Evidence for a Role of the Integrin VLA-4 in Lympho-hemopoiesis

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Summary

Adhesion molecules are probably required for retention of maturing lymphocyte precursors in bone marrow, where they closely interact with and are dependent on stromal cells. Lympho-myeloid cell lines avidly adhere to cloned stromal cell lines in culture and screening pairs of these resulted in a selection strategy for a new monoclonal antibody to a leukocyte adhesion molecule. Immunoprecipitation analyses and comparison to a previously described antibody showed that it recognizes the α_4 chain of the integrin, VLA-4. This antibody totally inhibited lymphopoiesis and retarded myelopoiesis in long-term bone marrow cultures. A similar selection strategy resulted in two additional antibodies which define a single 100-kD species on stromal cells. This stromal cell adhesion molecule is a potential counter-receptor/ligand for VLA-4 on murine lympho-myeloid cells. Our findings suggest a new role for VLA-4 in lymphoid progenitor–microenvironment interactions. Recognition molecules that function in cell migration and inflammation in peripheral tissues may be important for steady-state lymphopoiesis within bone marrow.

Rapid progress has been made in determining the molecular basis for cell-cell and cell-matrix interactions. A number of potential cell adhesion molecules have been identified in experimental models of bone marrow, where both types of interactions are probably essential for the orderly expansion, movement, and maturation of blood cell precursors (1–7). Members of the large integrin superfamily of cell adhesion/recognition molecules participate in many vital functions in development, hemostasis, and immune responses. Little is known about their possible involvement in lympho-hemopoiesis, but there are reports that erythroid and lymphoid precursors express fibronectin receptors (3, 4).

We previously obtained evidence that CD44 can function as a cell adhesion molecule on certain lymphoid cell lines by recognition of hyaluronate (8, 9). Antibodies to CD44 inhibit production of lymphoid and myeloid cells in long-term bone marrow cultures (LTBMC)[1], suggesting that this may be an essential cell interaction molecule. However, we found that the adhesion of some lympho-myeloid cell lines to cloned stromal cells could not be explained on the basis of hyaluronate recognition by CD44 and there was reason to believe that multiple cell adhesion mechanisms were involved (9, 10). We have now developed a satisfactory cell adhesion model to address this question, and used it to prepare two types of mAbs. The first, prepared by immunizing with lympho-myeloid cells, recognizes the integrin VLA-4. This antibody inhibited hemopoietic cell–stromal cell recognition and blocked lymphopoiesis in long term bone marrow cultures. Two additional antibodies were prepared by immunizing with adherent fibroblast-like cells and selection with a similar adhesion assay. The monomeric glycoprotein they recognize is also an adhesion molecule, but one with a quite different pattern of cellular representation. It is similar in size to human VCAM-1, which is thought to be a counter-receptor for VLA-4 (11). Thus, VLA-4, which is a well defined receptor for extracellular matrix and cell associated ligands may contribute to lymphopoiesis within the bone marrow microenvironment.

Materials and Methods

Cell Lines. The BM-2 B cell hybridoma and 70Z/3 pre-B lymphoma lines have been described (8, 12). The B lymphoma, WEHI 231, and the P815 mastocytoma lines were obtained through the American Type Culture Collection (ATCC) (Rockville, MD). The stromal cell clone +/+ 2.4 was initially derived as a control from the bone marrow of normal littermates of WCB6F$_1$/J.S1/Sl^d mice (13). Preliminary experiments established that certain lymphomyeloid cell lines bound avidly to it via a mechanism that was unrelated to CD44 (see Table 1). All cell lines were maintained in

[1] *Abbreviation used in this paper:* LTBMC, long-term bone marrow cultures.

RPMI 1640 supplemented with glutamine, 50 μM 2-ME, and 10% FCS.

Monoclonal Antibodies and Fibronectin Fragments. Fisher rats (Sasco, Omaha, NE) were immunized intraperitoneally with P815 for PS/2 or BALB/3T3 and +/+ 2.4 cells for M/K series antibodies. Spleen cells from these rats were then fused with Sp2/0 cells. Hybridoma supernatants were screened with a cell adhesion assay using P815 and BALB/3T3 for PS/2 or 70Z/3 and +/+ 2.4 for M/K series antibodies. Hybridoma cells producing blocking antibodies were cloned by limiting dilutions and reselected by the same procedure. PS/2 is IgG2b/κ, and M/K-1 and M/K-2 are both IgG1/κ. Control antibodies were KM 201 (IgG1) directed against CD44 (8), FD441.8 (IgG2b) specific for LFA-1 (obtained from ATCC), 14.8 (IgG2b) reactive with CD45R (14), KMC8.8 (IgG2a) directed against a bone marrow–derived stromal cell clone BMS2 (8), and R1-2 (IgG2b) recognizing Peyer's patch-specific homing receptor (15). All antibodies except R1-2 were semipurified out of ascitic fluid from SCID mice with ABx column chromatography (J.T. Baker Inc., Alexandria, VA). The culture supernatant from R1-2 was concentrated by ammonium sulfate precipitation. A rabbit serum against murine fibronectin and 40- and 120-kD fragments from human fibronectin was purchased from Telios, Inc. (San Diego, CA).

Cell Adhesion Assay. Cell lines were radiolabeled by incubating 2×10^7 cells/ml in complete medium (10% FCS RPMI 1640) with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C and then washed three times in complete medium. The +/+ 2.4 stromal cell clone was plated in 24-well plates (Corning Glass Works, Corning, NY) at 3×10^4 cells/well and allowed to grow overnight before the adhesion assay. The labeled cells (2×10^5/well) were added to the stromal cell layer and incubated 30 min at 37°C. The unbound cells were removed by three cycles of washing in prewarmed complete medium with vigorous agitation on a Minishaker (Dynatech Laboratory Inc., Alexandria, VA) for 30 s before each aspiration. Bound cells were lysed with 0.1 N NaOH, 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), and the ^{51}Cr was counted with a γ counter (Beckman Instruments Inc., Fullerton, CA). Percentages of bound cells were determined by the formula: percent bound = 100× [(cpm from bound cells)/(input cell associated cpm − spontaneously released cpm)]. Antibodies were added at the same time as the labeled hemopoietic cells.

LTBMC. LTBMC of B lineage cells were prepared according to methods detailed by Whitlock and Witte (16). Briefly, pooled cells from femora and tibiae were placed into 25-cm^2 flasks (6×10^6 cells/culture; Costar, Cambridge, MA). The culture medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and 5% FCS (Lot no. 1111885; Hyclone Laboratories, Logan, UT). LTBMC of myeloid cells were initiated and maintained by the methods described by Dexter et al. (17). Briefly, 10^7 bone marrow cells were cultured in 25-cm^2 flasks. The culture medium consisted of α-MEM (Gibco Laboratories), penicillin, streptomycin, 10^{-7} M hydrocortisone sodium salt, and 20% horse serum (Gibco Laboratories). These flasks were incubated in 5% CO_2 at 33°C. In both LTBMC, half of medium (3 ml) was replaced with fresh medium weekly. The antibodies (3 μg/ml) were added to the medium at the beginning of culture and with each feeding.

Cell Surface Biotinylation and Immunoprecipitation. Cells were surface labeled by a sulfosuccinimidobiotin (Sulfo-NHS-biotin) (Pierce Chemical Co., Rockford, IL) procedure. Briefly, after three washes in HBSS), cells were suspended in saline with 0.1 M Hepes, pH 8.0, (10×10^6/ml). Sulfo-NHS-biotin was dissolved in a small amount of saline and added to cell suspensions such that the final concentration of NHS-biotin was 0.1 mg/ml. After a 40-min incubation at room temperature with occasional shaking, cells were washed three times with chilled RPMI 1640. The cell lysates were prepared (5×10^7/ml) in a lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1% sodium azide. EDTA (3 mM) was used instead of $MgCl_2$ and $CaCl_2$ in the experiment indicated. Soybean trypsin inhibitor (10 μg/ml), aprotinin (1 U/ml), PMSF (1 mM), and Leupeptin (1 μg/ml) were added as protease inhibitors. After centrifugation, the lysates were precleared twice with 50 μl goat anti–rat IgG Sepharose 4B (Zymed Laboratories, San Francisco, CA) conjugated with normal rat IgG, and then immunoprecipitated with a mAb-conjugated goat anti–rat IgG Sepharose 4B for 2 h at 4°C with rotation. After washing three times in a buffer containing 50 mM Tris-HCl (pH 8.3), 0.6 M NaCl, 0.5% NP-40, and 0.1% sodium azide, the bound proteins were released by boiling for 5 min in a sample buffer containing 0.125 M Tris-HCl, 2% SDS, and 10% glycerol with or without 5% 2-ME, pH 6.8. SDS-PAGE was carried out as in our previous studies (8).

After electrophoresis, the gels were equilibrated for 30 min in a transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) and proteins were electrophoretically blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were soaked for 1 h in PBS containing 10% BSA, 0.05% Tween 20, and 0.1% thimerosal, and followed by a 60-min incubation with 0.1% avidin horse radish peroxidase (Bio-Rad Laboratories), 1% BSA, and 0.05% Tween20. After washing in PBS containing 0.05% Tween20, biotinylated proteins were visualized with PBS containing 0.6 mg/ml 4-chloro-1-Naphthol (Sigma Chemical Co.), 0.003% hydrogen peroxide, and 20% methanol.

Immunofluorescence Analyses. Cells were incubated for 20 min on ice with biotinylated PS/2 in RPMI 1640 supplemented with 5% FCS and 0.1% sodium azide. This was followed by two washes and incubation with fluoresceinated avidin (Zymed Laboratories) for an additional 20 min. Propidium iodide was added during the second incubation to detect dead cells. Labeled cells were then analyzed on an EPICS V flow cytometer (Coulter Electronics Inc., Hialeah, FL). Staining was similar for mAbs that were unlabeled with biotin except that FITC labeled mouse anti–rat Ig was used as a second reagent. For crossblocking experiments, cells were preincubated in saturating amounts of unlabeled antibodies for 20 min, washed three times, and then stained with biotinylated antibody.

Results

Derivation of a New mAb to VLA-4. Previous studies suggest that multiple cell adhesion molecules and ligands may contribute to the mutual recognition of lymphocyte precursors and the stromal cells that regulate their growth and differentiation (9). Selection of a given pair of stromal cells and lympho-myeloid cell lines that adhere to each other allows one or more adhesion molecules/ligands to be individually studied. For example, the avid binding of BM-2 hybridoma cells to the BMS2 stromal line requires CD44 and hyaluronate (9). We sought a new experimental model in which neither CD44 or cell bound hyaluronate were important. The results of one such analysis are shown in Table 1. While 70Z/3 (pre-B lymphoma), P815 (mastocytoma), and WEHI 231 (B lymphoma) cells all displayed measurable affinity for several adherent cell lines, this was resistant to inhibition by the KM201 mAb, which inhibits BM-2 binding (8). This cell adhesion

Table 1. *Two Cell Adhesion Mechanisms Distinguished by mAbs*

Cell line	mAb added				
	None	FD441.8	PS/2	KM 201	14.8
BM-2	62.9 ± 3.2	69.8 ± 3.6	50.8 ± 3.9	7.3 ± 1.1	66.2 ± 2.2
B815	58.8 ± 1.3	57.1 ± 1.6	16.7 ± 0.5	52.9 ± 0.9	ND
7OZ/3	45.5 ± 3.5	48.1 ± 3.6	15.9 ± 3.4	41.1 ± 3.2	47.5 ± 3.3
WEHI231	77.1 ± 1.6	64.7 ± 0.6	3.8 ± 0.2	67.6 ± 0.6	66.3 ± 2.3

A bone marrow stromal cell clone, +/+2.4 (13), was placed in 24-well plates (3×10^4 cells/well). Radiolabeled cell lines were added 1 d later with the indicated antibodies (10 μg/ml) and washed out after a 30-min incubation at 37°C. The results are presented as mean percentages of bound cells ± SE for quadruplicate determinations.

system then formed the basis for development and selection of two types of mAbs.

The PS/2 antibody was obtained by immunization of rats with P815 cells. It had a distinctly different pattern of inhibition from that previously found with anti-CD44 antibodies (8, 9) (Table 1). When used with the most ideal pair of adherent and nonadherent cells (P815 and +/+ 2.4 stromal cells), adhesion was blocked in a dose-dependent manner (Fig. 1). The 70Z/3 cell line was slightly less efficient in binding to this stromal cell clone, but it was similarly sensitive to inhibition by PS/2 (Table 1).

Cell surface labeling and immunoprecipitation were then done to characterize the glycoprotein recognized by PS/2 (Fig. 2). In the presence of divalent cations, and without reduction, two protein species were observed by SDS-PAGE of material from 70Z/3 cells. The small band was lost in the presence of a chelating agent, EDTA. This indicates that divalent cations are required for the noncovalent association of two chains to form a heterodimer. The epitope recognized by PS/2 must reside on the larger of the two chains. Their mobility was affected by reduction and their approximate mol wt were 130 and 150 kD.

The 130-kD band was also detectable when P815 cells were subjected to similar analysis (Fig. 2). However, with this cell

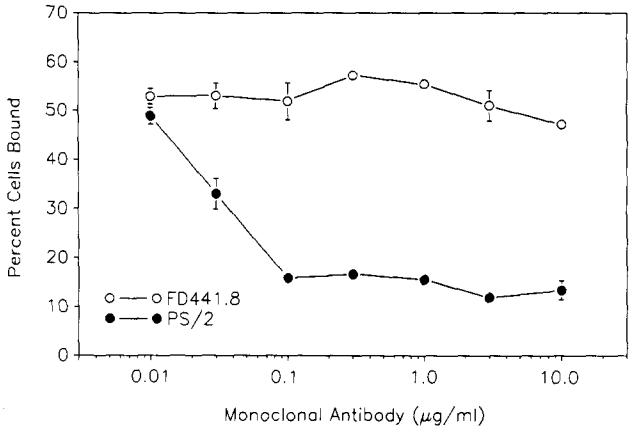

Figure 1. A new mAb blocks adhesion of lymphomyeloid cells to a stromal cell clone. An adherent layer was prepared with the +/+ 2.4 stromal cell clone and ^{51}Cr-labeled P815 cells were added the following day. Triplicate culture wells contained an antibody to LFA-1 (FD441.8), or PS/2 mAbs. After 15 or 30 min of incubation and vigorous washing, the fraction of adhering cells (mean ± SE) was determined by scintillation counting. Similar results were obtained in two other experiments with P815 and three experiments with 70Z/3 cells.

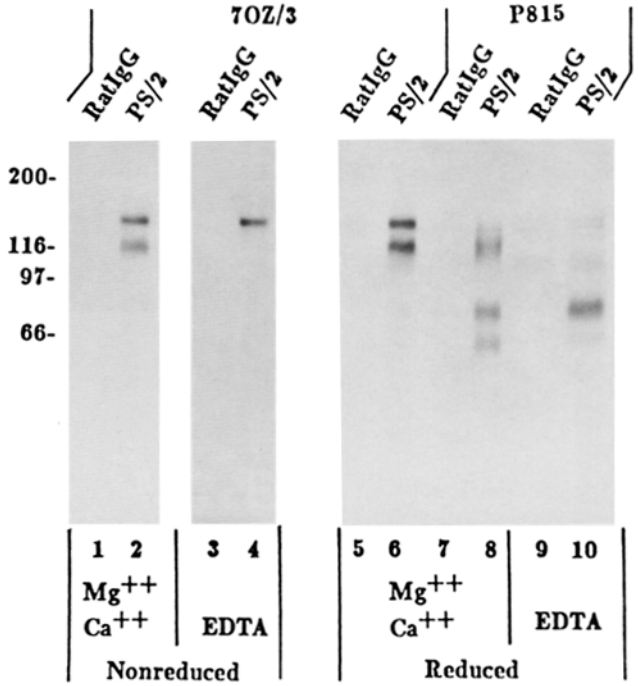

Figure 2. Immunoprecipitation of surface biotin-labeled material from 70Z/3 and P815 cells with PS/2 mAbs. The lysis buffer contained Triton X-100 and either divalent cations (lanes *1*, *2*, and *5–8*), or EDTA (lanes *3*, *4*, *9* and *10*). Antibody-coated beads were used to recover labeled glycoproteins which were subsequently run on 7.5% SDS PAGE, transferred to nitrocellulose, and developed with horseradish peroxidase-labeled avidin.

line, the larger species appeared to be more labile, and two small proteins of 85 and 70 kD were precipitated. Most of the smallest band was lost together with the 130 kD subunit when the isolation was done under divalent cation free conditions. Thus, the PS/2 epitope may be on the 85-kD polypeptide of P815 cells.

These immunochemical characteristics were very similar to the recently described murine VLA-4 integrin, as detected by the R1-2 mAb to the Peyer's Patch homing receptor, LPAM-1 (15) as well as antibodies to human VLA-4 (18). Identical sized bands were precipitated from 70Z/3 by R1-2 and PS/2 antibodies (Fig. 3 *A*) and flow cytometric analysis was performed to determine if similar epitopes were being recognized. The two antibodies gave similar, strong positive staining of 70Z/3, BM-2, WEHI-231, and P815 cells (not shown). When 70Z/3 cells were pretreated with unlabeled PS/2 before subsequent staining with biotinylated PS/2, complete blocking was achieved. Almost the same degree of interference was achieved with R1-2, while a control antibody against CD44 (KM201) had little effect (Fig. 3 *B*). Therefore, epitopes recognized by the PS/2 and R1-2 antibodies must be closely associated on the α_4 chain of VLA-4. That the epitopes are not identical is indicated by the fact that PS/2, but not R1-2, recognizes human cells (data not shown).

The antigen(s) recognized by PS/2 was absent or minimally detectable on stromal cells, whereas virtually all lymphomyeloid cells were positive. Bone marrow cells expressed the highest density (Fig. 4) and, in sections, it appeared to be concentrated at cell junctions (data not shown). In contrast to a previous report that human granulocytes lack VLA-4 (18), myeloid cells maintained under Dexter-type long-term bone marrow conditions were positive, as were lymphoid cells from Whitlock-Witte cultures (Fig. 4).

Inhibition of Long-term Cultures by Antibodies to VLA-4. Addition of mAbs to long-term cultures can be used to implicate cell surface glycoproteins in normal bone marrow functions (8). Whitlock-Witte cultures sustain growth of pre-B cells in association with an adherent layer (16). Production of lymphocytes was completely blocked by inclusion of PS/2 mAbs in the culture medium (Fig. 5; Fig. 6 *A* and *B*). A slightly different result was obtained when bone marrow was placed in culture under Dexter-type conditions, which favor the growth of myeloid and stem cells (17). When PS/2 was present in that circumstance, foci of surface adherent myeloid cells were in-frequent during the first few weeks. However,

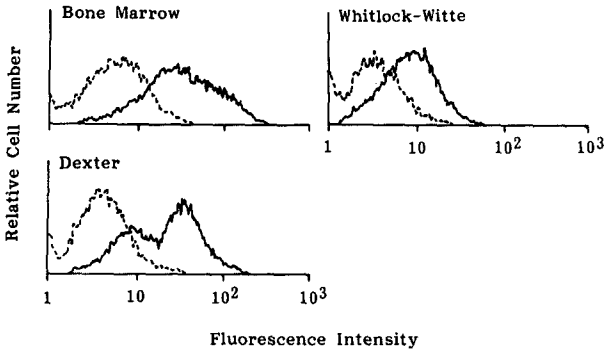

Figure 4. Expression of VLA-4 on lymphoid and myeloid cells. Flow cytometric analysis was performed on lymphoid cells obtained from Whitlock-Witte type LTBMC or myeloid cells present in fresh marrow cell suspensions or Dexter cultures. Dark curves represent the immunofluorescence resulting from biotin-labeled PS/2 antibody followed by treatment with fluorescein-labeled avidin. Light tracings depict background staining with only the second reagent.

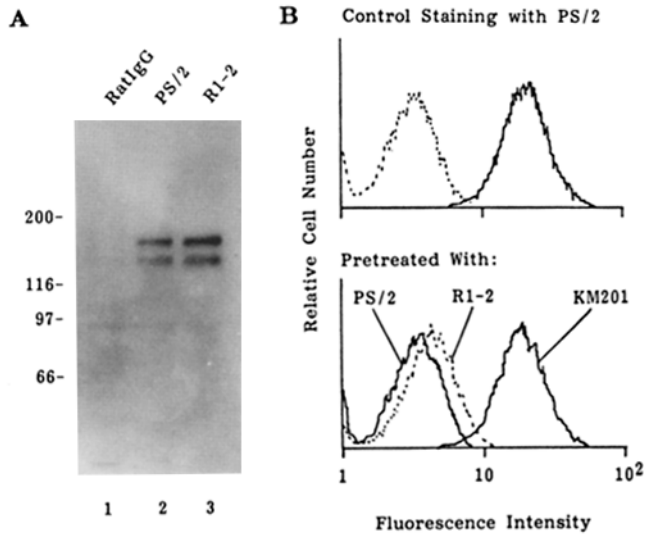

Figure 3. Crossblocking and immunoprecipitation with a previously characterized antibody to VLA-4. (*A*) 70Z/3 cells were surface labeled and precipitated with PS/2, R1-2, or control rat IgG before SDS-PAGE under reducing conditions. (*B*) The same cells were also analyzed by flow cytometry after immunofluorescent labeling with PS/2. The staining intensity achieved with PS/2 without pretreatment is shown in the upper panel. The lower panel shows the results obtained when the cells had been previously treated with unlabeled PS/2, R1-2, or KM 201 antibodies.

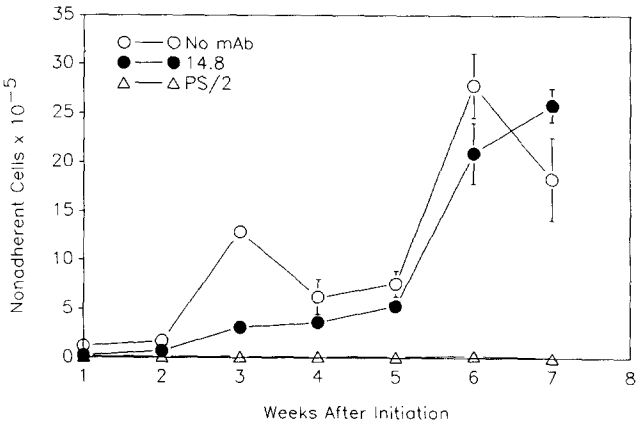

Figure 5. mAbs to VLA-4 block lymphopoiesis in LTBMC. Replicate cultures of murine bone marrow were prepared and maintained in the continuous presence of rat IgG2b antibodies to VLA-4 (PS/2), CD45R (14.8), or medium alone. Numbers of nonadherent cells collected at weekly intervals were counted by hemacytometer (mean cells per five flasks ± SE).

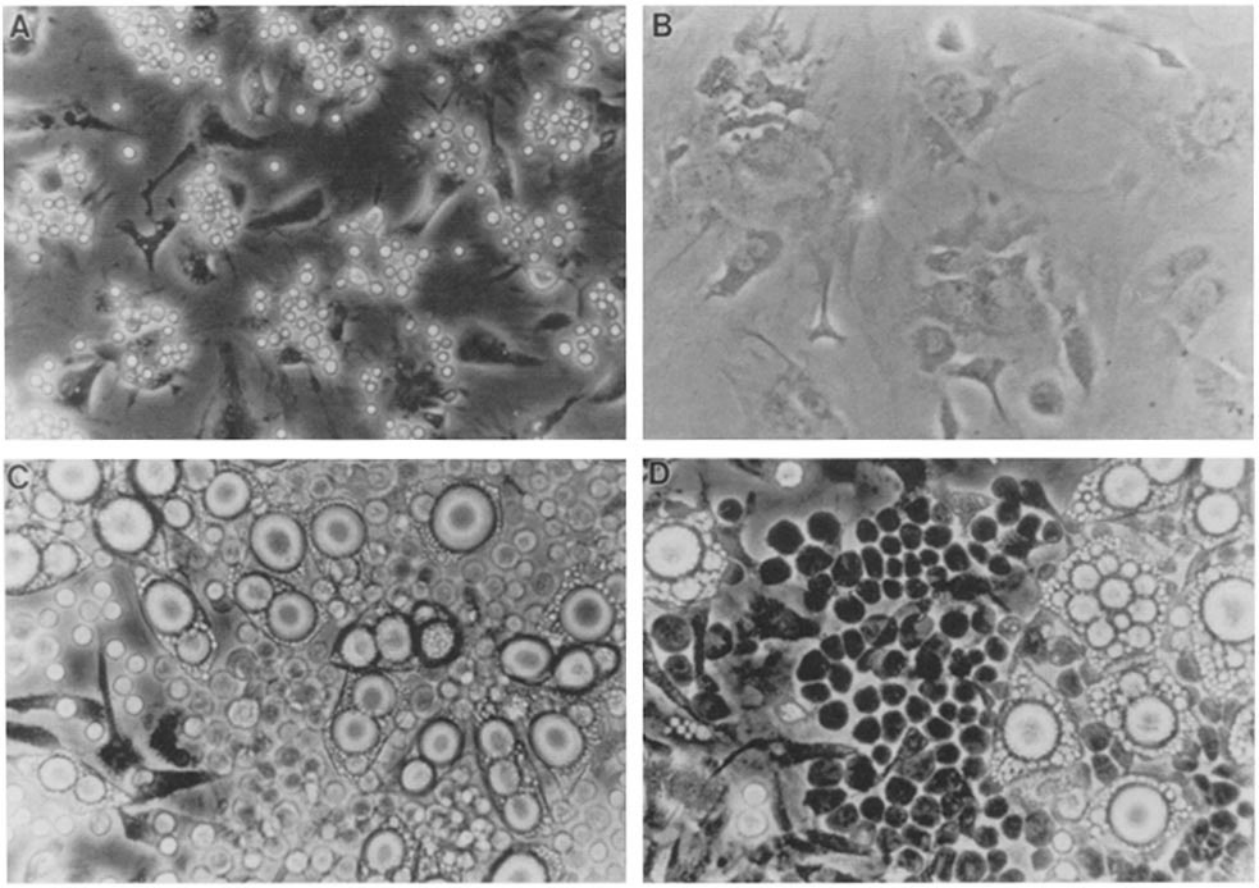

Figure 6. Effect of mAbs to VLA-4 on the morphology of LTBMC. Lymphocyte foci developed normally in Whitlock-Witte cultures containing the control 14.8 antibody (*A*), but not in the presence of PS/2 (*B*). Changes in the adherent stromal cells were not remarkable. Surface adherent myeloid cells were plentiful in Dexter cultures containing the control antibody to LFA-1 after 3 wk of culture (*C*). In contrast, hemopoietic cells were mostly restricted to flattened foci beneath the stromal cell layer in Dexter cultures containing PS/2 (*D*).

progenitor cells appeared to be present beneath the stromal cell layer (Fig. 6 *C*), which eventually generated nonadherent myeloid cells in normal numbers by 6 wk of culture (Fig. 7). Therefore, lymphopoiesis was completely sensitive, and myelopoiesis was retarded, in cultures containing this antibody.

The adherent layers of long-term cultures seemed unaffected by PS/2 antibodies (Fig. 6). In Dexter cultures, the usual complex arrangement of adipocytes, stromal cells, and macrophages was seen (Fig. 6 *C*). Similarly, the antibody had little influence on most colony assays involving lymphoid and myeloid progenitors (not shown). These included semisolid agar cultures with IL-7 (pre-B cell colonies; CFU-pre-B) and L cell–conditioned medium (CFU-c). When added to methylcellulose cultures containing lymphocyte clones over an adherent stromal cell layer, numbers of proliferating foci were normal. However, the colonies were much more compact than usual (not shown). This indicates that the antibody may induce spontaneous cell aggregation or impede lymphocyte migration.

Antibodies to an Adhesion Molecule on Stromal Cells. Previous reports suggest that integrins on lymphocyte precursors may function by recognition of fibronectin on microenvironmental elements (4). Furthermore, the CS-1/V25 region of fibronectin is known to be one ligand for VLA-4 (19, 20). However, in preliminary experiments, a rabbit antiserum to mouse fibronectin had no effect on the binding of P815, 70Z/3, or WEHI 231 cells to +/+ 2.4 stromal cells. Furthermore, 70Z/3 cells did not have the same avidity for dishes precoated with 40 kDa and 120 kDa human fibronectin fragments as they did for +/+ 2.4 cells. As previously reported (4), there was a relatively high background of binding to control BSA treated wells when serum free medium was used. The weak binding to fibronectin or BSA was not diminished by the PS/2 antibody (results not shown). These negative results prompted a search for other possible ligands for the VLA-4 expressed on stromal cell clones.

Rats were immunized with BALB/3T3 cells and boosted a final time with +/+ 2.4 stromal cells to elicit antibodies to adherent cell antigens. After fusion, hybrids were selected with a rapid screening assay involving the adherence of 70Z/3 cells to +/+ 2.4 cells. Two antibodies, M/K-1 and M/K-2, which were effective for blocking this system, were identified from >1,000 hybrids (Fig. 8). The inhibitions were substantial with P815, WEHI 231, and 70Z/3 cells, but always slightly less than those achieved with the PS/2 anti–VLA-4 antibody. While staining of +/+ 2.4 stromal cells was detectable by flow cytometry with these two new antibodies, the lympho-

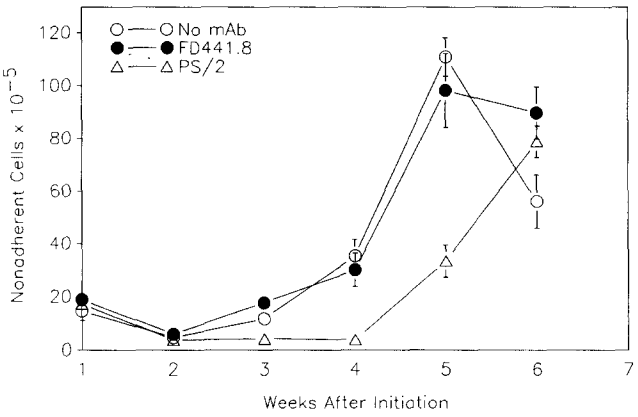

Figure 7. mAbs to VLA-4 retard myeloid cell production in Dexter-type LTBMC. Isotype matched antibodies were added as indicated at the beginning of culture and replenished with each feeding. Numbers of nonadherent cells were enumerated by hemacytometer counts at weekly intervals (mean of five flasks ± SE).

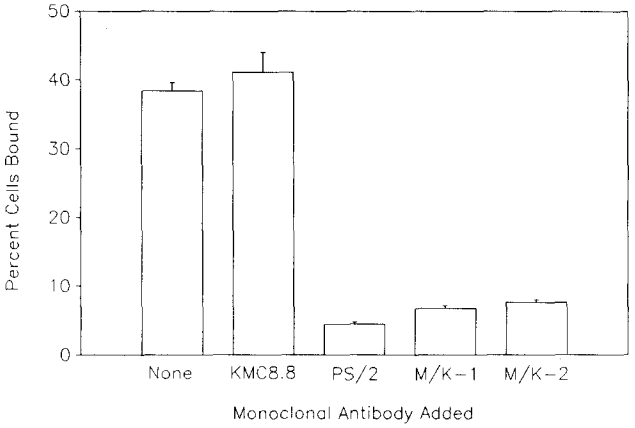

Figure 8. New mAbs to a stromal cell antigen inhibit adhesion of pre-B lymphoma cells to a stromal cell clone. A representative experiment is shown where chromium labeled 70Z/3 cells were added to wells containing +/+ 2.4 stromal cells in the presence of 10 μgm/ml of various mAbs. The new M/K series of antibodies were compared to a previously described antibody (KMC8.8) which recognizes stromal cells and myeloid cells (8), along with the PS/2 antibody to VLA-4.

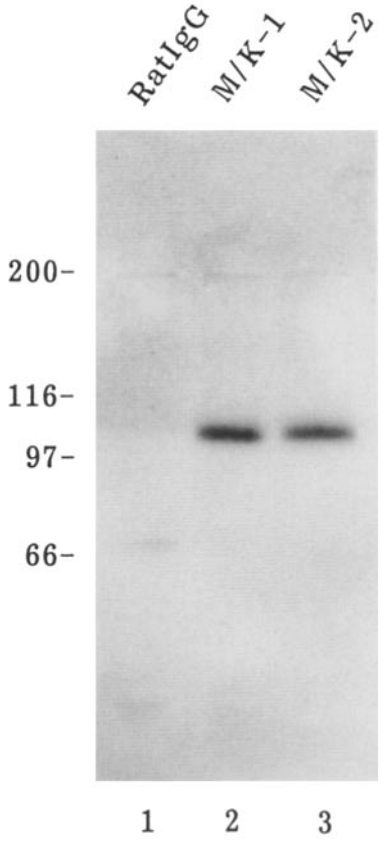

Figure 9. Immunoprecipitation of a stromal cell surface antigen by new mAbs. The +/+ 2.4 stromal cell clone was surface labeled with biotin and immunoprecipitated with M/K-1, M/K-2 antibodies, or normal rat IgG and subjected to SDS PAGE under reducing conditions. The single bands were also observed when nonreducing conditions were used, but the mobility corresponded to a size of ~92 kD (not shown).

myeloid cell lines (70Z/3, P815, and WEHI 231) were negative (data not shown). Thus, the M/K-1 and M/K-2 antibodies are directed to an adhesion molecule(s) preferentially expressed on stromal cells.

Immunoprecipitation of surface labeled material from +/+ 2.4 cells with the new antibodies resulted in a single band of ~100-kD under reducing conditions (Fig. 9) and 92 kD under nonreducing conditions (results not shown). No apparent difference was found between these two antibodies, which appear to recognize a monomeric glycoprotein on stromal cells. Staining with these reagents was not diminished by treatment with phosphatidyl inositol-specific phospholipase C (PI-PLC) (data not shown), indicating that the antigen is an integral membrane protein. Additional studies involving the M/K-1 and M/K-2 antibodies will be described elsewhere. However, it is important to note here that, in three independent experiments, both antibodies interfered with the adhesion of lymphocytes to stromal cells in LTBMC. Moreover, most of the lymphocytes quickly detached from established long-term cultures after addition of either the M/K series or PS/2 antibodies (Miyake et al., manuscript in preparation).

Discussion

Transient physical relationships exist between hemopoietic progenitor cells and the bone marrow microenvironment that provides signals required for their proliferation and differentiation. Studies with LTBMC indicate that CD44 and hyaluronate may mediate critical cell interactions in bone marrow, but that other cell surface glycoproteins and matrix constituents must also be involved (8, 9). We have now implicated a second interaction molecule, VLA-4, in B lymphopoiesis and prepared antibodies that identify a possible counter-receptor on stromal cells.

Any pair of adhesive cells may preferentially utilize only one of many possible cell adhesion mechanisms. This was the case with binding of the BM2 hybridoma to BMS2 stromal cells, where CD44 on the former recognized hyaluronate on the latter (8, 9). Treatment with either hyaluronidase or mAbs to CD44 virtually eliminated adhesion between these two cell lines. In contrast, we found that 70Z/3 (pre-B lymphoma), WEHI-231 (B lymphoma), and P815 (mastocytoma), cell lines avidly bound to another stromal cell clone, +/+ 2.4. Treatment with anti-CD44, or anti–LFA-1 antibodies had little effect on binding between these cell lines.

All of the lympho-myeloid cells used in these experiments express CD44. Furthermore, BM-2, whose binding was not inhibited by the PS/2 antibody, is positive for VLA-4 when stained with the same reagent. Thus, various cell lines bind well to the same stromal cell clone, albeit via different mechanisms. The CD44 density on BM-2 cells is high, relative to the other cells. This could permit it to preferentially bind hyaluronate, and the very large hyaluronate molecule could interfere with BM-2 recognition of another stromal cell ligand with its VLA-4. Unlike BM-2, neither 70Z/3 or P815 cells bound well to hyaluronate coated wells (10). Preferential utilization of one or another cell adhesion molecule could also result from their selective activation. CD44 and a number of integrins become functional on lymphocytes after stimulation with phorbol esters or physiologic stimuli (21–25). It is interesting to consider that developing hemopoietic cells might selectively modulate the functions of different adhesion molecules as they migrate within and exit from the bone marrow.

Immunoprecipitation analyses and comparison to the previously described R1-2 antibody identified the antigen recognized by PS/2 as the α_4 chain of the VLA-4 integrin heterodimer (15). Previous studies revealed that two additional proteins precipitate with this type of antibody and represent cleaved fragments from the α_4 subunit (15, 18, 19). Furthermore, the presence of these small polypeptides may be related to cell activation state (26, 27). The two fragments were always resolved in immunoprecipitates from P815, but not 70Z/3 cells. However, the functional significance is not obvious, because both cell lines bound well to stromal cells.

All cloned stromal cell lines which support lymphocyte growth in culture can also make myeloid colony stimulating factors (reviewed in reference 28). Furthermore, under appropriate culture conditions, the same stromal cells sustain growth and differentiation of myeloid progenitors (29). This suggests that some common components of the microenvironment are required for support of these distinct lineages of blood cells. A reciprocal relationship between pre-B cells and immature myeloid cells in cyclic neutropenia patients (30) is also consistent with there being common requirements. However, it is possible to differentially affect these lineages with culture conditions and other manipulations. For example, myelopoiesis is preferentially inhibited by low concentrations of TGF-β whereas the reverse is true with IFN-γ (reference 31 and unpublished observations).

The PS/2 antibody completely blocked lymphopoiesis when added to Whitlock-Witte type cultures, but only retarded the outgrowth of productive foci of myeloid cells in Dexter-type cultures. Bright and dull staining populations of PS/2-positive myeloid cells were observed by flow cytometry of normal Dexter cultures (Fig. 4). Lymphocytes taken from Whitlock-Witte cultures had an apparent density of VLA-4 equivalent to, or less than, that on myeloid cells. Therefore, the differential effect can not be explained by the absence of VLA-4 on myeloid cells. Further studies might reveal that early lymphoid and myeloid precursors from which the cultures derive differ in expression or use of VLA-4.

From studies largely done with human VLA-4, two potential ligand/counter-receptors have been described, fibronectin and VCAM-1. This integrin recognizes the CS-1/V25 domain of fibronectin (19, 20, 32) and a possible connection with lymphopoiesis is suggested by the finding that some murine pre-B cell lines and bone marrow B lineage cells bound to plates coated with a similar fibronectin fragment (4). Our experiments do not eliminate the possibility that lymphocytes adhere to the matrix partly through this mechanism. However, the apparent affinity of hemopoietic cells to fibronectin seemed too weak to explain the binding to stromal cell lines and this led to a search for other possible ligands on adherent cells. A similar selection strategy to that used to obtain the PS/2 antibody resulted in two additional mAbs (M/K-1 and M/K-2) which recognized a monomeric glycoprotein on stromal cells, but not on several lympho-myeloid cell lines. Since both PS/2 and M/K series antibodies inhibited adhesion in the same system, it is possible that the latter recognize a counter-receptor for VLA-4. However, formal proof would require molecular definition and manipulation of the 100-kD species precipitated by M/K-1 and M/K-2.

A second ligand for VLA-4 is inducible on inflamed human endothelial cells and designated VCAM-1 or INCAM-110 (11, 33–35). There is evidence this molecule is utilized for adhesion of leukocytes and it has been speculated to be involved in leukocyte extravasation or tumor metastasis, as well as retention of lymphocytes in germinal centers (36). The murine counterpart of VCAM-1 has not yet been defined and expression of VCAM-1 in human bone marrow has not been studied (37). Furthermore, there have been no formal descriptions of a human long-term culture system comparable to the one used here to study murine lymphopoiesis. The similarity in size between human VCAM-1 and the antigen detected by our M/K series antibodies is interesting and the possibility of a relationship between the two is under investigation. Thus far, we know that the antigen is expressed on one cultured endothelial cell line, where it can function as a lymphocyte adhesion molecule. Like VLA-4, it is easily detected in murine bone marrow sections, but present on only a subpopulation of large cells in bone marrow suspensions (Miyake et al., manuscript in preparation).

Kina et al. (38) prepared a polyclonal antibody to a thymus derived stromal cell clone, which also blocks adhesion of particular cell lines to stromal cells. Immunoprecipitation revealed that the same or closely related molecules are detected by the polyclonal and M/K series antibodies. This raises the interesting possibility that maturing thymocytes may use similar mechanisms for recognition of the microenvironment.

VLA-4 is the second cell adhesion molecule shown to be important for lymphopoiesis in long term culture experiments (8). However, while antibodies to CD44/Pgp-1 were equally effective at inhibiting lymphoid and nonlymphoid cultures, the anti-VLA-4 antibody studied here preferentially inhibited lymphopoiesis. The antibodies also differ with respect to their effects when added to previously established cultures. In that circumstance, lymphocyte deadhesion resulted from addition of PS/2, or M/K series, but not anti-CD44 antibodies (Miyake et al., manuscript in preparation). Exploitation of these experimental models should allow further dissection of critical cell recognition events in lymphopoietic microenvironments. Some of the most important adhesion molecules may be ones also required in extramedullary sites for leukocyte migration and responses to inflammation.

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