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Use of Monoclonal Antibodies, Morphology, and Cytochemistry to Probe the Cellular Heterogeneity of Acute Leukemia and Lymphoma[1]

Tucker W. LeBien,[2] Robert W. McKenna, Candice S. Abramson, Kazimiera J. Gajl-Peczalska, Mark E. Nesbit, Peter F. Coccia, Clara D. Bloomfield, Richard D. Brunning, and John H. Kersey

Departments of Laboratory Medicine and Pathology [T. W. L., R. W. M., C. S. A., K. J. G-P., R. D. B., J. H. K.], Pediatrics [M. E. N., P. F. C., J. H. K.], and Medicine [C. D. B], University of Minnesota Health Sciences Center, Minneapolis, Minnesota 55455

Abstract

A combined immunological, morphological, and cytochemical approach to the study of malignant cells in patients with acute leukemia and lymphoma is presented. Newly produced monoclonal antibodies that bind to antigens of human mononuclear cells (TA-1), or B-lymphocytes (BA-1) were used to study malignant cells from patients with acute lymphoblastic leukemia (ALL), acute myelocytic leukemia, acute myelomonocytic leukemia, and chronic lymphocytic leukemia. Results in lymphoid leukemia-lymphoma patients were compared with other immunological markers and indicate that the major groups of ALL and childhood non-Hodgkin's lymphoma are T-ALL, pre-T-ALL, pre-B-ALL, B-ALL, and non-T, non-B-ALL. In addition, each major group had multiple phenotypes when analyzed with seven immunological markers including the erythrocyte rosette receptor, surface immunoglobulin, cytoplasmic immunoglobulin M, the early lymphocyte-acute lymphoblastic leukemia antigen, monoclonal antibody TA-1, monoclonal antibody BA-1, and a monoclonal antibody against HLA-DR. While immunological heterogeneity was demonstrable within each group, distinct biological behavior was observed, with T-ALL and B-ALL generally presenting as "lymphomas" and the others presenting as "leukemias." Morphological analysis using the French-American-British classification provided independent information in the definition of groups with differing clinical behavior. Cytochemical analyses demonstrated focal paranuclear staining of leukemia cells with acid phosphatase in 73% of T-ALLs and 6% of non-T, non-B-ALLs.

Introduction

Investigations in recent years have been conducted in a number of centers related to cellular markers in the ALLs[3] and non-Hodgkin's lymphomas of childhood and adolescence. These investigations have utilized a variety of immunological (6, 8, 11, 14, 15, 30), morphological (3, 4), and cytochemical (7, 13, 20, 22, 24) methods and have revealed the previously unrecognized heterogeneity of this group of disorders. The purpose of this report is to review studies conducted at the University of Minnesota; these studies have concentrated on an improved definition of the cellular phenotypes in ALL and lymphoma with emphasis on a combined immunological, morphological, and cytochemical approach. Preliminary data obtained with monoclonal antibodies to human lymphocyte cell surface antigens will be presented, and the significance of these results will be discussed.

Materials and Methods

Patients. Fresh leukemia cells were obtained from patients seen in the Hematology/Oncology Section, Department of Pediatrics, and the Medical Oncology Section, Department of Medicine, both at the University of Minnesota. Cells from all patients were studied at the time of diagnosis and before treatment using immunological, morphological, and cytochemical methods. Studies of pediatric ALL and non-Hodgkin's lymphoma patients included a combined cellular phenotype and clinical analysis, while study of adult ALL patients was restricted to analysis of cellular phenotype only.

Heparinized bone marrow (and sometimes peripheral blood) was collected from ALL patients prior to therapy. In cases with primarily a lymphomatous presentation, nodal tissues were used. All immunological studies were done on viable malignant cell suspensions, and >50% malignant cells were required to consider a sample evaluable.

Immunological Characterization of Leukemia Cells. Immunological characterization of leukemia cells was carried out with several techniques that have been described in detail previously. In all instances, cells from a single patient were considered positive for a given marker if >25% of the leukemia cells were positive.

E receptors were detected using unsensitized sheep RBC as described previously (15). SIg was detected using direct immunofluorescence as described by Gajl-Peczalska *et al.* (9). CIgM staining was used to identify pre-B-cells and was done as described by LeBien *et al.* (17). Surface and cytoplasmic staining were examined with a Zeiss microscope equipped with Ploem epiillumination. Pre-B-cells were identified on the basis of IgM distribution largely in the perinuclear area or concentrated in the Golgi zone. The EL-ALLA was detected using a rabbit heterantiserum prepared against 3 M KCl extracts of the human pre-B-ALL cell line NALM-6-M1 (18). Human Ia-like antigens (HLA-DR, framework region) were detected using a monoclonal antibody kindly provided by Dr. John Hansen, University of Washington (12).

Production of Monoclonal Antibodies. Only a brief description will be given here, inasmuch as detailed reports on the production, maintenance, and characterization of our monoclonal antibodies have been presented elsewhere (1, 19).

BALB/c mice were immunized against either the T-lymphocyte leukemia cell line CCRF-HSB-2 (HSB-2) or the pre-B-ALL cell line NALM-6-M1. Spleen cells from individual immune mice were then fused with the BALB/c myeloma cell line P3-NSI-1-Ag4-1 (NS-1) using polyethylene glycol. NS-1 was obtained from Dr. Caesar Milstein through the facilities of the Salk Institute Cell Distribution Center, La Jolla, Calif. Fused cells were cultured in medium containing Littlefield's concentrations of hypoxanthine, aminopterin, and thymidine (21). The hybrido-

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[2] Recipient of a Young Investigator Award (CA-28526) from the National Cancer Institute. To whom requests for reprints should be addressed, at Department of Laboratory Medicine and Pathology, Box 609 Mayo, University of Minnesota, Minneapolis, Minn. 55455.

[3] The abbreviations used are; ALL, acute lymphoblastic leukemia; E-receptor, sheep RBC receptor; SIg, surface immunoglobulin; CIgM, cytoplasmic IgM; EL-ALLA, early lymphocyte-ALL antigen; ACP, acid phosphatase; CLL, chronic lymphocytic leukemia; AML, acute myelocytic leukemia; AMML, acute myelomonocytic leukemia; E-rosette, erythrocyte-forming rosette; T-ALL, thymus-derived acute lymphoblastic leukemia; B-ALL, bone marrow-derived acute lymphoblastic leukemia.

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mas of interest were eventually cloned by limiting dilution (0.5 cell/well) on nonadherent spleen cell feeders, and the clones were injected into pristane-primed mice to obtain high-titer ascitic fluid. Ascitic fluid was used as a source of monoclonal antibodies in this report.

Binding of monoclonal antibodies to leukemia cells was detected by indirect immunofluorescence. Briefly, target cells were incubated with appropriately diluted monoclonal antibody (or control ascitic fluid, obtained by giving pristane-primed mice injections of a nonsecreting hybridoma) for 40 min on ice. Cells were then washed and stained with goat anti-mouse immunoglobulin (Meloy Laboratories, Inc., Springfield, Va.) for an additional 40 min on ice. Cells were finally washed, mounted under glass coverslips, and examined under fluorescent microscopy.

Morphology and Cytochemistry. The morphology of the leukemia cells was studied in Wright's-Giemsa-stained bone marrow and blood smears and in bone marrow trephine biopsy sections. Cases were classified according to the FAB cooperative group morphological classification of ALL (4). Cytochemical studies were performed on bone marrow smears. Leukemia cells were evaluated for ACP using the naphthol ASBI phosphoric acid-fast garnet GBC method of Li *et al.* (20), for periodic acid-Schiff positivity by the McManus method (23), and for β-glucuronidase by the method of Lorbacher *et al.* (22).

Results

Immunological Markers. Table 1 shows a summary of the reactivity of monoclonal antibodies TA-1 and BA-1 with normal peripheral blood mononuclear cells. TA-1 binds to all peripheral blood T-cells and weakly with most monocytes, while BA-1 binds only to peripheral blood B-cells. Tables 2 to 4 show the results of an immunological analysis of 37 children and adults with leukemia. In Table 2, 18 newly diagnosed ALL patients were analyzed with 7 immunological markers, and the individual phenotypes are shown as enclosed boxes. The data clearly show the existence of 8 distinct phenotypes within the 18 patients studied. The most prevalent phenotype (Patients R. C., A. K., L. H., J. Z., P. S., J. G., D. R., and J. M.) was Dr^+, $EL\text{-}ALLA^+$, $BA\text{-}1^+$, accounting for 44% of the ALL tested. Three possible phenotypes of T-cell lineage are also shown. The first (Patients B. O., K. Y., and R. B.) was E^+, $TA\text{-}1^+$; the second (Patients B. S. and J. F.) was E^+, $TA\text{-}1^-$; and the third (Patient R. S.) was E^-, $TA\text{-}1^+$. The latter may be an example of the pre-T-cell phenotype. Cells from Patient J. T. were $CIgM^+$, DR^+, $EL\text{-}ALLA^+$, $BA\text{-}1^+$, consistent with the pre-B-cell phenotype. In addition, several other phenotypes were represented (Patients D. B., K. Y., and K. K.), each differing from the other with DR, EL-ALLA, or BA-1.

Table 3 shows the results obtained when 5 immunological markers were used to study CLL. The data show the existence of a single phenotype (SIg^+, DR^+, $BA\text{-}1^+$, $TA\text{-}1^-$, E^-), consistent with previous observations by ourselves and others indicating that the predominant cell involved in CLL is a B-lymphocyte.

Finally, Table 4 shows the results obtained when 5 immunological markers were used to study AML or AMML. Whereas AML patients were either DR^+ or DR^-, in no instance did cells from these patients react with monoclonal antibodies TA-1 or BA-1. In contrast, the 3 AMML patients exhibited heterogeneity in DR expression, but all 3 were reactive with TA-1. The reactivity of AMML patients with TA-1 is in accordance with data obtained using peripheral blood monocytes, the majority of which are also $TA\text{-}1^+$ (see Table 1).

Morphological and Cytochemical Analyses. Sixty-one newly diagnosed adults and children with ALL were studied in detail relative to morphology and cytochemistry of the leukemic cells. The results of these studies have been published previously (24). Cells from all patients who had complete morphological and cytochemical analysis were studied for E-rosette formation and SIg. Eleven of the 61 patients had blast cells that were E^+, 2 patients had cells that were SIg^+, and 48

Table 1

Reactivity of monoclonal antibodies TA-1 and BA-1 with peripheral blood mononuclear cells

Reactivity of TA-1 or BA-1 with T- or B-cells was determined by double fluorochrome staining. Cells were stained with rhodamine isothiocyanate goat anti-human immunoglobulin; washed; stained with TA-1, BA-1, or control ascitic fluid; washed; and stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. Reactivity with monocytes was determined by incubation of monocytes (obtained by adherence to fetal bovine serum-coated plates) with TA-1, BA-1, or control ascitic fluid, followed by washing and staining with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin.

	TA-1	BA-1
T-cells	P[a]	N
B-cells	N	P
Monocytes	P (weak)	N

[a] P, positive; N, negative.

Table 2

Immunological characterization of cells from patients with ALL

Patient	E	SIg	CIgM	DR[a,b]	EL-ALLA[c]	BA-1[b]	TA-1[b]
R. C.	N	N	N	P	P	P	N
A. K.	N	N	N	P	P	P	N
L. H.	N	N	N	P	P	P	N
J. Z.	N	N	N	P	P	P	N
P. S.	N	N	N	P	P	P	N
J. G.	N	N	N	P	P	P	N
D. R.	N	N	N	P	P	P	N
J. M.	N	N	N	P	P	P	N
B. O.	P	N	ND	N	N	N	P
K. Y.	P	N	ND	N	N	N	P
R. B	P	N	ND	N	N	N	P
B. S.	P	N	ND	N	N	N	N
J. F.	P	N	ND	N	N	N	N
R. S.	N	N	N	P	P	N	P
J. T.	N	N	P	P	P	P	N
D. B.	N	N	N	P	P	N	N
K. Y.	N	N	N	N	P	P	N
K. K.	N	N	N	P	N	P	N

[a] DR, HLA-DR (framework region); BA-1, B-cell antigen 1; TA-1, T-cell antigen 1; N, negative; P, positive; ND, not determined.
[b] Detected with monoclonal antibody.
[c] Detected with heterologous antiserum.

Table 3

Immunological characterization of cells from patients with CLL

Patient	SIg	DR[a,b]	BA-1[b]	TA-1[b]	E
H. G.	P	P	P	N	N
L. P.	P	P	P	N	N
J. C.	P	P	P	N	N
E. F.	P	P	P	N	N
T. F.	P	P	P	N	N
G. K.	P	P	P	N	N
L. P.	P	P	P	N	N
N. G.	P	P	P	N	N

[a] DR, HLA-DR (framework region); BA-1, B-cell antigen 1; TA-1, T-cell antigen 1; P, positive; N, negative.
[b] Detected with monoclonal antibody.

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Table 4
Immunological characterization of cells from patients with AML and AMML

Patient	Diagnosis	Sig	DR[a,b]	BA-1[b]	TA-1[b]	E
J. C.	AML	N	P	N	N	N
M. E.[c]	AML	N	P	N	N	N
T. W.	AML	N	P	N	N	N
D. G.	AML	N	P	N	N	N
C. G.	AML	N	N	N	N	N
P. P.[c]	AML	N	N	N	N	N
N. F.[c]	AML	N	N	N	N	N
T. B.	AML	N	N	N	N	N
C. K.	AMML	N	P	N	P	N
F. A.	AMML	N	P	N	P	N
C. L.	AMML	N	N	N	P	N

[a] DR, HLA-DR (framework region); BA-1, B-cell antigen 1; TA-1, T-cell antigen 1; N, negative; P, positive.
[b] Detected with monoclonal antibody.
[c] Cells from these patients were studied at relapse.

patients had cells that were E^-, Sig^-. When these cases were evaluated relative to FAB morphology, 9 of the E^+ cases were FAB-L1, 2 were FAB-L2, and none were FAB-L3. Both cases that were Sig^+ were FAB-L3. Of the 48 cases that were E^-, Sig^-, 39 were FAB-L1, 9 were FAB-L2, and none were FAB-L3. Sig and FAB-L3 morphology showed good correlation, but there did not appear to be a correlation between E-rosette positivity and FAB-L1 or FAB-L2 morphology. However, 10 of the 11 E^+ ALL cases were associated with the presence of a minor population of small leukemic cells which manifested markedly hyperchromatic nuclei that frequently exhibited striking nuclear convolution (25). Only 4 of the 48 E^-, Sig^- cases had these small leukemic cells with hyperchromatic convoluted nuclei (25).

Cytochemical studies on these same 61 patients demonstrated a positive correlation between E-rosette positivity and focal paranuclear staining with ACP. Eight of 11 patients with E^+ ALL had focal ACP staining, while only 3 of 48 patients with E^- ALL had this focal staining ($p = 0.005$) (24). β-glucuronidase staining was generally similar to ACP staining although results were more difficult to interpret. The periodic acid-Schiff stain showed no correlation with E-rosette positivity (24).

Major Cellular Groups of Childhood Lymphoid Leukemia-Lymphoma. Table 5 summarizes the profiles of the major immunological groups of ALL and lymphoma in childhood and adolescents (adults not included in this analysis) at the University of Minnesota. The major groups as shown are non-T, non-B-ALL, pre-B-ALL, pre-T-ALL, T-ALL/lymphoma, and B-ALL/lymphoma. It must be emphasized that this group of patients is not the same group discussed under "Morphological and Cytochemical Analyses." As reported previously by ourselves (18) and others (30), the EL-ALLA is expressed on 75 to 80% of non-T, non-B-ALL cases. Pre-B-ALL is shown as a closely related form of ALL with demonstrated EL-ALL antigen positivity and, like non-T, non-B-ALL, shows a leukemia rather than lymphoma presentation. Pre-T-ALL is defined in this analysis as leukemia with an E^-, $TA\text{-}1^+$ phenotype and thus far includes only one case. T-ALL includes those cases that are primarily E^+, $TA\text{-}1^+$. However, not all E^+ patients are $TA\text{-}1^+$ (see Table 2). This is not surprising, because TA-1 is detectable on only 67% of thymocytes and is, therefore, not reacting with all T-lymphocytes. As shown in Table 5, T-ALL frequently shows a population of small hyperchromatic convoluted cells (25). As noted, cells of this phenotype are generally ACP^+. Patients with T-ALL frequently present as lymphoma rather than leukemia and have relative sparing of bone marrow. In our experience, B-ALL in childhood always presents as lymphoma with characteristic Sig^+ cells and FAB-L3 morphology.

Discussion

This report decribes the multiparameter approach to the ALLs and lymphomas used at the University of Minnesota in recent years. This approach combines an immunological, morphological, cytochemical, and clinical analysis of patients with these diseases. One conclusion derived from this analysis, as reported previously (8, 14, 30), is the existence of a significant heterogeneity in diseases that were at one time treated as a single entity. Immunological probes have been particularly useful in unravelling this heterogeneity.

Most ALLs probably represent the malignant counterpart of lymphocytes residing in normal bone marrow, thymus, or lymph node. Several leukemias, such as the E^+ T-ALL or the Sig^+ B-ALL, clearly represent the malignant counterpart of a "mature" T- or B-lymphocyte. However, Greaves *et al.* (11) have suggested that the majority of ALLs are probably the malignant manifestation of cells (primarily lymphoid?) found in early stages of hematopoiesis. Several examples of leukemias representing "immature" lymphocytes have been reported, including pre-B ($CIgM^+$, Sig^-) (17, 31) and pre-T (T-cell antigen$^+$, E^-) (15, 26, 29). In the past, however, a major difficulty with immunological analysis was a lack of widely available standardized reagents. Antibodies prepared in the classical manner required multiple adsorption steps to attain operational specificity and were available only in limited quantity. With the advent of the monoclonal antibody methodology (16) has come the realization that the previous problems of specificity and availability can be overcome. The monoclonal antibodies developed in our laboratories and reported in this manuscript are examples of such reagents.

Evidence presented in this paper suggests that monoclonal antibodies TA-1 and BA-1 are detecting specificities present on mutually exclusive populations of human peripheral blood mononuclear cells. TA-1 binds primarily to peripheral blood T-lymphocytes and monocytes (19). The binding to monocytes does not appear to be via Fc receptors, because $F(ab')_2$ fragments prepared by pepsin digestion show the same binding specificity as does whole antibody. Reactivity of TA-1 with leukemia cells correlated well with normal cells. TA-1 reacted with ALL patients (3 of which were E^+ and therefore considered T-cell leukemias) and the malignant monocyte/macrophage found in AMML (3 of 3). Therefore, TA-1 appears to offer potential for assisting in the difficult distinction between AML and AMML.

Unlike TA-1, monoclonal antibody BA-1 reacts uniquely with normal peripheral blood B-lymphocytes and shows no reactivity with T-lymphocytes or monocytes. In studies reported elsewhere (1), BA-1 does not appear to be reacting with Sig or HLA-DR determinants. We are currently pursuing studies to determine whether BA-1 is detecting B-lymphocyte antigens previously described by others using heterologous antisera (2, 5, 10). Reactivity of BA-1 with peripheral blood B-lymphocytes clearly distinguishes it from antibody binding to the EL-ALLA.

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Table 5

Major groups of childhood lymphoid leukemia-lymphoma

	Non-T, non-B-ALL	Pre-B-ALL	Pre-T-ALL	T-ALL/lymphoma	B-ALL/lymphoma
Immunological markers	DR^{+a} EL-ALLA$^+$ (most) BA-1$^+$ (most)	CIgM$^+$ DR$^+$ EL-ALLA$^+$ BA-1$^+$	TA-1$^+$ E-rosette negative	TA-1$^\pm$ E-rosette positive	DR$^+$ Sig$^+$ BA-1$^+$
Morphology	FAB-L1 or FAB-L2	?	?	FAB-L1 or FAB-L2	FAB-L3
Cytochemistry	Focal ACP$^-$ Focal β-Glu$^-$ 70% PAS$^+$	Focal ACP$^-$ Focal β-Glu$^-$?	?	Focal ACP$^+$ Focal β-Glu$^+$ 90% PAS$^+$	Focal ACP$^-$ Focal β-Glu$^-$ PAS$^-$
Involvement					
Bone marrow	++++	++++		0 to ++++	0 to ++++
Blood	+ to ++++	+ to ++++	?	0 to ++++	0 to ++
Lymphoid tissue	0 to ++	0 to ++		++++	++++
Probable site of origin	Bone marrow	Bone marrow	Bone marrow	Thymus Lymph node Skin Bone marrow	Lymph node Gastrointestinal tract Bone marrow

[a] DR, HLA-DR locus; TA-1, T-cell antigen 1; BA-1, B-cell antigen 1; β-Glu, β-glucuronidase; PAS, periodic acid-Schiff; superior plus, positive; superior minus, negative; superior ±, intermediate.

Because BA-1 reacts with a significant number of ALL, it should be useful in our continuing efforts to unravel the immunological heterogeneity of this disease. This is already exemplified by data in Table 2 which shows 2 patients (R. S. and D. B.) who were EL-ALLA$^+$, BA-1$^-$ and one patient (K. K.) who was EL-ALLA$^-$, BA-1$^+$. A major question that must be addressed in the future is whether BA-1 is detecting leukemias which represent the malignant counterpart of normal cells committed to B-lymphocyte differentiation.

In conclusion, the results presented herein indicate that a variety of immunological, morphological, and cytochemical methods are useful in probing the heterogeneity of acute leukemias and lymphomas. We are particularly enthusiastic about the potential of monoclonal antibodies, as evidenced by the preliminary data in this report and other reports (27, 28, 32). With the appropriate exchange and utilization of these reagents in the future, the standardization of immunological data from center to center should become more feasible. Even at this early stage, our data and data from other centers indicate that patients' response to therapy and long-term prognosis can, to a certain extent, be predicted from multiparameter analyses. For example, patients with T-ALL and B-ALL have a shortened overall survival compared to patients with non-T, non-B-ALL (4, 24). FAB morphology has been shown to contribute independent prognostic information; *i.e.*, patients with non-T, non-B-ALL have a worse prognosis if they have L2 morphology than if they have L1 morphology (24). The contributing role of monoclonal antibodies TA-1 and BA-1 in the diagnosis and classification of human leukemias is currently under investigation.

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