Immunophenotyping of Acute Myeloid Leukemia Using Monoclonal Antibodies and the Alkaline Phosphatase–Antialkaline Phosphatase Technique

By Curtis A. Hanson, Kazimiera J. Gajl-Peczalska, Janet L. Parkin, and Richard D. Brunning

The leukemic cells from 41 cases of acute myeloid leukemia (AML) and 17 cases of acute lymphocytic leukemia (ALL) were immunophenotyped by the alkaline phosphatase–antialkaline phosphatase (APAAP) immunocytochemical technique utilizing eight monoclonal antibodies (MoAb) reactive with cells of myeloid origin and seven MoAb reactive with lymphoid antigens. Ninety percent of the cases of AML reacted with one or more of the pan-myeloid MoAb, My7, My9, or 20.3. Reactivity of the myeloid panel of MoAb showed some correlation with the French-American-British (FAB) classification of AML. Five of six cases of acute promyelocytic leukemia (APL) were HLA-DR negative; the one HLA-DR-positive APL had a minor population of HLA-DR-negative promyelocytes. OKMS and/or My4 reacted with 16 of 16 monocytic leukemias. No specific marker of early erythroid development was identified. AP3, a MoAb reactive with platelet glycoprotein (GPIIa), was specific for acute megakaryoblastic leukemia. Immunocytochemistry was also helpful in classifying seven cases of AML with equivocal or negative routine cytochemistry. Two cases of AML had minor populations of blasts detected by the APAAP technique that were immunologically distinct from the major blast population; these minor populations emerged as the predominant cell type at relapse. Two cases of ALL expressed multiple myeloid and lymphoid antigens. Two other cases that morphologically were ALL reacted with only myeloid MoAb: one consisted entirely of immature basophils on ultrastructural examination. Immunophenotyping results using the APAAP technique were comparable with those obtained with flow cytometry. The APAAP technique is a reliable method for immunophenotyping leukemia that complements other methods of immunologic evaluation. The primary advantages of this method include its use with routinely prepared blood and bone marrow smears and the ability to correlate immunocytochemical reactions with morphology.

March 1986; 41 of the patients were diagnosed as AML, and 17 were diagnosed as ALL. Studies on 32 AML and 12 ALL cases were performed on specimens taken prior to chemotherapy; 9 AML and 5 ALL cases were studied at relapse.

Morphologic classification was based on criteria established by the French-American-British (FAB) Cooperative Study Group. The 41 cases of AML were subclassified as follows: M1, 5 cases; M2, 10 cases; M3, 4 cases; M3 variant, 2 cases; M4, 12 cases; M5a, 1 case; M5b, 3 cases; M6, 3 cases, and M7, 1 case. Bone marrow specimens were prepared according to previously described methods. Cytochemical studies included myeloperoxidase (MPO), sudan black B (SBB), and nonspecific esterase (NSE). Terminal deoxynucleotidyl transferase (TdT) determination was performed on all specimens by indirect immunofluorescence. Ultrastructural evaluation included routine transmission electron microscopy and myeloperoxidase and platelet peroxidase ultracytochemistry.

Monoclonal antibodies. Sixteen MoAbs that react with cells of myeloid origin were initially evaluated; the following eight were selected because of their apparent utility in distinguishing AML from ALL and in establishing an immunophenotypic pattern correlating with the FAB classification of AML: HLA-DR, My7, My9, 20.3, My4, OKMS, OKT9, and AP3. Expression of HLA-DR, which is present on multiple cell types including myeloblasts, is thought to be absent from granulocytes beginning at the promyelocyte stage of differentiation. My7, My9, and 20.3 are antibodies reactive with most stages of granulocytic and monocytic differentiation.

From the Department of Laboratory Medicine and Pathology, University of Minnesota Health Sciences Center, Minneapolis.

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C.A.H. is presently at the Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109.

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is also expressed on erythroblasts. My4 and OKM5 are reactive with cells of monocytic origin; in addition, OKM5 reacts with erythroblasts, platelets, and megakaryocytes. OKT9, an antitransferrin receptor antibody, reacts with erythroid precursors, thymic lymphocytes, and high mitotic rate tumor proliferations. AP3, a MoAb that recognizes platelet glycoprotein IIa (GPIIa), can be identified on platelets, megakaryocytes and megakaryoblasts. The other eight myeloid MoAbs were not found to add significant differentiation data. Leu M4 and 20.2 reacted only with mature granulocytes. Leu M3, Leu M4, and OKM1 identified cells that were morphologically obvious as monocytes, neutrophil myelocytes, or segmented neutrophils. R10, an anti-glycoprotein A MoAb, was present on late basophilic, polychromatophilic and orthochromatophilic erythroblasts but did not react with proerythroblasts and myeloblasts. My10, which is present on hematopoietic stem cells, may be expressed on both lymphoblasts and myeloblasts.

A panel of seven lymphoid monoclonal antibodies, B4, BA1, J5, BA3, T11, OKT6, and Leu3, was used to determine B or T cell lineage.

Reagents. Alkaline phosphatase antialkaline phosphatase immunocomplexes were prepared as described by Cordell et al. Calf intestinal alkaline phosphatase (Sigma Chemicals, St Louis) was added at a final concentration of 5 mg/mL to tissue culture supernatant containing mouse monoclonal anti-alkaline phosphatase antibody, which was provided by Dr David Mason, Oxford, England. Unconjugated rabbit antimonoclonal Ig was obtained from Dakopatts (Santa Barbara, CA).

Naphthol-AS-BI, levamisole, fast red TR salt, and tinlma base were obtained from Sigma; 0.05 mol/L of Tris-buffered saline (TBS), pH 7.6, was used to dilute all antibodies and the APAAP enzyme complex and was also used as a wash for slides between incubation steps.

Naphthol-AS-BI (2.6 mg) was dissolved in 0.2 mL of dimethyl formamide (Matheson, Coleman, and Bell), followed by the addition of 9.8 mL of 0.1 mol/L of TBS, pH 8.2. Levamisole and fast red TR salt were added at a final concentration of 1 mmol/L and 1 mg/mL, respectively. The substrate solution was then filtered.

Immunocytochemical staining. Immunocytochemical studies were performed on routinely prepared, air-dried blood and bone marrow smears using the APAAP technique after the method of Cordell and colleagues. In brief, slides were fixed in a cold (4°C), 2.5% formalin-acetone mix for 30 to 60 seconds. Primary MoAb was applied for a 30-minute incubation period. Slides were then incubated for 30 minutes with rabbit anti-mouse Ig, the secondary antibody that forms a bridge between the primary MoAb and the APAAP complex, which was then applied for 60 minutes. Following the 60-minute incubation with the APAAP complex, the slides were reincubated with the rabbit anti-mouse antibody for 10 minutes and the APAAP complex for 30 minutes to enhance the intensity of the labeling reaction. This double-bridge procedure for enhancement of the reaction product was described by Vacca. All incubation procedures were performed in a moist chamber at room temperature and were followed by washing in TBS. The reaction product was developed using the alkaline phosphatase substrate, which was applied for 20 minutes. Slides were then rinsed in tap water, counterstained with Gill's hematoxylin, and coveredslipped with glycerin mount. The percentage of positive reacting cells was determined by the assessment of 300 nucleated cells. Positivity with a MoAb was defined as reactivity with >20% of the leukemic cells. There was some variability in the intensity of staining between antibodies and between the different cases. A positive reaction was defined as a cell having an intensity of staining greater than that seen in background residual myeloid cells and in a negative control specimen; equivocal staining was interpreted as a negative reaction. Mouse myeloma ascitic fluid, which contains all immunoglobulin subclasses, was used as a negative control antibody. The use of fixed cells in the APAAP procedure avoids nonspecific Fc binding problems; incubation of viable cells in solution with antibodies may lead to Fc binding of MoAb in monocytic leukemia with flow cytometric methods.

Thirty-three of the 41 cases of AML and 14 of the 17 cases of ALL were immunophenotyped by flow cytometry using EDTA blood and/or heparinized bone marrow aspirate samples obtained from the same bone marrow procedure as that used for the APAAP study. MoAbs used included HLA-DR and MCS2, a myeloid MoAb that recognizes an antigen similar to that identified by My7. The myeloid MoAbs My4, My7, My9, and OKM5 were used in the later stages of the study in 13 cases. Lymphoid MoAbs used in the flow cytometry portion of the study included BA1, BA3, 35.1 (sheep erythrocyte receptor antibody), and OKT6.

RESULTS

Acute myeloid leukemia. Thirty-seven of the 41 cases of AML (90%) reacted with 1 or more of the 3 myelomonocytic antibodies My7, My9, or 20.3 (Table 1); 11 of the cases reacted with all three antibodies. Thirty of the cases (73%) reacted with My7 and 25 (68%) with My9; 88% of the cases reacted with either My7 or My9. Four of the 41 cases did not react with any of these three antibodies; 1 was subclassified as FAB-M1, 2 were classified as M2, and 1 was classified as M7 (megakaryoblastic leukemia). The case of M7 reacted with AP3, an anti-GPIIIa MoAb. The three cases of nonreacting M1 and M2 were cytochemically positive with MPO and SBB. The one case of M1 had 16% myeloperoxidase-positive blasts; 12% blasts expressed My7, which was lower than our defined threshold of positivity with MoAb. One of the cases of M2 was a therapy-related myeloid leukemia. Myeloid antigen expression was also negative by flow cytometry in the other nonreacting case of M2.

The eight myeloid MoAbs were also evaluated for their ability to discriminate the FAB classification of AML (Table 1). Four immunophenotypic patterns correlated with the FAB subtypes. First, HLA-DR was negative in 5 of 6 cases

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<tr>
<th>Table 1. Cytochemistry and MoAb Reactivity (APAAP) in 41 Cases of Acute Myeloid Leukemia</th>
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<td>MoAb</td>
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†Number of cases tested if less than the total number of cases in that classification is shown in parentheses.

Numbers correspond to positive-reacting cases for each FAB type; positivity is defined as MoAb reactivity in >20% of leukemic cells.

Abbreviation: ND, not done.

*Positivity in the erythroleukemias is defined as MoAb reactivity in >20% of the myeloblasts.

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of acute promyelocytic leukemia (M3), including 2 cases of the hypogranular variant. The one positive HLA-DR case (Fig 1) consisted of a proliferation of typical-appearing promyelocytes with extensive coarse granulation; cells with multiple Auer rods were identified. Although the majority of promyelocytes in this case were HLA-DR positive, 23% of the promyelocytes were HLA-DR negative. HLA-DR reactivity was present in the majority of cases of AML-M1 and M2 (13 of 15) and in all the monocytic leukemias. Less than 20% of the blasts in two cases of M6 and in the one case of M7 were positive with HLA-DR. Second, all monocytic leukemias reacted with My4 and/or OKM5. In addition, five cases of M1 and M2 showed My4 reactivity on 26% to 52% of the leukemic cells. OKM5 positivity was also detected on erythroblasts and megakaryocytes. Third, OKT9 reacted with erythroblasts, including proerythroblasts, and some myeloblasts in the erythroleukemias; myeloblasts in two cases of M1 also reacted with OKT9. Fourth, AP3 was a specific marker for M7. A positive reaction with AP3 was only observed in the platelets, megakaryocytes, micromegakaryocytes, and megakaryoblasts. Blasts in the other sub-

types were uniformly negative with AP3. The one case of M7 was confirmed with platelet peroxidase ultracytochemistry.

Immunophenotyping was also compared with routine cytochemistry. Three cases classified as M1 by morphology were cytochemically equivocal or negative with MPO and SBB and showed positive reactivity with one or more of the myeloid MoAbs My7, My9, or 20.3; one was positive with OKM5 and My4, suggesting a monocytic component. Two cases of M4 and one of M5a (Fig 2) had equivocal or negative staining of the leukemic blasts with NSE. The blasts from all three cases reacted with My4 and OKM5, confirming the monocytic lineage; all cases had monocytic features by electron microscopy.

Two cases of AML-M6 had minor populations of megakaryoblasts and monoblasts, respectively, identified immunocytochemically at diagnosis. In both cases, the minor population emerged as the predominant leukemic cell type at relapse, megakaryoblastic in one (Fig 3) and monoblastic in the other.

None of the 41 cases of AML showed reactivity with the B lineage MoAb. Ten cases (24%) reacted with Leu-3, which is
thought to recognize a cytoplasmic antigen present in monocytes/macrophages. The intensity of staining was less than that usually found on Leu-3-positive helper T cells. Ten AML cases reacted with Leu-3; seven were FAB-M4 and three were FAB-M5.

Acute lymphocytic leukemia. Fourteen of the 17 cases of ALL reacted with one or more of the B cell lineage MoAb; one case exhibited a T cell phenotype. Four cases of ALL showed unusual immunophenotyping with the MoAb (Table 2). Virtually all blasts in two cases (nos. 1 and 2) reacted with both lymphoid (B lineage) and myeloid MoAb. A high percentage of the leukemic cells in case 1 had MPO-positive granules by ultracytochemistry; chromosome studies showed a normal 46XY karyotype; cytogenetic studies showed a normal 46XY karyotype. Electron microscopic evaluation of case 2 showed undifferentiated blasts with negative ultracytochemistry, compatible with an ALL; cytogenetic studies showed a 47XY,+21 karyotype. The intensity of staining with the lymphoid and myeloid MoAb did not differ in these two cases.

Two cases (nos. 3 and 4) classified as ALL by morphology and TdT positivity were negative with all the lymphoid MoAbs; both reacted with multiple myeloid MoAb (Fig 4). Ultrastructurally, case 3 consisted entirely of immature basophils (Fig 5); basophilic granules were not apparent by light microscopy. Cytogenetic studies showed a 46XY karyotype. Case 4 occurred in an 85-year-old man who had a leukocyte count of 446 x 10⁹/L and died within 12 hours of admission. No chromosome analysis was performed.

Correlation of immunocytochemistry with flow cytometry. There was good correlation of the immunophenotype results in the 47 cases evaluated with both the APAAP and flow cytometry techniques; there were no discrepancies in the classification of the leukemias between the two methods. Minor variations in the percentage of reacting cells were sometimes noted and were probably related to the different methods of specimen preparation.

**DISCUSSION**

The utility of MoAbs in the diagnosis and classification of malignancies of the hematopoietic system is well established. The initial studies with these markers primarily used flow cytometry or microscopic immunofluorescent techniques. The introduction of immunocytochemistry has considerably expanded the potential application of immunophenotyping acute leukemia. This increased utility relates primarily to the application of immunocytochemical methodology to routinely prepared blood and bone marrow smears and cytospin preparations. Immunochemistry allows direct visualization of the reactivity pattern of individual leukemic cells with the MoAb.

The diagnostic utility of MoAb in the evaluation of cases of acute leukemia, either by flow cytometry or immunocytochemistry, relates to two primary points: the distinction between AML and ALL and the correlation of patterns of antibody reactivity with the classification of AML. In addi-

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**Table 2. Four Cases of ALL With Unusual Immunophenotype**

<table>
<thead>
<tr>
<th>Case</th>
<th>DR</th>
<th>B4/RA1</th>
<th>CALLA</th>
<th>OKT6/T11</th>
<th>My7</th>
<th>My9</th>
<th>20.3</th>
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My4, OKM5, OKT9, and AP3 were negative in all cases studied. Abbreviation: ND, not done.

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**Fig 4.** Left: Bone marrow smear from a patient with acute lymphocytic leukemia showing lymphoid-appearing blasts. Right: Blasts from same patient showing My7-positive staining. Alkaline phosphatase-antialkaline phosphatase (APAAP), hematoxylin counterstain Original magnification x 1,000; current magnification x 500.

**Fig 5.** Electron micrograph of a peripheral blood leukemic blast from the same case as shown in Fig 4 illustrates peroxidase-positive granulation (arrows). Peroxidase activity is lacking in the nuclear envelope and rough endoplasmic reticulum. Lead citrate only Original magnification x 19,000; current magnification x 12,350. Inset: At higher magnification, the peroxidase-containing granules show a speckled pattern of positivity typical for basophil granules Original magnification x 39,000; current magnification x 25,350.
IMMUNOPHENOTYPING OF ACUTE MYELOID LEUKEMIA

The use of MoAbs in the evaluation of cases of AML is of more recent origin than in ALL. Because of the several possible differentiation patterns in the myeloid system, the results have not been quite as distinct as in the lymphocytic leukemias. A study of 196 AML patients from the Cancer and Leukemia Group B (CALGB) showed My9 reactivity in 70% and My7 expression in 57% of cases. Neame and colleagues found 87% of 75 cases of AML reacted with My9 and 75% with My7; 96% expressed My9 and/or My7. My7 and My9 also appear to be highly specific for myeloid leukemias. Griffin and co-workers found reactivity in only 1 of 109 cases of ALL with My7 and 2 of 109 with My9.34

The results from the present study demonstrate that, as in flow cytometry techniques, multiple pan-myeloid MoAb must be used in the evaluation of acute leukemias with immunocytochemical methodologies; 88% of the cases of AML in this series was positive with My7 and/or My9. The MoAb 20.3, which recognizes an antigen present on immature myeloid cells, monocytes, and erythroid cells, had a pattern of reactivity similar to that of My7 and My9. The blasts from three cases of AML did not react with any of the myeloid or lymphoid MoAb; MPO and SBB were positive in all three cases. These results underscore the importance of cytochemistry in the classification of AML.

None of the lymphoid MoAbs used in this study except Leu-3 reacted with any of the cases of AML. The lymphoid MoAb identified all but two cases of ALL diagnosed by morphology and TdT positivity; the blasts from both cases reacted with myeloid MoAb. One consisted of immature basophils on electron microscopy. The other case occurred in an elderly male with a marked leukocytosis; no ultrastructural or chromosome analysis was available.

The blasts from two cases of leukemia classified as ALL reacted with both myeloid and lymphoid MoAb. This pattern appears to be an infrequent finding, although some authors have reported 20% to 25% of cases of ALL and AML with mixed immunophenotypes. The significance of these findings is not clear; it has been reported that cases of ALL that react with myeloid MoAb have a poor response to induction chemotherapy. Both of the two cases in this study entered remission after the first course of induction chemotherapy.

The reactivity of Leu-3, a marker for helper T cells, with cells of monocytic origin has been previously described. Wood and associates showed that the normal expression of the Leu-3 antigen on monocytes appears to result from endogenous synthesis and is predominantly cytoplasmic in location. The APAAP technique, as opposed to routine flow cytometric methods, will detect such cytoplasmic expression as shown in this study.

Studies have shown that myeloid MoAb can be used to subclassify cases of AML. Using a large panel of MoAbs, Neame and colleagues found correlation of the immunophenotype and FAB classification in >80% of 75 cases of AML. Our results demonstrate that some correlation of immunophenotype with the FAB classification. The lack of reactivity of HLA-DR in the majority of cases of APL confirms previous observations that the promyelocyte is predominantly HLA-DR negative. One case of APL in the current study was HLA-DR positive; however, a small population of HLA-DR-negative promyelocytes was morphologically evident with use of the APAAP technique. This is consistent with the 27% of cases of APL with HLA-DR positivity in the CALGB report. In contrast to the CALGB finding of HLA-DR expression in two of three hypogranular variants of APL, the two hypogranular variants in the present study were HLA-DR negative. HLA-DR negativity was not exclusive to APL in the present series since 13% of M1 and M2 cases and 24% of cases of ALL were HLA-DR negative.

The antigens identified by My4 and OKM5 are expressed primarily in M4 and M5 AML; 14 of 16 cases of M4 and M5 were positive with My4 in this study. OKM5 is a MoAb reactive with monocyte determinants and is also expressed on platelets and nucleated RBCs. OKM5 was present in 14 of 16 (88%) cases of monocytic leukemia in this study; no other subset showed blast reactivity. The reactivity of OKM5 with platelets, megakaryocytes, and normoblasts did not lead to an interpretable difficulty because these cells could be morphologically distinguished with the APAAP technique. OKM5 positivity appeared to begin at the basophilic erythroblast stage; the proerythroblasts and myeloblasts in the erythroleukemias were OKM5 negative. Although some micro-megakaryocytes reacted with OKM5, no megakaryoblasts in the one case of M7 reacted.

No specific and sensitive marker of early erythroid development was identified. R10, an antiglycophorin A antibody, was found only on cells that were morphologically obvious as erythroblasts beginning at the basophilic stage; no proerythroblast or myeloblast reactivity was noted. This is consistent with other studies showing that this MoAb labels only post-CFU-E stages of development. As a result, R10 would be of limited value in cases of M6 with markedly immature RBC precursors. OKT9, a MoAb to the transferrin receptor, was present on some of the myeloblasts in addition to all the erythroblasts in the erythroleukemias. Blast reactivity with OKT9 was also found on myeloblasts in two cases of M1, however, and may be detected in some cases of ALL.

Previous reports showed that MoAbs against GPIIb/IIIa or IIa have utility in the diagnosis of M7. The ultrastructural detection of platelet peroxidase is probably the earliest marker of megakaryocytic differentiation and remains the diagnostic standard. Immunologic analysis with MoAb allows more rapid identification, however, at less expense.

Although antibody reactivity with anti-factor VIII antibodies may be observed in some cases of megakaryoblastic leukemia, reactivity with MoAb to GPIIb/IIIa is a more sensitive marker of megakaryoblasts than is reactivity with anti-factor VIII.

Although immunophenotyping may provide helpful confirmatory data in the classification of AML, the majority of cases in this study were readily classified on the basis of routine morphology and cytochemistry. All seven cases of AML with negative or equivocal routine cytochemistry
reacted with one or more of the myeloid MoAbs, however. These findings suggest that immunocytochemistry may be an important tool for identifying blasts of granulocytic or monocytic lineage when routine cytchemistry is negative.

The APAAP technique also facilitates morphological identification of immunologically heterogeneous cell populations. Two cases of untreated AML had low numbers, <5%, of blasts that were immunocytochemically different from the major population. The minor populations eventually emerged as the predominant cell type at relapse. Detection of such minor populations of leukemic cells may not be possible with flow cytometry because most MoAbs are not leukemia-restricted and react with normal myeloid elements.

This study and another report from this laboratory show that results obtained with APAAP immunocytochemistry are comparable to those obtained with flow cytometry; variations in percentages between methods occur in individual cases. Immunocytochemistry offers advantages over flow cytometry in that routinely prepared blood and marrow smears and cytopsin preparations may be used; this is useful when retrospective immunophenotyping may be desirable or when specimens and instrumentation for flow cytometry are not available. Interpretation of results is similar to the interpretation of myeloperoxidase or nonspecific esterase cytochemistry.

Use of APAAP immunocytochemistry requires strict adherence to high technical standards. The quality of reagents and antibodies must be assured, as in any immunophenotyping procedure; positive and negative controls must be evaluated in all specimen batches. The use of a dilute (2%) formalin-acetone mix for a short time (30 seconds) gives excellent cytomorphology of the cells and maintains surface and cytoplasmic antigenicity. Although antigenicity is usually maintained for several months if slides are stored with dessicant at room temperature (personal observation) or frozen at $-20^\circ\text{C}$, careful evaluation of controls is necessary when using stored material. Nonspecific reactivity must also be assessed; alkaline phosphatase activity in neutrophils can be effectively inhibited by levamisole. Endogenous phosphatase activity in macrophages can be diminished by maintaining an alkaline reaction (pH 8.2).

Immunocytochemistry with the APAAP technique is a relatively new but reliable procedure and is an important addition to currently used methods for evaluating acute leukemia.

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IMMUNOPHENOTYPING OF ACUTE MYELOID LEUKEMIA


Immunophenotyping of acute myeloid leukemia using monoclonal antibodies and the alkaline phosphatase-antialkaline phosphatase technique

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