Ultrastructural, Cell Membrane, and Cytogenetic Characteristics of B-Cell Leukemia, a Murine Model of Chronic Lymphocytic Leukemia

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ABSTRACT

A murine model of a spontaneous, transplantable BALB/c B-cell leukemia (BCL) is described. Extreme leukemia and splenomegaly develop in H-2d-compatible recipients of tumor cells. Tumor cells are medium to large lymphocytes that can be transformed to plasmacytoid cells following in vitro stimulation with lipopolysaccharide. Karyotypic analysis of transformed tumor cells reveals 36 chromosomes with several monosomes and 7 marker chromosomes. The ultrastructure of the tumor cells was studied using transmission and scanning electron microscopy. Although the appearance of tumor cells seems normal by morphological criteria, an impaired capping ability was documented using the fluorescein-conjugated concanavalin A-binding test. Impaired capping ability was documented before leukemia was overt as early as 1 to 3 days following inoculation of tumor cells. The B-cell leukemia (BCL) provides a useful murine model for the study of various aspects of human bone marrow-derived malignant disorders.

INTRODUCTION

BCL, a spontaneous B-cell leukemia originally described in 1978 by Slavin and Strober (22) in a 2-year-old female BALB/cKa (H-2d) mouse. The original tumor bearing mouse had high leukocyte counts (up to 200,000 lymphocytes/cu mm) and marked splenomegaly. Leukemic cells resembled human CLL cells, and uniform mature, medium to large lymphocytes were found in the spleen and peripheral blood. Positive cell surface determinants included monomeric IgM and IgD with monoclonal lambda light chain and a single idiotype, Fc receptors, H-2d alloantigens including H-2D and H-2K, and la antigens encoded by the E subregion (13, 22, 24, 27). Tumor cells expressed in vitro to LPS, a typical B-cell mitogen, with marked proliferation (22) and secretion of monoclonal IgM isotype antibodies. No BCL-specific cytogenetic markers were described in inbred mice are of the T-cell lineage (20). Although B-lymphocyte tumors have been reported in mice, these neoplasms were induced experimentally, using carcinogens or Abelson virus (1, 4, 9, 18). To the best of our knowledge, BCL is the first animal model of a spontaneous CLL-like B-cell tumor. Since most human lymphocytic lymphomas and leukemias appear to derive from the B-cell lineage (15), the BCL, may provide a useful animal model for the study of normal and neoplastic characteristics of B-cell immunobiology and B-cell tumor therapy. In the present report, we have further characterized the tumor cell ultrastructure, growth kinetics, and biology including studies on cell membrane characteristics and BCL-specific cytogenetic markers.

MATERIALS AND METHODS

Mice. Inbred male or female BALB/c (H-2d), C57BL/6 (H-2b), BALB/c × C57BL/6 F1 (H-2d/b), and C3H/He (H-2<sup>a</sup>/<sup>R</sup>) mice, 6 to 8 weeks old, were used throughout this study. All mice were kept in conventional animal facilities.

BCL, Tumor Maintenance. The BCL tumor was passaged and maintained in vivo by i.v. inoculation of 5 × 10<sup>6</sup> PBL obtained from leukemic mice into syngeneic BALB/c recipients.

Evaluation of Leukemogenesis. The appearance of leukemia was monitored by weekly PBL counts and evaluation of splenomegaly. Peripheral blood obtained from the retroorbital veins of tumor-bearing mice was collected in heparinized capillaries and diluted in 2% acetic acid. The leukocyte count was determined using a hemacytometer. Mice were sacrificed at different time intervals following BCL inoculation for kinetic evaluation of spleen size by weight and cell content.

Light Microscopy, Histochemistry. The following stains were used for cytocentrifuge preparations and smears of the leukemic cells: oil red O (3), Sudan black, peroxidase, periodic acid-Schiff (10), acid phosphatase (2), nonspecific esterase (a-naphthyl acetate method with and without fluoride inhibition), AS-D chloroacetate esterase (26), β-glucuronidase (7), and muramidase (lysozyme) (11).

Transmission Electron Microscopy. Cell pellets (6 to 8 × 10<sup>6</sup> cells) were fixed with cacodylate-buffered 1.25% glutaraldehyde (pH 7.3, 310 mosmol/l) for at least 1 hr at room temperature, then prepared for scanning electron microscopy by critical point drying using Freon 13 as described in earlier studies (17). The specimens were then coated with a thin layer of gold:palladium (1:1), critical point dried using Freon 13 as described in earlier studies (17).

Scanning Electron Microscopy. Six million cells were fixed in suspension in 1% phosphate-buffered glutaraldehyde (pH 7.3, 310 mosmol/l) for at least 1 hr at room temperature and then collected onto glass coverslips covered by poly-L-lysine as described by Sanders et al. (19). Coverslips with monolayers of cells were fixed for 1 hr longer at room temperature, then prepared for scanning electron microscopy by further dehydration in a graded series of ethanol, embedded in low-viscosity epoxy resin embedding medium according to the method of Spurr (23), and sectioned with a MT-2 Porter Blum microtome equipped with a diamond knife. Thin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and viewed with a Phillips EM-300 electron microscope.

Scanning Electron Microscopy. Six million cells were fixed in suspension in 1% phosphate-buffered glutaraldehyde (pH 7.3, 310 mosmol/l) for at least 1 hr at room temperature and then collected onto glass coverslips covered by poly-L-lysine as described in earlier studies (17). The specimens were then coated with a thin layer of gold:palladium and examined with a Jeol SM-35× scanning electron microscope.
Differential counts were performed from random areas on 500 cells. Cells were classified according to the presence of surface ridges and ruffles, microvilli, and blebs.

**Assay for Binding of F-Con A.** Peripheral blood was collected into heparinized glass tubes. Spleen cells were obtained by cutting spleen tissue into small pieces and teasing the cells into 0.1 M PBS, pH 7.2. Lymphocyte suspensions were layered on a Ficoll-Hypaque gradient (5). Contaminating erythrocytes were lysed with ammonium chloride, and the cells were washed twice in PBS.

F-Con A was prepared at a fluorescein:protein molar ratio of 1.86 (Miles-Yeda, Rehovot, Israel). From 1 to 2 x 10^6 cells were incubated with F-Con A at 100 µg/ml for 30 min at 37°C. The cells were washed in PBS, and the percentage of F-Con A capping was determined after counting a total of 300 to 400 cells using a Zeiss microscope with UV light source. Only single cells and small clumps (2 to 5 cells) were counted in order to determine the percentage of caps.

**Preparation of Cells for Cytogenetic Studies.** Spleen cells for cytogenetic analysis were obtained by teasing spleen fragments through a steel mesh into PBS. Cells were washed twice in F-10 medium (Grand Island Biological Co., Grand Island, N. Y.) and put into 0.075 M KCI for 30 min. In some instances, Colcemid (0.05 µg/ml) was added to the KCl solution simultaneously with the addition of the spleen cells.

Harvesting for chromosomal analysis was done by hypotonic treatment with 0.075 M KCl and fixation with methanol:acetic acid (3:1) using standard procedures. Chromosome preparations were done on wet slides and dried for a few sec on a hot plate (63°C). For chromosome counts, cells were stained with 3% Giemsa's stain (Gurr improved R66) in phosphate buffer, pH 6.8. Chromosome G-banding was performed in trypsin using a modification of the technique described by Klinger (12). Chromosomes were arranged according to the standard mouse karyotype (6). Marker analysis and specific breakpoints were determined using the nomenclature of Nessbitt and Francke (16).

**RESULTS**

**Morphology.** Light microscopy showed that many of these cells resembled normal large and medium-sized lymphocytes. Some of them had a more delicate nuclear chromatin, and nucleoli were evident. Most cells had a high nuclear:cytoplasmic ratio and a rim of basophilic cytoplasm. After incubation with LPS, cells appeared larger, and some had plasmacytoid features with more abundant basophilic cytoplasm.

**Transmission Electron Microscopy.** Typical ultrastructural features of lymphocytes were seen (Fig. 1). Most cells had nuclei with margined heterochromatin and evenly distributed euchromatin. In some cells, nuclear pockets and nucleoli were clearly evident, particularly after stimulation with LPS. The cytoplasm showed polyribosomes, some short strands of rough endoplasmic reticulum, and relatively large numbers of mitochondria. The mitochondria, which were frequently clustered together were large and often showed "ballooning" of the matrix with disruption of the cristae. This finding may, however, be due in part to fixation artifact. After LPS stimulation, a small proportion of the larger cells, estimated to be between 5 to 10% of the overall population showed plasmacytoid features with a well-developed Golgi apparatus and rough endoplasmic reticulum. These cells had eccentric nuclei with more delicate euchromatin and nucleoli (Fig. 2).

Scanning electron microscopy revealed that the cells were spherical, with varying numbers of short microvilli. Most cells had moderate to markedly villous surfaces and lacked ridge-like profiles and ruffles (Fig. 3).

**Cytochemical stains of unstimulated BCL1 cells including periodic acid-Schiff, Sudan black, peroxidase esterases, β-glucuronidase, acid phosphatase, and muramidase were negative.**

**Kinetic Growth of BCL1 Cells in the Blood.** Leukemia developed in all BALB/c mice inoculated with as few as 10 BCL1 cells. The kinetics of leukemia development in the peripheral blood as monitored by serial venous aspirates was clearly cell dose dependent as demonstrated in Chart 1. Maximal peripheral blood counts reached 540 x 10^6/cu mm. Kinetics of leukemia development was uniform in all groups receiving a similar BCL1 inoculum.

**Kinetic Development of BCL1 in the Spleen.** A significant increase in spleen weight occurred as early as 3 days following inoculation of 10^7 cells, before any peripheral leukemic infiltration was apparent (0.89 ± 0.01 (S.D.) as compared to 0.14 ± 0.01 g in normal mice). The spleen size has occasionally increased up to 50-fold reaching a cell content of up to 5 x 10^9 cells.

**Hematological Status of Leukemic Mice.** The hematological status of BCL1-inoculated mice revealed progressive anemia and thrombocytopenia concomitantly with the development of advanced leukemia. Anemia became more severe in long-term survivors as documented in Table 1. No overt tendency to bleeding or infections could be noted during the early stages of BCL1. Spontaneous bleeding (rectal) was occasionally noted during terminal stages.

**Histocompatibility-related Resistance to Leukemogenesis.** BCL1 tumor cells developed originally in BALB/c mice maintain surface H-2^a alloantigens including H-2D, H-2K, and la antigen (data not shown). Semiallogeneic BALB/c x C57BL/6 F1 (H-2^ab) mice were somewhat more resistant to BCL1 inoculation and showed a protracted disease course. Nevertheless, all F1 recipients of 10^7 tumor cells developed a typical leukemia as shown in Fig. 3. Completely allogeneic mouse strains (C57BL/6-H-2^k; C3H/HeJ-H-2^a) uniformly rejected the histoincompatible H-2^a tumor cells (Chart 2).

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**Table 1**

<table>
<thead>
<tr>
<th>Time interval following BCL1 inoculation</th>
<th>WBC/cu mm</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Platelets/cu mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>6,220 ± 3,360⁴</td>
<td>14.8 ± 1.9</td>
<td>708,800 ± 13,830</td>
</tr>
<tr>
<td>15 days</td>
<td>7,460 ± 1,053</td>
<td>15.8 ± 1.8</td>
<td>808,000 ± 212,683</td>
</tr>
<tr>
<td>30 days</td>
<td>73,800 ± 27,869</td>
<td>10.2 ± 0.9</td>
<td>224,000 ± 57,037</td>
</tr>
<tr>
<td>112 days</td>
<td>189,667 ± 144,223</td>
<td>7.0 ± 1.0</td>
<td>264,000 ± 108,166</td>
</tr>
</tbody>
</table>

⁴ Mean ± S.D.
Karyotypic Analysis of BCL\textsubscript{1}. Chromosomal analysis of spontaneous and LPS-induced mitoses were carried out in cultured whole blood, PBL, and spleen cells. No spontaneously dividing cells were found in the peripheral blood. Seventy-four metaphase plates were counted following in vitro stimulation of Ficoll-Hypaque-purified PBL with LPS. Five cells had 40 chromosomes, 62 cells had 36 chromosomes, 6 cells had 35 chromosomes including one robertsonian translocation, and one cell had only 34 chromosomes. Spontaneous mitoses were easily demonstrated in the spleen. Altogether, 139 cells were counted of which 23 had 40 chromosomes, 99 cells had 36 chromosomes, 11 cells had 35 chromosomes, and 6 cells had only 34 chromosomes. These results indicate that the majority of the cells representing the BCL\textsubscript{1} tumor cells consisted of 36 chromosomes. Only 27 chromosomes of the BCL\textsubscript{1} cells showed banding patterns corresponding to the normal banded mouse karyotype. Seven marker chromosomes were defined in addition to 2 deleted chromosomes, Nos. 14 and 15. A detailed analysis of the karyotype was published separately (25). A few residual normal diploid cells were also present.

**DISCUSSION**

The present investigations further characterize the spontaneous BALB/c B-cell leukemia which we described previously (22). The cytological features of the BCL\textsubscript{1} cells are essentially the same as those seen in human disorders of well-differentiated lymphocytic lymphoma and CLL. The main features of the BCL\textsubscript{1} include leukemia and splenomegaly with lesser involvement of lymph nodes, bone marrow, and other visceral organs (21). The neoplastic cells are mature, relatively large with a moderate amount of cytoplasmas. The morphology, the tissue distribution, and the immunological features of the tumor cells (high density of surface IgM, low density of surface IgD, and response to LPS) (14) all suggest that the BCL\textsubscript{1} represent a clonal expansion of early B-cells. In that respect, the BCL\textsubscript{1} disease closely resembles that of a subset of human CLL described by Galton et al. (8) as prolymphocytic leukemia. This group of patients in general shows a much more malignant type of CLL with marked splenomegaly and extremely high PBL counts. Interestingly, splenectomy seems to have a positive therapeutic effect in both the BCL\textsubscript{1} disease and in prolymphocytic CLL (14, 25).

The BCL\textsubscript{1} tumor cells appear to be unique in their vigorous response to in vitro stimulation with LPS. We have demonstrated that, following LPS stimulation, the BCL\textsubscript{1} cells undergo further proliferation and differentiation into larger cells that show plasmacytoid features with an eccentric immature nucleus and well-developed endoplasmic reticulum (Fig. 2).

The proliferative response of BCL\textsubscript{1} cells to LPS was also used for the study of cytogenetic characteristics of tumor cells. Chromosomal deletions and 7 defined marker chromosomes were found in most cells studied. Although the appearance of the BCL\textsubscript{1} cell surface seems to be normal by transmission and scanning electron microscopy, we have characterized some defects in the capping properties of the cell membrane using F-Con A. The appearance of lymphocytes with reduced cap-forming ability in the blood as early as 1 to 3 days after inoculation of tumor cells is not fully understood. However, it was previously shown that lymphocytes from patients with Hodgkin's and non-Hodgkin's lympho-
mas and CLL exhibited reduced cap-forming ability with F-Con A in the early stages of the disease and also during remission even though malignant cells were undetectable in the blood (28). Cell membrane defects may partially account for the unique homing patterns of B-cell tumors. We have previously demonstrated that BCL, cells home predominantly to the spleen (25), and others have reported that these tumor cells do not enter the thoracic duct circulation (14).

The BCL, tumor described here may serve as a model for studying the immunology and immunopathology of human well-differentiated lymphocytic lymphoma-leukemia variants. As a cell type of a particular clone with limited heterogeneity, the BCL, seems to be useful for biological and chemical analysis of various products associated with resting, proliferative, and secretory events following LPS stimulation. Also, the presence of a tumor-specific IgM\(\lambda\) idotype on the surface of BCL, cells makes it a useful model for studying new therapeutic modalities using the idiotypic marker as a target for selective immunotherapy with idotype-specific antibodies.

REFERENCES


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Fig. 1. Electron micrograph of leukemic lymphocytes showing cells with ample cytoplasm containing ribosomes and large mitochondria, which are at times clustered together with disruption of cristae. A few short strands of endoplasmic reticulum are seen. × 9,200.

Fig. 2. Larger leukemic cell from LPS-stimulated culture with eccentric immature nucleus and ample cytoplasm showing ribosomes and relatively well-developed endoplasmic reticulum. The cell has plasmacytoid features. × 7,600. A, Details of endoplasmic reticulum. × 10,860.

Fig. 3. Scanning electron microscopic photographs of spherical leukemic cells showing typical surface features, i.e., varying numbers of relatively short microvilli. × 8,700.
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