Myeloid Differentiation Antigen Defined by a Monoclonal Antibody

By Otto Majdic, Kristof Liszka, Dieter Lutz, and Walter Knapp

A monoclonal antibody of the IgM kappa type directed at a determinant on K-562 cells was produced and characterized. This hybridoma antibody, provisionally termed D5, defines a membrane-associated antigen that seems to be restricted, within the hemopoietic system, to the granulocytic cell lineage and possibly also to very immature erythroid and monocytic cells. Within the granulocytic cell lineage, D5 antibody reacts with all differentiation stages starting from the promyelocyte up to and including the mature granulocyte. Normal and malignant myeloblasts are only weakly positive or negative. Within the monocytic cell lineage, only fairly immature malignant cells as represented by cells from two patients with monoblastic leukemia and by two monoblastoid cell lines were found to be strongly D5 positive. Among the mature glass-adherent monocytic cells from normal peripheral blood, only very few cells react with D5 antibody. The fact that we have raised this antibody against cells (K-562) that are considered to represent very immature erythroid precursor cells would suggest that normal erythroid precursors should also be D5 positive. However, so far we are unable to detect such cells. Therefore, the proportion of D5-positive erythroid precursors resembling the K-562 cell type must either be very low in the bone marrow, or the D5 antigen is not present on normal erythroid precursor cells and only aberrantly expressed on K-562 cells. Mature erythrocytes are also D5 negative. There was no evidence that lymphoid precursors react with D5 antibody, in that all normal and malignant lymphoid cells tested so far were D5 negative. Further, platelets showed no reactivity with D5 antibody.

HUMAN LEUKOCYTE surface antigens are becoming increasingly important. While lymphocyte surface antigens and histocompatibility antigens have been studied extensively, relatively few research groups have investigated the antigenic characteristics of normal and malignant myeloid cell surfaces.

To our knowledge, all studies on membrane antigens of myeloid cells so far reported were performed with either appropriately absorbed heterologous or allogeneic antisera. In this article we present the results of our study with a monoclonal antibody reacting with a myeloid differentiation antigen.

MATERIALS AND METHODS

Production of Monoclonal Antibodies

Immunization. A 6-wk-old female BALB/c mouse was immunized with 3 x 10⁸ viable K-562 (see Table I) cells i.p. and the spleen was removed for hybridization 3 days later.

Somatic cell hybridization and growth of hybridomas. Spleenocytes (10⁶) were fused as described by Köhler and Milstein using 35% polyethylene glycol, mol wt 4000, with 10⁶ x 63-Ag8.654 BALB/c myeloma cells, a nonimmunoglobulin producing subclone of the mouse myeloma cell line P3-X63-Ag8.17

Selection of hybridomas. After cell fusion, cells were cultured in hypoxanthine, aminopterin, thymidine medium at 37°C with 5% CO₂ in a humid atmosphere. Two weeks later, the supernatants were tested for reactivity with surface antigen of K-562 cells by indirect trace binding assays as described by Williams et al. Briefly, 100 µl of each supernatant were incubated for 60 min with 10⁶ test target cells. After 3 washes, the cells were incubated for another 60 min with 0.1 µg ¹²⁵I-labeled rabbit F(ab')₂ anti-mouse F(ab')₂ antibodies (~5 x 10⁵ cpm), washed again 3 times, and counted for bound radioactivity.

Cloning. Selected hybridoma cultures were cloned and recloned by limiting dilution in the presence of feeder cells.19

In vivo propagation. Cultured hybridoma cells (5-10 x 10⁶) were injected i.p. into BALB/c adult mice that were treated 7-30 days previously with an i.p. injection of 2, 6, 10, 14-tetramethylpentadecan (Pris痰). Asecic fluid was harvested usually 10-20 days after hybridoma inoculation.

Characterization of D5-Reactivity

Cultured human cell lines. Nine established human hemopoietic cell lines (K-562, HL-60, THP-1, U-937, Raji, Kiet, Reh-6, CEM, Molt-4) were used to evaluate the cell lineage specificity of D5. The characteristics and the references of these lines are outlined with the results in Table 1.

Normal mononuclear cells (MNC). Peripheral blood MNC were isolated from heparinized blood of healthy volunteer donors by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. Tonsils were obtained immediately after tonsillectomy from children with subacute tonsillitis. Thymus specimens were obtained from patients who had portions of the thymus gland removed during corrective cardiac surgery. Spleens were obtained from patients with traumatic lesions of this organ. MNC from these tissues were prepared by teasing and filtration through nylon mesh.

MNC-subsets. T- and B-cell-enriched fractions were obtained essentially as described before by separating E-RFC from nonrosetting cells over a Ficoll-Hypaque gradient after previous removal of adherent cells. Adherent cells were isolated by scraping off with a rubber policeman those cells that after overnight culture at 37°C remained firmly attached to the bottoms of plastic Petri dishes after 3-5 washings with medium. In some experiments, MNC preparations were stained with toluidine blue and simultaneously fixed with paraformaldehyde (1% w/v) before subjecting them to immunofluorescence procedures. This staining and fixation procedure does not interfere with D5 binding and allows the identification of

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basophils on the basis of their reddish purple, metachromatic granules.

**Neutrophilic granulocytes.** These were separated simultaneously with erythrocytes from the MNC fraction by Ficoll-Hypaque (Pharmacia) density centrifugation and then freed from erythrocytes by hypotonic ammonium chloride lysis.

**Leukemic cells.** Leukemic cells were obtained from heparinized peripheral blood and bone marrow of leukemia patients at the time of diagnosis or relapse after informed consent had been secured. The diagnosis of leukemia was made using standard clinical, morphological, and cytochemical criteria. All neoplastic preparations selected for this study showed >75% abnormal cells. Isolated tumor cells were studied either freshly obtained or cryopreserved in 10% dimethyl sulfoxide and 20% fetal calf serum in the vapor phase of liquid nitrogen until the time of surface characterization. This could be done since initial studies with D5-positive cells had shown that the D5 antigen is stable to cryopreservation and freeze-thawing. The viability of all cryopreserved leukemic cell preparations included in this study exceeded 85%.

**Immunofluorescence.** The binding of D5 was assessed by indirect immunofluorescence with fluoresceinated rabbit F(ab')2 antibodies or by direct immunofluorescence using fluoresceinated isolated D5 hybridoma antibodies. Results of these investigations and the characterization of D5 antibody specificities on established human cell lines are shown in Table 1. Although D5 was obtained after hybridization of myeloma cells with spleen cells from a mouse previously immunized with K-562 cells, the reaction of D5 with Raji cells was much weaker and confined to a proportion of approximately 40% of all K-562 cells.

**RESULTS**

**Preliminary Characterization of Established Hybridomas**

After fusion, the cells were distributed in 144 culture wells, each containing 10⁶ spleen cells and 10⁵ myeloma cells in 1.5 ml HAT medium. After 2 wk, culture supernatants were tested for reactivity with surface antigens of K-562 cells (used for immunization) by indirect trace binding assays. The supernatants from 6 cultures clearly reacted with K-562 cells, but not with Raji or CEM cells. The hybrids in one of these cultures were then cloned by a limiting dilution procedure. One of the final clones (D5) that gave the same reaction pattern was propagated in vitro and used in all further studies. As revealed by immunofluorescence and by immunoelectrophoresis (kindly performed by J. Radl, Rijswijk, Holland), D5 monoclonal antibody proved to be of IgM kappa type.

**Characterization of Antibody Specificity on Established Human Cell Lines**

To further define the cell type with which D5 reacts, several human cell lines were tested for their reactivity with D5. Results of these investigations and the characteristics of the various cell lines used are shown in Table 1.

The most brilliant fluorescence and the highest proportion (96%) of positive cells was found with HL-60 cells, an established human promyelocytic cell line. Although D5 was obtained after hybridization of myeloma cells with spleen cells from a mouse previously immunized with K-562 cells, the reaction of D5 with K-562 cells was much weaker and confined to a proportion of approximately 40% of all K-562 cells.

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**Table 1. Characterization of D5 Antibody Specificity on Established Human Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line Designation</th>
<th>Cell Type</th>
<th>Origin</th>
<th>Percent D5 Positive</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-562</td>
<td>Myeloid/erythroid</td>
<td>CML blast crisis</td>
<td>49.6 ± 10.7</td>
<td>20</td>
</tr>
<tr>
<td>HL-60</td>
<td>Promyelocytic</td>
<td>Promyelocytic leukemia</td>
<td>93.6 ± 3.2</td>
<td>21</td>
</tr>
<tr>
<td>U-937</td>
<td>Immature monocyte</td>
<td>Histioytic lymphoma</td>
<td>81.6 ± 5.7</td>
<td>22</td>
</tr>
<tr>
<td>THP-1</td>
<td>Immature monocyte</td>
<td>AML</td>
<td>93.3 ± 3.5</td>
<td>23</td>
</tr>
<tr>
<td>Ralti</td>
<td>B lymphoid</td>
<td>Burkitt lymphoma</td>
<td>Negative</td>
<td>24</td>
</tr>
<tr>
<td>Kiet</td>
<td>B lymphoid</td>
<td>Normal B lymphocytes</td>
<td>Negative</td>
<td>25</td>
</tr>
<tr>
<td>Reh-6</td>
<td>Common ALL type</td>
<td>Common ALL</td>
<td>Negative</td>
<td>26</td>
</tr>
<tr>
<td>CEM</td>
<td>T lymphoid</td>
<td>T-ALL</td>
<td>Negative</td>
<td>27</td>
</tr>
<tr>
<td>Molt-4</td>
<td>T lymphoid</td>
<td>T-ALL</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD from 3 experiments.*
The difference in the staining intensity between HL-60 and K-562 cells is also illustrated in Fig. 1.

The histiocytic lymphoma cell line U 9372 and the monoblastoid cell line THP-1 gave only a slightly weaker staining than the HL-60 cell line, and the percentage of positive cells was found to be similarly high (82% and 94%, respectively).

No positive cells whatsoever were found after incubation of D5 conjugate with cell lines of B (Raji and Kiet), T (Molt-4 and CEM), or common ALL type (Reh-6).

This selective reaction pattern of D5 was not only observed with the direct and the indirect immunofluorescence technique, but also in a standard two-stage microcytotoxicity assay. Ascitic fluid from hybridoma bearing mice in the presence of rabbit complement killed virtually all HL-60 cells up to a dilution of 1:8000. At dilution 1:24000, 50% of the HL-60 cells were still lysed, whereas Reh-6 and CEM cells were unaffected by ascitic fluid dilutions of 1:100 (Fig. 2).

Characterization of D5 Antibody Specificity on Normal Human Leukocytes (Table 2)

In MNC preparations from the peripheral blood of healthy individuals, a small but regularly detectable
percentage of clearly D5-positive cells (1%–7%, mean 3.8%) was found. With control mouse IgM antibody (VIE-G4) or control ascitic fluid, we only rarely found a few (<1%) weakly fluorescent cells in these preparations but never such a clearcut fluorescence as observed with D5. These D5-positive cells were SIg negative and did not form rosettes with sheep red blood cells (E-RFC). In double staining experiments it could be shown that these D5-positive cells have no detectable surface Ia antigens and contain no cytoplasmic immunoglobulins. However, all these clearly D5-positive MNC do contain lysozyme in their cytoplasm. This would indicate that they belong to the myeloid or the monocytic cell lineage. Plastic-adherent MNC were, therefore, isolated and tested for D5 reactivity. However, only 2%–8% (mean 5%) of the plastic-adherent MNC fraction, which contained mainly monocytes, were D5 positive. The basophils, another lysozyme-positive cell type that is enriched in the MNC fraction, were also found to be D5 negative.

For the time being we can only state that in the MNC fraction from peripheral blood, a small but detectable proportion of cells are D5 positive. These cells have a mononuclear morphology. They are obviously not lymphocytic but contain easily detectable lysozyme in their cytoplasm. They do not express Ia antigens and are not enriched in the plastic-adherent fraction of peripheral blood MNC.

The staining of this small proportion of cells with D5 antibody seems to be specific and not to be due to Fe receptor binding, since two control preparations including a monoclonal mouse antibody of IgM class gave absolutely no comparable staining of peripheral blood MNC.

In contrast to the peripheral blood, absolutely no D5-positive cells were found in MNC preparations from thymus, spleen, and tonsil. Also completely negative were platelets and erythrocytes. A very bright staining of virtually all cells (91%–96%, mean 93%) was obtained when peripheral blood granulocytes were stained for D5. In the presence of rabbit complement, >90% of the granulocytes were also lysed by D5 ascitic fluid up to a dilution of 1:220,000 (Fig. 2).

High proportions of D5-positive cells (14%–94%, mean 54%) were also observed in bone marrow preparations from patients with nonhematologic diseases. The fluorescence intensity distribution pattern of such a patient is shown in Fig. 3D. In the peripheral blood as well as in the bone marrow, all strongly D5-positive cells contained cytoplasmic lysozyme. There were, however, also some lysozyme-negative cells in the bone marrow that were weakly D5 positive.

![Fig. 3. FACS analysis of mononuclear cells from patients and healthy controls. All preparations were assayed by direct immunofluorescence for reactivity with D5 monoclonal antibody. (A) Peripheral blood mononuclear cells (MNC) from a patient with chronic myeloid leukemia (stable phase). (B) Peripheral blood MNC from a patient with acute myeloid leukemia. (C) Peripheral blood MNC from a healthy control. (D) Bone marrow mononuclear cells from a patient with nonhematologic disease.](image-url)
MYELOID DIFFERENTIATION ANTIGEN

Table 3. Reactivity of D5 With Leukemia Cells

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number Studied</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML (stable phase)</td>
<td>7</td>
<td>7*</td>
</tr>
<tr>
<td>AML</td>
<td>9</td>
<td>5*</td>
</tr>
<tr>
<td>AMoL</td>
<td>2</td>
<td>2*</td>
</tr>
<tr>
<td>Monocytic leukemia</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CLL</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CALLA⁺, Ia⁺, E⁺</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>CALLA⁺, Ia⁻, E⁻</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

*Individual percentage values of D5-positive cases: CML—90%, 91%, 98%, 66%, 56%, 73%, 78% and 81% of all mononuclear cells; AML—75%, 35%, 15%, 42%, and 44% of blast cells; AMoL—86% and 56% of blast cells.

D5 Reactivity of Human Leukemic Cells (Table 3)

In none of the patients with lymphatic leukemia—10 patients with chronic lymphatic leukemia (CLL) of the B-cell type, 6 patients with acute lymphatic leukemia (ALL) of the common ALL type (Ia⁺, CALLA⁺, E⁻), and 2 patients with acute lymphatic leukemia of the T-cell type (Ia⁻, CALLA⁻, E⁺)—a positive reaction for the D5 antigen was observed.

Hairy cells in three patients with hairy cell leukemia were D5 negative.

A very strong staining for D5 was found on the majority (66%–98%, mean 84%) of leukemia cells from 8 patients with chronic myeloid leukemia (CML) who were in the stable phase of their disease. Among these D5-positive cells, as judged by phase contrast microscopy, all myeloid differentiation steps starting with the promyelocyte could be found. In some preparations, weakly stained myeloblasts could also be seen. A typical fluorescence intensity distribution pattern of D5-FITC-stained CML cells is shown in Fig. 3A. Among acute myeloid leukemia (AML) patients (>75% blasts), considerable heterogeneity of D5 staining was observed. In 4 patients, essentially no D5-positive myeloblasts could be observed. The FACS analysis of one such patient is shown in Fig. 3B. In the other 5 patients studied, the percentage of D5-positive blast cells was found to be 75%, 35%, 15%, 42%, and 44%, respectively. A strong reactivity of the majority of cells (86% and 56%) with D5 was also observed in 2 patients with acute monoblastic leukemia (AMoL), while the more mature looking cells of one patient with monocytic leukemia were D5 negative.

DISCUSSION

We have produced a monoclonal antibody (D5) that defines a membrane-associated antigen that seems to be restricted within the hemopoietic system to the granulocytic cell lineage and possibly also to very immature erythroid and monocytic cells.

Within the granulocytic cell lineage, the expression of this antigen seems to be maturation linked. The antibody strongly reacts with the more mature differentiation stages starting with the promyelocyte and including the mature granulocyte. Normal and malignant myeloblasts are negative or only weakly positive.

The opposite seems to be true for the monocytic cell lineage. Among the mature, strongly glass-adherent mononuclear cells from normal peripheral blood and from a patient with monocytic leukemia, only very few cells were found to be D5 positive. Fairly immature cells of the monococyte differentiation lineage, as represented by two patients with monoblastic leukemia, and the THP-1 and U-937 cell lines were found to be strongly D5 positive. If one accepts that malignant cells represent frozen states of normal cell differentiation and that U-937 and THP-1 cells truly represent immature monocytic cell as was suggested on the basis of their phenotypic characteristics, one has to conclude that within the monocytic cell lineage the expression of D5 antigen seems to be negatively linked to maturation, i.e., D5 antigen density decreases or is lost with maturation.

A similar relation might possibly also exist for the erythroid cell lineage. We have obtained this monoclonal antibody by fusing plasmocytoma cells with spleen cells from mice previously immunized with K-562 cells. The K-562 cell line was established in 1975 by Lozzio and Lozzio from the pleural fluid of a patient with chronic myeloid leukemia in blast crisis. The exact nature of this cell line remains unclear so far. The morphological and cytochemical features originally conformed best with that of immature nonlymphoid cells. The surface characteristics (absence of Ig, common ALL antigen, Ia-like antigen and C3 receptor, presence of Fc receptor, and minute amounts of HLA and β2-microglobulin) also seemed to exclude that K-562 represents cells from a CML blast crisis of common ALL type but rather very immature myeloid cells. The myeloid nature was also supported by the demonstration of group-specific human granulocyte antigens on the surface of K-562 cells. Analysis of the surface glycoprotein pattern of K-562 cells, however, did not show myeloid, but erythroid features, and the major surface glycoprotein of K-562 could be identified as the major red cell sialoglycoprotein, glycophorin-A. In addition, it could be shown that exposure of K-562 cells to butyric acid induces morphological and functional erythroid differentiation. This would indicate that K-562 cells represent fairly early maturation steps in erythroid differentia-
tion rather than being very immature myeloid cells. The fact that our D5 antibody was raised by immunizing with K-562 cells and the demonstration of D5 binding to K-562 surfaces suggests that this antigen might possibly also be present on erythroid precursor cells. The density of D5 antigen expression on K-562 cells is very low, however, and more mature and morphologically identifiable erythropoietic cells in bone marrow specimens were all negative. Double staining experiments for lysozyme and D5 also showed that practically all clearly D5-positive cells were also lysozyme positive. The proportion of D5-positive erythroid precursors resembling the K-562 cell type must, therefore, be very low in the bone marrow or the D5 antigen is normally not present on erythroid precursor cells and only aberrantly expressed on K-562 cells. So far no evidence was found that lymphoid precursor cells might also be D5 positive. Lymphoid cells of various origins were all D5 negative. This also applies to all lymphatic leukemias as well as all lymphoid cell lines tested. Thrombocytes and erythrocytes were also completely negative.

A striking finding in our studies was the observation that small but regularly detectable numbers (4% ± 1%) of normal peripheral blood mononuclear cells were D5 positive. These cells have no cytoplasmic or membrane immunoglobulins, do not express surface Ia antigens, and have no receptors for E-rosette formation. Most of them are lysozyme positive but not adherent, and as preliminary evidence suggests (data not shown), many of them seem to express Fc receptors. Further cell separation and functional tests will be required to establish the precise nature of these nonlymphoid, nonadherent mononuclear cells.

Changes of surface antigen expression during cell differentiation have recently been described in other systems. Probably the best studied example is the variable expression of Ia-like antigens in different cell lineages.37 This antigen originally was found within the hemopoietic system on B lymphocytes and monocytes/macrophages, similar selectively lost by the granulocytic, thrombocytic, and erythroid lineages during bone marrow differentiation, but further expressed by B cells and monocytes/macrophages. Similar selective losses of surface antigen expression during maturation and differentiation were also found with the help of hybridoma antibodies within the human T-cell lineage,38 and a variety of other examples almost certainly will follow. Hybridoma technology has made it possible to prepare a range of antibodies that should be able to define developmental and functional compartments of all cell lineages. So far, these studies in man have mainly been focused on the T-cell lineage.38 Clearly, much more work needs to be done with the other hemopoietic cell lineages; the hybridoma antibody described here can only be a beginning. We are now preparing a series of hybridoma antibodies against myeloid surface antigens. Together with the human myeloid cell lines which recently became available and can be made to differentiate in vitro into mature cells,39,40 it should be possible to reach a deeper understanding of the differentiation control in human myeloid leukemia and in a broader perspective of hemopoietic differentiation in general.

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