A VCAM-like Adhesion Molecule on Murine Bone Marrow Stromal Cells Mediates Binding of Lymphocyte Precursors in Culture

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Abstract. Two new mAbs (M/K-1 and M/K-2) define an adhesion molecule expressed on stromal cell clones derived from murine bone marrow. The protein is similar in size to a human endothelial cell adhesion molecule known as VCAM-1 or INCAM110. VCAM-1 is expressed on endothelial cells in inflammatory sites and recognized by the integrin VLA-4 expressed on lymphocytes and monocytes. The new stromal cell molecule is a candidate ligand for the VLA-4 expressed on immature B lineage lymphocytes and a possible homologue of human VCAM-1. We now report additional similarities in the distribution, structure, and function of these proteins. The M/K antibodies detected large cells in normal bone marrow, as well as rare cells in other tissues. The antigen was constitutively expressed and functioned as a cell adhesion molecule on cultured murine endothelial cells. It correlated with the presence of mRNA which hybridized to a human VCAM-1 cDNA probe. Partial NH_2 terminal amino acid sequencing of the murine protein revealed similarities to VCAM-1 and attachment of human lymphoma cells to murine endothelial cell lines was inhibited by the M/K antibodies. All of these observations suggest that the murine and human cell adhesion proteins may be related. The antibodies selectively interfered with B lymphocyte formation when included in long term bone marrow cultures. Moreover, they caused rapid detachment of lymphocytes from the adherent layer when added to preestablished cultures. The VCAM-like cell adhesion molecule on stromal cells and VLA-4 on lymphocyte precursors may both be important for B lymphocyte formation.

Long term bone marrow culture techniques make it possible to define molecular and cellular interactions which may regulate production of blood cells in vivo (28). These experimental approaches are particularly well developed for studies of mice, where bone marrow cultures can be used to selectively sustain growth of immature myeloid or lymphoid cells. Furthermore, cloned stromal cell lines are available to which cloned lymphoid cells attach and are stimulated to divide. Stromal cells are a source of many cytokines and it has been suggested that the production of one pre-B growth factor results from direct contact of immature lymphocytes with them (29, 61). Thus, cell adhesion molecules (CAMs)[1] on stromal cells could be important for signal transmission, as well as for mediating lymphocyte binding.

Many CAMs have been identified on erythroid, myeloid, and lymphoid progenitors, as well as on stromal elements with which they interact (5, 7, 19, 30, 32, 43, 50, 62). The murine equivalent of CD44 was the first one to be directly implicated in lymphocyte formation (35). mAbs to CD44 completely blocked formation of myeloid or lymphoid cells when continuously added to long term bone marrow cultures. However, CD44 does not seem to be important for binding of pre-B cells to stromal cells and mAbs were developed that define another pair of adhesion molecules (37). B lineage lymphocytes and their precursors express the integrin VLA-4 and antibodies (PS/2) to this molecule preferentially inhibited lymphopoiesis in culture.

Two antibodies (M/K-1 and M/K-2) prepared to stromal cells detect another cell adhesion molecule and it is similar in size to one of two known ligands for VLA-4. Transfection and other studies in humans revealed that VLA-4 recognizes a single-chain glycoprotein known as VCAM-1 or INCAM 110, whose expression on endothelial cells is dramatically induced by inflammatory agents (12, 16, 21, 47). It seemed possible that our M/K antibodies recognize a murine homologue of VCAM-1 and that this molecule functions in bone marrow as a ligand for the VLA-4 on pre-B cells. We now provide additional information about the tissue distribution, structure and function of this cell adhesion molecule.

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; EOMA, hemangioendothelioma; PI-PLC, phospholipase C.

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Materials and Methods

Cell Lines and mAbs

The murine hemangioendothelioma (EOMA) and aorta endothelial cell lines have been described (4, 40). They were verified on the basis of expression of acetylated LDL receptors and angiotensin converting enzyme. The +/+ 2.4 stromal cell clone was a gift of Dr. Joel S. Greenberger at the University of Massachusetts (Boston, MA) (3). All other cell lines were either obtained from the American Type Culture Collection (Rockville, MD) or derived in our laboratory and described in previous publications (37). Similarly, the derivation of mAbs has been described (35, 37). As in our previous studies, antibodies were purified from ascites fluid prepared in immunodeficient C.B-17 *scid/scid* mice.

Northern Blots

Total RNA was extracted and analyzed on Northern blots exactly as in our previous studies (17). The stringency of wash conditions were twice at room temperature (10 min each) and once for 30 min at 50°C in 2× SSC with 0.1% SDS. This was followed by two additional washes of 15 min each at 50°C in 0.1× SSC with 0.1% SDS.

Immunoprecipitation and Protein Sequencing

Up to 100 culture dishes (100 mm) of +/+ 2.4 stromal cells were harvested with a silicone policeman, pooled, and extracted with 1% Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 50 mM iodoacetamide, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% NaN_3, 10 μg/ml trypsin inhibitor, 1 μm/ml leupeptin, 1 U/ml aprotinin, and 1 mM PMSF. After centrifugation, the lysate was precleared twice with goat serum-coupled Affigel 10 (Biorad Laboratories, Cambridge, MA) beads and once with goat anti-rat Ig coupled Sepharose beads loaded with rat IgG. Specific complexes were then collected on goat anti-rat Ig Sepharose beads loaded with M/K-1.2 antibodies by incubation at 4°C overnight. These beads were then washed twice with 0.2% Triton X-100, 0.6 M NaCl, 50 mM Tris/HCl, pH 7.5, 0.1% NaN_3. Protein was eluted from the beads by boiling for 5 min in Laemmli-reducing SDS solution and subjected to PAGE on 7.5% gels. The protein was then electrophoretically transferred to polyvinyl difluoride membranes (Millipore Continental Water Systems, Bedford, MA) and visualized by Coomassie blue staining. NH_2-terminal amino acid sequence analyses were performed on a gas-phase protein sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, CA). These determinations were made by Dr. Ken Jackson in the Molecular Biology Resource Facility of the Saint Francis Hospital of Tulsa Medical Research Institute (Oklahoma City, OK). Each residue was detected 2–4 times in four completely independent experiments.

Immunofluorescence and Immunoperoxidase Staining

Cells in suspension were preincubated for 20 min on ice with mAbs that had been biotinylated or were unlabeled. After two washes, either fluorescein-labeled streptavidin (Zymed Laboratories, S. San Francisco, CA) or mouse anti-rat Ig was added for an additional incubation. Propidium iodide was added during this time and used as a gating parameter to exclude nonviable cells. Labeled cells were then evaluated by flow cytometry on either a Coulter EPICs or FACScan (Becton Dickinson & Co., Mountain View, CA). In some experiments, normal spleen, 70Z/3, or endothelioma cells were pretreated with phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*. This highly purified enzyme was a gift from Dr. Martin Low (Columbia University, New York) and was used as in our previous study (33). As positive controls, we monitored the release of Thy-1 and/or Ly-6 antigens. Tissues were removed for histology and snap frozen in O.C.T. embedding medium (Miles Laboratories Inc., Elkhart, IN) using a 2-methylbutane bath submerged in liquid N_2. Sections of 4–6 μm were fixed in acetone for 2 min at −20°C, washed in PBS, and treated with NaN_3 and H_2O_2 before blocking with BSA. They were then stained and processed with a kit from Zymed Laboratories. Bone marrow plugs were expelled with medium and processed the same way.

Cell Adhesion Assay

Cell lines were radiolabeled by incubation of 50 × 10^6 cells/ml in saline with 20% FCS and $Na_2{}^{51}CrO_4$ for 2 h at 37°C. They were then washed three times before addition of 10^5 cells to adherent cell layers. The latter were plated in 24 well plates at 3 × 10^4 cells/well and allowed to grow for two days before use in adhesion assays. After 30 min of coculture at 37°C, unbound cells were removed by three cycles of washing in prewarmed complete medium. Before each aspiration, plates were vigorously agitated on a Vortex mixer to which a plate adaptor was attached. Bound cells were lysed with 0.1 N NaOH, 0.1% Triton-X100 and the ^{51}Cr counted with a gamma counter (Hewlett Packard Co., Palo Alto, CA). Percentages of bound cells were determined by the formula: percent bound = ([cpm from bound cells]/[input cell associated cpm − spontaneously released cpm]) ×

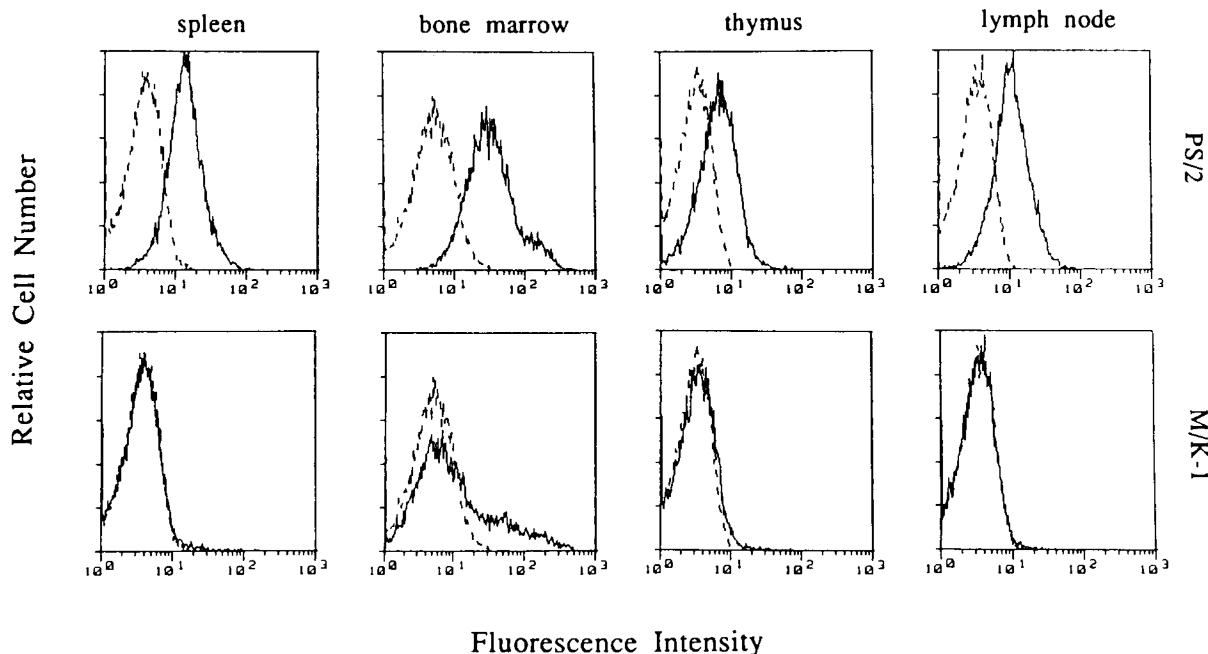

Figure 1. Differential patterns of expression of the integrin VLA-4 (detected by monoclonal PS/2 antibody) and a stromal cell adhesion molecule (M/K-1 antibody) on murine cell suspensions. Immunofluorescent staining and flow cytometry were performed as detailed in the Materials and Methods. Solid lines depict specific staining and negative control histograms are shown with dotted lines.

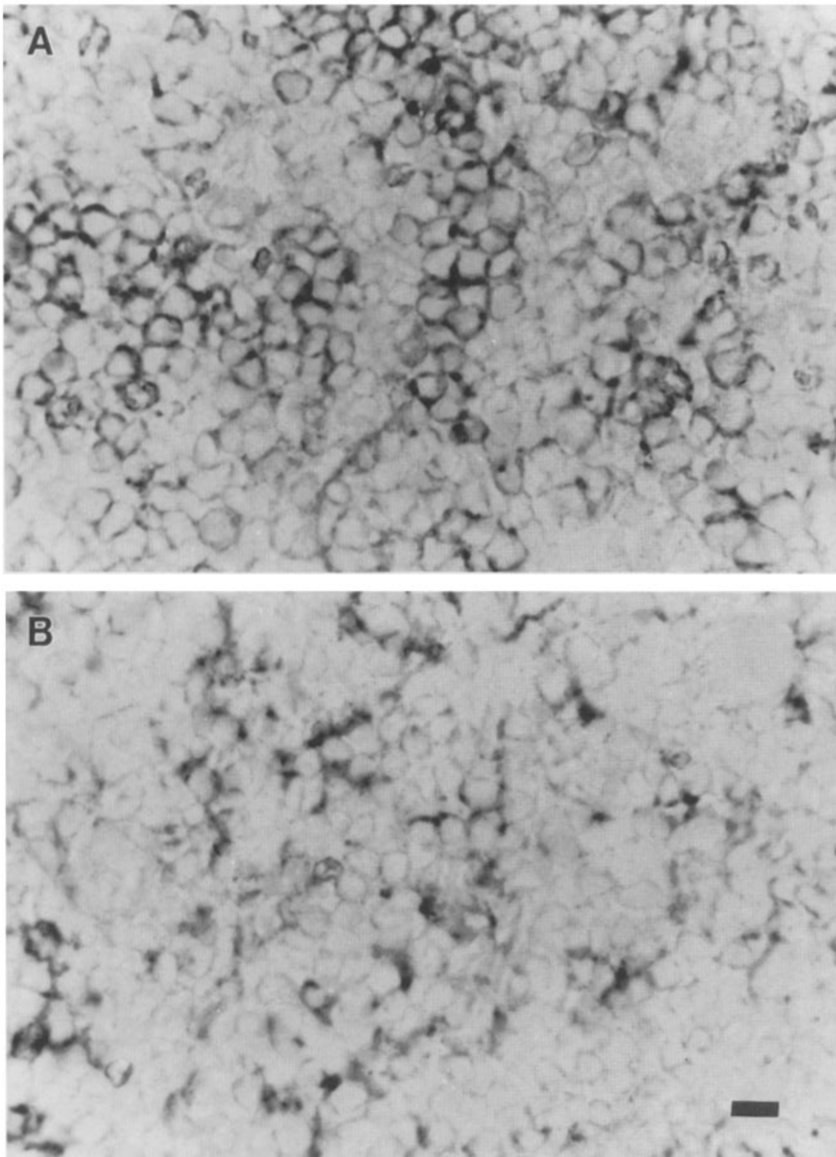

Figure 2. Both VLA-4 and the stromal cell antigen are constitutively expressed in murine bone marrow. Frozen sections of normal marrow plugs were analyzed by immunoperoxidase staining with a monoclonal antibody to VLA-4 (PS/2) or the stromal cell antigen (M/K-1). The background in control sections treated with an isotype matched, irrelevant mAb was extremely low (not shown). Bar, 10 μm.

100. Antibodies (10 μm/ml) were added at the same time as the ^{51}Cr-labeled lymphoma cells.

Long Term Cultures and Antibody Treatments

Long term bone marrow cultures were initiated and maintained exactly as in our previous studies (35, 37). These techniques were based on those developed by Whitlock and Witte (66). In some experiments, the indicated amounts of mAbs were added at the initiation of culture and with each feeding.

Detachment of Lymphocytes from Stromal Cells

Whitlock-Witte cultures were set up under standard conditions and maintained for at least 5 wk. At this time, numerous foci of lymphocytes were present attached to the adherent layers. All nonadherent and easily mobilized lymphocytes were removed by gentle agitation and aspiration. Then, 3 ml of medium containing either antibodies (5 μg/ml) or PI-PLC (added in 10-fold excess of that required for Thy-1 or Ly-6 release) was added to the flasks. In most experiments, the cultures were incubated at 37°C for 2 h before removing this medium and performing hemacytometer counts. Then 3 ml of 0.02% EDTA in PBS was added to each flask and held 10 min at room temperature to remove all remaining lymphocytes. These were also counted and the sum collected in both treatments was used as the number of total releasable lymphocytes. In one experiment, which only involved addition of antibodies, the flasks were first prechilled to 4°C.

Results

Tissue Distribution of a Stromal CAM

We previously prepared two mAbs (M/K-1 and M/K-2) which recognize an ~100-kD adhesion molecule expressed on a cloned stromal cell line (37). Cross-blocking experiments indicate that the antibodies detect very closely related, if not identical epitopes (data not shown). This stromal cell antigen is a possible murine homologue of human VCAM-1, which is known to be a ligand for the integrin VLA-4 (12).

Significant numbers of cells positive for the stromal cell antigen were only present in bone marrow suspensions (Fig. 1). This subpopulation had forward and right angle light scatter properties typical of large cells (data not shown). A reticular pattern was observed when bone marrow was sectioned and examined by immunoperoxidase staining (Fig. 2). In

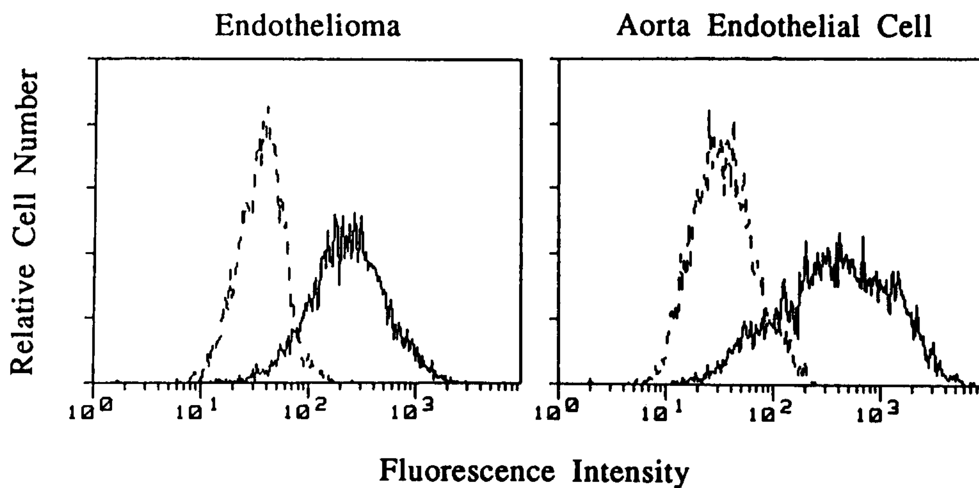

Figure 3. Two murine cell lines of endothelial origin express the stromal cell adhesion molecule. Viable suspensions of the cultured cells were stained and analyzed by flow cytometry. Histograms obtained with the M/K-1 antibody are given as solid lines and the irrelevant controls (FD441.8 antibody to LFA-1) are shown as dotted lines.

contrast, VLA-4 was detected by the PS/2 antibody on a majority of lymphohemopoietic cells in marrow, as well as in peripheral lymphoid tissues. A survey of other tissues revealed the Peyer's patches to be another site where the VCAM-like molecule was detected. In sections of lymph nodes, skin, spleen, and thymus, only very weak staining of widely scattered cells was obtained with the M/K antibodies. This included a small fraction of the follicular dendritic cells in germinal centers. Also, a subset of thymic macrophages appeared to be positive. VCAM-1/INCAM110 is known to be inducible on human endothelial cells (13, 45–47). Similarly, we found that the stromal cell antigen was constitutively expressed on two murine endothelial cell lines (Fig. 3). There have been no descriptions of VCAM-1 in human bone marrow. Otherwise, the distribution of the murine stromal cell antigen is generally consistent with that of human VCAM-1.

Northern blots were prepared with total RNA from a panel of cell lines and probed with human VCAM-1 cDNA (42). A transcript of ∼3.2 kb, as well as a less abundant species of ∼1.6 kb, was only detected in cell lines known to be positive for the stromal cell antigen by immunofluorescence analysis (Fig. 4). Hybridization was also found with samples of normal spleen. For comparison, the same blots were probed with ICAM-1 cDNA (58). There was an entirely different pattern of expression for this adhesion molecule and transcripts were never detected in stromal cells (Fig. 4 and data not shown). These results demonstrate that a cell adhesion molecule previously recognized on a murine stromal cell is constitutively expressed in bone marrow and faintly represented on reticular cells in other tissues. Like human VCAM-1, it can be made by endothelial cells and is present in concordance with mRNA which cross hybridizes with a human VCAM-1 probe.

Structural Similarities to VCAM-1

Treatment with PI-PLC substantially reduced the staining of stromal cells or endothelial cells with antibodies to Ly-6, an antigen known to be attached to the membrane via a glycosyl phosphatidylinositol linkage (46). In contrast, this treatment had no effect on the antigen detected by M/K antibodies or on VLA-4 (see Materials and Methods). Both of these may be conventional transmembrane proteins.

Partial NH_2-terminal sequencing was then attempted with immunoprecipitated protein and determinations were possible for amino acids in positions 3 through 8 (Fig. 5). Four of these are identical to the published sequence of human VCAM-1 (40) and one additional amino acid represents a conservative substitution (serine to threonine). Both amino acid changes could result from single nucleotide substitutions and no gaps or realignments were needed to reveal similarities in the sequences. These observations are consistent

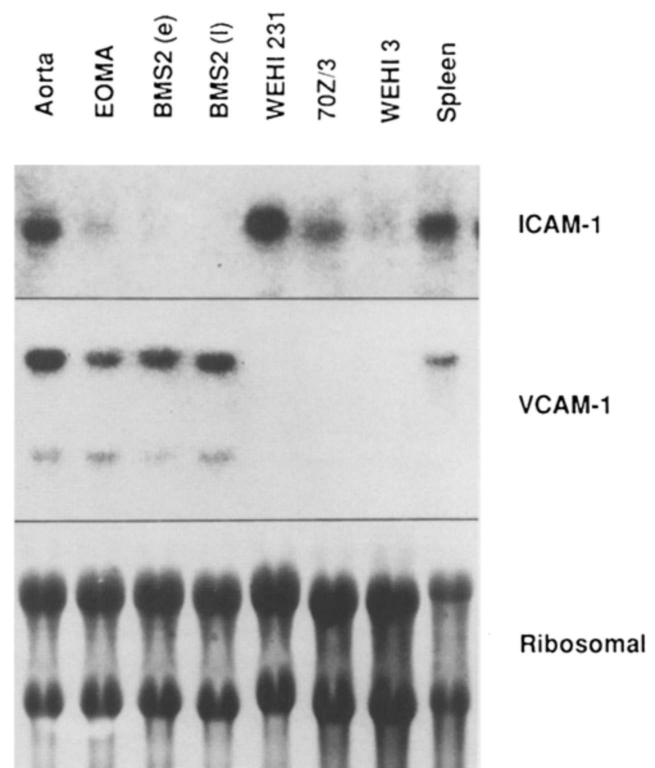

Figure 4. Selective expression of ICAM-1 and VCAM-1 mRNA's in murine tissues. Total RNA was prepared from aorta endothelial and endothelioma (*EOMA*) cell lines as well as early (*e*) and late (*l*) passages of the BMS2 stromal cell clone and used for Northern blot analysis. Preparations were also made from B lymphoma (W231), pre-B lymphoma (*70Z/3*), myelomonocytic leukemia (*WEHI3*), and normal BALB/c spleen cells. A photograph of the ethidium stained ribosomal RNA is also shown to demonstrate sample loading.

Human:	F	K	I E	T	T	P E
Mouse:	?	?	I E	I	S	P E

Figure 5. Partial NH_2-terminal amino acid sequence identity of human VCAM-1 and a murine stromal cell adhesion molecule. The published human sequence is shown beginning with the first residue after the signal peptide. Each murine amino acid was unambiguously detected at least twice in four determinations. The serine to threonine difference is indicated as a conservative change.

with the possibility that the M/K antibodies recognize a molecule related to VCAM-1.

Conservation of VCAM/VLA-4-mediated Cell Adhesion

A previous study demonstrated that murine melanoma cells bound to cytokine-activated human endothelial cells. This adhesion was blocked by an antibody to human VCAM-1/INCAM110 (47). The VLA-4 on murine melanoma cells might therefore be able to recognize human VCAM-1. We have now found that the reciprocal relationship is also possible (Fig. 6). Two human cell lines, Ramos and Nalm 6, readily attached to a murine endothelioma line and this recognition was significantly blocked by M/K and PS/2 antibodies. An antibody to murine CD44 (KM201) had no effect. Although PS/2 was developed and characterized as an antibody to murine VLA-4, it also has some affinity for human cells (37; and results not shown). It was consistently less effective than the M/K antibodies in blocking adhesion of human cells. Interaction of the human Ramos lymphoma to an endothelial line derived from murine aorta was similar to the results shown here, while binding of Nalm-6 to the same line was more avid, and less efficiently inhibited (data not shown). Nalm-6 also quickly migrated beneath adherent layers and this pseudo-emperipolesis was not inhibited by any antibodies tested. Therefore, human cell lines can recognize conserved adhesion ligands on murine endothelial cells. In the examples shown, the binding must have been largely mediated by human VLA-4 and a murine VCAM-like molecule.

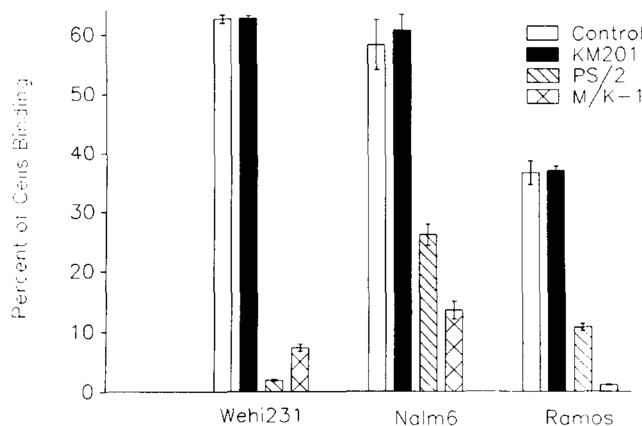

Figure 6. Adhesion of murine and human cell lines to a murine endothelioma is at least partially mediated by the VCAM-like stromal cell adhesion molecule. Murine B lymphoma (*Wehi231*), human pre-B leukemia (*Nalm6*), and human Burkitt lymphoma (*Ramos*) lines were ^{51}Cr labeled and used in the cell adhesion assay as described in the Materials and Methods. mAbs (10 μg/ml) to murine CD44 (*KM201*), VLA-4 (*PS/2*), and the VCAM-like antigen (*M/K-1*) were added as indicated. Note that the PS/2 antibody also has a weak affinity for human cells.

Table I. Inhibition of Whitlock/Witte Cultures by Addition of Monoclonal M/K Antibodies

Group	Antibody	Concentration	Positive/Total*
I	Control	3 μg/ml	5/5
	KMI-6	3 μg/ml	5/5
	M/K-1	3 μg/ml	5/5
	M/K-2	3 μg/ml	5/5
II	Control	5 μg/ml	6/6
	D7	5 μg/ml	5/6
	M/K-1	5 μg/ml	1/6
	M/K-2	5 μg/ml	2/6
III	Control	3 μg/ml	4/4
	KMI-6	3 μg/ml	4/4
	M/K-1	3 μg/ml	1/4
	M/K-2	3 μg/ml	0/4
IV	Control	5 μg/ml	4/4
	KMI-6	5 μg/ml	4/4
	M/K-1	5 μg/ml	0/4
	M/K-2	5 μg/ml	0/4

* Numbers of flasks with lymphocyte growth/total after 8 wk of culture. Long term bone marrow cultures were initiated and maintained in the continuous presence of mAbs at the indicated concentrations. Irrelevant control antibodies include D7 (anti-Ly-6) and KMI-6, which detects an abundant antigen of unknown function on stromal cells.

Participation of the VCAM-like Molecule in Marrow Cell Adhesion

Evidence for the importance of CD44 and VLA-4 in lympho-hemopoiesis was previously obtained by adding mAbs to long term bone marrow cultures (35, 37). Similar studies were done with the M/K series antibodies (Table I). In the first experiment, lymphocyte production was not prevented by including 3 μg/ml of antibodies in the culture medium. However, there was a striking effect on cell adhesion. A small number of nonadherent lymphocytes are always present in control cultures, but virtually all of the lymphocytes were easily mobilized by gently tapping those flasks which contained M/K antibodies. The only lymphocytes which remained associated with the adherent layer were in small foci beneath stromal cells (see Fig. 8). Lymphocytes that could be recovered from M/K antibody containing cultures were not unusual with respect to cell surface markers (results not shown). Another antibody, KMI6, detects a relatively abundant protein expressed on bone marrow stromal cells, but had no effect on cell adhesion assays or bone marrow cultures (unpublished observations). It was used throughout as a negative control. In subsequent experiments, where higher concentrations of M/K antibodies were usually added, lymphocyte production was totally inhibited in at least 75% of the culture flasks. The M/K antibodies had no effect on production of myeloid cells when added to Dexter-type long term bone marrow cultures (data not shown).

Preliminary experiments revealed that CD44 antibodies only inhibited lymphopoiesis when added during the first week of culture (data not shown). However, we found that lymphocyte adhesion to stromal cells could be disrupted at any stage by addition of the M/K or PS/2 antibodies (Fig.

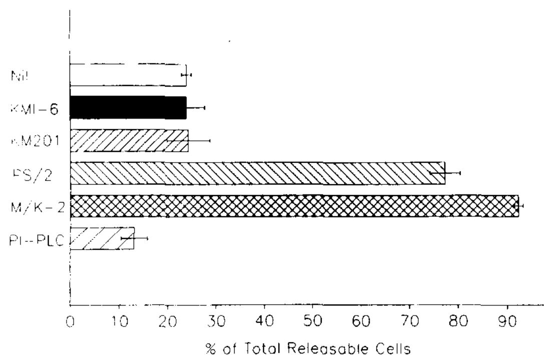

Figure 7. Lymphocyte detachment from established long term bone marrow cultures by mAbs. After removing any free-floating lymphocytes, medium was replaced with monoclonal antibodies (5 μg/ml) or PI-PLC. Numbers of cells liberated from the stromal cell layer during 2 h of subsequent incubation was determined as detailed in the Materials and Methods. Monoclonal KMI-6 antibody detects an abundant antigen on stromal cells which has no known function, KM201 recognizes murine CD44/Pgp-1, PS/2 detects VLA-4, and M/K-2 is directed to a VCAM-like stromal cell adhesion molecule. Data is shown from one of six similar experiments.

7). In six experiments, an average of 20.2 $\pm$ 1.6% of the lymphocytes spontaneously detached from the adherent layer during one cycle of medium replacement and a 2-h incubation. Similarly, 17.5 $\pm$ 3.5% of the cells were recovered when an anti-CD44 antibody (KM201) was present. However, an average of 70.9 $\pm$ 2.1% of the lymphocytes were liberated by anti-VLA-4 (PS/2) and 88.6 $\pm$ 1.4% were mobilized by the M/K (anti-VCAM) antibodies. The effect of these antibodies was readily apparent by microscopic examination of the cultures (Fig. 8). Focal adhesion of lymphocytes to stromal cells was disrupted and only the slightest agitation caused them to float into the medium. As in our previous studies (67), essentially all of the surface adherent lymphocytes were detached by EDTA treatment, suggesting divalent cations are important in the adhesion process. It is possible that the antibodies only delivered a stimulus to lymphocytes or stromal cells, such that lymphocytes are actively released. However, similar results were obtained in one experiment, where the entire procedure was performed at 4°C. Addition of a highly purified PI-PLC from B. thuringiensis did not result in significant lymphocyte detachment (average 18.2 $\pm$ 2.5% release). Therefore, requirements for lymphocyte adherence match the known properties of murine VLA-4 and the VCAM-like molecule. The effects on long term bone marrow cultures indicate that both CAMs may be critical to interactions between lymphocytes and stromal cells.

Discussion

These findings reveal structural and other similarities between a CAM expressed by murine stromal cells and one known as VCAM-1 or INCAM110 in humans. The latter is thought to function as a ligand for the integrin VLA-4 and to mediate important functions in inflamed peripheral tissues (12, 41, 42, 53). We now show that the murine VCAM-like antigen is constitutively expressed in bone marrow, where it appears to be recognized by VLA-4^{+} lymphocytes. Thus, the same pair of molecules which participate in immune related responses may be critical to normal lymphopoiesis.

VCAM-1/INCAM 110 was first identified as an inducible CAM on human endothelial cells which could mediate melanoma cell or lymphocyte binding (47, 48). Lymphocytes, monocytes, and natural killer cells may all recognize this molecule on stimulated endothelial cells or follicular dendritic cells of germinal centers (1, 8, 14, 20). The distribution of VCAM-1 in human bone marrow has not been described (59). However, information is accumulating about its expression in other human tissues, where it is particularly conspicuous in sites of inflammation (6, 49). Particular cytokines induce VCAM-1 on freshly isolated endothelial cells from humans and primates (34, 63). In this respect, endothelial cells from different organs appear to respond to different combinations of stimuli, suggesting there may be tissue specific regulation of expression.

The M/K series of antibodies was prepared by immunization with stromal cell clones derived from murine bone marrow (37). We have now demonstrated that antigen positive cells are present in normal bone marrow and that the antigen can be expressed on endothelial cells. Two established endothelial cell lines constitutively expressed this molecule, where it was a functional CAM. Furthermore, the M/K antibodies specifically recognize the endothelium of inflamed murine cardiac allografts (Pelletier, Sedmak, Miyake, Kincade, Ferguson and Orosz, manuscript submitted for publication). Interestingly, antibodies to VCAM-1/INCAM110 have recently been used to monitor inflammation in human cardiac transplants (6).

As another indication of concordance, cell lines which expressed the stromal cell antigen also contained mRNA which hybridized to a human VCAM-1 probe. Two types of transcripts have thus far been isolated from human cells, which differ only with respect to a 256 nucleotide insertion (23, 42, 45). Using a human cDNA probe, we detected hybridization to an ~1.6-kb RNA species in murine cell lines, in addition to the expected 3.2-kb transcript.

Similarities in NH_2 terminal amino acid sequence between human VCAM-1 and the murine stromal CAM would be consistent with a structural relationship between these proteins and we also obtained evidence for common functions. Certain human lymphoma lines have been previously shown to selectively utilize VLA-4 for endothelial cell recognition (12, 14). Antibody blocking experiments indicate that the attachment of Ramos and Nalm 6 cells to murine endothelioma cells must have been largely dependent on human VLA-4 and murine VCAM-1 (Fig. 6). Murine F16 melanoma cells adhere to activated human endothelial cells via VCAM-1 (47). Therefore, recognition between this pair of CAMs may work across species in either direction.

Immunoperoxidase staining revealed some expression of the VCAM-like antigen in sections of murine Peyer's patches and thymus. VLA-4 and the closely related LPAM-1 molecule are thought to direct lymphocyte traffic to murine Peyer's patches (24, 25) and it will be interesting to learn if a VCAM-like molecule functions as an adhesive ligand in that circumstance. Polyclonal antibodies prepared against thymic stromal cell clones by Kina and colleagues (27) recognize a similar CAM(s) to that detected by our M/K an-

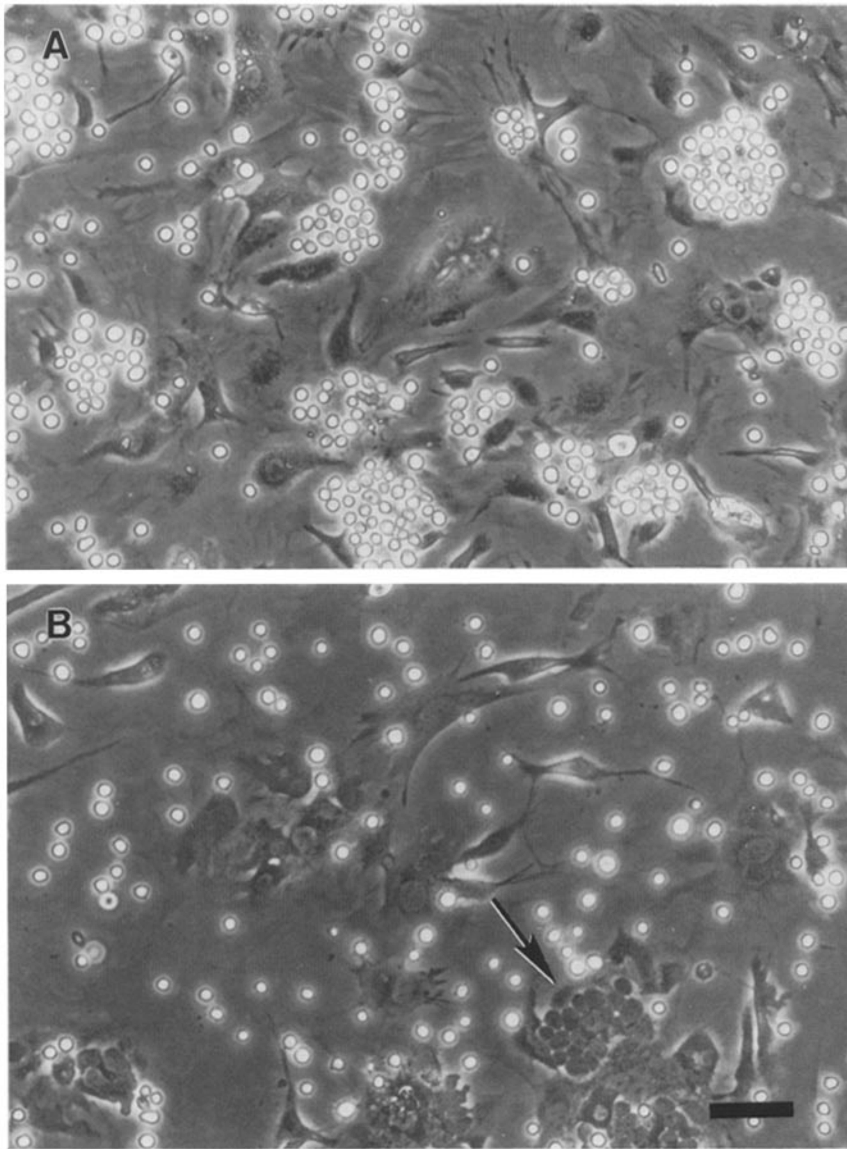

Figure 8. Effect of mAbs to the VCAM-like cell adhesion molecule on established bone marrow cultures. Control cultures received isotype matched irrelevant antibodies (*A*). With the exception of foci of lymphocytes located beneath the adherent layer (*arrow*), typical nests of lymphocytes on stromal cells are disrupted within 2 h of addition of M/K antibodies (*B*). Bar, 50 μm.

tibodies, raising the possibility that B and T lymphopoiesis have common features. However, additional adhesion molecules have been described in the thymus (10, 22, 57).

It is interesting to contrast the roles of CD44 and its ligands with the molecules addressed in this study. Production of myeloid and lymphoid cells were completely blocked by inclusion of anti-CD44 antibodies in the medium (35). However, this was only the case if the antibodies were present during the first week of culture and there was no effect on adhesion or other functions in established cultures. Ligands known to be recognized by CD44 include hyaluronate, mucosal vascular addressin, and collagens (9, 15, 31, 36, 44). One study indicated that collagens contribute to stromal cell layer functions (68) and anti-CD44 might interfere with some type of collagen recognition which is critical at the outset of culture.

Antibodies to VLA-4 and the VCAM-like molecule preferentially inhibited lymphopoiesis in culture (37), consistent with the notion that other specialized CAMs may be important for myeloid cell formation (7). In every experiment, lymphocyte outgrowth was completely prevented by anti-VLA-4 addition (37). In contrast, while lymphocyte detachment always occurred in cultures containing the M/K series antibodies, a fraction of the flasks continued to support lymphocyte growth (Table I). An apparent greater efficiency of inhibition by anti-VLA-4 might suggest that stromal cells express functional ligands for VLA-4 other than the VCAM-like molecule. It has been demonstrated that in addition to VCAM-1, VLA-4 recognizes the CS-1 peptide sequence of fibronectin (12, 16, 21). Fibronectin receptors have been described on lymphoma lines and B cell precursors, but their function in lymphopoiesis has not been investigated (5, 30, 52).

The fact that lymphocytes grew in a fraction of cultures in the presence of M/K antibodies suggests that their survival does not require rigid contact. However, gravity should bring them in close association with the stroma during incubation. Also, the antibodies could deliver a signal to stromal

cells similar to that normally achieved by lymphocyte adhesion. For example, contact between lymphocytes and stromal cells may stimulate production of IL-7, a pre-B cell growth factor (61). In this context, it will be interesting to learn if any of the CAMs play a signal transmitting role in bone marrow. Evidence is accumulating that VLA-4 and CD44 may function as receptors and/or accessory molecules on mature lymphocytes and monocytes (11, 26, 38, 54, 55, 65).

Disruption of binding of immature lymphocytes to stromal cells was virtually complete within 2 h of addition of antibodies to VLA-4 or the VCAM-like antigen (Figs. 7 and 8). While these observations indicate that VLA-4 and a VCAM-like molecule may be particularly important for B lymphopoiesis, CAMs frequently function in concert (51, 60). Further study is required to establish their importance in vivo and to determine if other molecules are involved. Another exciting area of investigation will be the modulation of functional adhesion during pre-B cell maturation. The timely and selective release of mature lymphocytes into the circulation and derangement of this in leukemia could involve such CAMs. Since mature lymphocytes can recognize VCAM-like molecules via VLA-4, the focus might be placed on expression or function of the stromal cell CAMs. However, there is evidence for nonfunctional VLA-4 on various cell types and the avidity of VLA-4 for fibronectin is immediately increased on T cells with activation (18, 24, 56, 64). Indeed, we have observed that some transformed VLA-4 positive pre-B cell lines do not recognize the VCAM-like adhesion ligand (unpublished observations). Conformational changes probably occur in the extracellular domains of other integrins in concert with changes in their affinities for ligand (2, 13, 39). Down regulation of VCAM expression or a reduction in the density and/or function of VLA-4 at an appropriate stage would permit detachment of mature lymphocytes from stromal cells.

Dr. Joel S. Greenberger of the University of Massachusetts (Boston, MA) provided a valuable cell line, Dr. Martin Low of Columbia University (New York) provided the PI-PLC and Drs. Lauralee Osborn of Biogen, Inc. (Cambridge, MA) and Adrienne A. Brian of the University of California (San Diego, CA) donated the cDNA probes. We also thank Ms. Margaret Robertson and Ms. Anna Henley for expert technical assistance and Drs. Linda Thompson, Carol Webb, Rod McEver, and Bart Frank for valuable advice. Dr. Ken Jackson of our Molecular Biology Resource Facility performed the amino acid sequencing.

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References

1. Allavena, P., C. Paganin, I. Martin-Padura, G. Peri, M. Gaboli, E. Dejana, P. C. Marchisio, and A. Mantovani. 1991. Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *J. Exp. Med.* 173:439–448.
2. Altieri, D. C., and T. S. Edgington. 1988. A monoclonal antibody reacting with distinct adhesion molecules defines a transition in the functional state of the receptor CD11b/CD18 (Mac 1). *J. Immunol.* 141:2656–2660.
3. Anklesaria, P., V. Klassen, M. A. Sakakeeny, T. J. FitzGerald, D. Harrison, M. E. Rybak, and J. S. Greenberger. 1987. Biological characterization of cloned permanent stromal cell lines from anemic $S1/S1^d$ mice and +/+ littermates. *Exp. Hematol.* 15:636–644.
4. Auerbach, R., W. C. Lu, E. Pardon, F. Gumkowski, G. Kaminska, and M. Kaminski. 1987. Specificity of adhesion between murine tumor cells and capillary endothelium: an *in vitro* correlate of preferential metastasis *in vivo*. *Cancer Res.* 47:1492–1496.
5. Bernardi, P., V. P. Patel, and H. F. Lodish. 1987. Lymphoid precursor cells adhere to two different sites on fibronectin. *J. Cell Biol.* 105:489–498.
6. Briscoe, D. M., F. J. Schoen, G. E. Rice, M. P. Bevilacqua, P. Ganz, and J. S. Pober. 1991. Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. *Transplantation.* 51:537–539.
7. Campbell, A. D., M. W. Long, and M. S. Wicha. 1987. Haemonectin, a bone marrow adhesion protein specific for cells of granulocyte lineage. *Nature (Lond.).* 329:744–746.
8. Carlos, T. M., B. R. Schwartz, N. L. Kovach, E. Yee, M. Rosso, L. Osborn, G. Chi-Rosso, B. Newman, R. Lobb, and J. M. Harlan. 1990. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. *Blood.* 76:965–970.
9. Carter, W. G., and E. A. Wayner. 1988. Characterization of the Class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263:4193–4201.
10. Couture, C., P. C. Patel, and E. F. Potworowski. 1990. A novel thymic epithelial adhesion molecule. *Eur. J. Immunol.* 20:2769–2773.
11. Denning, S. M., P. T. Le, K. H. Singer, and B. F. Haynes. 1990. Antibodies against the CD44 p80, lymphocyte homing receptor molecule augment human peripheral blood T cell activation. *J. Immunol.* 144: 7–15.
12. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/Fibronectin binding site. *Cell.* 60:577–584.
13. Figdor, C. G., Y. Van Kooyk, and G. D. Keizer. 1990. On the mode of action of LFA-1. *Immunology Today.* 11:277–280.
14. Freedman, A. S., J. M. Munro, G. E. Rice, M. P. Bevilacqua, C. Morimoto, B. W. McIntyre, K. Rhynhart, J. S. Pober, and L. M. Nadler. 1990. Adhesion of human B cells to germinal centers *in vitro* involves VLA-4 and INCAM-110. *Science (Wash. DC).* 249:1030–1033.
15. Gallatin, W. M., E. A. Wayner, P. A. Hoffman, T. St. John, E. C. Butcher, and W. G. Carter. 1989. Structural homology between lymphocyte receptors for high endothelium and class III extracellular matrix receptor. *Proc. Natl. Acad. Sci. USA.* 86:4654–4658.
16. Garcia-Pardo, A., E. A. Wayner, W. G. Carter, and O. C. Ferreira, Jr. 1990. Human B lymphocytes define an alternative mechanism of adhesion to fibronectin. The interaction of the $\alpha_4\beta_1$ integrin with the LHGPEI-LDVPST sequence of the type III connecting segment is sufficient to promote cell attachment. *J. Immunol.* 144:3361–3366.
17. Gimble, J. M., C. E. Pietrangeli, A. Henley, M. A. Dorheim, J. Silver, A. E. Namen, M. Takeichi, C. Goridis, and P. W. Kincade. 1989. Characterization of murine bone marrow and spleen derived stromal cells: analysis of leukocyte marker and growth factor mRNA transcript levels. *Blood.* 74:303–311.
18. Gismondi, A., S. Morrone, M. J. Humphries, M. Piccoli, L. Frati, and A. Santoni. 1991. Human natural killer cells express VLA-4 and VLA-5, which mediate their adhesion to fibronectin. *J. Immunol.* 146:384–392.
19. Gordon, M. Y., D. Clarke, J. Atkinson, and M. F. Greaves. 1990. Hemopoietic progenitor cell binding to the stromal microenvironment in vitro. *Exp. Hematol.* 18:837–842.
20. Graber, N., T. V. Gopal, D. Wilson, L. A. Beall, T. Polte, and W. Newman. 1990. T cells bind to cytokine-activated endothelial cells via a novel, inducible sialoglycoprotein and endothelial leukocyte adhesion molecule-1. *J. Immunol.* 145:819–830.
21. Guan, J. L., and R. O. Hynes. 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. *Cell.* 60:53–61.
22. He, H.-T., P. Naquet, D. Caillol, and M. Pierres. 1991. Thy-1 supports adhesion of mouse thymocytes to thymic epithelial cells through a Ca^{++}-independent mechanism. *J. Exp. Med.* 173:515–518.
23. Hession, C., R. Tizard, C. Vassallo, S. G. Schiffer, D. Goff, P. Moy, G. Chi-Rosso, S. Luhowskyj, R. Lobb, and L. Osborn. 1991. Cloning of an alternate form of VCAM1. *J. Biol. Chem.* 266:6682–6685.
24. Holzmann, B., and I. L. Weissman. 1989. Peyer's patch-specific lymphocyte homing receptors consist of a VLA-4-like alpha chain associated with either of two integrin beta chains, one of which is novel. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1735–1741.
25. Holzmann, B., B. W. McIntyre, and I. L. Weissman. 1989. Identification of a murine Peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an *a* chain homologous to human VLA-4*a*. *Cell.* 56:37–46.
26. Huet, S., H. Groux, B. Caillou, H. Valentin, A.-M. Prieur, and A. Bernard. 1989. CD44 contributes to T cell activation. *J. Immunol.* 143:798–801.
27. Kina, T., A. S. Majumdar, S. Heimfeld, H. Kaneshima, B. Holzmann, Y. Katsura, and I. L. Weissman. 1991. Identification of a 107-kD glycoprotein that mediates adhesion between stromal cells and hematolymphoid cells. *J. Exp. Med.* 173:373–381.
28. Kincade, P. W. 1987. Experimental models for understanding B lymphocyte formation. *Adv. Immunol.* 41:181–267.
29. Kincade, P. W., G. Lee, C. E. Pietrangeli, S.-I. Hayashi, and J. M. Gimble. 1988. Cells and molecules that regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* 7:111–143.
30. Lemoine, F. M., S. Dedhar, G. M. Lima, and C. J. Eaves. 1990.

Transformation-associated alterations in interactions between pre-B cells and fibronectin. *Blood.* 76:2311–2320.
31. Lesley, J., R. Schulte, and R. Hyman. 1990. Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. *Exp. Cell Res.* 187:224–233.
32. Liesveld, J. L., J. M. Winslow, M. C. Kempski, D. H. Ryan, J. K. Brennan, and C. N. Abboud. 1991. Adhesive interactions of normal and leukemic human CD34⁺ myeloid progenitors: role of marrow stromal, fibroblast, and cytomatrix components. *Exp. Hematol.* 19:63–70.
33. Low, M. G., J. Stiernberg, G. L. Waneck, R. A. Flavell, and P. W. Kincade. 1988. Cell-specific heterogeneity in sensitivity of phosphatidylinositol-anchored membrane antigens to release by phospholipase C. *J. Immunol. Methods.* 113:101–111.
34. Masinovsky, B., D. Urdal, and W. M. Gallatin. 1990. IL-4 acts synergistically with IL-1 Beta to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. *J. Immunol.* 145:2886–2895.
35. Miyake, K., K. L. Medina, S.-I. Hayashi, S. Ono, T. Hamaoka, and P. W. Kincade. 1990. Monoclonal antibodies to Pgp-1/CD44 block lymphohemopoiesis in long-term bone marrow cultures. *J. Exp. Med.* 171:477–488.
36. Miyake, K., C. B. Underhill, J. Lesley, and P. W. Kincade. 1990. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172:69–75.
37. Miyake, K., I. L. Weissman, J. S. Greenberger, and P. W. Kincade. 1991. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* 173:599–607.
38. Nojima, Y., M. J. Humphries, A. P. Mould, A. Komoriya, K. M. Yamada, S. F. Schlossman, and C. Morimoto. 1990. VLA-4 mediates CD3-dependent CD4⁺ T cell activation via the CS1 alternatively spliced domain of fibronectin. *J. Exp. Med.* 172:1185–1192.
39. O'Toole, T. E., J. C. Loftus, X. Du, A. A. Glass, Z. M. Ruggeri, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1990. Affinity modulation of the $alpha_{IIb}$ $beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Reg.* 1:883–893.
40. Obeso, J., J. Weber, and R. Auerbach. 1990. A hemangioendothelioma-derived cell line: its use as a model for the study of endothelial cell biology. *Lab. Invest.* 63:259–269.
41. Osborn, L. 1990. Leukocyte adhesion to endothelium in inflammation. *Cell.* 62:3–6.
42. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell.* 59:1203–1211.
43. Patel, V. P., and H. F. Lodish. 1986. The fibronectin receptor on mammalian erythroid precursor cells: characterization and developmental regulation. *J. Cell Biol.* 102:449–456.
44. Picker, L. J., M. Nakache, and E. C. Butcher. 1989. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J. Cell Biol.* 109:927–937.
45. Polte, T., W. Newman, and T. V. Gopal. 1990. Full length vascular cell adhesion molecule 1 (VCAM-1). *Nucleic Acids Res.* 18:5901.
46. Reiser, H., H. Oettgen, E. T. H. Yeh, C. Terhorst, M. G. Low, B. Benacerraf, and K. L. Rock. 1986. Structural characterization of the TAP molecule: a phosphatidylinositol-linked glycoprotein distinct from the T cell receptor/T3 complex and Thy-1. *Cell.* 47:365.
47. Rice, G. E., and M. P. Bevilacqua. 1989. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science (Wash. DC).* 246:1303–1306.
48. Rice, G. E., J. M. Munro, and M. P. Bevilacqua. 1990. Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism. *J. Exp. Med.* 171:1369–1374.
49. Rice, G. E., J. M. Munro, C. Corless, and M. P. Bevilacqua. 1990. Vascular and nonvascular expression of INCAM-110: A target for mononuclear leukocyte adhesion in normal and inflamed human tissues. *Am. J. Pathol.* 138:385–393.
50. Rosemblatt, M., M. H. Vuillet-Gaugler, C. Leroy, and L. Coulombel. 1991. Coexpression of two fibronectin receptors, VLA-4 and VLA-5, by immature human erythroblastic precursor cells. *J. Clin. Invest.* 87:6–11.
51. Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1–5.
52. Ryan, D. H., B. L. Nuccie, C. N. Abboud, and J. L. Liesveld. 1990. Maturation-dependent adhesion of human B cell precursors to the bone marrow microenvironment. *J. Immunol.* 145:477–484.
53. Schwartz, B. R., E. A. Wayner, T. M. Carlos, H. D. Ochs, and J. M. Harlan. 1990. Identification of surface proteins mediating adherence of CD11/CD18-deficient lymphoblastoid cells to cultured human endothelium. *J. Clin. Invest.* 85:2019–2022.
54. Shimizu, T., G. A. Van Seventer, R. Siraganian, L. Wahl, and S. Shaw. 1989. Dual role of the CD44 molecule in T cell adhesion and activation. *J. Immunol.* 143:2457–2463.
55. Shimizu, Y., G. A. Van Seventer, K. J. Horgan, and S. Shaw. 1990. Costimulation of proliferative responses of resting CD⁺ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. *J. Immunol.* 145:59–67.
56. Shimizu, Y., G. A. Van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (Beta 1) integrin receptors on T cells. *Nature (Lond.).* 345:250–253.
57. Singer, K. H., P. T. Le, S. M. Denning, L. P. Whichard, and B. F. Haynes. 1990. The role of adhesion molecules in epithelial-T-cell interactions in thymus and skin. *J. Invest. Dermatol.* 94:85S–90S.
58. Siu, G., S. M. Hedrick, and A. A. Brian. 1989. Isolation of the murine intercellular adhesion molecule 1 (ICAM-1) gene: ICAM-1 enhances antigen-specific T cell activation. *J. Immunol.* 143:3813–3820.
59. Soligo, D., R. Schiro, R. Luksch, G. Manara, N. Quirici, C. Parravicini, and G. L. Deliliers. 1990. Expression of integrins in human bone marrow. *Br. J. Haematol.* 76:323–332.
60. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:425–434.
61. Sudo, T., M. Ito, Y. Ogawa, M. Iizuka, H. Kodama, T. Kunisada, S.-I. Hayashi, M. Ogawa, K. Sakai, S. Nishikawa, and S.-I. Nishikawa. 1989. Interleukin 7 production and function in stromal cell-dependent B cell development. *J. Exp. Med.* 170:333–338.
62. Thomas, P. S., C. E. Pietrangeli, S.-I. Hayashi, M. Schachner, C. Goridis, M. G. Low, and P. W. Kincade. 1988. Demonstration of neural cell adhesion molecules on stromal cells which support lymphopoiesis. *Leukemia.* 2:171–175.
63. Thornhill, M. H., S. M. Wellicome, D. L. Mahiouz, J. S. S. Lanchbury, U. Kyan-aung, and D. O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and independent binding mechanisms. *J. Immunol.* 146:592–598.
64. Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.
65. Webb, D. S. A., Y. Shimizu, G. A. Van Seventer, S. Shaw, and T. L. Gerrard. 1990. LFA-3, CD44 and CD45: Physiologic triggers of human monocyte TNF and IL-1 release. *Science (Wash. DC).* 249:1295–1297.
66. Whitlock, C. A., and O. N. Witte. 1982. Long term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. USA.* 79:3608–3612.
67. Witte, P. L., M. Robinson, A. Henley, M. G. Low, D. L. Stiers, S. Perkins, R. A. Fleischman, and P. W. Kincade. 1987. Relationships between B-lineage lymphocytes and stromal cells in long term bone marrow cultures. *Eur. J. Immunol.* 17:1473–1484.
68. Zuckerman, K. S., R. K. Rhodes, D. D. Goodrum, V. R. Patel, B. Sparks, J. Wells, M. S. Wiche, and L. A. Mayo. 1985. Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long-term murine bone marrow cultures. *J. Clin. Invest.* 75:970–975.