

# Requirement for VLA-4 and VLA-5 Integrins in Lymphoma Cells Binding to and Migration beneath Stromal Cells in Culture

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**Abstract.** Physical interaction between human lymphomas and murine bone marrow derived stromal cells were studied. Nalm-6 pre-B cells adhered to BMS2 stromal cells and subsequently migrated beneath them, while Ramos Burkitt lymphoma cells, adhered but did not migrate. Four mAbs were established against Nalm-6 cells, which were able to block initial adhesion of Nalm-6 cells. Two of them were directed against the  $\alpha_4$  chain of VLA-4, and other two recognized the  $\beta_1$  chain of VLA integrins. Therefore, the initial adhesion of Ramos and Nalm-6 cells to BMS2 was largely mediated by the VLA-4 integrin expressed on lymphocytes. The corresponding ligand on stromal cells appears to be VCAM-1, because antibodies against murine VCAM-1 blocked the adhesion. However, antibodies against the  $\alpha$  chain of VLA-4 were not capable of blocking subsequent migration be-

neath stromal cells. In contrast, antibodies against the  $\beta$  chain of VLA integrins blocked the migration beneath stromal cells as well as the initial adhesion. Because a common  $\beta$  chain can be shared among integrins, the role of other VLA integrins in Nalm-6 cells migration was investigated. VLA-5 and VLA-6 as well as VLA-4 were expressed on Nalm-6 cells, but not on Ramos cells. Additional blocking experiments revealed that VLA-4 and VLA-5 are likely to work in concert to mediate the migration of Nalm-6 cells beneath stromal cells. Thus, particular VLA integrins appear to be responsible not only for lymphocyte adhesion but also for migration with respect to stromal cells. These findings may have implications for cell-cell interactions and directed migration of lymphocytes in bone marrow and other tissues.

**L**YMPHOCYTE progenitors grow and differentiate in bone marrow, where the microenvironment plays a crucial role in lymphopoiesis (10, 11). A stromal cell, for example, produces a number of factors affecting growth and differentiation of lymphohemopoietic progenitor cells (11). Some factors such as Steel factor (30), or macrophage colony-stimulating factor (CSF-1)<sup>1</sup> (27) are produced in either membrane bound or soluble forms, both of which may have biological activities (3, 27). The close contact of lymphohemopoietic progenitors with stromal cells could make growth factors, particularly membrane bound ones, more accessible.

We recently identified one, very late antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1) cell adhesion pathway that may be critical for the interaction between lymphocyte progenitors and stromal cells (14). These cell adhesion molecules (CAMs) are expressed in bone marrow and mAb treatment detached lymphoid cells from the stromal layer in long term bone marrow cultures (15). How-

ever, this cell adhesion pathway may not explain all the associations between B lymphocyte progenitor cells and stromal cells. Many pre-B lymphocyte cell lines, after initial adhesion to stromal cells, crawl beneath them (31). This phenomenon has been referred to as pseudoemperipolesis (7), and occurs with thymocyte (16), and B lymphocyte progenitors (31) as well as in mature lymphocytes (8) or granulocytes (25) interaction with endothelial cells in culture. It is possible that this striking in vitro phenomenon reflects molecular mechanisms used by lymphocyte progenitors to orientate themselves in bone marrow. Lymphocyte progenitors would not only associate with stromal cells, but migrate with respect to them as differentiation proceeds (9).

The present study has been focused on molecular mechanisms underlying pseudoemperipolesis, the migration beneath stromal cells. The human acute lymphocytic leukemia (ALL) cell of pre-B cell type, Nalm-6, adhered to a murine stromal cell clone BMS2 and subsequently migrated beneath them. Initial adhesion was largely blocked by antibodies against VLA-4 or VCAM-1, but the subsequent migration beneath was not.

A cell adhesion screening assay utilizing Nalm-6 and BMS2 resulted in four mAbs against Nalm-6. Two mAbs blocked the initial adhesion but not subsequent migration.

1. *Abbreviations used in this paper:* CAM, cell adhesion molecules; CSF, colony-stimulating factor; 2-ME, 2-mercaptoethanol; VCAM, vascular CAM; VLA, very late antigen.

These were found to be directed against the  $\alpha_4$  chain of VLA-4. Two other antibodies, which blocked both initial adhesion and subsequent transmigration, were against the  $\beta_1$  chain of VLA-4. Because  $\beta$  chains can be shared among integrins, other VLA integrins could be involved in migration beneath stromal cells. Combined addition of antibodies against VLA-4 and VLA-5 similarly blocked Nalm-6 cell migration as well as antibodies against  $\beta_1$ . Our results suggest that VLA-4 and VLA-5 expressed on B lymphocyte progenitors may work together to mediate recognition and orientation with respect to stromal cells.

## Materials and Methods

### Cells and Antibodies

Human Burkitt lymphoma cell, Ramos, and human erythroleukemia cell, K562 were obtained through JCRB (Japanese Cancer Research Resources Bank, Tokyo, Japan). Nalm-6 cells were generously provided by Dr. P. W. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK). BMS2 is a murine bone marrow-derived stromal cell clone (20). All cell lines were maintained in RPMI 1640 supplemented with 50  $\mu$ M 2-mercaptoethanol (2-ME), antibiotics, and 10% FCS.

BALB/c mice (Japan SLC Co., Shizuoka, Japan) were immunized with Nalm-6 cells and then spleen cells were fused with Sp2/0 cells. Hybridoma supernatants were screened with a cell adhesion assay using Nalm-6 cells and BMS2 cells. Hybridoma cells producing blocking antibodies were cloned by limiting dilution and reselected by the same procedure. Four mAbs were established. SG/73 and SG/19 are IgG<sub>1</sub>/ $\kappa$ . SG/7 is IgA/ $\kappa$ . SG/17 is IgG<sub>2a</sub>/ $\kappa$ . TS1/22 (mouse anti-human LFA-1: lymphocyte function associated antigen-1) hybridoma cells were obtained through American Type Culture Collection (ATCC) (Rockville, MD). Rat mAbs against murine VLA-4 (PS/2: IgG<sub>2b</sub>) and against murine VCAM-1 (M/K-1: IgG<sub>1</sub>) were described previously (14). These antibodies were purified from ascitic fluid prepared from severe combined immunodeficient (SCID) mice. The culture supernatant of GoH3 (rat anti human VLA-6) (26) was donated from Dr. Arnold Sonnenberg (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands). Mouse mAb against  $\beta_1$  chain (4B4) (17) was a kind gift from Dr. Chikao Morimoto (Dana Farber Cancer Institution, Boston, MA). Rat mAbs 13 and 16, which recognize integrin  $\beta_1$  and  $\alpha_5$ , respectively (1), were provided by Dr. Kenneth M. Yamada (National Cancer Institute, Bethesda, MD).

### Fibronectin and CS-1 Attachment Assay

Human plasma fibronectin (5  $\mu$ g/ml in PBS; Gibco Laboratories, Grand Islands, NY) and CS-1 peptide-conjugated rabbit IgG (1.5  $\mu$ g/ml in PBS) which was prepared as described by Nojima et al. (17), were plated on 96-well plates (Immulon II, Dynatech, Alexandria, VA). After 2 h of incubation at 37°C, the coated wells were washed twice with PBS, and then blocked with 1% BSA in PBS for 2 h at 37°C. Ramos cells ( $1 \times 10^5$ ) were preincubated with 10  $\mu$ g/ml of the indicated mAbs in serum-free medium for 30 min at 37°C, and then transferred to 96-well plates coated with fibronectin, CS-1-IgG, or BSA. After 1 h of incubation at 37°C, nonadherent cells were removed by addition and removal of prewarmed PBS two times. The number of residual cells bound to the wells was measured by adding 100  $\mu$ l of 0.5 mg/ml MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma Chemical Co., St. Louis, MO) in serum-free medium and incubating for 1 h at 37°C. The formazan precipitate formed was dissolved in 200  $\mu$ l of 0.4 N HCl in isopropanol and OD was measured at 560 nm on a plate reader. Percent adherence was calculated by the following formula: % adherence =  $(OD_{560}$  of experimental wells -  $OD_{560}$  of BSA-coated wells) / ( $OD_{560}$  of unwashed wells -  $OD_{560}$  of unwashed wells -  $OD_{560}$  of BSA-coated wells)  $\times 100$ .

### Cell Adhesion Assay

Cell lines were radiolabeled by incubating  $5 \times 10^7$  cells/ml in saline with 20% FCS and 100  $\mu$ Ci of  $Na_2^{51}CrO_4$  (Amersham Japan, Tokyo, Japan) for 2 h at 37°C and then washed three times in prewarmed complete medium (10% FCS RPMI 1640). The BMS2 stromal cell clone was plated in 24-well plates (Becton Dickinson Labware, Lincoln Park, NJ) at  $3 \times 10^4$  cells/well

and allowed to grow overnight before the adhesion assay. The labeled cells ( $1 \times 10^5$ /well) were added to the stromal layer and incubated 30 min at 37°C. The 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 X-100 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 =  $([cpm \text{ from bound cells}] - [input \text{ cell associated cpm} - \text{spontaneously released cpm}]) \times 100$ . Antibodies (10  $\mu$ g/ml) were added at the same time as the  $^{51}Cr$ -labeled lymphoma cells.

### Cell Surface Biotinylation and Immunoprecipitation

Cells were surface labeled by a sulfosuccinimidobiotin (Sulfo-NHS-biotin) (Pierce Chemical Co., Rockford, IL) procedure (14). Briefly, after three washes in HBSS, cells were suspended in saline with 0.1 N Hepes, pH 8.0 ( $50 \times 10^6$  cells/ml). Sulfo-NHS-biotin was dissolved in a small amount of saline and added to cell suspensions such that the final concentration of sulfo-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 ( $50 \times 10^6$  cells/ml) in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 0.1% sodium azide. Soybean trypsin inhibitor (10  $\mu$ g/ml), and PMSF (1 mM) were added as protease inhibitors. After centrifugation, the lysates were precleared twice with 50  $\mu$ l of Sepharose 4B conjugated with normal goat serum, and then immunoprecipitated with a mAb bound to goat anti-mouse IgG conjugated Sepharose 4B (Zymed Laboratories, San Francisco, CA) for 2 h at 4°C with rotation. Goat anti-rat IgG Sepharose 4B was used for mAb 13. After washing three times in the lysis buffer, the bound proteins were released by boiling for 5 min in a sample buffer containing 0.125 M Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol without 2-ME. SDS-PAGE was carried out as described (14).

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. 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% HRP-conjugated avidin (Bio-Rad Laboratories, Richmond, CA), 1% BSA, and 0.05% Tween 20. After washing in PBS with 0.05% Tween 20, biotinylated proteins were visualized with PBS containing 0.6 mg/ml 4-chloro-1-naphthol, 0.003% hydrogen peroxide, and 20% methanol.

### Immunofluorescence Analyses

Cells were incubated for 20 min on ice with antibodies. This was followed by two washes with RPMI 1640 supplemented with 3% FCS and 0.1% sodium azide, and incubated with FITC-labeled second reagents. Propidium iodide was added during the second incubation to detect dead cells. Labeled cells were then analyzed on a FAC-Scan (FAC-scan is a registered trademark of Becton Dickinson Co., Mountain View, CA). FITC conjugated goat anti-mouse IgG+A+M (Zymed Laboratories, San Francisco, CA) was used as an second reagent for mouse antibodies, FITC conjugated MAR18.5 (mAb against rat  $\kappa$  chain) for rat mAbs, or FITC-labeled avidin (Organon Teknika Co., West Chester, PA) for biotinylated antibodies. For cross-blocking experiments, cells were preincubated with saturation amounts of unlabeled mouse antibodies for 20 min, washed once, and then stained with either biotinylated antibodies (SG/7, SG/19, SG/17) or a rat antibody. FITC labeled avidin was used for biotinylated mAbs, or FITC-labeled MAR18.5 for a rat mAb.

### Migration Assay

BMS2 cells were plated in a 24-well plate at  $3 \times 10^4$  cells/well, and allowed to grow for 2 d before use in migration assays. Lymphoid cell lines ( $2 \times 10^5$ /well) were added to adherent cell layers, followed by overnight incubation (18 h), and then inspected by inverted phase contrast microscopy (Olympus, Tokyo, Japan), and photographed. For cross section of stromal cell monolayer culture, BMS2 cells were plated in cell culture insert with which cyclopore membrane (9-mm diam and 3- $\mu$ m pore size) (Falcon, Lincoln Park, NJ) was attached. After overnight culture with Nalm-6 cells, cyclopore membrane was removed from plastic, fixed in 6% formaldehyde in 0.1 M phosphate buffer (pH 7.3), embedded in paraffin. The section was stained with Toluidin blue.

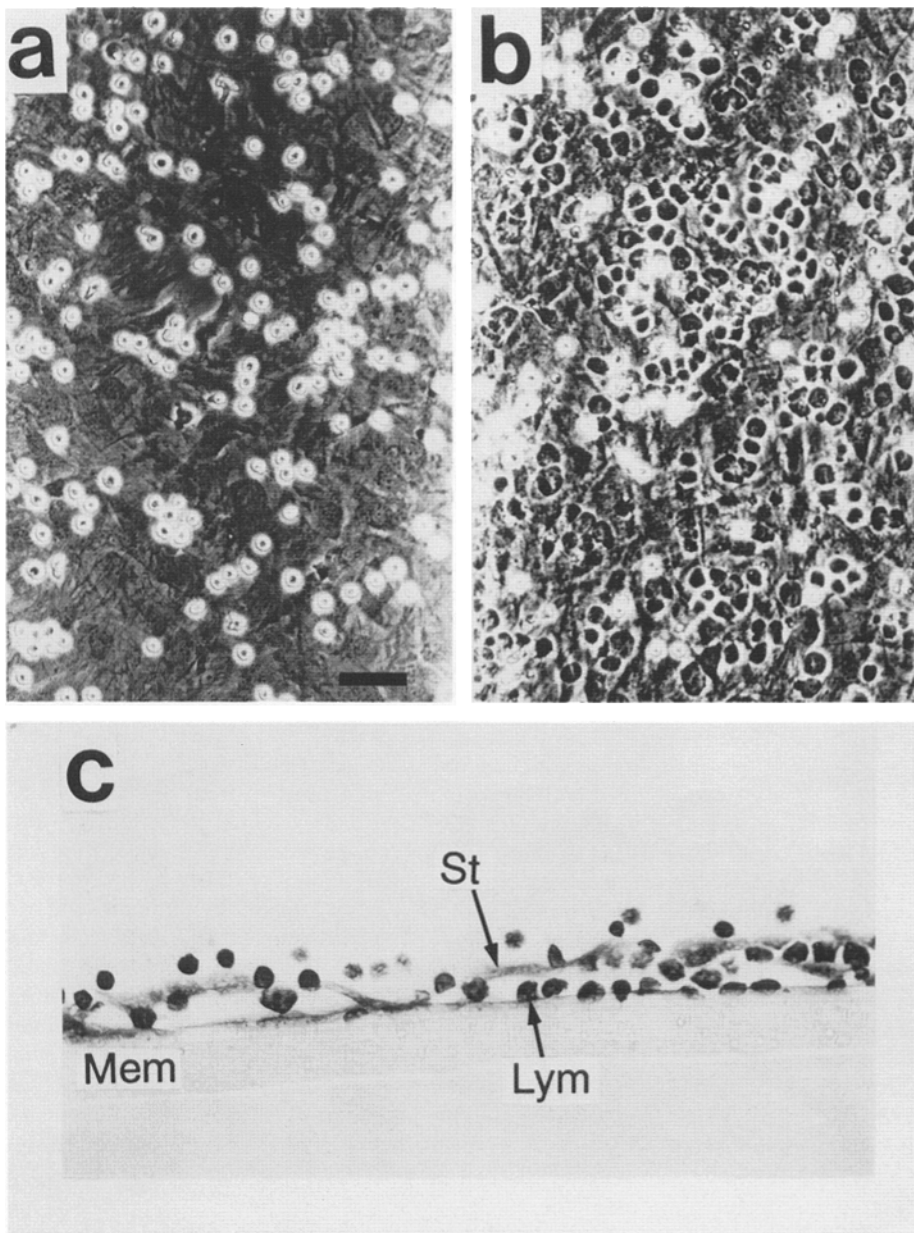
## Results

### *Nalm-6 Cells Migrate beneath Stromal Cells after Adhesion*

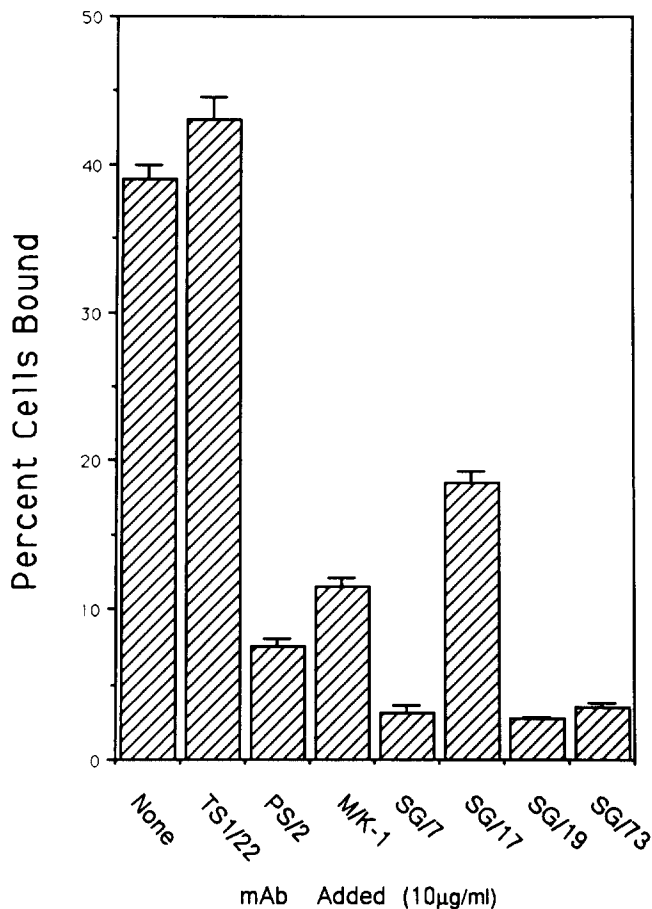
Some cell adhesion pathways are conserved between human and mice such that a human lymphoid cell line can adhere to a murine stromal cell line. This was the case with two human B lineage cell tumors, Ramos and Nalm-6, which readily attached to murine endothelial cell lines (15). This was largely mediated by the VLA-4/VCAM-1 cell adhesion pathway, because it was extensively blocked by both anti-VLA-4 and anti-VCAM-1 mAbs (15). The present study used a murine bone marrow-derived stromal cell clone BMS2, instead of endothelial cell lines. Both Ramos and Nalm-6 cells adhered to BMS2. Nalm-6 cells appeared, however, distinct from Ramos cells by a phase contrast microscopy (Fig. 1). While Ramos cell remained refractile, Nalm-6 cells turned

dark in several hours. This phenomenon has already been described with thymocytes (16), B lymphocyte precursors (31), mature T lymphocytes (8), and neutrophilic granulocytes (25). These reports showed that dark appearance reflected crawling beneath stromal cells or beneath endothelial cells. This was referred to as pseudoemperipolesis (7). The interaction between Nalm-6 cells and BMS2 cell looked like pseudoemperipolesis. To confirm this, cross section was carried out. Indeed, Nalm-6 cells migrated beneath stromal cell (Fig. 1 c). Moreover, dark cells appeared in the same focal plane as stromal cells, while refractile cells out of focus (data not shown), as is shown in neutrophilic granulocytes (25). These results showed that Nalm-6 cells crawled beneath stromal cells after initial adhesion, and turned dark under a phase contrast microscopy. Turning to the dark appearance would reflect the processes following initial adhesion, or migration beneath stromal cells.

Although initial adhesion was significantly blocked with



*Figure 1.* Adhesion of human B lineage cell tumors to murine stromal cells. Ramos cells (a) and Nalm-6 cells (b) were inoculated onto the BMS2 monolayer prepared in 24-well microplates, cultured overnight and photographed. A cross section of BMS2 monolayer was shown in c. BMS2 stromal cell monolayer (St) was formed above filter membrane (Mem), and several Nalm-6 lymphoma cells (Lym) were observed between them. Bar, 50  $\mu$ m.



**Figure 2.** New mAbs to Nalm-6 cells inhibited Ramos cell adhesion to stromal cells. Ramos cells were  $^{51}\text{Cr}$  labeled, and used in the cell adhesion assay as described in the Materials and Methods. mAbs (10  $\mu\text{g}/\text{ml}$ ) to LFA-1 (TS1/22), murine VCAM-1 (M/K-1), murine VLA-4 (PS/2) along with SG series antibodies were added simultaneously with Ramos cells. The values are presented as the means  $\pm$  SD for quadruplicate wells.

antibodies against VLA-4 or VCAM-1 (15), subsequent pseudoemperipolysis was not affected by these reagents (data not shown). Therefore other molecules could be involved in this phenomenon. To understand the molecular mechanism, mAbs were prepared against Nalm-6 cells.

#### **Antibodies Which Block Lymphocyte Adhesion to and Migration Beneath Stromal Cells**

The cell adhesion system using Nalm-6 and BMS2 cells was used for screening, and selection of four mAbs which blocked initial adhesion of Nalm-6 cell to BMS2. The effect of these antibodies on initial adhesion is shown in Fig. 2. Ramos cell adhesion to BMS2 was used instead of Nalm-6 cells, because the results are clearer due to the lack of subsequent migration. Similar results were obtained with Nalm-6 cells as in the previous report (15). This adhesion was mostly blocked by anti-murine VLA-4 antibody PS/2, or anti-VCAM-1 antibody M/K-1. As shown previously (15), PS/2 is crossreactive with human VLA-4. All SG series antibodies except SG/17 completely blocked this VLA-4/VCAM-1-dependent adhesion. VLA-4 is known to bind to fibronectin (5, 29), in addition to VCAM-1. As shown in Fig. 3, Ramos cells

also adhered to the CS-1 portion of fibronectin in a VLA-4 dependent manner. All of the SG series of monoclonal antibodies clearly blocked Ramos cell adhesion to intact fibronectin or a CS-1-IgG fragment (Fig. 3). These results suggested that the SG series antibodies may be reactive with VLA-4 integrin.

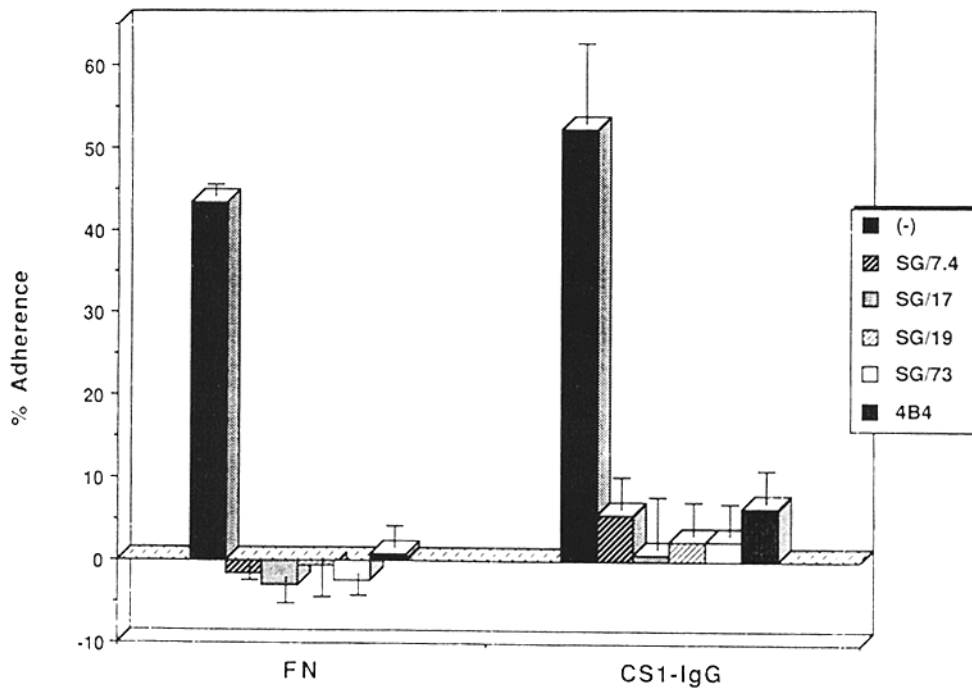
The effects of SG series antibodies on migration beneath stromal cells were then investigated. Nalm-6 cells were inoculated onto BMS2 cells together with antibodies, and incubated overnight. As shown in Fig. 4, both SG/7 and SG/19 completely kept Nalm-6 cells from turning dark, while SG/17 and SG/73 could not. Nalm-6 cells did not migrate in the presence of SG/7 or SG/19. This result discriminated SG/17 and SG/73, from SG/7 and SG/19.

#### **Immunochemical and Flow Cytometric Characterization of Molecules Recognized by SG Antibodies**

For further characterization of the SG series antibodies, immunoprecipitations were carried out. Three antibodies (SG/7, SG/17, and SG/19) precipitated similar sized peptides from Ramos cells. The approximate sizes of these peptides were 110 and 140 kD under nonreducing conditions (Fig. 5 A), and 130 and 150 kD under reducing conditions (data not shown). SG/73 precipitated similar sized molecules as the other three antibodies, but the precipitated bands were not as distinct (data not shown). The epitope it recognizes may be labile on a solubilized molecule. The sizes of these two peptides are compatible with those known for VLA-4 (6). We next analyzed the SG series antibodies by immunofluorescence on K562 cells, which express VLA-5 but not VLA-4 (data not shown). Neither SG/17 nor SG/73 stained K562 cells whereas SG/7 and SG/19 did (data not shown). These two antibodies were used for immunoprecipitation, and results are shown in Fig. 5 B. SG/7 and SG/19 precipitated similar sized molecules from K562 cells. The approximate sizes were 110 and 140 kDa under nonreducing conditions, and 130 kDa when reduced (data not shown). These immunoprecipitation results were very similar to those reported for VLA-5 (1). As expected, SG/17 did not precipitate any molecule from K562 cells (data not shown). SG/17 and SG/73 may be directed against the  $\alpha$  chain of VLA-4 whereas SG/7 and SG/19 may recognize the common  $\beta$  chain of VLA integrins.

Cross-blocking experiments were carried out to confirm the specificities of the SG series antibodies. The binding of PS/2 antibody was clearly blocked by pretreatment of Nalm-6 cells with the SG/73 antibody (data not shown). SG/73 and PS/2 antibodies may therefore recognize a shared or closely related epitope on the  $\alpha$  chain of VLA-4. In contrast, pretreatment with SG/17, SG/7, or SG/19 antibodies did not block the binding of PS/2 to Nalm-6 cells. SG/17 blocked VLA-4-dependent Ramos cell adhesion to VCAM-1 and fibronectin (Figs. 2 and 3), and precipitated similar sized molecules to VLA-4 (Fig. 5 A). Furthermore, SG/17 did not stain K562 cells. These results strongly supported the idea that SG/17 as well as SG/73 are directed against the  $\alpha$  chain of VLA-4. The finding that SG/17 did not block binding of PS/2 to Nalm-6 cells may be due to that the epitope recognized by SG/17 is distinct from that seen by SG/73 or PS/2 antibodies.

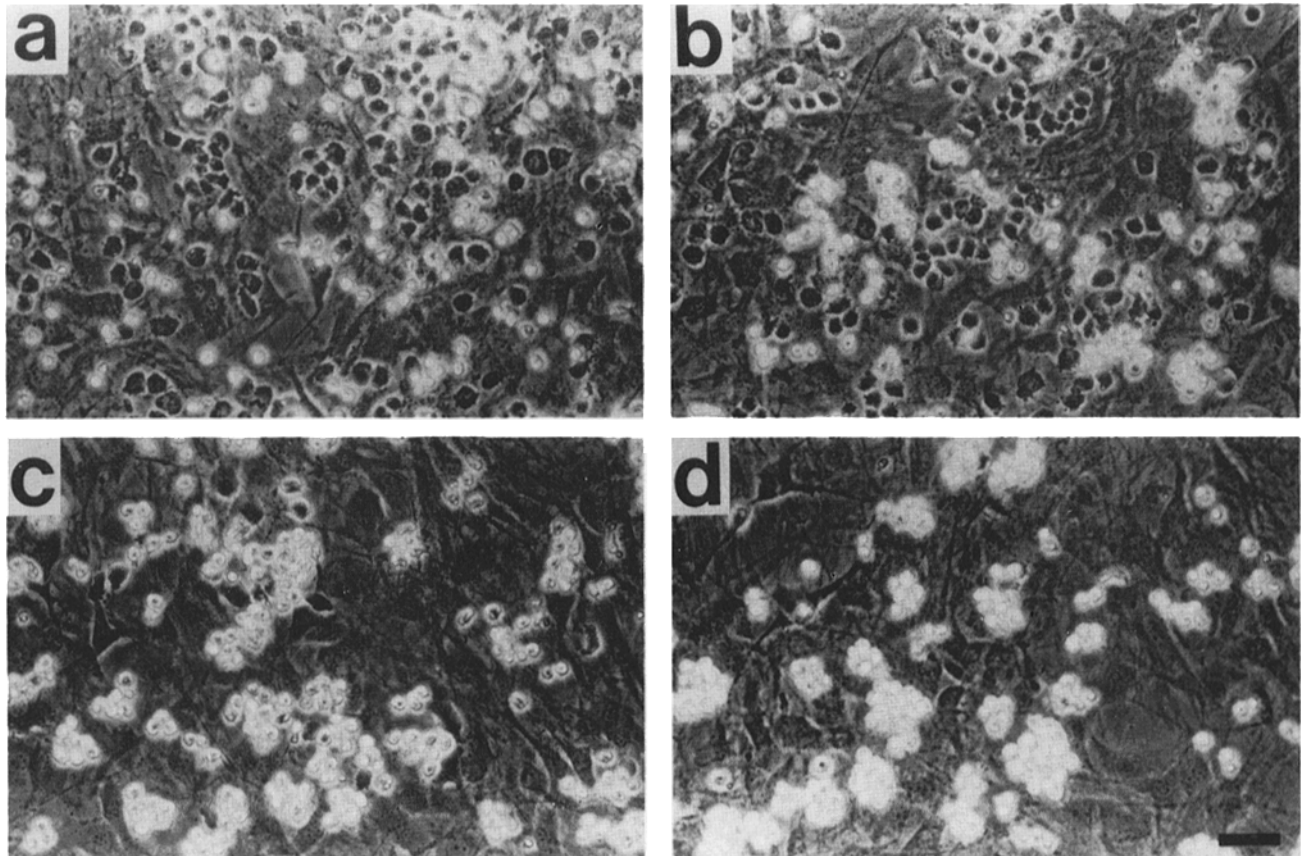
More detailed studies were then done with the SG/7 and



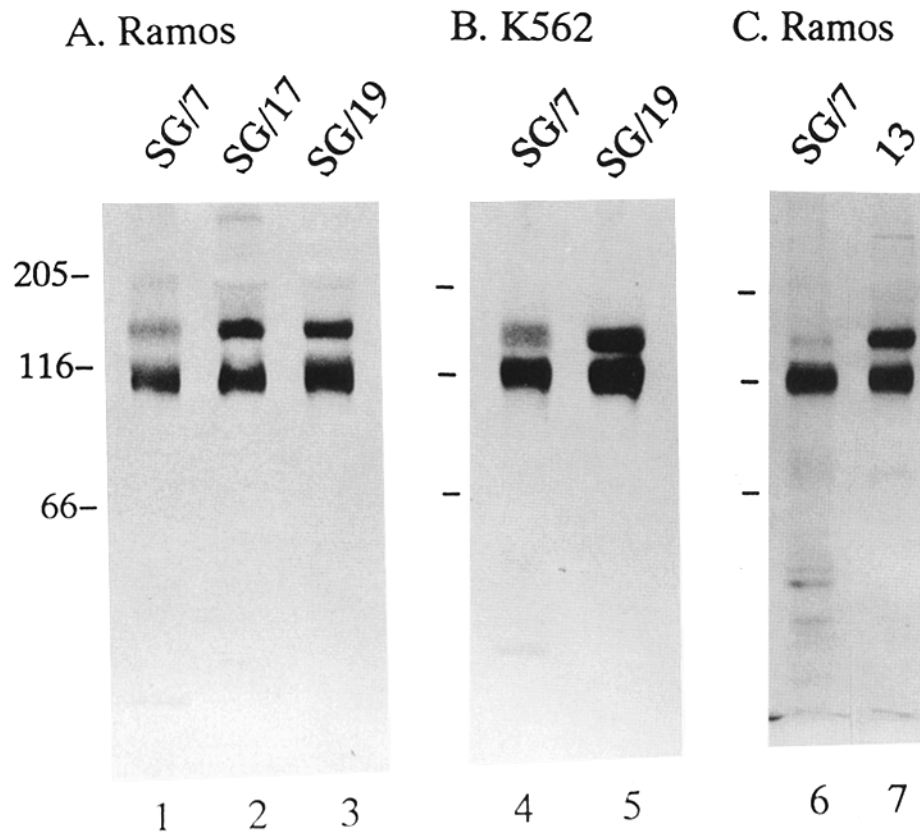
**Figure 3.** SG series antibodies blocked Ramos cells adhesion to fibronectin or CS1-IgG. Ramos cells were used in the cell attachment assay as described in the Materials and Methods. A mAb against  $\beta_1$  chain (4B4) was used along with SG series antibodies. The results are presented as the means  $\pm$  SD for triplicate determinations.

SG/19 antibodies. mAb 13, which was previously shown to be reactive with the  $\beta_1$  chain of VLA integrins (1), was used for immunoprecipitation and epitope analysis. As shown in Fig. 5 C, mAb 13 precipitated similar sized molecules to that

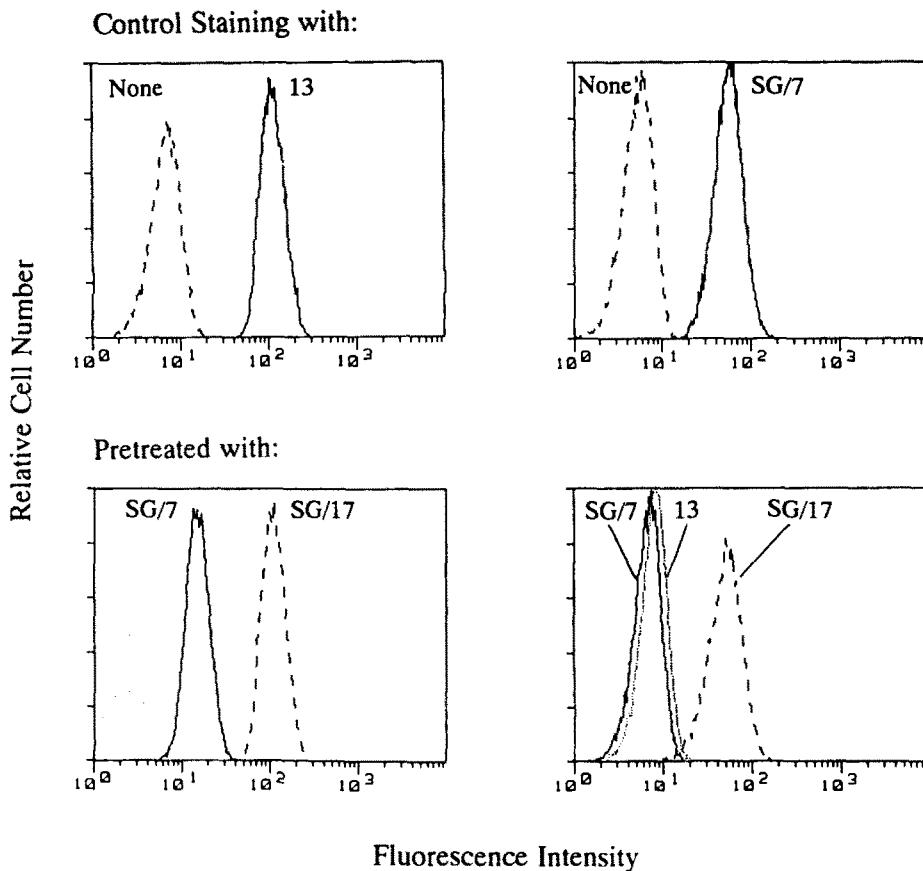
recognized by SG/7. The SG/7 blocked mAb 13's binding to Nalm-6 cells. A reciprocal experiment is the binding of SG/7 to Nalm-6, and this was also blocked by mAb 13 (Fig. 6). SG/19 precipitated similar sized molecules from Ramos or



**Figure 4.** Nalm-6 cell migration beneath stromal cells was blocked by SG/7 or SG/19 but not by SG/73. Nalm-6 cells were added to the BMS2 monolayer with SG series mAbs (10  $\mu$ g/ml). After overnight culture, cells were inspected by phase contrast microscopy. (a) Without antibody; (b) SG/73; (c) SG/7; (d) SG/19. SG/17 had no effect on Nalm-6 migration (data not shown). Bar, 50  $\mu$ m.



**Figure 5.** Immunoprecipitation of surface biotin-labeled materials from Ramos and K562 cells with SG series antibodies and mAb 13. Immunoprecipitation was carried out as described in Materials and Methods. Cell lysates from Ramos (lanes 1-3, 6 and 7) or K562 (lanes 4 and 5) were precleared and applied to beads which were conjugated with either SG/7 (lanes 1, 4, and 6), SG/17 (lane 2), SG/19 (lanes 2 and 4), or 13 (lane 7). Bound proteins were resolved on 8.2% SDS-PAGE under nonreduced conditions.



**Figure 6.** Cross-blocking analysis of SG/7 antibody with mAb 13. Nalm-6 cells were stained with 13 (left) or biotinylated SG/7 (right). Upper panels show control staining without pretreatment. Hatched lines show staining with second reagent only (fluoresceinated mouse anti-rat kappa antibody for mAb 13, and fluoresceinated avidin for SG/7). Lower panels show staining profiles with pretreatment of either SG/7, SG/17, or 13 as indicated.

K562 cells to that seen by SG/7 (Fig. 5, A and B), and cross-blocked 13's binding to Nalm-6 cells (data not shown). Moreover, SG/7 and SG/19 efficiently crossblocked each other's binding (data not shown), indicating that epitopes recognized by them are physically very close. SG/7 and SG/19 must therefore be reactive with the  $\beta_1$  component of VLA integrins. SG/7 however could be discriminated from SG/19 in that SG/7 recognized a divalent cation dependent epitope on the  $\beta_1$  chain of integrins (K. Miyake and M. Kimoto, manuscript in preparation). Moreover the band of  $\alpha$  chain is always more faint than that of  $\beta$  chain (Fig. 5). This antibody might disrupt an association of the two chains. In summary, the newly established mAbs recognize the  $\alpha$  chain (SG/17, SG/19) and  $\beta$  chain (SG/7, SG/19) of the VLA-4 molecule.

#### VLA-4 and VLA-5 Are Required for Nalm-6 Cell Migration beneath Stromal Cells

The crawling of Nalm-6 cells beneath stromal cells was affected by anti- $\beta_1$  antibodies but not by anti- $\alpha_4$  antibodies (Fig. 4). This suggested that VLA integrins other than VLA-4 was involved in this phenomenon, because the  $\beta_1$  chain is shared among VLA integrins (6, 23). Therefore the expression of VLA integrins on Nalm-6 cells was studied. Nalm-6 cells expressed VLA-4, -5, -6 (Fig. 7) but not VLA-1, -2, -3 (data not shown). Ramos cells expressed VLA-4 and VLA-3 but not VLA-1, -2, -5, or -6 (Fig. 7 and data not shown). The expression of VLA-5 and VLA-6 may explain the migration beneath stromal cells because Nalm-6 cells express these integrins and migrate, but Ramos cells do not express these integrins and do not migrate. The blocking effect of antibodies against VLA-4, -5, and -6 were investigated on Nalm-6 cell migration beneath stromal cells. The results are shown in

Fig. 8. Any single antibody against VLA-4, -5, or -6 did not block Nalm-6 cell migration when used alone (Fig. 8, b-d). Only the combined addition of the antibodies against VLA-4 and VLA-5 had a similar blocking effect as antibodies against  $\beta_1$  chain. Other combinations, which included an antibody against VLA-6, had no effect (Fig. 8, f and g). Nalm-6 cells are likely to use both VLA-4 and VLA-5 in concert for attachment to and migration beneath stromal cells. To examine the involvement of VCAM-1, an antibody against VCAM-1 was added alone or together with an antibody against VLA-5, but the migration was not blocked (data not shown).

#### Discussion

Cell surface molecules were implicated in the migration of a pre-B cell line, Nalm-6, beneath a murine stromal cell BMS2. Nalm-6 cells adhered to BMS2 cells and subsequently crawled beneath. Ramos cells, in contrast, did not migrate beneath but remained attached to stromal cell surface. Four mAbs were established, which could block Nalm-6 cell adhesion to BMS2. All these antibodies blocked VLA-4-dependent adhesion of Ramos cells to VCAM-1 and fibronectin. Two of them blocked Nalm-6 cell migration beneath stromal cells. Immunochemical and flow cytometric analyses revealed that these two antibodies were directed against the  $\beta_1$  chain of integrins, and two other antibodies were reactive with the  $\alpha$  chain of VLA-4. The expression of other VLA integrins were studied, because the  $\beta_1$  chain is shared with other VLA integrins (6, 23). Nalm-6 cells, indeed, expressed VLA-5 and VLA-6 as well as VLA-4, whereas Ramos cells expressed VLA-4 but not VLA-5 or VLA-6. The combined treatment with antibodies against VLA-4 and VLA-5 kept Nalm-6 cells from crawling beneath

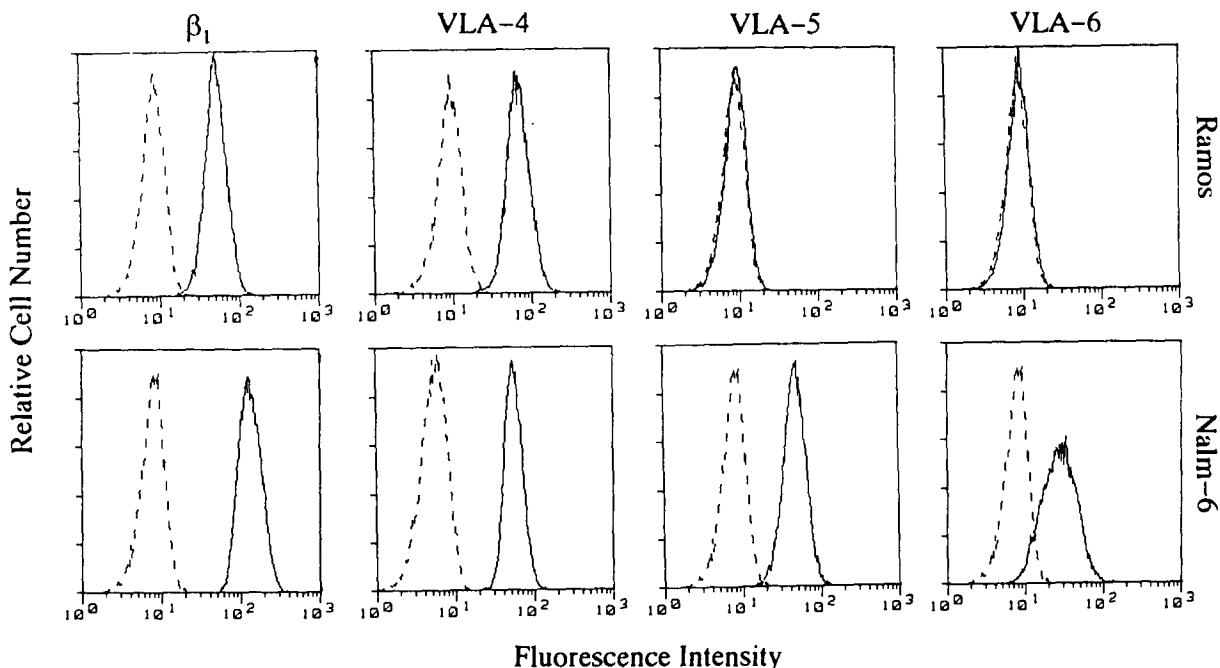
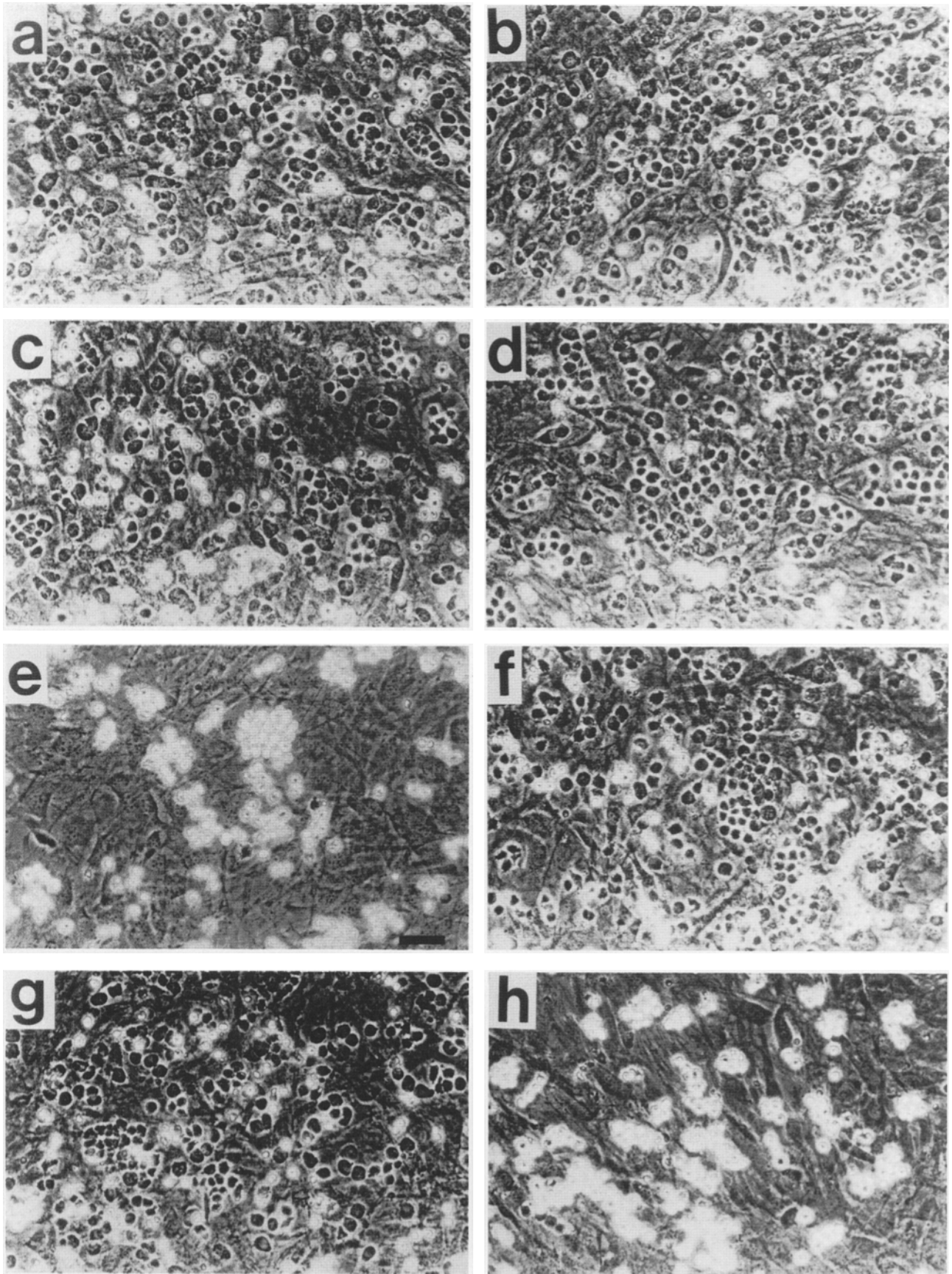


Figure 7. Differential patterns of expression of VLA-4, 5, 6 integrins on Ramos cells and Nalm-6 cells. Ramos cells and Nalm-6 cells were stained with 13 (anti- $\beta_1$  chain), SG/73 (anti-VLA-4), 16 (anti-VLA-5), or GoH3 (anti-VLA-6). Control profiles were stained with second reagent only and shown as hatched line.



**Figure 8.** Combined treatment with antibodies against VLA-4 and VLA-5 blocked Nalm-6 cells migration beneath stromal cells. Nalm-6 cells were inoculated onto BMS2 monolayers with various antibodies (10  $\mu\text{g/ml}$ ). The culture supernatant was used for GoH3. Photographs were taken by phase contrast microscopy after over-night culture. (a) Without antibody; (b) anti-VLA-4 antibody (SG/73); (c) anti-VLA-5



stromal cells. The antibody against VLA-6 even in combination with anti-VLA-4 or VLA-5 did not block the migration. Therefore VLA-4 and VLA-5 are likely to be employed in concert for Nalm-6 migration beneath stromal cells. The absence of VLA-5 on Ramos cells may explain the reason why Ramos cells did not migrate beneath stromal cells.

One of the important questions to be asked is the ligand(s) on stromal cells for VLA-4 and -5 during Nalm-6 cell migration. The migration of Nalm-6 cells was not blocked with an antibody against VCAM-1 alone or together with an antibody against VLA-5. Therefore other molecules are likely to work during Nalm-6 cell migration as a ligand for VLA-4, although the involvement of VCAM-1 during migration is not excluded yet. Fibronectin would be the first candidate, because VLA-4 as well as VLA-5 is known to be a receptor for fibronectin (6, 23). VLA-4 binds to the CS-1 portion of alternatively spliced fibronectin (5, 29), and VLA-5 binds to fibronectin in a RGD peptide dependent manner (6, 23). Many studies reported that progenitors of erythroid or lymphoid lineage attached to fibronectin but mature erythrocytes or lymphocytes did not (2, 4, 13, 19, 28). For example, B220<sup>+</sup> surface  $\mu^-$  cells from bone marrow avidly attached to fibronectin, whereas B220<sup>+</sup> surface  $\mu^+$  cells from bone marrow did not (13). The expression of VLA-4 and/or VLA-5 would explain these changes of affinity for fibronectin as is shown in erythroblastic precursor cells (22) or lymphoid precursor cells (24). The affinity for fibronectin by either VLA-4 or VLA-5 alone may not be enough for migration beneath stromal cells, because addition of antibody against either VLA-4 or VLA-5 could not completely inhibit migration. Higher affinity for fibronectin achieved through VLA-4 and VLA-5 could be needed for Nalm-6 cell migration. It is interesting in this context to note that fibronectin secretion and matrix assembly are polarized to the basolateral surface of endothelial cells (12). This might be the case with stromal cells. Nalm-6 cell migration might be guided by a gradient of fibronectin concentration from the apical to basal surface of stromal cells. Treatment with anti-fibronectin antibodies or peptides might be informative for further studies.

B lineage cells may regulate their migratory properties with maturation by controlling the expression or affinity of cell adhesion molecules. The present study compared human B cell lymphoma and pre-B ALL. The former just adheres to murine stromal cells, and expresses VLA-4 but not VLA-5. The latter, which expresses both VLA-4 and VLA-5, not only adheres but subsequently migrates beneath stromal cells. This difference may reflect maturation states of pre-B and B lymphocytes, because the VLA-5 integrin is reported to be expressed on CD10<sup>+</sup> B lymphocyte precursors in bone marrow but not on mature CD20<sup>+</sup> B lymphocytes in peripheral blood (24). VLA-4 remains expressed on mature B lymphocytes but in lower amount than that on B lymphocyte precursors in bone marrow. Resident B lymphocytes from lymphoid organs including tonsils and peripheral lymph nodes express less VLA-4 than B lymphocytes from peripheral blood (21).

C-kit has been reported to be the receptor for Steel factor and to transduce growth and/or differentiation signal on

hemopoietic progenitor cells (reviewed in 30). The c-kit positive cells in human bone marrow or fetal liver are highly enriched in progenitor cells of the erythroid or myeloid lineage, and express both VLA-4 and VLA-5, whereas c-kit negative cells in bone marrow express VLA-4 but not VLA-5 (18). Neither erythrocytes nor granulocytes in the circulation express VLA integrins (6). VLA integrin expression on erythroid or myeloid lineage cells may change with their maturation sequentially, namely from VLA-4 and -5 positive, through VLA-4 alone positive, and then to no VLA integrins. Steel factor, the ligand for c-kit, has a membrane bound form as well as a soluble form, and the former is suggested to be more important for physiologic roles in vivo than the latter (3). Progenitor cells might need cell adhesion molecules for receiving efficient stimulation from membrane bound growth factors. In this context, VLA-4 and VLA-5 may bring progenitor cells in tight association with stromal cells. As maturation proceeds, VLA-4 and VLA-5 may decrease in their expression on progenitor cells. It will be interesting to determine the signals required for changing affinities and/or expression of these integrins.

While studying physical interactions between lymphocytes and stromal cells, an ideal situation was found to discriminate between adhesion alone versus adhesion of lymphocytes followed by transmigration beneath stromal cells in culture. One human pre-B ALL cell line, Nalm-6, attached to and crawled beneath a monolayer of stromal cells whereas a more mature B cell lymphoma, Ramos, only attached to the upper surface. mAbs were prepared which blocked these events. Immunochemical and flow cytometric analyses were performed to determine which adhesion molecules were expressed and which ones were responsible for adhesion and transmigration. Initial adhesion to the stromal cell surface was largely dependent on VLA-4 expressed by the lymphoid cells and its recognition of VCAM-1 on the stromal cells. However, this receptor-ligand pair was not sufficient for subsequent migration beneath stromal cells. The latter phenomenon was dependent on the concerted action of VLA-4 and VLA-5 integrins. These findings may have implications for cell-cell interactions necessary for lymphocyte formation in bone marrow and directed migration of lymphocytes in other tissues.

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antibody (mAb 16); (d) anti-VLA-6 antibody (GoH3); (e) anti-VLA-4 and -5 (SG/73 + mAb16); (f) anti-VLA-4 and -6, (SG/73 + GoH3); (g) anti-VLA-5 and -6 (mAb 16 + GoH3); (h) anti-VLA-4, -5, and -6 (SG/73 + mAb 16 + GoH3). Bar, 50  $\mu$ m.

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