Multimarker Classification of Acute Lymphoblastic Leukemia: Evidence for Further T Subgroups and Evaluation of Their Clinical Significance

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Leukemic blasts from 339 consecutive patients (226 children, 113 adults) with acute leukemia negative for peroxidase and unspecified esterase, were tested in each case for the following surface markers: sheep erythrocyte receptor (E-R.), T-cell antigens (HuTLA), surface immunoglobulin (Slg), and “common” non-T, non-B ALL antigen (cALLA). A clear distinction of 6 subgroups of patients with acute lymphoblastic leukemia (ALL) was possible: (1) common null-cell or cALLA (cALLA +) in 119 patients; (2) intermediate-cell or c/T-ALL (cALLA +, HuTLA +) in 87 patients; (3) pre-T-cell or pre-T-ALL (HuTLA +) in 47 patients; (4) T-cell or T-ALL (HuTLA +, E-R +) in 48 patients; (5) B-cell or B-ALL (Slg +) in 6 patients (another phenotype recently defined by Vogler et al. was recognized in some patients of the c-ALL group, but was not analyzed consistently); and (6) pre-B-cell or pre-B-ALL (cALLA +, cytoplasmic Ig +). In 32 leukemias no surface marker could be demonstrated. Particular emphasis was put on various T phenotypes. Consecutive typing of ALL thus helped to establish the frequency of two phenotypes which so far were thought to be rather rare: HuTLA occurring together with cALLA (group 2) and HuTLA occurring without E-R (group 3). The measurement of T-antigen amounts by quantitative immunonautoradiography disclosed the existence of phenotypes of non-E-rosetting T leukaemias with more T antigen per cell than found on normal or leukemic T lymphocytes expressing the E-R. ALL subdivision was further substantiated by additional markers such as terminal deoxynucleotidyl transferase, Fc receptors for IgG or IgM, C3 receptors on T and/or other lymphoblasts. The disappearance of the stimulatory capacity of allogeneic lymphocytes on the more differentiated T phenotypes was measured in mixed lymphocyte culture (MLC). A strong acid phosphatase reaction and a marked reaction for acid esterase were mostly found in pre-T- and T-ALL, whereas periodic acid Schiff staining prevailed in blasts with cALLA. An analysis of clinical data at presentation revealed significant differences for cell counts, age distribution, and organomegaly for the four main subgroups. The highest remission and lowest relapse rates were observed in the c/T-ALL group by using life-table analysis, whereas the response to treatment of the pre-T- and T-ALL groups was significantly poorer. We conclude that a combined use of a number of tests is essential to reach a precise diagnosis of subclasses of ALL with different biologic and prognostic characteristics.

IMMUNOLOGIC STUDIES using T- and B-lymphocyte markers have established the classification of three groups of acute lymphoblastic leukemia (ALL): one group with T-cell membrane phenotype, one minor group of B-cell origin, and a major group without any conventional markers of lymphoid cells. The latter group, which was mostly recognized per exclusionem and called null-cell or non-T, non-B ALL, was positively identified by the demonstration of a specific antigen. Nevertheless, the origin of the malignant cells in this group, which includes more than 70% of the cases, remained questionable. The demonstration of Ia-like determinants that are expressed by B cells, but also by some cells of the myeloid series and by a subset of T cells, left the question as to the true cellular origin of non-T, non-B ALL unsettled. Recently, a differentiation along the B-cell axis was suggested in 4 of 22 patients by the demonstration of small amounts of cytoplasmic IgM in the lymphoblasts. A fourth group of ALL was thus defined, namely pre-B-cell leukemia, which dissected the pool of unclassified ALL. Several findings, however, suggest that in many patients non-T, non-B ALL cells may represent T-cell progenitors, for example, the demonstration of a high content of the leukemic cells for a peculiar DNA polymerase, the terminal deoxynucleotidyl transferase (TdT), shown to be restricted to the thymus and to a prothymocyte population in the bone marrow. Investigations with anti-T-cell sera have so far disclosed reactions with ALL cells that did not form spontaneous rosettes with sheep erythrocytes (E). Since the E-receptor is generally accepted as a differentiation marker of mature T lymphocytes, an incomplete T-phenotype was assumed in those cases. The aim of this study is to provide a detailed analysis of a great number of patients with acute leukemias by using various membrane markers together with routine morphology, cytochemistry, enzyme markers, and antigenic stimulation to allogeic lymphocytes. The reliability of individual tests has been critically evaluated, and the relative impor-
tance and specificity of individual markers was assessed. Special attention was paid to the recording of the expression of two independent T-cell differentiation markers, namely E-receptor and T-cell antigens, together with the cALL-antigen of non-T, non-B ALL blasts, which were investigated in every patient included in the study. The reaction with anti-T in a considerable part of common ALL with lymphoblasts identified by the ALL-antigen disclosed a new subgroup in which a differentiation versus the T-axis is suggested. In another part of acute leukemias, anti-T did provide a means for a prompt and reliable diagnosis of ALL also in those instances where leukemic cells were devoid of any other marker. The immunologic ALL subdivision is related to clinical data at presentation and to the response of polychemotherapy in children.

MATERIALS AND METHODS

Patients

From January 1977 through December 1979, we received heparinized blood and bone marrow samples from patients admitted to 12 hospitals in Munich and West Germany, that were participating in the study. The samples were taken from newly presented and untreated cases of acute leukemia. This study did not include samples of myelomonocytic leukemias, of preleukemic states, or of other bone-marrow-infiltrating tumors. Long-term lymphomatous disorders terminating in a leukemic phase, such as lymphosarcoma of adults, were also excluded. The hematologic diagnosis was made by the hematologists of the referring hospital, including routine morphology and cytochemistry. In each case, a May-Grünwald-Giemsa stained film was checked in addition by one of us (D.H.) who was unaware of the results of the immunologic typing. The patients tested were divided into adults (more than 15 yr old) and children.

Two chemotherapeutic regimens were used for the remission induction in children. Each protocol contained prednisone, vincristine, asparaginase, doxorubicin, cyclophosphamide, cytosinarabinoside, methotrexate, and 6-mercaptopurine. Allocation to one of the two protocols was at random. Central nervous system (CNS) prophylaxis was given in the form of 2400 rad to the cranium and 5 intrathecal injections of methotrexate.

Postnatal human thymus glands were obtained from children undergoing open heart surgery. Fetal thymuses were obtained from therapeutic abortions.

Cytotoxic Studies

Blood films and bone marrow smears were stained with May-Grünwald-Giemsa and periodic acid Schiff (PAS). Cytotoxic reactions were performed by standard techniques for the demonstration of acid phosphatase, acid esterase, naphthol-AS-acetate-esterase, and peroxidase.

Sample Preparation

Lymphocytes and blast cells from both the marrow and the blood were isolated by Ficoll-Isoopaque density gradient centrifugation, washed twice in Hanks' balanced salt solution (HBSS), and incubated at 37°C for 45 min in HBSS supplemented with 10% fetal calf serum to remove cytophilic antibody. Cells from solid tissue were prepared by teasing and filtration through a nylon mesh. Although in leukemic patients a high percentage of abnormal cells was usually present in the final cell suspension, careful examination of the cells was of utmost importance when residual T or B lymphocytes constituted a fair percentage of the final cell suspension. Bone marrow cell suspensions containing over 90% blasts were preferably examined. Cells that were not used for the basic marker program were frozen for further studies in a fractionated manner (−1°C/min) using media containing 10% dimethylsulfoxide and 10% fetal calf serum. The cells were kept in liquid nitrogen at −190°C.

Rosette Tests

Spontaneous rosette formation with untreated (E) or with S-(2-amino-ethyl)isothiouronium bromide hydrobromide (AET) treated sheep erythrocytes was performed according to Jondal et al. and Kaplan and Clark. In each case, both tests were performed. The modified test using AET-treated erythrocytes is more sensitive, and the rosettes formed are very stable and resistant to disruption by routine handling. The advantage of the original E-test is the possibility to judge the size and morphology of the rosette-forming cells after adding methylene blue. Wright-stained slides were prepared if more than 5% of the cells formed rosettes. E-receptors stable at 37°C were investigated by incubation of the rosetting mixture at 37°C for 1 hr. Spontaneous rosette formation with mouse red blood cells was performed according to Forbes and Zalewski.

C3 receptors were studied in a rosette assay using ox red blood cells (Eox) sensitized with an IgM-rich preparation of a rabbit anti-ox red blood cell serum and fresh AKR/J mouse serum as the source of complement, as described. Such Eox ACmo were shown to carry primarily C3d. Complement coating was monitored by rosette formation with Daudi cells, which are known to react with C3d. A mixed rosette assay using glutaraldehyde-fixed erythrocytes sensitized with 19s antibody-plus-mouse complement was performed as described. Fixed erythrocytes are easily recognized by staining with methylene blue. For demonstration of strong receptors for the Fc part of IgG (which are common on monocytes, some B-cells, and myelocytic cells), we applied an EA-rosetting test as described. Weak receptors for the Fc part of rabbit IgM or IgG were demonstrated according to Moretta et al. by rosette tests with ox red blood cells coated with a high concentration of antibody just below the agglutination point.

Antisera

Anti-human T-cell globulin was prepared from rabbit anti-human thymocyte globulin by extensive absorption with liver-kidney homogenate, chronic lymphatic leukemia cells of B-cell type obtained by a cell separator, and B-lymphoblastoid cell lines. The absorption and subsequent purification procedures and detailed investigations on the specificity and the reaction pattern have been published previously. Similarities and combined studies revealed that the antiseraum does not react with Ig-bearing cells, with myelocytic cells, with monocyctic cells, with brain cells, or with any other tissue cell except cells of the T-cell series. In functional tests, a cross-reaction with hemopoietic progenitors of the myelopoietic and erythropoietic series was also excluded. Immunoprecipitation studies have shown that the antibodies specifically precipitate four glycoproteins (GP 165, GP 160, GP 45, GP 25) from surface-labeled T lymphocytes and thymocytes. Anti-cALL globulin was prepared according to Greaves et al. by immunizing rabbits with non-T, non-B ALL cells coated with unabsorbed antithymocyte globulin. A quantity of 107 cells were injected subcutaneously and intravenously on days 14, 15, 16, and 23. Absorption and fractionation procedures and specificity testing were performed as described. Additional absorptions with a
lymphoblastoid B-cell line removed the remaining weak activities with myelopoietic and erythropoietic progenitors, the number of CFU-C-forming progenitors not being affected.⁸

Ig determinants were detected by using a polyvalent rabbit anti-human immunoglobulin. For surface immunoglobulin (SIg) studies, a directly fluoresceinated F(ab')₂ fragment was employed and proved to be especially useful for investigations of cell suspensions contaminated by myelomonocytic cells. Cytoplasmic immunoglobulins (Clg) were demonstrated after fixation of spread cells in cold methanol for 5 min, rehydration in phosphate-buffered saline (PBS), and incubation with anti-Ig reagents in a moist chamber according to Vogler et al.¹² The fluoresceinated goat anti-IgM antibody used for this purpose had a log 2 titer of 23 with IgM-coated erythrocytes in the passive hemagglutination test at a concentration of 1 mg/ml. Clg was tested only in some cases (see Results).

Serologic Test Systems

Cytotoxicity of the IgG-fractions of anti-T and anti-cALL sera was evaluated at various antisera dilutions by employing the complement-dependent dye-exclusion microtest as described by Mittal et al.⁹ This test, which needs only 5 x 10⁴ cells, was performed in all cases. For anti-T, plateau values of over 90% up to a titer of 1:256 were recorded for thymus cells and of 70–90% up to a titer of 1:64 for blood lymphocytes. Common ALL cells were lysed in general to 90% at a 1:128 titer of anti-cALL globulin. For immunofluorescence studies, we used the fluoresceinated IgG-fraction of a goat anti-rabbit F(ab')₂ antiserum. Indirect immunofluorescent stainings were performed after incubation with pepsin digest F(ab')₂, fragment preparations of rabbit antisera (anti-T, anti-cALL). Incubations were done at 4°C and were followed by three washings with HBSS containing 5% bovine serum albumin (BSA), and Na-azide. Suitable concentrations of reagents were established by testing T lymphocytes, thymus cells, common ALL cells, and CLL cells as negative controls. For evaluating stained cells, we used a Leitz Orthoplan microscope equipped with Ploem epi-illumination.

Quantitations of antibodies bound to cell samples were performed in some cases by complement fixation reaction according to a micromethod adapted to mononuclear cells as antigens.²⁸ The amount is measured by photometry of the hemolysis of sensitized sheep erythrocytes.

Quantitations of antibodies bound per single cell were performed in some cases by using microphotometry of silver grain densities on cells in autoradiographs after incubation with radiiodinated antibodies as described.¹¹ By comparing the grain densities over the cells and over a standard exposed simultaneously on the same slide, we obtained a calculation of absolute amounts of radioactivity.¹² Double-labelings were performed by mixing the cells with iodinated and peroxydase-conjugated antibodies. Autoradiography and counterstaining for peroxydase provided a persistent demonstration of two possible antigens on single cells.

Terminal Deoxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (TdT) enzyme levels were measured as described.¹³ A quantity of 5–10 x 10⁶ leukocytes were used in each test. Coded samples were sent in frozen state from Munich to London without communicating the results of immunologic and cytochemical studies.

Mixed Lymphocyte Culture

The stimulatory capacity of lymphoblasts of 21 ALL cases was investigated in a micromethod of the mixed lymphocyte culture (MLC) test. Peripheral blood lymphocytes of five unrelated HLA-D different blood donors served as responders. ALL lymphoblasts that were cryobiologically frozen at diagnosis, were thawed and x-irradiated with 2500 R (stimulating cells). A quantity of 10⁵ responding cells were mixed with 10⁴ stimulating cells in cell culture medium (RPMI 1640 plus antibiotics) enriched with 10% human AB-serum in round-bottom microtiter plates. After 96-hr incubation (5% CO₂ and 90% humidity) the cell mixtures were labeled with 1 μCi [³H]-thymidine (specific activity 5 Ci/m mole) for a further 16 hr. The triitated thymidine incorporation of triplicate cultures (arithmetic mean) in the specific MLC combinations was related to the response towards a pool of x-irradiated lymphocytes from 10 unrelated blood donors, which was taken as 100% relative response (RR). In preceding experiments it was established that MLCs between peripheral blood lymphocytes and ALL lymphoblasts exhibit an identical time course kinetic with regard to [³H]-thymidine incorporation as MLCs between lymphocytes from healthy donors.

Evaluation and Statistics

Statistical analysis of hematalogic data was performed by using Kruskal-Wallis one-way analysis of variance test results. The level of significance was calculated using normal two-tail approximation. For comparing clinical signs and remission rates, the Fisher exact test (two-tail) or chi-square test were used. Life-table analysis was performed by using the method of Cutler and Ederer.²⁴

RESULTS

Immunologic ALL Subset Classification

Two-hundred and twenty-six children and 113 adults over 15 yr with newly diagnosed acute lymphoblastic (ALL) or acute undifferentiated leukemia (AUL) were examined. All patients had replacement of normal bone marrow elements by leukemic cells that lacked the morphological criteria of myeloic or monocytic cells and were negative for peroxidase and unspecific esterase. We classified each case on the basis of blast expression of receptors for sheep erythrocytes (E-R), T-cell differentiation antigens (HuTLA), surface Ig (SIg), and common ALL antigen (cALLA). The results are given in Table I. In 32 patients, no marker was demonstrated. Such unclassified leukemias (AUL) were more frequent in adults, where they occurred in 23 of 113 patients, whereas only 9 of 216 children had AUL. This difference is statistically significant using the χ²-test. It should be noted that the clinical diagnosis of acute undifferentiated leukemia (AUL) based on morphological and cytochemical criteria (negative for peroxidase, NAS, and coarse granular PAS) was made more often. As shown in Table 2, 77 of 106 samples from 59 adults and 47 children that were labeled with AUL as the clinical diagnosis were typed as ALL by immunologic criteria, namely by the demonstration of E-R, HuTLA, SIg, or cALLA. Vice versa, only 3 patients with the clinical diagnosis of ALL lacked immunologic markers characteristic of ALL. In these patients, the diagnosis was mainly based on PAS-reaction, which is per se not unequivocal. Table 1 shows furthermore
that in 119 patients, only cALLA was demonstrated on the lymphoblasts. This phenotype, which is characteristic of the common ALL without B- or T-cell markers,\(^5\) prevailed, being recorded in 38% of the children and 30% of the adults. In a considerable number of other cases, namely in 87 patients, an additional reaction of the blast cells with anti-T was recorded. An intermediate phenotype of blast cells expressing both cALLA and T antigens was therefore assessed in 87 of 206 patients with cALLA-positive leukemia. This ALL subgroup, named c/T-ALL, was found in 30% of the children and 15% of the adults. This difference is statistically significant using the \(\chi^2\)-test.

In order to demonstrate that both antigens were expressed by single lymphoblasts, a double-labeling method using iodinated anti-cALL globulin and peroxidase-conjugated anti-T globulin was performed. In the autoradiographs counterstained for the enzyme, the occurrence of the intermediate cell phenotype was established, as shown in Fig. 1. Lymphoblasts with this phenotype prevailed in c/T-ALL, whereas blasts with T-cell antigen only or with cALLA only were demonstrable in low numbers.

E-rosette formation of the blast cells was observed in 48 patients. Of each specimen, an additional test using AET-treated erythrocytes was performed. With AET-treated erythrocytes, even larger proportions of blasts were capable of forming rosettes. This was especially helpful when the samples were tested after post handling. For discrimination from contaminating T lymphocytes, an arbitrary limit of 10% rosettes was applied for marrow samples, whereas E-rosettes of the blood were only considered in the situation of high leukocyte counts. In rare equivocal cases, the examination of Giemsa-stained preparations helped to confirm that lymphoblasts were the only cells that formed rosettes. In addition, the strong reaction with anti-T, giving brilliant rings in immunofluorescence and lysis at low antibody concentrations in cytotoxicity, also helped to discriminate T-blast cells from T lymphocytes. In all cases that were E-positive, a strong reaction with anti-T was characteristic. This subgroup with E-R and HuTLA is called T-ALL in this study. In some cases, E-R was demonstrable after 1 hr of incubation at 37°C. The latter subtype is also named Thy-ALL.

Lymphoblasts that expressed only T-cell antigens without an E-receptor were recorded in a relatively large number of cases, i.e., in 47 patients. In adults, 9 of 19 patients with blast cells of this phenotype were previously diagnosed as AUL according to hematologic criteria. The same number of children was typed for this so-called pre-T-cell form of ALL with incomplete T-cell phenotype as for the T-ALL group with both T-cell markers. Cases that had up to 10% E-rosetting cells were also included in this subgroup, since the phenotype of lymphoblasts that expressed only T-cell antigen prevailed in those cases.

In five patients, easily detectable surface immunoglobulins (Slg) were demonstrated on the blast cells. Ig-typing, which was performed as described,\(^23\) showed that in 4 cases monoclonal IgM of the lambda-type was expressed. In one child with monoclonal SlgM of high density providing a ring-like staining of 80% of the cells in immunofluorescence, a simultaneous expression of cALLA was demonstrated on 90% of the blasts. This phenotype was in contrast to the very faint surface stainings for Slg on a minority of

<table>
<thead>
<tr>
<th>Major Groups</th>
<th>Total (n = 339)</th>
<th>Children (n = 226)</th>
<th>Adults* (n = 113)</th>
<th>Phenotypic Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cALLA</td>
</tr>
<tr>
<td>AUL</td>
<td>32 (9%)</td>
<td>9 (4%)</td>
<td>23 (20%)</td>
<td>-</td>
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<tr>
<td>ALL</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1. cALL</td>
<td>119 (35%)</td>
<td>86 (38%)</td>
<td>33 (29%)</td>
<td>+</td>
</tr>
<tr>
<td>2. c/T-ALL</td>
<td>87 (26%)</td>
<td>70 (31%)</td>
<td>17 (15%)</td>
<td>+</td>
</tr>
<tr>
<td>3. Pre-T-ALL</td>
<td>47 (14%)</td>
<td>29 (13%)</td>
<td>19 (17%)</td>
<td>-</td>
</tr>
<tr>
<td>4. T-ALL</td>
<td>48 (14%)</td>
<td>29 (13%)</td>
<td>19 (17%)</td>
<td>-</td>
</tr>
<tr>
<td>5. B-ALL</td>
<td>5 (1%)</td>
<td>3 (1%)</td>
<td>2 (2%)</td>
<td>-</td>
</tr>
</tbody>
</table>

* > 15 yr.
† In one child, cALLA was demonstrated in addition to Slg.

Table 2. Comparison of Clinical and Immunologic Diagnosis of ALL and AUL in Acute Leukemias Negative for Peroxidase and Unspecific Esterase

<table>
<thead>
<tr>
<th>Patients</th>
<th>Clinical Diagnosis</th>
<th>Immunologic Diagnosis</th>
</tr>
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<tbody>
<tr>
<td>59 Adults</td>
<td>AUL*</td>
<td>38 ALL + 21 AUL†</td>
</tr>
<tr>
<td>54 Adults</td>
<td>ALL</td>
<td>52 ALL + 2 AUL</td>
</tr>
<tr>
<td>47 Children</td>
<td>AUL</td>
<td>39 ALL + 8 AUL</td>
</tr>
<tr>
<td>169 Children</td>
<td>ALL</td>
<td>168 ALL + 1 AUL</td>
</tr>
</tbody>
</table>

*Clinical diagnosis: acute undifferentiated leukemia, based on hematologic criteria.
†Immunologic diagnosis: acute unclassified leukemia (negative for E-R, Slg, T, cALLA).
MULTIMARKER CLASSIFICATION OF ALL

Fig. 1. Lymphoblasts of a patient with intermediate c/T form of ALL. The cells had been incubated with radioiodinated anti-cALL globulin and peroxidase-conjugated anti-T globulin. After enzyme reaction, autoradiographic procedure, and Hemalaun-staining, three kinds of cells were demonstrable: radioactive-labeled or cALL + (asterisk), enzyme-labeled or HuTLA +, and double-labeled or cALL + HuTLA + (arrow) blasts. The latter phenotype prevailed in the c/T form of ALL.

blast cells in some patients of the c-ALL group. Those barely detectable SIg stainings were not considered for the classification of this study. Likewise, demonstrations of cytoplasmic Ig(clg), which is characteristic of the pre-B-cell type of ALL,12 were not included, since only some of the patients were investigated.

Cytologic and Cytochemical Studies

Consistent morphological criteria related to the immunologic classification were not observed. Applying the FAB-classification,35 which discriminates three types (L1, L2, and L3) of ALL according to the degree of heterogeneity in the distribution among the leukemic cell population and the occurrence of individual cytologic features, we classified 40% of the cALL, 51% of the c/T-ALL, 38% of the pre-T-ALL, and 47% of the T-ALL as L1; 57% of the cALL, 45% of the c/T-ALL, 62% of the pre-T-ALL, and 47% of the T-ALL as L2. The five B-cell ALLs were all of the L3 type. The L1 type was rare in the other subgroups (3% of the cALL and 3% of the c/T-ALL).

The results of cytochemical studies are given in Table 3. The acid phosphatase reaction, assumed to label T-ALL,36 prevailed in the T and pre-T subgroups. However, a considerable number of leukemias with cALL-antigen also showed reactions to this enzyme. Positivity for PAS was recorded in the opposite way. The groups with cALL antigen more often had blasts with PAS than the subgroups negative for cALLA. Acid esterase, an enzyme assumed to label some cells of the T-cell series,37 was distributed in a similar way as acid phosphatase.

Terminal Deoxynucleotidyl Transferase

The results of TdT determinations are given in Fig. 2. Thirty-eight patients in the study were examined in comparison to six additional patients with other hematologic malignancies, namely acute myelomonocytic leukemia (AMML), chronic lymphocytic leukemia of the B-cell (B-CLL) and T-cell type (T-CLL), and prolymphocytic leukemia of the T-cell type (T-PL). TdT was detectable in 32 of 38 patients with ALL or

| Table 3. Cytochemical Reaction Pattern in Immunologic ALL Subgroups |
|-------------------|-----------------|------------------|
| ALL Subgroup      | PAS*            | Acid Phosphatase†| Acid Esterase† |
| cALL              | 13/33†          | 10/33            | 7/28           |
| c/T-ALL           | 10/33           | 11/32            | 7/29           |
| Pre-T-ALL         | 0/12            | 10/12            | 10/12          |
| T-ALL             | 2/15            | 13/15            | 7/14           |
| Significant       |                 |                  |                |
| differences§      |                 |                  |                |
| c-vs pre-T-ALL    |                 | c-vs pre-T-ALL   | c-vs pre-T-ALL |
| c-vs T-ALL        |                 | c-vs T-ALL       | c/T-vs pre-T-ALL|
| c/T-vs pre-T-ALL  |                 | c/T-vs T-ALL     |                |

*Coarse granular.
†Distinct positivity, granular, and paranuclear pattern of staining.
‡Positive cases/cases investigated.
§According to the χ² test.
AUL. There was a wide range of TdT activity in the various ALL subgroups. The mean value of TdT levels was highest in cALL, followed by c/T-ALL, T-ALL, and pre-T-ALL, while the single case with B-ALL was negative. It is interesting to note that the values for cALL were clustered in two groups, one with high levels and one with lower levels. Two cases in the former group, which were tested for cytoplasmic Ig, were negative, whereas one case tested in the low TdT group was clg-positive.

**Mixed Lymphocyte Culture Studies**

The blastogenic response of normal lymphocytes pooled from five donors to the different types of allogeneic lymphoblasts is shown in Fig. 3. The RR values produced by lymphoblasts with cALL antigen was significantly higher than the response induced by lymphoblasts without cALL antigen belonging to the pre-T and T-ALL subgroups \((p < 0.01)\). A relatively wide distribution of stimulation rates was recorded when c/T-lymphoblasts of various patients were used as stimulators. Quite different stimulation rates were obtained when chronic lymphatic leukemia cells (CLL) of the B or T type were used as allogeneic stimulators, where were investigated in comparison. The CLL cells were typed for B- or T-cell origin as described. \(^{38}\) Rosette formation was not observed in ALL with T antigen. However, in 2 of 16 patients having blasts with cALLA only, a rosetting phenomenon was observed with 11% or 27% blast cells from the marrow. In one case, cytoplasmic Ig was investigated and proved to be positive. The expression of receptors for complement protein—a feature that was formerly believed to label B but not T lymphocytes\(^{41}\) but was later demonstrated on a minority of T cells\(^{41}\)—was investigated in 210 patients. Here, it is interesting to note that this receptor was more prominent in cases without cALL antigen but with T markers (18 positive of 64), whereas lymphoblasts with cALL antigen and C3-R were demonstrated only in 14 patients of 132 (see Table 4). In mixed rosette assays, the simultaneous expression of E-R and C3-R on the same lymphoblast has already been reported previously.\(^{26}\)

Receptors for the Fc fragment of IgG, which are strongly expressed on B lymphocytes\(^{42}\) but also on other lymphatic cells,\(^{43}\) were investigated in 210 ALL patients. Those receptors (Fc-R) were demonstrable in 57 patients distributed among all subgroups (see Table
Fig. 3. Blastogenic response of normal lymphocytes pooled from five donors to the different phenotypes of ALL lymphoblasts in mixed lymphocyte culture (MLC). Each point represents the arithmetic mean of triplicate cultures with one ALL sample related to the response towards a pool of x-irradiated lymphocytes of 10 unrelated blood donors, which was taken as 100% relative response (RR). Cultures with CLL lymphocytes of B or T type were performed in comparison.

4). Recently developed techniques\textsuperscript{27} that demonstrate weak receptors for the Fc fragment of IgM or IgG occurring on T\textsuperscript{27} and B lymphocytes\textsuperscript{44} were applied in 13 patients in the study. As shown in Table 4, those low-affinity rosette-forming blasts were found in 6 patients for IgM and 10 patients for IgG. Fc IgM receptors were less pronounced in T-differentiated ALL.

In order to analyze the expression of T markers in the various forms of ALL with T differentiation, a quantitative investigation of single cells was undertaken by applying quantitative immunoautoradiography.\textsuperscript{31,32} This method provides the possibility to measure the number of antibodies bound to a single cell. The binding of anti-T was investigated in 18 ALL patients. The results are compared with measurement in 4 patients with T-CLL, in 12 normal donors for blood T lymphocytes, and in 8 thymus specimens from children and fetuses of less or more than 12 wk gestation (see Table 5).

Three categories with weak, medium, and strong expression of T antigen were selected for this purpose. T lymphocytes of the blood had an average binding of $157 \times 10^3$ antibodies/cell, the mean values of 200 measured cells ranging between $124 \times 10^3$ and $172 \times 10^3$ for 12 donors. Nearly twice this number of anti-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Samples & Cytoplasmic Ig & E mouse-R & C3-R & Fc-R & Fc IgM-R\textsuperscript{†} & Fc IgG-R\textsuperscript{†} \\
\hline
CALL & 4/19\textsuperscript{§} & 2/16 & 9/74 & 13/74 & 3/4 & 3/4 \\
\hline
c/T-ALL & 5/16 & 0/8 & 5/68 & 15/68 & 1/3 & 2/3 \\
\hline
Pre-T-ALL & 0/8 & 0/2 & 5/28 & 12/28 & 1/3 & 3/3 \\
\hline
T-ALL & 0/9 & 0/3 & 13/36 & 14/36 & 1/3 & 2/3 \\
\hline
B-ALL & 0/2 & 0/2 & 3/4 & 3/4 & NT & NT \\
\hline
B-CLL & 0/11 & 40/47 & 40/62 & 62/65 & 4/4 & 4/4 \\
\hline
T-CLL & NT & 0/8 & 1/8 & 2/8 & 3/3 & 2/3 \\
\hline
\end{tabular}
\caption{Expression\textsuperscript{•} of Various Markers in Different ALL Subgroups and in CLL of B- or T-Cell Type}
\end{table}

\textsuperscript{*} The threshold for a positive label was a reaction in more than 10% cells for a marker.
\textsuperscript{†} Indicator erythrocytes coated with high concentration of antibody.
\textsuperscript{\ddagger} Positive cases/cases investigated.
\textsuperscript{§} In this case, only 42% of blasts reacted with anti-T.
Table 5. Intensity of T-Cell Marker Expression on Leukemia Cells of Various Forms of T-Differentiated ALL and of T-CLL in Comparison to Normal Cells of the T-Cell Series

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. Investigated</th>
<th>No. of Mol. Anti-T Antibody/Cell*</th>
<th>E-R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50-100 x 10^3</td>
<td>100-200 x 10^3</td>
</tr>
<tr>
<td>c/T-ALL</td>
<td>5X</td>
<td>XX†</td>
<td>XXX</td>
</tr>
<tr>
<td>Pre-T-ALL</td>
<td>6X</td>
<td>X</td>
<td>XXXX</td>
</tr>
<tr>
<td>T-ALL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subtype A</td>
<td>5X</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Subtype B</td>
<td>3X</td>
<td>0</td>
<td>XX</td>
</tr>
<tr>
<td>T-CLL</td>
<td>4X</td>
<td>XXX</td>
<td>X</td>
</tr>
<tr>
<td>T lymphocytes (blood)</td>
<td>12X</td>
<td>0</td>
<td>12X</td>
</tr>
<tr>
<td>Thymocytes</td>
<td>4X</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fetal thymocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12 wk</td>
<td>2X</td>
<td>XX positive†</td>
<td></td>
</tr>
<tr>
<td>&gt;12 wk</td>
<td>2X</td>
<td>XX positive†</td>
<td></td>
</tr>
</tbody>
</table>

* Measured by photometric immunautoradiography. Mean values of 200 cells were recorded for each sample.
† Each X represents the mean value of the sample of one patient.
‡ Tested only by immunofluorescence.

bodies were bound to thymocytes, which had an average of 296 x 10^3 molecules on the surface as measured in 4 samples. A relatively low number of antibodies was bound on T cells of T-CLL, as already described. Lymphoblasts of intermediate c/T phenotype had a wide range of low and medium amounts of T antigens, whereas common ALL cells were completely negative (not shown in Table 5). Lymphoblasts of the pre-T-cell type had somewhat more antigens, most of them in the medium range. ALL patients investigated with

Fig. 4. Age and sex distribution in 300 patients with newly diagnosed ALL. A subgrouping according to the immunologic phenotype of the leukemia cell is performed.
Table 6. Relationship Between Immunologic ALL Type and Clinical Signs at Diagnosis for Children With ALL

<table>
<thead>
<tr>
<th>Feature</th>
<th>Total</th>
<th>cALL</th>
<th>c/T-ALL</th>
<th>Pre-T-ALL</th>
<th>T-ALL</th>
<th>Significant Differences*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>132</td>
<td>57</td>
<td>37</td>
<td>15</td>
<td>23</td>
<td>—</td>
</tr>
<tr>
<td>Mediastinal enlargement</td>
<td>13</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>9</td>
<td>T- vs c; c/T-; pre-T-ALL</td>
</tr>
<tr>
<td>Lymph node enlargement</td>
<td>72</td>
<td>35</td>
<td>14</td>
<td>10</td>
<td>13</td>
<td>c- vs c/T-ALL</td>
</tr>
<tr>
<td>Spleen enlargement</td>
<td>87</td>
<td>37</td>
<td>20</td>
<td>11</td>
<td>19</td>
<td>c/T- vs T-ALL</td>
</tr>
<tr>
<td>Liver enlargement</td>
<td>94</td>
<td>39</td>
<td>24</td>
<td>11</td>
<td>20</td>
<td>c- vs T-ALL</td>
</tr>
<tr>
<td>White blood count (x 10^9/liter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>54</td>
<td>33</td>
<td>20</td>
<td>82</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;100</td>
<td>21</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>12</td>
<td>c- vs T-ALL</td>
</tr>
<tr>
<td>10–99</td>
<td>51</td>
<td>24</td>
<td>15</td>
<td>7</td>
<td>5</td>
<td>c/T- vs T-; pre-T-ALL</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>29</td>
<td>21</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin t (g/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;9</td>
<td>47</td>
<td>22</td>
<td>11</td>
<td>2</td>
<td>12</td>
<td>c/T- vs T-ALL</td>
</tr>
<tr>
<td>&lt;9</td>
<td>69</td>
<td>30</td>
<td>22</td>
<td>9</td>
<td>8</td>
<td>Pre-T- vs T-ALL</td>
</tr>
<tr>
<td>Platelet count t (x 10^9/liter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;90</td>
<td>32</td>
<td>8</td>
<td>11</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>&lt;90</td>
<td>88</td>
<td>45</td>
<td>24</td>
<td>7</td>
<td>12</td>
<td>c- vs c/T-ALL</td>
</tr>
</tbody>
</table>

*Fisher exact test for organ signs; Kruskal-Wallis one-way analysis of variance test results was used for hematologic data.
†Data from 116 patients.
‡Data from 120 patients.

E-rosettes at 37°C had large amounts of T antigens with an average of 265 × 10^3, which is quite similar to the figure determined for thymocytes. The avid E-receptor was also demonstrated on thymus cells. In some cases of this T-ALL subgroup, a C3-R was demonstrable. C3-R were also expressed by lymphoblasts of the pre-T group and by fetal thymocytes up to the 12th week of gestation. The latter cells resembled pre-T lymphoblasts by having T antigens without an E-receptor, as demonstrated by immunofluorescence in two samples.

Clinical Studies

The age and sex distribution of the patients are given in Fig. 4 on the basis of the ALL subgroup. T-ALL did not occur in patients over 35 yr. A great majority of the patients with T-ALL were in between 10 and 20 yr, whereas the other forms had a peak incidence around 5–20 yr. The male sex predominated in the forms without cALL antigen, i.e., the pre-T and T-ALL. Differences in sex and age distribution were statistically significant between cALLA-positive and cALLA-negative ALL.

The relationship between immunologic classification and clinical and hematologic data upon presentation of the disease was investigated in the first 132 children in the study. The results are given in Table 6. Thirteen children had a predominantly anterosuperior mediastinal widening on their admission chest x-rays, 9 of those patients being affected by T-ALL, 2 by pre-T-ALL, and 2 by c/T-ALL. This symptom was therefore significantly associated to T-ALL with E-R. Lymph node enlargement was less pronounced in children with c/T-ALL compared to those with c-ALL. A splenomegaly was evident in most children with T-ALL, but significantly less pronounced in c/T-ALL. Liver enlargement was significantly less often found in c-ALL compared to T-ALL. The mean values of leukocyte counts were more elevated in pre-T-ALL and T-ALL. The statistical analysis of the hematologic data of all patients in the Kruskal-Wallis test revealed significant differences according to...
immunologic subgroup for leukocytes, platelets, and hemoglobin, as shown in Table 6.

The response to treatment was analyzed by the life table method in 127 children treated with two different 8-drug protocols, as shown in Table 7. Allocation to one of the protocols was at random and depended on the hospital of admittance. The c/T-ALL form had the highest remission rate, followed by c-ALL, T-ALL, and pre-T-ALL. The difference in the remission rate was significant between c/T and pre-T-ALL according to the $\chi^2$-test. The rate of relapse was lowest in c/T-ALL followed by c-ALL, pre-T-ALL, and T-ALL. Disease-free survival curves observed in the life-table analyses are plotted in Fig. 5 according to the immunologic subset. The difference in disease-free survival between the c/T- and pre-T or T-ALL patients shown in Fig. 5 is statistically significant using the $\chi^2$-test. The differences between cALL and pre-T or T-ALL were not significant.

**DISCUSSION**

The experimentally reproducible identification of similar cell surface markers on acute lymphoblastic leukemia (ALL) cells by immunologic methods has allowed classification of this malignancy into T-cell, so-called null-cell (non-T, non-B), and pre-B-cell or B-cell types. In addition to disclosing that ALL is an very heterogeneous disorder, such an immunologic classification has proved to be of prognostic significance. The majority of ALL, however, still cannot be clearly allocated to T or B differentiation. Comparative studies have demonstrated that the phenotype of these cells resembles early lymphatic progenitors demonstrable in rare numbers in the bone marrow and fetal organs. In this way, a pre-B-cell feature of the leukemia cells has been recognized in some of patients belonging to the null cell group.

On the other hand, several findings suggest that in many patients, non-T, non-B ALL cells may represent T-cell progenitors. In addition to the biochemical demonstration of the thymic enzyme terminal transferase in ALL, a reaction of samples that were E-receptor-negative with antithymocyte sera favored the assumption of a T differentiation. In this study, a division of those leukemias into two subgroups was performed by using specifically absorbed heterologous antisera against thymus cells and ALL cells devoid of B and T markers. One group of leukemias was thus defined by reaction of the lymphoblasts with both antisera, and another group by lymphoblasts reacting with anti-T only.

In some cases of the latter group, C3 receptors were demonstrable. The phenotype therefore resembles fetal thymus cells, up to the 12th week of gestation, which were E-negative but did react with anti-T and had C3-R. C3-R have already been demonstrated in fetal thymus. It is interesting to note that C3-R was also demonstrated on some ALL cells with E-R, whereby the expression of C3-R was interpreted as a label for a T-precursor cell attribute. As shown in Table 5, the antigenic quantities were quite similar in the case of T lymphoblasts with E-R at 37°C and in the case of thymus cells. A pre-T-cell attribute of the T-positive E-R-negative cell phenotype has been demonstrated in a fraction of bone marrow lymphocytes and in a minority of blood lymphocytes that
can be induced to rosette sheep erythrocytes after incubation with thymosin.\textsuperscript{54}

For the other group with lymphoblasts expressing both T and cALL antigen, a normal counterpart has not been described so far. A technical obstacle is constituted by the fact that double labelings are mandatory for demonstration in a situation where the cells to be suspected are extremely rare. The cALL antigen is described in some rare cells of the bone marrow of children, where it only has a weak expression.\textsuperscript{57} A convincing argument for the existence of such intermediate phenotypes is the establishment of lymphoblastoid cell lines with the continuous existence of cells with T and cALL antigen, as recently described.\textsuperscript{58} On four of six T-cell leukemia lines analyzed, Minowada et al.\textsuperscript{58} demonstrated a variable but definite expression of cALL antigen in crossabsorption experiments.

In our opinion, these newly described phenotypic subgroups of ALL may represent clonal cell populations arrested at different stages of maturation versus T. The most immature cell of this lineage may be the cell with T and cALL antigen, an intermediate phenotype between null and T-cell ALL. The next line is a cell phenotype with T antigen only. A consecutive expression of C3-R may be the link to a cell type with T antigen, C3-R, and E-R at 37°C. The latter phenotype is followed by a cell type lacking C3-R, but preserving T antigen and E-R, which is demonstrable at 4°C. This cell type already resembles the familiar phenotype of mature T lymphocytes. This putative line of differentiation is supported by T-antigen quantitations, as shown in Table 5. A schematic summary of the findings including other markers is given in Fig. 6.

Comparison with clinical data provided evidence that the new classification is of biologic relevance. Significant differences in age distribution (Fig. 4) and in presentation features were found (see Table 6). For instance, the pre-T and T-ALL group are different in their hemoglobin level at diagnosis. The higher hemoglobin levels in E-rosette-positive ALL have been interpreted as a late blockade of erythropoiesis by a tumor arising outside the marrow.\textsuperscript{7} Accordingly, the low Hb values of the pre-T form could be taken as an argument indicative of marrow origin, as is assumed for the other E-negative forms.

A decisive criterion for the evaluation of an ALL classification is its relationship to therapy. In spite of major advances in the treatment, about one-half of the children involved still die of their disease.\textsuperscript{59} Simone et al.\textsuperscript{45} related eight pretreatment characteristics to a poor clinical course. Two of these prognostic features (mediastinal mass, high white count) have shown to be strongly related to the E-rosette-positive form of ALL.\textsuperscript{45-49} This does not mean that the prognostic value of the T-marker can be substituted by clinical characteristics. In the study of Chessells et al.,\textsuperscript{49} the duration of complete remission was shorter for the group with E-receptor than for the group with leukocyte counts over 100,000. Immunologic markers of prognostic value should therefore be included in the battery of prognostic factors. In our study, the demonstration of T antigen without E-R or cALL antigen also appears to be associated with a worse response to treatment. In this connection it is of interest to note that in the British study,\textsuperscript{49} a poor response to initial induction therapy was noted for a so-called null-cell group consisting of blast cells without E-receptor, surface Ig, or ALL antigen. Some leukemias with only T-antigen must also be suspected in this group, since a test for anti-T was not performed in this study. In our study, too, a relatively low remission rate was clearly evident in the pre-T-cell group. In contrast, the demonstration of T antigen together with cALL antigen seems to label a subgroup that has a comparatively better prognosis (see Table 7). Obviously, the T antigen discloses in this situation a T-differentiated subset out of the cALL antigen labeled “non-T, non-B” ALL group, which is associated with a good response to current therapy. On the other hand, Brouet et al.\textsuperscript{50} have recently suggested that a sizable portion of patients with pre-B-cell leukemia, which belongs to the other part of the “non-T, non-B” ALL pool (see Table 4), do indeed carry high-risk factors.

A still unsettled finding is the fact that ALL

![Fig. 6. Expression intensity of various markers in different phenotypes of ALL blasts that are ordered in a putative line of differentiation. For abbreviations see text.](image-url)
lymphoblasts, which appear to be more differentiated according to immunologic markers, are associated with a poorer prognosis. This is especially true in the situation of B-ALL with lymphoblasts that express easily identifiable surface Ig. In our study, as in others, no continuous response was registered for the five patients with B-ALL. In two children, a prominent infiltration of the gut was demonstrated at autopsy. However, also the forms with more progressed T differentiation, as assumed by demonstration of the E-receptor, are associated with a less favorable course of the disease. The mitotic indices in children with lymphoblasts with T marker were shown to be higher than in children with null lymphoblasts. Kinetic studies have demonstrated that the proportion of E-positive marrow blasts has a larger growth fraction. Another argument for the less controlled growth of E+ blasts could be that the more mature “normal” phenotype provides a passport to escape immunologic self-surveillance. The MLC-data (see Fig. 3) clearly demonstrate that allogeneic immune mechanisms are also less stimulated the more T-differentiated the stimulator blast cell becomes. This can be best explained by assuming that the so-called LD determinants responsible for stimulation of allogeneic lymphocytes are merely expressed on B cells but not on T cells. The clear-cut difference in the stimulatory capacity of CLL cells of the B or T type, as shown in Fig. 3, fits well into this interpretation. It can be speculated that autologous immune mecha-

nisms are also less stimulated by a leukemia cell with a phenotype resembling a mature T lymphocyte. The low stimulation capacity of blast cells with E-receptor has already been described by others.

Considering the importance of the reaction of blast cells with anti-T sera in this study, which is shown to be crucial to a favorable prognosis in connection with a simultaneous expression of cALL antigen, but for an unfavorable prognosis without cALL antigen, two points of view should be discussed. First, evidence accumulated by this study and others stresses the need for a whole battery of tests, the results being considered together before the leukemic cell type is decided on. This is important for a position to tailor treatment to cell type. Second, standardization of diagnostic antisera like anti-T is mandatory. This will hopefully be achieved by monoclonal antibody productions. Those monoclonal anti-T antibodies that are already defined in various specificities for dissecting T-cell heterogeneity are very promising for future work in the serologically standardized classification in ALL.

ACKNOWLEDGMENT

We are especially grateful to the hematologists who participated in the study (Dr. G.F. Wündisch, Dr. R.J. Haas, Dr. B. Janka, Dr. Ch. Bender-Götze, Dr. D. Hoelzer, Dr. J. Wahlen) for sending samples and providing detailed clinical observations on their patients, the results of which will be fully collated with other clinical data on a later occasion. We thank G. Schwarzkopf for help in statistical analyses. We are grateful to our technicians and fellows whose invaluable assistance has made this work possible.

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Multimarker classification of acute lymphoblastic leukemia: evidence for further T subgroups and evaluation of their clinical significance

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