Immunohistochemical Characterization of a 183 KD Myeloid-Specific–DNA-Binding Protein in B5 Fixed, Paraffin-Embedded Tissues, and Bone Marrow Aspirates by Monoclonal Antibody BM-1

By Alan L. Epstein, Michael Samoszuk, Efstatios Statopoulos, Gregory S. Naeye, Charles V. Clevenger, Susan Weil, and Robert J. Marder

A monoclonal antibody, designated BM-1, which is reactive in B5 formalin-fixed, paraffin-embedded tissues, has been generated against a cytoplasmic and nuclear antigen expressed in human myeloid precursor cells and derived leukemias. Using the avidin-biotin-complex immunoperoxidase procedure, BM-1 was found to stain selectively myeloid precursor cells in normal bone marrow and mature granulocytes in the blood. In a screen of 26 normal adult and fetal human organs fixed in B5 formalin, BM-1 was negative in all nonhematopoietic tissues with the exception of tissue granulocytes and scattered cells in the peripheral cortex of the thymus. Likewise a screen of 30 solid tumor cell lines including a spectrum of carcinomas, sarcomas, and neural-derived tumors was negative. BM-1 was also negative with 21 T and B cell lymphomas and 11 Hodgkin’s disease tumors. A preliminary study of tumors of the hematopoietic system revealed that BM-1 was reactive with M2 and M3 acute myelogenous leukemias (AML), chronic myelogenous leukemias (CML) and myelomonocytic leukemias, and granulocytic sarcomas. M1, M4, M5, and M6 AML clot preparations were negative in this study, indicating that BM-1 may have a role in the histopathologic diagnosis of myelogenous leukemia. Myeloid leukemic cell lines HL-60, ML-2, KG1, and TPH-1-0 showed BM-1 nuclear and/or cytoplasmic reactivity in a subpopulation of cells, but erythroid and lymphoid leukemias and all lymphoma cell lines were negative. Immunoperoxidase studies of a panel of fetal tissues showed BM-1 positive cells in the peripheral cortex of the thymus and portal myelopoietic regions of the liver at 18 weeks gestation. Finally, DNA-cellulose and solid phase radioimmunoadsorb (RIA) techniques developed in our laboratory demonstrate that the BM-1 antigenic domain is reactive only after binding to eukaryotic but not prokaryotic single- or double-stranded DNA. Immunoblot techniques using a DNA-cellulose purified protein sample revealed that BM-1 recognizes a 183 kD protein. These studies indicate that BM-1 is recognizing a myeloid-specific antigen that, because of its DNA binding characteristics, may have an important role in the differentiation of myeloid cells at the molecular level.

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A LARGE NUMBER of monoclonal antibodies to myeloid-specific–cell-surface antigens have been described.1–3 Some of these reagents have proven useful as markers of myeloid differentiation and have greatly improved our ability to characterize and diagnose acute myelogenous leukemia (AML) subtypes.4–5 In a previous report6 we described the generation of three monoclonal antibodies (MoAbs) to myeloid-specific nuclear antigens expressed in differentiating human myeloid leukemia cells and demonstrated that MoAbs to internal cellular antigens may be both cell- and maturation-specific in nature. In the present study a new myeloid-specific MoAb, designated BM-1, is described, which is directed against a DNA-binding protein expressed in the cytoplasm and nucleus of normal and malignant myeloid cells. Unlike other reported markers, BM-1 appears to be restricted in its reactivity to M2 and M3 AML as identified by the French–American–British (FAB) classification.7 In addition, BM-1 retains its immunoreactivity in B5-fixed, paraffin-embedded tissue sections, including those prepared from bone marrow aspirate clot preparations, and therefore appears to have diagnostic utility as a tumor marker for myeloid leukemias and granulocytic sarcomas. Because of its restricted expression in M2 and M3 AML, BM-1 may identify a maturation-specific antigen that can be used to study the differentiation and transformation of human myeloid cells.

MATERIALS AND METHODS

Cell lines and tissues. A complete list of the lymphoid and solid tumor cell lines used in these experiments is shown in a previous publication.8 Tumor cell lines designated with the SW prefix were obtained from Dr William McCombs, III, at the Scott and White Memorial Hospital, Temple, Texas. All of the cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 U/mL penicillin-G, and 100 μg/mL streptomycin sulfate. The cell lines were cultured in a well-humidified 5% CO2 incubator and were routinely passaged twice per week.

For the immunohistochemical studies, human tissues fixed with B5 formalin were obtained from biopsies performed on patients for diagnostic procedures at the Los Angeles County–University of Southern California Hospital and Northwestern Memorial Hospital. Fetal tissues (gestational ages 18 to 22 weeks) obtained from abortuses were fixed in B5 formalin prior to paraffin-embedding procedures. Bone marrow aspirates from leukemia and lymphoma patients were prepared as clot preparations and fixed in B5 formalin prior to paraffin-embedding procedures. All patients participating in this study were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

Antigen preparation. Nuclei from human peripheral blood mononuclear cells were prepared as previously described.9
Immunoassays. One-milliliter aliquots of the nuclear preparations were thawed, sonicated to reduce viscosity, and emulsified in 1.5 mL of complete Freund's adjuvant by using two glass syringes and a 20-gauge microemulsifying needle (Biolog, Inc, Lake Havasu City, AZ). Three 10-week-old Balb/c female mice were injected subcutaneously (SC) at multiple sites by using a 22-gauge needle and glass syringe. Two weeks later the mice were reinoculated as above, except the nuclear extracts were prepared in incomplete adjuvant. Ten days later the mice received a third inoculation of antigen, this time without adjuvant and by intravenous (IV) injection. Four days later the mice were killed by cervical dislocation and the spleens removed by aseptic techniques.

Cell fusion and cloning procedures. Spleen cells were fused with 8-azaguanine-resistant mouse myeloma NS-1 cells at a ratio of 5:1 respectively by using 40% polyethylene glycol 1540 MW (J.T. Baker, Phillipsburg, NJ), as described by Frazekas de St Groth and Scheidegger. Culture supernatants from wells with active cell growth were tested by indirect immunofluorescence microscopy with fixed-cell preparations as described below. Positive cultures were cloned on 0.5% Noble agar (Difco Laboratories, Detroit) containing RPMI 1640 medium, 20% FCS, and antibiotics. In the first method single-stranded and double-stranded DNA celluloses were prepared by the procedure of Moss et al using calf thymus DNA (Sigma Chemical Co, St. Louis, MO) in transfer buffer (24 mmol/L Tris, pH 8.0), each tube was incubated at 4°C for one hour with 10 mg/mL bovine serum albumin in phosphate-buffered saline (PBS), the wells were blocked for one hour with 10 mg/mL bovine serum albumin in PBS at 4°C. The wells were then incubated with 50 µL MoAb supernatant for one hour at 4°C. After six washes the wells were finally incubated with 100,000 cpm of 125I-labeled goat antimouse IgGs for one hour at 4°C. The wells were then washed 6 times and counted in a gamma counter.

Immunoblot procedure. A chronic myelogenous leukemia (CML) biopsy that expressed a high level of the BM-1 antigen, as determined by DNA cellulose RIA, was used in an immunoblot procedure to identify the mol wt of the BM-1 antigen. Other MoAbs with known reactivities were used as controls to verify the specificity of the antigen-antibody reactions. Whole cell lysates were dissolved in nonreducing sodium dodecyl sulfate (SDS) sample buffer and electrophoresed in 5% to 10% SDS-polyacrylamide gradient gels at 20 mA for four hours. Alternatively, myeloid cell lysates produced in Ca/PIPES buffer as described above were prepared on DNA-cellulose using FPLC chromatography (Pharmacia, Piscataway, NJ). Details of this procedure will be presented in a separate publication. Antigen-containing fractions were identified by DNA-cellulose RIA, as described above. Positive fractions were combined and concentrated by ultrafiltration using 25,000 mW cutoff Centricon membrane cones (Amicon, Danvers, MA) before being electrophoresed using the same conditions described above. The separated proteins were transferred to nitrocellulose (Millipore grade HA, 0.45 µm) using a semidydro electrophoretic apparatus (Sartorius, West Coast Scientific Co, Inglewood, CA) in transfer buffer (24 mmol/L Tris-HCl, pH 8.3, 192 mmol/L glycine, 20% methanol) at 160 mA for three hours at room temperature. Lanes containing the whole cell lysate and mol wt markers (BioRad, Richmond, CA) were stained for ten minutes with 0.1% amido black and destained in 10% glacial acetic acid and 5% methanol. The remaining lanes were blocked with 0.25% Tween-20 in PBS for one hour at room temperature and washed in 0.05% Tween-20 in PBS (wash buffer). The lanes were then incubated for one hour with BM-1 supernatant ( neat) and washed 3 times with wash buffer for 10-minute intervals. Five micrograms of biotinylated goat antimouse IgG (Vector Laboratories, Burlingame, CA) diluted in 1 mg/mL bovine serum albumin in PBS was then added for one hour and then washed as above. After this step the lanes were incubated with avidin-biotin-complex (Vector Laboratories) using 100 µL of each reagent in 0.3 mol/L NaCl for 30 minutes followed by three washes. Finally, the lanes were developed with 6 µg 4-chloro-1-napthol (Sigma Chemical Co, St Louis, predissolved in 2 mL methanol) diluted in 10 mL PBS containing 5 µL of 30% H2O2 for 15 minutes followed by repeated washings.

RESULTS

Generation of MoAb BM-1. Hybridoma clone BM-1 was produced by the fusion of mouse myeloma NS-1 cells and Balb/c splenocytes obtained from a mouse hyperimmunized with nuclei from human peripheral blood mononuclear cells. Isotypic analysis revealed that MoAb BM-1 is of the IgG, heavy chain subclass. BM-1 was initially identified by indirect immunofluorescence techniques with the use of
Table 1. Reactivity of BM-1 With Human Myeloid Leukemia Cell Lines by Indirect Immunofluorescence

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>BM-1*</th>
</tr>
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<tbody>
<tr>
<td>HEL92.1 (erythroid)</td>
<td>---†</td>
</tr>
<tr>
<td>K562 (erythroid-CML)</td>
<td>---</td>
</tr>
<tr>
<td>HL-60 (promyelocytic)</td>
<td>+ 5% cytoplasmic, 5%-20% nuclear</td>
</tr>
<tr>
<td>KG1 (myeloid)</td>
<td>5% nuclear</td>
</tr>
<tr>
<td>ML-2 (myeloid)</td>
<td>+</td>
</tr>
<tr>
<td>TPH-1-0 (monocytic)</td>
<td>+ 10% nuclear</td>
</tr>
</tbody>
</table>

*Fixed-cell indirect immunofluorescence assay.
†Data expressed as (−) negative; (+) positive.

Indirect immunofluorescence studies with established human tumor cell lines. Indirect immunofluorescence studies revealed that BM-1 was negative with all T, B, and undifferentiated lymphomas and leukemias, myeloma cell lines, and Epstein-Barr virus (EBV)-transformed lymphoblastoid cultures. In addition, BM-1 was negative with 30 solid tumor cell lines derived from a wide variety of human tumor types. As shown in Table 1, BM-1 stained a small percentage of HL-60, ML-2, KG1, and TPH-1-0 cells. The paraformaldehyde-acetone-fixed cell preparations from peripheral blood.

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![Fig 1](image_url)

Fig 1. Immunoperoxidase staining reactivity of MoAb BM-1 on B5-fixed, paraffin-embedded tissue and bone marrow aspirate clot sections. (Counterstained with hematoxylin). (A) BM-1 staining of M1 AML bone marrow aspirate clot preparation showing no reactivity with leukemia population. A BM-1 positive cell in upper right hand corner may be a residual, normal, myeloid precursor cell and acts as an internal positive control for this specimen (magnification ×936). (B) BM-1 staining of M2 AML bone marrow aspirate clot section showing strong reactivity of BM-1 with leukemic cells (magnification ×836). (C) BM-1 staining of CML bone marrow clot section showing strong positive staining of the majority of leukemic cells (magnification ×793). (D) BM-1 staining of granulocytic sarcoma biopsy showing strong cytoplasmic staining of malignant cells (magnification ×790). (E) BM-1 staining of adult thymus revealing antigen-positive cells at the periphery of the cortex (magnification ×124). (F) BM-1 staining of fetal thymus again revealing BM-1 positivity at the peripheral cortex and in the interlobular spaces (magnification ×122). (G) BM-1 staining of fetal liver identifying sites of early myelopoiesis at the portal areas (magnification ×266). (H) BM-1 staining of normal adult bone marrow showing selective staining of myeloid precursor cells around fat globules (magnification ×720). (I) BM-1 staining of adult kidney with a granulocytic infiltrate in the glomerulus (magnification ×403).
Immunoperoxidase studies using B5-fixed, paraffin-embedded tissues from human leukemias, lymphomas, and normal biopsies. The immunoperoxidase staining reactivity of BM-1 on B5-fixed, paraffin-embedded clot preparations from acute and chronic leukemias and multiple myelomas is presented in Table 2. In the acute leukemias 8/10 cases with M2 and M3 subtypes were positive, while the other subtypes were negatively stained. All five cases of CML were strongly positive, while those derived from lymphoid cells were uniformly negative. Acute lymphocytic leukemia (ALL) and myeloma were also negative. The staining reactivities of BM-1 with M1 and M2 AML and CML cases are shown in Fig 1A, B, and C respectively. From these studies BM-1 was found to stain the cytoplasm of the leukemia cells, and only minor staining of the nucleus was noted in some cases.

In Table 3 the reactivity of BM-1 on human lymphomas and related diseases is shown. All 32 cases of Hodgkin’s and histiocytic lymphoma; DHL, diffuse histiocytic lymphoma. The immunoperoxidase staining reactivity of BM-1 with normal biopsies.

**Table 3. Reactivity of BM-1 With Human Lymphomas and Related Diseases in B5-Fixed, Paraffin-Embedded Tissues**

<table>
<thead>
<tr>
<th>Lymphoma* or Disease</th>
<th>BM-1</th>
</tr>
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<tbody>
<tr>
<td>WDLL</td>
<td>0/2†</td>
</tr>
<tr>
<td>PDL</td>
<td>0/4</td>
</tr>
<tr>
<td>Mixed</td>
<td>0/4</td>
</tr>
<tr>
<td>DHL</td>
<td>0/6</td>
</tr>
<tr>
<td>Undifferentiated (Burkitt’s)</td>
<td>0/1</td>
</tr>
<tr>
<td>T cell</td>
<td>0/2</td>
</tr>
<tr>
<td>Mycosis fungoides</td>
<td>0/2</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>0/11</td>
</tr>
<tr>
<td>CML in spleen</td>
<td>1/1</td>
</tr>
<tr>
<td>Gaucher’s disease</td>
<td>0/1</td>
</tr>
<tr>
<td>Granulocytic sarcoma</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Abbreviations: WDLL, Well-differentiated lymphocytic lymphoma; PDL, poorly-differentiated lymphocytic lymphoma; mixed, mixed lymphocytic and histiocytic lymphoma; DHL, diffuse histiocytic lymphoma.

†Number of positive cases/total number of cases.

**Table 4. DNA-Binding RIAs**

<table>
<thead>
<tr>
<th>MoAb</th>
<th>DNA Cellulose RIA</th>
<th>Solid Phase RIA</th>
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<tbody>
<tr>
<td></td>
<td>Lymphoid Lysate</td>
<td>Myeloid Lysate</td>
</tr>
<tr>
<td></td>
<td>+ SS + DS - DNA + SS + DS</td>
<td>+ DNA + SS + DS + E coli/DNA + PBR322/DNA</td>
</tr>
</tbody>
</table>

**Abbreviations:** + SS, with single-stranded calf thymus DNA; + DS, with double-stranded calf thymus DNA.

*Data expressed as – : background cpm; + : 5× background cpm; ++ : 10× background cpm.

Finally, the immunoreactivity of BM-1 was shown to be variable in tissues using formalin and other nonprecipitating fixatives. Decalcification of bone marrow biopsies also showed variable staining by BM-1, indicating that this step is potentially detrimental to BM-1 reactivity. Cryostat sections of human tissues and leukemic samples, however, showed the same staining intensity and pattern of reactivity as B5-fixed tissue specimens for BM-1.

**Biochemical characterization of the BM-1 antigen.** Routine immunoblotting procedures using myeloid whole-cell lysates failed to identify the mol wt of the antigen recognized by BM-1. However, as shown in Table 4, DNA-binding RIAs using single- and double-stranded calf thymus DNA and a cellular lysate prepared from CML cells showed that BM-1 recognizes a DNA-binding protein. Lysates from nonmyeloid sources produced negative results, as did a panel of 30 antinuclear MoAbs generated in our laboratory (data not shown). For comparison, myeloid-specific antinuclear MoAbs 825-18, 882-17, and 888-54 and NS-1 control supernatants were run in parallel with BM-1 and showed no positivity in this assay with the CML lysate. DNA from prokaryotic sources also failed to support BM-1 binding in the presence of the CML lysate. Identical results were obtained using both DNA-cellulose and solid-phase RIAs. These data strongly support the interpretation that BM-1 identifies a DNA-binding protein whose active antigenic site is present when bound to eukaryotic DNA. As shown sche-
Fig 2. Diagrammatic sketch of DNA-cellulose RIA offering one interpretation of experimental results. In this assay myeloid cell lysates were reacted with single- or double-stranded eukaryotic DNA bound to cellulose beads followed by BM-1 binding. A 125I-goat antimouse IgG secondary antiserum was then used to quantitate the amount of BM-1 bound to the DNA-cellulose beads. Upon binding of the BM-1 antigen to the DNA, the antigenic site becomes accessible, possibly by a conformational change in the molecular structure as shown here. Free p183 in solution is not in the right conformation and is therefore not able to bind to BM-1.

Table 5. Characteristics of MoAb BM-1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Immunogen</td>
<td>Peripheral blood mononuclear cell nucleus</td>
</tr>
<tr>
<td>Isotype</td>
<td>IgG</td>
</tr>
<tr>
<td>Antigen</td>
<td>183-kD DNA-binding protein</td>
</tr>
<tr>
<td>Antigen site</td>
<td>Cytoplasm and nucleus</td>
</tr>
<tr>
<td>Bone marrow reactivity</td>
<td>Myeloid precursors</td>
</tr>
<tr>
<td>Lymphoid reactivity</td>
<td>None</td>
</tr>
<tr>
<td>Adult and fetal tissue reactivity</td>
<td>Tissue granulocytes; scattered cells in peripheral cortex and interlobular septae of adult and fetal thymus; portal regions of fetal liver (18 weeks).</td>
</tr>
<tr>
<td>Tumor specificity</td>
<td>M2, M3, AML, CML and CMMOL; granulocytic sarcoma</td>
</tr>
</tbody>
</table>
MONOCLONAL ANTIBODY BM-1

1129

human tissues and leukemic samples have been confirmed in cryostat sections, signifying that the staining reactivity of BM-1 in paraffin sections is consistent with that of other methods of tissue preparation. Because of its ability to stain paraffin-embedded tissue sections and its apparent specificity for myeloid leukemias, BM-1 may have diagnostic potential in the identification of granulocytic sarcomas. As discussed by Whitcomb et al, the diagnosis of granulocytic sarcoma may be difficult and may be confused with lymphoid malignancies. A specific marker, such as BM-1, for myeloid-derived tumors that retains its reactivity in routine pathologic specimens would be an important new tool for the diagnostician.

The second major finding is that BM-1 appears to recognize a novel myeloid-specific maturation antigen that is not expressed in other human tissues or malignancies. Because of its cytoplasmic and nuclear localization, its ability to stain paraffin-embedded tissues, and its DNA-binding properties, the 183-kD BM-1 antigen is readily distinguishable from other reported myeloid-associated antigens. Earlier studies by Goldberger et al and Briggs et al have revealed the presence of myeloid-associated—nonhistone nuclear antigens using polyclonal antibodies to dehistonized chromatin preparations. Likewise Whittingham et al identified a granulocytic nuclear antigen using sera from patients with rheumatoid arthritis and autoimmune, chronic, active hepatitis. More recently our own laboratory reported on the characterization of three MoAbs to myeloid-specific nuclear antigens and showed that these antigens were expressed during different periods of cell maturation after induction of myeloid leukemia cell lines with 1,25 dehydroxyvitamin D3 and dimethylsulfoxide. Finally, Fitz-Gibbon et al have used MoAbs to identify cytoplasmic antigens associated with granules expressed in myeloid cells. All of these studies indicate that myeloid-specific antigens exist not only on the cell surface but also within the cytoplasm and nucleus and that these antigens may be useful in characterizing myeloid maturation. Nuclear antigens, in particular, may be associated with DNA sequences or RNA processing within the nucleus required for the expression of myeloid-specific antigens. In this context the BM-1 antigen, which has been shown to have DNA-binding properties, is worthy of further study, and its mode of action within the nucleus may represent an important model of cellular differentiation.

The third major result of this study is that BM-1 appears to be restricted in its immunoreactivity to M2 and M3 AML. Although other combinations of MoAbs have been shown to have subset specificity, BM-1 alone has this specificity among the acute leukemias. Our study therefore appears to provide additional proof for the biological basis of the FAB classification. As suggested by Browman et al, Drexler and Minowada, and Neame et al and as recently reviewed by Crocker and Burnett, cytochemistry and immunophenotyping greatly improve the diagnostic utility of the FAB classification of AML. With the addition of the BM-1 MoAb, further improvements in typing AML may be possible.

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