Preparation and Characterization of Monoclonal Antibodies Recognizing Two Distinct Differentiation Antigens (Pro-Im1, Pro-Im2) on Early Hematopoietic Cells

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A series of monoclonal antibodies recognizing myeloid differentiation antigens were prepared by immunizing Balb/c mice with HL-60 cells. Hybrids secreting antibodies reactive with HL-60 cells but unreactive with peripheral blood mononuclear cells were isolated and further cloned. One clone was found to produce an IgG2a antibody recognizing an 85,000-dalton molecular weight surface glycoprotein, and a second clone was found to produce an IgM antibody recognizing a heat-stable determinant present on a glycolipid. We have termed these antigens Pro-Im1 and Pro-Im2, respectively (Pro for using HL-60 promyelocytes as an immunogen and Im for the presence of these antigens on immature cells). aPro-Im1 and aPro-Im2 were used to investigate the surface expression and tissue distribution of these two antigens. Pro-Im1 and Pro-Im2 were found to be brightly expressed on a fraction of fetal liver hematopoietic and bone marrow cells. Both antibodies mediated complement-dependent inhibition of CFU-GM, BFU-E, and CFU-GM/GEMM formation assayed by soft agar colony and burst formation, indicating the expression of these antigens by early hematopoietic precursor cells. This was further confirmed by the induction of HL-60 cells by TPA to differentiate into more mature monocytes and macrophages, accompanied by the loss of both antigens. Pro-Im1 and Pro-Im2 were absent from peripheral blood monocytes, erythrocytes, and platelets, but Pro-Im2 was expressed on granulocytes. Both antigens were absent from thymocytes and peripheral T cells. Cytofluorographic analysis suggested their absence from peripheral blood B cells but that both were expressed on a minority of tissue B cells. Analysis of 150 cases of various myeloid and lymphoid malignancies demonstrated Pro-Im1 and Pro-Im2 expression on myeloblasts and promyelocytes from some acute myelogenous leukemias as well as some B cell malignancies, suggesting that these antigens are shared by early hematopoietic cells and a subset of B cells.

MONOCLONAL ANTIBODIES produced by mouse hybridomas have led to rapid advances in the understanding of normal and malignant human lymphoid cells. Similar understanding of human myeloid cells will be facilitated by the development of a series of monoclonal antibody probes to myeloid cell surface antigens. Recently, several cell lines of the myeloid series and monoclonal antibodies directed at myeloid differentiation antigens have been developed. The HL-60 myeloid cell line was isolated from the peripheral blood leukocytes of an adult female with acute promyelocytic leukemia. Most cells from this line have the morphology of myeloblasts and promyelocytes. These cells can be induced to differentiate into mature myeloid cells or monocytes by certain chemical agents and by a "physiologic" lymphocyte conditioned medium. These observations indicate that the HL-60 line will be useful both in the development of monoclonal antibodies reactive with myeloid/monocytic differentiation antigens and in studies of the control of myeloid/monocytic cell differentiation.

Toward this end, we have developed and characterized two monoclonal antibodies, aPro-Im1 and aPro-Im2, which recognize distinctive differentiation antigens present on the HL-60 cell line as well as on early hematopoietic cells. We provide a biochemical analysis of the Pro-Im1 and Pro-Im2 antigens, their distribution on normal hematopoietic cells, malignant myeloid/monocytic and lymphoid cells, and on non-lymphoid tissues, and discuss their relatedness to current concepts of progenitor cell differentiation.

MATERIALS AND METHODS

Immunization and Somatic Cell Hybridization

Balb/c mice were immunized intraperitoneally with $10^8$ exponentially growing, washed HL-60 cells once per week for four weeks. Splenectomy was performed three days after the final immunization. Cell hybridization was performed using the technique of Kohler and Milstein. Fused cells were washed, then suspended in hypoxanthine aminopterine thymidine (HAT) medium and distributed into 96-well plates. Cultures were fed with HT medium on days 9 and 13. Macroscopic hybridomas were observed in most of the wells by days 10 to 21.

Antibody Screening and Cloning of Hybridized Cells

Approximately 10 to 21 days later, supernatants were collected from wells with hybrid cell growth and tested by indirect immuno-

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fluorescence in the presence of antibody reactive with HL-60 cells. Supernatants reactive with HL-60 cells were absorbed by excess peripheral blood mononuclear cells to remove antibodies directed at common antigens shared between peripheral blood mononuclear cells and the HL-60 cells. Hybrids from wells that contained antibodies specific for HL-60 cells were harvested and cloned by a limiting-dilution method in the presence of feeder cells. Selected clones were subsequently recloned and maintained by intraperitoneal injection of $10^6$ cells into Balb/c mice primed with pristine for ascites development.

**Cell Surface Iodination, Immunoprecipitation, and Two-Dimensional Gel Electrophoresis**

Cells from the immunizing cell line HL-60 and ML1, a myeloblastic cell line, were radiolabeled externally with $^{125}$I by the laccoperoxidase-catalyzed reaction according to the technique of Marchalonis et al. The cells were lysed with Nonidet P-40 detergent (Sigma Chemical Co, St Louis, Mo) and immunoprecipitated with the monoclonal antibodies aPro-Iml and aPro-Im2, respectively. The resulting immunoprecipitates were eluted by boiling the beads either with 4 mol/L urea and 2% sodium dodecyl sulfate (SDS) in 0.1 mol/L TRIS HCl (pH 8.0) buffer and performing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 1.5 mm thick slab gels with a 5% gel and 20% gradient of acrylamide, or boiling the beads with 4 mol/L urea in 0.1 mol/L TRIS HCl (pH 8.0) buffer and performing a two-dimensional gel analysis according to the technique of Bushkin et al modified from the technique of O'Farrell. Briefly, first-dimensional resolution was performed on isoelectric focusing (IEF) slab gels constructed with pH 3.5 to 10 ampholines (actual gradient, 9.0–3.8). The slabs were then cut into strips for the second-dimension resolution by SDS-PAGE. Marker proteins of known molecular weight ($\beta$-galactosidase, phosphorylase-b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, $\alpha$-lactalbumin and bovine heart cytochrome c) were co-run on each SDS-PAGE gel. The gels were then stained, dried on filter paper under vacuum, and autoradiographed on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY).

**Phorbol Ester Induced Differentiation**

HL-60 cells were cultured in a 5% CO$_2$ humidified atmosphere at 37°C in the presence of freshly prepared 1.6 x $10^8$ mol/L 12-O-tetradecanoylphorbol 13-acetate (TPA) (originally stored frozen in acetone at mmol/L). At each time point of study, adherent cells were resuspended by scraping them with a plastic policeman. Dead cells were sedimented out by Ficoll-Hypaque density gradient centrifugation. The harvested cells were examined for their expression of the Pro-Im1 and Pro-Im2 antigens and other surface markers by indirect immunofluorescence.

**Peripheral Blood and Tissue Specimens**

Samples of heparinized venous blood and bone marrow cells were obtained from normal healthy volunteers who gave informed consent. Samples of heparinized venous blood and aspirated bone marrow were collected at the time of diagnosis or at relapse from patients with chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), acute monocyctic leukemia (AMoL), chronic myelogenous leukemia (CML) and multiple myeloma. These diagnoses were made by employing standard clinical, morphologic, and cytochemical criteria. Eighty percent or more of the cells in each one of these cases was malignant by cytomorphologic criteria.

Representative portions of lymph nodes were obtained under sterile conditions from the surgical specimens of patients undergoing diagnostic evaluation for malignant lymphoma. These specimens were classified according to conventional histopathologic criteria as benign lymphoid hyperplasia or malignant lymphoma; the latter were further classified according to the system of Rappaport. Normal human thymus was obtained from patients having portions of the thymus gland removed during the course of corrective cardiac surgery. Portions of spleen were obtained from patients undergoing splenectomy for traumatic laceration. Tonsils were obtained from children undergoing tonsillectomy for chronic tonsilitis. Portions of fetal liver were obtained during the course of postmortem dissection of spontaneously and therapeutically aborted fetuses. Representative portions of a variety of normal and pathologic nonlymphoid tissues (skin, lung, kidney, brain, liver, paratid, thyroid, pancreas, breast, testis, uterus, prostate, gastrointestinal tract) were similarly obtained from surgical specimens removed during the course of standard operative procedures. All specimens were immediately transported to the laboratory in tissue culture medium RPMI 1640 (Gibco, Grand Island, NY).

**Mononuclear Cell Isolation**

Cell suspensions were prepared from the lymphoid tissues by teasing apart the tissue in RPMI 1640 until the cells were separated from the connective tissue stroma. Cell suspensions with a viability less than 70%, as determined by trypan blue dye exclusion, were discarded. A mononuclear cell suspension of greater than 95% viability and free of contaminating erythrocytes was prepared from each lymphoid tissue, peripheral blood, and bone marrow specimen by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation.

**Peripheral Blood Fractionation**

Peripheral blood mononuclear cells were further fractionated as follows: Granulocytes were isolated from the cell pellet by hypotonic NH$_4$Cl lysis of the erythrocyte population. Monocytes (Mφ) were isolated from the mononuclear cell suspension by plating the mononuclear cells at a density of $5 \times 10^6$ cells per milliliter in RPMI 1640 with 10% fetal calf serum on Petri dishes for two hours. Nonadherent cells were removed by vigorous lavage. The adherent cells were recovered after incubation for one hour at 37°C in RPMI 1640 containing 0.1% EDTA. Greater than 90% of the recovered adherent cells had morphologic characteristics of Mφ. The remaining lymphocytes, depleted of Mφ by adherence on Petri dishes, were separated into E rosette-forming (T) cells and non-E rosette-forming (B) cells by reacting them with sheep erythrocytes and fractionating them on Ficoll-Hypaque density gradients.

**Cryostat Tissue Sections**

Representative portions of the various nonlymphoid tissues were cut into 4 x 4 x 2 mm blocks, placed in OCT embedding compound (Lab Tech, Ill) in airtight Beem capsules (Pelco, Calif), snap frozen in a mixture of isopentane and dry ice, and stored at $-80^\circ$C. When needed, the bottom of the frozen capsule was cut with a razor blade and the tissue was removed without thawing and mounted on a chuck containing OCT embedding medium. Cryostat sections 4 µm thick were cut on American Optical Cryostat, immediately fixed in acetone for five seconds, air-dried briefly, and then stored at $-20^\circ$C until the time of staining.

**Lymphocyte Marker Analysis**

Fluorescein conjugated rabbit anti-human immunoglobulin F(ab')$_2$ antibody fragments monospecific for $\delta$, $\mu$, $\gamma$, $\alpha$, and $\lambda$ determinants were prepared as previously described. Cell surface and cytoplasmic immunoglobulin were demonstrated by direct
immunofluorescence.43,45 Cytophilic uptake of IgG as a cause of nonspecific immunofluorescent staining was avoided by incubating the cells for two hours to overnight at 37°C before immunofluorescent staining. Spontaneous sheep erythrocyte (E) rosette formation was assayed with Vibrio cholerae Neumaminidase (VCN Type V, Sigma Chemical Co, St Louis, Mo) treated sheep erythrocytes at 37°C.7 Terminal deoxynucleotidyl transferase (TdT) was demonstrated by indirect immunofluorescence (Bethesda Research Laboratories, Gaithersburg, Md). Common acute lymphoblastic leukemia antigen (cALLa) was detected with monoclonal antibody J5 ( Coulter Electronics, Hialeah, Fla).

Surface Immunofluorescence

Reactivity of the monoclonal antibodies with the various cell populations was assayed by indirect immunofluorescence using fluorescein conjugated goat (F(ab)') anti-mouse IgG (G/M FITC) (Cappel Laboratories, Cochranville, Pa) and analyzed with either a Leitz Ortholux microscope with phase contrast optics and polem illumination or on a cytofluorograph (Model FACS IV, Becton Dickinson, Mountainview, Calif).

Tissue Section Immunoperoxidase

Cell surface membrane determinants reactive with aPro-1m1 and aPro-1m2 were demonstrated in tissue section by an avidin-biotin-complex immunoperoxidase technique, as previously described in detail.39

Antibody-Dependent Complement-Mediated Inhibition of CFU-GM, BFU-E, and CFU-GEMM Formation

Unseparated nucleated bone marrow cells or cells of density less than 1.077 g/mL were obtained after separation with Ficoll-Hypaque, suspended in Iscove's modified Dulbecco's medium (IMDM, Gibco) containing 10% fetal calf serum (FCS) and kept at 4°C until being used for treatment or culture. Antibody-dependent, complement-mediated cytolysis was performed as previously described by Andrew et al.21

Colony-Forming Assays

(A) Colony (>50 cells per aggregate) and cluster (three to 50 cells per aggregate) formation of bone marrow cells was stimulated by exogenously supplied colony stimulatory factors. About 0.5 nonadherent low density or 7.5 x 10^5 low density human bone marrow cells or what was left after antibody and complement treatment were suspended in 1 mL of 1.3% agar culture medium (Difco Laboratories, Detroit, Mi) that included McCoy's 5A medium supplemented with additional essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate and contained 10% heat-inactivated (36°C for 30 minutes) fetal calf serum (Microbiological Associates, Walkersville, Md). No background colonies and only rare clusters (up to 20) were observed under these conditions when no exogenous stimulatory factors were supplied. CSFs were present in medium conditioned by the GCT cell line.

The approximate percentages of colony and cluster types stimulated by GCT-CM after seven days of incubation were 40% neutrophils, 10% eosinophils, 30% macrophages, and 20% neutrophil-macrophages. These results were in relatively good agreement with those published by others.

(B) The colony assay for human CFU-GEMM was carried out according to the procedure of Fauser and Messner.46,47 Bone marrow cells were plated at 2 x 10^5, or what was left after antibody and complement treatment, in 35-mm Lux standard tissue culture dishes containing a 1-mL mixture of IMDM, 0.8% methylcellulose, 30% fetal bovine serum, 5% medium conditioned by leukocytes from patients with hemachromatosis in the presence of 1% phytohemagglutinin (PHA-LCM) and 5 x 10^-2 mol/L 2-mercaptoethanol. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO2 in air. One unit of a step-III preparation of sheep plasma erythropoietin (Connaught Labs, Ltd, Willowdale, Ontario, Canada) was added to each dish on day 4 and 5 unless otherwise noted. Colonies were scored with an inverted microscope after 13 to 14 days of incubation and were further identified after staining with benzidine in the dishes or after picking out colonies with a fine pipette, putting on a glass slide and staining with Wright-Giemsa to confirm their mixed nature. Mixed colonies usually contained erythroid, granulocytic and monocytic cells and sometimes contained megakaryocytes.

(C) BFU-Es were scored from these same plates. In some cases, and where specifically stated, BFU-Es were scored from plates cultured as previously reported.27 Six to ten plates were scored per treatment point.

RESULTS

Development and Initial Characterization of Monoclonal Antibodies Directed Against Surface Antigens on HL-60 Cells

As described above, mouse splenocyte-myeloma cell hybrids producing antibodies that reacted with HL-60 cells but not with peripheral blood mononuclear cells were screened by indirect immunofluorescence on a cytofluorograph. Two such antibodies, designated aPro-1m1 (an IgG2a) and aPro-1m2 (an IgM) that reacted strongly with HL-60 cells (Fig 1A) but not with peripheral blood mononuclear cells (Fig 1B) were cloned six times in vitro and implanted subcutaneously into pristine primed Balb/c mice. The resulting ascetic fluid exhibited detectable reactivity by both indirect immunofluorescence and complement-dependent cytotoxicity to HL-60 cells at titers as high as 1:10^6.
Molecular Characterization of the Target Antigens Recognized by αPro-Im1 and αPro-Im2

Both HL-60, a promyelocytic line, and ML-1, a myeloblastic line, cells were radiolabeled externally with 125I by the lactoperoxidase-catalyzed reaction, lysed with Nonidet P-40 detergent, and the resulting solubilized surface membrane proteins reacted with αPro-Im1 and αPro-Im2, respectively, as described above. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and subsequently by two-dimensional gel electrophoresis involving isoelectric focusing in the first dimension and SDS-PAGE under reducing conditions in the second dimension. The SDS-PAGE gel pattern of immunoprecipitated materials obtained from surface 125I-labeled ML-1 cells shown in Fig 2A. In lane a, αPro-Im1 precipitated under nonreducing conditions a polypeptide chain with molecular weight of 85,000 daltons from ML-1 cells, whereas αPro-Im2 did not precipitate any discernible components (Fig 2A, lane b). Further analysis of antigen Pro-Im1 under reducing conditions by 2D gel electrophoresis revealed that Pro-Im1 is a neutral molecule that has an isoelectric point in the range of 6.0 to 7.0 (Fig 2B). Repeated attempts to precipitate antigen Pro-Im2 from both HL-60 and ML-1 cells failed, suggesting that the antigen recognized by αPro-Im2 may not be a surface protein in nature. When HL-60 target cells were heated for one minute at 100 °C, αPro-Im2 still gave bright staining of these cells, whereas staining by αPro-Im1 disappeared entirely, suggesting that the antigenic determinant recognized by αPro-Im2 is heat stable, and nonimmunoprecipitable, possibly a glycolipid in nature. Inhibition of anti-HL-60 activity as detected by αPro-Im2 with various carbohydrate determinants by indirect immunofluorescence analysis on a cytofluorograph has indicated that only the Lea determinant exhibits a partial inhibitory effect. Further analysis of the biochemical nature of the Pro-Im2 antigen is currently underway.

Loss of Pro-Im1 and Pro-Im2 Antigens Following Induction of HL-60 Cell Differentiation Into Monocytic Cells by TPA

Earlier studies have shown that HL-60 promyelocytic leukemic cells acquire monocyte-macrophage characteristics as a result of in vitro differentiation induced by lymphocyte conditioned medium29 or by phorbol diester.27 We have examined the expression of Pro-Im1 and Pro-Im2 by the HL-60 cells following TPA treatment. In the presence of 1.6 × 10−9 mol/L TPA (Fig 3B) both Pro-Im1 and Pro-Im2 antigens disappeared rapidly (within 24 hours) from the HL-60 cells when compared with the control cultures (Fig 3A). Meanwhile, the 24-hour culture of TPA-treated HL-60 cells began to express OKM1 antigen associated with the surface expression of complement receptors and the acquisition of phagocytic capability by these TPA-treated HL-60 cells.

Expression of Pro-Im1 and Pro-Im2 on Normal Hematopoietic Elements from Peripheral Blood and Lymphoid Tissues

αPro-Im1 and αPro-Im2 were initially selected for their strong positive reactivity with HL-60 cells (Fig 1A) and their negative reactivity with peripheral blood mononuclear cells (Fig 1B). Nonetheless, we chose to perform a further specificity analysis using mononuclear cell suspensions prepared from peripheral blood, thymus, lymph node, tonsil, and spleen. We also prepared fractions of monocyte-enriched adherent cells, E rosette-enriched T cells and E rosette-depleted nonadherent B and null cells from lymphoid tissues and peripheral blood and granulocyte, erythrocyte, and platelet fractions from peripheral blood. The number of positive cells for each marker was enumerated by indirect immunofluorescence and flow cytometry (Table 1). Monocyte enriched adherent cells (Fig 1C),
Fluorescence Intensity

EARLY HEMATOPOIETIC CELL SURFACE ANTIGENS

Table 1. Comparative Percentage of Normal Human Tissue Lymphocytes That Express HLA-DR Antigen, Surface Immunoglobulin, Pro-Im1, Pro-Im2, T3, and E Rosette Receptor

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Specimens</th>
<th>Unseparated MNC</th>
<th>E Rosette-Enriched</th>
<th>E Rosette-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>2</td>
<td>33 25 18 14 65 69</td>
<td>2 95 1 1</td>
<td>95 2 35 28</td>
</tr>
<tr>
<td>Tonsil</td>
<td>5</td>
<td>52 45 18 12 44 46</td>
<td>2 95 2 1</td>
<td>95 1 37 30</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>42 30 15 8 55 56</td>
<td>2 95 1 2</td>
<td>95 2 32 25</td>
</tr>
<tr>
<td>Thymus</td>
<td>5</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 30 &gt;95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures represent the percentage of cells expressing each marker.

thymocytes, E rosette-enriched T cells (Fig 1D), erythrocytes and platelets were unreactive with αPro-Im1 and αPro-Im2. However, αPro-Im2 but not αPro-Im1 gave very bright staining with virtually all peripheral blood granulocytes (Fig 1F). Moreover, Pro-Im1-positive cells (3% to 10%) were found in the non-E rosette forming, nonadherent cell population isolated from peripheral blood (Fig 1E). This cell population, however, did not express Pro-Im2 (Fig 1E). Further analysis of these Pro-Im1-bearing cells revealed that they were copurified with the peripheral blood dendritic cells in the low-density, slg-negative, nonadherent and non-E rosette-forming cell population (80% HLA-DR⁺, 30% Pro-Im1⁺). Pro-Im1 and Pro-Im2 antigens were also found to be expressed on a fraction of cells from the E rosette-depleted, B cell-enriched fraction isolated from lymph node, tonsil, and spleen (Table 1). Pro-Im1 and Pro-Im2 positive populations appeared to be entirely contained within the DR⁺ positive cell population. This finding gave us a clear indication that both Pro-Im1 and Pro-Im2 are also expressed on subpopulations of B lymphocytes.

Expression of Pro-Im1 and Pro-Im2 on Normal Liver Hematopoietic and Bone Marrow Cells

Both antigens were screened for their expression on early hematopoietic cells. Cryostat tissue sections prepared from three fetal livers (10, 15, and 20 weeks gestational age) and bone marrow cells from six normal volunteers were examined. Scattered large, immature hematopoietic cells, occurring singly and in small clusters in the hepatic sinusoids of all three fetal livers were found to be reactive with αPro-Im1 and αPro-Im2. It was not possible to precisely identify the lineage of these Pro-Im1 and Pro-Im2 positive cells in cryostat sections by cytomorphologic criteria. These positive hematopoietic cells most likely represent myeloid progenitor cells and possibly pre-B cells. Among the six normal bone marrow cell preparations examined, αPro-Im1 was found to react with 25% to 40% of the bone marrow cells, whereas αPro-Im2 was found to react with 15% to 25% of these cells (Fig 4). The bone marrow cell preparations examined usually contained <5% Leu-4/T3⁺, 5% to 8% HLA-DR⁺, and 3% to 7% OKM1⁺ cells. Most of the cells were of myeloid lineage including <1% myeloblasts, 1% to 2% promyelocytes, 5% to 15% myelocytes, 15% to 20% metamyelocytes, and 40% to 50% polymorphonuclear cells. In one representative experiment, the Pro-Im1-positive cells consisted of 1% myeloblasts, 7% promyelocytes, 43% myelocytes, 20% metamyelocytes, 5% polymorphonuclear cells and <2% monocytes; and the Pro-Im2-positive cells isolated by FACS IV consisted of 1% myeloblasts, 6% promyelocytes, 26% myelocytes, 28% metamyelocytes, 40% polymorphonuclear cells, and <2% monocytes. In addition, functional assays involving colony and burst formation of granulocytes, macrophages, erythroid cells, etc, under the influence of colony stimulating factor(s) and erythropoietin were used to examine the complement-dependent cytotoxicity effects of these two antibodies so as to permit evaluation for the presence or absence of Pro-Im1 and Pro-Im2 antigen(s) on these hematopoietic precursor cells.

Reactivity of αPro-Im1 and αPro-Im2 With Committed Hematopoietic Progenitor Cells

The reactivity of each antibody with committed hematopoietic precursors including CFU-GMs and BFU-Es was assayed in negative-selection experiments by inhibition of colony growth after treatment of bone marrow cells with antibody in the presence of complement. Granulocyte colony formation by precursor cells that are present in unseparated normal bone marrow cells was significantly inhibited by pretreatment with

Fig 4. Expression of Pro-Im1 (A) and Pro-Im2 (B) on normal human bone marrow cells detected by indirect immunofluorescence analysis. (-----) control using normal mouse IgG; (jagged line) αPro-Im antibodies.
αPro-Im1 and αPro-Im2 in the presence of complement.

Table 2 summarizes the averaged results of two parallel assays in one representative experiment. For each culture, $2 \times 10^5$ unseparated bone marrow cells under the stimulation of colony stimulating factor gave rise to 185 colonies (0.09%) or 394 colonies plus clusters (0.2%), as shown in the medium control. Mere treatment of cells with antibodies such as αHLA-DR (IgG2a), αLeu-1 (IgG2a), αPro-Im1 (IgG2a) and αPro-Im2 (IgM) before culture gave rise to comparable numbers of colonies and clusters. Pretreatment of the cells with complement alone similarly did not result in inhibition of colony formation. However, pretreatment of cells with lytic αHLA-DR, αPro-Im1, or αPro-Im2 plus complement resulted in significant inhibition of granulocyte-macrophage colony formation, with inhibition up to 50%, 63%, and 81%, respectively. In each experiment, inhibition of clusters was comparable to that of the colonies. Pretreatment of cells with lytic αLeu-1 antibody did not give significant inhibition.

Erythroid colony formation scored at day 14 by precursor cells that are present in unseparated normal bone marrow cells was also significantly inhibited by pretreatment with αPro-Im1 and αPro-Im2 in the presence of complement (Table 2). For each culture, $2 \times 10^5$ unseparated bone marrow cells under the stimulation of erythropoietin gave rise to 56 colonies of burst formation. Treatment of the cells with antibodies alone did not give significant inhibition. However, pretreatment of cells with αHLA-DR, αPro-Im1, or αPro-Im2 plus complement provided a significant inhibition of mixed colony formation. Treatment with αHLA-DR gave 50% inhibition. Treatment with αPro-Im1 gave 94% inhibition and treatment with αPro-Im2 gave 98% inhibition, a near complete inhibition of mixed colony formation.

**Table 2. Inhibition of CFU-GM, BFU-E, and CFU-GEMM Formation by Treatment of Normal Bone Marrow Cells With Monoclonal Antibodies and Complement**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU-GM (%)</th>
<th>colonies (%)</th>
<th>colonies + clusters (%)</th>
<th>colonies (%)</th>
<th>colonies + clusters (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>185 ± 7</td>
<td>394 ± 7</td>
<td>56 ± 3</td>
<td>15 ± 1</td>
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<tr>
<td>Anti-HLA-DR</td>
<td>181 ± 3(−2)</td>
<td>374 ± 6(−5)</td>
<td>55 ± 3(−2)</td>
<td>14 ± 1(−7)</td>
<td></td>
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<tr>
<td>Anti-Leu-1</td>
<td>179 ± 4(−3)</td>
<td>390 ± 5(−1)</td>
<td>52 ± 3(−7)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Anti-Pro-Im1</td>
<td>175 ± 9(−5)</td>
<td>356 ± 13(−10)</td>
<td>47 ± 3(−16)</td>
<td>11 ± 1(−27)</td>
<td></td>
</tr>
<tr>
<td>Anti-Pro-Im2</td>
<td>178 ± 5(−3)</td>
<td>365 ± 9(−7)</td>
<td>50 ± 4(−11)</td>
<td>13 ± 2(−13)</td>
<td></td>
</tr>
<tr>
<td>Medium + complement</td>
<td>203 ± 11</td>
<td>452 ± 9</td>
<td>52 ± 4</td>
<td>14 ± 1</td>
<td></td>
</tr>
<tr>
<td>Anti-HLA-DR + complement</td>
<td>102 ± 4(−50)</td>
<td>241 ± 6(−47)</td>
<td>20 ± 2(−62)</td>
<td>7 ± 1(−50)</td>
<td></td>
</tr>
<tr>
<td>Anti-Leu-1 + complement</td>
<td>195 ± 8(−3)</td>
<td>408 ± 11(−10)</td>
<td>48 ± 4(−7)</td>
<td>ND</td>
<td></td>
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<tr>
<td>Anti-Pro-Im1 + complement</td>
<td>75 ± 10(−63)</td>
<td>215 ± 22(−52)</td>
<td>18 ± 1(−65)</td>
<td>0.8 ± 0.5(−94)</td>
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<tr>
<td>Anti-Pro-Im2 + complement</td>
<td>38 ± 6(−81)</td>
<td>113 ± 8(−75)</td>
<td>6 ± 2(−88)</td>
<td>0.2 ± 0.1(−98)</td>
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</table>

*2 $\times 10^5$ unseparated bone marrow cells were plated in each culture. Colonies for CFU-GM were scored on day 7, and colonies for BFU-E as well as CFU-GEMM were scored on day 14.

**Reactivity of αPro-Im1 and αPro-Im2 With Pluripotential Hematopoietic Stem Cells**

Parallel experiments were carried out to examine whether Pro-Im1 and Pro-Im2 are present on multipotent stem cells through the complement-dependent inhibition of the mixed colony formation (CFU-GEMM). As shown in Table 2, for each culture, $2 \times 10^5$ unseparated bone marrow cells gave rise to 15 mixed colonies when scored on day 14 with an inverted microscope. These colonies were further identified by picking out each of them with a fine pipette, placing them on a glass slide, and staining them with Wright-Giemsa to confirm their mixed cell nature. These mixed colonies usually contained erythrocytes, granulocytes, and monocytes and sometimes contained megakaryocytes. Mere treatment of cells with antibodies alone did not give significant inhibition. However, pretreatment of cells with αHLA-DR, αPro-Im1, or αPro-Im2 plus complement provided a significant inhibition of mixed colony formation. Treatment with αHLA-DR gave 50% inhibition. Treatment with αPro-Im1 gave 94% inhibition and treatment with αPro-Im2 gave 98% inhibition, a near complete inhibition of mixed colony formation.

**Expression of Pro-Im1 and Pro-Im2 on Myeloid and Lymphoid Malignancies**

Mononuclear cell suspensions prepared from peripheral blood, bone marrow, and lymph node specimens obtained from 150 patients with high count leukemia or extensively involved by malignant lymphoma were investigated for their expression of Pro-Im1 and Pro-Im2 (Table 3). In each case, more than 80% of the isolated cells were identifiable as malignant. The cell population of a given case was considered positive if more than 20% of the malignant cells were reactive with the said monoclonal antibody. Neoplastic cells...
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Phenotype</th>
<th>Cell of Origin</th>
<th>No. Cases Studied</th>
<th>No. Cases Expressing</th>
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<tr>
<td>Acute myeloblastic leukemia (M1)</td>
<td>HLA-DR+ S1g- E-</td>
<td>Myeloid</td>
<td>18</td>
<td>5*</td>
</tr>
<tr>
<td></td>
<td>cALLa- TdT+</td>
<td></td>
<td></td>
<td>6*</td>
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<td>HLA-DR+ S1g- E-</td>
<td>Myeloid</td>
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<td>(acute phase)</td>
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*Neoplastic cells from only two AML (M1) cases co-expressed Pro-Im1 and Pro-Im2

Table 3. Summary of Number of Cases of Various Hematopoietic Malignancies That Express Pro-Im1 and Pro-Im2

Expression of Pro-Im1 and Pro-Im2 on Nonlymphoid Tissues

Cryostat tissue sections prepared from a variety of normal and malignant nonlymphoid tissues were examined for their reactivity with αPro-Im1 and αPro-Im2. Fifteen percent of the neoplastic cells in one case were Pro-Im1+, and 22% of the neoplastic cells in another case were Pro-Im2+. Interestingly, all three cases of ALL were atypical in that they lacked the B lymphocyte associated antigens BL1 and BL2.α Pro-Im1 and αPro-Im2 reacted with the neoplastic B cells from six and five of 19 cases of B-chronic lymphocytic leukemia (HLA-DR+, S1g+, E-), respectively. Similarly, a variable percentage of the neoplastic cells from six and seven of 13 cases of non-Hodgkin B cell lymphomas (HLA-DR+, S1g+, E-), expressed Pro-Im1 and Pro-Im2, respectively. In contrast, malignant plasma cells isolated from seven patients with multiple myeloma or plasma cell leukemia were Pro-Im1 and Pro-Im2 negative. The expression of Pro-Im1 and Pro-Im2 by certain B cell malignancies is further evidence that these antigens are shared by early hematopoietic cells and a subset of B cells.

Expression of Pro-Im1 and Pro-Im2 on Nonlymphoid Tissues

Cryostat tissue sections prepared from a variety of normal and malignant nonlymphoid tissues were examined for their reactivity with αPro-Im1 and αPro-Im2. αPro-Im1 reacted strongly with cerebral cortical and cerebellar vascular endothelium but not with endothelial cells in other organs. As anticipated from cell suspensions studies, αPro-Im2 reacted strongly with granulocytes. αPro-Im1 and αPro-Im2 were found to react only very weakly or not at all on the
epithelium and mucosa of the organs studied here. Thus, the Pro-Im antigens have a very narrow tissue distribution.

DISCUSSION

In an effort to identify antigens present on early hematopoietic cells and employ them as markers for these cells, we used HL-60, a cell line containing both myeloblasts and promyelocytes, as immunogen for the development of monoclonal antibodies specifically reactive with HL-60 cells but not peripheral blood mononuclear cells. Two such antibodies that display certain unique characteristics were developed. An IgG2a antibody (αPro-Im1) recognizes a glycoprotein with a molecular weight of 85,000 daltons. An IgM antibody (αPro-Im2) recognizes a heat stable nonimmunoprecipitable glycolipid.

Initially, we used these two monoclonal antibodies to study the phenotypic changes of HL-60 cells before and after TPA treatment, since the HL-60 cell line has been used as a model to study the control of myeloid cell maturation. HL-60 cells can develop into either granulocytic or monocytic cells when exposed to different inducing agents. Chemicals such as DMSO promote granulocytic differentiation while phorbol diesters and certain lymphokines induce monocytic maturation. Results obtained in our study indicate that both Pro-Im1 and Pro-Im2 disappear rapidly from the HL-60 cells after TPA induction, which is accompanied by the surface expression of the monocytic antigen OKM1, complement receptors, and the acquisition of phagocytic capability by these cells. This observation suggests that both Pro-Im1 and Pro-Im2 antigens are present on cells in early stages of myeloid differentiation. This notion was further supported by our finding that αPro-Im1 and αPro-Im2 react with normal human bone marrow cells and fetal liver hematopoietic cells.

The fact that Pro-Im1 and Pro-Im2 are present on a sizable fraction of fetal liver hematopoietic and bone marrow cells and that their expression on HL-60 cells is lost following TPA induction prompted us to examine their possible presence on various hematopoietic precursor cells. The progenitor cells (CFU-GM and BFU-E) that give rise to granulocyte-macrophage and erythroid colonies, respectively, appear to be generated from pluripotential stem cells. In addition, the progenitor cells CFU-GEMM that give rise to mixed colonies of granulocytes, erythrocytes, monocytes and megakaryocytes have been suggested to represent human multipotential stem cells or a subpopulation of stem cells. Our experiments involving complement-mediated inhibition of CFU-GM and BFU-E colonies and CFU-GEMM mixed colony formation in the presence of various monoclonal antibodies further suggest that both Pro-Im1 and Pro-Im2 are expressed on the blood precursor as well as stem cells, although a definitive conclusion on this point awaits the direct isolation of these cells by both monoclonal antibodies with stem cell activity.

We also investigated the expression of Pro-Im1 and Pro-Im2 antigens on the myeloid malignancies. Most classifications of acute myelogenous leukemia are based on morphologic and cytochemical features. The joint French-American-British (FAB) group proposed criteria for the classification of AML have been widely accepted. Six subtypes of AML are identified: M1 and M2 are progressive levels of myeloid maturation; M3, promyelocytic leukemia; M4 and M5, myelomonocytic and monocytic variants; and M6, erythroleukemia. The relationship between normal myeloid cells and myeloid leukemic cells classified by the FAB system is at present unclear, due partly to the difficulty in characterizing the normal myeloid counterparts. If myeloid leukemia cell surface antigens reflect those of the normal myeloid cell stage from which they are derived, then utilization of the appropriate monoclonal antibodies that recognize myeloid differentiation antigens could facilitate subclassification of the myeloid leukemias and more precisely characterize normal myeloid differentiation.

A clonal proliferation of mature granulocytes and their progenitors has been observed in CML. Most cases of CML evolve to an acute phase or “blast crisis” (CML-BC), which is characterized by decreasing cell maturity and additional chromosomal abnormalities. Two types of CML-BC have been described—myeloid CML-BC and lymphoid CML-BC. Myeloid CML-BC cells have phenotypic markers similar to AML, whereas lymphoid CML-BC cells phenotypically resemble common type ALL cells. Among the 42 cases of myelogenous leukemia that we examined, we found Pro-Im1 to be expressed on the neoplastic cells in 12 of 32 cases of M1, M2, M3, and M4 morphology and on the cells in two of six cases of myeloid CML-BC. Pro-Im2 expression was generally more restricted to M1-AML and to myeloid CML-BC. This observation might be explained by the postulate that the dominant leukemia cell type in AML may not necessarily represent a maturation arrest at the level at which malignant transformation has occurred and that AML blasts may be induced to differentiate in vitro and possibly in vivo. In CML, the target cell for malignant transformation may be a multipotential stem cell. When the chronic phase terminates into an acute phase, a relatively primitive cell type predomi-
nates with the malignant pluripotent cells losing their capacity to differentiate normally during the blast crisis.

Examination of Pro-Im1 and Pro-Im2 expression on normal hematopoietic elements from peripheral blood and lymphoid tissues indicates that the Pro-Im1 and Pro-Im2 antigens are absent from adherent monocytes, thymocytes, and E rosette-enriched T cells. Although the original selection criteria used to screen these antibodies included lack of expression of Pro-Im1 and Pro-Im2 on peripheral blood mononuclear cells, more careful examination of all lymphocyte populations revealed the unexpected expression of Pro-Im1 on a minor fraction (3% to 10%) of peripheral blood nonadherent, non-E rosette forming cells, as well as the expression of both Pro-Im1 and Pro-Im2 on a subset of lymphoid tissue B cells.

The expression of Pro-Im1 and Pro-Im2 on lymphoid malignancies correlated with their expression on normal mononuclear cells. None of 26 T cell malignancies expressed either Pro-Im1 or Pro-Im2. Among the 43 cases of cALLa- common type ALL (cALL) cells examined, only three cases were found to express either Pro-Im1 or Pro-Im2. Interestingly, these three Pro-Im1/Pro-Im2 positive cases were different from the other 40 cases in that they were both BL1 and BL2. Whether the Pro-Im1/Pro-Im2 positive cells in these two ALL cases represent precursor lymphoid stem cells that can further differentiate into pre-B common ALL type cells bearing BL1 and BL2 antigens remains to be answered. Finally, both Pro-Im1 and Pro-Im2 antigens were found to be expressed, though weakly, by variable percentages of neoplastic cells isolated from certain B lymphoid malignancies with the HLA-DR+ S1g-cALLa- E phenotype. This makes the Pro-Im1 and Pro-Im2 antigens “jumping antigens” with respect to B lymphoid differentiation. The early lymphoid stem cells, like other hematopoietic precursor cells initially express both antigens, lose both antigens when the cells mature into committed pre-B or pre-T lymphoid cells, and then regain their expression along the B cell differentiation pathway. Similar findings were obtained for Pro-Im2 along the myeloid differentiation pathway. Pro-Im2 is originally expressed by pluriptotent stem cell(s) and by some committed CFU-GM and BFU-E cells, is lost when these cells develop into more mature myeloid cells as well as monocytes, and is regained upon differentiation into terminal stage granulocytes.

It is unlikely that Pro-Im1 or Pro-Im2, based upon their molecular nature and their cellular expression, are identical to other recently described myeloid antigens. Among the many myeloid antigens described, Pro-Im1 resembles most the one defined by monoclonal antibody L1B2, whereas Pro-Im2 resembles most the one defined by monoclonal antibody 1G10 with the exception that both Pro-Im1 and Pro-Im2 are also expressed on B lymphocyte lineage. It appears that Pro-Im1 and Pro-Im2 antigens are the first group of antigens reported to be expressed on untreated HL-60 cells but not on TPA-treated HL-60 cells that have a predominant expression on early human hematopoietic cells which is shared by a subset of human B cells in their intermediate stages of development.

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Preparation and characterization of monoclonal antibodies recognizing two distinct differentiation antigens (Pro-Im1, Pro-Im2) on early hematopoietic cells

R Peng, A Al-Katib, DM 2d Knowles, L Lu, H Broxmeyer, B Tolidjian, JW Chiao, B Koziner and CY Wang