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THE DISTINCT LEUKOCYTE INTEGRINS OF MOUSE SPLEEN DENDRITIC CELLS AS IDENTIFIED WITH NEW HAMSTER MONOCLONAL ANTIBODIES

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The use of mAbs to surface antigens has identified several molecules, other than the TCR-CD3 complex and peptide-presenting MHC products, which contribute to the interaction of APC and T lymphocytes. Most notable are mAbs against CD4 and CD8 (1, 2), and lymphocyte function-associated antigen 1 (CD11a, LFA-1)¹ (3) and its ligand, intercellular adhesion molecule 1 (CD54, ICAM-1) (4). Each mAb inhibits antigen-driven T cell proliferation when antigen is presented by a variety of APC, including one of the most potent, dendritic cells (5).

An early step in a primary immune response is the association of T cells and APC in discrete, long-lived (days), aggregates (6, 7). However, none of the known molecules on either T cells or dendritic cells appears essential for the early stages of cluster formation in mice, primarily because rat mAbs directed against these molecules do not block the formation of sizeable aggregates in the murine MLR (5). For example, mAbs to LFA-1 inhibit the binding between T cells and activated B cells but fail to inhibit initial dendritic-T cell clustering (8).

Recently, when the rat has proved to be a difficult host for preparing mAb to structurally conserved murine molecules, several investigators have used Armenian hamsters as a host (9–11). We adopted a similar approach to search for mAbs that would inhibit dendritic-T cell clustering or would bind to dendritic cells in a novel assay using the FACS. We report here the isolation of two new anti-mouse leukocyte integrin mAbs: 2E6, which recognizes a determinant common to all members of the $\beta 2$ integrin family and N418, which identifies a distinct p150,90 member of the family, possibly CD11c. Using these new reagents, we describe the distinct expression and function of leukocyte integrins on spleen dendritic cells.

Materials and Methods

Animals

BALB/C $\times$ DBA/2 F₁ [C $\times$ D2 F₁, H-2^d], C57Bl/6 $\times$ DBA/2 F₁ [B6 $\times$ D2 F₁, H-2^{bxd}], and C3H/He [H-2^k] mice of both sexes, from the Trudeau Institute (Saranac, NY), were used at 8–12 wk of age. Adult female Armenian hamsters [*C. migratorius*] were purchased from Cytogen Research and Development (Cambridge, MA).

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Culture Medium

RPMI 1640 (Gibco Laboratories, Grand Island, NY) was supplemented with 20 $\mu\text{g}/\text{ml}$ gentamicin, 5×10^{-5} M 2-ME, and 10% heat-inactivated FCS (R10; Hazelton, Lenexa, KS), and used for maintaining hybridomas and primary cell cultures. For selecting hybridomas, DMEM with high glucose (Gibco Laboratories) was supplemented with 20% FCS, HAT, 2-ME, and antibiotics (HAT medium).

Cell Populations

Murine spleen dendritic cells were prepared by a multistep technique (12). Briefly, spleens were sequentially digested with collagenase, floated on dense BSA, adhered to tissue culture plastic, washed free of nonadherent cells, cultured overnight, and depleted of contaminating FcR^+ B cells and macrophages by rosetting with antibody-coated sheep RBC. The dendritic cells were routinely >95% pure as judged by FACS analysis (Results) (8, 12).

B cells were purified from spleen suspensions by depleting dendritic cells and macrophages by passage over columns of Sephadex G10 (Pharmacia Fine Chemicals, Piscataway, NJ), and then depleting T cells by treatment with a cocktail of anti-T cell reagents (anti-Ly-1, anti-Thy-1, anti-Ly-2) and rabbit complement (Pel-Freez Biologicals, Rogers, AR). Activated B cells were prepared by culturing purified B cells with polyclonal goat anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA) coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals) at 5 $\mu\text{g}/\text{ml}$ for 2–3 d. The anti-Ig blasts were enriched on Ficoll-Hypaque gradients (Pharmacia), and shown to be >95% B blasts with similar levels of class II MHC products to dendritic cells (8).

CD4^+ T cells were nylon wool nonadherent, mesenteric lymph node cells that had been treated with anti-Ia and anti-Ly-2 mAbs plus complement to deplete accessory cells and CD8^+ T cells.

Peritoneal exudate cells were harvested from mice 4 d after an injection of 1–1.5 ml i.p. of thioglycolate (Difco Laboratories, Detroit, MI).

Immunization and Fusion

Two fusions were performed using hamster spleen cells that had been primed to mouse spleen dendritic cells. In the first, which yielded the 2E6 anti-CD18 reagent, 5×10^6 dendritic cells were given intraperitoneally on day –41 and –3 before fusing. In the second, which yielded the N418 anti p150,90 and N22 anti-MHC class II mAb, the hamsters were primed with 5×10^6 dendritic cells intraperitoneally on day –220 and boosted with 1.3×10^6 cells intraperitoneally on day –10 and 5×10^6 cells intracardiac on day –3.

For both fusions, on day 0 the SP 2/0 myeloma cells were mixed with hamster spleen cells at a ratio of 1:4 and fused with 50% (wt/vol) polyethylene glycol for 90 s. After slowly diluting the fusion mixture with a total of 15 ml of warm medium over a total of 8 min, the cells were either further diluted in HAT medium (clones N418, N22) for direct plating or were first centrifuged and then gently resuspended in HAT medium for plating (clone 2E6). $2\text{--}3 \times 10^4$ myeloma cells were plated per well in 96-well flat-bottomed plates (microwells; Corning Glass Works, Corning, NY). The wells were fed two to four times over a 3-wk period before screening the culture supernatants.

Hybridoma Screening

Three assays were used to select hybridomas of interest. (a) To test if the hybridoma supernatant (25% vol/vol) would inhibit the formation of dendritic-T cell clusters in the primary MLR, 3×10^4 dendritic cells and 3×10^5 allogeneic T cells were cultured in flat-bottomed microtest wells and clustering was assessed at 24 h by microscopic observation. (b) Supernatant was added to a cryostat section of spleen followed by labeling with a peroxidase (POX)-conjugated anti-hamster Ig (in the presence of 1% mouse serum) or with biotin-RG7 plus avidin-biotin-peroxidase complexes (see below). (c) Selective binding to spleen dendritic cells was evaluated with a new FACS approach. Purified dendritic cells were labeled with FITC (2 $\mu\text{g}/\text{ml}$ 20 min) as described (13) and mixed with unlabeled mesenteric lymph node cells at a ratio of 1:10. 10^5 cells of the mixture were added to round-bottomed wells containing 100 μl of hybridoma culture supernatant. Bound mAbs were detected with anti-hamster Ig

and a PE-label (see below), so that two-color FACS analysis would identify mAbs that stained dendritic cells (positive in both FITC and PE channels) but not the other leukocytes (negative in both channels).

Hybridoma Cloning

Hybridomas were cloned by limiting dilution (14). Wells positive for hybridoma growth were rescreened for staining of spleen sections (see Results). The 2E6 anti-CD18 cells have been stable secretors, but the N418 anti-CD11c line has required periodic recloning to prevent overgrowth by nonsecreting clones.

FACS Staining

10^5 to 5×10^5 cells per sample were stained in round-bottomed multiwell plates. Washing was carried out by centrifuging the plates two to three times between staining steps with PBS containing 1% BSA and 0.02% NaN₃. Binding of hamster mAbs was visualized with a three-step technique involving hybridoma supernatants, biotinylated mouse anti-rat κ chain (mAb RG7/7.6, at 2–3 $\mu\text{g/ml}$) that crossreacts with Armenian hamster κ chains (15), and PE-streptavidin (Biomedica Corp., Foster City, CA; 3 $\mu\text{g/ml}$). Alternatively, the hamster mAb was stained with a FITC or biotin conjugate of a goat anti-hamster Ig (Vector Laboratories, Burlingame, CA). Binding of rat mAbs used a two-step technique in which the second reagent was FITC-mouse anti-rat Ig (Boehringer-Mannheim Biochemicals, Indianapolis, IN; 3 $\mu\text{g/ml}$). All samples were analyzed on a FACScan (Becton-Dickinson, Mountain View, CA).

Production, Purification, and Modification of mAbs

Rat mAbs RA3-6B2.1 (anti-B220), F4/80 (anti-macrophage), M1/70 (anti-CD 11b/CR3), FD441.8 (anti-CD11a/LFA-1), B21.2 (anti-I-A^{b,d}), GK1.5 (anti-CD4), and 3.155 (anti-CD8/Lyt-2) were all grown in our laboratory and used as culture supernatants for staining and blocking experiments (16). Hamster mAbs, in addition to the new reagents described here, included 2C11 (anti-CD3) (10) and S4B6.1 (anti-murine IL-2) (11), which were provided by Drs. J. Bluestone (University of Chicago, Chicago, IL) and T. Mossman (DNAX Research Institute, Palo Alto, CA) and maintained in our lab. For Ig purification from many hybridomas (2E6, M1/70, and FD441.8), cells were grown to maximal density in serum-free medium (Nutridoma-SP, Boehringer-Mannheim), and the mAb was precipitated with 50% saturated $[\text{NH}_4]_2\text{SO}_4$ for 2 h at 4°C. This technique provided Ig of high purity as judged by Coomassie staining on SDS-PAGE. For purification of mAb RG7/7.6 (17), ascites that had been precipitated with $[\text{NH}_4]_2\text{SO}_4$ (kindly provided by M. Birkeland and E. Puré), was further purified by protein A chromatography. The N418 Ig was purified from hybridoma culture supernatant using a protein A column (Pierce Chemical Co., Rockford, IL).

Purified mAbs were coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals) at a ratio of 2 mg/ml packed beads as per manufacturers instructions. Purified RG7/7.6 was coupled with biotin-*N*-hydroxysuccinimide (Zymed Laboratories, San Francisco, CA) at a weight ratio of 1:1 and then dialyzed extensively against PD (PBS without Ca^{2+} or Mg^{2+}).

An F(ab')_2 fragment of 2E6.1 was generated by incubating 5 mg purified mAb with 0.5 ml pepsin gel (Pierce Chemical Co.), as per manufacturer's instructions, for 4 h at 37°C with constant agitation. Undigested molecules were subsequently removed by passage over immobilized protein A columns. The F(ab')_2 fragment was judged free of contaminating intact IgG by analysis on SDS-PAGE and Coomassie staining.

Immunoprecipitation

$1.5\text{--}3 \times 10^7$ cells were washed two times in RPMI and one time in PD with 1 mg/ml glucose. Cells were resuspended in 1 ml of the latter buffer and iodinated by the sequential addition of glucose (to 20 mM), 3 mCi ^{125}I (Na^{125}I ; New England Nuclear, Boston, MA) 20 μg LPO, and glucose oxidase (18). After 15 min on ice, cells were washed three times and then lysed on ice with buffer containing 1% NP-40, 1% aprotinin, 1 mM PMSE, and 1 mM DFP (added fresh) for 10 min. Cell debris and nuclei were pelleted at 10,000 rpm for 10 min. $0.5\text{--}1 \times 10^7$ cpm were precipitated per sample after first preclearing the lysate three times with normal rat Ig and RG7-Sepharose, or rat Ig coupled directly to Sepharose. Samples were precipi-

tated either directly with an equal volume of antibody coupled to Sepharose or indirectly with equal volumes of antibody supernatant followed by RG7-Sepharose. After incubating at 4°C with shaking, Sepharose pellets were washed two times with PD, once with high salt azide (HSA, 0.6 M NaCl, 0.0125 M KPO₄, 0.02% NaN₃, pH 7.4), once with mixed detergent buffer (0.05% NP-40, 0.1% SDS, 0.3 M NaCl, 10 mM Tris pH 8.6), once with HSA, and twice with PD. Pellets were resuspended in 2× sample buffer (2% SDS, 10% glycerol, 80 mM Tris pH 6.8, with bromophenol blue), boiled, and subjected to SDS-PAGE and autoradiography with enhancing screens.

Immunocytochemistry

Fresh tissue was embedded in OCT (Miles Inc., Elkhart, IN) and 10- μ m-thick sections were applied to 10-well slides (Carlson Scientific Inc., Peotone, IL). Slides were fixed in acetone (10 min, RT) and stained by the sequential addition of primary mAb supernatant, biotin-RG7/7.6, avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc.), and 3,3'-diaminobenzidine tetrahydrochloride (Polysciences, Inc., Warrington, PA) (19). Slides were mounted in PD-glycerol for photography.

Functional Effects of mAbs on Leukocyte Integrin Function

C3bi Binding. Terasaki microwells were coated with 20% FCS in PBS and then elicited peritoneal cells were applied (5 μ l/well at 10⁶/ml in PBS with 1% BSA) for 40 min at 37°C. After washing, hybridoma culture supernatants were added to the wells for 10 min on ice. Then sheep RBC coated with C3bi (20) were applied (5 μ l/well at 10⁸/ml) and incubated for 10 min on ice plus a further 30 min at 37°C. The plates were washed by gently swirling and inverting in PBS, and fixed with 2.5% glutaraldehyde for scoring. The attachment index was the total number of attached RBC per number of macrophages counted $\times$ 100.

Homotypic Clustering. This assay was performed according to Rothlein and Springer (21). Anti-Ig-activated B cells were added to 96-well flat-bottomed plates with 50 ng/ml PMA (Sigma Chemical Co., St. Louis, MO). mAbs were added at 25% vol/vol. The cultures were monitored under an inverted microscope and photographed after 2 h at 37°C.

MLR Clustering and Proliferation. Purified dendritic cells or B blasts, irradiated with 1,500 rad ¹³⁷Cs, were added in graded doses to 3 $\times$ 10⁵ CD4⁺, allogeneic T cells in 96-well plates (8). Purified mAb or hybridoma culture supernatant was added at the start of the MLR. The cultures were photographed at 24 h of culture. 1 μ Ci/well [³H]TdR was added for the last 8 h as noted. The wells were harvested with a Skatron automatic harvester (Skatron, Inc., Sterling, VA) to be counted with a Betaplate scintillation counter (LKB Wallac, Finland).

Results

Selection of Distinctive Hamster Anti-mouse Leukocyte mAb. Several approaches were used to screen hybridomas that were generated from dendritic cell immune, hamster spleen. One approach, the blocking of dendritic-T cell clustering in the primary MLR, did not yield a single active mAb from $\sim$ 1,500 wells in two separate fusions. Another screen, immunolabeling of mouse spleen sections, provided a large number of reagents that stained in a fashion that had not been noted previously with a large panel of anti-mouse leukocyte mAb (19). A frequent pattern, exemplified by 2E6, was reactivity with many cell types in red pulp especially granulocytes, as well as many cells in white pulp, particularly T cells. 2E6 proved to be an anti-leukocyte integrin mAb, recognizing a determinant shared by all members of the leukocyte integrin family (see below). Another frequent immunolabeling pattern was identical to that seen with standard anti-mouse MHC class II mAb. This also will be illustrated below.

A third screening approach involved selective binding to dendritic cells in a two-color FACS assay. FITC-labeled dendritic cells were mixed at 1:20 with unlabeled

mesenteric lymph node cells, and aliquots of the mixture were exposed to the hybridoma test supernatants. Reactive mAbs were visualized with a biotinylated secondary reagent followed by PE-streptavidin. The most frequent pattern was a lack of reactivity with either FITC labeled or unlabeled cells (Fig. 1 *a*). Another frequent pattern was strong reactivity with dendritic cells and a subpopulation of node cells. In many cases, as exemplified by N22 in Fig. 1 *b*, these mAb later proved to be anti-MHC class II by immunoprecipitation and/or by comparison with existing anti-class II reagents in immunolabeling procedures. The most intriguing pattern, exemplified by N418 (Fig. 1 *c*) was clear staining of dendritic but not lymph node cells. While N418 proved to identify a distinct p150,90 integrin (see below), most other "dendritic cell specific" candidates proved to react with a fine extracellular matrix around blood vessels and scattered about red and white pulp. These were not pursued further.

Molecular Characterization of N418 and 2E6. Immunoprecipitation of ^{125}I -labeled spleen cell lysate with 2E6 revealed a predominant heterodimer of approximate molecular mass 180, 90 kD, with some minor bands at 150–160 kD (Fig. 2, *top left*, lane 2). This pattern was reminiscent of those immunoprecipitated by already characterized rat mAbs to murine LFA-1 and CR3 (22), and their human counterparts (23). These molecules are members of a family of leukocyte $\beta 2$ integrins, each a heterodimer with a distinct α chain (ranging from 180 to 150 kD) but an identical β chain (90–95 kD). Indeed, the 180, 90 kD heterodimer precipitated by 2E6 comigrated with bands precipitated by the anti-LFA-1 α chain mAb (FD441.8). Much less protein was precipitated from the spleen cell lysate with anti-CR3 α chain [M1/70]. This is consistent with the fact that lymphocytes, the major cell population released from the spleen, express predominately the LFA-1, CD11a integrin. Sequential immunoprecipitation (Fig. 2, *top right*) proved that 2E6 and anti-LFA-1 identified the same major heterodimers on spleen cells.

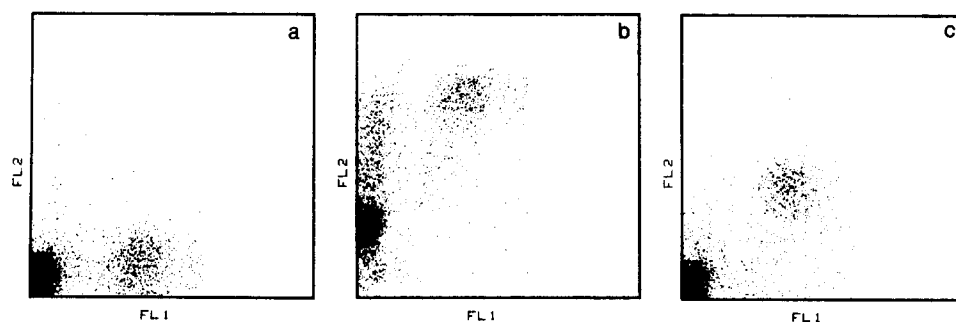


FIGURE 1. FACSscan profiles representing typical patterns of staining from screening hybridoma supernatants. EA⁺ dendritic cells were prelabeled with FITC as described and mixed with bulk mesenteric lymph node cells at a ratio of 1 dendritic cell to 10 lymph node cells. 1×10^5 cells were stained per microwell with 100 μl of test hybridoma supernatant. Immunoreactivity was detected with a 3 layer technique involving biotin-RG7/7.6 followed by PE-streptavidin (see Methods). Cells were analyzed on a FACSscan. Results are presented as single event dot plots of fluorescence intensity 1 (FITC) vs. fluorescence intensity 2 (PE). The axes span 4 $\log_{10}$ decades. (A) Nonreactive hybridoma supernatant; (B) anti-class II MHC-reactive mAb, N22; (C) dendritic cell-reactive mAb, N418.

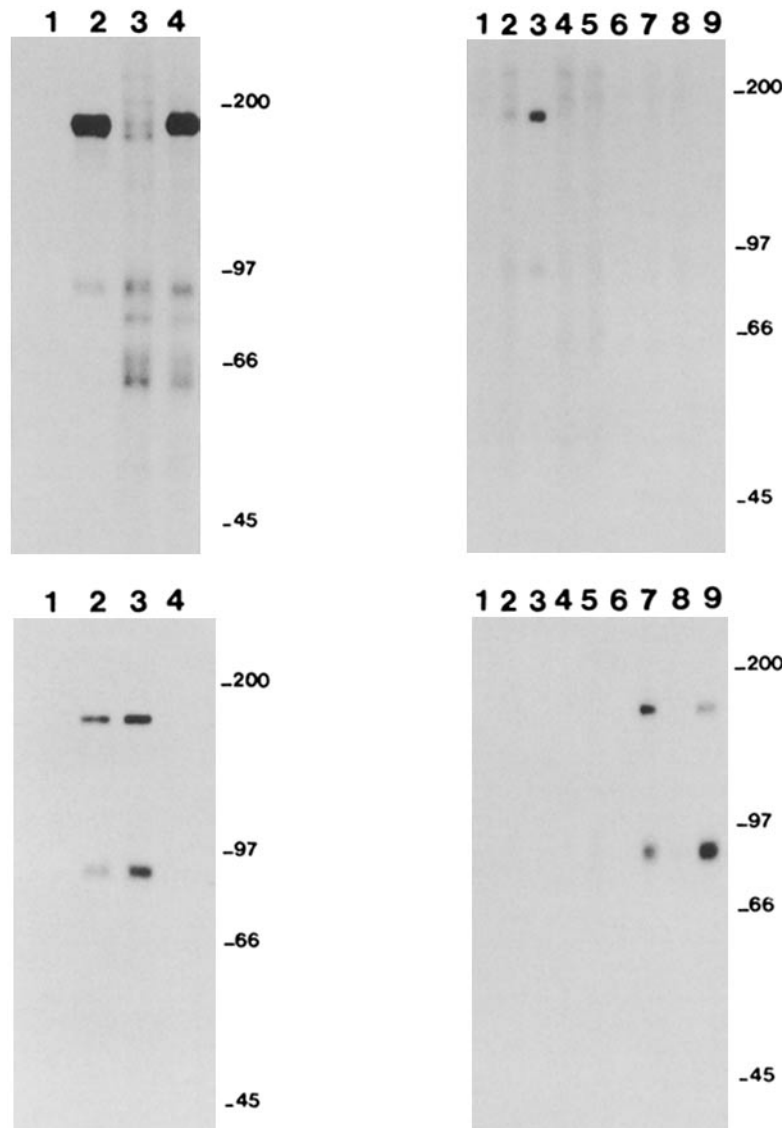


FIGURE 2. Immunoprecipitation of ^{125}I -labeled spleen cell lysate and peritoneal macrophage lysate with anti-integrin mAbs. 3×10^7 spleen leukocytes (*top*) or 2.2×10^7 thioglycolate elicited peritoneal cells (*bottom*) were labeled with ^{125}I . Immunoprecipitation of cell lysates was performed as described in Materials and Methods. Samples were subjected to SDS-PAGE and autoradiography. Molecular weights correspond to the positions of marker proteins stained with Coomassie blue. (*Left*) Primary immunoprecipitation run on 6% SDS-PAGE. (Lane 1) Normal rat Ig; (lane 2) 2E6; (lane 3) M1/70 (anti-CD11b); (lane 4) FD441.8 (anti-CD11a). (*Right*) Sequential immunoprecipitation of spleen (*top*) and peritoneal (*bottom*) lysates. Samples were precleared with the first mAb, then precipitated with a second mAb and run on 6% SDS-PAGE. (lane 1) M1/70 followed by M1/70, (lane 2) M1/70 followed by FD441.8, (lane 3) M1/70 followed by 2E6, (lane 4) 2E6 followed by M1/70, (lane 5) 2E6 followed by FD441.8, (lane 6) 2E6 followed by 2E6, (lane 7) FD441.8 followed by M1/70, (lane 8) FD441.8 followed by FD441.8, (lane 9) FD441.8 followed by 2E6.

In contrast, the major integrin on peritoneal macrophages was a 170, 90 kD heterodimer precipitated by mAb M1/70 but not by FD441.8 (Fig. 2, *bottom left*), as expected (22). Again the M1/70 heterodimer comigrated with 2E6, and sequential immunoprecipitation (Fig. 2, *bottom right*) confirmed the identity of these two heterodimers. Based on these results, and additional blocking experiments below, we concluded that 2E6 recognizes a determinant common to the LFA-1 (CD11a) and CR3 (CD11b) members of the leukocyte $\beta 2$ integrin family, most likely the common CD18 β chain.

Since mAb 2E6 was raised against spleen dendritic cells and stained these cells strongly (see below), we performed comparable studies on iodinated spleen dendritic cells. 2E6 precipitated LFA-1 and CR3 α chains from the dendritic cell preparation, but the predominant species was a 150, 90 kD heterodimer (Fig. 3, lane *b*). The

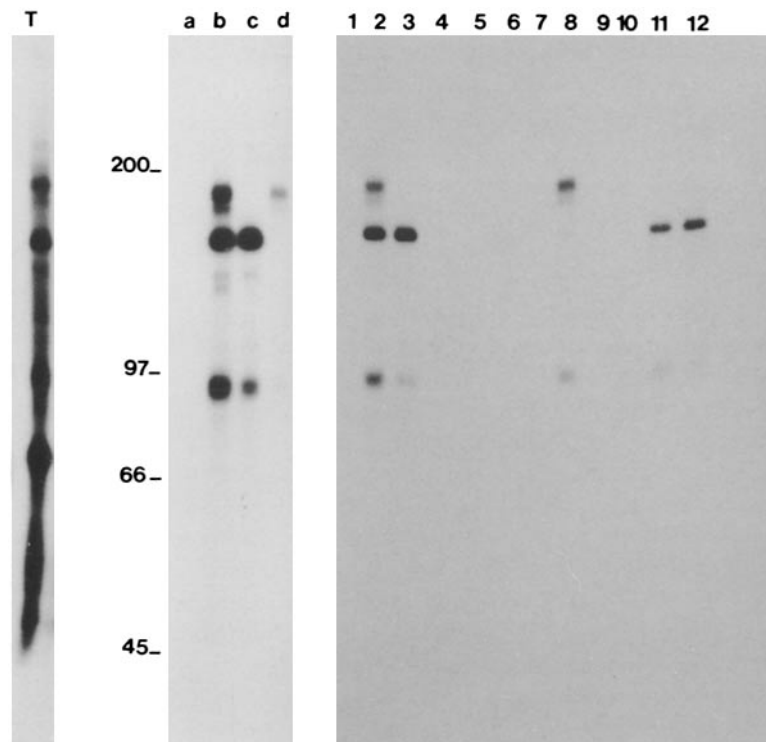


FIGURE 3. Immunoprecipitation of ^{125}I -labeled spleen dendritic cells with anti-integrin mAbs. (Lane 1) 7×10^7 EA $^{-}$ spleen dendritic cells were surface labeled with ^{125}I . Samples were immunoprecipitated as described in Materials and Methods and subjected to 6% SDS-PAGE and autoradiography. (*Left*) Lane T is total surface-labeled dendritic cell lysate. (*Middle*) Primary immunoprecipitations. (a) S4B6 (anti-IL-2) control hamster IgG, (b) 2E6, (c) N418, (d) FD441.8 plus M1/70 (both mAbs added simultaneously). (*Right*) Sequential immunoprecipitation. Samples were precleared with the first mAb then immunoprecipitated with the second mAb. (Lane 1) S4B6 followed by S4B6, (lane 2) S4B6 followed by 2E6, (lane 3) S4B6 followed by N418, (lane 4) 2E6 followed by S4B6, (lane 5) 2E6 followed by 2E6, (lane 6) 2E6 followed by N418, (lane 7) N418 followed by S4B6, (lane 8) N418 followed by 2E6, (lane 9) N418 followed by N418, (lane 10) FD441.8 + M1/70 followed by S4B6, (lane 11) FD441.8 + M1/70 followed by 2E6, (lane 12) FD441.8 + M1/70 followed by N418.

150 kD α chain was distinct, since preclearing with anti-LFA-1 and CR3 α chains eliminated the 180 and 170 kD α chains upon reprecipitation with 2E6, but did not remove the 150 kD band (Fig. 3, lane 11). Intriguingly, the bands at 150 and 90 kD are among the major iodinated species on dendritic cells (Fig. 3, lane T).

When the more restricted anti-dendritic cell mAb N418 was tested, the mAb failed to precipitate any bands from total spleen lysate (not shown) but precipitated a 150, 90 kD heterodimer from dendritic cell lysate (Fig. 3, lane c). This heterodimer comigrated with the major bands precipitated by 2E6. Reciprocal preclearing experiments demonstrated that the heterodimers precipitated by 2E6 and N418 were identical. Since N418 failed to precipitate any of the higher molecular mass integrin α chains, we conclude that N418 recognizes the α chain of a 150, 90 kD integrin, while 2E6 recognizes the common β chain.

FACS Analysis. Since mAb 2E6 reacted with all members of the leukocyte integrin family, it was not surprising that its cellular distribution was extensive. FACS analyses (Fig. 4) showed that 2E6 strongly stained a variety of leukocytes, including spleen dendritic cells, activated B blasts, and peritoneal macrophages. However, while lymphocytes and macrophages stained as strongly with 2E6 as with anti-LFA-1 (lymphoblasts) or anti-CR3 (macrophages), dendritic cells stained more strongly with 2E6 than with either anti-LFA-1 or anti-CR3. The N418 mAb, which recognized a third, distinct, β_2 integrin, stained the spleen dendritic cells but not the lymphocytes and macrophages (Fig. 4). As little as 0.1 μ g/ml of Ig provided optimal staining of dendritic cells, so that antibody was not limiting in these assays. Therefore the FACS data again showed that the 150, 90 kD integrin primarily was found on dendritic cells in the populations we studied.

Immunocytochemistry. Spleen sections were stained with a panel of mAbs to known mouse leukocyte antigens (16) to identify the antigens B220 (B cells), CD3/4/8 (T cells), F4/80 (red pulp macrophages), RB6 (granulocytes), SER-4 (marginal zone macrophages), and B21-2 (MHC class II). These mAb, as described before (19, 24), outlined the B and T cell areas of the white pulp, the marginal zone separating white and red pulp, and the macrophage/granulocyte-rich red pulp. With these mAbs as guides, it was noted that 2E6 and N418, the new mAbs to leukocyte integrins, each stained in a pattern that had not previously been described.

2E6 the presumptive anti-CD18 reagent produced extensive staining of both white and red pulp (Fig. 5, *top right*). In the white pulp, the lymphocytes in the T-dependent area stained more strongly than in the B area. In the red pulp, both granulocytes and macrophages were stained. The 2E6 stain was, for the most part, a composite of the stain seen with the CD11a (LFA-1 or F441.8) and CD11b (CR3 or M1/70) mAb. Anti-CD11a stained lymphocytes, particularly T cells around the white pulp central artery, while M1/70 stained granulocytes in the red pulp, and a web of macrophages in the marginal zone (Fig. 5, *top*).

N418 anti-p150,90 stained the white and red pulp differently from the other anti-integrin reagents. In the white pulp, staining was restricted to dendritic profiles within the T area, with few or no profiles in the B area (Fig. 5, compare bottom left and right). The N418 stain was different from N22 anti-class II that stained all B cells in addition to dendritic profiles in the T area (Fig. 5, *bottom middle*). The staining by N418 of dendritic cells in the T area was expected, since T areas contain "interdigitating cells" that have the cytology and phenotype of isolated dendritic cells (19, 25).

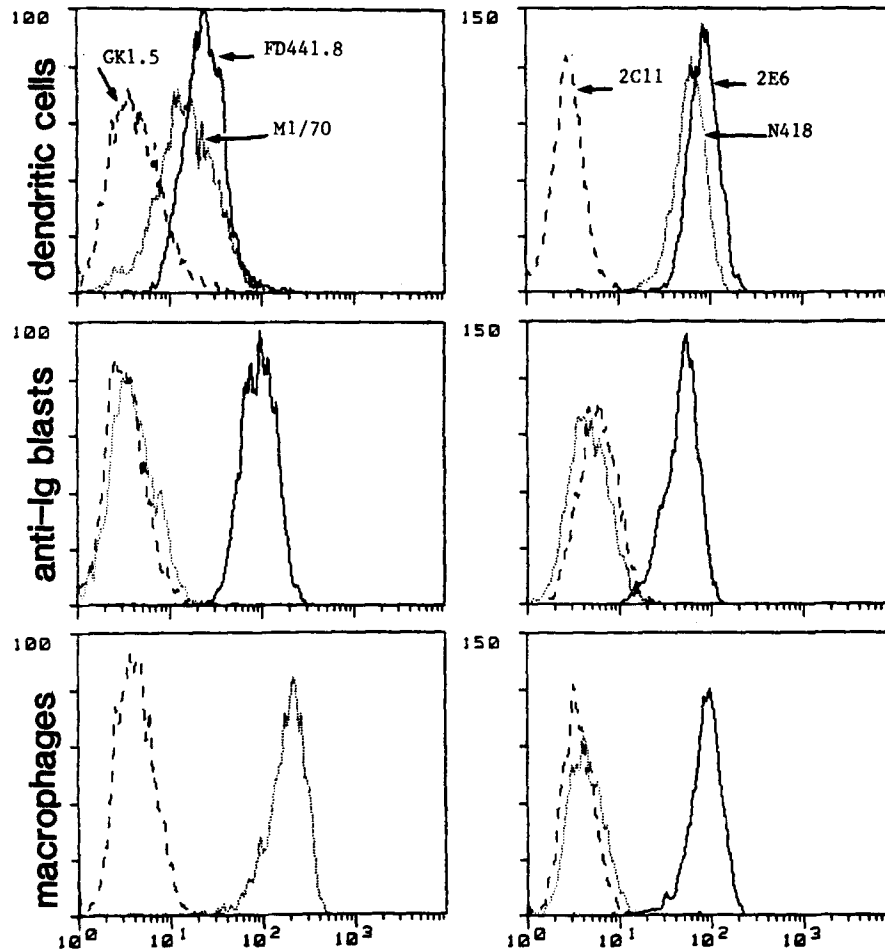


FIGURE 4. FACS analyses (frequency vs. fluorescence histograms) to demonstrate the distribution of leukocyte integrins on peritoneal macrophages, spleen B blasts, and spleen dendritic cells. Cx1D2 F1 dendritic cells (*top*), anti-Ig blasts (*middle*), and macrophages (*bottom*) were stained with a series of rat (*left*) or hamster (*right*) anti-mouse mAbs. The rat mAbs were M1/70 (anti-CD3), FD441.8 (anti-LFA-1), and GK1.5 (anti-CD4, control rat IgG). These mAbs were visualized with a secondary FITC-mouse anti-rat Ig. The hamster mAbs were 2E6, N418, and 2C11 (anti-CD3, control hamster Ig), and were visualized with a secondary FITC-rabbit anti-hamster Ig.

In the red pulp [not shown], N418 did not stain granulocytes, but did stain scattered dendritic profiles that were much less numerous than the number of F4/80 positive red pulp macrophages.

In the marginal zone between red and white pulp, N418 produced a distinct staining pattern. To interpret the pattern at low power (Fig. 6, *top*), serial sections were stained with the SER-4 mAb (24) to outline the marginal zone macrophages, which were in turn surrounded by the F4/80⁺ macrophages of the red pulp as described (19) (Fig. 6, *top*). In contrast to most prior descriptions of the marginal zone as a continuous rim of macrophages, here identified with the SER-4 mAb, we found the marginal zone to be interrupted by regions containing nests of N418⁺ cells but very

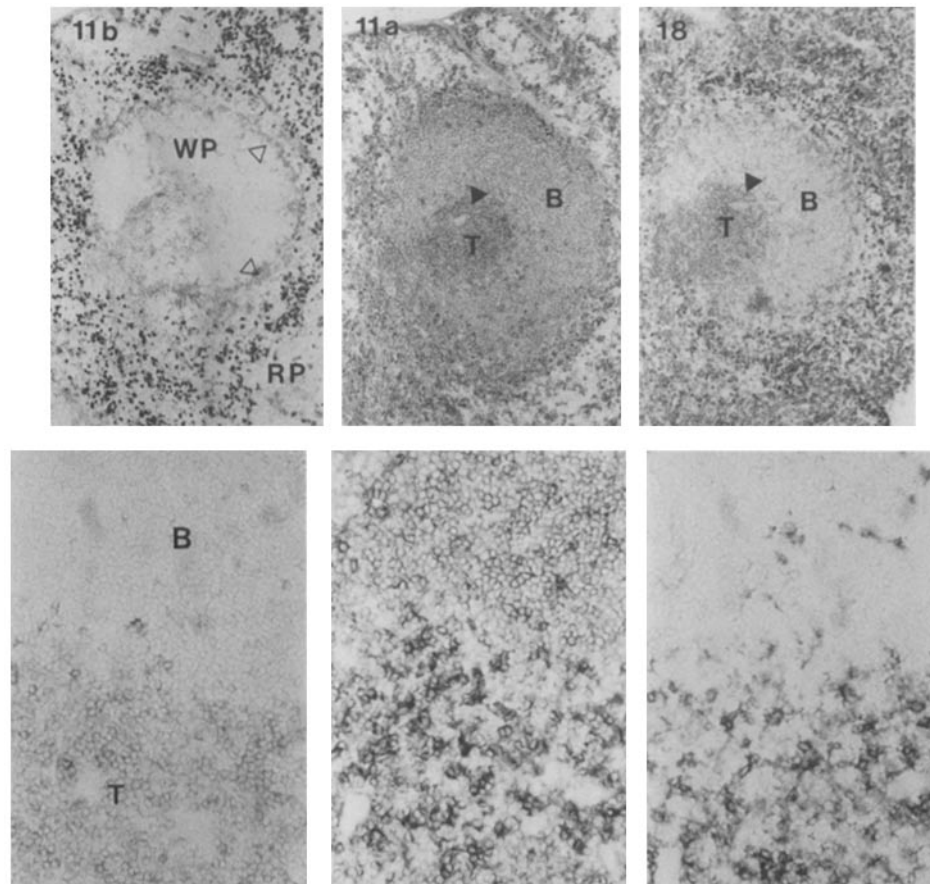


FIGURE 5. Immunolabeling of spleen sections with mAb to leukocyte integrins. Adjacent sections are shown here. (*Top*) Comparison of mAb to CD11a (LFA-1), CD11b (CR3), and CD18 (2E6). $\times 50$. Each micrograph includes a white pulp nodule (WP), with a central arterial vessel (black arrowhead) surrounded by red pulp (RP). The central region of the white pulp contains a T area (T) surrounded by a B area (B), as evidenced by staining adjacent sections with mAb to T and B cells (not shown). M1/70, anti-CR3 or CD11b stains marginal zone macrophages at the border of white and red pulp (open arrowheads) and red pulp granulocytes. FD441.8, anti-LFA-1 or CD11a stains white pulp lymphocytes, particularly T cells around the central artery (closed arrowhead). 2E6 anti-CD18 stains the white pulp like anti-CD11a except that the labeling is less intense. This is because an anti-rat rather than an anti-hamster Ig was used in staining these sections. 2E6 also stains the red pulp considerably. At higher power, it is evident that both granulocytes and the network of red pulp macrophages are stained. (*Bottom*) Immunolabeling of splenic white pulp with hamster anti-mouse leukocyte mAb. Adjacent sections consisting of a T area (T) and B area (B) are shown. $\times 200$. (*Left*) 2C11 anti-CD3 outlines the T cells in the periarterial sheaths of the white pulp. (*Middle*) N22 anti-MHC class II stains most of the B cells in the white pulp follicles, and many Ia-rich dendritic profiles in the T area. (*Right*) N418 anti-p150,90 stains the T area, but the pattern is more similar to an anti-Ia, dendritic cell stain (compare with N22 anti-Ia, *middle*) than to an anti-T cell stain (compare with 2C11, *left*).

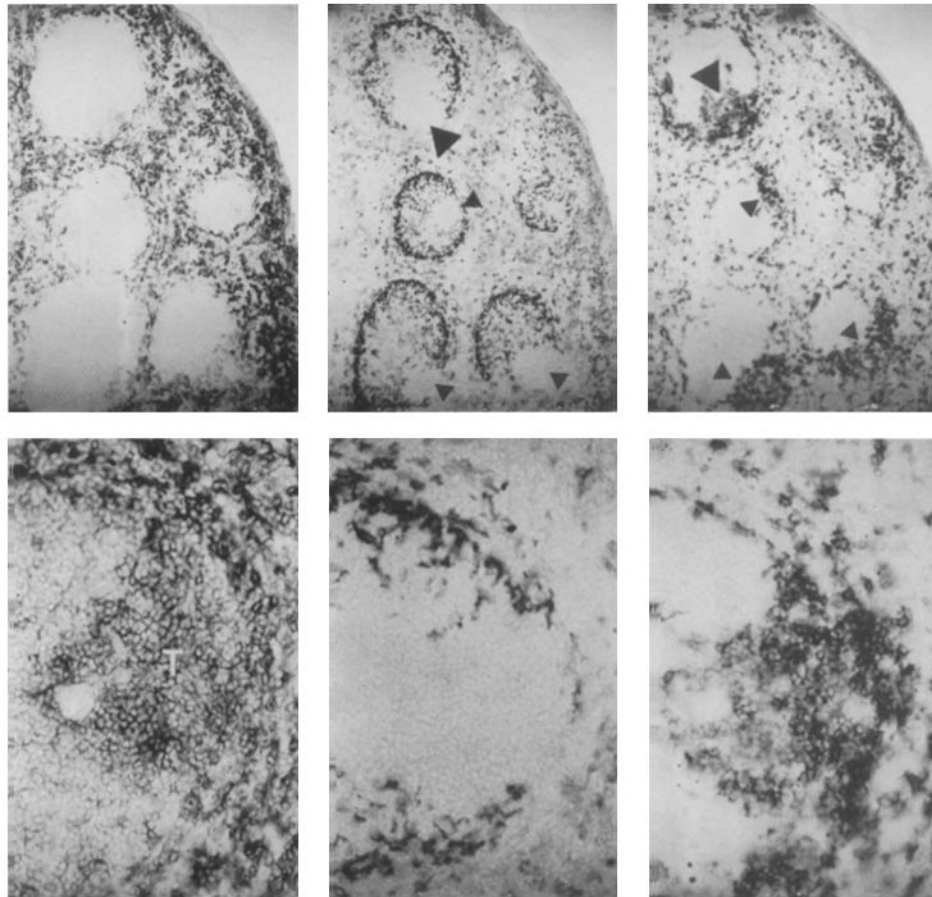


FIGURE 6. Identification of nests of N418⁺ presumptive dendritic cells in macrophage-free regions at the margin of white and red pulp. (*Top*) Adjacent sections are stained at low power ($\times 50$) to identify N418⁺ cells at the white-red pulp border. F4/80 (*left*) primarily stains macrophages in the red pulp, thereby highlighting five white pulp nodules. SER-4 (*middle*) stains marginal zone macrophages that lie just internal to the red pulp macrophages. The marginal zone of four of the five white pulp nodules in the section is punctuated by a macrophage-free region (*arrowheads*), which is the major site in which N418 stains (*right*). (*Bottom*) Adjacent sections, at higher power ($\times 200$), of one of the white-red pulp junctions depicted with the large closed arrowhead in the top panels. 2E6 anti-CD18 (*left*) is used here to show a collection of CD18-rich T cells at the white-red pulp junction. This region is not occupied by macrophages, as shown with the SER-4 mAb (*middle*), but is occupied by densely stained, N418⁺ dendritic cells (*right*).

few macrophages that stained for SER-4 or F4/80 (Fig. 6). At higher power, there was little or no overlap between the cells stained by N418 and those stained by SER-4 (Fig. 6, *bottom*) and F4/80 (not shown). However, the sections through the N418-stained areas contained either penetrating arterial vessels and or collections of T cells (Fig. 6, *bottom*). This pattern [see Discussion] of N418 staining is consistent with recent observations of Austyn and colleagues who noted that when dendritic

cells were injected intravenously they entered the marginal zone and then homed into the periarterial T-dependent areas of the white pulp (26).

2E6 Inhibits the Function of Lymphocyte and Macrophage Integrins. Several cell-cell binding assays are available that are known to depend on integrin function. The N418 anti-p150,90 mAb did not show any demonstrable effect in all of the assay systems we tested (not shown). Conceivably, the N418 epitope is not close enough to the active integrin binding site to influence function. 2E6, however, had marked inhibitory effects in cell-cell binding assays. One such assay involves the homotypic aggregates that develop between activated B lymphocytes; this aggregation is enhanced after treatment with phorbol esters (21). mAb M1/70 anti-CD11b failed to inhibit, but FD441.8 anti-CD11a and 2E6 anti-CD18 were completely inhibitory (not shown).

Heterotypic cell aggregates were generated between B blasts and T cells in a primary MLR (8). Again both mAb FD441.8 and 2E6 strongly blocked binding, while mAb M1/70 did not (Fig. 7, *bottom*). It is to be noted that Fc receptors on B blasts were not necessary for the inhibition with 2E6, since the F(ab')₂ fragment of 2E6 similarly inhibited the B cell-T cell MLR clustering (not shown). In addition, preincubation experiments indicated that the blocking effect of the mAbs was solely due to the function of LFA-1 on the T cell; i.e., blocking of B blast LFA-1 molecules did not reduce the MLR clustering but coating the T cells with mAb was inhibitory (not shown).

Red cells that have been opsonized with the C3bi fragment of the complement cascade (EC3bi) bind to macrophages. This binding is mediated by the CR3 molecule (27) and is inhibited by the M1/70 mAb (28). 2E6 inhibited the binding of EC3bi to macrophages, consistent with its reactivity with the common integrin β chain, while FD448.1 and control Ig failed to inhibit (not shown). These results, which are presented in detail elsewhere (29), provided functional evidence that 2E6 recognizes a common leukocyte integrin determinant found on macrophages, B cells, and T cells.

Role of Integrins in Dendritic Cell Function. Previous results in our laboratory indicated that mAbs to CR3 and LFA-1 did not reduce the initial clustering of dendritic cells and primary T cells at the onset of a cell-mediated immune response. Since we had identified a third integrin that was expressed at high levels on dendritic cells, we wished to evaluate its contribution to dendritic cell-T cell interactions. N418 anti-p150,90 did not block dendritic cell-T cell binding, but we did not know whether the N418 mAb bound to an active site on the 150, 90 kD molecule. Since it was clear from the functional studies on lymphocytes and macrophages that 2E6 mAb blocked the function of two other integrins, it might therefore be expected to block the function of the 150, 90 kD molecule on dendritic cells. To measure the contributions of leukocyte integrins in dendritic cell function we recorded the effects of 2E6 and other anti-integrin mAbs both early and late in the primary MLR.

After 24 h of coculture in the MLR, dendritic cells and resting T cells formed discrete clusters that were readily visible under the inverted microscope. These aggregates were sizeable (5–20 cells in diameter) at 24 h with dendritic-T cell ratios as low as 1:10 or 1:30. At a ratio of 1:3, we repeatedly failed to observe any differences in the size or frequency of clusters in the presence of any of the test mAbs, including 2E6 (Fig. 7, *top*). This failure to observe any inhibition was all the more striking because, at this same ratio of B blasts to T cells in the MLR, both 2E6 and FD441.8 markedly reduced the clustering (Fig. 7, *bottom*).

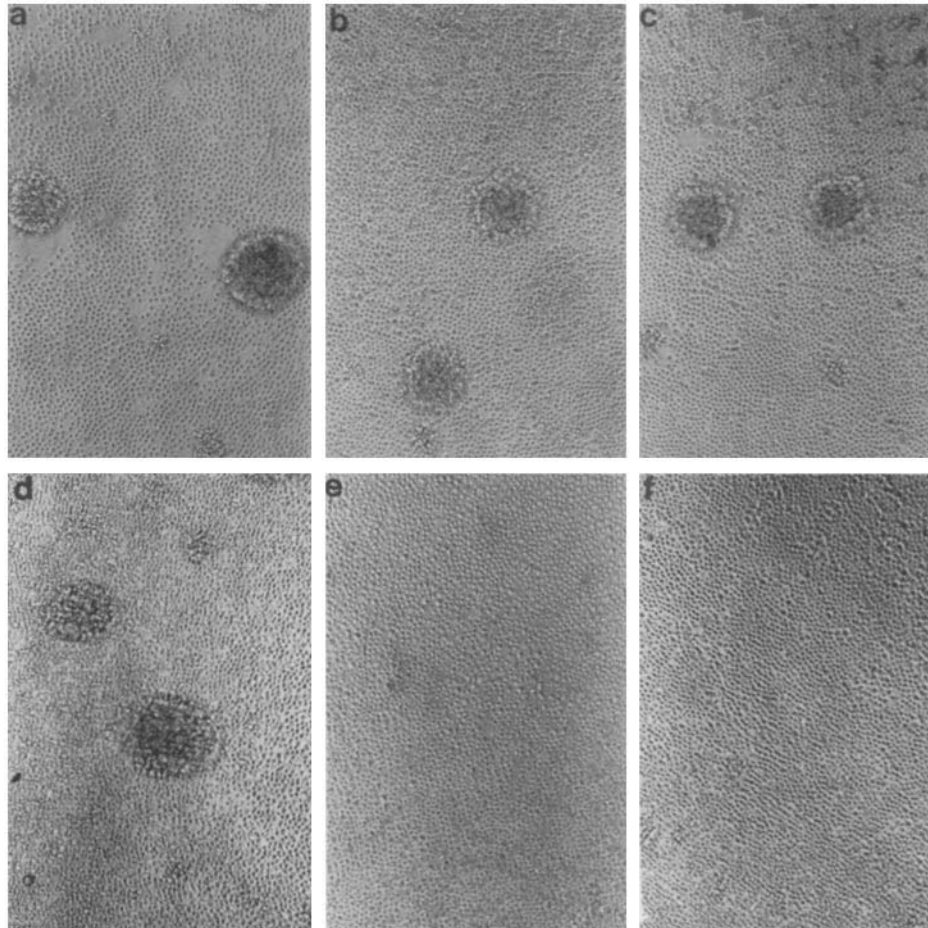


FIGURE 7. (*Top*) Blocking of dendritic cell-T cell binding in the MLR with anti-leukocyte integrin mAbs. 10^5 CxD2 F1 dendritic cells were added to 3×10^5 C3H CD4⁺ T cells in microwells in the presence of 25% vol/vol hybridoma supernatants. (*Bottom*) Blocking of anti-Ig blast-T cell binding in the MLR with anti-integrin mAbs. 10^5 CxD2 anti-Ig blasts were added in microwells to 3×10^5 C3H CD4⁺ T cells in the presence of 25% vol/vol hybridoma supernatants. The cultures were photographed at 20 h of the MLR at $\times 20$. (*a, d*) M1/70, (*b, e*) FD441.8, (*c, f*) 2E6.

As the dendritic-T cell ratio was lowered in the MLR, one could begin to visualize a reduction in the size of the clusters at 24 h, particularly in the presence of anti-LFA-1 (not shown). This result could indicate that at lower dendritic cell doses, a ligand for CD11a plays a role in the formation of APC-T cell contacts. The result also is consistent with a role for LFA-1 in stabilizing clusters once they are formed, as previously described (5, 8). mAbs that block the stabilizing role of LFA-1 in the cluster would be expected to increase the dissociation rate of such clusters. It follows that an increased dissociation rate would be visually more apparent in those cultures with smaller clusters, i.e., those clusters formed at lower numbers of dendritic cells.

As a later measure of integrin function in the MLR, we pulsed the cultures with [³H]TdR at 72–80 h to assess the level of DNA synthesis in the T cells. As previ-

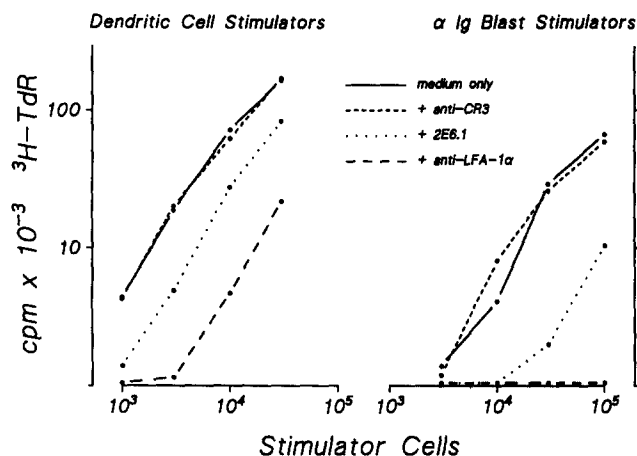


FIGURE 8. Blocking of T cell proliferation in the MLR with anti-integrin mAbs. B6xD2 F1 anti-Ig blasts (*right*) and dendritic cells (*left*) were cultured in graded doses with 3×10^5 CxD2 F1 CD4⁺ T cells. At the start of the MLR, hybridoma supernatants or control R10 medium were added at 12.5% vol/vol. Cultures were pulsed from 69 to 77 h.

ously observed (5, 8), anti-LFA-1 inhibited T cell proliferation over the entire dose-response (Fig. 8). mAb 2E6 also blocked, but less effectively than anti-LFA-1 mAb.

Discussion

The Hamster as a Host to Prepare mAb to Dendritic Cells. A more thorough analysis of the tissue distribution and function of dendritic cells has been hampered by the paucity of cell-restricted mAbs. Ideally, such mAbs would readily label dendritic cells in tissue sections, aid in their purification from cell suspensions, and help identify molecules important for the distinct aspects of dendritic cell function.

Previous attempts to raise such mAbs by immunizing rats with murine dendritic cells have been unsuccessful except for the 33D1 reagent (30). A major problem encountered in our experience is the abundance of mAbs to the products of mouse MHC genes. In one such immunization and fusion, anti-class II MHC mAbs represented up to 50% of all the positive hybridomas (our unpublished data). Also, it might be expected that dendritic cell-restricted molecules are conserved among closely related species. For these reasons, the rat may be a suboptimal host for raising anti-murine dendritic cell mAbs.

Both of these problems seem to be reduced by immunizing a more phylogenetically distant species, the hamster. Hamsters have been used by others to raise the first mAb to many murine molecules (9–11). In our two hamster anti-mouse dendritic cell fusions, anti-class II MHC mAbs were apparent, but their frequency was about 10%. Furthermore, mAbs to new specificities were detected, particularly the 2E6 and N418 anti-integrins described here.

Besides hamster hosts, we also used the FACScan to screen hybridoma supernatants. This assay (Fig. 1) is more sensitive than microscopic fluorescence and requires much smaller numbers of dendritic cells. By two-color analyses, we could search for dendritic cell-reactive and -specific mAbs.

Tentative Identification of the 2E6 and N418 Epitopes as CD18 and CD11c. Initial immunoprecipitation experiments with 2E6 and N418 clearly indicated that these mAbs recognized members of the leukocyte integrin family. 2E6 immunoprecipitated the well-characterized CD11a (LFA-1) and CD11b (CR3 receptor) heterodimers, which

are known (22, 23) to be the major integrins on lymphocytes and macrophages respectively (Fig. 2). Moreover 2E6 blocked leukocyte adhesion events that are mediated by these integrins. Although we were unable to blot the shared 90 kD CD18 integrin β chain with 2E6, it seems likely that this chain carries the epitope. 2E6 and anti-human CD18 mAbs both behave in an identical fashion with respect to the molecules recognized in immunoprecipitation and adhesion assays (27). Only a single comparable reagent to 2E6, clone M18, has been reported in mice (22), but to our knowledge this reagent has not been used subsequently.

The N418 mAb recognizes a distinct p150,90 heterodimer that is precipitated by 2E6 but not by anti-CD11a and CD11b (Fig. 3). Therefore, N418 likely recognizes an epitope on the p150 α chain. Tentatively N418 is an anti-CD11c reagent since CD11c is the only other known leukocyte integrin and in man has an apparent molecular mass of 150 kD. An alternative is that N418 recognizes a "CD11d" α chain, but there is little evidence for a fourth type of leukocyte integrin.

The Tissue Distribution of the N418 Epitope. Low levels of CD11c are expressed on human granulocytes and monocytes, and higher levels on macrophages that have been cultured long term or observed in tissue sections (31, 32). However, the studies in man have not addressed the question of CD11c on dendritic cells. We have yet to see strong reactivity of N418 with mouse macrophages and granulocytes (manuscript in preparation). For example, it reacts weakly or not at all with cultured peritoneal macrophages or blood monocytes, with bone marrow granulocytes, or with macrophage populations in tissue sections e.g., liver Kupffer cells and alveolar macrophages (our unpublished observations). This could mean that human and mouse CD11c have distinct tissue distributions, that dendritic cells express a novel form of the CD11 integrins, or that N418 recognizes a dendritic cell-restricted epitope on a p150 backbone that is shared with phagocytes.

Especially intriguing is the localization of N418⁺ cells in spleen. In T areas, N418 stained profiles that most likely are the interdigitating cells of these regions. Interdigitating cells are similar to isolated spleen dendritic cells in many respects (19, 25, 26), but it has been difficult to obtain mAbs like N418 that clearly react with both dendritic populations and not with most other cell types. Anti-MHC class II reagents like N22 (Fig. 5, *bottom*) are in our hands the only reagents that strongly stain isolated dendritic cells and can distinguish interdigitating cells from T lymphocytes in situ.

In addition to staining in the central region of the white pulp, N418 stained even more intensely at the periphery of the white pulp nodule. We were able to show that these foci of N418⁺ cells had few profiles with the markers of marginal zone (24) and red pulp (19, 33) macrophages. The N418⁺ foci corresponded to regions where arterial vessels and T cells also interrupted the marginal zone (Fig. 6). Therefore, it would seem that most dendritic cells in spleen are largely concentrated in regions where T cells enter into the white pulp nodule. This would place these APC in a position to select antigen-specific recirculating T cells and/or to move more deeply into the white pulp area.

The Distinct Integrins of Spleen Dendritic Cells. It is evident from this study that the leukocyte integrins on mouse spleen dendritic cells are abundant and distinct from other cells. While CD11a and CD11b can be detected on dendritic cells, the data with the 2E6 and N418 mAbs indicate that the p150,90 heterodimer is the most abun-

dant leukocyte integrin on these cells. Therefore spleen dendritic cells differ from most splenic lymphocytes and phagocytes which mainly express CD11a and CD11b, respectively.

CD11c in man is not well characterized functionally. A recent report presents evidence for a function in binding particles opsonized with C3bi (31). The CD11a and CD11b molecules have known adhesion functions. CD11a contributes to the homotypic binding of lymphocytes (21), and to the heterotypic binding of T to B cells (8) and of CTL to their targets (3). CD11b mediates the adhesion of phagocytes to particles opsonized with C3bi (28) and other ligands (34–36), and to endothelium (37).

We therefore tested if N418 would block one key binding function of dendritic cells, i.e., to T cells in the MLR. No block was observed. This could mean that N418 fails to recognize a functional domain needed for this binding. However, 2E6, which recognizes all integrins including p150,90 and blocks CD11a and CD11b functions including B–T cell clustering in the MLR, has little effect on dendritic–T cell clustering. Therefore, while leukocyte integrins can contribute to the function of dendritic–T cell clusters, they appear to do so after an initial CD11/18-independent binding event.

The failure of anti-CD11 and CD18 reagents to substantially reduce the initial dendritic–T cell clustering in a primary response like the MLR, perhaps is not surprising. The function of leukocyte integrins is regulated not merely by expression at the cell surface but by exposure to stimuli like PMA (20, 21) and other more physiologic ligands (38, 39). Also, the known ligands of the integrins, ICAM-1 and ICAM-2, are regulated at the level of expression but again by cytokines (40). Given the need for “activation” of integrins and their ligands, how, one might ask, would molecules like LFA-1 or ICAM be activated on a resting T cell, which is the cell the dendritic cell must bind to initiate the immune response?

An alternative role for the p150 major dendritic cell integrin is suggested by the work of Austyn et al. (26). They documented the capacity of spleen dendritic cells to home to the T areas of lymphoid organs, possibly by an initial binding step to the marginal zone region of spleen. p150,90 may function to guide dendritic cells into the marginal zone and/or into the T area. This homing function may contribute to the capacity of dendritic cells to initiate immune responses *in situ* (41). We are designing experiments to test if the N418 mAb would block dendritic cell migration and implicate p150,90 in their distinct homing properties.

Summary

Hybridoma fusions with hamster hosts were undertaken to generate mAbs to mouse spleen dendritic cells. Two mAb were obtained and used to uncover the distinct integrins of these APC. One, 2E6, bound a determinant common to all members of the CD11/CD18 family, most likely the shared 90 kD CD18 β chain. 2E6 immunoprecipitated the characteristic β_2 integrin heterodimers from lymphocytes (p180, 90; CD11a) and macrophages (p170, 90; CD11b), but from dendritic cells, a p150,90 (presumably CD11c) integrin was the predominant species. 2E6 inhibited the binding function of the CD11a and CD11b integrins on B cells and macrophages in appropriate assays, but 2E6 exerted little or no inhibition on the clustering of dendritic cells to T cells early in primary MLR, suggesting a CD11/CD18-independent mechanism for this binding. The second mAb, N418, precipitated a 150, 90 kD heterodimer that shared the 2E6 CD18 epitope. This N418 epitope may be the murine

homologue of the previously characterized human CD11c molecule, but the epitope was only detected on dendritic cells. N418 did not react with peritoneal macrophages, anti-Ig-induced spleen B blasts, or bulk lymph node cells. When used to stain sections of spleen, N418 stained dendritic cells in the T-dependent areas, much like anti-class II mAbs that were also generated in these fusions. In addition, N418 revealed nests of dendritic cells that punctuated the rim of marginal zone macrophages between red and white pulp. This localization positioned most dendritic cells at regions where arterial vessels and T cells enter the white pulp. We conclude that the p150, 90 heterodimer is the major $\beta 2$ integrin of spleen dendritic cells, and we speculate that it may function to localize these APC at sites that permit access to the recirculating pool of resting T cells.

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