A Comparison of Surface Marker Analysis and FAB Classification in Acute Myeloid Leukemia

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Surface marker analysis with rosette tests and a large panel of xenoantisera and monoclonal antibodies was done on the malignant cells of 55 patients with acute myeloid leukemia (AML). The diagnosis was made on morphological and cytochemical grounds, and the leukemias were classified according to the quantified FAB criteria. The marker tests included the E- and EA-rosette test, immunofluorescence with rabbit-polycional antisera against human Ig, x, and λ light chains, thymocytes, granulocytes, erythrocytes, platelets, lysozyme, (leukemic) myeloblasts, the common ALL antigen, SB cell-line cells (anti-la), and a mouse anti-la serum. The monoclonal mouse antibodies applied were anti-T-cell antibody (3A1), two anti-granulocyte-monocyte antibodies (OKM1 and B2.12), four antigranulocyte antibodies (MI/N1, UJ 308, B4.3, and B13.9), an antiplatelet antibody (C17.28), anti-HLA heavy chains (w6/32. HLK), anti-la antigen (OK11), and OKT10. AML cells from many patients lacked the expression of myeloid markers, and we found that a correlation existed between the relative maturity of the leukemia subtype and the extent of positivity for these markers. Surface marker analysis discriminated poorly between the “myeloid” and “monocytoid” subtypes: OKT10 and the “T-cell marker” 3A1 were often expressed on AML cells. In two cases of AML, there was an unexpected expression of platelet antigens with the monoclonal antiplatelet antibody. One of them, classified as M1, was ultrastructurally a megakaryoblastic proliferation with a positive reaction for platelet peroxidase. Only with the help of computerized analysis, it was possible to prove a clear correlation between the surface marker profile and the FAB classification.

During the last 10 yr, immunologic techniques defining B, T, and null lymphocytes have elucidated the heterogeneity and the cellular origin of the malignant lymphocytes in acute lymphoblastic leukemia (ALL). Previously, indistinguishable and clinically relevant subgroups of ALL are now being recognized.4,5 In acute myeloid leukemia (AML), classification and recognition of relevant subgroups are presently based only on cytologic and cytochemical techniques.6,10 Immunologic typing has not been feasible because specific markers for the various myeloid cell types were not (yet) available. In fact, most literature on this subject deals with the expression of rather unspecific markers on the cells of AML patients.11,15 However, recently this situation has changed and xenoantibodies (polyclonal and especially monoclonal) specific for antigens on cells of the granulocytic, monocytic, erythrocytic, and thrombocytic series have been raised.3,5,6,16,17,19,20

In this report, we present the results of an attempt to characterize the cells of AML patients with such antibodies. The results were related to those of internationally accepted FAB classification of leukemia performed in a quantitative way, as described by van Rhenen et al.7

MATERIALS AND METHODS

Morphological Characterization

Over a period of 4 yr, blood and/or bone marrow samples from 55 patients suffering from acute myeloid leukemia (AML) were collected. All peripheral blood and bone marrow smears were reviewed independently by two of us for classification according to the FAB criteria,7 as further quantified by van Rhenen et al.7

The smears had to be adequately prepared and stained with May-Grünwald-Giemsa, Sudan black, and α-naphthyl acetate esterase. Differential counts of at least 100 cells in the peripheral blood and of 500 cells in the bone marrow were performed.

Cell Isolation

For surface marker analysis, blood and/or bone marrow samples from patients were anticoagulated with EDTA or heparin and were processed within 24 hr. The leukemic cells were isolated from the blood and bone marrow by Ficoll-Isoaque density gradient centrifugation (d = 1.077 g/cu cm).

After isolation, all samples used for immunologic investigation contained more than 80% leukemic cells. The cells were tested fresh or after storage in liquid nitrogen.21

Rosette Tests and Xenoantisera

The following rabbit antisera were used: antigranulocyte serum,16 antimylloblast serum, which was raised against leukemic myeloblasts and rendered specific for the myelomonocytic series with selective absorptions (red cells, platelets, and lymphocytes), antierythrocyte,16 antiplatelet,16 antithymocyte, and anti-common-ALL serum.22

Furthermore, we applied anti-SB (anti-la),23 antilysozyme (Dakopatts, Copenhagen, Denmark), FITC-labeled F(ab')2 fragments of a rabbit anti-human Ig serum (K26-H 26-5-F, CLB, Amsterdam, The Netherlands), and anti-kappa and anti-lambda light-chain sera (Dakopatts, Copenhagen, Denmark), also rabbit antisera. We also

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used a mouse allo-anti-la serum (CLS), which reacts with all human HLA-DR antigens.24

For the detection of the Fc receptor for IgG, the EA-rosette test was used (human OR,R red cells sensitized with human IgG anti-Rh-D). The E-rosette test was applied as well.

**Monoclonal Mouse Antibodies**

Monoclonal antibodies (McAb) of the Orthoclone series were a gift from Dr. R. Verbruggen (Ortho Diagnostics, Beersel, Belgium). For our present investigations, we used OKT10 (THY) directed against an antigen with a mol wt of 42,000-44,000; 0KM1, directed against an antigen on granulocytes, monocytes, and macrophages; and OKI1 (FRA), directed against the la molecule. Furthermore, we applied the McAb 3A1, a gift from Dr. A.S. Fauci (NIH, Bethesda, Md.), which recognizes an antigen with a mol wt of 40,000 on the cells of the T-cell lymphoblastoid cell line HSB-2 and on a population of peripheral blood T cells; the McAb W6/32, a gift from Dr. P. Wernet (University of Tübingen, West Germany), which recognizes a common determinant on the heavy chain of HLA-A, B, and C antigen molecules was used as a positive control. McAb MI/N1 and UJ 308 were gifts from Dr. J.T. Kemshead (Imperial Cancer Research Fund, London, UK), raised against neuroblastoma cell line cells (CHP 100), but reactive not only with these cells but also with myeloid cells.32

The McAb B2.12, B4.3, B13.9, and C17.28 were produced in our own laboratory. Fusion, screening of culture supernatants of hybrid cultures for antibody activity against various test cells (by an ELISA technique), and cloning of hybrid cells by limiting dilution [with human endothelial culture supernatant (HECS)], were performed as previously described.33 The first three McAb, obtained as side products of an attempt to raise T-cell-specific antibodies, react strongly with granulocytes, but not with platelets or red blood cells. B2.12 (IgM) reacts also with monocytes and with a small population of lymphocytes too, whereas B4.3 (IgM) and B13.9 (IgG1) do not (Landsorp, unpublished observation).

McAb C17.28 is a platelet-specific McAb (IgG1), raised in our laboratory by immunizing a mouse with human peripheral blood lymphocytes and directed against the platelet glycoprotein IIb (Teterevo, unpublished observations).

In this study, ascites fluid from mice injected with cloned hybrid cells was used. A summary of the reactivity of the McAb with lymphocytes too, whereas B4.3 (IgM) and B13.9 (IgG1) do not (Landsorp, unpublished observation).

### Table 1. Reactivity of McAb With Peripheral Blood Cells of Healthy Individuals (The Percentage of Cells Reacting Positively Is Given)

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>RBC*</th>
<th>Platelets†</th>
<th>Lymphocytes‡</th>
<th>Monocytes§</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6/32HLK</td>
<td>0%</td>
<td>100% +</td>
<td>100% +</td>
<td>100% +</td>
<td>100% +</td>
</tr>
<tr>
<td>B4.3</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100% +</td>
</tr>
<tr>
<td>B13.9</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100% +</td>
</tr>
<tr>
<td>MI/N1</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100% +</td>
</tr>
<tr>
<td>UJ 308</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>100% +</td>
</tr>
<tr>
<td>B2.12</td>
<td>0%</td>
<td>0%</td>
<td>5%-10% +</td>
<td>100% +</td>
<td>100% +</td>
</tr>
<tr>
<td>OKM1</td>
<td>0%</td>
<td>0%</td>
<td>5%-10% +</td>
<td>100% +</td>
<td>100% +</td>
</tr>
<tr>
<td>OK1</td>
<td>0%</td>
<td>0%</td>
<td>10%-15% +</td>
<td>60% +</td>
<td>0%</td>
</tr>
<tr>
<td>C17.28</td>
<td>0%</td>
<td>100% +</td>
<td>2%-5% +</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>OKT10</td>
<td>0%</td>
<td>0%</td>
<td>75% +</td>
<td>40% (+)</td>
<td>0%</td>
</tr>
<tr>
<td>3A1</td>
<td>0%</td>
<td>0%</td>
<td>75% +</td>
<td>40% (+)</td>
<td>0%</td>
</tr>
</tbody>
</table>

*In the indirect antiglobulin test.
†In the indirect immunofluorescence test on cell suspensions of ≥90% purity.

**Immunofluorescence Test**

All serologic tests with the polyclonal antisera and the monoclonal antibodies (except with anti-Ig, κ, and λ sera that were directly FITC-labeled) were done in the indirect immunofluorescence test (IIFT). The cells were washed twice with phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA), fixed with 1% paraformaldehyde (PFA, 0.11 M), and washed again twice with PBS/BSA. PFA fixation diminished aspecific staining in the IIFT (unpublished observations). Thirty microliters of this cell suspension (10-15 × 10⁶ cells/ml) were mixed with 30 μl of antibody (poly- or monoclonal) in an optimal dilution in PBS/BSA and incubated for 30 min at room temperature (RT). The optimal dilution was determined in pilot experiments with purified positively and negatively reacting cells (peripheral blood cells, human cell line cells, etc.). It was defined as the highest dilution that still gave maximal fluorescence with the positive cells and no fluorescence with the negative cells. Concerning the McAb not produced in our laboratory, the dilution indicated by the manufacturer was used. The cells were washed twice with PBS/BSA and mixed with 50 μl of an FITC-labeled antiliglobulin reagent. As antiglobulin reagent in the assays with the rabbit antisera, we used FITC-labeled swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) diluted 1:100 in PBS/BSA. As antiglobulin reagent in the tests with McAb and with CLS, we used an FITC-labeled goat anti-mouse IgG (GM 17-1-F, CLB, Amsterdam, The Netherlands) diluted 1:80 in PBS/BSA.

The cells were incubated with the conjugates for 30 min at RT and washed twice with PBS/BSA. After the last washing, the supernatant was removed, and the cells were resuspended in one drop of a 70% (v/v) glycerol solution in PBS, mounted on a slide, and covered with a coverslip. The slides were examined in a Leitz Orthoplan fluorescence microscope and read independently by two of us.

Because a low percentage (less than 20%) of normal cells was often present in the leukemic cell samples, the results of these tests were considered positive if more than 20% of the cells showed a positive fluorescence.

For the detection of cytoplasmic antigens, such as lysozyme, we applied the cytocentrifuge technique. Briefly, 50 μl of a (PFA-fixed) cell suspension of 2-5 × 10⁶ cells/ml were spun on a slide for 10 min at 1000 rpm in a Shandon-Elliott cytocentrifuge, which was prespun shortly at maximal speed with PBS containing 10% BSA. The preparations were air-dried for minimally 1 hr and then investigated in the IIFT or stored at −20°C for later use. Further procedures were the same as in the IIFT on cell suspensions.

**Statistical Evaluation**

Computerized evaluation of the reaction patterns of the various antibodies (both conventional and monoclonal) was done with a
Table 2. Classification of 55 Patients With AML According to the FAB Criteria

<table>
<thead>
<tr>
<th>FAB</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5A</th>
<th>M5B</th>
<th>M6</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>16</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

Standardized Canonical Discriminant Function Analysis of the Statistical Package for Social Science (standard SSPS program: discriminant analysis), using the SARA computer of the University of Amsterdam for a classification in the previously defined FAB groups on the basis of the results of the immunologic characterization of the AML cells.

RESULTS

The results of the classification according to the FAB criteria in 55 patients with AML are shown in Table 2. We compared the results of the quantified FAB classification in 55 patients with AML with the percentage of patients in each FAB group whose cells reacted positively (>20%) with the polyclonal antisera, the McAb against antigens of the myeloid series (Fig. 1) and the other markers (Fig. 2). The McAb against antigens of the myeloid series were considered myeloid-specific, because no reaction with ALL or B-CLL cells was found (data not shown).

We also tested the cells in the E-rosette test with antithymocyte and with anti-cALL serum; the results were negative in all, confirming their nonlymphoid nature.

Regarding the McAb specific for granulocytes, generally, an increase in the percentage of patients whose cells were reactive was seen with increasing maturation (M1–M3, M5A–M5B) with the possible exception of M6. The M1 and especially the MSA group behaved as immature in this respect.

The same as with the McAb specific for granulocytes was seen with the polyclonal antisera reacting with normal granulocytes and monocytes. However, the two McAb reactive with both granulocytes and monocytes (OKM1 and B2.12) showed no real difference in the frequency of positivity between the various FAB subgroups, except in M5B, where the cells of many patients reacted positively. In the M6 and M5A groups, only few leukemias reacted positively. The cells of one patient in the M1 and of one patient in the M5B group reacted positively with the McAb C17.28 directed against a platelet antigen and with the antiplatelet serum. The cells of these patients were also OKM1- and B2.12 positive. In the M2 and the M3 group, the cells of some patients reacted positively with the antiplatelet serum but negatively with the McAb against platelets. This may indicate that the antiplatelet serum is less specific and still contains contaminating antibodies of unknown specificity.

The three antisera (a.SB, CLS and OKI1) against Ia-like determinants showed a decreased reactivity with the cells of patients with more differentiated leukemias (M3, M4, and M6), all those in the M3 group being negative with these sera (even less than...
The McAb 3A1, reported to detect antigens on thymocytes, both reacted positively with the cells of a large number of patients. In the M5A group, the cells of all patients were positive with OKT10.

The antierthrocyte serum was positive with the cells of two patients with erythroleukemia (M6) and with one of the patients with promyelocytic leukemia (M3). The cells of all patients were positive with the anti-HLA serum (W6/32.HLK).

The number of patients whose cells did not express any antigenic marker of the myeloid lineage is shown in Table 3. This was often true for immature AML (M1 and M5A). The cells of all these patients expressed the antigens detected by the OKT10 reagent and/or antibodies against the Ia-like antigen.

A computerized canonical discriminant function analysis was made to see whether it was possible to classify the cells on the basis of their immunologic reaction pattern into the various FAB subgroups. The highest probability for the various FAB-group subgroups was calculated, and this resulted in a classification identical with the standard FAB classification in 69% of the cases (Table 4).

Table 3. Number (and Percentage) of Patients Whose Cells Only Expressed the OKT10 and Ia-Like Antigens Distributed in the Various FAB Groups

<table>
<thead>
<tr>
<th>FAB Group</th>
<th>OKT10- and/or Ia-Positive Only</th>
<th>Positive for Myelomonocytic Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>4 (44%)</td>
<td>5 (56%)</td>
</tr>
<tr>
<td>M2</td>
<td>2 (12%)</td>
<td>14 (88%)</td>
</tr>
<tr>
<td>M3</td>
<td>0 (0%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>M4</td>
<td>0 (0%)</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>M5A</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>M5B</td>
<td>1 (7%)</td>
<td>13 (93%)</td>
</tr>
<tr>
<td>M6</td>
<td>0 (0%)</td>
<td>3 (100%)*</td>
</tr>
</tbody>
</table>

*One positive for erythroid markers only.

10% of the cells reactive) as with anti-Ig and antilysozyme serum. The cells of patients in the M6 group were negative for CLS and OKT11, but in 2 of 3 cases, positive with the aSB serum. This discrepancy indicates that, apart from anti-Ia antibodies, extra antibodies of yet unknown specificity are present in this serum.

The finding in the M3 group that the cells of none of the patients reacted with any of the three anti-Ia sera confirms the observation of Winchester et al., who showed that the normal promyelocyte is predominantly la negative. This result may be explained by a loss of the la antigen during the differentiation of myeloblasts to promyelocytes. Thus, la negativity seems to be specific for AML type M3. Of interest is that we also found in these cases negativity for the lysozyme marker.

With regard to the Fc-IgG receptor (EA) and membrane-bound immunoglobulin (mIg), our results, especially in M5A and M5B (monocytic leukemia), are similar to those reported in the literature, where the immunoglobulin was found to be polyclonal IgG of exogenous origin and Fc-IgG receptor-bound. There was a discrepancy between the results of the number of patients' cells expressing an Fc-IgG receptor and those with detectable mIg, especially in M2 and M3. This was also found by Fernandez et al. and may be due to differences in the affinity of the Fc-IgG receptors.

Also, the antigens recognized by the McAb that were applied showed no specificity for particular FAB subgroups. This was to be expected for those McAb that recognize both granulocyte and monocyte antigens. However, we found, as did Majdic et al., that McAb with specificity for antigens on granulocytes only also often showed a positive reaction with cells of relatively mature monocytic origin (M5B). This indicates that these antigens are normally present on more
immature monocytes, or that we are, here too, dealing with aberrant expression of these antigens owing to the leukemic nature of the cells.

A positive correlation was found between the reactivity of the leukemic cells with many of the xeno- and McAb used and the stage of maturation of these cells. Thus, most of the antibodies used in these investigations probably recognize maturation antigens. On the contrary, markers such as the Ia antigen and the antigen recognized by OKT10 were more often positive on the cells of patients with immature leukemias.

Our results confirm the discriminating value of the quantified FAB criteria,7 which separate the immature (and relatively mature subtypes), especially within the monocytic leukemias. In M5A, almost no reactivity was seen with the antibodies against the myelomonocytic antigens, whereas most of the cells of the patients in the M5B group did react. The M1 and M5A group were predominantly OKT10- and Ia-positive, a phenotype frequently seen in acute (immunologically) undefined leukemia (AUL), reflecting its immature nature.21,26

We found an unexpected expression of platelet-specific antigens on the cells of one patient in the M1 and one in the M5B group, as detected with the McAb C17.28. In the M1 patient, electromicroscopy (kindly performed by Dr. D. Catovsky) showed that the cells of this patient were in fact megakaryoblasts, with a positive platelet peroxidase reaction, which explains this result. However, the cells of the M5B patient were clearly of myeloid origin in this technique. Possibly, here again, the immature cells still express platelet antigens, or, as mentioned above, we are dealing with aberrant expression owing to the leukemic character of the cells.

Our finding that the McAb 3A1, which is described as being T-cell-specific, reacted with the cells of about half of the patients with AML is not so unexpected, because Haynes et al.30 had already described weak reactions with the cells of the erythroblastoid cell line K562 and with the cells of the myeloblastoid cell line HL-60, and we found it to react also weakly with monocytes (Table 1).

When a computerized FAB classification was calculated on the basis of the immunologic profiles of the patients’ cells, a concordant classification was observed in 69%. This supports the validity of the FAB classification which, as the marker analysis, is also based predominantly on the stage of maturation of the leukemic cells. The stage of maturation seems to be a clinically important parameter in AML in relation to prognosis.9 Although it is presently less discriminatory, it is clear from our results that immunologic characterization can be an important tool in addition to the FAB classification to establish the stage of maturation of the leukemia.

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