Adhesion-Dependent Survival of Normal and Leukemic Human B Lymphoblasts on Bone Marrow Stromal Cells

By Atsushi Manabe, K. Gopal Murti, Elaine Coustan-Smith, Masa-aki Kumagai, Frederick G. Behm, Susana C. Raimondi, and Dario Campana

We investigated the requirement for intimate contact between bone marrow stroma and B lymphoblasts from normal donors and children with leukemia. By scanning electron microscopy, both normal and leukemic cells seeded onto stroma were surrounded by folds of stromal cells or were linked to the stroma by fine tendrils and uropods. Separation of normal B progenitors from stroma by use of microporous membranes led to significantly lower cell recoveries compared with results when contact was unimpeaded. For instance, 22.5% ± 1.8% (mean ± SEM) of CD19+ , CD34+ cells (most immature subset) were recovered after a 7-day culture directly on stroma, compared with 5.2% ± 0.7% after growth on membranes (P < .001 by Student’s t test). In 6 of 11 cases of B-lineage acute lymphoblastic leukemia, separation of progenitors from stroma resulted in apoptosis and a greater than 60% reduction in cell recovery. In the remaining 5 cases, however, this effect was much less pronounced, with reductions in cell recoveries ranging from 48.5% to less than 1% (median, 39.0%) of control values. Inhibition of very late antigen-4, a surface molecule critical for adhesion of B lymphoblasts to stroma, was associated with a greater loss of normal CD34+ B progenitors compared with that for equivalent leukemic cells. These results establish direct contact with stroma as a survival requirement of normal B lymphoblasts and show marked heterogeneity in stromal dependency among B-lineage leukemic cells.

THE HOMEOSTATIC CONTROL of human B-lineage lymphopoiesis requires close coordination of survival, proliferation, and differentiation signals. The critical stimuli have not yet been completely identified; although in late fetal and postnatal life, they are known to be produced by the bone marrow (BM) microenvironment. In the absence of BM stromal support, immature B cells from acute lymphoblastic leukemia (ALL) samples rapidly die as a result of apoptosis, much like cytokine-dependent cell lines cultured in the absence of essential growth factors. The ability to generate BM-derived stromal layers in vitro and to establish favorable culture conditions for human immature B cells

© 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Cells. BM samples were taken, with informed consent accord- ing to the Declaration of Helsinki and approval from the Institutional Review Board, from 8 healthy BM transplant donors, aged 2 to 13 years (median, 9 years), and from 11 patients with newly diagnosed B-lineage ALL, aged 7 months to 18 years (median, 7 years; see Table 1). In each case of ALL, greater than 80% of the blasts were positive for CD19, CD22, class II antigens, and terminal deoxynucleotidyl transferase. Mononuclear cells were collected after centrifugation on a Lymphoprep density gradient (Nycomed, Oslo, Norway), washed three times in phosphate-buffered saline (PBS) and once in AIM-V medium (GIBCO, Grand Island, NY; Cat. No. 320-2055A). Fresh leukemic cells and normal BM lymphoblasts were placed in culture within 4 hours of collection. In some experiments, cryopreserved samples of B-lineage ALL were used immediately after thawing. Comparative experiments showed no differences in B-lymphoblast recovery when fresh or cryopre- served samples were cultured on stromal layers (Manabe et al. and A. Manabe and D. Campana, unpublished observations, March 1992). Normal CD19+ BM cells were obtained by use of CD19-immunomagnetic beads (Dynal, Oslo, Norway). Cells were detached from the beads using a goat antiserum to mouse Fab fragments (DETAChAHEAD; Dynal). These procedures, performed according to the manufacturer’s instructions, yielded cell populations containing 91% to 98% CD19+ cells, as assessed by flow cy-
tometry (see below). The cells' viability consistently exceeded 90% by trypan-blue dye exclusion. No significant differences in survival of B-cell progenitors were seen when cultures of unseparated and CD19-enriched normal BM B cells were compared, or in similar experiments performed with ALL cells (data not shown), indicating that CD19-immunomagnetic separation has no harmful effects on immature B cells.

To obtain BM stromal cells, we collected mononuclear cells from 6 normal BM donors, aged 7 to 31 (median, 17) years. The cells were separated as described above and washed three times in RPMI-1640 (Whittaker Bioproducts Inc, Walkersville, MD; Cat. No. 14-901A). Stromal layers were prepared in 24- and 96-well plates (Costar, Cambridge, MA; Cat. No. 3424 and 3596), as described earlier. The only modification in the published procedure was the use of RPMI-1640 with 10% fetal calf serum (FCS). Cultures were incubated at 37°C, 5% CO2, and 90% humidity, for 7 days. Culture supernatants were harvested and used without modification or were concentrated sixfold after centrifugation in 3-kD cut-off microconcentrators (Amicon, Beverly, MA; Cat. No. 4202).

Cell culture studies. Before each experiment, we removed the media from cultures of BM stroma and washed the adherent cells seven times with AIM-V medium, consisting of Dulbecco's modified Eagle's medium, Ham Nutrient Mixture F12, HEPES buffer, human serum albumin, human transferrin, recombinant human insulin, L-glutamine, and antibiotics. No cytokine or animal serum was added to the medium. Leukemic and normal CD19+ cells were resuspended in AIM-V medium at a final concentration of 1.5 x 10^6/mL. Then, 200 μL of the suspension was placed in the wells of a 96-well flat-bottomed tissue culture plate or seeded onto BM stromal cells in identical plates. In parallel experiments, cells were seeded onto 6.5-mm Transwell diffusion chambers (Costar, Cat. No. 3413) placed into stroma-coated 24-well plates. These diffusion chambers have a microporous membrane (pore size = 0.4 μm) at the bottom with a surface area similar to that of the wells in the 96-well plates (0.33 x 0.32 cm²). All cell cultures were incubated at 37°C in 5% CO2 with 90% humidity. To inhibit VLA-4-mediated adhesion, we used a CD9 antibody to the integrin β chain (4B4; Coulter, Hialeah, FL; Cat. No. 6603131) and a CD49d antibody to the α chain (HP2.1; AMAC, Westbrook, ME; Cat. No. 0764). In these experiments, an isotype-matched unrelated antibody (Becton Dickinson, San José, CA) was used as a control. All reagents were diazylated against PBS and passed through a 0.22-μm filter; they were used at a final concentration of 1 to 5 μg/mL. At the termination of cultures, cells were harvested by vigorous pipetting. Microscopic examination of the plates ensured that all cells in the wells (ie, adherent and nonadherent) were recovered. Cells were suspended in PBS without Ca++ and Mg++ and passed through a 19-gauge needle to disrupt clumps. All experiments were performed in duplicate.

Scanning electron microscopy. Stromal cells were prepared for scanning electron microscopy (SEM) on Falcon Cyclopore membrane cell culture inserts (Becton Dickinson, Lincoln Park, NJ; Cat No. 3090) that were placed in Falcon multiwell tissue culture dishes containing RPMI plus additives, as described above. After seven washes in AIM-V medium, the inserts were transferred to wells containing AIM-V and seeded with immature B cells. The SEM studies included normal CD19+ BM cells, purified as above, and depleted of mature lymphocytes by treating with a CD37 monoclonal antibody (MoAb; RFB7; Sera-Lab, Crawley Down, Sussex, UK; Cat. No. MAS264b)9 and rabbit complement, as well as leukemic lymphoblasts from two cases of B-lineage ALL. After 3 to 5 days of culture, the inserts with the membrane bearing the cultures were washed with PBS and immersed in 2.5% glutaraldehyde in PBS for 1 hour. The membrane was rinsed with PBS containing 7% sucrose, postfixed in 2% glacial acetic acid for 1 hour, rinsed in water, passed through ethanol gradients (30%, 70%, 85%, 95%, and 100%) for 5 minutes each, and placed in fresh 100% ethanol for an additional 5 minutes. The preparations were dried by the critical point method in a Autosamdi-840 (Tousimis Research Corp, Rockville, MD), mounted on a specimen holder, and coated with gold in a sputter-coater (Denton Vacuum Inc, Cherry Hill, NJ). Cells were examined in the scanning node of a 1200 EXII TEMSCAN electron microscope (Japanese Electron Optics Limited Corp, Tokyo, Japan).

DNA fragmentation assay and flow cytometry. Cells were examined for evidence of apoptosis by DNA gel electrophoresis, as described in Manabe et al.3 In this assay, fragmented and intact DNA are separated by centrifugation. Electrophoresis of fragmented and intact DNA was always performed in parallel to ensure equal loading. Viable cells were enumerated by flow cytometry.3 Briefly, cells harvested after different times of culture were washed three times in PBS containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA). To assess cell survival, we resuspended cells representing all conditions of a given experiment in identical amounts of PBSA. Aliquots of the suspensions were placed in Falcon tubes (Becton Dickinson, Lincoln Park, NJ; Cat No. 2052) and incubated with CD19 (Leu 12) MoAb conjugated to fluorescein isothiocyanate (Leu 12 FITC; Becton Dickinson) or with a combination of CD19 conjugated to peridinin chlorophyll protein (Leu12 PerCP; Becton Dickinson), CD34 conjugated to phycoerythrin (HPCA-2 PE; Becton Dickinson) and a mixture of goat anti-human IgG + A light chain antiserum conjugated to FITC (Southern Biotechnology Associates, Birmingham, AL). Isotype-matched nonreactive antibodies were used as a control. After two washes in PBSA, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a FACScan flow cytometer with Lysis II software (Becton Dickinson). In each analysis, we designed gates around the area of the light-scatter dot plot where the vast majority of cells were found at the beginning of the cultures. Then, the cultured cells with predetermined light-scattering properties were enumerated by counting the number of events passing through the gate a period of time (eg, 30 seconds). The result was corrected for the distribution of cells expressing different immunophenotypes.3,12

Percentage of cell survival was calculated with the formula: (no. of cells in test sample) x 100/(no. of cells in control sample). Cell sorting experiments have indicated that apoptotic DNA fragmentation is confined to cells outside the gates drawn for viable cell counting (Swat et al9 and A. Manabe and D. Campana, unpublished observations, February 1992). Cell cycle analysis was performed by labeling cells prestained with CD19 FITC (Becton Dickinson) or CD10 FITC (Dako, Carpinteria, CA) with propidium iodide and determining their DNA content by flow cytometry (FACSscan with CellFit software; Becton Dickinson) as described in Campana et al.22

RESULTS

SEM of progenitor cell-stroma interaction. By SEM, stromal layers consisted of large elongated cells that formed a contiguous three-dimensional matrix with few interstitial spaces (Fig 1A). Large adipocytes were recognizable by their spherical lipid droplets. The stromal layer topography was interrupted by a few areas that contained large blanket cells (Fig 1B) with thin cytoplasmic folds overlapping clusters of lymphoid cells, giving a "cobblestone" appearance when observed by light microscopy. Macrophages were also evident in these regions.

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
Both normal and leukemic (cases No. 1 and 11; Table 1) immature B cells seeded onto stromal layers were readily recognized by their size (average diameter, 6 μm), morphology, and abundance. Some blast cells were spherical, whereas others had an ameboid shape, suggesting movement on the stromal cells. Lymphoblasts were linked to stromal elements by fine tendrils (filopods or microspikes; Fig 1C) or uropods (Fig 1D). Normal lymphoblasts were indistinguishable from leukemic cells by gross appearance (Fig 1E) and relation to stromal layers (Fig 1F and G). These results show intimate B progenitor-stroma contact and suggest that leukemic B lymphoblasts interact with BM stromal layers as avidly as do normal blasts.

Requirement for direct contact with stromal layers. We previously showed that stromal layers support the survival of B lymphoblasts in serum-free medium.$^{1,2}$ Without stromal layers, most of the cells died by apoptosis within 48 to 72 hours of culture. To investigate the necessity for direct contact with stromal layers, we used triple-color immunofluorescence and flow cytometry to quantify cell survival at different stages of normal B-cell differentiation. Cells were stained with antibodies to CD19, CD34, and surface Ig (sIg) simultaneously, permitting recognition of relatively immature (CD34⁺) and mature (sIg⁺) subpopulations as well as a third group of cells (CD19⁺, CD34⁻, and sIg⁻), most likely at an intermediate level of differentiation (Fig 2). In all sam-
Table 1. Patient Characteristics at Diagnosis

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Age (yr)</th>
<th>WBC (10⁹/L)</th>
<th>Percentage S-Phase</th>
<th>Immunophenotype†</th>
<th>CD34</th>
<th>CD10</th>
<th>CD20</th>
<th>Cases</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>264</td>
<td>3.5</td>
<td></td>
<td>95</td>
<td>96</td>
<td>53</td>
<td>&lt;1</td>
<td>46,XY,t(8;22)(q34;11)</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>17</td>
<td>NA</td>
<td></td>
<td>89</td>
<td>93</td>
<td>3</td>
<td>&lt;1</td>
<td>47,XY,del(12)(p11)</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>14</td>
<td>1.4</td>
<td></td>
<td>89</td>
<td>95</td>
<td>1</td>
<td>&lt;1</td>
<td>45,X,-X,del(12)(p11)</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>9</td>
<td>4.8</td>
<td></td>
<td>78</td>
<td>91</td>
<td>5</td>
<td>&lt;1</td>
<td>46,XY,del(12)(p11)</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>75</td>
<td>3.1</td>
<td></td>
<td>90</td>
<td>93</td>
<td>3</td>
<td>&lt;1</td>
<td>46,XX,del(11)(q23)</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>587</td>
<td>3.0</td>
<td></td>
<td>90</td>
<td>95</td>
<td>67</td>
<td>&lt;1</td>
<td>46,XY,t(9;22)(q34;11)</td>
</tr>
<tr>
<td>7</td>
<td>&lt;1</td>
<td>68</td>
<td>2.0</td>
<td></td>
<td>15</td>
<td>8</td>
<td>4</td>
<td>40</td>
<td>46,XY,t(4;11)(q21;11)</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>367</td>
<td>9.9</td>
<td></td>
<td>10</td>
<td>21</td>
<td>&lt;1</td>
<td>67</td>
<td>46,XY,t(11;19)(p23;13)</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>1</td>
<td>6.5</td>
<td></td>
<td>87</td>
<td>87</td>
<td>6</td>
<td>&lt;1</td>
<td>+6,XX,+X,del(1)(p11),+4,+6,+8,+</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>86</td>
<td>3.3</td>
<td></td>
<td>&lt;1</td>
<td>95</td>
<td>42</td>
<td>97</td>
<td>+10,+14,+17,+18,+21,+mar</td>
</tr>
<tr>
<td>11</td>
<td>&lt;1</td>
<td>96</td>
<td>8.5</td>
<td></td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>82</td>
<td>46,XY,t(4;11)(q21;12)</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.
† All patients but no. 4 were subjects of previous studies (Manabe et al. and Campana et al.).

In each case, greater than 80% of cells were class II+, CD19*, CD22*, and TdT+. The data are percentages of mononucleated cells expressing the indicated antigen.

The total number of normal CD19+ cells recovered after 7 days of culture on stromal layers was 42.3% ± 4.2% (mean ± SEM) of those originally seeded. Culturing purified CD19+ cells on microporous membranes placed on BM stromal layers led to significant decreases in cells' recovery. For instance, in the CD19+, CD34+, slg- subpopulation (most immature), the mean ± SEM recovery of cells grown directly on stroma was 22.5% ± 1.8%, compared with 5.2% ± 0.7% when progenitors were separated from stroma by use of a diffusion chamber (P < .001 by Student's t-test; Table 2). Among cells with a CD19+, CD34-, slg- phenotype (intermediate development), the results were 46.4% ± 7.1% versus 18.9% ± 3.7% (P = .006). To determine whether the reduction in cell numbers seen in diffusion chambers was caused by suppression of proliferative activity, we measured the size of viable cells recovered after 7 days of culture. Mean ± SEM forward scatter value of CD19+, slg- progenitor cells cultured in contact with stroma was 336 ± 22 versus 371 ± 32 of those cultured in diffusion chambers (Fig 3). Thus, there was no decrease in the proportions of large, presumably proliferating, cells in the diffusion chambers, suggesting that the decline in cell numbers under these culture conditions was mainly caused by a higher rate of cell death.

The ability of leukemic cells that are phenotypically equivalent to normal B-cell progenitors to survive free from direct contact with stromal layers was assessed in 11 cases of B-lineage ALL. After 7 days of culture on stromal layers, the percentage of recovered CD19+ cells ranged from 53.8% to 178.0% (median, 93.7%; Table 3), confirming our previous observations of a prolonged survival of ALL blasts on stromal layers as compared with that of their normal counterparts. Imposing a microporous membrane between the

---

Fig 2. Phenotypic characterization of normal B-cell differentiation after 7 days of culture on stromal layers. Normal BM CD19+ cells were purified and cultured on stromal layers. Cells were labeled with CD19 PerCP, anti-lg FITC, and CD34 PE (see Materials and Methods). Triple-color labeled samples were analyzed by flow cytometry. After 7 days of culture the mean ± SEM percentage of CD19 expression among nonapoptotic cells was 91.8% ± 1.8% (left panel). Some of these were slg+ (most mature; middle panel, quadrant 2), whereas others were CD34+ (most immature; right panel, quadrant 2). No CD34+ , slg+ cells were seen (data not shown). Quadrants were drawn to enclose all cells labeled with fluorochrome-conjugated isotype-matched nonreactive antibodies within quadrant 3 (bottom left corner of dot plots).
Table 2. Percentage of Normal BM B-Cell Subsets Recovered From Direct or Indirect Culture on Stromal Layers

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>CD19⁺, CD34⁺, slg⁻</th>
<th>CD19⁺, CD34⁺, slg⁺</th>
<th>CD19⁺, CD34⁻, slg⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contact</td>
<td>No Contact</td>
<td>Contact</td>
</tr>
<tr>
<td>1</td>
<td>23.7</td>
<td>6.2</td>
<td>28.8</td>
</tr>
<tr>
<td>2</td>
<td>18.5</td>
<td>3.6</td>
<td>79.6</td>
</tr>
<tr>
<td>3</td>
<td>23.8</td>
<td>8.2</td>
<td>48.4</td>
</tr>
<tr>
<td>4</td>
<td>30.1</td>
<td>5.3</td>
<td>38.7</td>
</tr>
<tr>
<td>5</td>
<td>20.6</td>
<td>3.8</td>
<td>41.7</td>
</tr>
<tr>
<td>6</td>
<td>18.4</td>
<td>4.4</td>
<td>41.4</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>22.5 ± 1.8</td>
<td>5.2 ± 0.7*</td>
<td>46.4 ± 7.1</td>
</tr>
</tbody>
</table>

Values refer to nonapoptotic cells recovered after 7 days of culture as compared with those originally seeded. Different subsets were identified by triple-color flow cytometry (see the Materials and Methods and Fig 3). At the beginning of the cultures, 18.8% ± 3.1% (mean ± SEM) of CD19⁺ cells were CD34⁺, slg⁻, whereas 53.9% ± 4.1% and 25.5% ± 3.2% were CD34⁻, slg⁻ and CD34⁻, slg⁺, respectively. Cultures termed "No Contact" were performed in diffusion chambers inserted in wells that contained confluent stromal layers (see the Materials and Methods).

* P < .001 by t-test.
† P = .006.
‡ P = .017.

cells and stromal layers resulted in a greater than 60% reduction in cell recovery in 6 cases, analogous to the results with normal progenitors. The increased loss of cells in diffusion chambers was attributed to apoptosis, based on results of a DNA fragmentation assay performed after 48 hours of culture (Fig 4). In the remaining 5 cases, culture in diffusion chambers produced appreciably lower reductions in cell recovery, from 48.5% to less than 1% (median, 39.0%; Table 3). The number of cells remaining in diffusion chambers did not correlate with the presenting white blood cell count, the percentage of cells in S phase, the age, or the karyotype (Table 1). These findings indicate that the lymphoblasts in some cases of ALL have a lower requirement for direct stroma contact than do their normal counterparts.

Continued survival of leukemic B lymphoblasts in diffusion chambers suggests that stromal cells secrete survival factors that diffuse through microporous membranes. We tested this possibility with "stroma-conditioned" culture supernatant, prepared from stromal layers that had been incubated in AIM-V medium for 7 days. Daily addition of 12.5%, sixfold-concentrated stroma-conditioned medium (a quantity that yielded the best results in titration experiments; data not shown) resulted in cell recoveries that were 85.6% (case no. 1) and 48.7% (case no. 5) of those achieved in direct contact with stroma, as compared with 0.8% and 27.6% when the conditioned medium was added only once at the beginning of culture, and 0.7% and 9.8% in tests performed without stroma conditioning. In the latter tests, the values are the means of duplicate experiments and refer to nonapoptotic CD19⁺ leukemic cells recovered after 7 days of culture, compared with those originally seeded.

* Diffusion chambers were inserted in wells containing confluent stromal layers (see the Materials and Methods). Numbers in parentheses are the percentages of cells recovered in diffusion chambers relative to the results of parallel cultures in which contact with stroma was allowed.

Table 3. Percentage of B-Lineage ALL Cells Recovered From Direct or Indirect Culture on Stromal Layers

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Contact</th>
<th>No Contact*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>178.0</td>
<td>181.5 (+2.0)</td>
</tr>
<tr>
<td>2</td>
<td>57.7</td>
<td>36.8 (-27.2)</td>
</tr>
<tr>
<td>3</td>
<td>91.0</td>
<td>55.5 (-39.0)</td>
</tr>
<tr>
<td>4</td>
<td>93.7</td>
<td>55.0 (-41.3)</td>
</tr>
<tr>
<td>5</td>
<td>61.4</td>
<td>31.6 (-48.5)</td>
</tr>
<tr>
<td>6</td>
<td>102.5</td>
<td>38.4 (-61.6)</td>
</tr>
<tr>
<td>7</td>
<td>53.8</td>
<td>18.1 (-66.3)</td>
</tr>
<tr>
<td>8</td>
<td>127.7</td>
<td>33.5 (-73.9)</td>
</tr>
<tr>
<td>9</td>
<td>106.0</td>
<td>21.2 (-80.0)</td>
</tr>
<tr>
<td>10</td>
<td>143.0</td>
<td>21.0 (-85.3)</td>
</tr>
<tr>
<td>11</td>
<td>70.2</td>
<td>8.9 (-87.3)</td>
</tr>
</tbody>
</table>

Values are the means of duplicate experiments and refer to nonapoptotic CD19⁺ leukemic cells recovered after 7 days of culture, compared with those originally seeded.

* Diffusion chambers were inserted in wells containing confluent stromal layers (see the Materials and Methods). Numbers in parentheses are the percentages of cells recovered in diffusion chambers relative to the results of parallel cultures in which contact with stroma was allowed.

Fig 3. Size analysis of CD19⁺, slg⁻ normal immature B cells (sample no. 4; Table 2) after 7 days of culture with (top) and without (bottom) contact with stromal layers. FSC values were slightly increased in cultures performed in diffusion chambers, suggesting persistence of proliferative activity (see Results).
ADHESION-DEPENDENT SURVIVAL OF B LYMPHOBLASTS

Fig 4. B lymphoblasts die by apoptosis after 48 hours of culture when separated from stroma. Shown is gel electrophoresis of fragmented DNA, separated from intact DNA by centrifugation (see Materials and Methods). When B-lineage ALL cells were cultured in the absence of stroma, DNA fragmentation rapidly occurred (second lane). Signs of apoptosis were also seen when cells were cultured in diffusion chambers, which allowed free flow of stroma-produced growth factors (third lane), but they were abolished by culture in direct contact with stroma layers (fourth lane). The first lane contains a molecular size marker (123 bp; Gibco BRL, Gaithersburg, MD).

occurrence of cell death by apoptosis was indicated by morphologic changes (eg, nuclear fragmentation) and by characteristic shifts in the cells’ light scattering properties, consisting of lower forward scatter and higher side scatter (data not shown; see Manabe et al and Campana et al).

Effects of anti-VLA-4 antibodies on progenitor cell survival. The natural ligands of VLA-4 are fibronectin and VCAM-1, found in abundance in the extracellular matrix and cell membranes of stromal components. Therefore, we investigated whether addition of anti-VLA-4 antibodies (CD29 and CD49d, directed against the VLA-4β and VLA-4α, respectively) to cultures of normal B-cell progenitors would affect the survival of such cells on stromal layers. Both reagents clearly reduced the number of immature CD34+ B cells. In four separate experiments, the mean ± SEM cell recovery in the presence of CD29 was 34.5% ± 8.6% of the control values (no antibody; Fig 5), whereas addition of isotype-matched control antibody in parallel cultures did not affect cell recovery. By contrast, the number of more mature slg− cells recovered after CD29 addition was 95.1% ± 7.6% of the control (Fig 5). Among cells at an intermediate stage of differentiation (CD19+, CD34−, slg−), the relative cell recovery was 74.4% ± 7.8%. Addition of CD49d in three experiments yielded the following cell recoveries relative to control values: 45.1% ± 10.6% for CD34+ cells; 69.6% ± 10.3% for CD19+, CD34−, slg− cells; and 81.9% ± 4.3% for slg+ lymphocytes (Fig 5). Thus, the effects of anti-VLA-4 were particularly important for the most immature normal B-cell precursors.

We tested whether the inhibitory effects of anti-VLA-4 antibodies on immature B cells could be caused by suppression of their proliferative activity. Cell size of CD19+, slg− progenitors was identical in cultures with and without CD29 (forward scatter [FSC] = 315 ± 11). In two experiments, DNA content analysis of CD10+ immature B cells was examined after 2 days of cultures. Among CD10+ cells with normal DNA content (DNA index, ≥1.0), the cumulative percentage of cells in S, G2, and M phases in the control samples was 11.1% (S = 9.9%; G2/M = 1.2%) and 14.4% (S = 11.5%; G2/M = 2.9%). In the parallel cultures with CD29, the results were 12.3% (S = 10.6%; G2/M = 1.7%) and 16.3% (S = 13.7%; G2/M = 2.6%). CD10+ cells with a DNA index less than 1.0, probably undergoing apoptosis (Darzynkiewicz et al), were 15% and 41% in the control samples. In cultures containing CD29, the proportion of these cells was higher (27% and 59%), suggesting increased cell death rate rather than decreased proliferation as the cause of the diminished cell numbers.

The effects of CD29 antibody on cultures of leukemic B lymphoblasts were heterogeneous. In 5 of 10 cases, cell recovery was not reduced after 7 days of culture, whereas, in the remaining cases, it ranged from 58.8% to 86.7% (median, 80.9%) of control values (Fig 5). Direct comparison of mean ± SEM cell recoveries in the presence of anti-VLA-4β (relative to those of parallel control cultures) among phenotypically equivalent normal and leukemic CD34+ B lymphoblasts (cases no. 1 to 6, >75% CD34+ cells; see Table I) showed striking differences. 32.9% ± 11.9% (normal) versus 97.0% ± 2.9% (leukemia), P < .001. Thus, despite considerable variability among cases, leukemic lymphoblasts appear less dependent on VLA-4–mediated adhesion than do their normal counterparts. In each of the 10 cases of ALL tested, greater than 80% of the blasts were CD29+; hence, the level of expression of this molecule was not a factor in the effects of the antibody. In confirmatory experiments, the effects of CD49d were tested in 5 ALL cases; the results paralleled those seen with CD29 (Fig 5). The observed decrease in cell numbers was probably caused by impaired survival, rather than by decreased proliferative activity, because the addition of CD29 or CD49d did not alter the percentages of cells in various phases of the cell cycle in tests performed after 1, 3, and 5 days of culture (Fig 6).

DISCUSSION

In this study, we present evidence that adhesion to stromal layers is critical for the survival of normal immature B cells and for leukemic lymphoblasts in a proportion of cases of ALL. SEM showed a tight attachment of immature B cells to stromal components. In some areas of the stromal layer, B lymphoblasts were surrounded by folds of stromal cells, analogous to observations of murine B cells growing in Whitlock-Witte cultures of murine B cells. This intimate association also recalls observations of close attachment of
Effects of adding anti-VLA-4β (CD29; □) and anti-VLA-4α (CD49d; □) antibody on survival of subsets of normal CD19+ BM cells and on leukemic CD19+ lymphoblasts. Bars indicate the mean percentage of cell recoveries after 7 days of culture relative to those seen in parallel cultures without antibody. For normal B cells, the results of 4 (for CD29) and 3 (for CD49d) experiments are shown and SEM is indicated. No cells from case no. 9 were available for these studies.

Stromal layers provide crucial survival factors for B-cell progenitors; however, it is not clear whether intimate progenitor-stroma contact is an essential feature of this dependency. Indeed, Ryan et al10 have shown selective adhesion of immature B cells to BM-derived fibroblasts. To address whether direct contact with stromal layers is necessary for lymphoblast survival, we used diffusion chambers in which a microporous membrane separates lymphoid cells from stroma but allows a free flow of stroma-secreted soluble factors. In contrast to findings with myeloid cells,13 direct cell-cell contact was clearly beneficial to B-lineage BM cells (Table 2). We suggest that the demise of immature B cells that follows their untimely separation from stromal layers is mainly caused by cell death rather than by a decrease in proliferation, as has been observed for growth factor-dependent cells.4,7 This interpretation is supported by the observation that the proportions of large CD19+ sIg- cells remaining after culture in diffusion chambers were analogous or even higher than those observed in tests where contact with stroma was allowed, suggesting the persistence of proliferative activity. In addition, DNA fragmentation assays with ALL cells directly documented the occurrence of apoptosis under those culture conditions. These results are in agreement with reports indicating that the early stages of murine B-cell production in the BM depend on a close association with stromal cells.32,33

Adhesion of immature B cells to stroma is mediated by the integrin VLA-4.13 In contrast to other adhesion proteins (eg, leukocyte function antigen-1, CD44, and L-selectin), VLA-4 expression is most intense at an early stage of B-lymphoid development, declining progressively as differentiation proceeds.13 Therefore, we thought it reasonable to assess the progenitor-stroma relationship by using anti-VLA-4 antibodies to block adhesion. Among normal BM B cells, the most immature population (CD34+) showed the greatest adverse effects from perturbation of VLA-4; cell recovery under these culture conditions was as poor as that for equivalent cells in diffusion chambers. We think that this was caused by impaired cell survival, rather than by accelerated differentiation, because numbers of more mature cells did not increase in cultures containing CD29 or CD49d. In addition, in cultures of normal BM B cells containing CD29, numbers of CD10+ cells with abnormally lower DNA content increased. Low DNA stainability, caused by DNA fragmentation and diffusion of low-molecular-weight DNA content.
product from the cells, is typical of cells undergoing apoptosis.23 Finally, in cultures of both normal and leukemic cells, addition of anti-VLA-4 antibodies did not abolish cells' proliferative activity, as determined by cell size and DNA content analysis. Thus, we speculate that perturbation of VLA-4-mediated binding to stroma triggers apoptosis in immature B cells.

The inhibitory effects of anti-VLA-4 antibodies on immature B-cell recovery seen in our study resembled those reported in studies of colony formation by human B-cell progenitors,24 even more drastic effects were obtained in cultures of murine B cells, where anti-VLA-4 reagents completely abolished lymphopoesis.15 Adhesion of more mature B cells to BM fibroblasts is only slightly affected by the addition of anti-VLA-4 antibodies.13 In our study, recovery of more mature cells was scarcely influenced by the addition of these reagents to the cultures. In summary, these findings indicate that stromal factors crucial for the survival of normal B-cell progenitors are either membrane-bound or concentrated in the extracellular matrix (or both), and that the role of VLA-4 molecules may be to maintain immature B cells in close proximity to the growth factors necessary for their survival and proliferation. However, we cannot rule out the possibility that ligation of VLA-4, which not only inhibits cell adhesion to stromal elements but may also induce tyrosine phosphorylation and homotypic aggregation in immature B cells,34 may exert direct cytotoxicity on normal B-cell progenitors.

Abnormal survival requirements offer an attractive explanation for the aberrant growth patterns of leukemic B lymphoblasts. Indeed, the prolonged survival of neoplastic B cells in extramedullary sites in patients suggests that they have a reduced dependency on stroma. By comparison with normal B-cell progenitors, the leukemic cells from 5 of our 11 cases of B-lineage ALL in the present study were clearly less dependent on contact with stroma for survival. Moreover, in the same cases, inactivation of VLA-4 with CD49d and/or CD29 antibodies failed to decrease cell recovery. In cases where VLA-4 inhibition did cause cell losses, the decreases were never as pronounced as those seen in diffusion chambers, suggesting that factors other than VLA-4-mediated adhesion account for the stroma dependency of leukemic cells. Thus, at least in a proportion of cases of B-lineage ALL, the leukemic cells have reduced requirements for contact with BM stroma. These observations recall the study of Gordon et al,15 who showed that clonogenic cells in Philadelphia chromosome-positive chronic myelogenous leukemia grow well on stromal layers unable to sustain normal hematopoietic progenitors.

This study supports the general hypothesis that leukemic cells can survive free of signals that regulate normal B-cell growth. In our small series of patients, there were no obvious correlations between the stroma dependency of leukemic cells and initial clinicopathologic (eg, white blood cell count) or cellular (eg, karyotype) features, nor was it possible to relate the culture characteristics of these cases to treatment outcome, because of the short follow-up times. However, it is reasonable to suggest that B-lineage ALL cells with reduced stroma dependency, the survival and expansion of which can be supported by diffusible factors, would have the ability to grow in extramedullary sites.

ACKNOWLEDGMENT

We thank J. Gilbert for critical review and editorial assistance.

REFERENCES


Adhesion-dependent survival of normal and leukemic human B lymphoblasts on bone marrow stromal cells

A Manabe, KG Murti, E Coustan-Smith, M Kumagai, FG Behm, SC Raimondi and D Campana