Adhesion Molecule Expression on B-Cell Chronic Lymphocytic Leukemia Cells: Malignant Cell Phenotypes Define Distinct Disease Subsets

By Giulio De Rossi, Daniela Zarcone, Francesca Mauro, Giannamaria Cerruti, Claudya Tenca, Antonio Puccetti, Franco Mandelli, and Carlo E. Grossi

Expression of surface adhesion molecules of the Ig superfamily (CD54 and CD58), of the integrin family (β1, β2, and β3 chains), of the selectin family (L-selectin), and of the lymphocyte homing receptor (CD44) was analyzed on B-cell chronic lymphocytic leukemia (B-CLL) cells from 74 patients. The aim of the study was the definition of phenotypically distinct disease subsets and the correlation of adhesion molecule phenotypes with clinical parameters. Expression of CD58 on B-CLL cells defined more advanced disease stages. In comparison with β chain-positive cases, patients whose cells did not express β1, β2, and β3 integrin chains fell into the most favorable prognostic group, with lower lymphocytosis and the absence of splenomegaly, diffuse bone marrow infiltration, and therapy requirement. A novel finding was the expression of β2 chains on cells from a minority (12 of 74) of B-CLL cases. β2 chains were always coexpressed with β1 and β3 chains. Two-color immunofluorescence analyses of adhesion molecules such as α5β1 integrin (LeuM5) and L-selectin (Leu8) showed that these markers were detectable on variable proportions of leukemic cells, thus confirming the intraclonal phenotypic heterogeneity of B-CLL. Differences in the intensity of CD44 expression were also shown among the various B-CLL clones. Finally, no major variations were shown by comparison of adhesion molecule phenotypes of leukemic cells simultaneously obtained from blood and bone marrow, and of CD5- versus CD5+ clones.

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MOST LYMPHOPROLIFERATIVE disorders are monoclonal expansions of B or T cells at different stages of maturation that evolve as leukemia or lymphoma.1 Malignant cells proliferate within the tissue of origin and some of them reach the circulation and spread via blood and lymph vessels. In a further step of tumor progression, these cells extravasate and seed into other tissues.2,3 Adhesive interactions between neoplastic cells, endothelial cells, and extracellular matrix (ECM) proteins are crucial for these sequential events to occur.4,5 Expression of adhesion molecules of the Ig superfamily, of the integrin family, of the selectin family, and of the “lymphocyte homing receptor,” CD44, has been investigated in lymphoproliferative disorders.6-18 However, a complete pattern of adhesion molecule expression in lymphoid malignancy has not yet emerged, and conflicting data on the prognostic significance of these phenotypic features have been reported.15,16

B-cell chronic lymphocytic leukemia (B-CLL) is a lymphoproliferative disorder with a high degree of biologic heterogeneity.1,20 This lymphoid malignancy originates from a CD5+ normal B-cell counterpart that may represent a distinct subset of B cells.21,22 However, in less than 10% of the cases, B-CLL cells do not express CD5.19,21 Other phenotypic features may be heterogeneous among B-CLL cases, such as expression of β2 integrin molecules (CD11b/CD18 and CD11c/CD18) found on myelomonocytic cells and on hairy cell leukemia (HCL) cells.23,24 Expression of CD11b/CD18 has been associated with the ability of B-CLL cells to produce interleukin-1,25 whereas expression of CD11c/CD18 appears to define a B-CLL variant with distinctive clinical features.25,26 Further phenotypic heterogeneity of B-CLL cells is due to maturation differences among malignant clones and to intraclonal maturation, as shown by ultrastructural and cytochemical analyses.27

Clinical heterogeneity of B-CLL is also well defined and prognosis is often unpredictable.19,28 Many patients do not require treatment and are unlikely to die of leukemia. In contrast, other patients present with rapidly progressive disease and have a median survival of less than 2 years. So far, a clear correlation between the surface antigenic phenotype of leukemic cells and the clinical behavior has not been defined.

In the present study, we have investigated the expression of a variety of adhesion molecules on cells from 74 B-CLL patients. Surface molecules of the Ig superfamily (CD54/intercellular adhesion molecule-1 [ICAM-1] and CD58/lymphotocyt function-associated antigen 3 [LFA-3]); of the β1, β2, and β3 integrin family; of the selectin family (L-selectin/Leu8); and of CD44 have been analyzed by immunofluorescence and flow cytometry (see the literature29-34 for reviews on these molecules). Expression of adhesion molecules has allowed distinction of phenotypically diverse groups of patients and clinical differences have emerged among some of these groups.

MATERIALS AND METHODS

Patients. Seventy-four patients were evaluated in our Division from September 1990 through December 1991. The mean age of the patients was 62 years, with a range between 41 and 85 years. There was one 19-year-old female. The male to female ratio was 40 to 34. B-CLL was diagnosed according to generally accepted criteria that included peripheral blood lymphocytosis greater than 5,000/μL and bone marrow lymphocytosis greater than 30%.28 Diagnosis was confirmed by immunophenotypic analysis of surface Ig light chain and...
were studied when receiving the CHL plus PDN regimen.

At the time of study, 63 of 74 patients had never been treated. However, at a later time, 23 of the 63 patients had to start treatment due to a second neoplasia, and 1 from myocardial infarction.

Clinical evaluation of the patients included staging according to Rai et al. and Binet et al., peripheral blood lymphocytosis, evaluation of liver, spleen, and lymph node enlargement (aided by computed tomography [CAT] and echotomography), and lymphocyte doubling time (LDT).

The pattern of bone marrow infiltration was evaluated in bone marrow biopsies of 52 of 74 patients. Biopsy was not performed in 22 patients due to advanced age, cardiovascular risk factor, or refusal of informed consent.

Immunophenotypic analyses. Mononuclear cells were isolated from heparinized peripheral blood samples by Ficoll-Hypaque density gradient centrifugation. When, in addition to peripheral blood samples, bone marrow aspirates were also available (7 of 74 patients), single-cell suspensions of bone marrow cells were centrifuged on Ficoll-Hypaque density gradients to recover mononuclear cells. Freshly isolated mononuclear cells were used for immunophenotypic analyses. Further immunophenotypic and biochemical studies were performed using frozen cell aliquots stored in liquid nitrogen.

Cells were incubated with mouse monoclonal antibodies (MoAbs) to a variety of surface molecules (as indicated in Table 1), followed by affinity-purified, fluorescein isothiocyanate (FITC)-labeled goat antimouse Ig (Southern Biotechnology Associates, Birmingham, AL). In other experiments, biotinylated MoAbs were used, followed by FITC-streptavidin.

Two-color immunofluorescence analyses were performed using FITC-labeled and phycoerythrin (PE)-labeled mouse MoAbs (Table 1). Controls were provided by cells incubated directly with the secondary reagent, omitting the primary reagent, and by cells incubated with an unrelated MoAb of an isotype identical to that of the test MoAb (anti-CD62, IgG1; anti-CD10, IgG2a; anti-CD69, IgG2b), followed by the labeled secondary reagent (Table 1). These antibodies were used as negative controls.

Cells were analyzed by one-color or two-color fluorescence using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and setting gates on the lymphocyte populations. Analysis by two-color fluorescence was restricted to those markers that appeared equifrequent as for their attribution to B-CLL cells or to residual T cells. However, this was seldom the case because in the majority of the patients (53 of 74) the percentage of leukemic cells was greater than 70%.

Immunoprecipitation. B-CLL cells were washed twice in cold phosphate-buffered saline (PBS) and resuspended in PBS with 10 mmol/L glucose. Surface proteins were labeled with sulfo succinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin; Pierce, Rockford, IL). Ten million cells and 2.5 mg of biotin were used per experiment. Labeled cells were lysed with 10 mmol/L Tris, 150 mmol/L NaCl, and 1% NP-40, pH 7.5, containing protease inhibitors. Lysates were centrifuged for 10 minutes at 10,000g. The supernatant was preclarified twice with 50 μL of beads coated with antimouse IgG (Calbiochem, San Diego, CA). Beads were sedimented by centrifugation for 2 minutes at 10,000g and the supernatant was used for immunoprecipitation. Ten micromolar of monoclonal anti-CD61 MoAb (Immunotech) was added to each sample and incubated overnight at 4°C. Fifty microliters of bead-immobilized antimouse IgG was added to each tube and the samples were rotated for 1 hour at 4°C. After centrifugation for 2 minutes at 10,000g, beads were washed three times with cold PBS and twice with 10 mmol/L Tris, 2 mmol/L L-EGTA, 0.1% sodium dodecyl sulfate (SDS), pH 7.5, followed by elution with sample buffer. Eluates were run on 7.5% polyacrylamide gel under nonreducing conditions. Polypeptides separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to ELET, 0.1% sodium dodecyl sulfate (SDS), pH 7.5, containing protease inhibitors. Proteins were detected with 5-bromo-4-chloro-indolyl phosphate and nitroblue tetrazolium (Sigma, St Louis, MO) as substrate.

Statistical analyses. Each single clinical and phenotypic parameter has been matched with all of the others using the statistical technique of cross tab. We used the Pearson x2 test (SPSS/PC) to define the significance of each cross and analyzed the percentages in each tab. When the probability value was less than 0.05, the hypothesis that the two examined variables are independent was rejected.

The Student's t-test was used to evaluate statistical differences in the mean lymphocyte counts among various phenotypic groups.

RESULTS

Seventy-four B-CLL patients were considered in this study. In all of the samples, leukemic cells were enumerated via the expression of HLA-DR and residual T cells were identified through the expression of surface CD3 molecules.

In 52 of 74 patients, we determined percentages of HLA-DR+ and residual T cells that were greater than 70%. In the remaining patients, these percentages ranged from 50% to 70%. In the majority of the patients (67 of 74), residual T cells were less than 20%. Consequently, the threshold of positivity for each marker was set at greater than 20%. In the 7 cases that had greater than 20% T cells, the phenotypes of leukemic cells were assessed by two-color immunofluorescence analysis. No difference in the fluorescence intensity was observed between freshly iso-

<table>
<thead>
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<th>MoAb</th>
<th>Isotype</th>
<th>Specificity</th>
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</tr>
<tr>
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<td>IgG 2a</td>
<td>CD58 (LFA-3)</td>
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</tr>
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<td>Leu M5 (PE)</td>
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<td>IOT 8 (FITC, PE)</td>
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<td>L-selectin</td>
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<td>IMM</td>
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</table>

Abbreviations: BD, Becton Dickinson (San Jose, CA); IMM, Immunotech (Marseille-Luminy, France); CO, Coulter (Hialeah, FL).

* Fluorochromes in parentheses refer to directly labeled MoAb used for two-color immunofluorescence analyses.
† Unrelated MoAb used as isotype-matched negative controls.
ADHESION MOLECULES ON B-CLL CELLS

lateral and frozen cells, even for molecules, such as L-selectin, that shed from the cell surface.11

Expression of CD54 (ICAM-1) and CD58 (LFA-3) on B-CLL cells. CD54 was detected on B-CLL cells from 13 of 74 patients; CD58 was present on cells from 6 of 13 CD54 cases and was never found independently of CD54 expression. In 2 of 3 patients whose cells expressed CD54, peripheral blood and bone marrow samples were available simultaneously. In both cell samples, CD54 and CD58 were expressed at similar proportions.

Expression of $\beta_1$ (CD29), $\beta_2$ (CD18), and $\beta_3$ (CD61) integrins on B-CLL cells. We analyzed the surface expression of $\beta_1$, $\beta_2$, and $\beta_3$ integrin chains on cells from 74 B-CLL cases. In addition, we determined the expression of $\alpha_k$ (CD11a, LFA-1), $\alpha_M$ (CD11b, Mac-1), and $\alpha_\xi$ (CD11c, Leu M5), the $\alpha$ chains known to associate with leukocyte ($\beta_2$) integrins.26,30

Expression of $\beta_1$, $\beta_2$, and $\beta_3$ integrin chains on B-CLL cells allowed us to distinguish three groups of B-CLL cases in which cells did not express detectable surface $\beta_1$, $\beta_2$, and $\beta_3$ chains; or expressed $\beta_1$ and/or $\beta_2$, but not $\beta_3$ integrin chains; or else were positive for all three of the $\beta$ chains (Table 2 and Fig 1).

Due to the sensitivity threshold of the indirect immunofluorescence technique routinely used to determine integrin $\beta$ chain expression and to assess whether a negative result could indicate absence of a given $\beta$ chain or its expression at a very low density, we tested different detection systems. In one approach, labeled antimouse Ig isotype (IgG2a and IgG1) antibodies were used as secondary reagents to detect $\beta$ chain expression. Fluorescence-activated cell sorter (FACS) analyses did not show differences in fluorescence intensity when these reagents were compared with secondary antitotal mouse Ig antibodies (data not shown). In contrast, significant differences in $\beta$ chain expression on B-CLL cells were shown when results from indirect immunofluorescence assays were compared with those obtained using biotinylated primary mouse MoAb followed by fluorescein-labeled streptavidin (Fig 2).

While confirming previous results showing that, in most B-CLL cases, $\beta_1$ and $\beta_2$ integrin chains are expressed on the surface of malignant B cells,13,44-46 we observed that, in 12 of 74 patients (Table 2), B-CLL cells also expressed surface $\beta_3$ chains detectable by indirect immunofluorescence (Fig 1). Lymphocytes from 4 patients, 3 positive for surface $\beta_3$ chains and 1 negative by FACS analysis, were labeled with biotin and immunoprecipitated with anti-CD61 MoAb followed by SDS-PAGE. A band of 90 Kd was present in the three cases whose cells expressed $\beta_3$ chains detectable by immunofluorescence. No band was detected in the $\beta_1$ case (Fig 3). An unidentified band of approximately 68 Kd found in two cases (lanes B and C in Fig 2) could be a proteolytic fragment of the 90-Kd polypeptide. In all of the $\beta_1^-$ cases we also found coexpression of surface $\beta_1$ and $\beta_2$ chains.

To rule out that detection of $\beta_3$ chains could have been due to platelets adherent nonspecifically to the malignant cells, we stained B-CLL cells from three cases with antibodies to the platelet-specific glycoprotein (GP) Ib-IIa (CD41a). Results were consistently negative. This finding is also reinforced by the lack of staining of B-CLL cells from all cases with MoAb to CD62 (P-selectin), a marker of activated platelets and endothelial cells.30 Finally, no adherent platelets were detected in Giemsa-stained cytocentrifuge preparations of B-CLL cells.

Analysis of $\beta_1$ and $\beta_2$ chain expression allowed us to distinguish a group of patients whose cells did not express detectable $\beta_1$ and $\beta_2$ chains. These cases (30 of 74) were also negative for $\beta_3$ chains. In 32 of 74 patients, $\beta_1$ (5 of 32), $\beta_2$ (9 of 32), or, more commonly, both chains (18 of 32) were found on B-CLL cells at detectable amounts in the absence of $\beta_3$ chain expression (Table 2). In all of the cases, $\beta_2$ chains were coexpressed with $\alpha_k$ chains to form the CD11a/CD18 integrin complex.

Expression of $\beta$ integrin chains was determined by indirect immunofluorescence and flow cytometric analysis. Thus, negative means undetectable using this technique. See text and Fig 2 for analysis and comment on these data.

Abbreviation: NS, nonsignificant.

| Table 2. Integir $\beta$ Chain Expression and Clinical Features at the Time of Study |
|---------------------------------|----------|----------|----------------|----------------|
| $\beta_1$, $\beta_2$, $\beta_3$ | $\beta_1$, $\beta_2$, $\beta_3$ | $\beta_1$, $\beta_2$, $\beta_3$ | $\beta_1$, $\beta_2$, $\beta_3$ | $\beta_1$, $\beta_2$, $\beta_3$ |
| Patients | 30 | 32 | 12 | |
| Sex (M/F) | 18/12 | 17/15 | 5/7 | |
| Age (Median) | 62 | 61 | 63.5 | |
| Rai stages 3-4 and Binet stages B-C | 9/30 | 10/32 | 3/12 | |
| LDT <12 mo | 6/26 | 9/24 | 4/10 | |
| Splenomegaly | 11/30 | 21/32 | 10/12 | |
| Peripheral blood lymphocytes (x10^3/μL) | 28.9 ± 23.0 | 44.8 ± 44.0 | 30.9 ± 26.4 | |
| Diffuse bone marrow pattern | 3/22 | 6/21 | 5/11 | |
| Therapy requirement | 10/30 | 15/32 | 9/12 | |

Expression of $\beta$ integrin chains was determined by indirect immunofluorescence and flow cytometric analysis. Thus, negative means undetectable using this technique. See text and Fig 2 for analysis and comment on these data.

Abbreviation: NS, nonsignificant.
Fig 1. Detection of $\beta_1$, $\beta_2$, and $\beta_3$ integrin chains on cells from three representative B-CLL cases. Mouse MoAb anti-3 chain was used, followed by FITC-labeled goat antimouse lg antibodies. Left row: $\beta_1^+$, $\beta_2^+$, $\beta_3^+$ case. Middle row: a case in which most cells express detectable $\beta_1$ and $\beta_2$, but not $\beta_3$ chains. Right row: a case positive for all of the three $\beta$ chains analyzed. HLA-DR and CD3 are used as markers for leukemic B cells and for residual T cells, respectively. Percentages of positive cells are reported at the top right corner of each profile.

(LFA-1) heterodimer; $\alpha_{M}$ (CD11b, OKM1/Mac1) and $\alpha_{X}$ (CD11c, LeuM5) were detected, respectively, on $\beta_2^+$ cells from 1 of 41 and 14 of 41 patients. Cells from the latter group of patients were analyzed by two-color direct immunofluorescence to determine percentages of leukemic (HLA-DR$^+$) cells also expressing LeuM5. Figure 4 shows three representative cases in which HLA-DR$^+$ cells also expressed LeuM5 at variable proportions. Notably, $\beta_3$ was found in 6 of 14 cases in which cells were LeuM5$^+$. The difference in $\beta_3$ expression on cells from LeuM5$^+$ versus LeuM5$^-$ cases was highly significant ($P = .0027$).

In seven cases in which blood and bone marrow samples were simultaneously available, we found that expression of integrin $\beta$ chains was identical in cells from five patients. $\beta_1$ chains were detectable on blood but not on bone marrow B-CLL cells in one case. The opposite occurred in another case.

Expression of L-selectin on B-CLL cells. L-selectin (Leu8) was detectable on the surface of B-CLL cells in 21 of 74 cases. Variable proportions of leukemic cells were positive for L-selectin in the various cases (Fig 5). We did not find significant differences in the frequency of L-selectin-positive cases among the patient groups expressing different sets of $\beta$ chains. In one of seven cases in which blood and marrow samples were tested simultaneously, L-selectin was detected on blood but not on bone marrow cells. No difference was observed in the remaining cases.

Expression of CD44 on B-CLL cells. CD44 was detectable on B-CLL cells from all patients but one. The intensity of CD44 expression on leukemic cells was compared with that of residual T cells by two-color immunofluorescence. As shown in Fig 6, CD44 was expressed on CD3$^+$ cells at higher density than on HLA-DR$^+$ (B-CLL) cells. However, in several cases, both cell types expressed CD44 at the same density. This allowed us to distinguish three groups of patients according to high (16 of 73), intermediate (18 of 73), or low (39 of 73) CD44 expression. No difference was found when CD44 expression was analyzed on blood and bone marrow B-CLL cells.

Correlation between CD5 and adhesion molecule expression on B-CLL cells. The adhesion molecule profile was evaluated in the 17 patients whose cells were CD5$^-$ versus 57 CD5$^+$ cases. We found an increased incidence ($P = .049$) of CD5$^+$ cases in the LeuM5$^+$ group.

Correlation between adhesion molecule expression and clinical features. Analysis of adhesion molecule expression allowed for the distinction of subsets of patients whose cells
did or did not express the various types of adhesive receptors at detectable levels. All of the phenotypic differences were related to clinical parameters, namely, stage of disease according to Rai and Binet, LDT, peripheral blood lymphocytosis, splenomegaly, hepatomegaly, nodal involvement, bone marrow infiltration pattern, and therapy requirement.

Patients whose cells expressed CD58 were more frequently assigned to the Rai 3 and 4 and Binet B and C stages ($P = .038$) and exhibited a higher incidence of splenomegaly ($P = .025$). As for CD54, we did not find significant correlations with the evaluated clinical parameters.

Several correlations between B-CLL cell phenotype and clinical features were found when integrin $\beta$ chain expression was analyzed (Table 2). Cases lacking $\beta_1$, $\beta_2$, and $\beta_3$ chain expression had a significantly lower incidence of splenomegaly ($P = .004$). When cases lacking all of the analyzed $\beta$ chains were compared with those expressing all three $\beta$ chains, it was found that the former had a significantly lower incidence of diffuse bone marrow infiltration ($P = .044$) and of therapy requirement ($P = .027$) (Table 2). Among the three groups, $\beta$ chain-negative cases had the lowest peripheral blood lymphocytosis, but these data were not statistically significant ($P = .08$).

In view of the possibility that $\alpha_3\beta_2$ (LeuM5) expression distinguishes a subset of patients with a more aggressive disease course or variants of chronic lymphoproliferative disorders, we examined the clinical features of 14 LeuM5$^+$ versus 60 LeuM5$^-$ cases (Table 3). Patients in the former group presented with a significantly higher lymphocytosis ($P = .021$) and need for therapy ($P = .006$).

L-selectin was detected on cells from 21 of 74 cases. No significant correlation has been observed between clinical parameters and Leu8 expression.

As for the variable intensity of CD44 expression on B-CLL cells, the only clinical difference among the three groups was that between CD44low and CD44high cases. A significantly increased incidence of diffuse bone marrow infiltration was found in the latter group ($P = .012$).

**DISCUSSION**

B-CLL is a disorder with a high degree of biologic and clinical heterogeneity. Surface molecules mediating cell-
to-cell and cell-to-ECM adhesion could be good candidates for phenotypic to clinical correlations in view of their role for tumor cell spreading and metastasis. 46

Expression of adhesion molecules may also explain certain functional characteristics of B-CLL cells, such as their ability to adhere to substrates. A previous study has shown that B-CLL cells express $\beta_1$ and $\beta_2$ integrin chains and that these molecules codistribute with cytoskeletal proteins at specific adhesion sites called podosomes. 42 In the same study, $\beta_3$ integrin chains were undetectable and, quite intriguingly, no $\alpha$ chains could be shown in association with $\beta_1$ and $\beta_2$ chains. This raises the important issue of the detection of surface adhesion molecules. Experiments shown in Fig 2 clearly demonstrate that assessment of surface integrin expression (as well as that of other adhesion molecules) on B-CLL cells largely rests upon the detection system that is used. Therefore, negativity or positivity for surface adhesion molecules is of relative value, and is probably attributable to the surface density of any given molecule rather than to its presence or absence. This may hamper a possible comparison of phenotypic analyses from different laboratories and may explain some conflicting data reported in the literature. However, we believe that our classification of B-CLL cases into phenotypic subsets (as shown, for example, in Table 2) reflects true phenotypic differences and that it lends itself to the possibility of clinical correlations provided that cells from all cases are analyzed using the same reagents and the same detection system.

Adhesion molecules of the Ig superfamily (CD54 and CD58) were found in a minority of B-CLL cases. As for other cell types (eg, endothelial cells and keratinocytes), this may reflect a state of cell activation induced by cytokines produced by the malignant B cells or by other cell types. 47

While confirming previous studies describing the expression of $\beta_1$ and $\beta_2$ integrin chains on B-CLL cells, 23,42-44 we found that cells from 12 of 74 B-CLL patients also expressed $\beta_3$ chains, always in association with $\beta_1$ and $\beta_2$ chains. This novel finding was confirmed by both flow cytometric and

Fig 4. Two-color immunofluorescence analysis of HLA-DR and $\alpha_\beta_2$ chain (LeuM5) coexpression on B-CLL cells from three distinct patients. Variable percentages of double-positive cells are determined in each case (percentage values are reported in square 2). Double-negative cells in square 3 correspond to residual T cells.

Fig 5. Two-color immunofluorescence analysis of HLA-DR and L-selectin (Leu8) coexpression on B-CLL cells from three distinct patients. Variable percentages of double-positive cells are shown in the three cases (percentage values are reported in square 2). Double-negative cells in square 3 correspond to residual T cells.
biochemical analyses and we could rule out that it was due to adherent platelets (see Results). Therefore, we could define distinct groups of patients according to the expression of \( \beta_1, \beta_2, \) and \( \beta_3 \) integrin chains.

No major differences were noted when expression of the above described adhesion molecules was analyzed on B-CLL cells simultaneously obtained from blood and bone marrow or when CD5\(^+\) clones were compared with CD5\(^-\) clones, with the exception of a significantly higher incidence of LeuM5\(^+\) clones in the latter group.

The high degree of biologic and clinical heterogeneity of B-CLL has prompted numerous studies aimed at the detection of risk factors. These studies have been addressed both at the definition of phenotypic heterogeneity of B-CLL cells and of several clinical parameters. We selected groups of patients with distinctive adhesion molecule phenotypes. These groups were evaluated in relation to a variety of clinical features. Staging according to Rai and to Binet, LTD, and the bone marrow histologic pattern have been considered important prognostic parameters in survival analyses of B-CLL.\(^{35-38}\) Furthermore, therapy requirement is related to the appearance of negative risk factors and/or to disease progression. Therefore, we considered the need for therapy as a relevant prognostic variable. Evaluation of survival time could not be taken into account because at least one-half of the patients had a follow-up period shorter than 3 years.

The main conclusions that can be drawn from a correlation between adhesion molecule phenotype and clinical features are the following. Patients whose cells do not express integrin \( \beta \) chains (\( \beta_1, \beta_2, \) and \( \beta_3 \)) show the most favorable clinical features as compared with the other groups (Table 2). Expression of \( \alpha_\beta_2 \) integrin (LeuM5) has allowed for the detection of a B-CLL subset with clinical features similar to those of HCL and of other types of B-CLL lymphoproliferative disorders.\(^{35,36,45}\) In the 14 LeuM5\(^+\) cases of our series, we did not find most of the features described in the above studies. However, patients whose malignant cells expressed LeuM5 differed from LeuM5\(^-\) cases for a significant incidence of higher lymphocytosis and of therapy requirement (Table 3).

Furthermore, expression of CD58 was detected in cases with higher incidence of splenomegaly and advanced Rai/Binet stages. Diffuse bone marrow infiltration was significantly more frequent in patients whose cells expressed CD44\(^{\text{high}}\). This is consistent with the finding that CD44 me-

### Table 3. CD11c/CD18 (LeuM5) Expression and Clinical Features at the Time of Study

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<th>Patients</th>
<th>LeuM5(^-)</th>
<th>LeuM5(^+)</th>
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<td>6/14</td>
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<td>Peripheral blood lymphocytes (( &gt;10^4/\mu L ))</td>
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<td>55.3 ± 49.6</td>
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<td>Diffuse bone marrow pattern</td>
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<td>6/10</td>
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<tr>
<td>Therapy requirement</td>
<td>23/60</td>
<td>11/14</td>
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</table>

Abbreviation: NS, nonsignificant.

Fig 6. Expression of CD44 on B-CLL cells and on residual T cells from two distinct patients. B-CLL cells were identified as HLA-DR\(^+\), whereas T cells were labeled with MoAb to CD3. Top row: B-CLL cells (left) and T cells (right) express CD44 at the same intensity. Bottom row: B-CLL cells express CD44 at lower density in comparison to that determined on T cells. Percentages of double-positive cells are reported at the top right corner.
diates adhesion of maturing B cells to bone marrow stromal cells. Analyses of the relationships of clinical features with cell phenotypes have their major constraint in the long median survival of B-CLL patients. However, LDT and therapy requirement are evidently dynamic variables and will therefore be evaluated in the long run.

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REFERENCES

32. Butcher EC: Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. Cell 67:1033, 1991

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