MURINE T CELLS EXPRESS A CELL SURFACE RECEPTOR FOR MULTIPLE EXTRACELLULAR MATRIX PROTEINS

Identification and Characterization with Monoclonal Antibodies

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The integrin supergene family of cell adhesion molecules (ISCAM)[1] includes several cell surface heterodimers that are involved in both the attachment of cells to extracellular matrix (ECM) proteins (1, 2) and in cell-cell interactions (3). The heterodimers are composed of noncovalently linked α and β chains with molecular masses of ~130–210 kD and 95–130 kD, respectively, as determined by SDS-PAGE under nonreducing conditions. The α chain, in some cases, is post-translationally processed to yield a disulfide-bonded dimer composed of a larger fragment and a ~25–30 kD subunit (4). Three distinct families of human integrins have been defined based on the association of a common β chain with distinct α chains (1–3). One family, which was originally termed the very late activation (VLA) antigens, was identified by mAbs that selectively reacted with lymphoid antigens induced by prolonged antigen or mitogen stimulation in vitro (5). Recent studies have shown that this family is composed of at least six distinct α chains associated with a common β chain (β_1), and several of these dimers are expressed on resting and activated lymphocytes as well as certain nonlymphoid cells (6, 7). Previously, cell surface receptors for ECM proteins, such as fibronectin and vitronectin, had been determined to be heterodimers with biochemical features similar to members of the VLA family (8, 9). It is now clear that several members (VLA-2, VLA-3, and VLA-5) of this family are cell surface receptors for ECM proteins (2, 10). In those cases where the ECM protein-receptor interaction has been characterized, the interaction can be frequently inhibited by peptides containing the tripeptide, Arg-Gly-Asp (RGD) (11). The second ISCAM family includes the leukocyte adhesion proteins (LAP) LFA-1, Mac-1, and p150,95 (the distinct human α chains have been named CD11a,b,c, and the common β_2 chain, CD18) (3). In contrast to the other two ISCAM families, one member of the LAP family, LFA-1, appears to be primarily involved in cell-cell interactions (3) rather than cell-ECM protein interactions. Members of the third family of the ISCAM

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[1] *Abbreviations used in this paper:* DETC, dendritic epidermal T cell; ECM, extracellular matrix; ISCAM, integrin supergene family of cell adhesion molecules; LAP, leukocyte adhesion proteins; PAA, protein A-Agarose; PGA, protein G-Agarose; VLA, very late activation.

use the β_3 chain, and include the vitronectin receptor (9) and the platelet gpIIb/IIIa complex (12).

The studies in this report are derived from our attempts to produce mAbs specific for cell surface antigens and receptors expressed by a panel of murine dendritic epidermal T cell (DETC) lines and hybridomas that bear the TCR-γ/δ (13–16). We identified a hamster and a rat mAb that selectively reacted with DETC by FACS analysis and that immunoprecipitated a heterodimeric structure that strongly resembled members of the integrin family. We demonstrate here that these mAbs recognize distinct epitopes on the same α chain, that DETC lines bear ECM protein receptors, and that these mAbs inhibit the attachment of DETC to ECM proteins. Since the antigen identified by these mAbs can be induced on peripheral T cells after prolonged in vitro stimulation with mitogens, and is expressed by certain T cell clones, we postulate that this antigen plays a physiologic role in T cell function.

Materials and Methods

Media. All B cell hybridomas were cultured in DME with glucose 4.5 g/liter (Biofluids, Rockville, MD) supplemented with 10–20% FCS (Biofluids), 10mM Hepes, nonessential amino acids (0.1 mM), L-glutamine (300 μg/ml), sodium pyruvate (1 mM), penicillin G (100 U/ml), streptomycin (100 μg/ml), and 2-ME (5×10^{-5} M; Aldrich Chemical Co., Milwaukee, WI). Supplemented RPMI 1640 (Biofluids) contains 10% FCS, Hepes, L-glutamine, penicillin, and streptomycin at the above concentrations.

Animals. Armenian hamsters were purchased from Dr. G. Yerganian of Cytogen Research and Development (West Roxbury, MA). All mouse strains and Lewis rat were supplied by the Division of Research Services, National Institutes of Health (Bethesda, MD).

Reagents and Cell Lines. The cloned DETC lines Y93A, Y245, T245, T93B, and T184 have been previously described (13–15). The DETC hybridomas, T195/BW and T245/BW 1C6.F11, are the fusion products of a DETC line and BW5147, and have been previously described (15, 16). The HAT-sensitive nonsecretory murine myeloma Sp2/0-Ag14 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The following cell lines were obtained from ATCC except where indicated and have been previously described: T cell lines CTL-L (17), EL-4, BW5147; T cell clones DB14, AE7, and F1.A2 (generously provided by M. Jenkins, NIH) (18–20), D10.G41 (21), OE4 (generously provided by G. Trenn, NIH) (22); IL-3-dependent cell lines 32DC3, DA-1, and FDCP-1 (generously provided by J. Ihle, Frederick Cancer Research Facility, Frederick, MD) (23, 24); sarcoma Meth A (provided by E. Gregg, NIH) (25); fibroblast lines NIH 3T3 and BALB 3T3; B cell hybridoma LS 102.9; monocyte/macrophage P388D_1; mastocytoma P815. The hamster mAb H1.2F3 (26), and the rat mAbs 7D4 (anti-IL-2R, reference 27) and 2.4G2 (anti-Fc receptor, 28), and MAR 18.5 (mouse anti-rat κ 29) have been described. Human rIL-2 was generously provided by the Cetus Corp. (Emeryville, CA). The antisera specific for murine TCR Cγ1 gene product have been described (14).

Immunization Protocol and Production in mAb. Armenian hamsters were initially immunized with 2×10^7 Y93A cells emulsified in CFA. The hamsters were then boosted twice with 2×10^7 viable Y93A cells every 2–3 wk, with the final boost 3 d before fusion. All injections were given intraperitoneally. Two hamster spleens were harvested, and cell suspensions were fused to the HAT-sensitive nonsecretory murine myeloma SP2/0-Ag14 with 50% PEG as previously described (26). Approximately 2×10^5 cells/well were plated in 96-well tissue culture plates (Costar, Cambridge, MA) in 100 μl medium, and the resultant hybridomas were selected in HAT-containing media for 14 d as previously described. Approximately 17 d after fusion, 100-μl aliquots of growing wells were used to stain the immunizing cell line and FACS analysis was performed. H9.2B8 was identified as a mAb that was reactive with the immunogen and nonreactive with CTL-L. Wells of interest were cloned by limiting dilution until stable antibody-producing lines were established. The clones were screened by FACS analysis. The

rat mAb 8.18E12 was produced and identified by the same protocol as outlined above, except that a male Lewis rat was immunized with Y245 cells and the resulting hybridomas were screened for reactivity with Y245 cells and the $CD3^-$ thymoma, BW5147.

The mAb H9.2B8 was affinity purified from spent cell supernatant on a protein A-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ). Optimal conditions for purification are binding to protein A-Sepharose at pH 8.6 and elution at pH 4.3. Ascitic fluid containing the mAb 8.18E12 was collected from pristane-primed nude mice injected with the 8.18E12 hybridoma. The mouse anti-rat κ mAb, MAR 18.5 that had been purified by protein A-Sepharose affinity chromatography of spent culture supernatant, was coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals) according to the manufacturer's instructions. The mAb 8.18E12 was affinity purified from mouse ascitic fluid with this MAR 18.5-Sepharose column by binding at pH 8.6 and elution at pH 4.3.

Immunoprecipitation Analysis. The Y245, Y93A, and T245 cell lines, which grow adherent to plastic, were harvested from tissue culture flasks with PBS containing EDTA 0.02% (Versene, Gibco Laboratories, Grand Island, NY), washed with PBS, then surface labeled with $Na^{125}I$ by the lactoperoxidase method as described previously (14, 15). The cells were washed with PBS, lysed with 0.5% NP-40 at 10^8 cells/ml for 30 min at 0°C, and centrifuged at 13,000 *g* for 15 min. After preclearance overnight at 4°C with 100 μl normal rabbit serum, 100 μl protein A-Agarose (PAA; Bethesda Research Laboratories, Gaithersburg, MD) and 100 μl protein G-Agarose (PGA; Genex, Gaithersburg, MD), the lysates were subjected to specific immunoprecipitations by incubation of 100 μl of lysate with 10 μl of purified mAb (10 μg) for 60 min followed by an additional 60-min incubation with 10 μl of PAA or PGA (for mAb 8.18E12) beads. The immunoprecipitates were washed three times with 0.5 ml of lysis buffer, eluted from the beads, and analyzed on SDS-PAGE (12% acrylamide) gels in the presence or absence of 5% 2-ME. For the sequential immunoprecipitation experiments, the precleared lysates were cleared of material reactive with a particular mAb by five serial incubations with mAb and agarose beads, followed by pelleting of beads, and reincubation of supernatants with mAb and agarose beads. For the "off-diagonal" gels, immunoprecipitates were run under nonreducing conditions for 12 h in an SDS-PAGE (12% acrylamide) cylindrical gel at 0.4 mA/gel. The gel was equilibrated in 2-ME, then layered on a SDS-PAGE slab gel and run for 12 h at 7 mA/gel. Immunoprecipitation of the individual chains was achieved by incubation of the precleared lysate with a mAb and PAA or PGA beads as above, elution of the immunoprecipitates under nonreducing conditions (0.05 M Tris, 1% SDS at 80°C for 10 min), or elution from beads under reducing and alkylating conditions (0.05 M Tris, 1% SDS, 0.002 M DTT for 5 min at 67°C followed by an additional 15-min incubation at room temperature with 0.02 M iodoacetamide). The supernatants were then mixed with 400 μl of 2.5% NP-40, 0.01 M Tris, and reprecipitated with the appropriate mAb as described above then analyzed on SDS-PAGE under nonreducing or reducing conditions as appropriate. The gels were dried, and labeled proteins were visualized by exposure to Kodak XAR-5 radiographic film at −70°C.

Preparation of Activated Cell Populations. Spleen cell suspensions were prepared from C3H/HeN mice and were passed through a sterile nylon wool column as previously described (26). The nonadherent cells, enriched for T cells, were cultured in supplemented RPMI medium at 10^6 cell/ml with Con A (5 μg/ml, Pharmacia Fine Chemicals) in 24-well plates (Costar) for 4 d. Beginning on day 4, the cells were fed supplemented RPMI containing human rIL-2 (20 U/ml) with medium changes every 3–4 d.

FACS Analysis. Adherent DETC cell lines and hybridomas were detached from tissue culture flasks with EDTA 0.02% in PBS (Versene 1:5,000; Gibco Laboratories, Grand Island, NY), and washed in FACS buffer (ice-cold HBSS without phenol red, containing 3% FCS and 0.1% sodium azide). C3H/HeN thymocyte, mesenteric lymph node, spleen, and bone marrow cell suspensions were prepared as previously described (26) and washed in FACS buffer. Concentrations of antibody that had been previously determined to be saturating were added to 0.5×10^6 to 1×10^6 cells. After a 30-min incubation on ice, the cells were washed and appropriate directly FITC-labeled second antibodies were added. In the case of the hamster antibodies, FITC-$F(ab')_2$ goat anti-hamster Ig (Cappel Laboratories, Malvern, PA) and for the rat mAb, FITC-MAR 18.5 were used. After another 30-min incubation on ice and

final washes, 10,000 cells were analyzed on a FACSCAN flow cytometer (Becton Dickinson & Co., Sunnyvale, CA). Dead cells were excluded from analysis by propidium iodide staining, and debris were excluded by light scatter parameters.

For the binding inhibition assays, cells were prepared for FACS analysis as described above, washed in FACS buffer, and incubated with saturating concentrations of unlabeled mAb for 15 min on ice. The appropriate concentration of the directly fluoresceinated mAb was then added for an additional 30 min on ice. After three washes, the cells were analyzed as described above.

Adhesion Assay. Stock preparations of rat fibronectin (Calbiochem-Behring Corp., La Jolla, CA), human fibronectin (Calbiochem), rat collagen I (Sigma Chemical, St. Louis, MO), rat fibrinogen (Sigma), and human vitronectin (Calbiochem), ovalbumin (Sigma), mouse laminin (Collaborative Research, Bedford, MA), collagen IV (Sigma) were diluted in 0.05 M Tris-buffered saline, pH 9.8. The protein solutions were distributed in 96-well tissue culture flat-bottomed (Costar) plates at 50 μ/well. After an overnight incubation at 37°C, the coated wells were washed three times with PBS to remove nonimmobilized protein. To saturate the remaining protein binding sites, 100 μl of adhesion media (serum-free RPMI 1640 with BSA 2.5 mg/ml; Sigma) was added and the plates were incubated at 37°C for 2 h.

Subconfluent Y93A or T195/BW cultures were labeled with 5 μCi/ml [^{3}H]TdR overnight at 37°C. The supernatant was discarded and adherent cells were detached by exposure to 0.02% EDTA in PBS for 5 min. The cells were washed three times in adhesion media. If preincubation was required for the assay, 4×10^4 cells in 100 μl adhesion media were distributed to U-bottomed 96-well plates (Costar) containing affinity-purified mAb H9.2B8, 8.18E12, RGDS (Calbiochem), RGES (Peninsula Laboratories Inc., Belmont, CA), or control hamster mAb (H1.2F3), or control rat mAb (anti-IL-2R, 7D4) at 4°C for 20–30 min. Both control mAbs bind to antigens expressed by the DETC lines used for adhesion assays. The labeled cells were then transferred to 96-well plates previously coated with ECM or control proteins. The plates were centrifuged at 500 rpm for 10–20 s to lightly settle the cells to the bottom surface and were incubated at 37°C for 3–4 h. Nonadherent cells were removed from the wells by addition and removal of 100 μl adhesion media three times, and finally, 50 μl of adhesion media was added to each well. The adherent cells were harvested onto glass fiber strips with the PhD cell harvester (Cambridge Technology, Watertown, MA) and radioactivity was assessed by liquid scintillation counting. All samples were performed in triplicate. Percent adherence is defined as the remaining cpm divided by the total input cpm (derived from wells that were not washed). There is a linear correlation between the number of cells in the wells and the cpm measured.

Results

Production of mAbs H9.2B8 and 8.18E12. To generate mAbs specific for cell surface antigens and receptors expressed by DETC lines, we immunized rats and hamsters with DETC lines and fused the immune spleen cells with SP2/0-Ag14. The resultant antibody producing hybridomas were screened by FACS analysis for positive reactivity with the immunogen, and negative reactivity with another T cell line. The rat mAb 8.18E12 was derived by immunization with the DETC cell line, Y245; it reacted with the immunogen, but not with the BW5147 cell line. Similarly, the hamster mAb H9.2B8 was derived by immunization with another DETC cell line, Y93A, and it reacted with the immunogen, but not with the IL-2-dependent CTL-L cell line. Both 8.18E12 and H9.2B8 were cloned by limiting dilution several times in order to produce stable antibody producing hybridomas. Both mAb were purified by affinity chromatography for use in the studies reported here.

Biochemical Analysis of the Antigen Recognized by mAbs H9.2B8 and 8.18E12. The mAbs H9.2B8 and 8.18E12 immunoprecipitated, from lysates of ^{125}I surface-labeled Y93A cells, a similar complex that consisted of 140- and 95-kD bands under nonreducing

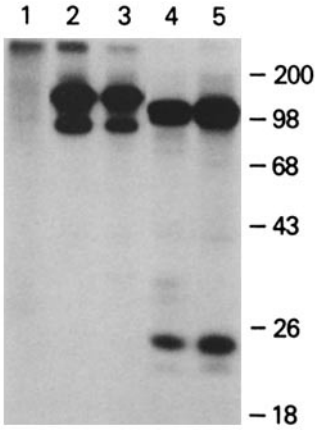

FIGURE 1. Immunoprecipitation analysis with mAbs H9.2B8 and 8.18E12. Lysates of ^{125}I cell surface labeled Y93A cells were immunoprecipitated with normal rabbit serum (lane 1), mAb H9.2B8 (lanes 2 and 4), or mAb 8.18E12 (lanes 3 and 5) and analyzed on SDS-PAGE under nonreducing conditions (lanes 1-3) or reducing conditions (lanes 4 and 5). The Mr is indicated on the right ($\times 10^{-3}$).

conditions that dissociated into 120-, 100-, and 23-kD bands in the presence of 2-ME (Fig. 1). This result suggested that two of the three chains seen in the reducing gel are disulfide linked. Furthermore, the 95-kD band appeared to migrate as a 100-kD band under reducing conditions, a result that suggested the presence of intrachain disulfide bonds. Similar results were obtained with the Y245 and T245 cell lines (data not shown).

To clarify the relationship between the subunits reactive with the mAbs, the H9.2B8 immunoprecipitate was electrophoresed in a cylindrical gel under nonreducing conditions, equilibrated with 2-ME, and run under reducing conditions on an SDS-PAGE slab gel (Fig. 2). On such "off-diagonal gels," disulfide-linked dimers run below the diagonal in the same vertical plane, whereas subunits with intrachain disulfide bonds may run above the diagonal. The 140-kD species, seen under nonreducing conditions, thus appeared to be composed of 120- and 23-kD subunits that are disulfide linked since they ran in the same vertical plane. The 95-kD protein migrated on or slightly above the diagonal, separate from the 120/23-kD subunits. Identical results were obtained with the mAb 8.18E12 (data not shown). These results suggest that

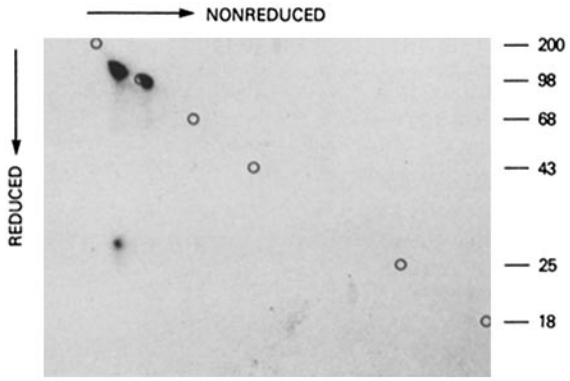

FIGURE 2. "Off-diagonal" gel analysis of the antigen recognized by H9.2B8. Lysates of ^{125}I labeled Y93A cells were immunoprecipitated with H9.2B8 and subjected to SDS-PAGE separation in cylindrical gel under nonreducing conditions (horizontal), then equilibrated with 2-ME and run under reducing conditions in the second dimension (vertical) on an SDS-PAGE slab gel.

the 95-kD protein noncovalently interacts with the 120/23-kD subunits and contains intrachain disulfide bonds.

mAb H9.2B8 and 8.18E12 Both Recognize the Same 120 kD α Chain Fragment But Bind to Distinct Epitopes. Since both mAb H9.2B8 and 8.18E12 immunoprecipitated a similar antigen complex, the relationship between the determinants reactive with each mAb was examined in more detail. The identity of the complex precipitated by each mAb was confirmed by sequential immunoprecipitations. Fig. 3 demonstrates that five steps of sequential preclearing of cell lysates with mAb H9.2B8 eliminated reactivity on subsequent immunoprecipitation with mAb H9.2B8 (Fig. 3, lane *2*). This precleared lysate no longer contained material reactive with mAb 8.18E12 (Fig. 3, lane *3*), but control immunoprecipitates with anti-Cγ1 antisera were unchanged (Fig. 3, lane *8*) from immunoprecipitates before preclearance (Fig. 3, lane *7*). Identical results were obtained when the initial lysates were precleared with the mAb 8.18E12 (Fig. 3, lanes *4–6*, *9*, and *10*). Thus, these data demonstrate that the mAbs H9.2B8 and 8.18E12 are specifically reactive with the same antigen complex, a 140- and 95-kD heterodimer that resembles the α and β chains, respectively, of the integrin family.

To determine which subunit is reactive with each mAb, immunoprecipitations were first performed with either mAb alone. The immunoprecipitates were then analyzed on SDS-PAGE under nonreducing conditions (H9.2B8, Fig. 4, lane *2*; 8.18E12, lane *3*) or the precipitates were dissolved in SDS and immunoprecipitated again with the same mAb (H9.2B8, Fig. 4, lane *4*; 8.18E12, lane *5*) before SDS-PAGE analysis under nonreducing conditions. It is clear that both mAbs selectively reacted with the 140-kD α chain since the second immunoprecipitates contained only the 140-kD chain to the exclusion of the other chains. Furthermore, if the H9.2B8 precipitate was dissolved in SDS and reprecipitated with mAb 8.18E12 (Fig. 4, lane *6*) or

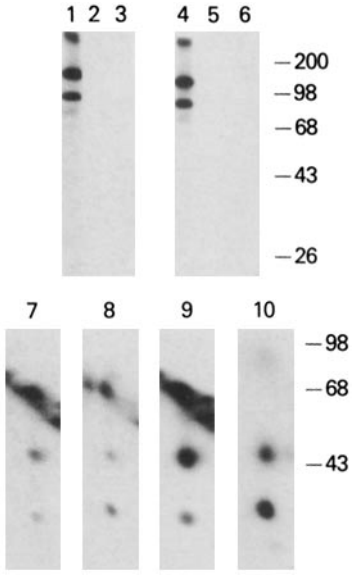

FIGURE 3. Sequential immunoprecipitations with mAbs H9.B8 and 8.18E12. Lysates of ^{125}I cell surface labeled T245 cells were immunoprecipitated with mAb H9.2B8 (lane 1) or serially precleared five times with mAb H9.2B8 (lanes 2–3) then immunoprecipitated with mAb H9.2B8 (lane 2) or mAb 8.18E12 (lane 3). Similarly, lysates were immunoprecipitated with mAb 8.18E12 (lane 4) or serially precleared five times with mAb 8.18E12 (lanes 5–6) then immunoprecipitated with mAb 8.18E12 (lane 5) or mAb H9.2B8 (lane 6). Control immunoprecipitates were performed with the anti-T cell receptor antisera specific for Cγ1 gene product (lanes 7–10) before (lanes 7 and 9) and after (lanes 8 and 10) preclearance with mAb H9.2B8 (lanes 7, 8) or 8.18E12 (lanes 9, 10) and analyzed on an "off-diagonal" gel. For lanes 7–10, only the relevant portion of the gel is shown.

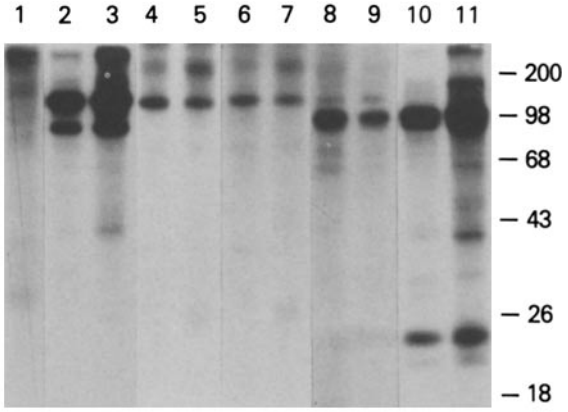

FIGURE 4. mAb H9.2B8 and 8.18E12 recognize the same 120-kD α chain fragment. Lysates of ^{125}I-labeled Y93A cells were subjected to immunoprecipitation as follows, with conditions of SDS-PAGE given in parenthesis (nonreducing [NR] vs reducing, [R]). (Lane *1*) Normal rabbit serum; (lane *2*) immunoprecipitate with mAb H9.2B8 (NR); (lane *3*) immunoprecipitate with mAb 8.18E12 (NR); (lane *4*) immunoprecipitate with mAb H9.2B8, precipitate dissolved in SDS and reimmunoprecipitated with mAb H9.2B8, (NR); (lane *5*) immunoprecipitate with mAb 8.18E12, precipitate dissolved in SDS and reprecipitated with mAb 8.18E12 (NR); (lane *6*) immunoprecipitate with mAb H9.2B8, immunoprecipitate dissolved in SDS and reimmunoprecipitated with mAb 8.18E12, (NR); (lane *7*) immunoprecipitate with mAb 8.18E12, precipitate dissolved in SDS and reimmunoprecipitated with mAb H9.2B8 (NR); (lane *8*) same as lane *4* except immunoprecipitate dissolved, reduced, alkylated, then reimmunoprecipitated with mAb H9.2B8 (R); (lane *9*) same as lane *5* except immunoprecipitate dissolved, reduced, alkylated, then reimmunoprecipitated with mAb 8.18E12 (R); (lane *10*) same as lane *2* except R; (lane *11*) same as lane *3* except R.

vice versa (Fig. 4, lane *7*), the 140-kD chain was precipitated. These data demonstrate that each mAb is reactive with the same α chain. To determine which of the α chain fragments is reactive with the mAbs, immunoprecipitates were run on SDS-PAGE under reducing conditions (H9.2B8, Fig. 4, lane *10*; 8.18E12, lane *11*) or the precipitates were dissolved in SDS, reduced, alkylated to prevent reassociation of disulfide bonded subunits, then immunoprecipitated again with the same mAb and analyzed under reducing conditions (H9.2B8, Fig. 4, lane *8*; 8.18E12, lane *9*). This analysis demonstrated that each mAb specifically reacts with the 120-kD subunit of the 140-kD α chain.

Although the above data demonstrate that both mAbs react with the identical larger α chain fragment, binding inhibition studies, performed by FACS (Fig. 5), demon-

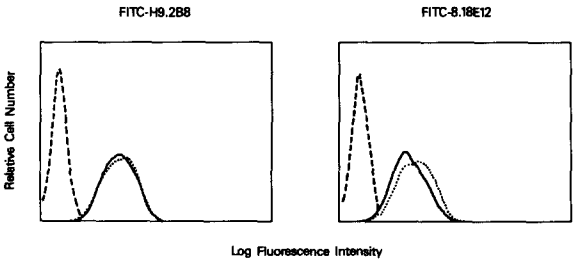

FIGURE 5. FACS binding inhibition assay with mAb H9.2b8 or 8.18E12. (*Left*) Y93A cells were preincubated with FMF buffer alone (*dotted line*), unlabeled mAb H9.2B8 (*dashed line*), or unlabeled mAb 8.18E12 (*solid line*) then stained with FITC-H9.2B8. (*Right*) Y93A cells were preincubated with FACS buffer alone (*dotted line*), unlabeled mAb 8.18E12 (*dashed line*), or mAb H9.2B8 (*solid line*) then stained with FITC-8.18E12. Cells were then analyzed by FACS as described. Staining with irrelevant antibody was identical to dashed line and is not shown.

strated that the binding of directly fluoresceinated mAb H9.2B8 to Y93A cells was blocked by preincubation with unlabeled H9.2B8, but not by unlabeled mAb 8.18E12, even though unlabeled mAb 8.18E12 completely blocked the binding of directly fluorescinated 8.18E12. Moreover, unlabeled H9.2B8 did not inhibit the binding of FITC-8.18E12. Taken together, these data demonstrate that the mAbs bind to distinct epitopes on the larger fragment of the same α chain.

Tissue and Cell Line Distribution and Induction of the Expression of the Antigen Complex Recognized by mAbs H9.2B8 and 8.18E12. By FACS analysis, the antigen complex recognized by mAb H9.2B8 is not expressed by resting cells in spleen, lymph node, or thymus cell suspensions, but a significant number (30–40%) of bone marrow cells display the antigen complex (Fig. 6). The staining with mAb 8.18E12 was consistently less bright than that seen with mAb H9.2B8, but there was always a concordance in reactivity with either mAb. By FACS analysis, the mAbs H9.2B8 and 8.18E12 were reactive with all DETC lines and hybridomas tested (Table I). Several T clones and tumor lines were also reactive, but the IL-2-dependent CTL-L line was not, even though a CTL clone, OE4, was positive. The antigen was also present on two fibroblast lines, NIH 3T3 and BALB 3T3, but was absent on the fibrosarcoma, Meth A. In most cases, the intensity of mAb H9.2B8 staining was greater than mAb 8.18E12, and in the remaining instances, the low level reactivity with mAb H9.2B8 was correlated with a lack of detectable staining with mAb 8.18E12.

Since certain of the integrins identified on human T cells (VLA-1 and VLA-2) are expressed only after 2–3 wk after activation by Con A and maintenance in IL-2 (5), the reactivity of splenic T blasts with mAb H9.2B8 was determined by serial FACS analysis after Con A stimulation. Whereas the activation antigens Ly-6 and IL-2R were clearly detectable within 1–2 d after stimulation (27, 30, and data not shown), definite expression of the complex recognized by H9.2B8 was not detectable on day 4 and did not occur until at least day 7 after stimulation (Fig. 7). This expression then persisted until the final day of analysis (day 28). Identical results were obtained with mAb 8.18E12 (data not shown). Since the kinetics of expression and the activation protocol are similar to that described for expression of the VLA antigens VLA-1 and VLA-2 (5), these data establish that the antigen recognized by H9.2B8 and 8.18E12 are expressed in a similar manner i.e., as late activation an-

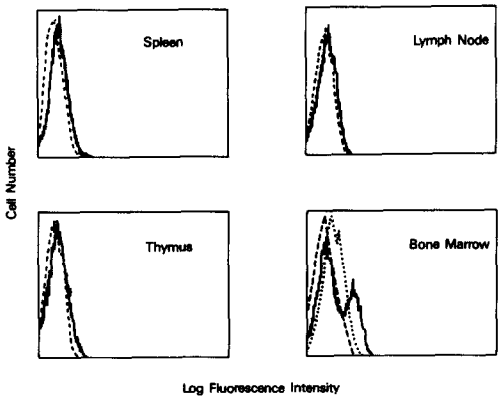

FIGURE 6. FACS analysis of tissue distribution of antigen complex recognized by mAb H9.2B8 or 8.18E12. C3H/HeN spleen, lymph node, thymus, or bone marrow suspensions were stained with FITC-H9.2B8 (*solid line*) or FITC-8.18E12 (*dotted line*) then subjected to FACS analysis. Control staining with irrelevant mAb is indicated by the dashed line. In the spleen, lymph node, and thymus panels, the dotted line is obscured by the solid line.

TABLE I

FACS Reactivity of mAbs H9.2B8 and 8.18E12

Cells	H9.2B8	8.18E12
DETC lines and hybridomas:		
Y93A	+ + +	+ + +
Y245	+ + +	+ + +
T245	+ +	+
T93B	+ +	+
T184	+ +	+
T195/BW	+ +	+
T245/BW	+	+/−
T cell clones:		
DB14	−	−
AE7	+	+
F1.A2	+	+
D10.G41	−	−
OE4	+ +	+
T cell lines/tumors:		
EL4	+	+/−
BW5147	+	−
CTL-L	−	−
Others:		
32DC3	−	−
DA-1	−	−
FDCP-1	−	−
MethA	−	−
NIH 3T3	+	−
BALB 3T3	+	−
SP2/0	−	−
LS	+	−
$P388D_1$	−	−
P815	−	−

Cell lines were stained with FITC-H9.2B8 or FITC-8.18E12 in the presence of mAb 2.4G2 and analyzed with a FACScan flow cytometer. In all cases, all cells were uniformly reactive (or nonreactive) with the mAbs. The relative staining intensity of the cell lines on a log scale is denoted: + + +, strongly positive; + +, moderately positive; +, low level positive; +/−, borderline positive; −, no different than irrelevant control antibody staining.

tigens. Furthermore, expression of this antigen occurred 14 d after alloantigen activation (data not shown), demonstrating that this late expression can occur after another stimulus.

DETC Lines Express ECM Protein Receptors. Both the molecular properties and the FACS expression data strongly suggested that the antigen identified by the two mAbs might represent the murine homologue of one of the human receptors for ECM proteins. In addition, both of the DETC lines that served as immunogens, as well as all other DETC lines and hybridomas derived in our laboratory, grow as monolayers that are firmly adherent to plastic tissue culture vessels, but that can readily be detached by Ca^{2+} chelators (Stingl, G., and W. Yokoyama, unpublished observations). To

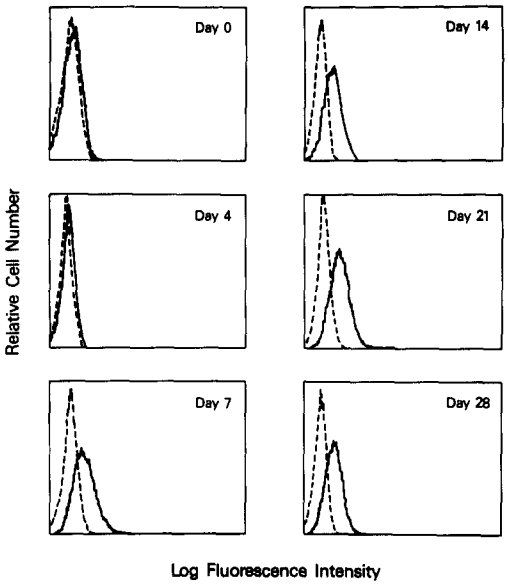

FIGURE 7. Mitogen induction of expression of antigens recognized by mAb H9.2B8. Splenic T cells were stimulated with Con A 5 μg/ml then analyzed with FITC-H9.2B8 (*solid lines*) on the indicated days after stimulation with day 0 being the initial day of culture. On day 4, the cells were washed and cultured in rIL-2 (20 U/ml). Background staining with an irrelevant mAb is indicated by the dashed line.

demonstrate that the antigen identified by the mAbs was involved in the binding to specific ECM proteins, we devised an assay to quantitate the adhesion of cells to ECM proteins. Proliferating DETC lines or hybridomas were labeled overnight with [^{3}H]TdR, detached from culture flasks, washed to remove unincorporated isotope, and then incubated in microtiter plates that had been precoated with irrelevant or ECM proteins. After a 3–4-h incubation period, the wells were washed to remove nonadherent DETC. The remaining adherent cells were harvested onto glass fiber filters, and the amount of [^{3}H]TdR remaining on the filters quantitated by scintillation counting.

The Y93A cell line specifically bound to human fibronectin, rat fibrinogen, and at lower levels to human vitronectin, but not to the control protein ovalbumin (Fig. 8). Adhesion was proportional to the protein concentration in the coating buffer, and at fibrinogen, fibronectin, and vitronectin concentrations above ~6.25 μg/ml, the binding appeared to be maximal, possibly due to saturation of protein binding

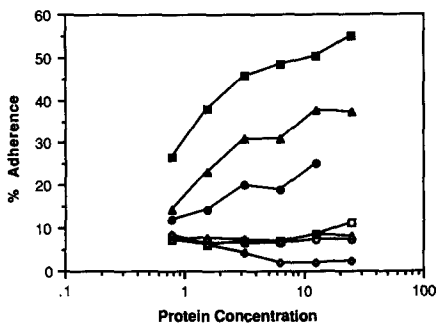

FIGURE 8. Adhesion of Y93A cells to ECM-protein. ^{3}H-labeled Y93A cells were incubated in wells previously coated with increasing concentrations of the ECM proteins as indicated. The wells were washed and the cells remaining were harvested onto glass filter disks with a PHD cell harvester. The disks were counted by liquid scintillation counter and percent adherence was determined as described in Materials and Methods. (■) Fibrinogen, (▲) fibronectin, (●) vitronectin, (□) collagen I, (○) collagen IV, (△) ovalbumin, (◇) laminin.

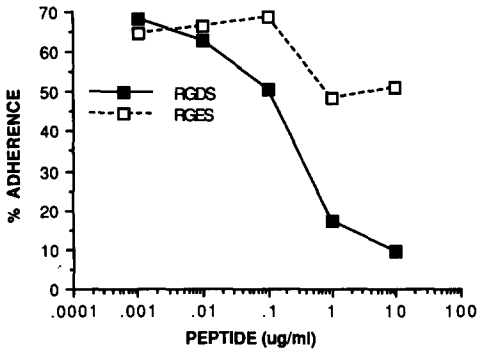

FIGURE 9. Blockade of adhesion to fibronectin by RGD-containing peptide. Y93A cells were assayed for adhesion to wells coated with constant amounts of rat fibronectin (10 μg/ml) as described in Fig. 9 after preincubation with and in the continuous presence of increasing concentrations of the tetrapeptides RGDS or RGES as indicated.

sites on the plastic surface. The binding of the DETC cell lines to fibrinogen always appeared to be greater than or equal to the binding to human fibronectin. The binding to human vitronectin was not observed in all studies and always appeared to be less than the binding to fibronectin. Comparable results were obtained with rat fibronectin (Figs. 10 and 11, data not shown). There was no demonstrable specific binding to collagen I and IV or laminin (Fig. 8), even at coating concentrations of 100 μg/ml (data not shown). Similar results were obtained with the DETC line Y245, and the hybridoma T195/BW (data not shown).

Since the interaction between fibronectin and its cellular receptor can be specifically blocked by peptides that contain the tripeptide sequence RGD, and since the binding of other ECM proteins to their receptors can be frequently inhibited by the same tripeptide (11), we performed the binding studies in the presence of increasing concentrations of the tetrapeptide RGDS or in the presence of a control tetrapeptide (RGES) that does not affect adhesion to fibronectin (11). The tetrapeptide RGDS inhibited in a dose-dependent manner the binding of Y93A cells to wells coated with a constant amount of rat fibronectin, while no inhibition was seen in the presence of the control peptide (Fig. 9). Similar results were obtained with Y245 cell line and the T195/BW hybridoma (Fig. 11 and data not shown).

The Binding of DETC Hybridoma and Lines to Fibronectin, Fibrinogen, and Vitronectin is Blocked by the mAbs H9.2B8 and 8.18E12. Neither mAb H9.2B8 nor 8.18E12 alone had any significant effect on T195/BW adhesion to rat fibronectin despite mAb concentrations of up to 50 μg/ml (Fig. 10). However, since the mAbs bind to distinct

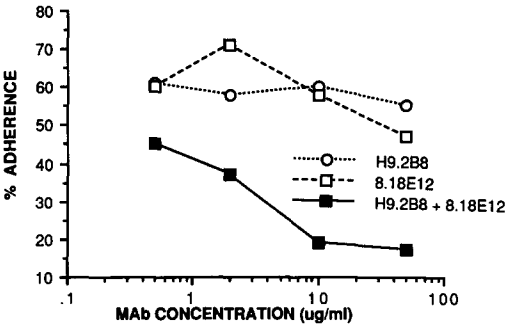

FIGURE 10. Blockade of adhesion to fibronectin with mAb H9.2B8 and 8.18E12. Y93A cells were assayed for adhesion to rat fibronectin-coated wells as described in Fig. 9, after preincubation with and in the continuous presence of increasing concentrations of the mAbs H9.2B8 and/or 8.18E12, as indicated.

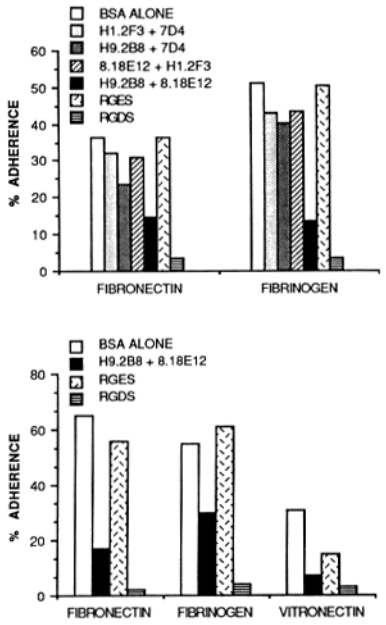

FIGURE 11. Blockade of adhesion to other ECM proteins by RGD and mAb H9.2B8 and 8.18E12. The adhesion of Y93A cells (*top*) or T195/BW cells (*bottom*) to wells coated with the substrates (10 μg/ml in coating buffer) indicated were assayed as described in Fig. 9. The cells were incubated with RGDS or RGES (10 μg/ml) as described in Fig. 9 or with mAb H9.2B8 and/or 8.18E12 (10 μg/ml) as described in Fig. 10. In addition, control incubations with rat mAb 7D4 (10 μg/ml) and/or hamster mAb H1.2F3 (10 μg/ml) are included in the top panel.

epitopes on the same 120-kD α chain fragment (Figs. 3 and 4), they were examined together for their effect on adhesion. In combination, mAbs H9.2B8 and 8.18E12 inhibited the adhesion of T195/BW to rat fibronectin at mAb concentrations of 10 μg/ml each. In addition to fibronectin, the adhesion of Y93A cells to rat fibrinogen (Fig. 11, *top*) and the adhesion of the T195/BW hybridoma to rat fibrinogen and human vitronectin (Fig. 11, *bottom*) were inhibited by RGDS, as expected, and by the combination of the mAbs H9.2B8 and 8.18E12. Incubation of the cells with RGES or either H9.2B8 or 8.18E12 alone or with control rat and hamster mAbs (Fig. 11, *top*) did not inhibit adhesion. Comparable findings were obtained for the DETC line Y245 (data not shown). Taken together, these data demonstrate that the binding of the DETC lines and hybridoma to all three ECM proteins (fibronectin, fibrinogen, and vitronectin) can be inhibited by the combination of mAbs H9.2B8 and 8.18E12.

Discussion

The results of our biochemical and functional studies strongly suggest that the mAbs H9.2B8 and 8.18E12 identify a murine integrin involved in the binding of cells to ECM proteins. mAbs H9.2B8 and 8.18E12 immunoprecipitate a complex that consists of a 140-kD chain that is composed of a 120- and a 23-kD subunit that are disulfide linked. The 140-kD chain is noncovalently associated with a 95-kD chain. This pattern of cell surface proteins is characteristic of the α and the β chain, respectively, of certain ISCAM members (1–3, 11).

We have described a simple, quantitative assay for cell adhesion to ECM proteins. While previous reports used similar methods for immobilization of ECM proteins to plastic surfaces, many of the studies use manual cell counting methods to quantitate the assay. The method we describe here used microtiter plates, is semi-automated,

quantitative, and is amenable to large experiments. We have used this assay to determine that murine DETC lines and a DETC hybridoma adhere to the ECM proteins, fibronectin, fibrinogen, and at lower levels, to vitronectin. The lower level of binding to human vitronectin may be due to a lower affinity for vitronectin binding to plastic or to a species difference, although the cells appear to adhere equally well to rat and human fibronectin. Since DETC cell adherence to these ECM proteins can be blocked by a tetrapeptide containing the RGD sequence, these data are compatible with previously reported experiments that suggest that many of the integrin-ligand interactions are inhibitable by this peptide sequence (11). Nucleotide sequence analysis of human integrins has demonstrated the presence of α chain domains characteristic of cation binding proteins (11, 31). Previous studies have demonstrated that Ca^{2+} binds to the IIb (α) subunit of the platelet gpIIb/IIIa receptor (32) and that cations affect the interaction of these receptors with their ligands (33). These data are consistent with our observation that the DETC lines, which are extremely adherent to plastic tissue culture flasks, may be readily detached with cation chelators like EDTA. While it is possible that the culture media, i.e, FCS, contains ECM proteins like vitronectin (previously named serum spreading factor), these observations are also compatible with the possibility that our DETC cell lines produce an ECM protein to which the DETC cells attach via their integrins. In this regard, it has been demonstrated that certain murine T cell clones can produce fibronectin in vitro (34). The precise character of the ECM protein produced by the DETC cell lines, if any, is under investigation.

We have established that the mAbs H9.2B8 and 8.18E12 recognize an integrin because these mAbs, in combination, inhibit the attachment of cells to the ECM proteins. While it is fortuitious that such a combination of mAbs was produced inasmuch as we did not screen for such a functional effect, the mAbs are of different species origin and were made against different cell lines. By sequential immunoprecipitation experiments, it is clear that these mAbs recognize the same antigenic complex and bind to the same larger α chain fragment. However, the mAbs bind to distinct epitopes since neither inhibits the binding of the other by FACS analysis. The requirement for both mAbs to block adherence suggests that both bind separate epitopes near the receptor-ligand binding site and that blockade occurs by steric hinderance. An alternative view would be that the two distinct antigenic epitopes bind to two separate sites on the fibronectin molecule and that both fibronectin sites are necessary for complete cell attachment, a concept supported by the recent observation documenting that cells can attach to two different sites on the fibronectin molecule (35, 36). The ability of the mAbs to block the adhesion of the DETC cell lines to fibronectin, fibrinogen, and vitronectin suggests that a single receptor complex is responsible for the interaction of the DETC with these substrates. However, additional RGD-dependent integrins that also mediate binding to these substrates cannot be excluded, as the mAb blockade does not appear to be as complete as the RGD blockade.

The precise molecular relationship of the integrin recognized by the mAbs H9.2B8 and 8.18E12 to the proposed classification system of human integrins (1) cannot be determined with certainty because of the species difference and because similar studies have not been reported for all of the described integrins. Furthermore, no lymphocyte integrin has been described that binds to fibronectin, fibrinogen, and vitronectin.

However, there are distinctive features that have been reported for the human integrins that tend to suggest that the DETC integrin is a member of the β_1 or β_3 family. It appears that the DETC integrin is not any of the described members of the LAP (β_2) family, an antigen and receptor system well characterized on murine cells (3), because none of the described α chains in the murine or human β_2 family have been noted to dissociate into two fragments, and none have a similar tissue distribution or ligand specificity. While the DETC integrin bears some resemblance to certain members of the β_1 family, specifically VLA-1 and VLA-2, in that we demonstrated that the DETC integrin is similarly a late activation antigen (5), the DETC integrin two fragment α chain resembles instead the structure of the VLA-3 and VLA-5 human integrins (2). The ligand binding experiments seem to exclude homology to human VLA-5 since this receptor apparently binds only fibronectin (2). While the human VLA-3 complex binds to fibronectin, it also binds to collagen and laminin but has not been described to bind to fibrinogen or vitronectin (2). Therefore, none of the VLA antigens have been reported to have all three properties, i.e., ligand specificity, antigen expression, and two fragment α chain, in common with the DETC integrin. An alternative view is to consider the possibility that the DETC integrin is a member of the β_3 family; both human members, platelet gp IIb/IIIa and the vitronectin receptor, have the two-fragment α chain structure (2, 9, 12, 31, 37). Although the vitronectin receptor reportedly binds only to vitronectin (9), the platelet gp IIb/IIIa complex has been shown to bind multiple ligands, including fibronectin, fibrinogen, vitronectin, and Von Willebrand factor, but not collagen (12, 38); this adhesion pattern is similar to the pattern that we have established for the DETC integrins. Nevertheless, while human $\beta 1$ family members are expressed by both platelets and lymphocytes (38–40), the lymphocyte expression of platelet gpIIb/IIIa has not been convincingly documented. Thus, although further detailed biochemical and molecular analysis will be required to fully resolve the relationship of the murine integrin described here and human integrins, this is the first demonstration of a lymphocyte receptor that binds to fibronectin, fibrinogen, and vitronectin.

Although all DETC lines examined express the integrin recognized by mAbs H9.2B8 and 8.18E12 (Table I; Kehn, P., and W. M. Yokoyama, data not shown), we have been unable to demonstrate staining with these mAbs in situ on fresh epidermal sheets (Elbe, A., and G. Stingl, data not shown). Since this integrin is expressed by splenic T cell blasts after mitogen or allostimulation and by several T cell clones, its expression appears to reflect a state of T cell activation, similar to that initially described for the VLA antigens, rather than specificity for DETC. In this regard, the relatively late and prolonged expression of these integrins after T cell stimulation is reminiscent of that reported for the Pgp-1 antigen. Pgp-1 antigen expression in vivo has been correlated with prior activation, i.e., as a marker of memory cells (41). The possible relationship between the expression of the integrin recognized by mAb H9.2B8 and 8.18E12 and T memory cells is presently under investigation.

The role of integrins that bind ECM proteins in T cell ontogeny and function has not been thoroughly explored. It is likely that these receptors are involved in lymphocyte trafficking during T cell ontogeny, since in the developing avian thymus, precursor hemopoietic cells and thymocytes display fibronectin receptors that are required for the cells to traverse a basement membrane barrier (42). Murine thymocyte subset analysis (43) has shown that the fibronectin receptor-bearing cells be-

long predominantly to the cells that bear the phenotype $CD4^-$, $CD8^-$ (double negative) and large $CD4^+$, $CD8^+$ rather than small $CD4^+$, $CD8^+$ or single positives ($CD4^+$ or $CD8^+$). Since the double-negative subset can give rise to all of the thymocyte subpopulations (44), these data suggest that fibronectin binding plays a role during the early phases of thymocyte development. Moreover, other studies suggest that such integrins may be involved in trafficking during the immune response inasmuch as ECM proteins are actively synthesized by inflammatory cells (45) and macrophages (46). Furthermore, VLA^+ T cells are found in the synovial fluid of chronically inflamed rheumatoid arthritis joints (47) and other sites of inflammation (48). Further dissection of the biochemical and molecular features of the murine integrins should aid the analysis of the physiological role of integrins in T cell ontogeny and in the in vivo immune response, studies that are particularly well suited to murine studies.

Summary

Cell–cell and cell–extracellular (ECM) protein interactions are mediated through heterodimers termed integrins. We have demonstrated that dendritic epidermal T cell (DETC) lines adhere to the ECM proteins, fibronectin, fibrinogen, and vitronectin but not to collagen, laminin, or control proteins. This adhesion was blocked by the tetrapeptide RGDS, but not the control peptide, RGES. We have derived a hamster mAb H9.2B8, and a rat mAb, 8.18E12, from immunizations with DETC lines. The mAbs in combination, but not individually, specifically inhibited the adhesion of DETC lines to fibronectin, fibrinogen, and vitronectin. Immunoprecipitation analysis revealed that both mAbs reacted with a heterodimer composed of noncovalently linked 140- and 95-kD subunits. The 140-kD subunit can be reduced to 120- and 23-kD fragments. Although the two mAbs did not cross-compete for binding to DETC, sequential immunoprecipitation studies indicated that they react with the same 120-kD fragment. While all DETC cell lines and several T cell clones were reactive with the mAbs, the mAbs were not reactive with normal spleen, lymph node, thymus, or skin. Stimulation of splenic T cells with Con A or allogeneic cells induced mAb reactivity after 1 wk in vitro. These data demonstrate that a single lymphocyte receptor, with biochemical features characteristic of integrins, mediates RGD-dependent binding to the ECM proteins, fibronectin, fibrinogen, and vitronectin. Furthermore, since this integrin is expressed by long-term activated T cells, this receptor may play a physiological role in T cell function.

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