Myelomonocytic Myeloma Cell Line (LB 84-1)

By B.G.M. Durie, T.M. Grogan, C. Spier, E. Vela, V. Baum, M.A. Rodriquez, and Y. Frutiger

A human cell line (LB 84-1) has been established from the bone marrow of a patient with Bence-Jones myeloma. Coexpression of plasma cell (Leu 17[CD38]) and myelomonocytic antigens (Leu M1[CD15], Leu MS [CD11c], MY7 [CD13] plus butyrate and chloracetate esterase) proved to be an unusual but sustained feature of this cell line. The plasma cell phenotype with multinuclearity was retained. Shared major chromosomal abnormalities (del [5] [p14], t[5:7] [q35:7], del [6] [q21], and del 7[q32]) between the direct and cell line karyotypes affirmed the LB 84-1 cell as being derived from the original patient myeloma clone. The mechanisms potentially responsible for the aberrant coexpressed phenotype are discussed. This myelomonocytic myeloma cell line will hopefully prove to be a valuable tool for the study of the genotypic and phenotypic evolution of human myeloma.

CASE REPORT

A 45-year-old woman presented in January 1983 with bone pain and a constellation of abnormal laboratory findings, subsequently shown to be associated with multiple myeloma. The skeletal survey showed multiple lytic bone lesions, and the bone marrow showed 80% plasma cells. The serum contained an IgA monoclonal protein, and the urine contained 240 mg/24h of κ Bence-Jones proteinuria. Treatment with a Southwest Oncology Group protocol, using VMCP-VBAP,18 produced >75% regression by June 1983. In March 1984, however, she relapsed and was found to have flagrantly progressive multiple myeloma, with multiple new lytic bone lesions. The hemoglobin was 9.7 g/dL, hematocrit 29.3%, WBC 2,900/μL, with an unremarkable differential. No circulating plasmablasts nor myeloblasts were noted. The platelet count was 100,000/μL. The serum β2 microglobulin was 8.7 mg/L. The total serum protein level was 5.9 g/dL, with the serum albumin being 4.5 g/dL. There was only a tiny IgA monoclonal spike remaining in the serum at a level of 124 mg/dL. The total IgG level was low at 270 g/dL, and the IgM level was low at 25 mg/dL. However, massive Bence-Jones proteinuria had developed with 9.1 g/24 h of protein in the urine, which was 87% free κ light chains. A type of Bence-Jones myeloma escape phenomenon had therefore occurred.

With appropriate informed consent, bone marrow aspiration and biopsy were performed. The bone marrow contained 62% plasma cells. The tritiated thymidine labelling index of bone marrow plasma cells was 4%. The morphology of the plasma cells in the direct specimen, is illustrated in Panel A, of Fig 1. Multinucleated plasma cells (with as many as three to four nuclei/cell) were prominent. Of note, this tendency to multinuclearity was a feature of the subsequently established cell line. Heparinized aliquots of the direct bone marrow aspirate were used for a variety of special studies, including phenotyping, cytogenetic analysis, and in vitro culture studies. The direct chromosome analyses were very abnormal. Figure 2 shows a representative karyotype from the 27
metaphases prepared. Alternate chemotherapy was initiated, but despite several approaches rapid disease progression occurred, and the patient died 2 months later, in May 1984 (total survival 16 months).

MATERIALS AND METHODS

With appropriate informed consent, approximately 96 million bone marrow cells were obtained in the heparinized aspirate material and used for the culture studies. After initial hypaque-ficoll centrifugation, the bone marrow plasma cells were set up in Falcon culture flasks at a concentration of $5 \times 10^5$ cells/mL of culture medium. The standard culture medium used was RPMI 1640 medium, supplemented with 15% fetal calf serum, 100 INU/mL of penicillin, and 50 $\mu$g/mL of streptomycin. The medium was changed approximately twice weekly. The Falcon flask cultures were established using specially prepared M3 medium as previously reported. After 5 weeks in culture, during which time serial aliquots were used for ongoing evaluation and studies, good sustained culture growth was obtained from which serial aliquots could then subsequently be frozen for detailed analyses. Serial passaging with regular freezing of aliquots has been possible up to the present time. There has been remarkable stability in the karyotypic and other features of the established cell line with serial passaging.

Cytogenetic studies. Cells from the original bone marrow were suspended in 5 mL of Fresh RPMI 1640 medium (GIBCO, Grand Island, NY) containing 15% fetal calf serum, L-glutamine, penicillin, and streptomycin for 24 hours. Colcemid (GIBCO, Lawrence, Mass) (10 $\mu$g/mL) was added for the last one hour of incubation, with cells harvested and banded as previously described. Cyto- genetic nomenclature used to describe the results followed the recommendations of the International system for Human Cytogenetic Nomenclature (ISCN). When the origin of marker chromosomes could not be clearly identified, they were designated as unidentified markers.

Immunoglobulin gene rearrangement analysis. High molecular weight DNA was extracted from a frozen aliquot of myeloma cell culture material by using quainium diethyranate buffer and standard methods. DNA was digested with HindIII, Eco R1, or BamH1, size-fractionated on 0.6% agarose gels, and transferred by Southern blotting to nitrocellulose. Cloned genomic DNA fragments containing the immunoglobulin heavy-chain joining region (JH) light chain constant region (CA) or $\kappa$ light-chain constant region (Cx), kindly provided by Dr Philip Leder, were labelled with deoxyctydine triphosphate (dCTP) [\alpha$^32$P] by nick translation to an activity of at least $10^8$ cpm/$\mu$g. After hybridization with the probes, the filters were washed under conditions of high stringency, and autoradiography was performed.

Transmission electron microscopy. The myeloma cells from direct and cultured material were initially fixed in suspension for one hour at room temperature with 1.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) and then spun in conical plastic tubes at 2,500 rpm for ten minutes in a Sorvall (Dupont, Wilmington, DE) GLC-1 swinging-bucket table-top centrifuge. The resulting pellets were fixed with 3% glutaraldehyde in 0.1 mol/L phosphate for one hour at 4°C and then postfixed for 1½ hours in 1% OsO$_4$ in a 0.1 mol/L phosphate buffer. The pellets were dehydrated through a graded series of ethanol and embedded in Spurr’s Electron Microscopy Sciences, Ft. Washington, PA) low-viscosity epoxy resin. One-micron sections were cut and stained with toluidine blue and examined with a light microscope. Thin sections were cut with a DuPont diamond knife using a DuPont/Sorvall MT-5000 ultramicrotome and mounted on uncoated 200-mesh copper grids. Ultrathin sections were stained with a lead citrate and uranyl acetate and examined with a Hitachi (Hitachi Scientific Instruments, Mt View, CA) HU-12 electron microscope.

Cytchemistry. The myeloma cells were evaluated for acid phosphatase and $\beta$-glucuronidase ($\beta$-gluc) using routine methods as previously published. Immunologic marker studies. Cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) slide preparations of the myeloma cell material were used for immunologic evaluation. Biotin-avidin conjugation and horseradish peroxidase labeling with diaminobenzidine tetrahydrochloride (DAB) as the detection agent were used, as previously reported. Briefly, cytocentrifuge slides were fixed in acetone at 4°C for ten minutes, air-dried at room temperature, and then hydrated in phosphate-buffered saline (PBS) at pH 7.4 for ten minutes. After a second PBS wash, a second-stage reagent, biotin-
conjugated Fab₂ goat anti-mouse IgG (Tago, Burlingame, CA) was added, followed by a 15-minute incubation and a three-minute PBS wash. The cytocentrifuge slides were next incubated with avidin D conjugated with horseradish peroxidase (Vector Laboratories, Norwalk, CN). After another 15-minute incubation period, slides were again washed twice with PBS. Next, each slide was incubated with a DAB solution consisting of 3 mg DAB/mL PBS, along with 0.010 mL of 30% H₂O₂. After the five-minute incubation in DAB solution, slides were washed in PBS for three minutes and rinsed in distilled water. Slides were incubated in a copper sulfate solution for five minutes, rinsed with PBS, dipped in distilled water, and counterstained with methylene blue for five minutes, followed by a brief 100% ethanol dip, two changes of xylene, and coverslipping with Permount (Fisher Scientific, Fair Lawn, NJ).

Both B- and T-cell lymphoid antibodies, myeloid/monocytic antibodies, and antibodies assessing the activation and proliferative status of the cells in question were employed as the primary antibodies. The mouse monoclonal antibodies used (with International workshop Cluster group designations in parenthesis) included the following. To detect immunoglobulins, κ and λ for light chains, μ, γ, α (α₁ and α₂), and δ for JH (Becton Dickinson, Mountain View, CA); to detect B-antigens: B1 (CD20), B4 (CD19) (Coulter Immunology, Hialeah, FL), Leu 12 (CD19), Leu 14 (CD22), Leu 16 (CD20) (Becton Dickinson); B2 (CD3, CD21, Coulter Immunology); CR1 (complement receptor, CD21, Becton Dickinson); plasma cell antigens: PCA-1 (Coulter); PC-1 (kindly supplied by Drs Kenneth Anderson and Lee Nadler, Dana Farber Institute, Boston, MA); Leu 17 (CD38) (Becton Dickinson). For T-antigen detection, the following were used: Leu 1 (CD5), Leu 2 (CD8), Leu 3 (CD4), Leu 4 (CD3), Leu 5 (CD2), Leu 6 (CD1), Leu 7, Leu 9, (CD7), and Leu 11b (CD16) (Becton Dickinson). For myeloid/monocytic antibody detection, the following were used: Leu M1 (CD15), Leu M3, (CD14), Leu M5 (CD11c) (Becton Dickinson), and MY-7 (CD13) (Coulter Immunology). Other antibodies used included IL-2 (interleukin-2, CD25), HLA-DR (Ia), TRF (transferrin receptor), CALLA (CD10), Leu 8, Leu 15 (CR3, CD11), HPCA-1 (human progenitor cell antigen) (Becton Dickinson), Ki-67 (proliferative antigen), DRC-1 (dendritic reticulum cell), and LC (leukocyte common antigen (CD45) (Dakopatts, Copenhagen, Denmark). A negative control slide, substituting mouse ascitic fluid (Bethesda Research Laboratories, Gaithersburg, MD) for the primary antibody was included with each test battery. Reactive lymph nodes were used as positive control specimens.

**Flow cytometry.** Using an indirect immunofluorescence technique (IF), mouse monoclonal antibodies were used to study the cells, by a previously described technique.⁵ The cultured cells were washed in RPMI medium and then stained with trypan blue to determine viability. They were washed and resuspended in PBS (pH 7.4). The antibodies used were the same as for the immunoperoxidase studies, except for Leu 6, Leu 7, and Tdt. The cells were incubated with the antibodies for 30 minutes at 4°C in the dark, washed with PBS, the PBS aspirated, and the secondary goat antimouse IgG-fluorescein isothiocyanate (FITC) applied. This incubated for 30 minutes at 4°C in the dark. After washing in cold
PBS twice, the cells were kept on ice in the dark and immediately analyzed. The cells were not fixed. The cells were analyzed using a Coulter EPICS V flow cytometer (EPICS Division, Coulter Immunology) using the standard technique.11 The data were analyzed by the Immunology program of the MDADS (Multiparameter Data Acquisition and Display System, EPICS Division, Coulter Immunology) computer. The coplot was created by using multiple 1 parameter histograms on the EASY 88 (Extended Analysis System with Intel 80/88 Microprocessor, EPICS Division, Coulter Immunology) computer system (Fig 3).

RESULTS

Cell culture morphology, cytochemistry, and electron microscopy. Figure 1 B shows the morphology of the plasma cells obtained from the early weeks of culture. It can be seen that the plasma cell morphology with multinuclearity is remarkably similar in the original (A) and culture material (B). At the electron microscopic level the cells had features of immature B cells plus double membrane bound mitochondria seen typically in steroid secretory cells, and certainly unusual for plasma or myeloma cells.

The plasma cell phenotype of the LB 84-1 cell line has been retained with multiple cell passages. The tritiated thymidine labeling index of the LB 84-1 line is consistently high (41% to 49%) with a generation time (Tc) of 34.2 hours by the BrDu sister chromatin exchange method as previously reported.1

Karyotype analysis. Figure 2A shows a representative metaphase from the direct marrow material, and Fig 2B shows the common structural abnormalities found in the LB 84-1 cell line material. The symbols indicate the presence of five specific structural abnormalities found in both the direct and cell line material.

Table 1 provides a complete summary of the karyotypic findings in the direct specimen and cell line. The most striking feature was the consistent finding of identical cytogenetic abnormalities in the direct chromosome testing, and the review of 18 karyotypes from the cell line cultures. Specific numeric abnormalities found in both samples were the loss of the X chromosome as well as chromosomes 17 (CD38) positivity, a striking and unexpected feature was the consistent finding of identical cytogenetic abnormalities found in both direct and cell line material. The most striking feature was the 7q-chromosome abnormality, which we have previously documented in association with enhanced expression of P-glycoprotein.22 Using the specific C21 probe, and in immunocytochemical technique, the significant expression of P-glycoprotein was documented in this cell line LB 84-1, associated with the 7q-chromosome abnormality.22

Drug sensitivity testing. The cell line material was tested for sensitivity or resistance to a variety of chemotherapeutic and biologic agents. Consistent with the increased level of P-glycoprotein detected by the immunocytochemical method, the cells were resistant to exposure to adriamycin at a level similar to that we previously documented in 8226 myeloma cells tenfold resistant to adriamycin (R10).23 As we have noted for the 8226 R10 line made tenfold resistant to adriamycin, there was unexpected or paradoxical sensitivity to TNFα, a2b interferon, and the glucocorticoids dexamethasone and methylprednisolone.24

Immunotyping studies. The complete immunotyping analysis from the cell culture material is summarized in Table 2. Antigens positive in the direct marrow are also identified. In addition to the B1, B4, PCA-1, PC-1, and Leu 17 (CD38) positivity, a striking and unexpected feature was the myelomonocytic phenotype detected, including positivity for butyrate esterase and chloracetate esterase, Leu M1 (CD15), Leu M5 (CD11c), and leucocyte common antigen (CD45), in addition to some Ia antigen (HAL-DR) positivity. Both the direct marrow and the LB 84-1 cell line demonstrated very weak positivity for IgA heavy chain (κ). The direct bone marrow material was also positive for κ light chain, whereas the cell line was negative for κ but weakly positive for λ. There was no secretion of IgA, κ, or λ by the cell line.

To further evaluate the document the coexpression of myeloma or B-cell associated antigens and the myelomonocytic antigens, flow cytometric analysis was carried out (Fig 3). It can be seen that there was consistent coexpression of plasma cell (PC 99% and PCA-1 100%) and myelomonocytic antigens (MY-7 99% and Leu M1 99%). Multiparameter analysis is shown in the preceding report4 (Figs 2 and 3 and text of that manuscript).

DNA studies. Analysis of immunoglobulin gene rearrangement in DNA from the LB 84-1 cell line showed clear
Table 1. Clonal Cytogenetic Abnormalities in Direct and Cell Line Material (LB 84-1)

<table>
<thead>
<tr>
<th>Numeric</th>
<th>Structural</th>
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<tbody>
<tr>
<td>Direct</td>
<td>Cell Line</td>
</tr>
<tr>
<td>- X</td>
<td>del (1) (p36)</td>
</tr>
<tr>
<td>- 2</td>
<td>t(1;?) (p36)</td>
</tr>
<tr>
<td>- 4</td>
<td>t(2;?) (p25;?)</td>
</tr>
<tr>
<td>- 8</td>
<td>del (3) (q22)</td>
</tr>
<tr>
<td>- 9</td>
<td>dup (3) (q26 q29)</td>
</tr>
<tr>
<td>- 10</td>
<td>del (5) (p14)</td>
</tr>
<tr>
<td>+ 11</td>
<td>t(5;?) (q35;?)</td>
</tr>
<tr>
<td>- 13</td>
<td>del (6) (q21)</td>
</tr>
<tr>
<td>- 14*</td>
<td>del (7) (q32)</td>
</tr>
<tr>
<td>- 16</td>
<td>+ 21</td>
</tr>
<tr>
<td>- 17</td>
<td>t (14;?) (q32;?)</td>
</tr>
<tr>
<td>+ 18</td>
<td>del (6) (q21)</td>
</tr>
<tr>
<td>- 21</td>
<td>ring</td>
</tr>
<tr>
<td>- 22</td>
<td>del (7) (q31)</td>
</tr>
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</table>

Hypodiploid AA 22 metaphases evaluated

Hyperdiploid AA 55 metaphases evaluated

Abbreviation: AA, all metaphases abnormal.
Note: All abnormalities in bold type found in common between direct and cell line material.
*Clonal loss of chromosome 14 in both direct and cell line material.

Studies for Epstein-Barr virus (EBV). The direct and cell line material was negative for EBV by both DNA (Southern) techniques and by in situ hybridization. Cell line LB 84-1 was probed for the presence of EBV, with an EB viral DNA repeating sequence, IR-V, a 3.1 Kb BAM-HI restricted internal repeating sequence of B95-8 EBV, cloned into pBR322 (probe kindly supplied by J. Pagano, NC), as well as a second EBV probe, pDH3, a HindIII-Bgl II fragment (positions 166.481-169-033) of B95-8 EBV cloned into pAT53 (probe kindly supplied by P. Farrell, Cambridge, England). The commercially biotin-labelled EB viral probe (PATHO-GENE, BY ENZO Biochemicals, New York City) was probed for the presence of EBV in LB 84-1 cell line material. Both alleles of chromosome 14 were deleted in most karyotypes.

Table 2. Hybrid Immunophenotype of Myelomonocytic Myeloma Cell Line (LB 84-1)

<table>
<thead>
<tr>
<th>B/Plasma Cell Antigens</th>
<th>Myelomonocytic Myeloma</th>
<th>Leu 3 (CD4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 (CD20)</td>
<td>Leu M1 (CD 15)</td>
<td></td>
</tr>
<tr>
<td>B2 (CD19)</td>
<td>Leu M3</td>
<td>HLA-DR (Ia)</td>
</tr>
<tr>
<td></td>
<td>Leu M5 (CD 11c)</td>
<td></td>
</tr>
<tr>
<td>PCA-1*</td>
<td>MY7 (CD13)</td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>Butyrate esterase†‡</td>
<td>Ki-67†</td>
</tr>
<tr>
<td>Leu 17 (CD 38)*</td>
<td>Chloracetate esterase*: receptor (TRF)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucocyte common (CD 45)*</td>
<td></td>
</tr>
</tbody>
</table>

Note: >90% of cells in LB 84-1 cell line positive for all antigens listed; see description of antigens in materials and methods.
*Also positive in direct bone marrow material. Other antigens not tested initially and unfortunately no more material available.
†Not done on direct material, but tritiated thymidine LI% = 4%.
‡Corresponds to peroxidase positivity.

Fig 4. This shows the DNA from several myeloma cell lines, following EcoRI digestion and probed with the 3.4 kilobase (KB) fragment of Cl. Size markers and the T3M4 cell line with the germ line configuration are shown for comparison. There is clear rearrangement of the Cl for LB 84-1, as well as for LB 84-5 and LB 84-2, compared to germ line (T3M4).
Other ancillary studies. A number of other aspects of cell line LB 84-1 are currently being investigated in detail. No abnormality of the first c-myc exon has been detected (Pvu II site).

DISCUSSION

We have documented the establishment of an unusual myeloma cell line with coexpression of plasma cell and myelomonocytic antigens. This myelomonocytic myeloma line corresponds to the phenotype demonstrated in the preceding report which details the results of 16 patients with myelomonocytic myeloma. In an additional manuscript, aberrant coexpression of T-cell antigens is also described. Myeloid or myelomonocytic coantigen expression in plasma cells has not been previously well documented. Nonetheless, there has been a puzzling association between multiple myeloma and a variety of myeloid disorders of the bone marrow, including acute myelogenous leukemia, polycytemia rubra vera, chronic neutrophilic leukemia, and megakaryoblastic leukemia. In no instance has it been clearly documented that the myeloid and the myeloma disease processes were derived from the same original stem cell compartment. The parallel or sequential association of these disease processes remains unexplained. However, both in this current case and in the others reported separately, there was not evidence of a discrete myeloid process, but rather emergence of a clone coexpressing myelomonocytic and plasma cell antigens.

The striking feature illustrated by cell line LB 84-1 was therefore the persistent coexpression of both the plasma cell and myeloid antigens (Fig 3). The detailed studies with the cell line material have allowed investigation of possible mechanisms involved. Of major interest is the complex karyotype including the loss of chromosome 14 from both the direct and cell culture karyotypes. This corresponded to the loss of the whole molecule immunoglobulin synthesis in the patient and also by the cultured cells. It also accounted for the inability to demonstrate any J_H rearrangement. This illustrates a possible mechanism for the process of “Bence-Jones escape,” which has been documented both in patients and in animal model systems. The occurrence of the five major chromosomal abnormalities (Fig 2) in both the direct bone marrow material, and in the LB 84-1 cell line, affirms beyond reasonable doubt that the cell line is indeed derived from the predominant myelomonocytic myeloma clone present in the original bone marrow. In addition, the striking myelomonocytic phenotype occurred in both the direct material and in the cell line with serial passaging. Coexpression of the plasma cell and the myelomonocytic antigens in the majority (> 90%) of the cells is a characteristic feature. The puzzling aspect is the more capricious expression of the immunoglobulin phenotype. Both the direct and the cell line material expressed IgA weakly in a minor component of the cells corresponding to minimal IgA secretion in the patient at the time of flagrant relapse. It should be noted that with further progression of the relapse, this IgA essentially disappeared from the serum. The expression of k light chain is more problematic in that the direct material did indeed show k expression, which could not be detected in the LB 84-1 cell line. Whether this lack of expression of k in vitro represents a geneotypic or phenotypic event is unclear. Lambda (\(\lambda\)) expression in vitro corresponded to \(\lambda\) chain rearrangement documented at the DNA level (Fig 4). As recent studies have shown, the translational and post-translational regulation of both light chain and heavy chain synthesis and secretion are extremely complex and susceptible to mutational change or modulation. It is not hard to envisage some derangement in the k synthesis and secretion in the sub clone that resulted in the LB 84-1 cell line. In separate studies, induction of a myelomonocytic phenotype in the 8,226 myeloma cell line with increased expression of the \(\lambda\) phenotype. Obviously further molecular studies are necessary to clarify this type of process.

Why the deregulated plasma cells in this particular instance also expressed myelomonocytic antigens remains unexplained. A recent linear theory of hematopoietic cell differentiation may account for the aberrant myelomonocytic antigens in already committed cells. In this theory, which is supported by in vitro and clinical data, there is sequential commitment during hematopoietic differentiation, first to megakaryocytic differentiation, followed by erythrocytic, then neutrophilic and monocytic differentiation, before lineage commitment to B-cell development. This theory therefore incorporates the concept that cells that end up with a B-cell commitment have undergone prior gene organization or rearrangement such that they could be committed to myeloid, erythroid, or megakaryocytic development. If this is true, it could account for the aberrant coexpression of these phenotypes in deregulated malignant plasma cells. As noted above, we have recently documented myelomonocytic co-antigen expression in the myeloma cell line 8226 (\(\lambda\) Bence Jones), following exposure to 1,25-dihydroxyvitamin D_3, which provides an additional model system to study the mechanisms involved.

The coexpression of P-glycoprotein is of importance in evaluating the mechanisms of drug-resistance. The cell line is resistant in a pattern similar to that achieved with the 8226 myeloma cell line made resistant to adriamycin by in vitro exposure to adriamycin for a 10-month period. The spontaneous occurrence of such drug resistance in a patient who received adriamycin in vivo provides an opportunity to study the natural evolution of this type of drug resistance process. The pharmacology and genetics of this process are being further studied.

The established cell line LB 84-1 therefore provides a unique tool for the evaluation of various genetic processes that occur in association with disease progression and drug resistance in the evolution of advanced multiple myeloma. A variety of very complex processes are involved, including changes in immunoglobulin genes, myeloid genes, and genes associated with the acquisition of drug resistance. Hopefully more detailed studies can provide insights into the mechanisms involved.
REFERENCES


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