Interaction of Leukocyte Integrins with Ligand Is Necessary but Not Sufficient for Function

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Abstract. The leukocyte integrins (CD11/CD18 or β_2-type integrins) are expressed exclusively on leukocytes and participate in many adhesion-dependent functions (Arnaout, M.A. 1990. *Blood.* 75:1037–1050; Springer, T. A. 1990. *Nature. (Lond.)* 346:425–434; Dustin, M. L., and T. S. Springer. 1991. *Annu. Rev. Immunol.* 9:27–66). The avidity of leukocyte integrin binding to their ligands or counter-receptors is dependent upon response to intracellular signals (Wright, S. D., and B. C. Meyer. 1986. *J. Immunol.* 136:1759–1764; Dustin, M. A., and T. S. Springer. 1989. *Nature (Lond.).* 341:619–624). We have investigated the effects of a novel mAb (mAb 24) which defines a leukocyte integrin α subunit epitope that is Mg^{2+}-dependent and may be used as a "reporter" of the activation state of these receptors (Dransfield, I., and N. Hogg. 1989. *EMBO (Eur. Mol. Biol. Organ) J.* 8:3759–3765; Dransfield, I., A.-M. Buckle, and N. Hogg. 1990. *Immunol. Rev.* 114:29–44; Dransfield, I., C. Cabañas, A. Craig, and N. Hogg. 1992. *J. Cell Biol.*) Data is presented to show that this mAb inhibits monocyte-dependent, antigen-specific T cell proliferation and IL-2–activated natural killer cell assays which are both dependent on lymphocyte function–associated antigen-1 (LFA-1), and complement receptor type 3 (CR3)-mediated neutrophil chemotaxis to f-Met-Leu-Phe. This inhibitory effect is not caused by the prevention of receptor/ligand binding because LFA-1/ICAM-1, LFA-1/ICAM-2,3 and CR3/iC3b interactions are, under activating conditions, promoted rather than blocked by mAb 24. As it does not interfere with mitogen-stimulated T cell proliferation, it is unlikely that mAb 24 transduces a "negative" or antiproliferative signal to the T cells to which it is bound. Using a model system of transient activation of LFA-1, we have found that mAb 24 prevents "deadhesion" of receptor/ligand pairs, possibly locking leukocyte integrins in an "active" conformation. It is speculated that inhibition of leukocyte integrin function by this mAb reflects the necessity for dynamic leukocyte integrin/ligand interactions.

THE three leukocyte integrins, lymphocyte function–associated antigen-1 (LFA-1), complement receptor type 3 (CR3)/Mac-1, and p150,95 are heterodimeric molecules each comprising a unique α subunit noncovalently associated with a common β_2 subunit (Sanchez-Madrid et al., 1983). The roles of these molecules in immune functions are best characterized for LFA-1(CD11a/CD18) and CR3-(CD11b/CD18). Antibody blocking studies have demonstrated a key role for LFA-1 in many cellular interactions of leukocytes. CR3 is important in functions such as myeloid cell phagocytosis, adherence to activated endothelium and chemotaxis (Arnaout, 1990; Springer, 1990; Smith et al., 1989). These activities are thought to represent in vitro correlates of the response to tissue inflammation.

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1. *Abbreviations used in this paper*: CR3, complement receptor type 3; ICAM, intercellular adhesion molecule; iC3b-E, iC3b-coated erythrocytes; LAK, lymphokine-activated killer; LFA-1, lymphocyte function–associated antigen-1; PBMC, peripheral blood mononuclear cells; PdBu, phorbol-12,13-dibutyrate; PHA, phytohaemagglutinin.

The avidity of CR3 for ligand can be regulated by cellular activation as shown by studies using phorbol esters and other stimulants such as f-Met-Leu-Phe (Wright and Meyer, 1986; Buyon et al., 1988; Lo et al., 1989). In addition, the interaction of LFA-1 with its ligand, termed intercellular adhesion molecule-1 (ICAM-1, CD54) (Rothlein et al., 1986), is transiently induced in response to T cell receptor cross-linking using CD3 mAbs, thus, providing a mechanism for strengthening initial antigen-specific T cell-target interactions (Dustin and Springer, 1989; van Kooyk et al., 1989). Activation with phorbol ester or through other T cell surface molecules and their ligands (e.g., CD2/LFA-3) results in a more prolonged LFA-1/ICAM-1 interaction (van Kooyk et al., 1989). As similar results using T cells have been obtained for VLA-4, VLA-5, and VLA-6 (Shimizu et al., 1990), different integrins may employ common activation pathways.

We have recently described a unique mAb (24) that binds to an epitope present on the α subunits of all three leukocyte integrins (Dransfield and Hogg, 1989; Dransfield et al., 1990, 1991). Recognition of this epitope in the intact heterodimer is Mg^{2+}-dependent and for intact cells, expression parallels receptor activity. We have therefore suggested that

control of the affinity of Mg^{2+} binding represents a potential mechanism for regulation of receptor function (Dransfield and Hogg, 1989). The effect of mAb 24 on leukocyte integrin function has been analyzed using three systems which are leukocyte integrin-dependent, namely, antigen presentation by monocytes to T cells (Dougherty and Hogg, 1987) and lymphokine-activated killer (LAK) cell cytotoxicity assays (Robertson et al., 1990) which are both LFA-1 dependent plus f-Met-Leu-Phe-stimulated chemotaxis of neutrophils, a response that has been found to be CR3-dependent (Anderson et al., 1986). These functions were inhibited by mAb 24, implicating the recognized epitope as a functionally important region of the leukocyte integrins. Surprisingly, when activated leukocyte integrins are binding to ligand, mAb 24 promoted rather than inhibited, indicating that functional effects of antibody occur after receptor occupancy. Studies using a model system for analysis of sequential activation and deactivation of LFA-1 function suggest that mAb 24 acts to prevent deactivation events, possibly by locking LFA-1 in the ligand-bound "active" form.

Materials and Methods

Monoclonal Antibodies

Antibodies used in this study were mAb 24 (Hogg and Selvendran, 1985; Dransfield and Hogg, 1989), 38 (CD11a) (Dransfield and Hogg, 1989), 44 (CD11b) (Malhotra et al., 1986), 14 (CD8) and MOPC21, a gift from Dr. Gordon Ross (University of Louisville, Louisville, KY). MAb 15.2 was characterized as an ICAM-1 mAb (CD54) by positive staining of ICAM-1-transfected L cells, immunoprecipitation, cell binding studies, and blocking of LFA-1/ICAM-1-dependent adhesion assays as reported herein. All mAbs were purified by protein A chromatography (Ey et al., 1978). Fab' fragments of mAb 24 were prepared by pepsin digestion (1% wt/wt) of intact IgG1 mAb 24 in order to generate $(Fab')_2$ fragments which were subsequently reduced with 0.01 M cysteine for 1 h at room temperature and blocked for a further hour with 0.03 M iodoacetamide. Any intact IgG and $(Fab')_2$ fragments were separated from Fab'24 by extensive adsorption with protein A-coupled Sepharose followed by fractionation using gel filtration chromatography. Analysis of 10 μg of Fab'24 by SDS-PAGE showed it to be free of any such contaminants (results not shown).

Cells and Cell Lines

The lymphoblastoid B cell line JY (Rothlein et al., 1986), Burkitt's lymphoma cell line Daudi (Robertson et al., 1990), T cell lymphoma cell lines SKW3 and J6 (Rothlein et al., 1986; Staunton et al., 1989) were maintained in RPMI 1640 plus 10% FCS. Activated T cells were expanded from unstimulated peripheral blood mononuclear cells (PBMC) by treatment with phytohaemagglutinin (PHA; 10 μg/ml; Pharmacia Fine Chemicals, Piscataway, NJ) and phorbol-12,13-dibutyrate (PdBu; 50 nM; Calbiochem-Behring Corp., La Jolla, CA) for 48 h. Cells were washed and cultured in RPMI 1640 containing 10% FCS and 10% supernatant from the IL-2 producing cell line MLA. After 1-2 wk of culture, quiescence was induced by removal of IL-2 from the culture medium for 2-3 d. The resulting quiescent T cells serve as a model for analysis of T cell activation (Cantrell and Smith, 1984; Cantrell et al., 1985).

Generation of Antigen- and Mitogen-induced Proliferative Responses

PBMC (2×10^5 cells) were cultured in 200 μl of RPMI-1640 containing 5% heat-inactivated, single donor human AB serum in 96-well flat bottomed tissue culture plates (Becton-Dickinson) in the presence of influenza virus at 1 μg/ml (Dougherty and Hogg, 1987; Dougherty et al., 1988) or mitogens PHA and pokeweed mitogen (Sigma Chemical Co.) at doses of 1 or 0.1 μg/ml. After titration to ensure use of saturating concentrations of antibody, the mAbs 14 (CD8), 44(CD11b), 38 (CD11a), and mAb 24 were used either as purified IgG at 50 μg/ml or as Fab' fragments 20 μg/ml (mAb 24 only). Cultures were pulsed with 1 μCi/well ^{3}H-thymidine for 4 h on day 6 for antigen responses and on day 3 for mitogen responses.

Lymphokine-activated Killer Cell Assay

The LAK cell assay was performed essentially as described by Groscurth et al. (1990) and Robertson et al. (1990) with some modification. Daudi cells were used as targets and were labeled with 0.1 mCi of ^{51}Cr for 90 min in 500 μl of RPMI 2% FCS at 37°C. After two washes in RPMI 1640, the cells were incubated at 37°C for an additional period of 60 min, washed again, and resuspended in RPMI 1640. T cells cultured for 13 or 14 d in the presence of IL-2 (see Cells and Cell Lines) were used as effector cells. The cytotoxicity assay was performed in quadruplicate in 96-well U-bottom plates (Flow Laboratories Inc., McClean, VA), by adding 4×10^4 labeled target cells/well plus various concentrations of effector cells resulting in effector to target cell ratios ranging from 60:1 to 3.7:1 in a final volume of 150 μl. For antibody inhibition studies, purified anti-CD11a mAb 38, mAb 24 and control mAb MOPC 21 were used at 50 μg/ml. After centrifugation at 50 *g* for 2 min, the plates were incubated at 37°C for 5 h. Finally, the plates were centrifuged again for 2 min at 50 *g* and 37.5 μl of supernatant from each well were removed and counted. The relative percentage of lysis was calculated using the formula:

$$100 \times \frac{\text{experimental } ^{51}\text{CR release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{total } ^{51}\text{Cr release} - \text{spontaneous release}}$$

Neutrophil Chemotaxis

Neutrophil chemotaxis was measured using the sub-agarose method (Nelson et al., 1975; Chenoweth et al., 1979) with some modifications. Neutrophils were pretreated for 10 min with mAbs 14, 44, 38, and 24 either as ascitic fluid (1:50) or purified mAbs (IgG, 35 μg/ml or Fab'24, 20 μg/ml). Clean glass microscope slides were dipped in 0.5% gelatin (Sigma Chemical Co.), rinsed and dried, covered with 5 ml of molten 1% agarose (Indubiose)/0.25% gelatin in medium 199 (Flow Laboratories) supplemented with 1% BSA and finally cut with a pattern-former giving two sets of three wells/slide. The neutrophils (8 μl at 5×10^7/ml in medium 199/1% BSA) were added to the central wells and 8 μl aliquots of medium 199/1% BSA, plus or minus f-Met-Leu-Phe (final concentration 10^{-7} M; Sigma Chemical Co.) were added to the two outer wells, respectively. The slides were incubated for 2 h at 37°C, fixed for 18 h in 2.5% glutaraldehyde and stained with Diff-Quik Staining Set (Merz & Dade, Switzerland). Migration distances of duplicate samples were measured by determining the leading edge of neutrophil mobility for both directed and random migration using a Zeiss 14 microscope with 160-fold magnification. Chemotactic response was defined as the specific migration toward the chemoattractant f-Met-Leu-Phe.

Cellular Aggregation Assay

1.5×10^5 JY, SKW3, J6 cell lines or quiescent cultured peripheral blood T cells (see later) in RPMI 1640 containing 10% FCS were added to 96-well tissue culture plates and aggregation was observed after incubation at 37°C for varying periods of time. In some samples, aggregation was induced by addition of 20 nM PdBu. Percentage of aggregating cells was assessed by enumeration of aggregate formation as follows (total number of cells added − number of free cells)/total number of cells added × 100. Each assay was performed in triplicate and a minimum of 400 cells were counted for each sample. mAbs 38 (CD11a), 15.2 (CD54), MHM23 (CD18), and mAb 24 were added before the induction of aggregation and used either as purified IgG (20 μg/ml) or as ascitic fluid (1:100). These amounts were at saturating concentrations as determined by flow cytometric analysis.

iC3b Rosette Formation

Monocyte-derived macrophages were prepared by seeding PBMC into 96-well plates at 2×10^6/ml in RPMI/1% pooled human AB serum. After 1 h, nonadherent cells were removed and adherent monocytes cultured for 4 d in RPMI/1% pooled AB serum before use in binding assays. iC3b-coated erythrocytes (iC3b-E) were prepared from C3b-E (a generous gift of Drs. G. Ross and R. B. Sim) as previously described (Malhotra et al., 1986). iC3b-E were washed twice in $HGVB^{++}$ buffer (20 mM Hepes, pH 7.4, 0.14 M glucose, 71 mM NaCl, 1 g/l gelatin, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and resuspended at 3×10^9/ml in $HGVB^{++}$ and 0.2 mCi of ^{51}Cr (Amersham Inc., Amersham, UK) added. After labeling for 1 h, cells were washed twice in $HGVB^{++}$ before use. 6×10^7 labeled iC3b-E were added to each well containing monocyte-derived macrophages and plates were

spun at 200 *g* for 30 s to bring cells into contact. After incubation at 37°C for 45 min, unbound iC3b-E were removed by washing and surface-bound iC3b-E lysed using 0.84% NH_4Cl and counted for ^{51}Cr using a Beta-plate counter (LKB Instruments, Inc., Uppsala, Sweden). For antibody inhibition studies macrophages were preincubated with saturating concentrations of ascitic fluids (typically 1:100 dilution) of mAbs 44 (CD11b), MHM23 (CD18), or 24. Fab' fragments of mAb 24 were used at a final concentration of 20 μg/ml.

Binding of T Cells to ICAM-1

T cells (>90% CD3 positive) were prepared from PBMC by passage over a nylon wool column of cells that were nonadherent to plastic (Julius et al., 1973) or were expanded in culture with IL-2 (see Cells and Cell Lines). These cells were labeled at 3×10^7 cells/ml in RPMI/10% FCS with 1 mCi of ^{51}Cr (Amersham Corp.), incubated for 30 min on ice in 3 μg/ml of biotinylated mAb UCHT1 (Beverley and Callard, 1981) washed once in ice-cold medium, and then incubated a further 30 min on ice in 10 μg/ml of goat antibiotin antibodies (Sigma Chemical Co.). Alternatively, using a second system of CD3 cross-linking, T cells were incubated with UCHT1 (IgG2a isotype; 1/5 tissue culture supernatant) on ice for 20–30 min before washing and further incubation with goat anti-mouse IgG2a antibodies (1/100; Nordic Immunology, Maidenhead, UK) using similar conditions. These labeled cells (5×10^5 cells/well) were added to 96-well tissue culture plates containing either confluent monolayers of ICAM-1-transfected CHO cells (a gift from D. Sansom and J. Trowsdale, Imperial Cancer Research Fund) or recombinant ICAM-1Fc protein at 160 ng/well as previously described (Dransfield et al., 1992) with the addition of mAb 24 IgG (20 μg/ml) or 24 Fab' fragments (10 μg/ml) in the presence or absence of anti-LFA-1 α subunit mAb 38 (15 μg/ml) or anti-ICAM-1 mAb 15.2 (15 μg/ml). Plates were then centrifuged at 100 *g* for 1 min and incubated for 10, 20, 40, or 110 min at 37°C. Wells were washed five times in warm RPMI-1640 before cell lysis with PBS containing 1% NP-40. Lysates were counted using a Betaplate counter (LKB Instruments, Inc.).

Results

LFA-1-dependent T Cell Proliferative Responses Are Inhibited by mAb 24

The proliferative response of PBMC to the antigen influenza virus is inhibited by mAb 24 and anti-CD11a mAb 38 but not by other control mAbs such as anti-CD11b mAb 44 or CD8 mAb 14 (Fig. 1 *a*). Inhibition of these responses by mAb 24 may occur as a result of cross-linking of surface integrin receptors through the use of whole IgG. However, we show in this study that Fab' fragments as well as intact mAb 24 are inhibitors of leukocyte integrin function, indicating that the observed inhibition is not a result of mAb-induced receptor clustering. In contrast to the effects of mAb 24 upon antigen-specific T cell proliferation, when PBMCs were treated with PHA (Fig. 1 *b*) or pokeweed mitogen (Fig. 1 *c*), at 1 μg/ml, there was no inhibition of T cell responses by mAb 24 (or by CD11a mAb 38). Therefore, mAb 24 was neither cytotoxic for these cells nor responsible for transducing regulatory signals preventing proliferation.

mAb 24 Affects the Size of Cellular Aggregates during Antigen Stimulation

When PBMCs are stimulated over a 6-d time course, with antigens such as influenza virus and purified protein derivative of tuberculin, large aggregates of proliferating cells form (Fig. 2 *a*). Such aggregation can be prevented by CD11a mAb (Fig. 2 *b*) and is not seen in control cultures without added antigen. In the antigen-stimulated samples cultured in the presence of mAb 24, there is formation of small aggregates over the initial time period of 8–10 h not seen in the absence of 24 (Fig. 2 *c*). These small aggregates remain constant in appearance over the assay time period. Furthermore, they do not form in the presence of mAb 24 when CD11a mAb is also present, indicating that LFA-1–dependent adhesions are required (data not shown). These observations suggest that mAb 24 affects normal cellular motility and/or cell–cell contact resulting in an increase in size of cellular aggregates during stimulation by antigen. As a consequence, further progression of the proliferative response may be inhibited.

LAK Cell Cytotoxicity Is Inhibited by mAb 24 at Low E/T Ratios

T lymphoblasts cultured for 12–14 d in the presence of IL-2 were incubated for 5 h with Daudi cells at effector/target (E/T) cell ratios ranging from 60:1 to 3.7:1. At the highest E/T ratios the cytotoxic interaction was only partly inhibited by a CD11a mAb and not by mAb 24 (data not shown). At

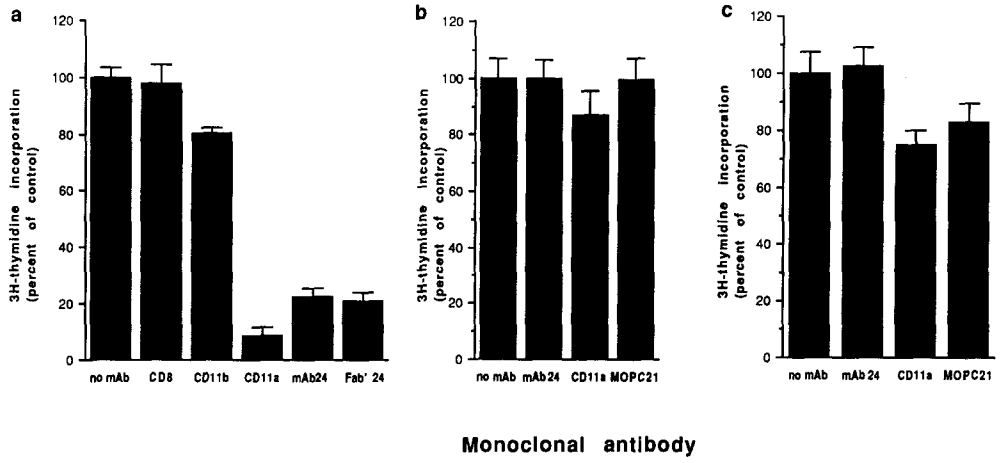
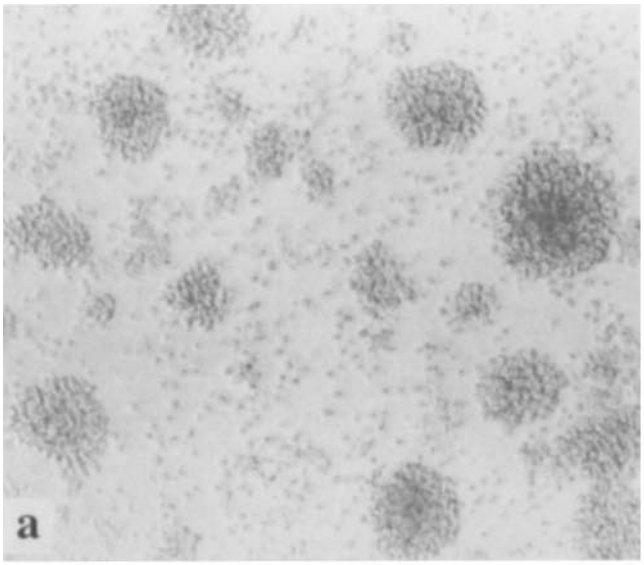
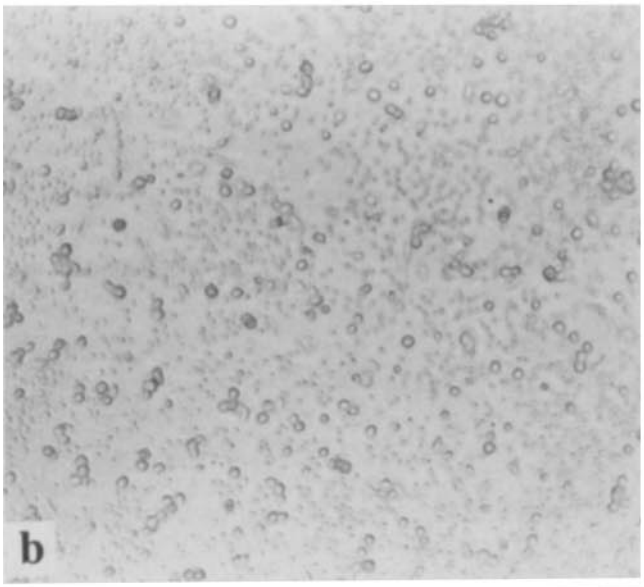


Figure 1. Effect of mAb 24 on antigen-specific T cell proliferation. (*a*) The proliferative response of PBMC to the antigen influenza virus, was inhibited by mAb 24, Fab'24, and by CD11a mAb 38, but not by CD11b mAb 44 or CD8 mAb 14. The mitogenic response of PBMC to (*b*) PHA and (*c*) to pokeweed mitogen was not inhibited by mAb 24, CD11a mAb 38, or control mAb MOPC 21. In the representative experiment shown, the donor response to influenza virus yielded 65,168 ± 6,071 cpm, to PHA yielded 66,040 ± 4,452 and to pokeweed mitogen, 36,303 ± 2,493. The results of quadruplicate samples are expressed as a percentage of the total counts ± SE.





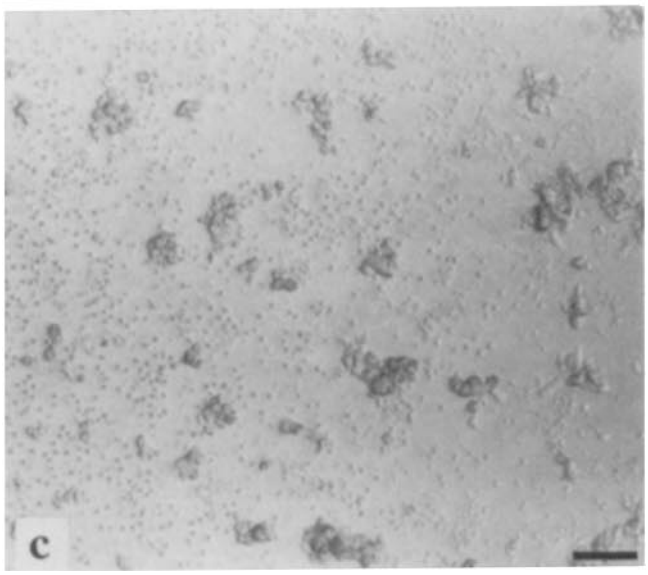


Figure 2. Aggregate formation during response of PBMC to antigen. (*a*) Aggregates formed by PBMC after 6 d of exposure to antigen, purified protein derivative of tuberculin. (*b*) Aggregate formation is inhibited by CD11a mAb 38 indicative of LFA-1 dependence. (*c*) Small clusters formed in the presence of mAb 24 maintain constant appearance throughout the 6-d period. Bar, 100 μm.

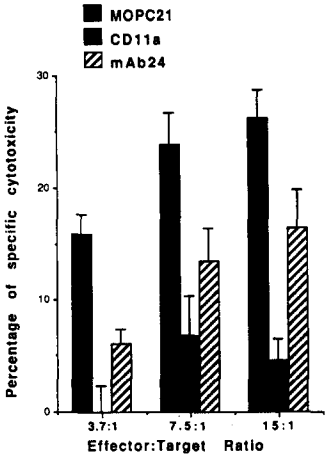


Figure 3. Effect of mAb 24 on LAK activity. The LAK activity of T cells cultured in IL-2 for 14 d against Daudi cells was inhibited by mAb 24. The LFA-1 dependence of this LAK activity is demonstrated with anti-CD11a mAb 38. The mAb MOPC 21 was used as a control.

lower E/T ratios (15:1 and particularly at 3.7:1) the assay became CD11a-dependent and could be correspondingly blocked by mAb 24 ($n = 5$). A typical experiment is shown in Fig. 3. This result also suggests that mAb 24 might affect cell motility or reduce opportunities for cell–cell contact under circumstances where effector cells were limiting.

Neutrophil Chemotaxis Is Inhibited by mAb 24

The directed movement of neutrophils toward the chemoattractant f-Met-Leu-Phe is inhibited by mAbs specific for the leukocyte integrin CR3 (Anderson et al., 1986). A subagarose assay (Nelson et al., 1975; Chenoweth et al., 1979) has been used to assess the effects of mAb 24 upon CR3-dependent neutrophil chemotaxis. Inhibition of neutrophil f-Met-Leu-Phe–directed migration was observed with use of CD11b mAb 44, mAb 24 and Fab'24 but not with CD11a mAb38 nor CD8 mAb14 (Fig. 4, *a* and *b*). Therefore, mAb 24 is able to inhibit both LFA-1–dependent functions and also the activity of CR3, consistent with the observation that all three CD11 α subunits express the 24 epitope.

mAb 24 Does Not Block Leukocyte Integrin Binding to Ligand

To analyze more directly the possibility that mAb 24 might affect the ability of leukocyte integrins to interact with ligand, we analyzed the effects of mAb 24 upon integrin/ligand binding using phorbol ester–induced aggregation of the JY and SKW3 cell lines which were LFA-1/ICAM-1 and LFA-1/ICAM-2,3-dependent, respectively, (Rothlein et al., 1986; Staunton et al., 1989; de Fougerolles et al., 1991) and binding of iC3b-E to tissue culture–matured monocytes which is CR3/iC3b dependent. Although anti-LFA-1 mAb blocked the LFA-1–mediated aggregation assays (Fig. 5, *a* and *b*) and anti-CR3 mAb inhibited the CR3-mediated rosetting assay (Fig. 5 *c*), no inhibition was observed with mAb 24 indicating that this mAb does not prevent receptor/ligand binding.

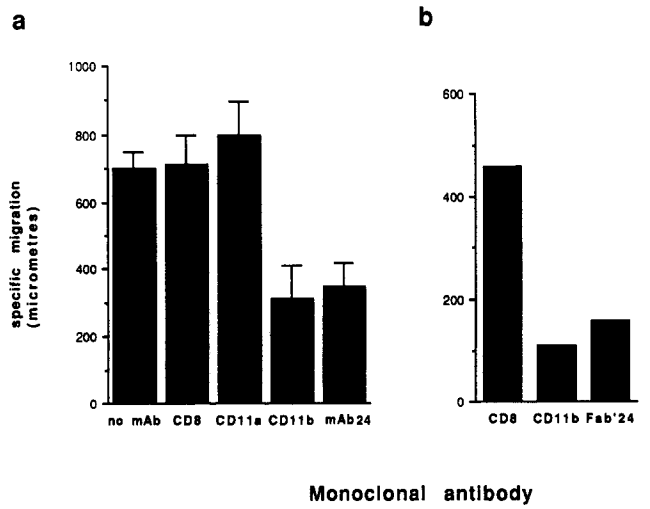


Figure 4. Effect of mAb 24 on neutrophil chemotaxis stimulated by f-Met-Leu-Phe. The chemotactic response of neutrophils, using the sub-agarose assay, was inhibited by mAb 24, Fab' 24, and CD11a mAb 44, but not by CD11a mAb 38 or CD8 mAb14. In *a*, mAbs were used as ascitic fluid (1:50); in *b* CD8 mAb 14 and CD11b mAb 44 were used as purified IgG at 35 μg/ml and Fab'24 at 20 μg/ml. Results in *a* are the mean ± SE of four separate experiments and in *b* the mean of two experiments.

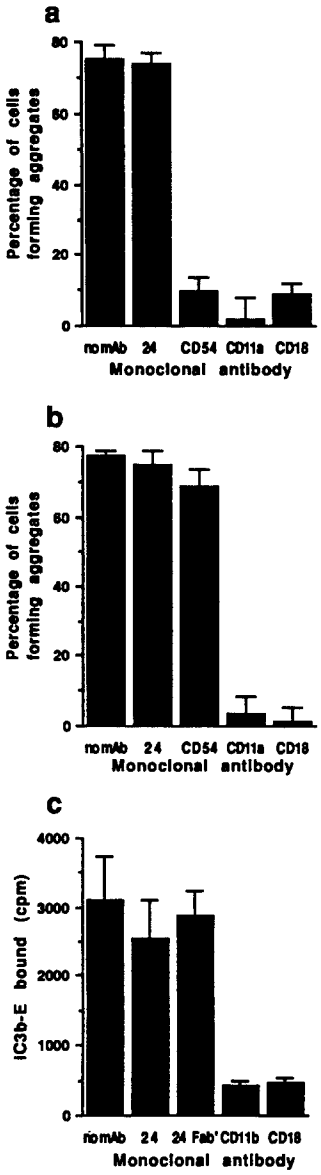


Figure 5. Effect of mAb 24 on LFA-1/ICAM-1,2,3-dependent aggregation and on macrophage/iC3b-coated erythrocyte CR3-dependent rosetting. (*a*) Phorbol ester-induced LFA-1/ICAM-1-dependent aggregation of JY lymphoblastoid cell line is inhibited by CD11a, CD18, and CD54 (ICAM-1) mAbs, but not inhibited by mAb 24. (*b*) Phorbol ester-induced LFA-1/ICAM-2,3-dependent aggregation of SKW3 T cell lymphoma cell line is inhibited by CD11a, CD18 mAbs but not by mAb 24 or CD54 mAbs. (*c*) Binding of iC3b-E by monocyte-derived macrophages (CR3-dependent) is inhibited by CD11b, CD18 mAbs but not by mAb 24. Results, representative of at least three experiments, are expressed as mean ± SD of triplicates.

mAb 24 Enhances Aggregate Formation in PdBu-treated Cells

Although mAb 24 did not inhibit leukocyte integrin/ligand interaction, it remained to be tested whether the negative effects of mAb 24 on leukocyte function were caused by an enhancement of leukocyte integrin/ligand binding. Therefore, mAb 24 was first tested for ability to directly cause the aggregation of a T cell line J6, which expresses both LFA-1 and ICAM-1 (Hogg and Horton, 1987) but grows in single cell suspension. mAb 24 (50 μg/ml) did not cause aggregate formation in these cells over a time period of 22 h (data not shown). Secondly, we wished to test the effect of mAb 24 on a cell line that undergoes spontaneous LFA-1/ICAM-1-dependent aggregation. Therefore, mAb 24 (50 μg/ml) or PdBu was added to dispersed cultures of the B cell line JY and the cells observed for any alteration in the rate of cellular aggregation. Fig. 6 shows that the rate of spontaneous aggregation is enhanced by PdBu over a 2-h period. Similarly an enhanced rate of aggregate formation is observed in the presence of mAb 24, comparable to that induced by PdBu. It was therefore concluded that mAb 24 was not able to induce LFA-1 activation de novo but was able to promote that which was already underway.

In a similar type of experiment, quiescent T cells at 1.5 × 10^8 cells/ml were incubated without (Fig. 7, *a* and *b*) or with 20 nM PdBu (Fig. 7, *c–f*) and the effect of mAb 24 (50 μg/ml) on aggregate formation was observed. There were no aggregates visible in control cultures without or with mAb 24 (Fig. 7, *a* and *b*) or in cultures with 20 nM PdBu at 30 min (Fig. 7 *c*). In this system, pronounced aggregation was observed only after 90 min in the presence of PdBu (Fig. 7 *e*). However, when quiescent T cells were cultured in the presence of mAb 24 and PdBu, cellular aggregates were observed at the earlier time point of 30 min (Fig. 7 *d*). After 90 min, aggregates formed in the presence of both PdBu and mAb 24 were similar in appearance to those in the presence of PdBu alone (compare Fig. 7, *e* and *f*). Thus, in a second system, mAb 24 hastened the formation of cellular aggregates only under circumstances where LFA-1 was in an activated form.

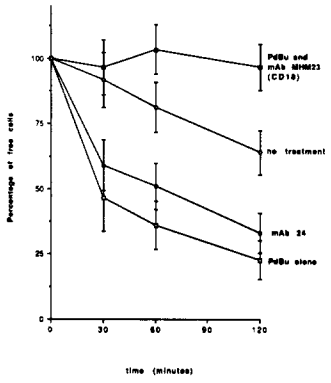


Figure 6. Influence of mAb 24 on spontaneous aggregation of JY cell line. After disruption to single cell suspension, JY cells spontaneously aggregate (-○-). The rate of aggregation is enhanced by mAb 24 (-●-) and PdBu (20nM) (-□-) and is inhibited by CD18 mAb MHM 23 (-■-). Results, representative of at least three experiments, are expressed as the mean ± SD of triplicates.

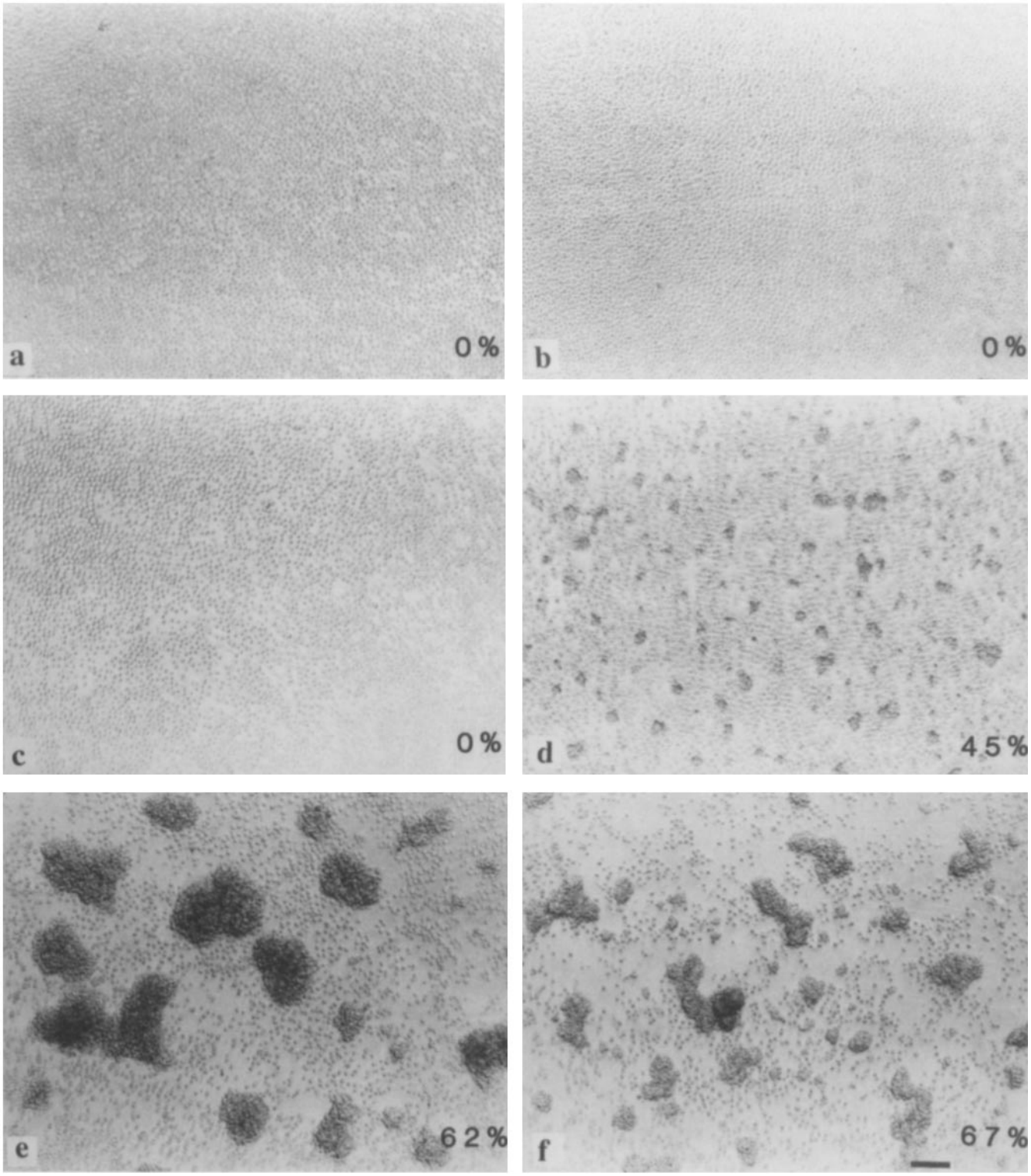


Figure 7. Influence of mAb 24 on PdBu-induced aggregation of quiescent T cells. (*a*) Control culture of quiescent T cells (*b*) with mAb 24 (50 μg/ml), 30 min (*c*) PdBu (20 nM) alone, 30 min (*d*) PdBu and mAb 24, 30 min (*e*) PdBu alone, 90 min. (*f*) PdBu and mAb 24, 90 min. The percentage of cells participating in aggregates has been indicated for each experimental condition. Bar, 150 μm.

Transient Increase in LFA-1 Avidity Is Prolonged by mAb 24

To further explore the effect of mAb 24 on leukocyte integrin function in the absence of blockade of ligand binding, we have utilized a system for analyzing LFA-1–dependent adhesion of T cells to ICAM-1 which serves as a model of activation and subsequent deactivation of LFA-1 ligand binding activity (Dustin and Springer, 1989). Two types of experiments were performed. Either freshly isolated T cells or IL-2 cul-

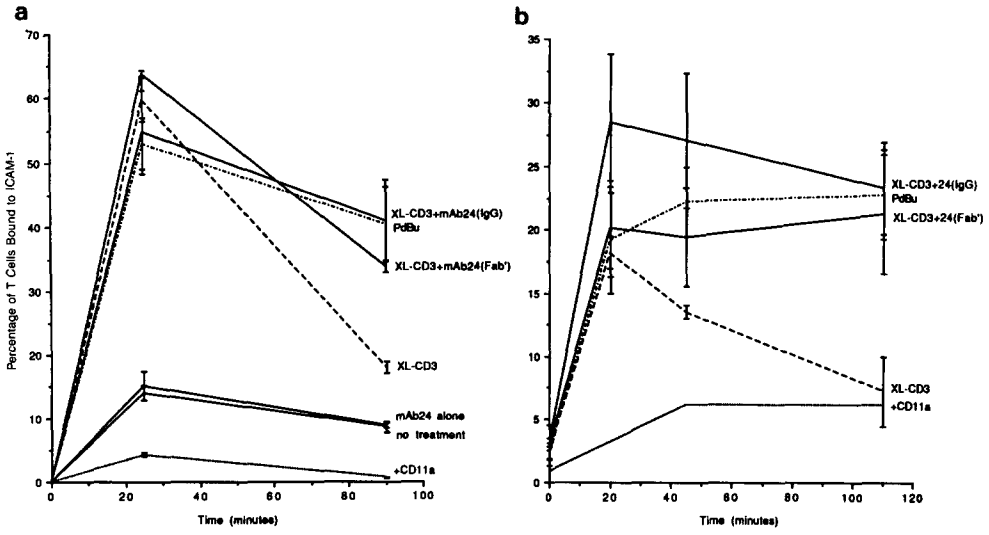


Figure 8. Reversible LFA-1-dependent T cell binding to ICAM-1 induced by CD3 cross-linking is prevented by mAb 24. Cross-linking of CD3 caused rapid, transient adhesion of (*a*) cultured T cells adhering to ICAM-1Fc or (*b*) freshly derived T cells adhering to ICAM-1 expressing CHO cells (*XL-CD3*) in contrast to prolonged adhesion observed in response to treatment with PdBu (*PdBu*). Cross-linking of CD3 in the presence of mAb 24 IgG (*XL-CD3 + 24[IgG]*) or Fab' fragments (*XL-CD3 + 24[Fab']*) prolonged activated T cell binding. Addition of Fab' fragments of mAb 24 alone did not induce T cell adhesion (*mAb24 alone*). Specific adherence was blocked by CD11a mAb38 (*+CD11a*) and CD54 mAb15.2 (data not shown) which reduced adherence to the level of untreated samples (*no treatment*). Binding of T cells to untransfected CHO cells was not detectable. Results (mean of triplicates ± SD) are representative of a minimum of four separate experiments.

tured T cell blasts were tested for binding to either ICAM-1 transfected CHO cells or to recombinant ICAM-1Fc. Rapid, transient adhesion of T cells to ICAM-1 was observed upon crosslinking of the TcR using CD3 mAbs, in contrast to the prolonged adhesion induced by the phorbol ester PdBu (Fig. 8, *a* and *b*). We consistently found that cultured T cells bound with greater efficiency than freshly isolated T cells. Specific T cell binding was blocked by CD11a mAb 38 or ICAM-1 mAb 15.2 (see Fig. 8 legend). Addition of whole IgG as well as Fab' fragments of mAb 24 had a profound effect upon the duration of adhesion induced by TcR cross-linking. Thus in the presence of mAb 24, similar or frequently enhanced levels of binding were reached within 20'–25', but reduction in the numbers of T cells bound to ICAM-1 after prolonged incubation was not observed. Fab' fragments of mAb 24 in the absence of cross-linked CD3 had no effect on T cell adhesiveness. These results demonstrate that the presence of mAb 24 prevents the de-adhesion of LFA-1 and ICAM-1. Our speculation is that mAb 24 maintains LFA-1 in the activated state and therefore inhibits post-receptor occupancy changes required for release of ligand.

Discussion

Leukocyte integrins are transiently activated through intracellular signalling pathways enabling increased avidity of binding to occur to counter receptors or ligands. To gain understanding of this activation process, we have further analyzed the effects on function of a novel mAb 24, which recognizes a Mg^{2+}-dependent epitope present on all three leukocyte integrin α subunits (Dransfield and Hogg, 1989). mAb 24, as intact IgG or Fab' fragments is able to inhibit antigen-specific T cell proliferation and LAK cell cytotoxicity, which are LFA-1-dependent, and f-Met-Leu-Phe-stimulated chemotaxis, which is a CR3-dependent process. There are several possible explanations for the inhibitory effects of this mAb. In some studies, mAbs to the $\beta 2$ subunit of leukocyte integrins have been shown to transduce negative regulatory signals to T cells (van Noesel et al., 1988) and a similar mechanism might be operative for mAb 24. Alternatively, mAb 24 might be cytotoxic for functioning cells. However, analysis of T cell responsiveness induced by mitogen showed that proliferation was unaffected by the presence of mAb 24 indicating that binding to its α subunit epitope was not delivering an antiproliferative signal.

Another possibility is that the inhibitory effects of mAb 24 on function might occur as a result of blockade of receptor/ligand binding. Peptide ligands, RGD and fibrinogen γ chain sequences, can be cross-linked to the putative cation binding domains of the $\beta 3$ integrins, VNR and IIb/IIIa (D'Souza et al., 1990; Smith and Cheresh, 1990; Smith and Cheresh, 1991). These observations have suggested that for integrins, divalent cation binding and interaction with ligand are intimately associated. However, the lack of an effect of mAb 24 on LFA-1- or CR3-dependent binding assays, suggests that the leukocyte integrins may interact with ligand in a different manner to RGD-specific integrins. Another possibility is that the 24 epitope, which behaves as an indicator of Mg^{2+} binding, is not physically located within the putative cation binding domains. The localization of this epitope is under investigation.

Although the inhibitory effect upon function of mAb 24 does not occur as a result of blocking receptor/ligand binding, it was of interest to know whether the mAb promoted this type of interaction. There was no induction of aggregation when mAb 24 was added to J6 T cell line growing in single cell suspension, indicating that it does not directly induce LFA-1/ligand binding on these cells. However, when mAb 24 was added to JY cells undergoing spontaneous aggregation or PdBu-stimulated T cell aggregation, it was able to enhance LFA-1-dependent interactions which were already underway. These observations, coupled with previous work showing that the 24 epitope is expressed only when LFA-1 is functionally active (Dransfield and Hogg, 1989;

Dransfield et al., 1990), suggest that mAb 24 is able to promote an increase in LFA-1–dependent binding to ligand only following prior activation of LFA-1. One explanation might be that mAb 24 induces intracellular signals, enabling LFA-1 to combine with ligand with increased avidity. Such activity has been suggested for other LFA-1 α subunit mAbs that enhance proliferation, rather than inhibit function (van Noesel, 1988; Carrera et al., 1988; Wacholtz et al. 1989; Pardi et al., 1989). However, inhibitory effects on function might occur if mAb 24 acts extracellularly by blocking conformational alterations resulting from activation.

To test these possibilities, we analyzed the effect of mAb 24 in an assay that allows analysis of activation and subsequent deactivation of LFA-1 binding activity to ICAM-1 (Dustin and Springer, 1989). mAb 24 had no effect on the rate of initial adhesion of T cells. However, the resulting prolongation of the initial adhesion phase by mAb 24 suggests that, by interacting with its epitope, mAb 24 prevents de-adhesion of LFA-1 and ICAM-1. It can be speculated that LFA-1 is unable to dissociate from ICAM-1 because an essential conformational alteration of the α and β subunits is prevented. Further evidence for this suggestion comes from the formation of small aggregates in antigen-stimulated cultures of peripheral blood cells in the presence of mAb 24. These aggregates form after a few hours exposure to antigen and subsequently appear to remain stable in size. One possibility is that access of other T cells to adherent antigen–presenting cells is prevented, thus precluding further stimulatory events. Similarly the ability of mAb 24 to prevent LAK cytotoxicity increased as the effector/target cell ratio decreased which could be explained by an increased dependence on effector cell recycling. Finally it can be speculated that neutrophils moving on a substrate in a chemotactic gradient might accomplish this end at least partly via an attachment-release mechanism involving CR3 as has been demonstrated for neutrophil binding to endothelium (Wright and Meyer, 1986; Lo et al., 1989).

Recognition of leukocyte integrin α/β heterodimers by mAb 24 is unique. As mAb 24 does not induce adhesion, it differs from mAbs recognizing LFA-1 directly (Keizer et al., 1988; van Kooyk et al., 1991) or indirectly (Nong et al., 1989; Mourad et al., 1990; Lauener et al., 1990; Koopman et al., 1990), which increase avidity of LFA-1 binding to ligand. The inhibitory effects of mAb 24 on leukocyte integrin function contrast with anti-LFA-1 mAb NKI-L16 that acts extracellularly to promote ligand binding activity (Keizer et al., 1988; van Kooyk et al., 1991). This might be explained if mAb 24 prevents release of ligand from activated LFA-1 rather than, as observed for NKI-L16, promotes initial stages of LFA-1/ligand interaction. The formation of large aggregates in the presence of NKI-L16 would not preclude dynamic interaction of leukocytes necessary for functions such as antigen presentation. In contrast, the functional effects of mAb 24 may be a direct result of prevention of such dynamic interaction. In this respect, mAb 24 is more similar to mAbs recognizing ligand-occupied β3 integrins described by Ginsberg and colleagues (Frelinger et al., 1988, 1990). They observed that these mAbs, which by definition do not prevent ligand binding, inhibit IIIb/IIIa-dependent functions such as clot retraction, possibly by preventing release of ligand.

As mAb 24 does not interfere with binding of LFA-1 or CR3 to ligand, the inhibition of chemotaxis and antigen-specific T cell proliferation by this mAb occurs in spite of engagement of leukocyte integrin with ligand. This may be because receptors are unable to dissociate from ligand in the presence of mAb 24 as is observed in the transient LFA-1 activation system triggered by CD3 cross-linking. Thus it may be that mAb 24 causes leukocyte integrins to retain an "activated" conformation, preventing a return to the "nonactivated" state. As a consequence, leukocyte integrin–dependent functions are inhibited, implying a requirement for dynamic receptor/ligand binding in these processes. In summary, we present evidence that conformational alterations as detected by mAb 24 are a requirement for function in leukocyte integrin–mediated processes.

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