Electrophoretic and Cytochemical Characterization of Alpha-Naphthyl Acetate Esterases in Acute Myeloid Leukemia: Relationships With Membrane Receptor and Monocyte-Specific Antigen Expression


Alpha-naphthyl acetate esterases (ANAE) were examined by cytochemical and isoelectric focusing (IEF) techniques in 48 cases of acute myeloid leukemia that were classified by conventional morphological criteria. Four main types of ANAE isoenzyme patterns were found by IEF, and comparisons with the expression of membrane receptors (Fc-IgG and C3b) and monocyte-specific antigens (UCHM1, UCHALF, and E11) suggest relationships between ANAE isoenzyme synthesis and distinct myeloid maturational stages. The results further indicate that the blast cells of acute myelomonocytic leukemia (AMML) may represent an immature variant of monocytic leukemia (AMoL) and that morphological examination alone is inadequate in the assessment of monocytic differentiation in acute myeloid leukemias. Inhibition studies of cytochemical ANAE activity with sodium fluoride (NaF) show that the presence of NaF-sensitive or NaF-resistant ANAE enzymes is often unrelated to the diagnostic category of acute leukemia. The results of this study are examined in relation to current concepts of myeloid differentiation, and the application of these findings to the subclassification of acute myeloid leukemias is discussed.

MATERIALS AND METHODS

Patients Studied

Cryopreserved blast cells from patients with acute nonlymphoid leukemia were obtained from the Imperial Cancer Research Fund, Department of Medical Oncology, at St Bartholomew's Hospital, London. In all cases, the blast cells comprised more than 95% of the nucleated cells examined. For the purpose of this study, the cases were classified morphologically into three main groups according to the French-American-British proposals. Group 1 represented acute myeloblastic leukemia (AML; FAB groups M1 and M2, n = 16), group 2 represented acute myelomonocytic leukemia (AMML; FAB group M4, n = 18), and group 3 represented acute monocytic leukemia (AMoL; FAB group M5, n = 14).

Monoclonal Antibody Studies

The presence of UCHM1, UCHALF, and E11 determinants were examined on blast cells from 40 cases using a FACS IV. RPMI, supplemented with 10% fetal calf serum or a combination of anti-HLe-1 (common leukocyte antigen) and anti-HLA-A, B, C, was employed as the negative or positive control, respectively. The FACS negative and positive windows were set using the appropriate controls, and cases with background staining 10% above that obtained with the negative control were excluded from the study. Monoclonal antibodies UCHM1 and UCHALF stain approximately
80% of normal monocytes, as well as the blast cells, from most cases of AMML and AMoL. The E11 determinant is found on blast cells from nearly all cases of AMoL and a smaller proportion of AMML, UCHM1, UCHALF, and E11 are nonreactive with blast cells from acute myeloblastic leukemia.

The remaining 8 cases (all AML) were examined for monocytic components with monoclonal antibodies Mo2 (Coulter Electronics, Hialeah, FL) and 63D3 (Bethesda Research Laboratories, Bethesda, MD). These antibodies specifically detect antigens found on normal adherent monocytes and on some blasts from AMML and AMoL. No significant reactivity is found with normal granulocytes and blast cells from AML.

**Extraction and Biochemical Assay of Leukocyte ANAE Enzymes**

Leukemic cells were pelleted by centrifugation and resuspended in a minimal volume of 20 mM n-morpholino ethane sulfonic acid (MES) buffer, pH 6.4, containing 20% glycerol. Cytoplasmic enzymes were extracted from the blast cells by sonication for 30 sec, followed by the addition of Triton-X to a final concentration of 1%.

After 5 min, the suspension was centrifuged at 5,000 g for 20 min, and the supernatant, containing extracted cytoplasmic enzymes, was removed and stored at -20°C. It was not necessary to quantitate leukemic blasts prior to sonication and enzyme extraction, as the cellular ANAE enzyme activities were expressed as a function of sonicate protein concentrations in all cases.

The spectrophotometric assay of α-naphthyl acetate esterases was determined by the rate of α-naphthyl acetate hydrolysis by leukocyte esterases at 235 nm. An esterase unit was defined as the amount of ANAE enzyme that hydrolyzed 1.0μmole of substrate in 1 min at 37°C. Protein concentrations of leukocyte sonicates were determined by the method of Lowry et al.

**Isoelectric Focusing (IEF) of ANAE Isoenzymes**

Enzyme extracts were focused on 5% polyacrylamide gels containing 2.4% ampholine (LKB) for 1,500 V/hr (LKB Multiphor 2103). Gel temperature was stabilized with a glass cooling plate and the pH gradient measured, following electrophoresis, by surface pH electrode. Gels were stained for ANAE and the enzyme patterns compared with those obtained from normal peripheral blood and bone marrow leukocyte fractions (manuscript in preparation). The volume of sample focused represented a range of 1-25 mU of ANAE enzyme activity.

**Cytochemistry**

ANAE cytochemical activity was demonstrated on cytocentrifuged leukemic blast cell monolayers with the substrate-diazonium salt combination of α-naphthyl acetate and hexazotized pararosanilin. In addition, the effect of sodium fluoride (NaF) at a concentration of 1.0 mg/ml on the cytochemical activity of ANAE was also assessed.

**Demonstration of Membrane Fc-IgG and C3b Receptors**

Blast cell membrane receptors for the Fc of IgG and C3b were assessed by rosetting techniques with IgG-coated and C3b-coated ox erythrocytes (oxE), as previously described.

**Statistics**

Statistical comparisons were made using the nonparametric Mann-Whitney (two-tailed) rank sum test.

**Results**

**ANAE Isoenzyme Patterns of Leukemic Blasts in Acute Myeloid Leukemias**

In this study of acute myeloid leukemia, four main types of ANAE isoenzyme patterns were obtained by IEF (Figs. 1 and 2). The first, which is subsequently referred to as a G-type pattern, was characterized by the presence of variable numbers of ANAE isoenzymes with isoelectric points (pls) between 6.2 and 8.1. The other three types of IEF pattern, designated Mon1, Mon2, and Mon3, were characterized by the presence of ANAE isoenzymes with pls between 5.5 and 6.1, in addition to the isoenzymes found in the G pattern. Mon1 patterns were defined as having a weak concentration of monocyt-associated isoenzymes (often in the form of two weak enzyme bands); Mon2 patterns showed moderate to high concentrations of four monocytic isoenzymes; and Mon3 patterns were characterized by the presence of six isoenzyme bands. The G pattern of ANAE isoenzymes was the same as that observed with normal peripheral blood granulocytes and bone marrow myeloblast fractions, although some variation in the number of isoenzyme bands was observed, particularly in the pl range 6.2–7.0. Isoelectric focusing of cytoplasmic ANAE isoenzymes from two normal peripheral blood monocyte fractions revealed the presence of four major isoenzyme components within the pl range 5.5–6.1 (Mon2 pattern), in...
addition to minor isoenzyme components with pls above 6.2. Treatment of enzyme extracts with neuraminidase (5 U/ml; Sigma, St. Louis, MO) did not alter the distribution of the different monocytic isoenzyme bands, suggesting that their minor differences in pl were not due to small variations in molecular charge caused by the action of cellular glycosidases.

ANAE and Monoclonal Antibody Studies in Acute Myeloblastic Leukemia (AML: FAB Groups M1 and M2)

IEF patterns of ANAE isoenzymes were examined in 16 cases of AML classified by conventional morphological criteria. Twelve of 16 cases showed a G-type IEF pattern and 4 cases a Mon1 pattern (Table I). There appeared to be no clear correlation between cytochemical staining of the blast cells and ANAE-IEF pattern type. Of the four G-IEF cases showing significant cytochemical ANAE activity, two (STO and CHR) showed NaF-sensitive weak granular reactions, and the others (CH-62 and BAN) showed NaF-resistant weak-diffuse staining patterns.

The presence of monocyte-associated antigens was insignificant in 13/16 of the AML cases, and of the 3 with significant numbers of UCHM1 and UCHALF antigen-positive blasts, 2 showed a G-type ANAE-IEF pattern. The ANAE concentrations of leukemic blasts in this group ranged from 5 to 54 mU/mg protein.

Table 1. Acid Alpha-Naphthyl Acetate Esterase and Monoclonal Antibody Studies in Acute Myeloblastic Leukemias (FAB Groups M1 and M2) Classified by Conventional Morphological Examination

<table>
<thead>
<tr>
<th>Patients</th>
<th>Monoclonal Studies</th>
<th>ANAE Positive Blasts †</th>
<th>ANAE Assay (mU/mg) ‡</th>
<th>IEF Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UCHM1</td>
<td>UCHALF</td>
<td>E11</td>
<td>Mo2</td>
</tr>
<tr>
<td>HO-25</td>
<td>&lt;10</td>
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<td>WA-61</td>
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<td>CH-62</td>
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<td>BEV</td>
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<td>PAR</td>
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<td>BAN</td>
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</tr>
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<td>FR-63</td>
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<td>22</td>
<td>&lt;10</td>
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</tr>
<tr>
<td>PU-65</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Antimonocyte: clone 63D3 (Bethesda Research Laboratories).
†Percentage ANAE-positive blasts: In the absence of NaF/in the presence of NaF (1.0 mg/ml). G indicates granular or focal type of reaction, all other reactions tending to be weak diffuse. NS indicates no significant reaction.
‡ANAE enzyme activity of leukocyte sonicate expressed as mU/mg protein. Results of monoclonal antibody studies are shown as percentages of positive cells.
NT, not tested.
Table 2. Acid Alpha-Naphthyl Acetate Esterase and Monoclonal Antibody Studies in Acute Myelomonocytic Leukemia (AMML: FAB Group M4)

The cytochemical ANAE reaction was moderate to strong diffuse in 11/18 cases, weak diffuse in 3, granular in 1, and insignificant in the remainder. There were no apparent relationships between cytochemical ANAE reactivity, cytochemical sensitivity of ANAE to NaF, and ANAE-IEF patterns (Table 2). Monoclonal antibody studies demonstrated that the AMML cases with G-type IEF patterns showed a minor, but consistent, UCHM1/UCHALF-positive blast cell component. Further analysis revealed that the AMML patients with Mon-type ANAE isoenzymes showed, with the notable exception of one case (DE-48), increased proportions of UCHM1-positive blasts (mean 25.8%) compared with the cases with G-type IEF patterns (mean 15.9%). It is also of interest that 4/6 cases with G-type patterns showed insignificant proportions of E11-positive blasts, whereas all but one of the Mon-ANAE IEF group had a significant E11-positive component (mean percent E11-positive blasts ± SE: G group 8.2 ± 9.3, Mon group 23.9 ± 12.4; p = 0.05).

The ANAE concentration of leukemic blasts in this AMML group ranged from 7 to 109 mU/mg protein, with a mean value of 40.6.

ANAE and Monoclonal Antibody Studies in Acute Monocytic Leukemia (AMoL: FAB Group M5)

All cases in this group showed a significant proportion of both cytochemically ANAE and UCHM1/UCHALF-positive blasts (Table 3). Isoelectric focusing of cytoplasmic esterases showed 2/14 cases with Mon1, 5/14 with Mon2, and 7/14 with Mon3 patterns.

The cytochemical reactions for ANAE were predominantly NaF-sensitive in all cases, and the percentages of ANAE-positive and UCHM1/UCHALF-positive blasts were generally in good agreement. However, of the four cases with insignificant E11-positive blast cell components, three showed Mon3-type ANAE-IEF patterns.

The ANAE concentrations of leukemic blasts in this group of patients ranged from 12 to 154 mU/mg protein, with a mean value of 68.6.

Monoclonal Antibody and NaF-Resistant Esterase Studies in Acute Myeloid Leukemias Categorized According to IEF Pattern

Forty cases of acute myeloid leukemia were grouped according to their ANAE-IEF patterns into G (n = 11), Mon1 (n = 9), Mon2 (n = 7), and Mon3 (n = 13)
in order to assess relationships with the expression of monococyte-specific antigens and the presence of NaF-resistant ANAE-positive blasts. The results (Table 4) show that there are significant increases in the percentages of UCHM1/UCHALF antigen-positive blasts between the G-IEF and Mon1-IEF groups and also between the Mon1-IEF and Mon2/3-IEF groups. However, there appears to be no significant differences in the expression of monocytic antigens between the Mon2-IEF and Mon3-IEF groups. The expression of the E11 antigen is similar in all groups with demonstrable monocytic ANAE isoenzymes, but is considerably lower in the G-type IEF group.

The increasing proportions of monocytic antigen-positive blasts between the G-IEF, Mon1-IEF, and Mon2/3-IEF-defined groups of acute myeloid leukemia suggest that qualitative changes in ANAE synthesis may be related to different stages of monocytic differentiation. In addition, the quantitative increases in specific monocytic ANAE isoenzyme synthesis, which appear to accompany these changes concomitantly, result in decreased proportions of NaF-resistant ANAE-positive blasts.

Expression of Membrane Fc-IgG and C3b Receptors by Blast Cells in Acute Myeloid Leukemias

Membrane Fc-IgG and C3b receptors were determined by rosette assay in 33 cases of acute myeloid leukemias (M1, M2, M4, and M5) that were categorized into four groups on the basis of ANAE-IEF and monoclonal antibody studies. These were (a) G-type IEF, UCHM1/UCHALF-positive component <10%.

<table>
<thead>
<tr>
<th>Monoclonal Studies</th>
<th>Percent ANAE-Positive Blasts*</th>
<th>Percent ANAE-Positive, NaF-Resistant Blasts†</th>
<th>ANAE Assay (mU/mg)‡</th>
<th>IEF Pattern</th>
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<tbody>
<tr>
<td>Patients</td>
<td>UCHM1</td>
<td>UCHALF</td>
<td>E11</td>
<td>NS</td>
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<td>LO-1</td>
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<td>69</td>
<td>76</td>
<td>91</td>
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<td>BE-69</td>
<td>24</td>
<td>22</td>
<td>&lt;10</td>
<td>82</td>
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</table>

*Percentage ANAE-positive blasts. All reactions are of moderate to strong diffuse type.
†Percentage ANAE-positive blasts in the presence of 1.0 mg/ml sodium fluoride (NaF). NS indicates no significant ANAE cytochemical reaction.
‡ANAE enzyme assay of leukocyte sonicate expressed as mU/mg protein.

Results of monoclonal antibodies are shown as percentages of positive cells. NT, not tested.

<table>
<thead>
<tr>
<th>IEF Pattern</th>
<th>Monoclonal Studies</th>
<th>ANAE Assay (mU/mg)†</th>
<th>Percentages of ANAE-Positive Blasts That Are NaF-Resistant (1.0 mg/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>14.0 (1.9)</td>
<td>15.4 (2.7)</td>
<td>5.9 (2.2)</td>
</tr>
<tr>
<td>n = 11</td>
<td></td>
<td></td>
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<tr>
<td>Mon1</td>
<td>25.3 (6.0)</td>
<td>24.3 (6.1)</td>
<td>17.9 (5.8)</td>
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<tr>
<td>n = 9</td>
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<td></td>
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<tr>
<td>Mon2</td>
<td>40.8 (8.5)</td>
<td>41.1 (8.8)</td>
<td>20.4 (6.5)</td>
</tr>
<tr>
<td>n = 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mon3</td>
<td>46.1 (6.9)</td>
<td>39.2 (7.3)</td>
<td>18.3 (5.6)</td>
</tr>
<tr>
<td>n = 13</td>
<td></td>
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</table>

Results are shown as mean percentages of positive blasts with standard errors (SE) given in parentheses.
*Represents the mean ANAE enzyme activity of leukocyte sonicates expressed as mU/mg protein.
†Indicates the proportion of ANAE-positive blasts reacting in the presence of sodium fluoride (NaF) expressed as a percentage of the proportion of ANAE-positive blasts stained in the absence of NaF.
DISCUSSION

This study has examined the distribution of ANAE isoenzymes in acute myeloid leukemias in which monocytic components were further assessed by monoclonal antibody and membrane receptor studies. Acute leukemias were investigated, in common with previous studies,17-20 as models for myeloid differentiation because they offer unique opportunities for examining immature cell characteristics by variable degrees of maturation arrest. While leukemogenesis may result in aberrant or asynchronous phenotypic expression,21,22 the blast cells in many cases of leukemia are considered to retain lineage fidelity23-25 and express developmental markers appropriate to their normal maturational counterparts. This study, therefore, in addition to assessing both cytoplasmic and membrane characteristics of morphologically categorized leukemic blasts, also examines the findings in relation to early granulocytic and monocytic differentiation.

The cytochemical and immunologic features of the acute myeloid leukemias within the FAB-defined groups are summarized in Table 6. The morphological diagnosis of AML was supported in 10/16 of the cases examined, in that no evidence of monocytic involvement or differentiation was found. The other 6 cases, however, showed minor but significant monocytic blast cell components by ANAE isoenzyme or immunologic investigations. Of the AMML cases, 17/18 had significant monocytic antigen (MonAg) positive populations, whereas the blasts in nearly 56% of these same cases showed weak or undetectable monocyte-associated ANAE isoenzymes by IEF. In contrast, the proportions of antigen-positive and ANAE-positive blasts in AMoL were generally in good agreement, and isoelectric focusing of cytoplasmic ANAE enzymes revealed the presence of Mon2/3 patterns in 12/14 cases.

Of interest is the finding of five cases of acute

(b) G-type IEF, antigen-positive component 10%-30%; (c) Mon-type IEF, antigen-positive component 10%-30%; and (d) Mon-type IEF with more than 30% antigen-positive blasts.

The results (Table 5) show that significant increases in membrane receptor activities accompany monocytic components. This study, therefore, in addition to assessing both cytoplasmic and membrane characteristics of morphologically categorized leukemic blasts, also examines the findings in relation to early granulocytic and monocytic differentiation.

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myeloid leukemia in which the proportions of MonAg blasts were low despite the demonstration of Mon-type ANAE isoenzymes by IEF. Weak monocytic components (Mon1) were found in three cases (OD-58, DAW, and PU-65) morphologically classified as AML (M1/M2) with <10% MonAg blasts. The other two cases, an AMML (DE-48) and AMoL (RO-16) with <10% and 26% weak UCHM1-positive/UCHALF-negative blasts, respectively, showed high concentrations (Mon3) of monocytic ANAE isoenzymes. Although the apparent AML cases with Mon1 ANAE-IEF patterns may be due to slightly disordered expression of membrane antigens and cytoplasmic ANAE, the antigen-negative AMML and AMoL cases are of more importance. While aberrant expression of monocytic ANAE isoenzymes by cells of committed granulocytic lineage, such as that found in well differentiated granulocytes in some primary and secondary myelodysplastic syndromes, remains a possibility, the distinctive morphology and high ANAE activities suggest that these cases may represent malignant expansions of normal UCHM1, UCHALF monocytes.

Characterization of cytoplasmic ANAE by IEF reveals that isoenzymes with a pl above 6.2 are found in both granulocytic and monocytic cells and that monocytic differentiation is specifically associated with the additional synthesis of distinct isoenzymes (MonANAE) within the pl range of 5.5–6.1. In this study of acute myeloid leukemias, G-type IEF patterns were found in 12/16 cases of AML, 6/18 cases of AMML, but not in AMoL. The leukemic cases with G-type ANAE patterns in the AMML group differ, however, from AML cases with similar IEF patterns by the presence of significantly higher proportions of MonAg blasts. These results, when interpreted in the context of myeloid differentiation, therefore suggest that expression of lineage-specific antigens precedes cytoplasmic MonANAE synthesis and that the MonAg MonANAE blasts found in some cases of AMML may correspond to an early stage of monocytic differentiation. It is also evident from these studies that membrane Fc-IgG receptor expression, associated with monocytic involvement in the leukemic process, correlates well with MonAg expression and that detectable increases in blast cell receptor affinities may occur prior to the appearance of cytoplasmic MonANAE.

Cases of acute leukemia in which Mon2 or Mon3 ANAE-IEF patterns were obtained appeared to show similar proportions of MonAg blasts (Table 4), and the relationships between these qualitatively different isoenzyme patterns is unclear. However, the unequivocal association of these particular ANAE-IEF patterns with AMoL suggests that the blast cells in this leukemic category are relatively well differentiated. In contrast, Mon1 patterns that are characterized by the presence of weak monocytic ANAE-IEF bands were found in 4/16 cases of AML, 4/18 cases of AMML, and 2/14 cases of AMoL. Of these 10 cases, 7 showed significant proportions of MonAg blasts, and it is considered that this ANAE isoenzyme pattern reflects, in terms of monocyte differentiation-linked ANAE isoenzyme synthesis, relative cytoplasmic immaturity.

There is increasing evidence to indicate that blast cells at the M1 and M2 levels of maturation may not be irreversibly committed to granulocytic differentiation. It is further suggested that commitment to the granulocytic or monocytic lineage may occur as late as the promyelocyte stage and that immature cells that show apparent lineage restriction by expression of granulocytic or monocytic characteristics may not be irreversibly committed and that there may be a transitional period in which changes in maturational direction can be effected by alterations in extracellular stimuli. Acute myelomonocytic leukemias (M4) are characterized by blast cells showing some evidence of monocytic differentiation together with a proportion of cells showing apparent granulocytic differentiation, as adjudged by the presence of chloroacetate esterases. Cytochemical studies of AMML blasts in this and previous studies, however, suggest that their ANAE reactivity is generally weaker than in AMoL, although some cases show strong ANAE-reacting cells. The cytochemical heterogeneity of ANAE reactions in AMML is confirmed by IEF whereby all four main pattern types were obtained. It is proposed therefore that AMML represents a maturational spectrum intermediate between the myeloblast (MonAg, MonANAE), both normal and leukemic, and the “monoblast” of AMoL (MonAg, MonANAE). The presence of chloroacetate esterases in a proportion of AMML blasts is not inconsistent with this hypothesis and may reflect their uncommitted nature, rather than indicating a mixture of blasts irreversibly committed to either granulocytic or monocytic differentiation.

Sodium fluoride (NaF) has long been employed in cytochemistry for the distinction of monocytic esterases from those present in granulocytes. The use of this inhibitor in conjunction with naphthol AS-D acetate (NASDAQ) clearly defines the NaF-resistant chloroacetate esterases of the granulocytic series, but the present investigation demonstrates that its application in differentiating monocytic and granulocytic ANAE isoenzymes is far less reliable. Indeed, it is evident from the observations in this study that the presence of NaF-sensitive or NaF-resistant ANAE enzymes was often unrelated to diagnostic category. While mono-
cytic leukemias (M5) usually showed strong cytochemical ANAE reactions that were predominantly NaF-sensitive, the ANAE cytochemistry of AML and AMML blasts was extremely variable. It is considered, therefore, that NaF inhibition studies of ANAE reactions have little diagnostic application in the assessment of monocytic involvement in acute myeloid leukemias.

In conclusion, this study has examined the expression of membrane antigens, membrane receptors, and cytoplasmic ANAE isoenzymes by blast cells in acute myeloid leukemias. The investigation demonstrates that monocytic differentiation by blast cells, particularly in the AML and AMML morphological categories, is more reliably assessed by immunologic and electrophoretic studies. In addition, the results provide further evidence for the heterogeneity of conventional morphological categories of acute myeloid leukemia and indicate that accuracy of leukemic classification may not always be achieved by morphology and cytochemistry alone.

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Electrophoretic and cytochemical characterization of alpha-naphthyl acetate esterases in acute myeloid leukemia: relationships with membrane receptor and monocyte-specific antigen expression

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