Surface Marker Analysis of Acute Myeloblastic Leukemia:
Identification of Differentiation-Associated Phenotypes


A series of monoclonal antibodies reactive with normal myeloid cells at different stages of differentiation (anti-
MY4, -MY7, -MY8, -Mo1, -la) were used to characterize the leukemic cells of 70 patients with acute myeloblastic leukemia (AML). Sixty-two of the leukemias expressed a phenotype corresponding to a recognizable immature normal myeloid cell. These 62 cases could be divided into 4 phenotype groups, corresponding approximately to the normal CFU-C (group I, 21%), myeloblast (group II, 26%), promyelocyte (group III, 8%), and promonocyte (group IV, 45%). Morphological subtyping of these leukemias tended to agree with the immunologic phenotype, particularly with more "differentiated" morphological subtypes, such as acute monocytic leukemia or acute promyelocytic leukemia. However, each phenotype group contained more than one morphological type of AML, indicating that the level of differentiation of the surface membrane of AML cells may not always be concordant with morphology. The phenotype groups were also analyzed with respect to cytochemical staining patterns, age, the presence of Auer rods, and complete remission rates. Statistically significant differences among the groups were noted in the distribution of myeloperoxidase staining, nonspecific esterase staining, and Auer rods. The complete remission rates varied from 60% (groups III and IV) to 88% (group II). These results suggest that surface marker analysis in AML may be used as a highly reproducible classification system that will provide additional information about the leukemic cells in conjunction with morphological analysis.

A TTEMPTS TO DEFINE clinically useful classification systems for acute myeloblastic leukemia (AML) have become particularly important because of the recent development of highly effective chemotherapy. Most classification schemes of AML take advantage of the morphological similarity of the leukemic cell to a normal counterpart cell, such as a myeloblast or promyelocyte. This type of analysis of morphology has been particularly well suited to AML because of the wide range of morphological variation among the leukemic cells. For example, the French-American-British (FAB) Cooperative Group scheme recognizes six (M1-M6) morphological variants in acute nonlymphocytic leukemia that reflect the degree of maturation of the leukemic cell. Although there do not appear to be major prognostic differences among the subtypes, some investigators have reported a slightly lower response rate or survival rate with the monocytic (M5) variant and longer remissions in promyelocytic (M3) patients. Cytochemical and biochemical techniques have been added to morphological analysis in an attempt to further define such subgroups and to improve diagnostic reproducibility. However, many patients are difficult to classify in any group, or have features of more than one group, and agreement among reviewers is not always concordant.

It has recently been demonstrated that monoclonal antibodies reactive with lineage-restricted antigens can be reliably used to diagnose acute lymphoblastic leukemia (ALL) and that such an immunologic analysis can provide prognostically useful information. In this article, we describe the use of a series of monoclonal antibodies reactive with differentiation antigens of myeloid cells to investigate the heterogeneity of AML. This series of antibodies can identify several different stages of early normal myeloid and monocyte differentiation, and expression of these antigens by AML cells may, in part, be a reflection of the level of maturity of those cells. The identification of different stages of AML cell differentiation through the use of these antibodies allows the development of a classification system that can provide information not obtainable by morphology or cytochemistry.

MATERIALS AND METHODS

Monoclonal Antibodies

Production and characterization of murine monoclonal antibodies anti-MY4, -MY7 and -MY8 have been previously described. Briefly, splenocytes from mice immunized with AML cells were fused with the P3/NS1/1-Ag4-1 myeloma cell line by the method of Kohler and Milstein. Hybrid clones were screened for the production of monoclonal immunoglobulin reactive with the immunizing cells by an indirect immunofluorescence assay, recloned by limiting dilution, and passaged as ascites tumors in pristane-primed BALB/c mice. The anti-Mo1 antibody was provided by Dr. Robert Todd.
(Dana-Farber Cancer Institute), and anti-I2 (anti-la) by Dr. Lee
Nadler (Dana-Farber Cancer Institute).

**Immunofluorescence Assays**

Antibody reactivity with test cells was determined by indirect immunofluorescence. A sample of 10^6 test cells was incubated (4°C, 30 min) with 100 μl of antibody (ascites fluid diluted 1:250 in minimal essential medium containing 2.5% human AB serum), washed 2 times, and further incubated with fluorescein-conjugated goat anti-mouse Ig (Tago, Burlingame, CA). After 2 additional wash steps, fluorescent antibody-coated cells were detected on a flow cytometer (FACS 1, Becton Dickinson, Mountain View, CA; or EPICS V, Coulter Electronics, Hialeah, FL). Background fluorescence, determined by using a nonreactive monoclonal IgG antibody, was subtracted.

**Isolation of Human Cell Fractions**

Granulocytes, monocytes, T cells, B cells, erythrocytes, and platelets were prepared from the peripheral blood of normal donors by standard techniques, as previously described. Natural killer (NK) cells were assayed in a 4-hr chromium release assay using K562 cells as targets. Bone marrow was obtained from healthy volunteers by aspiration into heparin-containing syringes. A mononuclear cell suspension was prepared by Ficoll-Hypaque sedimentation.

**Fluorescence-Activated Cell Sorting (FACS)**

The distribution of each antigen on immature bone marrow myeloid cells was determined by separation of marrow mononuclear cell suspensions into antigen-positive and antigen-negative cell fractions by FACS, as previously described. Cytocentrifuge smears of each fraction were stained with Wright’s stain, and a differential cell count of 200 cells was obtained.

**Hematopoietic Progenitor Cell Assays**

Antibody reactivity with hematopoietic progenitor cells was determined by sterile separation of normal bone marrow cells into antigen-positive and antigen-negative cell fractions by FACS. The appropriate colony assays were then performed with the cells in each fraction. In multiple experiments, recovery of progenitor cells after sorting was similar to recovery of total cells. The committed granulocyte-monocyte colony-forming cell (CFU-C) was assayed in agar by the method of Pike and Robinson. The erythroid burst-forming unit (BFU-E) and colony-forming unit (CFU-E) were determined as described by Clark and Houseman.

**Leukemia Samples**

Peripheral blood or bone marrow aspirate cells from 70 patients with AML were collected into sterile heparin-containing syringes prior to the initiation of antileukemic treatment. Mononuclear cells were prepared by Ficoll-Hypaque sedimentation (1.077 g/cu cm), and a Wright’s stained cytocentrifuge smear examined to determine the percentage of leukemic blasts. All patients had >50% blasts in the analyzed sample and usually >75%. Leukemic cells were cryopreserved in 10% dimethylsulfoxide in the vapor phase of liquid nitrogen. Immunofluorescence assays were performed on cryopreserved samples of all patients except those with acute promyelocytic leukemia (APL), which were analyzed immediately because of rapid progression. These leukemias showed no monocytic morphological features and were generally MPO+ and NSE-. Acute myelomonocytic leukemias (AMML) showed morphological features of partial monocytic differentiation (equivalent to FAB type M4). These leukemias tended to be MPO+ and NSE+. Acute monocytic leukemias (AMoL, equivalent to FAB M5) were MPO- and NSE+. Acute hypergranular promyelocytic leukemia (APL, equivalent to FAB M3) were MPO+ and NSE-. No patients with erythroleukemia (M6) were tested. The presence of Auer rods was determined on a Wright’s stained smear. A complete remission was defined as <5% blasts in the marrow aspirate, lack of circulating leukemic cells, and a return of normal levels of hematopoiesis.

**Statistical Analysis**

The influence of morphology, cytochemistry, and clinical characteristics on expression of these cell surface antigens was evaluated by chi square analysis. Antigens were studied individually, as well as within groups noted to be expressed frequently by the study population.

**RESULTS**

**Immunologic Phenotype of Immature Normal Myeloid Cells**

The distribution of Ia, MY4, MY7, MY8, and Mol antigens was determined by fluorescence-activated cell sorting of aliquots of bone marrow stained with each antibody and is depicted in Fig. 1. As shown, the differentiation of normal myeloid cells is accompanied by an orderly, gradual acquisition (or loss) of the surface structures defined by these antibodies. It is therefore possible to determine the surface antigen “phenotype” of myeloid cells at each level of differentiation. For example, CFU-C cells are predominantly Ia+ and MY7+, but lack MY4, MY8, and Mol antigens. With granulocyte maturation beyond the CFU-C stage, MY8 and Mol antigens are gradually acquired, while the antigen density of MY7 and Ia decreases. The promyelocyte lacks detectable Ia antigen, but MY7, MY8, and Mol antigens continue to be expressed.

During monocyte differentiation, however, Ia antigen is retained. The promonocyte is difficult to identify morphologically, particularly after cell sorting, and expression of antigens on this cell type cannot be determined with certainty. Ia and MY7 antigens, which are expressed by both the CFU-C and the monocyte, would presumably also be expressed by any intermediate cells (promonocytes). Cell sorting experiments confirm that cells with the appearance of...
promonocytes (large diameter, NSE+, high nuclear/cytoplasmic ratio) are Ia+ and MY7+. Similarly, MY4, MY8, and Mo1 are not expressed by CFU-C, but are acquired at some point in cellular development between CFU-C and the peripheral blood monocyte. Cell sorting experiments suggest that some, but not all, promonocytes express each of these antigens.

The information summarized in Fig. 1 is based on FACS analysis for each antigen of marrow samples from 3–6 individual normal donors. There appears to be little variation among normal individuals with respect to expression of these antigens on bone marrow cells. Antigen expression on the CFU-C cell has been confirmed by complement lysis experiments for those antibodies that are lytic (anti-MY7 is not lytic) and by a sensitive immune rosette technique for all antibodies. Expression of these antigens on nonmyeloid cells has been previously reported. MY4, MY7, MY8, and Mo1 antigens are not detectable on peripheral blood B cells, T cells, platelets, or erythrocytes, or on bone marrow erythrocyte progenitors (BFU-E and CFU-E) or megakaryocytes. Mo1 antigen appears to be associated with a receptor for a complement component (C3bi) and is also expressed on large granular lymphocytes having natural killer cell activity. Ia antigen is also expressed by B cells, activated T cells, and other hematopoietic stem cells, including BFU-E. MY4, MY7, MY8, and Mo1 are not expressed by acute lymphoblastic or chronic lymphocytic leukemias.

Using this series of monoclonal antibodies, it can be seen from Fig. 1 that 4 distinct stages of early myeloid differentiation are defined by unique surface antigen phenotypes. The most immature myeloid cell (group I, “CFU-C”) corresponds to the phenotype of the normal CFU-C (Ia+, MY7+ but lacking MY4, MY8, and Mo1). Group II (“myeloblast”) is defined by the acquisition of detectable levels of MY8 and Mo1 antigens. As the myeloblast matures to the promyelocyte stage, Ia antigen is lost, defining group III (“promyelocyte”). A fourth group (“promonocyte-mono- cell”) is defined by the acquisition of detectable levels of MY4 antigen, along with expression of Ia, MY7, MY8, and Mo1 antigens. It should be noted that group I and group II can be distinguished immunologically and functionally, but are not distinguishable morphologically, as the CFU-C cells cannot be morphologically identified with certainty. The labels “myeloblast,” “promyelocyte,” “promonocyte,” and “CFU-C” are used only to indicate a representative normal cell that would express each of the immunologically defined phenotypes.

**Phenotype Analysis of AML Patients**

Seventy patients with AML were analyzed for surface marker expression prior to initiation of therapy. The patients were classified as AML, AMML, AMoL, or APL by standard criteria, using both morphology and cytochemical stains (Table 1). Thirty-six patients had AML, 25 had AMML, 6 had AMoL, and 3 APL. The age, cytochemical staining, presenting white
count, and complete remission rate of these patients are shown in Table 1. Overall, 70% of patients were adults, 91% were MPO+, 58% were NSE+, 67% had Auer rods, 21% presented with WBC >100,000, and 71% went into complete remission. This group is comparable to previously reported groups from our institution and other centers.

The expression of Ia, MY4, MY7, MY8, and Mol antigens on AML patients is shown in Table 2. Overall, 89% of patients were Ia+, 41% were MY4+, 84% were MY7+, 57% were MY8+, and 63% were Mol+. For a given antigen, 20% fluorescent cells was considered "positive" and <20% was considered "negative." However, positive antigens were usually expressed on 50%–100% of the leukemic cells, while negative antigens were detected on <5%. In most cases, positive antigens were brightly fluorescent (high antigen density), with two exceptions. The expression of MY7 in AMoL was less bright in its fluorescence that the expression of MY7 in other leukemic types. Also, the expression of MY8 and Mol in APL was typically less than in most AMLs (M1 and M2) and AMMLs. It should be noted, particularly in the AML and AMML subtypes, that there was considerable heterogeneity of antigen expression.

In order to examine the approximate "differentiation" state of AML cells, the surface antigen phenotypes of individual leukemic patients were compared to the phenotype groups that were previously determined to identify stages of early normal myeloid cell maturation (Fig. 1 and Table 3). Sixty-two of the 70 patients expressed a phenotype compatible with one of these 4 groups, while 8 patients had phenotypes that did not fit the pattern of a normal counterpart cell. The clinical and laboratory parameters in the 62 leukemic patients have been analyzed with respect to the 4 immunologically defined groups to determine the relationship of this immunologic classification system with standard morphologic classification. Thirteen patients expressed a phenotype equivalent to the normal CFU-C (Group I, Table 3). Eight-five percent of these patients had leukemic cells that were considered to be morphologically AML (M1 or M2), while 15% had cells that were morphologically classified as AMML. Group II ("myeloblast") included 16 patients of whom 63% had AML and 37% had AMML. Group III ("promyelocyte") included all of the patients with APL, one patient felt to have AML, and one patient with AMML. The AMML patient was difficult to classify with certainty, as the cells had features of both M3 and M4. The largest group (group IV, "promonocyte") included 28 patients of whom 28% had AML, 50% had AMML, and 22% had AMoL. All of the patients with AMoL studied had the phenotype of group IV. Thus, there was a tendency for patients considered morphologically to have a less differentiated form of leukemia to have a less differentiated phenotype as well. Of note, unique phenotypes were observed in AMoL and APL. However, every immunologic group contained patients from more than one morphological group. The 8 patients whose cells did not have a phenotype that fit the immunologic classification were also heterogeneous. Six patients had AML, while 2 had AMML.

For this analysis, the MY8 and Mol antigens were considered as one group (MY8/Mol) because of their very similar expression on normal myeloid cells (Fig. 1). Expression of either Mol or MY8 or both was considered as evidence of partial differentiation. Seventy-three percent of the patients considered MY8/ Mol + expressed both MY8 and Mol, 17% expressed only Mol, and 10% expressed only MY8. No signifi-

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>AML</th>
<th>AMML</th>
<th>APL</th>
<th>AMoL</th>
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<tr>
<td>Number</td>
<td>36</td>
<td>25</td>
<td>3</td>
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<td>68</td>
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Table 1. AML Study Population

<table>
<thead>
<tr>
<th>Morphological Diagnosis</th>
<th>Number</th>
<th>Age 0-17</th>
<th>Age 18-60</th>
<th>MPO*</th>
<th>NSE</th>
<th>Auer Rod</th>
<th>WBC &gt;100K</th>
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<td>36</td>
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<td>70</td>
<td>91</td>
<td>58</td>
<td>67</td>
<td>21</td>
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* MPO, myeloperoxidase; NSE, alpha naphthylacetate esterase; WBC >100K, white blood cell count greater than 100,000/cu mm; CR, complete remission.

Table 2. Expression of Surface Antigens by Morphological Diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Ia</th>
<th>MY4</th>
<th>MY7</th>
<th>MY8</th>
<th>Mol</th>
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<tr>
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<td>92</td>
<td>81</td>
<td>39</td>
<td>50</td>
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<tr>
<td>AMML</td>
<td>92</td>
<td>56</td>
<td>84</td>
<td>76</td>
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<tr>
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<td>92</td>
<td>84</td>
<td>100</td>
<td>83</td>
<td>100</td>
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<tr>
<td>AMoL</td>
<td>86</td>
<td>100</td>
<td>100</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>89</td>
<td>41</td>
<td>84</td>
<td>57</td>
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</table>

* Morphologically or cytochemical criteria.
† An immunofluorescence assay was considered positive when >20% of test cells were fluorescent by FACS analysis. Generally, 50%–100% of leukemic cells were fluorescent in a positive AML.
‡ The antigen density tended to be low (dim fluorescence) compared to expression in other types of leukemia.
Table 3. Differentiation Levels in AML

<table>
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<tr>
<th>Phenotype Group</th>
<th>Approximate Stage of Differentiation</th>
<th>Phenotype</th>
<th>No. of Patients</th>
<th>Morphology</th>
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<td></td>
<td></td>
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<td>MY7</td>
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<tr>
<td>I</td>
<td>CFU-C</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>Myeloblast</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>III</td>
<td>Promyelocyte</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IV</td>
<td>Promonocyte</td>
<td>+</td>
<td>+</td>
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*Chi square analysis comparing distribution of morphological types in a given phenotype group with the total population.
†MY8 and Mol antigens were considered as a group because of their similar distribution on normal cells. See text for explanation.

Table 4 presents an analysis of the other clinical and laboratory parameters studied in these 62 patients. The majority of patients in each immunologic group were adult (range 54%–100%). Only 6 of the 62 cases were MPO–, but all 6 were found in group IV. NSE was detected in 0% (group III) to 76% (group IV) of cases. Auer rods were found in the majority of cases, except in group IV. Similar numbers of patients with high presenting white cell counts (>100,000/cu mm) were found in all groups. The highest complete remission (CR) rate (88%) was seen in group II, and the lowest (60%) in groups III and IV. When each group was compared to the total population, however, the difference in CR rates did not reach statistical significance.

DISCUSSION

Acute myeloblastic leukemia has long been recognized as a heterogeneous disease. The leukemic cells of different patients often show morphological features characteristic of different stages of myeloid differentiation. The classification schemes of AML are largely based on these morphological differences, which presumably reflect the maturation of the leukemic cell.1,2 We have previously described the production of monoclonal antibodies that react with specific differentiation-linked antigens of myeloid cells.9,10,21 The results presented here show that it is possible to trace the development of early myeloid cells through the use of anti-MY4, -MY7, -MY8, -Mo1, and -Ia to detect differentiation-associated changes on the cell surface. The patterns of reactivity of these monoclonal antibodies identify four different stages of differentiation that correspond approximately to the normal CFU-C, myeloblast, promonocyte, and promyelocyte. Since many AML cells morphologically resemble myeloblasts, promyelocytes, or promonocytes, it was of interest to determine the relationship between the level of morphological differentiation and the state of maturation determined by the cell surface antigen phenotype. Seventy patients with AML were analyzed with respect to morphology, cytochemistry, and surface antigens. Sixty-two of the 70 patients expressed a phenotype that was identical to one of the four phenotypes characteristic of normal immature myeloid cells. The most immature cell phenotype (group I, “CFU-C”) was characterized by the expression of Ia and MY7 antigens, but lacked MY4, MY8, or Mo1. This group included 13 AML patients, of whom 85% were considered by morphological criteria as having AML (M1 or M2). Group II (“myeloblast”) was characterized by the expression of Ia, MY7, and Mo1 or MY8 antigens, but lacked MY4. The majority of the 16 patients in this group had leukemic cells that were thought morphologically to be AML, but 37% were considered AMML. The smallest group (III, “pro-
myelocyte") included all of the patients with APL and was characterized by loss of Ia antigen with continued expression of MY7. The largest group (IV, "promonocytic") included 28 patients and was characterized by the expression of Ia, MY4, MY7, MY8, and Mol antigens. Seventy-eight percent of patients were considered to have monocytic morphology (AMML or AMoL), while 22% were considered to have AML. This analysis shows that although there is a tendency for the morphology to correlate with the surface antigen phenotype, each phenotypic group contains patients having different morphological types. This suggests that the surface antigen phenotype reflects a related, but somewhat different, view of the state of the leukemic cell differentiation than does morphology, thereby providing useful supplemental information to the standard morphological classification. Another advantage of immunodiagnosis is the high degree of reproducibility and objectivity of the technique, not always encountered in morphological classification systems in AML.3,5

In order to further evaluate the utility of an immunologic classification scheme in AML, the phenotype groups were analyzed to determine the distribution of patients by age, presence of marked leukocytosis, myeloperoxidase activity, nonspecific esterase activity, presence of Auer rods, and complete remission rate. The majority of patients in each group were adults, and the fraction of patients presenting with high WBC was similar in each group. NSE positivity was lowest in groups I and III (CFU-C and promyelocyte) and highest in group IV (promonocyte). Group IV had the lowest level of myeloperoxidase activity and also Auer rods. The latter finding is of interest because of the previous reports of the presence of Auer rods being prognostically favorable.6 The complete remission rate varied from 60% (groups III and IV) to a high of 88% (group II). However, this trend was not statistically significant. With larger numbers of patients, phenotype analysis in AML may prove useful in the identification of patient groups with particularly good or bad response to treatment. It will also be important to determine in the future if there are any significant differences in duration of remission among any of the phenotype groups. Finally, certain chromosome abnormalities have been described in association with specific morphological types in AML. For example, t(15q;17) is associated with APL.26 t(8;21) with FAB type M2,27,28 and rearrangement of the long arm of chromosome 11 with AMoL.29 It is likely that specific surface antigen phenotypes will similarly be associated with certain chromosomal abnormalities.

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Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes

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