Heterogeneity of Immunologic Markers and Surface Morphology in Childhood Lymphoblastic Lymphoma

By Elaine S. Jaffe, Raul C. Braylan, Michael M. Frank, Ira Green, and Costan W. Berard

The neoplastic cells from seven patients with childhood lymphoblastic lymphoma were studied for cell surface markers and surface morphology in the scanning electron microscope (SEM). The cells were studied for surface immunoglobulin (SIg), complement receptors (EAC), receptors for cytophilic antibody (IgGEA), and non-immune rosette formation with sheep red blood cells (E). In one patient the cells exclusively bound E, suggesting a T-lymphocytic origin. In two patients the cells bound EAC, but demonstrated no other B-lymphocytic markers. In two patients no markers were detected, and in two patients receptors for both E and EAC were demonstrated. Additional studies in one of these patients permitted simultaneous demonstration of both markers on the same neoplastic cells. The neoplastic cells were also examined by SEM after fixation and critical point dehydration. No consistent surface morphology was observed. In four patients the cells were predominately smooth, whereas in two patients variable numbers of surface microvilli were present. A correlation of the surface features with membrane markers could not be established. A comparison of the surface markers with clinical and cytologic features revealed clinical homogeneity in spite of the heterogeneous immunologic markers. This heterogeneity was most likely a reflection of neoplastic alteration and disordered differentiation of the cells. The observation of complement receptors on the cells of four cases is a feature not previously reported in this disease and should be investigated in other presumed T-cell malignancies.

CHILDHOOD LYMPHOBLASTIC LYMPHOMA or childhood lymphosarcoma (CLSA), a morphological entity most common in children and young adults, has been suggested to be of thymus-derived (T) lymphocytic origin.2,3 The presence of an anterior mediastinal mass in many of these patients had indicated clinically a possible thymic origin for these tumors. These neoplasms also seem to involve preferentially the thymic-dependent portions of the lymphoreticular system, the paracortex of lymph nodes, and the periarteriolar lymphoid sheaths of the spleen. In addition, T-lymphocytic markers have been demonstrated on the neoplastic cells in some cases.2,3 We have studied the membrane markers and scanning electron microscopic (SEM) appearance of the neoplastic cells from seven cases and have attempted to correlate these features with the presence or absence of involvement of the mediastinum, bone marrow, peripheral blood, or cerebrospinal fluid.
Table 1. Clinical Characteristics of Childhood Lymphoblastic Lymphoma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)/Sex</th>
<th>Disease at Dx.</th>
<th>BM/PB at Dx.</th>
<th>Time to PB + (mo)</th>
<th>WBC at Onset of Leukemia (10^9/L)</th>
<th>Blasts at Onset (%)</th>
<th>CSF A/D</th>
<th>Time (mo)</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.J.</td>
<td>14/M</td>
<td>Ing. LN</td>
<td>0/0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>At/od</td>
<td>18</td>
</tr>
<tr>
<td>L.K.</td>
<td>23/M</td>
<td>MED Scal. LN</td>
<td>+/+</td>
<td>0</td>
<td>33.8</td>
<td>68</td>
<td>+</td>
<td>D</td>
<td>13</td>
</tr>
<tr>
<td>R.B.</td>
<td>18/M</td>
<td>MED</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.T.</td>
<td>12/F</td>
<td>MED Cerv., Supracl., Scal. LN</td>
<td>0/0</td>
<td>10</td>
<td>5.6</td>
<td>29</td>
<td>0</td>
<td>D</td>
<td>43</td>
</tr>
<tr>
<td>T.A.</td>
<td>22/M</td>
<td>MED</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.R.*</td>
<td>19/M</td>
<td>MED Cerv., Supracl.</td>
<td>+/0</td>
<td>22</td>
<td>56.0</td>
<td>90</td>
<td>+</td>
<td>D</td>
<td>42</td>
</tr>
<tr>
<td>P.B.*</td>
<td>18/M</td>
<td>MED Cerv. LN</td>
<td>0/0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>AEd</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

*Less than 4 mo of follow-up.

Abbreviations: Dx., diagnosis; Ax., axillary; Cerv., cervical; Ing., inguinal; LN, lymph nodes; MED, mediastinum; Scal., scalene; Supracl., supraclavicular; BM, bone marrow; PB, peripheral blood; NE, not examined; WBC, white blood cell count; CSF, cerebrospinal fluid; A, alive; D, dead; ed, with disease; od, without disease.

**MATERIALS AND METHODS**

**Patient Population**

Seven patients with childhood lymphoblastic lymphoma were studied. All patients included in the study presented with peripheral lymphadenopathy and/or a mediastinal mass. The diagnoses were established by lymph node biopsy, which showed in paraffin sections classical pathologic features, i.e., replacement by a diffuse infiltrate composed of primitive blast cells. Individual cells demonstrated round to oval nuclei, larger than those of a normal lymphocyte, with finely distributed chromatin and inconspicuous nucleoli. In imprints and smear preparations stained with Wright's stain, the cells exhibited blastic chromatin, small nucleoli, and scant cytoplasm. Only one patient (L.K.) had blasts in the peripheral blood at diagnosis. Clinical features are summarized in Table 1. The cells studied were obtained prior to any therapy in three patients (D.J., J.R., P.B.) and during relapse in the other four. None of the patients was undergoing chemotherapy or radiotherapy at the time the cell sample was obtained. Control studies were performed on mononuclear cells isolated from normal peripheral blood.

**Preparation of Cell Suspensions**

Neoplastic cells for study were obtained from either lymph node (LN), bone marrow (BM), peripheral blood (PB), cerebrospinal fluid (CSF), or pleural fluid (PF), as indicated in Table 2. Morphology was examined on Wright-stained Shandon cytocentrifuge (Shandon Scientific Co., Sewickley, Pa.) preparations. The neoplastic cells as described above comprised at least 90%, of the population studied in all instances, and were readily distinguishable from normal lymphocytes. Viability was determined by trypan blue exclusion and exceeded 90%, in all cases.

PB and BM were purified through a Ficoll-Hypaque gradient. The cells were washed three times in RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.), and brought to the appropriate concentration for subsequent studies (see below).

A pellet was obtained from 20 ml of CSF or PF and mixed with 20 ml of a pH 7.4 ammonium chloride buffer (0.155 M NH₄Cl, 0.01 M KHCO₃, 0.1 M EDTA) at 0°C for 5 min to lyse contaminating erythrocytes. The remaining white cells were washed three times in serum-free medium and brought to a concentration of 2.5 x 10⁶.

A portion of the lymph node tissue was minced finely in RPMI-1640 and filtered through a stainless steel wire mesh. A pellet was obtained and red cells were lysed with ammonium chloride as above. The cells were washed three times in medium and brought to the appropriate concentration. The remaining tissue was fixed and used for routine histopathologic diagnosis.
Table 2. Immunologic Markers in Childhood Lymphoblastic Lymphoma

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Med. Mass</th>
<th>Source†</th>
<th>E(%)</th>
<th>EAC(%)</th>
<th>IgGEA(%)</th>
<th>Sig(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.J.</td>
<td>LN</td>
<td>75</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>85</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>L.K.</td>
<td>PB</td>
<td>10</td>
<td>9</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>R.B.</td>
<td>BM</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P.T.</td>
<td>PB</td>
<td>7</td>
<td>85</td>
<td>3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>T.A.</td>
<td>PB</td>
<td>80</td>
<td>70</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>J.R.</td>
<td>LN</td>
<td>7</td>
<td>30</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P.B.</td>
<td>PF</td>
<td>32</td>
<td>34</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>PB</td>
<td>65</td>
<td>16</td>
<td>18</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

*Populations studied contained at least 90% neoplastic cells.
†LN, lymph node; CSF, cerebrospinal fluid; PB, peripheral blood; BM, bone marrow; PF, pleural fluid.
¶Mean values from nine subjects studied.

Detection of EAC and IgGEA Rosette-forming Cells

The rosette assays for cells bearing complement receptors (EAC) or receptors for cytophilic antibody (IgGEA) were performed as previously described with slight modification. In short, equal volumes of the white cell suspensions (2.5 x 10⁶/ml) and the IgGEA, EAC, or IgMEA suspensions (1 x 10⁸/ml) were mixed in plastic tubes, centrifuged at 200 g for 10 min, and then incubated at 37°C for 30 min. The pellet was resuspended, an aliquot mixed with trypan blue, and the percentage of rosetted cells determined. Only viable cells were counted. A receptor for heterologous IgM has not been identified on human cells. Thus, IgMEA should not bind to any cell and serves as a control reagent. Conditions producing E rosette formation, i.e., 37°C-4°C, were never used, and IgMEA rosette formation was never observed. For cytologic identification of rosetted cells, Shandon cytocentrifuge preparations were stained with Wright's stain. Neoplastic cells composed at least 90% of the population studied in all instances. Cytocentrifuge smears of IgMEA controls were always examined, and pseudorosette formation was never observed.

The percentage of monocytes and/or histiocytes was determined by incubation of the cell suspension with latex particles (Dow Diagnosis, Indianapolis, Ind.) or performance of the non-specific esterase reaction on the cytocentrifuge preparations. The proportion of such cells present in the population was always less than 5%.

Detection of E-Rosette-forming Cells

Determination of E-rosette-forming cells, a T-cell characteristic, was done as previously described. Equal volumes of white cells (2.5 x 10⁶/ml) and sheep red blood cells (SRBC) (1 x 10⁸/ml) previously treated with neuraminidase were mixed and incubated under appropriate conditions. For cytologic identification of rosetted cells, Shandon cytocentrifuge preparations were stained with Wright’s stain.

Simultaneous Detection of EAC and E Rosettes

The cells of patient T.A. were studied for combined rosette formation. A modification of the method of Shevach et al. was used. E-rosette formation was detected using fluorescein-conjugated SRBC that were subsequently treated with neuraminidase [E₄(F)]. The EAC reagent was prepared using unconjugated SRBC, first treated with a 2 mg/ml solution of trypsin as described by Weiner et al. To detect EAC and E rosettes simultaneously, equal volumes of the white cells, E₄(F), and E₄AC reagents were mixed and incubated under appropriate conditions for both E and EAC-rosette formation. The cells were incubated in suspension for 15 min at 37°C, centrifuged for 10 min at 200 rpm, and incubated for 30 min at 37°C; the supernatant was then removed and replaced with 50% fetal calf serum, and the cells were further incubated for 18 hr at 4°C. The suspension was examined alternately by tungsten and ultraviolet illumination, and the percentages of E₄(F), E₄AC, and combined rosettes were determined.
Detection of Immunoglobulin-bearing Cells

Detection was performed as previously described. Briefly, $10^6$ white cells in 0.05 ml of medium were incubated in ice with 0.05 ml of a fluoresceinated polyvalent goat antiserum against human gamma, alpha, mu, kappa, and lambda chains (Meloy Laboratories, Springfield, Va.). The activity and specificity of this antiserum was tested by immunodiffusion using the appropriate purified human antigens. After three washings, the cells were resuspended in phosphate-buffered glycerine and examined with a Leitz microscope equipped with a vertical illuminator and phase contrast optics.

SEM Studies

In a single case (P.T.), washed, unfixed cells in suspension were collected on 0.45-μm pore size Flotronic filter membranes (Flotronic Inc., Spring House, Pa.) and fixed within minutes after the filtration procedure. In the other cases, the cells were fixed in suspension and subsequently either filtered onto Flotronic membranes or allowed to settle onto poly-L-lysine-coated cover slips. In all cases, the cells were fixed with 2.5% glutaraldehyde in Sorensen's buffer for at least 24 hr. The preparations were then rinsed in buffer, dehydrated by graded alcohols, dried by the critical point technique, and coated with gold-palladium on a rotating-tilting stage. The samples were examined with an ETEC Autoscan (ETEC Corp., Hayward, Calif.). Peripheral blood mononuclear cells from ten normal volunteers were fixed in suspension and processed as previously described.

RESULTS

Surface Markers

Previous studies in our laboratory and elsewhere have demonstrated that EAC is bound to both B lymphocytes and peripheral blood monocytes. A small proportion (<5%) of E-rosette-forming cells (T cells) also bind EAC. IgG EA is bound preferentially to monocytes, but in concentrated cellular suspension as used in this study (i.e., incubation after centrifugation), some lymphocytes, mainly B lymphocytes, also bind IgG EA. Easily detectable surface immunoglobulin (SIg) is a B cell characteristic. T cells are identified by E-rosette formation. The percentages of cells in normal peripheral blood with each of these markers are listed in Table 2.

In Table 2, the cell surface markers of the seven cases studied are enumerated. In addition, the rosetted cell suspensions were examined on Wright-stained cytocentrifuge preparations for cytologic identification of the receptor positive and negative cells. In only one patient (D.J.) were T-cell markers demonstrated exclusively. The neoplastic cells from both lymph nodes and CSF formed only E rosettes. In two patients (L.K., R.B.) the neoplastic cells appeared to be null cells with no demonstrable markers. In two other patients (P.T., J.R.) the cells had receptors for EAC, but no other B-cell markers were present. Although in one patient (J.R.) the percentage of EAC-rosetting cells was low (30%), all such cells were cytologically neoplastic. In one patient (T.A.) the cells were demonstrated to bear simultaneously receptors for EAC and E, with 87% of the cells binding both $E_N(F)$ and $E_A(C)$ (Fig. 1). In another patient (P.B.) the neoplastic cells, as verified by cytocentrifuge preparations, also formed rosettes with both E and EAC. However, sufficient cells were not obtained to permit simultaneous assay for both markers.
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Fig. 1. A cell from patient T.A. simultaneously binding E7AC and EN(F). x 930. (A) Viewed by tungsten illumination, the adherent red cells surround the entire circumference of the cell. (B) Viewed by ultraviolet illumination, only the fluoresceinated red cells (EN(F)) are seen and form the upper border of the rosette.

Scanning Electron Microscopy

Although some variability in cellular size and surface morphology was observed in all cases, within each case the surface architecture was relatively consistent. Completely smooth cells predominated in one case (P.T.) in which the cells were fixed after filtration. In four cases in which the cells were fixed in suspension, the cells also had predominantly smooth surfaces, but with a few short stub-like projections or small blebs. In one case (D.J.) most cells had predominantly villous surfaces, with considerable variation in number of microvilli among individual cells. In patient J.R. there was also a predominant villous population, but numerous cells with ridges or blebs or with smooth or bumpy surfaces were also observed. Representative photographs of cells from four different patients are shown in Fig. 2.

Normal peripheral blood mononuclear cells fixed in suspension showed less than 4% smooth cells. The majority of the cells showed numerous microvilli; a few cells (6%) showed ruffles and ridges.

DISCUSSION

Our studies indicate a heterogeneity of surface membrane characteristics not previously reported in childhood lymphoblastic lymphoma. All cases published to date have shown the exclusive presence of T-cell markers with an absence of other markers. We had only one case in which this pattern was present. In two of our cases the cells appeared to be null cells with no detectable markers. Furthermore, four cases had complement receptors, a marker not previously described in this disease. In two of these cases receptors for both EAC and E were present, and in one such case it was possible to demonstrate both markers on the same neoplastic cells using a simultaneous assay procedure. In two cases receptors for EAC were present without other markers.
Although these data might suggest diverse origins for the tumors, this heterogeneity is more likely a consequence of neoplastic transformation and disordered differentiation of the cells. While receptors for EAC are usually a feature of B lymphocytes, monocytes, or granulocytes, the neoplastic cells had no other characteristics of the above cell types, based on studies of surface markers, cytochemistry, and ultrastructural surface morphology. Furthermore, there is evidence that certain abnormal T-cell populations may also bear complement receptors\(^{10,21}\) and still be demonstrated to be thymic-derived by virtue of E-rosette formation\(^{10,21}\) or lysis by an anti-human T-cell serum.\(^{22}\) In addition, the cells of patient P.T., although possessing complement receptors, still exhibited preferential localization to the thymic-dependent area of the lympho-
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reticular system. In a lymph node biopsy from this patient the cells diffusely populated the paracortex but spared the follicles. Lymph node biopsies of the other patients studied showed diffuse effacement, and preferential paracortical or follicular distributions could not be determined.

At least four cases of lymphoid neoplasia have been reported previously in which markers for both B and T lymphocytes were present simultaneously. In one case the T and B markers were present on two different populations, whereas in the other three the markers were present simultaneously on the same neoplastic cells. We present here a case (T.A.) in which a neoplastic lymphoid population has been demonstrated to bear both B- and T-cell markers. A small percentage of normal peripheral blood lymphocytes also bear both B- and T-cell markers, and these neoplasias may be derived from such cells. Alternatively, this duality may be a manifestation of neoplastic transformation with abnormal differentiation or derepression.

Cells from the seven cases were processed for scanning electron microscopy to compare surface morphology with immunologic markers. No correlations could be established, but within each case the surface morphology was relatively consistent. In patient P.T. the observed surface architecture (entirely smooth) may have been artifactual, since the cells were allowed to settle onto a solid substrate before fixation. This procedure may be responsible for rapid changes in surface morphology. In the remaining cases the cells were fixed in suspension, thus preventing subsequent architectural modifications. When normal human peripheral blood mononuclear cells are fixed and prepared under similar conditions, over 96% of the cells exhibit villous or ruffled surfaces, both in our laboratory and others. However, in five of our cases most cells had a surface morphology that departed significantly from the norm, being either predominately smooth or markedly irregular. These abnormalities in surface morphology may reflect lack of differentiation, high mitotic rate, neoplastic transformation, or other unknown factors.

In two cases (J.R., D.J.) the cells had predominantly villous surfaces. Contrary to our expectations from the studies of Polliack et al., one such case displayed T-cell markers exclusively. It was also noted that the two cases with predominantly villous cells were the only two in which the cells were obtained from lymph nodes. In the remaining cases the cells were obtained from either peripheral blood or bone marrow, or in one instance, pleural fluid. It is possible that microenvironment or other factors may alter the surface of neoplastic or normal cells.

The surface markers were also compared with clinical characteristics, including the presence or absence of a mediastinal mass; involvement of bone marrow, peripheral blood, or cerebrospinal fluid; and peripheral blast counts at the time of leukemic progression (Table 1). In spite of the heterogeneity of the surface markers and surface morphology, these cases were both clinically and cytologically a homogeneous group. The presence or absence of markers did not appear to have clinical significance in these patients, and did not correlate with any of the clinical parameters investigated. Thus, it should be cautioned that although T-cell markers may frequently be demonstrable in this disease, their presence or absence should not be used as a criterion for diagnosis.
It does appear from both previous clinical studies and these cases that the presence of a mediastinal mass is a major determining factor in predicting progression to leukemia. In all of our patients with mediastinal tumors followed for over 4 mo, bone marrow and peripheral blood involvement developed, usually within 1 yr. The one patient without a mediastinal mass has remained aleukemic and free of demonstrable marrow involvement for over 16 mo of follow-up. Contrary to the expected association between mediastinal tumor and T-cell markers, this one patient without a mediastinal mass was the only one in whom exclusively T-cell markers were demonstrated.

Childhood lymphosarcoma (CLSA) shares many cytologic and clinical features with a particular subgroup of acute lymphoblastic leukemia (ALL), in which the blast cells frequently can be demonstrated to form E rosettes. On the basis of both clinical and immunologic data, it has been suggested that these diseases are in fact closely related and may represent different clinical manifestations of the same neoplastic process. Although E rosetting was not a consistent feature in our series, the clinical similarities are maintained. Males predominated (6/7) with an age range older (12–23) than that in typical childhood ALL. Cerebrospinal fluid involvement was frequent and occurred in four of five patients followed over 4 mo. In fact, because of the high risk of developing involvement of the central nervous system (CNS), the last two patients studied were given prophylactic therapy to the CNS. A mediastinal mass was demonstrable in six of seven patients. The short survival times for these patients (Table 1) are also in keeping with the relatively poor prognosis of E-positive ALL. E-positive ALL has, in addition, been said to present with higher peripheral blast counts frequently greater than 100,000 cells/cu mm. Although leukemia did develop in four of our patients, their peripheral counts were not markedly elevated. This observation may reflect only a different distribution of disease; tissue involvement, particularly of lymph nodes, is a conspicuous feature of CLSA, whereas in ALL the cells populate primarily the bone marrow and peripheral blood. These differences in distribution may reflect subtle differences in these cells not yet detected.

The presence of complement receptors, identified in four of seven of our cases, is a feature not previously reported in CLSA and described in only one instance of ALL. Furthermore, in most large series of ALL studied for surface markers, their presence has not been investigated. The identification of complement receptors on the MOLT cell line (derived from ALL) suggests that they might be present more frequently than anticipated. In fact, in a recent study from one of the above groups, an addendum indicates that preliminary studies for detection of complement receptors have revealed at least three positive cases. However, whether this marker will have the same clinical relevance in ALL as the E-rosette phenomenon remains to be determined. It should also be emphasized that, although the cells from three cases demonstrated complement receptors, there was no supportive evidence to suggest that these cells were of B-lymphocytic origin, and, in fact, some evidence indicated a T-cell origin. The presence of complement receptors in neoplastic and reactive T cells requires further investigation.
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Addendum

Since submission of this manuscript, complement receptors have been reported on the neoplastic lymphoid cells of cases similar to ours by two other groups.38,39

ACKNOWLEDGMENT

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