported properties of plasma fibronectin (50). The concentration of fibronectin was determined by absorbance at 280 nM (1 mg/ml = OD 1.3) (27).

Radiolabeling of Fibronectin. The fibronectin was labeled with ^{125}I by a modified Chloramine-T procedure, as previously described (27). Greater than 90% of the radioactivity was precipitated by 10% trichloroacetic acid, and the radiolabeling does not alter the affinity of fibronectin binding to an integrin (23). Aliquots were stored at -70°C after the addition of BSA to 1% final concentration.

Binding Assays. The binding of ^{125}I-labeled fibronectin was performed as previously described (27). The standard buffer used in the assays was modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO$_3$, 1 mg/ml BSA, 1 mg/ml glucose, and 2 mM MgCl$_2$), pH 7.4, and the incubation time was 30 min at 37°C. A typical binding assay, performed in a 1.5-ml microcentrifuge tube (Sarstedt, Germany), was in a 200 μl vol composed of 120 μl of cells (2 × 10⁶/tube), 40 μl of radiolabeled fibronectin, and 40 μl of stimulus (8A2 or PMA) and/or inhibitor (EDTA, excess cold fibronectin, or monoclonal antibody). After the incubation, 50-μl aliquots were layered in triplicate onto 300 μl of 20% sucrose in the same buffer in microfuge tubes (West Coast Scientific Inc., Hayward, CA), and centrifuged for 3 min at 12,000 rpm in a microfuge (model 11; Beckman Instruments, Fullerton, CA). The tips were amputated and counted, and the bound protein was calculated from the specific activity of the ligand (assuming a molecular weight for fibronectin of 440,000 daltons). The residual bound radioactivity in the presence of either 50× excess cold fibronectin or 5 mM EDTA was subtracted as nonspecific binding. The data was fitted to equilibrium binding models using the LIGAND program (51).

Assays of Cell Adhesion to Immobilized Fibronectin. Cell adhesion to immobilized fibronectin was quantified as previously described (27). Fibronectin (50 μl/well) at the concentrations indicated in the text was coated onto a 96-well Immulon II plate (Dynatech Laboratories, Inc., Chantilly, VA) for 2 h at 37°C, and then the wells were blocked with 1% BSA in PBS for 1 h at 37°C. The buffer used in the adhesion assay was the same as in the binding assays. In the assays using soluble fibronectin the cells were preincubated with 8A2 or PMA and soluble fibronectin for 30 min at room temperature, before addition to the wells. Where indicated, cells were preincubated with cytochalasin D for 30 min at 37°C. At the commencement of the assay the cells (concentration 10⁶ cells/ml) were added to the wells in 100-μl aliquots with appro-

priate concentrations of stimulus or inhibitor (PMA, 8A2, blocking antibody). After a 30-min incubation at 37°C, the nonadherent cells were washed off with 3 rounds of gentle pipetting. The residual adherent cells were checked by visual inspection, and quantified with a colorimetric reaction using endogenous cellular acid phosphatase activity (52). Optical densities were determined using an ELISA plate reader (Molecular Devices Corp., Menlo Park, CA) with a 410-nM filter. Background values, determined in wells coated with 1% BSA alone, were subtracted from each point. Adherence was expressed as a percentage of the number of cells originally added to each well, and determined from a standard curve generated using known numbers of cells.

Photography. Peripheral blood T lymphocytes were purified and allowed to attach to the bottom of microtitre wells coated with fibronectin (concentration 5 μg/ml) for 30 min at 37°C. Before washing they were photographed at 200× magnification using an inverted phase contrast microscope (model CK-2, Olympus, Tokyo, Japan) with a Polaroid camera attachment.

Results

PMA and the Anti-β1 Monoclonal Antibody 8A2 Stimulate T Cell Adhesion to Fibronectin by Different Mechanisms. To investigate the regulation of T lymphoid cell adhesion to fibronectin we first studied the cell line Molt-4. The fibronectin receptors expressed by these cells are α4β1 and α5β1, and their relative levels on the cell surface as determined by flow cytometry were comparable to those on peripheral blood T lymphocytes (Fig. 1). Molt-4 cells adhered slightly to fibronectin, and both PMA and antibody 8A2 induced a dose-dependent increase in the adhesion (Fig. 2 a). Similar effects were observed with purified peripheral blood T lymphocytes (not shown). Basal and stimulated adhesion were both dependent on the presence of bound fibronectin and completely inhibited by a combination of blocking monoclonal antibodies directed against α4β1 and α5β1, as previously described (28). To investigate the mechanism of increased adhesion we measured the effect of these stimuli on the affinity of soluble fibronectin binding. Surprisingly, PMA failed to stimulate
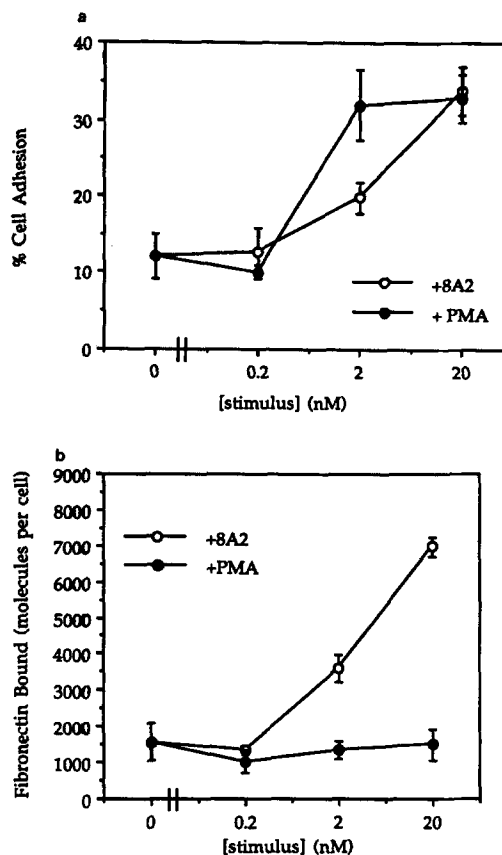
Figure 2. PMA induces Molt-4 adhesion to immobilized fibronectin without increasing the affinity of binding of soluble fibronectin. (a) The effect of increasing concentrations of PMA and 8A2 on adhesion of Molt-4 cells to fibronectin (2.5 μg/ml) was determined as described in Materials and Methods. The results are expressed as the percentage of the cells added to each well that remain adherent after washing, and are the mean ± SD of three determinations. (b) The effect of the same concentrations of PMA and 8A2 on the binding of ^{125}I-labeled soluble fibronectin to Molt-4 cells was determined as described in Materials and Methods. The results shown are the estimated specific binding, and are the mean ± SD of three determinations.
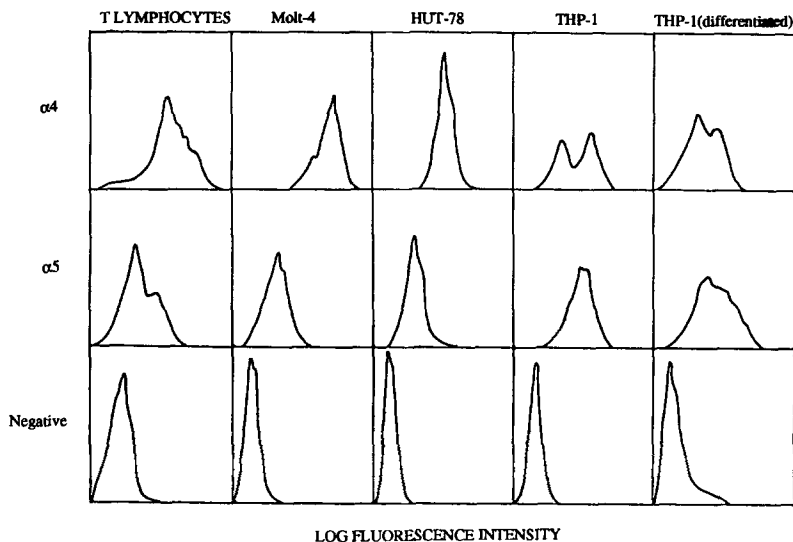
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Figure 1. Flow cytometric analysis of the expression of integrin α4 (using HP2/1) and α5 (using Ab16) chains by purified peripheral blood T lymphocytes, Molt-4 cells, HUT-78 cells, THP-1 cells, and THP-1 cells after 48 h of differentiation with PMA. Flow cytometry was performed as described in the Materials and Methods.
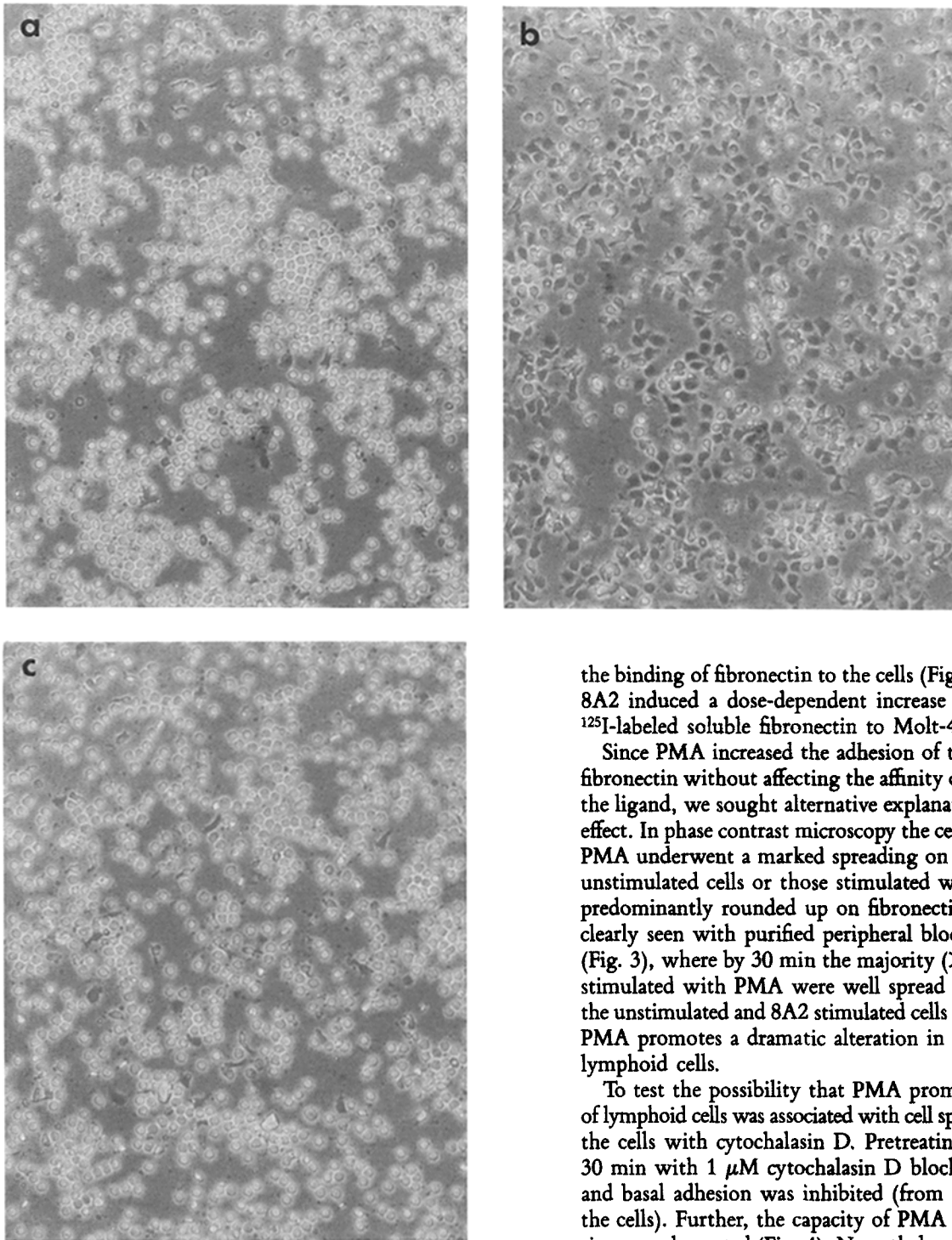
Figure 3. PMA selectively induces the spreading of peripheral blood T lymphocytes on immobilized fibronectin. Human peripheral blood T lymphocytes were isolated as described in Materials and Methods, and allowed to adhere to immobilized fibronectin (5 µg/ml) for 30 min at 37°C in the absence of stimulus (a), or in the presence of PMA (b) or 8A2 (c). The cells were photographed using an inverted phase contrast microscope (CK-2; Olympus, Tokyo, Japan) with a Polaroid camera attachment.

the binding of fibronectin to the cells (Fig. 2 b). In contrast, 8A2 induced a dose-dependent increase in the binding of ^{125}I-labeled soluble fibronectin to Molt-4 cells.

Since PMA increased the adhesion of the Molt-4 cells to fibronectin without affecting the affinity of the receptors for the ligand, we sought alternative explanations for the PMA effect. In phase contrast microscopy the cells stimulated with PMA underwent a marked spreading on fibronectin, while unstimulated cells or those stimulated with 8A2 remained predominantly rounded up on fibronectin. This was most clearly seen with purified peripheral blood T lymphocytes (Fig. 3), where by 30 min the majority (>65%) of the cells stimulated with PMA were well spread whereas <10% of the unstimulated and 8A2 stimulated cells were spread. Thus, PMA promotes a dramatic alteration in the shape of these lymphoid cells.

To test the possibility that PMA promotion of adhesion of lymphoid cells was associated with cell spreading, we treated the cells with cytochalasin D. Pretreating Molt-4 cells for 30 min with 1 µM cytochalasin D blocked cell spreading, and basal adhesion was inhibited (from 19 ± 5 to 0% of the cells). Further, the capacity of PMA to stimulate adhesion was abrogated (Fig. 4). Nevertheless, the 8A2 antibody still induced increased adhesion.

Based on the foregoing results, PMA and 8A2 appear to exert their effects on different steps in the adhesion process. To test this hypothesis, we examined their combined effect on adhesion. In the presence of limiting amounts of immobilized fibronectin, the combination of maximal doses of PMA and 8A2 increased adhesion more effectively than either alone (Fig. 5). The combination of 8A2 and PMA had no greater effect on the binding of soluble fibronectin than 8A2 alone
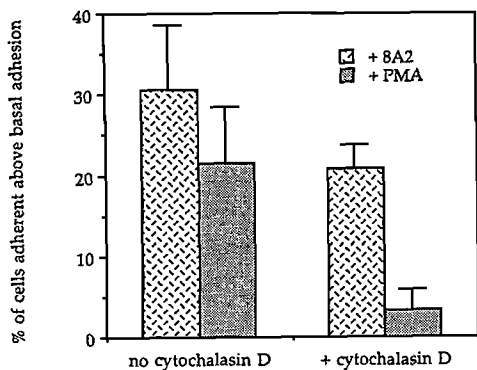
Figure 4. Cytochalasin D pretreatment of Molt-4 cells selectively inhibits PMA-induced adhesion to immobilized fibronectin. Cells were preincubated with cytochalasin D (1 μM) for 30 min at 37°C, and then the effects of PMA (20 nM) and 8A2 (20 nM) on adhesion to fibronectin (2.5 μg/ml) determined as described in Materials and Methods. In the absence of stimulus 19 ± 5% of the cells remained adherent without cytochalasin D, and 0% of the cells remained after cytochalasin D pretreatment. The results are expressed as the number of cells adherent above the basal level of adhesion, and are the mean ± SD of three determinations.
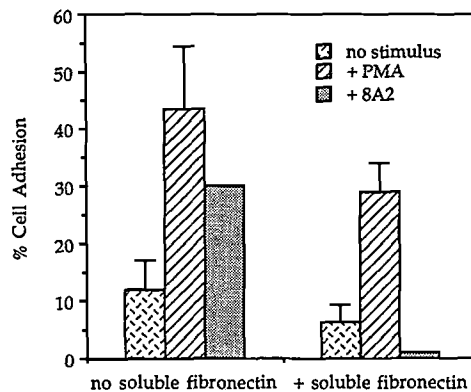

Figure 6. PMA and 8A2 have opposite effects on adhesion of Molt-4 cells to immobilized fibronectin in the presence of a physiological concentration of soluble fibronectin. Cells were preincubated for 30 min at 37°C with PMA (20 nM), 8A2 (20 nM), or neither, and with or without 750 nM soluble fibronectin. Adhesion to immobilized fibronectin (2.5 μg/ml) was then determined as described in Materials and Methods. The results are expressed as the percentage of the cells added to each well that remain adherent after washing, and are the mean ± SD of three determinations.

(3308 ± 321 fibronectin molecules bound per cell with both vs. 3296 ± 71 with 8A2 alone).

PMA and 8A2 Have Opposite Effects on Adhesion in the Presence of Physiological Concentrations of Soluble Fibronectin. In the foregoing experiments we characterized two mechanisms of promoting cell adhesion to fibronectin. PMA increased adhesion by altering cell shape, whereas 8A2 increased the affinity of the receptors for the fibronectin. We next investigated the biological implications of these alternative mechanisms by performing the adhesion assay in the presence of a physiological plasma concentration of soluble fibronectin (750 nM) (Fig. 6). In the absence of soluble fibronectin, PMA induced a fourfold (11–43%) increase in adhesion and 8A2 induced a threefold increase (to 30%). Addition of soluble fibronectin profoundly altered the adhesion response to 8A2. Under these conditions, 8A2 inhibited adhesion (7–1%). In
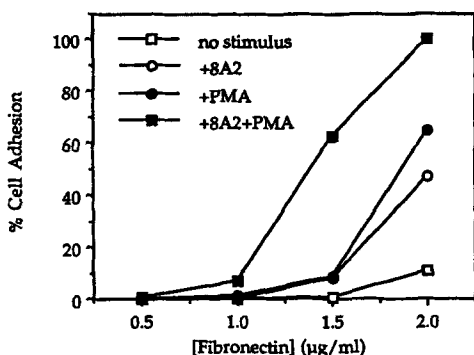

Figure 5. PMA and 8A2 have additive effects on the adhesion of Molt-4 cells to immobilized fibronectin. Adhesion of Molt-4 cells to increasing concentrations of fibronectin in the absence of stimulus, or in the presence of PMA (20 nM), 8A2 (20 nM), or both together was determined as described in Materials and Methods.

contrast, PMA stimulation still resulted in a fourfold enhancement of adhesion (7–29%).

Physiologic Modulation of the Affinity of α5β1. The foregoing experiment suggested that high affinity fibronectin receptors do not promote adhesion to immobilized fibronectin in the presence of physiologic concentrations of soluble fibronectin. To gain insight into the potential functions of high affinity receptors, we assessed the physiological circumstances in which they were induced.

We first studied the monocytic cell line THP-1, which takes on a number of macrophage-like characteristics when induced to differentiate by treatment with phorbol esters (53, 54). These include changing from a suspension to an adherent phenotype in culture, with a moderate degree of spreading. THP-1 cells express approximately equal amounts of α4β1 and α5β1, and the relative amounts did not significantly change with differentiation (Fig. 1). Adhesion of the cells to fibronectin was blocked by a combination of antibodies directed against these two receptors (not shown).

Undifferentiated THP-1 cells bound soluble fibronectin with low affinity (K_d = 919 nM), and the addition of a saturating concentration of 8A2 IgG (20 nM) resulted in an ∼50-fold higher fibronectin binding affinity (K_d = 18.2 nM) (not shown). The binding of soluble fibronectin was almost completely dependent on α5β1, as an antibody directed against this receptor (Ab 16) inhibited binding by ∼90%, whereas an anti-α4β1 monoclonal antibody (HP2/1) had little effect (Fig. 7 a). In other experiments, we found that Ramos B lymphoid cells, which express α4β1 and not α5β1, did not bind fibronectin with high affinity in physiological buffers, even in the presence of 8A2 (Faull, R., unpublished observations).

48 h after addition of 100 nM PMA, THP-1 cells bound soluble fibronectin with high affinity (K_d = 10.2 nM) to ∼2.7 × 10⁴ binding sites per cell (Fig. 8). Again the
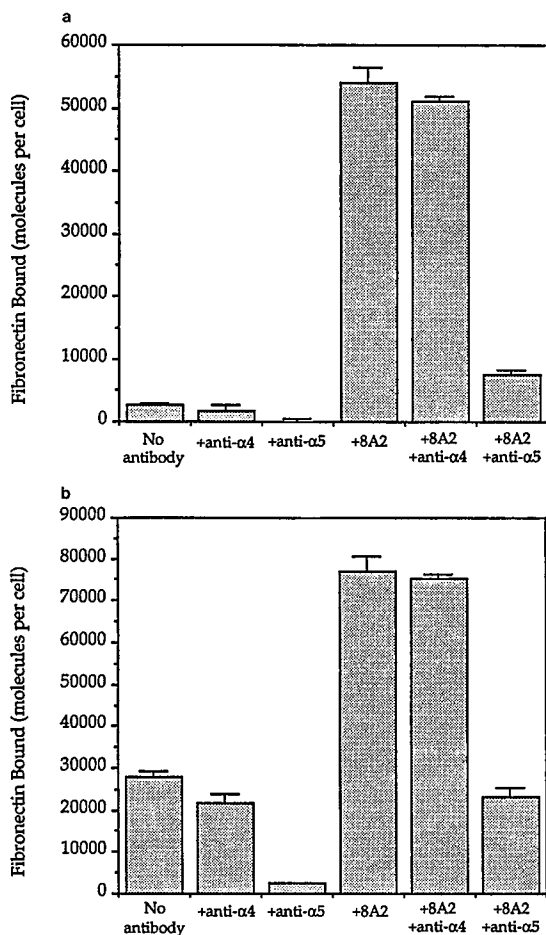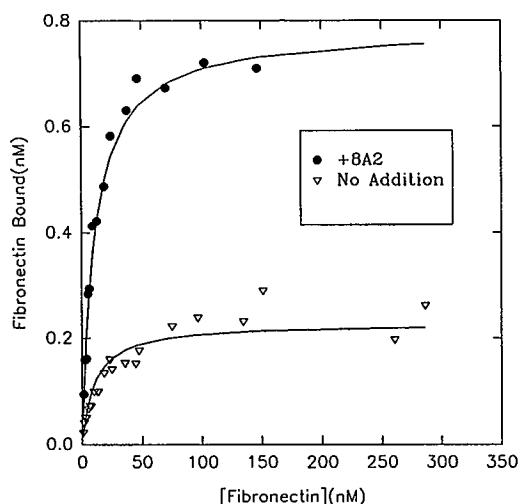
Figure 8. Specific binding of increasing concentrations of soluble fibronectin to differentiated THP-1 cells, either in the presence or absence of 8A2 (20 nM whole IgG). Cells were differentiated and the binding of ^{125}I-labeled soluble fibronectin was determined as described in Materials and Methods. The results shown are the estimated specific binding.

Figure 7. Binding of soluble fibronectin to (a) undifferentiated and (b) differentiated THP-1 cells is primarily dependent on $\alpha5\beta1$. (a) Fibronectin binding to undifferentiated THP-1 cells in the presence or absence of 8A2 (20 nM), and blocking monoclonal antibodies against $\alpha4\beta1$ (HP2/1) or $\alpha5\beta1$ (Ab 16). (b) Binding to differentiated THP-1 cells in the presence of the same antibodies. Cells were differentiated and the binding of ^{125}I-labeled soluble fibronectin was determined as described in Materials and Methods. The results shown are the estimated specific binding, and are the mean ± SD of three determinations.

fibronectin was predominantly bound to $\alpha5\beta1$ (Fig. 7 b). In the presence of 8A2 (20 nM) the soluble fibronectin was bound with similar affinity (K_d = 9.34 nM), but the number of high affinity binding sites increased ~3.4-fold (9.4 × 10⁴ binding sites/cell). The increase in number of high affinity binding sites was highly significant by constrained parameter (51) curve fitting (F = 160.83, df = 31, P = 0). In contrast, the difference in receptor affinity was not significant (F = .16, df = 31, P = 0.692). At higher input concentrations of radiolabeled fibronectin, binding to a separate population of low affinity receptors could also be detected (not shown). The 8A2-induced binding of fibronectin was still predominantly to $\alpha5\beta1$ rather than to another receptor (Fig. 7 b). The 8A2 had no significant effect on the number of $\alpha5\beta1$ receptors expressed on the cell surface, as determined by flow cytometry using Ab16 (mean fluorescence index

without 8A2 = 19.2 and with 8A2 = 20.2; see Materials and Methods). Thus, differentiation of THP-1 cells results in induction of $\alpha5\beta1$ with high affinity for fibronectin. Nevertheless, ~70% of the $\alpha5\beta1$ are still in the low affinity state.

We next used the T lymphoid cell line HUT-78 in order to examine the relationship between adhesion to immobilized fibronectin and binding of soluble fibronectin after cell activation by cross-linking the TCR. The HUT-78 cells express $\alpha4\beta1$ and $\alpha5\beta1$ as their predominant fibronectin receptors, and the ratio of the two is similar to that found on peripheral blood T lymphocytes (Fig. 1). Stimulation with either 8A2 or by cross-linking the TCR enhanced HUT-78 adhesion to fibronectin (Fig. 9 a), and neither stimulus induced visible changes in cell spreading. The unstimulated and stimulated adhesion were blocked by a combination of antibodies against $\alpha4\beta1$ and $\alpha5\beta1$, analogous to the Molt-4 cells (not shown). Both TCR cross-linking and 8A2 also increased the binding of soluble fibronectin to the cells (Fig. 9 b). In contrast, PMA had no such effect (data not shown).

Discussion

We draw the following conclusions from these experiments: (a) T lymphoid cell adhesion to fibronectin can be stimulated either by increasing the affinity of fibronectin binding to receptors or by altering events that occur following receptor occupancy. (b) Increasing the affinity of fibronectin receptors in the presence of soluble fibronectin may decrease cell adhesion. In contrast, postoccupancy mechanisms of increasing adhesion are unaffected by soluble fibronectin. (c) Differentiation of monocytic cells or stimulation of T lymphoid cells by cross-linking their TCR induces high affinity binding of soluble fibronectin. (d) Differentiated monocytic cells express
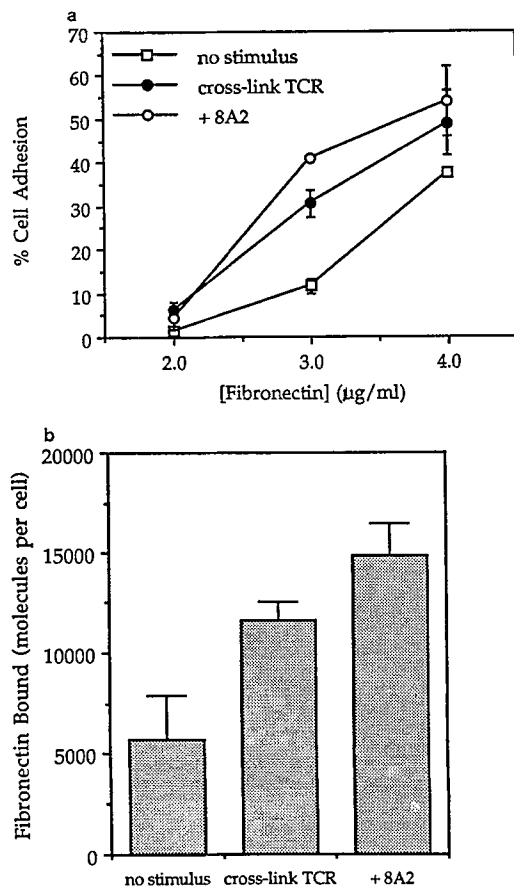
Figure 9. Cross-linking the TCR increases adhesion of the T lymphoid cell line HUT-78 to immobilized fibronectin and increases the binding of soluble fibronectin. Cell stimulation by cross-linking the TCR was performed as described in Materials and Methods. (a) The effects of cross-linking the TCR or 8A2 (20 nM) on the adhesion of HUT-78 cells to increasing concentrations of immobilized fibronectin were determined as described in Materials and Methods. The results are expressed as the percentage of the cells added to each well that remain adherent after washing, and are the mean ± SD of three determinations. (b) The effects of cross-linking the TCR or 8A2 (20 nM) on the binding of 100 nM ^{125}I-labeled fibronectin to HUT-78 cells were determined as described in Materials and Methods. The results shown are the estimated specific binding, and are the mean ± SD of three determinations.

two populations of fibronectin receptors: a minority in a high affinity state and the majority in a low affinity state. Thus they will continue to adhere in the presence of physiological concentrations of soluble fibronectin.

Adhesion of T lymphoid cells to fibronectin can be increased by two distinct mechanisms. The first is through conversion of the receptors to a high affinity form. Secondly, stimuli such as PMA enhance adhesion without any measurable change in the affinity for soluble fibronectin, and the increase in adhesion is associated with an increase in cell spreading. Cytochalasin D blocked the change in cell shape and also abrogated the increased adhesion. The 8A2 and PMA had an additive effect on cell adhesion, showing that in situations of limiting availability of immobilized ligand optimal cell adhesion requires both high affinity receptors and efficient post-

receptor occupancy events. This is analogous to $\alpha IIb\beta3$, where the "inactive" (low affinity) form is competent for adhesion to immobilized fibrinogen (55, 56) but does not bind soluble fibrinogen. Cytoskeletal organization and subsequent cell spreading dramatically increase the resistance of cells to shear stresses, resulting in stronger adhesion under experimental conditions (57–59). Phorbol esters are known to affect these events. For example, they stimulate associations between integrins and the cytoskeleton (60), and induce shape changes in neutrophils (61), and both PMA-induced macrophage adhesion to laminin (62) and $\alpha L\beta2$-mediated intercellular adhesion (63, 64) are dependent on cytoskeletal assembly. In addition, PMA stimulation of adhesion of Chinese hamster ovary cells to fibronectin does not depend on an increase in the affinity for soluble fibronectin (65). Activation of T lymphocytes by cross-linking their TCR also stimulates association between integrins and the cytoskeleton (66).

Nevertheless, while brief stimulation of these cells with PMA increases their adhesion to fibronectin without activating their fibronectin receptors, it is known that PMA stimulation induces high affinity forms of the integrins $\alpha IIb\beta3$ on platelets (24) and $\alpha M\beta2$ on monocytes (25) and neutrophils (67). This conformational sensitivity to PMA may be integrin dependent, and not a general property of all members of the family. Alternatively, the effects of PMA may be cell-type dependent, and it is significant that $\alpha IIb\beta3$ cannot be activated by PMA (or other platelet agonists) when expressed by other cells (e.g., Chinese hamster ovary cells) (68).

The net influence of soluble fibronectin on stimulated cell adhesion is dependent on the mechanism of stimulation. This has potential implications for the regulation of cell adhesion in vivo, as cells within the circulation are continuously exposed to soluble fibronectin at a concentration of 450–900 nM (69, 70). Fibronectin is also abundant in the extravascular fluids, although the concentration has only been measured in limited situations (e.g., synovial fluid, amniotic fluid, pleural effusions) (15). At the concentration of soluble fibronectin used in these experiments (750 nM) there is a modest inhibition of unstimulated adhesion due to partial occupancy of the low affinity receptors. The receptors remain in a low affinity state in the presence of PMA, so that soluble fibronectin also causes modest inhibition. Nevertheless, there remains a clearcut increase in adhesion compared to the unstimulated cells. In marked contrast, the 8A2 blocks adhesion, as >90% (calculated) of the high affinity receptors are occupied at this concentration of soluble fibronectin. Hence, high affinity fibronectin receptors do not mediate adhesion in the presence of plasma concentrations of soluble fibronectin, suggesting that they have an alternative function. Interestingly, high affinity fibronectin binding occurs almost exclusively to $\alpha5\beta1$, and the $\alpha4\beta1$ remains a low affinity receptor. Therefore $\alpha4\beta1$ remains available to mediate cell adhesion to both fibronectin and vascular cell adhesion molecule 1 in the presence of soluble fibronectin, and PMA can still increase $\alpha4\beta1$-mediated adhesion. This implies that cells use the low affinity receptors for adhesion under these circumstances, and that adhesion can still be stimulated by an effect on the post-receptor occupancy events. This is analogous to the observation that

stiffening of neutrophils, due to organization of the cytoskeleton induced by chemoattractants, may underlie their sequestration in lung and other capillaries during acute inflammation (71).

Both of the physiological inducers of high affinity fibronectin binding that we have studied are models for events that occur as those cell types (monocytes and T lymphocytes) become tissue resident during inflammation. This suggests that the high affinity fibronectin receptors have a specific role in this process. One possibility is that they promote assembly of a fibronectin matrix, enabling the cells to persist at the site of inflammation. For example, differentiation of F-9 teratocarcinoma stem cells in culture by retinoic acid is accompanied by the accumulation of fibrillar fibronectin deposits (72), suggesting that the fibronectin receptors have become competent to bind soluble fibronectin. In contrast, transformation of chicken embryo fibroblasts with Rous sarcoma virus is associated with loss of cell surface fibronectin and decreased interaction of the receptors with talin and fibronectin (73, 74).

The heterogeneity of the $\alpha 5\beta 1$ on the differentiated THP-1 cells suggests that it is important that the cells maintain a population of low affinity $\alpha 5\beta 1$ receptors, possibly for adhesion and subsequent detachment during cell migration. Evidence for functional heterogeneity of integrins has been reported in other studies. For example, cultured human endothelial cells express integrins (including $\alpha 5\beta 1$) on both their basal and luminal aspects (75), but only assemble a fibronectin matrix at their basolateral surfaces. This suggests that only the receptors at this site are capable of binding soluble fibronectin with high affinity (76). In another study, two distinct populations of $\beta 1$ integrins were identified on chicken embryo fibroblasts using a differential extraction procedure (77), and the fraction associated with focal contacts exhibited significantly higher phosphorylation. Finally, heterogeneity is not confined to the $\beta 1$ integrins, as an active subpopulation of $\alpha M\beta 2$ was defined on neutrophils using a monoclonal antibody that binds to an activation-specific epitope (67).

References

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