

# Establishment of a New Epstein-Barr Virus-Immortalized Cell Line From Chronic Lymphocytic Leukemia With Trisomy of Chromosome 12 That Produces Monoclonal IgM Against a Sheep RBC Antigen

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Leukemia cells from a patient with chronic lymphocytic leukemia (CLL) were found to bind sheep RBC (SRBC) through their monoclonal surface IgM. A lymphoblastoid cell line was obtained by immortalization of leukemic cells with Epstein-Barr virus (EBV). Cultured leukemic cells were found to have a supernumerary chromosome 12, an

abnormality typical of CLL of the B cell type. To our knowledge, this is the first EBV-immortalized cell line from B-CLL cells of known SRBC specificity and the third reported CLL cell line carrying trisomy of chromosome 12. © 1988 by Grune & Stratton, Inc.

**C**HRONIC LYMPHOCYTIC LEUKEMIA (CLL) B cells are, in general, highly refractory to transformation by Epstein-Barr virus (EBV),<sup>1</sup> and therefore, relatively few EBV-immortalized cell lines originating from CLL cells have been described.<sup>1-4</sup> In this paper we report the establishment of a novel EBV-transformed B cell line from the leukemic cells of a CLL patient that expressed surface  $\mu$ k immunoglobulin. A distinctive feature of these leukemic cells, which makes this cell line unique, is that their surface IgM reacted with a sheep RBC (SRBC) antigen. Specific binding of SRBC was maintained by the EBV-transformed cells, which indicated their leukemic origin. This was further confirmed by karyotypic analysis showing that the immortalized cells carried trisomy of chromosome 12, a cytogenetic abnormality typical of CLL of the B cell type.<sup>5</sup>

## MATERIALS AND METHODS

**Patient history.** The patient was a 56-year-old female who presented with fatigue, hepatosplenomegaly, and moderately enlarged axillary lymph nodes. Her hemoglobin concentration was 10 g/dL, and the platelet count was 210,000/ $\mu$ L. Her WBC count was 32,000/ $\mu$ L, with 8% neutrophils and 92% morphologically mature small lymphocytes.

A blood sample, which was used for phenotyping and for EBV transformation, was sent to our laboratory from the referring hospital before chemotherapy was begun.

**EBV transformation.** The patient's mononuclear cells were isolated from peripheral blood onto a Ficoll-Isopaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) according to standard procedures. The cells were incubated at  $2 \times 10^6$ /mL in RPMI 1640 (Flow Laboratories, McLean, VA) containing 20% fetal calf serum (Flow) and antibiotics. To obtain immortalization of leukemic B cells by EBV, the cultures were supplemented with 30% (final concentration) of the supernatant from the B95-8 cell line, which contained highly transforming EBV (kindly provided by Dr G. Ragona, Rome). After 24 hours of incubation at 37°C in 5% CO<sub>2</sub>, the cells were washed and cultured under the same conditions in EBV-free medium. Transformation was evident after ten days by the appearance of proliferating blastlike cells growing in large clumps.

**Cell phenotyping.** Phenotypic characterization of fresh and EBV-immortalized leukemic cells was performed by immunofluorescence. Surface immunoglobulin heavy and light chains were detected by commercial fluorescein-labeled goat monospecific antibodies (Nordic Immunology Tilburg, The Netherlands). Monoclonal antibody (MoAb) phenotyping, performed by indirect immunofluorescence as previously described<sup>6</sup> included OKT3, OKT4, OKT8, OKT10, OKT11, OKB2, (Ortho Pharmaceutical Corp, Raritan, NJ), and Leu-1 (Becton Dickinson, Sunnyvale, CA) MoAbs.

Rosette formation with erythrocytes from sheep or from different

animal sources was detected by a standard assay.<sup>6</sup> For sheep rosette-forming cell (SRFC) inhibition experiments,  $10^6$  cells were suspended in 100  $\mu$ L undiluted goat antisera to IgM, IgG, or IgA (specific antibody concentrations: 1.7, 2.6, and 3.3 mg/mL, respectively) (Kallestadt Laboratories, Austin, TX), incubated for one hour at 4°C, washed, and assayed for sheep rosette formation.

**In vitro immunoglobulin secretion.** Secretion of immunoglobulins into the culture supernatant was assayed by a sensitive enzyme-linked immunoassay (ELISA) performed as previously described.<sup>6</sup> Five-day culture supernatants were assayed both directly and after adsorption with an equal volume of sheep or rabbit erythrocytes (90 minutes at 4°C). EBV-immortalized B cell lines from normal individuals served as controls. The secretion of anti-SRBC IgM by cultured leukemic cells was assayed by a direct hemolytic plaque assay as previously described.<sup>7</sup> In some experiments, the cells were cultured in the presence of phorbol ester (phorbol 12-myristate-13-acetate, 0.01 to 1  $\mu$ g/mL) (Sigma Chemical Co, St Louis) for 48 hours before the plaque assay.<sup>8</sup>

**Cytogenetic investigations.** Exponentially growing cells were incubated for 3 hours in Colcemid (GIBCO, Grand Island, NY; 8 ng/mL) and then washed, incubated in hypotonic buffer, and fixed. Chromosomes were differentially stained by the QFQ-banding technique.

## RESULTS

The patient's fresh leukemic cells had a phenotype typical of B lymphocytes (ie, surface IgM $\kappa$ , B2-positive). However, the formation of SRBC rosettes prompted a further evaluation of T cell markers. This analysis confirmed the B cell nature of the leukemic cells and the absence of the T cell surface receptor for SRBC (detected with the T11 MoAb) (Table 1). Thus, the binding of SRBC was most likely due to the antibody activity of surface IgM.

To be able to perform an extensive analysis of these

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**Table 1. Phenotype of Fresh and EBV-Immortalized Leukemic Cells**

|             | Fresh Cells | Cell Line |
|-------------|-------------|-----------|
| SRFC (37°C) | 87          | 55        |
| IgG         | 2           | 1         |
| IgA         | ND          | 0         |
| IgM         | 84          | 68        |
| IgD         | ND          | 0         |
| $\kappa$    | 85          | 54        |
| $\lambda$   | 0           | 0         |
| B2          | 82          | 0         |
| T3          | 5           | 0         |
| T4          | 4           | ND        |
| T8          | 2           | ND        |
| T10         | ND          | 0         |
| T11         | 3           | 0         |
| Leu-1       | ND          | 0         |

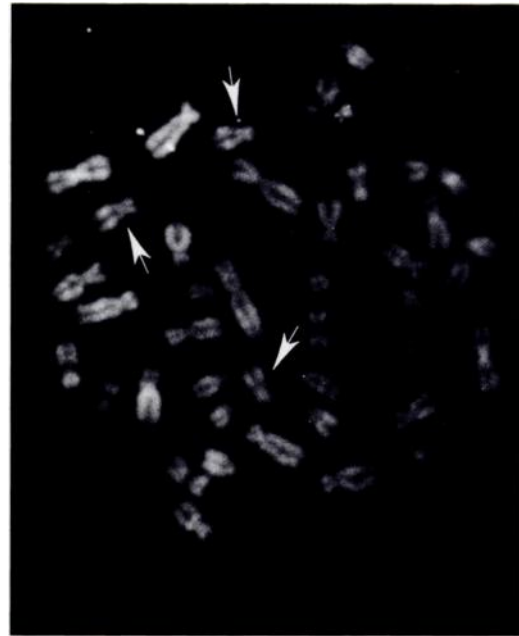
Abbreviations: ND, not determined.

leukemic cells, we successfully attempted to generate an EBV-transformed cell line from them. After transformation and stabilization for continuous *in vitro* growth of the patient's peripheral blood B cells, cultured lymphoblastoid cells were shown to retain the property of binding SRBC (50% to 80% SRFC in different experiments). Similar results were obtained when SRFC assays were performed at +4°C or at +37°C. No rosettes were formed with chicken, horse, or rabbit erythrocytes under the same experimental conditions. The percentage of cells binding SRBC was reduced from 53% to 24% by preincubation of the cells with a monospecific antiserum to human IgM but not with antisera against IgG or IgA, thus confirming that sheep rosette formation was due to surface IgM.

The phenotype of fresh leukemic cells differed from that of EBV-immortalized cells in that the B2 antigen was no longer detectable at the cell surface in the latter (Table 1). This finding is not surprising. In fact, despite the fact that only B-2 positive B cells can be immortalized by EBV<sup>9</sup> (the B2 molecule appears to correspond to the receptor for EBV),<sup>10</sup> about half of the normal and neoplastic EBV-transformed lymphoblastoid cell lines are B2-positive<sup>11</sup>; furthermore, among these, only a fraction of the cells bear the B2 antigen.<sup>11</sup> This discrepancy is most likely explained by the fact that EBV transformation itself inhibits the expression of this molecule.

Evidence for the leukemic origin of the EBV-transformed cell line was also provided by the presence of trisomy 12 (Fig 1), the most common chromosomal abnormality in B-CLL.<sup>5</sup> All the 73 metaphases whose Q-banded karyotypes were reconstructed showed a supernumerary chromosome no. 12. Out of these, 41 were 47,XX,12+, whereas the remaining 32 showed a reduced chromosome number ranging from 46 to 41. The more represented class (21 metaphases) was characterized by 46 chromosomes with random chromosome losses, preferentially involving small-sized chromosomes as expected.

The EBV-transformed leukemic cells secreted significant amounts of IgM in the culture medium. These IgM appeared to be SRBC specific because they were almost completely adsorbed by sheep but not rabbit RBC (Table 2). Converse-



**Fig 1. A QFQ-banded metaphase from a B-CLL cell line. Arrows indicate chromosomes no. 12.**

ly, we were unable to demonstrate the formation of direct hemolytic plaques, even after preincubation with phorbol ester; incubation with phorbol ester also failed to induce the acquisition of plasmacytoid features such as intracytoplasmic Ig<sup>12</sup> (data not shown). The most likely explanation for the discrepancy between the ELISA and the plaque-forming cell data is that the amount of IgM secreted by each cultured cell was too small to generate a direct hemolytic plaque under our experimental conditions.

## DISCUSSION

B cell tumors bearing surface immunoglobulin directed against SRBC are not exceedingly rare because several such cases have been reported in the literature.<sup>6,13-15</sup> It should be noted that the patient described here is distinct from similar cases previously reported by us.<sup>7,13</sup> A cell line derived from a lymphoma producing surface IgM to SRBC has also been described,<sup>15</sup> but these cells could be maintained only by *in vivo* passaging in nude mice. To our knowledge, this is the first lymphoblastoid cell line derived from B-CLL cells of

**Table 2. In Vitro Immunoglobulin Biosynthesis by EBV-Immortalized Leukemic Cells**

|          | IgM*       |       |       | IgG*       |      |      |
|----------|------------|-------|-------|------------|------|------|
|          | Unadsorbed | SRBC  | RRBC  | Unadsorbed | SRBC | RRBC |
| EBV-CLL† | 839        | 71    | 845   | 3          | 0    | 2    |
| LCL-73‡  | 6,750      | 6,430 | 5,980 | 49         | ND   | ND   |
| LCL-74‡  | 5,200      | ND    | ND    | 31         | ND   | ND   |
| LCL-PL1‡ | 5,650      | ND    | ND    | 15         | ND   | ND   |

Abbreviation: ND, not determined.

\*Nanograms per milliliters of culture supernatant, unadsorbed or adsorbed with sheep or rabbit (RRBC) erythrocytes.

†EBV-immortalized leukemic cells.

‡Normal EBV-immortalized lymphoblastoid cell line.

known antigen specificity. The availability of cell lines (Tsutsumi et al<sup>15</sup> and this report) or hybridomas<sup>14</sup> derived from these tumors might prove useful for the molecular characterization of the relevant SRBC antigens and, in turn, to explore whether they produce immunoglobulins reacting with a common SRBC determinant. In fact, it has been shown that several murine lymphomas of the CH series bind SRBC through surface immunoglobulins with common specificity for phosphatidyl choline.<sup>16</sup>

We have previously shown that the interaction of the antigen with surface immunoglobulin affected the *in vitro* differentiation of fresh CLL cells with SRBC specificity.<sup>7</sup> Thus, these cells might help to further investigate the antigen-induced modulation of leukemic cells.

At the cytogenetic analysis, all the 73 metaphases examined had trisomy 12, which confirmed their virtually uniform leukemic origin. Very few EBV-immortalized B-CLL cell lines have been reported thus far due to the remarkable refractoriness of CLL B cells to transformation by this virus.<sup>14</sup> In particular, only two other EBV-transformed continuous cell lines have been established, to our knowledge, from B-CLL cells carrying trisomy 12.<sup>2,3</sup> This chromosomal abnormality is, by far, the most common finding in CLL of the B cell type.<sup>5</sup> Additional lymphoblastoid cell lines have been established from the blood of a patient with B-CLL by Levitt et al.<sup>17</sup> These lines arose spontaneously (ie, without the addition of exogenous EBV), were EBV nuclear antigen-positive, and displayed some interesting features. The latter included unusual morphology, phagocytic activity, and chromosome abnormalities (including 14q+) without trisomy 12. However, the relationship of these cells to the leukemic cells remains unclear. Interestingly, trisomy 12 has also been

observed in two EBV-transformed cell lines established from B prolymphocytic leukemia cells.<sup>18,19</sup> These findings emphasize the significance of this abnormality in leukemogenesis as well as the relationship between B-CLL and prolymphocytic leukemia.

The factor(s) responsible for neoplastic transformation in B-CLL are presently unknown. It has been suggested that an autocrine mechanism might support the abnormal proliferation of B-CLL cells.<sup>4</sup> However, the significance of trisomy 12 is not understood. In this respect, our finding that the random loss of chromosomes observed in cultured B-CLL cells never involved chromosome 12 suggests that trisomy of this chromosome might confer a selective growth advantage. It is possible that a gene-dosage effect leads to increased or dysregulated expression of a chromosome 12-encoded oncogene, which results in the development of this type of neoplasia.<sup>20</sup> Increased expression of known cellular oncogenes has been observed in several tumors or cell lines derived from them, apparently as the result of gene amplification.<sup>21-23</sup> No known oncogenes have been mapped thus far on human chromosome 12.<sup>24</sup> Thus, the availability of this and other leukemic cell lines carrying trisomy 12 may provide suitable material for attempting to identify, eg, by subtractive hybridization<sup>25</sup> with chromosomally normal EBV-transformed B cell lines, a still unknown oncogene encoded on chromosome 12, possibly located at the region q13-q22.<sup>26</sup>

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