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Characterization of a New Non-Hodgkin's Lymphoma Cell Line (NCEB-1) With a Chromosomal (11:14) Translocation [t(11:14)(q13;q32)]

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A new cell line, NCEB-1, was established by Epstein-Barr virus (EBV) transformation of peripheral blood mononuclear cells from a patient with centroblastic-centrocytic diffuse lymphoma expressing IgM λ. The transformed cells were lymphoblastoid, with many cells showing a plasmacytoid morphology. The NCEB-1 cells had cytoplasmic Ig (CyIg), with loss of the surface Ig (SIg) expression. Cytogenetic analysis of the cell line demonstrated two clones with variations: a hypodiploid clone, with a complex karyotype including a t(11;14)(q13;q32) similar to the original tumor cells, and a near tetraploid clone with the same markers. Southern blot analysis of DNA from the patient's neoplastic cells and NCEB-1 demonstrated identical Ig heavy chain gene rearrangement, confirming the origin of the cell line. The cell line was not tumorigenic when tested in an in vitro assay using immunosuppressed mice. NCEB-1 has been in continuous culture for 9 months and will be valuable for the in vivo study of non-Hodgkin's lymphoma and EBV transformation.

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CYTOGENETIC analysis of tumors from patients with non-Hodgkin's lymphoma has revealed nonrandom chromosome abnormalities, some of which are related to the histologic subgroups.[1-3] In particular, the different types of 14q+ chromosomes show different distributions. The t(14;18)(q32;q21) is found in about 85% of follicular lymphomas. The t(8;14)(q24;q32) or its variants are found in virtually all Burkitt's lymphomas and many other high-grade lymphomas. A t(11;14)(q13;q32), found in 4% of low- to intermediate-grade lymphomas, is associated with small-cell lymphocytic lymphoma, although it has been reported in a variety of other lymphomas and chronic lymphocytic leukemia.[2,4] The oncogenes *c-myc*, *bcl*I, and *bcl*II, located on chromosomes 8, 11, and 18, respectively, have been shown to be translocated to the Ig heavy chain region on chromosome 14 in these tumors.[4-7] The t(11;14)(q23;q32) has been described in lymphoma and appears to be associated with high-grade tumors.[8,9]

Further studies on low- to intermediate-grade lymphomas are restricted because of the difficulty in establishing permanent cell lines comparable to those derived from high-grade tumors such as Burkitt's lymphoma.[10] The Epstein-Barr virus (EBV) has been used to produce cell lines from patients with chronic lymphocytic leukemia (CLL) and follicular lymphomas; the neoplastic origin of the EBV-transformed cells was confirmed using cytogenetic and molecular markers.[11,12] We have established a new EBV-transformed lymphoma cell line with an (11;14)(q13;q32) chromosome translocation. Analysis of the Ig heavy chain gene rearrangement confirmed that the cell line was derived from the patient's neoplastic cells. The morphology and the immunophenotype of the cell line suggest that the original lymphoma differentiated during EBV transformation. The cell line was not tumorigenic in immunosuppressed mice; this may have been due to the stage of differentiation.

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MATERIALS AND METHODS

Patient. A 57-year-old male was referred in April 1987 with a WBC count of 568 × 10^9/L with over 95% abnormal lymphocytes. The patient had diffuse palpable lymphadenopathy and hepatosplenomegaly. Bone marrow biopsy showed a diffuse infiltration with small cleaved cells (centrocytes) and large blast cells. A lymph node biopsy showed a diffuse centroblastic-centrocytic lymphoma (Kiel), intermediate grage (WF).[13] Immunophenotyping of the peripheral blood and the lymph node demonstrated light chain restriction (IgMλ). The patient was treated initially with combined chemotherapy with a good response, but returned 4 months later with aggressive disease and died.

Cell culture. Mononuclear cells were separated from peripheral blood by Ficoll-Hypaque (FH) centrifugation and incubated with the supernatant from the EBV-infected marmoset cell line B95-8 for two hours.[14] The cells were washed twice and seeded at 1 × 10^5/well in 96-well microplates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) and cultured in RPMI 1640 culture medium with 20% fetal calf serum (FCS) and 20 mmol/L of L-glutamine. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2. Clumps of cells were present in all wells after 3 weeks. Individual wells were transferred to 24-well plates (Linbro; Flow Labs, Rickmansworth, England), seeded with mouse peritoneal macrophages, and 4 weeks later were transferred to 25-cm^2 tissue culture flasks (Nunclon; InterMed, Denmark) without a feeder layer.

Characterization of the lymphoma and NCEB-1. Blood films were stained with May-Grünwald-Giemsa (MGG). Paraffin sections of the lymph node were stained with hematoxylin and eosin (H&E). Cytospin preparations of the NCEB-1 cell line were stained with MGG, periodic acid-Schiff (PAS), and nonspecific esterase, using α-naphthyl acetate.

Immunophenotyping was done as follows: cell suspensions from the patient and NCEB-1 were analyzed by indirect immunofluorescence performed with murine monoclonal antibodies (MoAb) using an Epics C flow cytometer. Cytocentrifuge preparations and frozen sections of the lymph node biopsy were examined using a standard indirect immunoperoxidase technique.[15] Cells were tested with MoAbs recognizing the cluster of differentiation (CD) antigens.[16] These included the T-cell–associated antigens CD3 (UCHT;SAPU) and CD5 (RFT-1, SAPU); B-cell–associated antigens CD19 (HD-

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37, DAKO), CD 21 (RFB6, SAPU), and CD 23 (MHM6; J. Gordon, Birmingham, England); and the interleukin-2 receptor CD 25 (IL-2R, DAKO). The other antibodies used were anti-DR (L243, ATCC); and anti-Igs anti-IgM FITC (Kallestad), anti-κ, and anti-λ (SAPU). In addition, the lymph node was stained with MoAbs recognizing CD9 (BA2, Hybritech), CALLA, CD10 (J5 Coulter), and OKTIO.[17] The second antibody used in immunofluorescence was a sheep antimouse IgG (Fab) FITC conjugated (Sigma Chemical Co, St Louis) and a rabbit antimouse conjugated to horseradish peroxidase (DAKO) for the immunoperoxidase technique. The Epstein-Barr nuclear antigen (EBNA) was detected using an indirect anticompliment immunofluorescence assay as described by Reedman and Klein.[18] Terminal deoxynucleotidyl transferase (Tdt) was assayed by indirect immunofluorescence (Pharmacia Inc, Piscataway, NJ).

Electron microscopy. Cells were fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.3), dehydrated in ethanol, and embedded in araldite resin. Sections were cut on a LKB V Ultratone (LKB, Cambridge, England), stained with uranyl acetate and lead citrate, and viewed under a Philips EM3000 electron microscope.

DNA Analysis. High molecular weight (mol wt) DNA was extracted by standard techniques. Ten to 15 μg was digested with the restriction enzymes *Bam*HI and *Hind*III, separated on 0.8% agarose gel by electrophoresis, and transferred to Hybond-N (Amersham Corp, Arlington Heights, IL) by the Southern technique.[19] Hybridization with nick-translated 32p-probes was followed by washing with 0.1 × sodium chloride and sodium citrate (SSC) and autoradiography at −70°C. The 2.5-kilobase (kb) *Eco*RI-*Bgl*II fragment of the joining region of the heavy chain gene was used as the JH probe and was a kind gift from T. Rabbits (MCR, Cambridge).[20]

Cytogenetics. The karyotype in the peripheral blood of the patient at the time of diagnosis was established from 12-0-tetradecanoylphorbol-13-acetate (TPA)-stimulated cultures.[21] NCEB-1 cells were set up at 3×10^5/mL and 28 hours later were blocked with 10^{-7} mol/L fluorodeoxyuridine and 4×10^{-6} mol/L uridine. After 17 hours they were released with 10^{-3} mol/L thymidine and cultured for a further 5.5 hours, with 0.1 μg/mL colcemid being added for the last 30 minutes. Standard cytogenetic techniques were used for harvesting and slide making. Metaphase chromosome preparations were G-banded using Wright's stain.[22]

Tumorigenicity assay. Irradiated, thymectomized CBA mice were prepared as described by Morten et al.[23] The mice were injected with 1×10^7 cells bilaterally in the lower groins from exponentially growing cultures. The animals were examined weekly for 12 weeks for tumor formation defined as a progressively growing mass. The acute lymphoblastic leukemia cell line (Reh) was used as a positive control.[24]

RESULTS

Cell culture. Three weeks after incubating the patient's lymphoma cells with EBV, clumps of lymphoblastoid cells were present in all wells. These cells were initially cultured with mouse peritoneal macrophages, and as proliferation increased, the cells were transferred to 25-cm² flasks without feeders. The cell line, designated NCEB-1, grew as typical lymphoblastoid cells in clumps with a doubling time of 72 to 96 hours growing in RPMI 1640 medium with 15% FCS.

Cytology. The cytologic appearance of the patient's lymphoma cells in blood, the histology of the lymph node biopsy, and the cytology of the NCEB-1 cell line are shown in Fig 1. The peripheral blood smear showed a mixture of small cleaved cells (centrocytes) and large blasts (centroblasts), which made up the majority of abnormal cells. The lymph node biopsy demonstrated a diffuse infiltration with small cleaved cells and large blast cells.

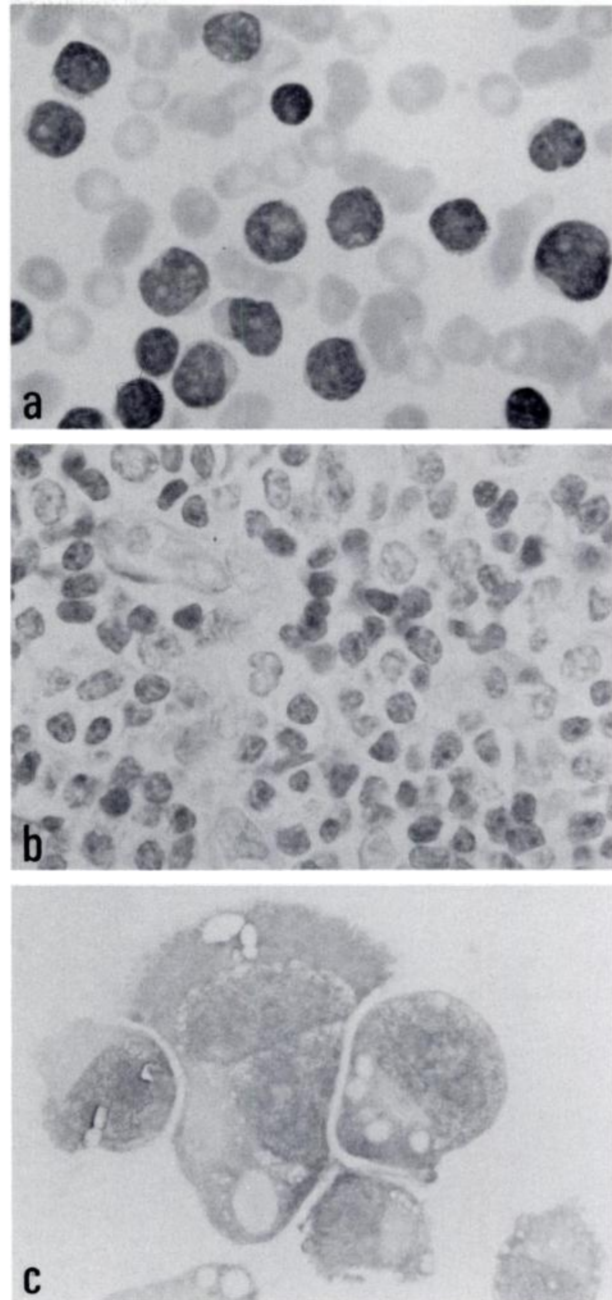

Fig 1. Photomicrographs of patient's lymphoma cells at diagnosis and NCEB-1. (A) peripheral blood smear (MGG). Original magnification ×400). (B) Section of lymph node biopsy (H & E). Original magnification ×200. (C) Cytospin of NCEB-1 (MGG). Original magnification ×900.

The morphology of the cell line was pleomorphic with typical lymphoblasts, plasmacytoid cells, and multinucleated giant cells. The NCEB-1 cells were PAS positive, but α-naphthyl acetate negative. The electron microscopy demonstrated the plasmacytoid morphology in the majority of cells, with abundant rough endoplasmic reticulum (RER)

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and a well-developed Golgi apparatus. Others were more lymphoblastoid with numerous mitochondria (Fig 2).

Immunophenotyping. NCEB-1 cells were strongly positive for EBNA, but were negative for Tdt. The results of the immunophenotyping using immunofluorescence and immunoperoxidase techniques are detailed in Table 1. The patient's cells were strongly positive for IgMλ, HLA-DR, CD5, 19, 21, 23, and 25. In addition to the results shown in Table 1, the lymph node biopsy was negative for CD9, 10, and OKT10.

The EBV-transformed cell line showed the same light chain restriction (IgMλ) as the patient's cells. However,

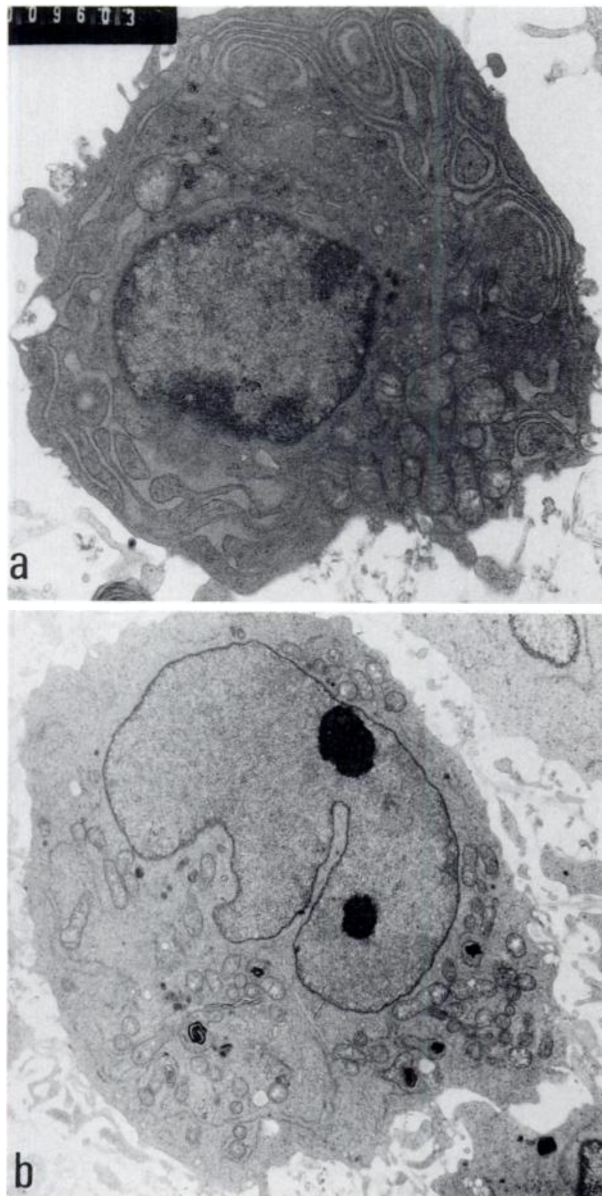

Fig 2. Transmission electron micrographs of NCEB-1. (A) Plasmacytoid cell with abundant rough endoplasmic reticulum. Original magnification ×10,300. (B) A less differentiated cell from the same culture. Original magnification ×4,500.

Table 1. Immunophenotype of the Patient's Lymphoma Cells and the NCEB-1 Cell Line

	Patient		Cell Line	
CD/Ag	Blood Surface (IF%)	Lymph Node (IP%)	Surface (IF%)	Surface and Cytoplasmic (IP%)
IgM	57	90	29	95
K	13	0	0	0
λ	87	90	0	100 cp
DR	95	90	94	95
CD3	0	20	0	0
CD5	26	90	8	20
CD19	NT	90	80	90
CD20	NT	NT	95	NT
CD21	NT	50	75	50
CD23	0	50	70	95
CD25	95	20	10	50

Abbreviations: IF, immunofluorescence; IP, immunoperoxidase; cp, cytoplasmic; NT, not tested.

there was complete loss of SIg, but with strong CyIg expression seen on immunoperoxidase staining. NCEB-1 was positive for HLA-Dr, CD5, 19, 21, 23, and 25.

Chromosome analysis. A total of 25 metaphases from both the patient and the cell line were examined. The NCEB-1 cells were studied after 4 months in culture, and 100% were abnormal with a hypodiploid clone and a near tetraploid clone with variations. The representative karyotypes from the patient's blood and the NCEB-1 cell line are as follows: Patient: 45,X,−Y,−15,−17,t(5;12)(p14;q12), der (8) t(8;?)(p11.2;?),t(9;?;13)(q32;?;q14), der (10) t(10;15)(q24;q15), t(11;14)(q13;q32), + ring, + mar (Fig 3A). NCEB-1 cell line: 43, x,−y,−12,−15,−17, der (5) t(5;12)(p14;q12), der (8) t(8;?)(p11.2;?), t(9;?;13) (q32;?;q14), der (10)t(10;15)(q24;q15), t(11;14)(q13;32), + mar (Fig 3B).

Southern blot. DNA from the patient's peripheral blood and the NCEB-1 cell line were analyzed for Ig gene rearrangement using the heavy chain probe (JH). Figure 4 demonstrates that the tumor cells and NCEB-1 show identical rearrangement using the restriction enzyme *Hin*dIII. The germ line band, which is seen in the placental control, was not detected in the patient or cell line samples.

Tumorigenicity assay. Four irradiated thymectomized mice were each injected subcutaneously in both groins with 1×10^7 NCEB-1 cells. After 12 weeks no tumors were detected. The leukemia cell line (Reh) produced tumors 2 weeks after injection.

DISCUSSION

We have described a new cell line derived from a patient with a diffuse centroblastic-centrocytic lymphoma. The clonal origin of the EBV-transformed cells was demonstrated by the chromosomal markers (Fig 3) and supported by the identical Ig heavy chain gene rearrangements seen in DNA samples from the original tumor and the established cell line (Fig 4). Both heavy chain alleles appeared to be rearranged when compared with the germ line seen in the placental control. Presumably one fragment represents the derivative of the 14q+ chromosome involved in the t(11;14), while the other fragment represents the normal chromosome 14 with

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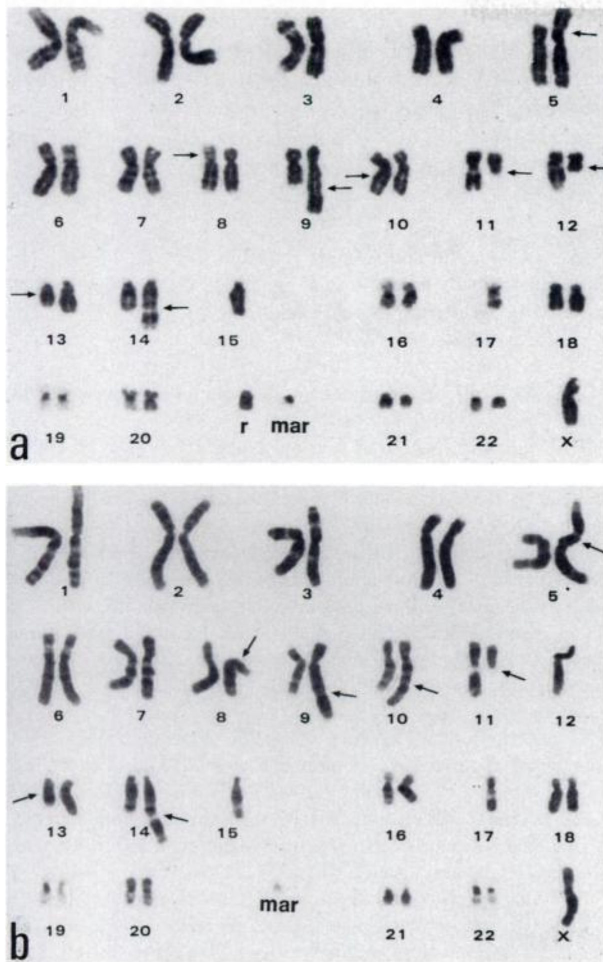

Fig 3. A comparison of the karyotypes from the patient's lymphoma cells (A) and the cell line (B). Arrows denote abnormalities (see text). Mar, marker; r, ring chromosome.

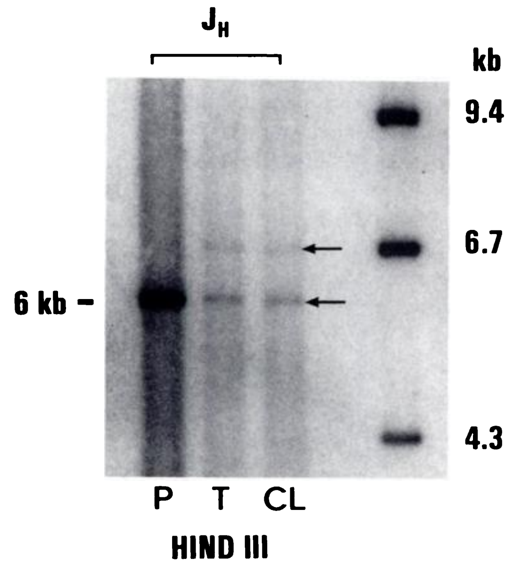

Fig 4. Autoradiograph of Southern blot of Ig genes from the patient's tumor (T) and the cell line (CL). Placental (P) DNA digests were used as a control. The dash marks indicate the germ-line position, and the arrows denote the position of the rearranged alleles. Genomic DNA digested with *Hin*dIII was hybridized to the probe for the joining region of the Ig heavy chain (J_H) gene.

an effectively rearranged V/D/J region, which is responsible for the IgM production.

The success of this EBV transformation was mainly due to the very low proportion of normal B cells in the initial sample. In samples with a larger percentage of normal cells, EBV-transformed normal cells tend to outgrow the abnormal cells.[11] We have recently transformed cells from two patients with CLL. Both cell lines showed clonal chromosomal markers in >50% of cells early in transformation. However, 2 months later, all metaphases examined in both cell lines were normal (unpublished data).

The use of a feeder layer was thought to have promoted the growth of the early lymphoblastoid cells. Many mouse cell lines are infected with type-C viruses. Although we have no evidence that our mouse peritoneal macrophages were infected with a xenotropic virus, human monocytes or conditioned medium should be used instead of murine feeders because of the potential of these viruses to enhance the growth of human cells.[25]

The plasmacytoid morphology and the absence of surface Ig expression suggest that NCEB-1 represents a subset of the patient's tumor cells that had plasmacytoid features, or more likely, NCEB-1 differentiated from the original lymphoma during EBV transformation. Another new cell line, FL-18-EB, recently established by EBV transformation of a follicular lymphoma, showed 15% SIg and 32% CyIg. FL-18, a spontaneous cell line from the same patient, had 75% SIg, while the patient's neoplastic cells weakly expressed SIg.[26] The loss of surface Ig and the development of strong cytoplasmic expression has also been described in a new cell line derived from EBV transformation of blood mononuclear cells from a patient with CLL.[12] Unlike most CLLs, the cells were strongly SIg positive and had a weak expression of CD5. Caligaris-Cappio et al suggested the SIg+, CD5− phenotype may characterize a cell more likely to transform with EBV. There appears to be an association between the ability to transform with EBV and a cell's expression of SIg together with the complement (C3d) receptor, CR-2.[27] Our patient's cells were SIg+, CD21+, but also expressed CD5, which continued to be expressed in the transformed cell line.

Non-Hodgkin's lymphoma cell lines with 11;14 translocations have been described previously, but all appear to involve the breakpoint q23 on chromosome 11. SU-DHL-11 and RC-K8 were derived spontaneously from patients with diffuse histiocytic lymphomas, with the latter showing Ig gene rearrangement.[8,9] Both cell lines, unlike NCEB-1, were tumorigenic in immunosuppressed mice, and cytogenetic analysis demonstrated (11;14)(q23;q32) translocations. The oncogene *c-ets-1* is located on chromosome 11 at band q23-24.[28] Increased expression of c-ets-1 in lymphoid malignancies with translocations involving the breakpoint q23 on chromosome 11 has not been demonstrated. The break at q13 on chromosome 11, which is seen in NCEB-1, involves the *bcl*I oncogene.[4] The translocation of *bcl*I to the Ig heavy

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chain gene region at band q32 on chromosome 14 is thought to deregulate the *bcl*I oncogene and contribute to cellular transformation. Tumorigenicity, as measured by in vitro and in vivo assays, and the involvement of *bcl*I or ets-1 in t(11;14) has not been studied in detail. NCEB-1 was not tumorigenic in our in vivo assay in immunosuppressed mice. This could be related to its slower growth rate in vitro and to the differentiation secondary to EBV transformation. Clinically, lymphomas and CLL with a t(11;14)(q13;32) may have a poor prognosis, as seen in our patient and in recent studies of CLL and intermediate lymphocytic lymphoma.[29]

In summary, we have described a new cell line derived from a patient with a diffuse centroblastic-centrocytic lymphoma. EBV transformation appears to be a valuable method for establishing cell lines from CLLs and lymphomas, but the cellular differentiation that occurs during transformation may affect tumorigenicity.

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