Differentiation-Linked Expression of Cell Surface Markers on HL-60 Leukemic Cells

By M. A. Boss, D. Delia, J. B. Robinson, and M. F. Greaves

The cell surface antigenic phenotype of HL-60, a human acute promyelocytic leukemia cell line, has been analyzed before and after maturation induction with dimethylsulfoxide (DMSO) using a panel of markers including a "library" of monoclonal antibodies and "conventional" antisera in conjunction with the fluorescence-activated cell sorter. HL-60 cells express granulocyte and "leukocyte" differentiation antigens but not antigens of the lymphoid, platelet, and erythroid lineages. DMSO-induced morphological maturation was found to be associated with a decrease in the proportion of cells in mitotic cycle, induction of C3d receptors, increased expression of granulocytic and leukocyte antigens, and diminished expression of HLA-A,B,C and β2-microglobulin determinants. HL-60 cells have no detectable expression of HLA-DR-associated determinants as assayed by rabbit anti-p28,33 monoclonal anti-HLA-DR (monomorphic determinant), and HLA-DRw typing alloantisera. The relationship of these changes in cell surface properties to normal granulocytic differentiation is discussed.

The HL-60 cell line was isolated from the peripheral blood leukocytes of an adult female with acute promyelocytic leukemia. Most cells had the morphology of myeloblasts and promyelocytes, but more mature granulocytes were also seen. Dimethylsulfoxide (DMSO), other polar compounds, and tumor-promoting agents, as well as a "physiologic" stimulus-colony stimulation factor (CSF-G) were shown to induce morphological maturation into neutrophilic granulocytes. Only a small proportion of cells, however, matured into fully segmented neutrophils. Induced HL-60 cells possess a number of immunologic, biochemical, and functional characteristics of mature granulocytic cells, including expression of a 130,000-dalton membrane glycoprotein, complement receptors, phagocytosis, and response to chemoattractants. These observations indicate that the HL-60 line may provide an excellent model system to analyze the control of gene expression and functional activity during granulocyte differentiation.

We report the detailed antigenic phenotype of resting and induced HL-60 cells using a "library" of monoclonal antibodies in addition to other immuno logic markers.

MATERIALS AND METHODS

Cells

The HL-60 cell line was provided by R. C. Gallo (National Institutes of Health, Bethesda, Md.). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were seeded at 10^6/ml in Nunc Petri dishes (90 mm diameter) with 10 ml/culture. For induction, 1.25% DMSO (Fisons) was added to fresh cultures with no subsequent additions or subculturing. Cells were analyzed morphologically by staining cyto centrifuge smears with Wright's stain. In the numbers presented here (except in Table 2), those for immature cells represent the combined percentages of myeloblasts and promyelocytes. The numbers for mature cells are the combined percentages of myelocytes, metamyelocytes, and neutrophils. The lymphoid leukemia

Table 1. Monoclonal (Hybridoma) Antibodies Used to Characterize HL-60 Cells

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Characterization</th>
</tr>
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<tbody>
<tr>
<td>NA134</td>
<td>Cortical thymocyte 45K polyepitide</td>
</tr>
<tr>
<td>AN51</td>
<td>Platelets*</td>
</tr>
<tr>
<td>P17/F12</td>
<td>Mature T lymphocytes†</td>
</tr>
<tr>
<td>LICR.-LON.</td>
<td>Glycophorin‡</td>
</tr>
<tr>
<td>R10</td>
<td></td>
</tr>
<tr>
<td>1/6A</td>
<td>Band III†</td>
</tr>
<tr>
<td>2D1</td>
<td>Leukocytes†</td>
</tr>
<tr>
<td>GT-1</td>
<td>Granulocytic cells†</td>
</tr>
<tr>
<td>W6/32</td>
<td>HLA-A,B,C framework **</td>
</tr>
<tr>
<td>PA2.6</td>
<td></td>
</tr>
<tr>
<td>BB5</td>
<td>B2-microglobulin</td>
</tr>
<tr>
<td>EC3</td>
<td></td>
</tr>
<tr>
<td>DA2</td>
<td>HLA-DR framework **</td>
</tr>
<tr>
<td>P1153/3</td>
<td>Neuroblastoma†</td>
</tr>
</tbody>
</table>

pc, Personal communication.
*Reacts (by immunofluorescence with platelets but not with other hemopoietic cells, monocytes, lymphocytes, granulocytes, and erythrocytes (A. McMichael, personal communication).
†Reacts with effectively all E (sheep) rosetting T cells in blood and tonsils and with a subset of thymocytes.
‡Reacts with all leucocytes but not erythrocytes or erythroid progenitors. Mature T and B cells and granulocytes react more strongly than their precursors.
§Reacts with granulocytes but not lymphocytes or red cells.
¶Detects nonleucic monomorphic determinants.
††Raised against neuroblastoma cells but reacts also with B lymphocytes, presumptive lymphocyte precursors in bone marrow, and ALL cells (Greaves, M. F. and Kennett, R. unpublished observations).
RESULTS

The HL-60 line has been maintained in our laboratory for approximately 18 mo during which time the resting cultures contained varying proportions of agranular blasts and promyelocytes.

As shown in Table 2, the addition of 1.25% DMSO to HL-60 cultures consistently induced morphological maturation of granulocytic cells. Our results differ from those previously reported in that the uninduced cultures contain a consistently higher proportion of

<table>
<thead>
<tr>
<th>Day</th>
<th>Blasts</th>
<th>Promyelocytes</th>
<th>Myelocytes</th>
<th>Metamyelocytes</th>
<th>Neutrophils</th>
<th>Cell Cycle % S + (G2M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.4-51.5</td>
<td>13.0-79.0</td>
<td>0-36.2</td>
<td>0-10.0</td>
<td>0-1.0</td>
<td>37.0-47.0</td>
</tr>
<tr>
<td>(27.7)*</td>
<td>(56.3)</td>
<td>(11.2)</td>
<td>(1.9)</td>
<td>(0.14)</td>
<td>(42.0)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.0-30.0</td>
<td>6.0-30.0</td>
<td>22.0-66.0</td>
<td>2.8-26.0</td>
<td>0-0.5</td>
<td>—</td>
</tr>
<tr>
<td>(17.9)</td>
<td>(17.9)</td>
<td>(38.0)</td>
<td>(11.9)</td>
<td>(0.25)</td>
<td></td>
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</tr>
<tr>
<td>4-7</td>
<td>0-22.0</td>
<td>2.2-46.4</td>
<td>15.0-58.0</td>
<td>7.5-66.2</td>
<td>0-3.0</td>
<td>25.0-27.0</td>
</tr>
<tr>
<td>(6.4)</td>
<td>(20.4)</td>
<td>(39.3)</td>
<td>(25.7)</td>
<td>(0.42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-11</td>
<td>0-2.2</td>
<td>0.5-52.2</td>
<td>7.4-55.8</td>
<td>14.2-64.2</td>
<td>0-1.6</td>
<td>26.0</td>
</tr>
<tr>
<td>(1.2)</td>
<td>(19.7)</td>
<td>(29.4)</td>
<td>(31.6)</td>
<td>(1.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0.5</td>
<td>42.5</td>
<td>54.5</td>
<td>2.0</td>
<td>18.0</td>
</tr>
<tr>
<td>21</td>
<td>1.0</td>
<td>7.0</td>
<td>41.5</td>
<td>15.5</td>
<td>35.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Differentials performed on cytocentrifuge preparations stained with May-Grünwald-Giemsa.

*Average of 9 experiments; mean in parentheses.

lines Nalm-1 and Reh were maintained and characterized as previously described.7-9

Reagents

The antisera used were: F(ab')2 rabbit anti-chronic granulocytic leukemia (CGL) raised as described previously for antisera to myelomonocytic cells;16 F(ab')2 rabbit anti-acute lymphoblastic leukemia (ALL);3,12 chicken and rabbit anti-p28,33 (Ia-like);3,4 goat anti-glycophorin-TRITC (Atlantic Antibodies, Maine); and F(ab')2 sheep anti-human Ig-FITC. A number of monoclonal antibodies (mouse ascitic Ig) were also employed and are listed in Table 1. TdT was assayed both by immunofluorescence on smeared fixed cells using a rabbit anti-TdT serum supplied by F. Bollum15 and by enzyme assay (kindly carried out by K. Ganeshaguru, Royal Free Hospital, London.16

Immunofluorescence

The binding of rabbit and mouse antibodies to cell surfaces was assessed by indirect immunofluorescence on viable cells in suspension using affinity purified and fluoresceinated F(ab')2 fragments of goat antibodies directed against F(ab')2 of rabbit Ig or similarly prepared goat anti-mouse Ig, for detecting mouse hybridoma (monoclonal) antibodies. Direct immunofluorescence was used for goat anti-glycophorin and sheep anti-human Ig. Both antiglobulins were cross absorbed with insolubilized human Ig.

Binding of cholera toxin (Schwartz-Mann) was detected with fluorescein-labeled rabbit IgG F(ab')2 antitoxin.17 Fluorescence was evaluated using a standard 16 Zeiss fluorescence microscope with epi-illuminescence and also with the Fluorescence Activated Cell Sorter (FACS-I, Becton Dickinson, Mountain View, Calif.).18,19

Fc and Complement Receptors

Fc and C3 receptors were determined by the erythrocyte rosette technique20 using ox erythrocyte sensitization with rabbit anti-ox red blood cell (RBC) IgG for assaying Fc receptors or ox erythrocytes sensitized with rabbit anti-ox RBC IgM plus zymosan-absorbed human complement for assaying C3(d) receptors. A control of IgM only treated ox RBC was used for C3 receptors and a control of ox RBC alone used for Fcγ receptors.

Cell Cycle Analysis

The proportion of HL-60 cells in different phases of the cell cycle was investigated by mithramycin staining and flow cytofluorimetry (using FACS-I) as previously described.21

Fig. 1. Cell cycle analysis of resting HL-60 and DMSO-induced HL-60 cells. HL-60 cells were cultured for 6 days with 1.25% DMSO (cf. Table 2). Fixed cells were labeled with mithramycin (see Materials and Methods) and analyzed by flow cytofluorimetry (FACS). Vertical axis: relative cell number. Horizontal axis: relative fluorescence intensity and DNA content.
The cell surface phenotype of HL-60 cells was investigated by immunofluorescence and rosette assays and compared with that of two lymphoid leukemic cell lines (Table 3, Fig. 2).

HL-60 cells lacked detectable cell surface immunoglobulin, cALL antigen, glycoporphin, band III, platelet antigen, and the human thymocyte or T-cell-specific antigens (Table 3). The possible expression of Ia-like or HLA-DR antigens was investigated using antisera raised against purified glycoproteins (p28,33), human (allo)anti-HLA-DR reagents, and monoclonal (DA2) anti-HLA-DR (framework). No cells were stained or killed with any of these reagents (see Fig. 2). A similar negative result was previously reported by Gallagher et al.4 using complement-
Fig. 2. FACS analysis of HL-60 cell surface antigenic phenotype. Uninduced HL-60 cells stained as viable cells in suspension with different antisera (cf. Tables 1 and 3). Vertical axis: relative fluorescence intensity. Horizontal axis: relative cell size (light scatter). HLA-A,B,C (W6/32 monoclonal). HLA-DR (DA2 monoclonal); ALL (rabbit anti-cALL serum). oo Cell populations stained or unstained by anti-CGL and separated for morphological analysis (see text). FACS settings: laser 200 mW; photomultiplier tube 700 v; fluorescence gain 16/1 (high sensitivity for all analyses) except for HLA-A,B,C and β2-microglobulin staining where 2/1 was used.

dependent cytotoxicity with rabbit and monkey antisera.

All cells expressed a granulocytic antigen defined by a monoclonal antibody and cholera toxin receptors (GM1 monosialoganglioside). Forty-three percent of uninduced cells were Fcγ receptor positive but lacked complement receptors. No TdT activity was found by either enzymatic or immunofluorescence assay. On DMSO-mediated induction of maturation, no difference was seen with these markers, except for complement receptors that appeared on 45% of cells by day 3 and 59% after 6 days.

In resting cultures, a discrete subpopulation of 11%-34% cells stained with the rabbit anti-CGL antisera (Fig. 2). The positive and negative cells were isolated by means of the FACS. In this experiment, the unseparated cultures contained 13% of cells that were relatively mature (i.e., myelocytes, metamyelocytes, and neutrophils). In the antigen positive (+) fraction separated by the FACS (see Fig. 2), 72% of the cells were mature, whereas in the antigen negative (−) fraction, only 4% cells were at comparable stages of maturation, over 90% being blasts and promyelocytes.

When DMSO-induced cells were stained with anti-CGL, a higher percentage of cells were stained (50%) (Fig. 3). The induced cells were also separated into antigen positive and negative fractions for morphological analysis. Again, the granulocytic antigen positive fraction contained mainly mature cells (73%) while the granulocytic antigen negative fraction had only few mature cells (18%).

Fig. 3. Increased expression of a granulocytic antigen on HL-60 following DMSO-induced maturation. HL-60 cells cultured for 8 days with DMSO. Vertical axis: relative cell number. Horizontal axis: relative fluorescence. A: Antibody [F(ab')2 anti-CGL]. C: Control [normal rabbit Ig F(ab')2]. o o Stained and unstained cells selected (using windows indicated by vertical lines) and separated by FACS for morphological analysis (see text). Fluorescence gain used: 16/1. Log scale on horizontal axis.
Increased expression of granulocytic antigen was not evident until after 6-7 days incubation with DMSO, when 32%–54% cells were labeled. A similar maturation-linked increased antigenic expression was observed with the monoclonal antibody 2D1, which has been shown to react with all leukocytes but more strongly with mature lymphocytes and polymorphs.

All uninduced cells were labeled very brightly by the monoclonal anti-HLA reagent W6/32 with a considerable range of intensity (see Figs. 2 and 4; note decrease of fluorescence gain setting of FACS indicating high intensity of staining, approximately 8 times that seen with anti-CGL). On induction with DMSO, a population with a reduced expression was detectable (Fig. 4). Cells from an induced culture were separated into HLA “bright” (+) and “dim” (−) fractions (see Fig. 4). The percentages of mature cells found were 22.5% in unseparated population, 13% in the bright population, and 44.5% in W6/32 dim population. If metamyelocytes and segmented neutrophils only were considered (myelocytes being omitted), 7.5% were found in the unseparated population, 0.5% in the bright, and 29.5% in the dim fractions, indicating an enrichment of the more mature cells in the population with the weaker expression of HLA. A similar maturation-linked alteration of antigenic expression was seen with β2-microglobulin using the BB5 monoclonal antibody (Fig. 4).

**DISCUSSION**

These studies have shown that the morphological and functional maturation of HL-60 promyelocytic leukemia cells as induced by DMSO involved exit from the cell cycle (and/or increased G1 or transit time) and a coordinated series of alterations in the cell surface, which include induction of C3 receptors, an increased expression of granulocytic and leukocyte differentiation antigens, and a corresponding decrease in HLA-A,B,C, and β2-microglobulin. Previous studies using human alloantisera have documented the very low density of HLA antigens on normal human polymorphonuclear leukocytes. Studies with monoclonal anti-HLA (W6/−A B C monomorphic determinant, as used in this study) have shown that normal myeloid precursors in bone marrow have a much higher density of HLA antigen than mature polymorphonuclear leukocytes as judged by immunoperoxidase staining or autoradiography. We assume that in our experiments the diminished but still substantial level of HLA expression seen following induction of maturation reflects in part at least that only a very small proportion of cells were mature neutrophils.

HL-60 cells in uninduced cultures do not express monoclonal antibody-defined antigenic markers of the lymphoid, platelet, or erythroid lineages. In common with other (noncultured) acute promyelocytic leukemias, they lack detectable HLA-DR or Ia-like antigens, which are, however, usually expressed on granulocytic progenitors, i.e., some (but not all) myeloblasts and CFU-GM, and acute myeloblastic leukemia cells. Since in our subline of HL-60, many of the resting cells had a blast-like rather than promyelocytic morphology, it might have been anticipated that these cells would be HLA-DR positive. There are several possible explanations for the lack of HLA-DR expression on uninduced HL-60 cells. Myeloblasts are undoubtedly heterogeneous and not all myeloblasts in AML or normal marrow are HLA-DR positive. HLA-DR expression might generally be restricted to the less mature myeloblasts in addition to which proliferation...
rate might influence the timing of expression. An alternative explanation is that the HLA-DR present on myeloblasts in vivo is not a product of these cells (i.e., absorbed from other HLA-DR-releasing cells) and is therefore not synthesized by HL-60 blast cells. Although this latter view seems unlikely, there is at present no direct evidence that granulocytic precursors synthesize HLA-DR.

These phenotypic features indicate fidelity of antigenic expression both quantitatively and qualitatively in accord with the HL-60 cells' granulocytic lineage and maturational status and further substantiate the validity of using this cell line to study some aspects of normal granulocyte biology. HL-60 cells and other granulocytic leukemias do, however, have incomplete maturation (with normal colony-stimulating factor or "nonphysiologic" inducers) and defective expression of granulocyte membrane alloantigens as in CGL\(^4\) (H.J.V.D. Reijden, personal communication). These features plus the chromosome changes recorded\(^4\) emphasize that despite their broad conservation of normal phenotype HL-60 cells do have phenotypic features that are associated with leukemia.

Finally, since HL-60 are said to differentiate into monocytes/macrophages when cultured with phorbol esters\(^38,39\) it will be of interest to compare the membrane phenotype of those induced cells to that described in this report with DMSO-induced granulocytic maturation.

ACKNOWLEDGMENT

We are particularly grateful to those colleagues listed in Table I who kindly provided monoclonal antibodies and also to Dr. F. Bollum for providing anti-TdT and Dr. R. Gallo for the HL-60 cell line. Professor H. Festenstein's laboratory kindly provided HLA-DR\(w\) typing facilities. We also acknowledge the technical help of D. R. Sutherland and W. Verbi, and thank J. Riggs for typing the manuscript.

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