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ESTABLISHMENT AND CHARACTERIZATION OF AN EBNA-NEGATIVE HUMAN LYMPHOMA CELL LINE (BHL-89)

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ABSTRACT

Background. **The aim of the work was to establish human malignant lymphomas in culture, in order to study the biological characteristics and drug sensitivity of lymphomas of human lymphoid origin.**

Materials and Methods. **Lymph nodes of patients were explanted and kept in cultures using conventional tissue culture methods. Cytogenetic methods were used for karyotype analysis. Clonogenic assay was applied to test drug sensitivity. The tumorigenic capacity of the cells was determined by inoculating them into immunosuppressed mice. Immunological and other markers were examined with conventional techniques.**

Results. **A cell line, BHL-89, was established in culture from a patient with B-cell type non-Hodgkin's malignant lymphoma. Cells started to grow after a few days without a feeder layer in stationary suspension. The population doubling time was 48 h. The cells were hyperploid, and non-random aberrations were +1, –15, +14q+. Cloning efficiency in soft agar was found to be as high as 50-60%. The cells expressed markers characteristic of early B cells. The BHL-89 cells were Epstein-Barr nuclear antigen (EBNA) negative. They produced tumors when 10^7 cells were injected into immunosuppressed mice. The cells were sensitive to dibromodulcitol (Elobromol), an alkylating antitumor drug, and resistant to the phorbol ester TPA.**

Conclusions. **The established EBNA-negative BHL-89 cell line has a few unique characteristics, e.g. rapid establishment without feeder cells, origin from the lymph node of an adult patient, high clonogenicity in soft agar, and resistance to TPA. The cell line is suitable for studying the nature of B lymphomas and testing compounds against lymphoproliferative disorders.**

Key words: human B cell line, chromosome markers, EBV, tumorigenicity, drug sensitivity

It is generally known that lymphoblastoid cell lines and Epstein-Barr virus (EBV)-positive lymphomas of the Burkitt-type are readily established in culture,[1,2] while EBV-negative lymphomas are difficult to establish *in vitro*, and such *in vitro* lines are relatively rare.[1,3-10] The EBV-negative lymphoma lines published so far have different characteristics. Most of them have low cloning efficiency in soft agar, at or below 1%, and some of them do not produce tumors in athymic mice. We began a joint study of human lymphomas with clinicians in order to establish and characterize various malignant cell lines of lymphoid origin. We succeeded in establishing several lines that proved to be of the lymphoblastoid type.[11] We describe here the characteristics of a new EBNA-negative human malignant lymphoma line.

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Materials and Methods

The patient was a 56-year-old woman who had a large tumor on the upper arm. The diagnosis of the axillary lymph node tumor was non-Hodgkin's malignant lymphoma of diffuse, small cleaved and large cell type (intermediate-grade lymphoma).

A piece of the lymph node tumor was chopped, rinsed with phosphate balanced salt solution, and small pieces of it were put in test tubes. The firmly attached tissue fragments were then immersed in a medium consisting of RPMI 1640 (GIBCO) plus 15% fetal calf serum (Phylaxia, Budapest). No feeder cells were necessary for establishment. The cells were maintained in glass or plastic flasks (NUNC). Two years after explantation, the RPMI 1640 medium was changed to HAM F-12 (GIBCO). Subculturing was performed twice a week by 1:1 or 1:2 dilution.

Population doubling time (DT) was estimated by counting cells on 4 consecutive days in a hemocytometer. Labelling index was determined by pulse-labelling cells with ^{3}H-thymidine for 10 min, and the ratio of labelled to unlabelled cells was determined using radioautography. Colony formation was determined by suspending 500 or 1000 cells in medium containing 10 μM mercaptoethanol and 0.25% agar (Noble agar, DIFCO) in 50 mm diameter plastic Petri dishes (NUNC). No basal layer was used. Colonies containing at least 50 cells were counted after 3 weeks.[12]

The tumorigenic property of cells was tested as previously described.[13] Briefly, 10^{7} cells were injected subcutaneously into immunosuppressed mice. Immunosuppression was achieved by thymectomy of 6-week-old CBA/Ca mice, ^{60}Co whole body irradiation with a 9.5 Gy lethal dose and syngeneic bone marrow transplantation within 24 h. For chromosome analysis, exponentially growing cells were treated with vinblastine, 5 ng/mL, for 1 h and Giemsa-banded by the acetic-saline-Giemsa (ASG) method. Cytochemical characteristics of BHL-89 cells were studied with a panel of stains as described by Sundström and Nilsson.[14] Immunological markers were examined with our indirect immunofluorescence method as described elsewhere.[15] The B cell-associated reagents were CD10 (J5), CD19 (B4), CD20 (B1) and CD21 (B2) from Coulter Corp., CD22 (RFB4) from Royal Free Hospital (G. Jánossy), sIgM-FITC from Heintel. Other monoclonal antibodies, included CD3 (OKT3), CD4 (OKT4) and CD8 (OKT8) from Ortho (New Jersey, USA), CD14 (MO2), anti-HLA-DR (Becton-Dickinson, Mountain View, USA), CD33 (MY9) from Coulter Corp. The intracytoplasmic CD3 and CD22 stains were also used.[16] For Tdt, rabbit anti-human Tdt reagents (Sera-Lab) with goat anti-rabbit-FITC second reagents (Sera-Lab) were used. Other monoclonals were supplied by the authors shown in the footnote. Expression of the bcl-2 protein by BHL-89 cells was detected by Western blot analysis.[17] BHL-89 cells were suspended in lysis buffer (10.0 mM Tris, pH 7.4, 150.0 mM NaCl, 1% Triton X 100, 1.0 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. The debris was removed by centrifugation at 14,000 g for 30 min. Aliquots of the supernatant containing soluble proteins were analyzed by SDS-PAGE (12% gels) transferred to nitrocellulose filters. The bcl-2 protein was detected by using bcl-2-specific antisera (Dako). The presence of Epstein-Barr virus nuclear antigen (EBNA) in the cells was evaluated by the method of Reedman and Klein.[18] Each smear of cells was thoroughly examined and each percentage value was computed from at least 1000 cells.[19] The drug sensitivity of cells was studied with the antitumor alkylating agent dibromodulcitol (DBD, Elobromol, Mitolactol, NSC-104800, Chinoin Pharmaceutical Factory, Budapest, Hungary), using a clonogenic assay as described previously.[12] The dose at which 50% of the cells survived was the inhibition concentration 50 (IC_{50}). The influence of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) on growth characteristics and on changes in several immunological markers of BHL-89 cells was studied by treating them with doses between 5 ng-1 μg/mL. TPA was obtained from SIGMA.

Results

Cells began to divide and proliferate a few days after explantation as seen by the presence

of mitotic figures in smears. The first subculture was made 3 weeks after explantation. By then cells were in continuous proliferation. Single cells and clumps were present in the suspension cultures. The population doubling time was 4-5 days during the early period following explantation, and later it stabilized at 48 hours. Twenty percent of the cells were labelled with ^{3}H-thymidine, suggesting that the low rate of S phase cells was in accordance with the slow rate of cell proliferation. The cells were round with a large nucleus and a narrow, basophilic cytoplasm (Figure 1). Some cells had 2 nuclei. The chromatin was homogeneously dispersed.

Colony formation of cells in soft agar fluctuated between 20 and 60% during long-term cultivation. Then it stabilized between 50 and 60% in medium containing 2-mercaptoethanol. Subcutaneous inoculation of 10^{7} cells into immunosuppressed mice resulted in a 100% *take* of tumors. The histological picture was similar to that of the patient's tumor, and it was a malignant lymphoma.

The chromosome number was established from 20 counted cells. Of these, 12 cells contained 47 chromosomes (modal number). Clonal aberrations were studied in 8 karyotypes. Trisomy 1 was found in 6 cells, monosomy 15 in 5 cells and +14q+ in 8 cells (Table 1). A representative karyotype with clonal aberrations is shown in Figure 2.

The cytochemical profile of BHL-89 cells was practically negative, or weakly positive with the reactions tested (Table 2). The cells were characterized by weak, non-specific esterase activity, sensitive to NaF inhibition. Immunological markers of BHL-89 cells are shown in Table 3. Cells expressed markers characteristic of early B cells and were positive for sIgM, icDC22 and Tdt. Some monoclonal reactions specific for the B lineage (BB1, Fellows, McConnels) carried out by J. Gordon (Karolinska Institütet, Stockholm)

Table 1. Cytogenetic abnormalities of BHL-89 cells.

	Chromosome number		
# of counted cells	#45	#46	#47
20	2	6	12
# of karyotyped cells	*Trisomy*	*Changes in*	*Monosomy*
	#1	#14	#15
8	6	7	5

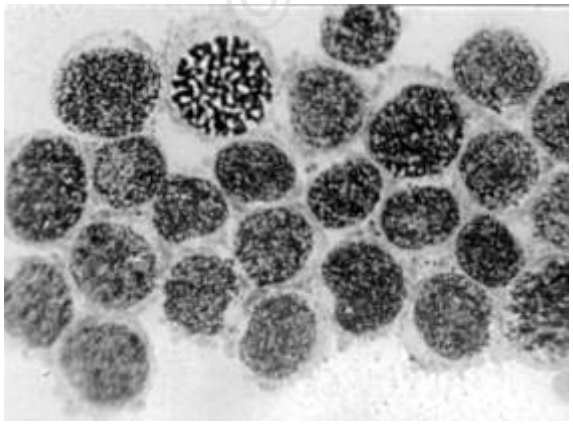

Figure 1. Cytocentrifuge preparation of BHL-89 cells stained with May-Grünwald-Giemsa. Magnification x 100.

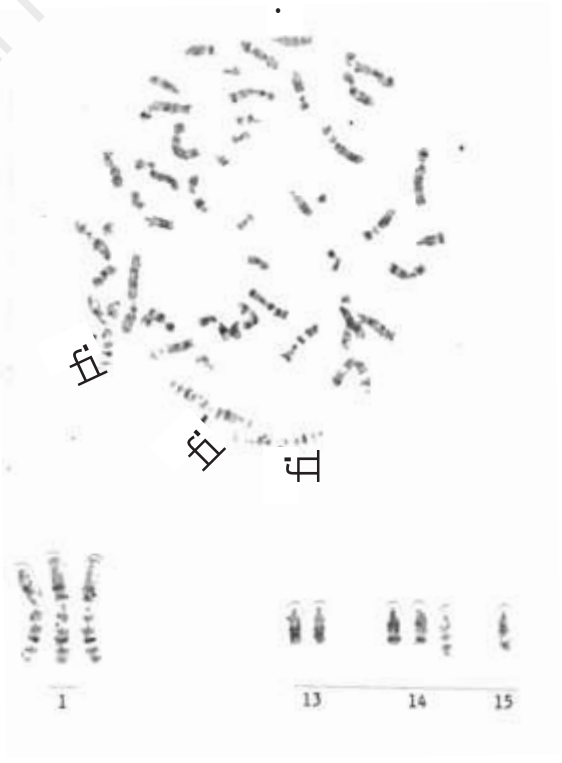

Figure 2. A representative chromosome plate of BHL-89 cells (G-banding). Three long arrows point to chromosomes #1, one short arrow to the #+14q+ marker. Lower line: chromosome #1 and chromosomes #13, #14 and #15 including the #+14q+ marker and #15 monosomy.

Table 2. Cytochemical reactions of BHL-89 cells.

α-naphthyl acetate esterase	
without NaF inhibition	+
with NaF inhibition	±
Acid α-naphthyl acetate esterase	±
Acid phosphatase	0
Periodic acid Schiff's	0-±
Peroxidase	0
Naphthol AS-D chloroacetate esterase	
without NaF inhibition	±
with NaF inhibition	0

Table 3. Immunological markers of BHL-89 cells.

Parameter	*Result*	*Parameter*	*Result*
CD14	0	CD10	0
CD3	0	CD9	7.8%
CD2	0	CD71	1.0%
CD1	0	CD23	0
CD11a	0	HLA-ABC	21.2%
HLA-DR	0	CD16	0
CD20	0	sIgM	94.0%
CD21	0	CD22 (i.cytopl.)	48.3%
CD19	0	Tdt	50.1%
CD13	0	BB1*	54.0%
CD33	0	Fellows*	77.0%
		McConnels*	0-34%

*B-cell monoclonals (Dr. John Gordon, personal communication, Karolinska Institütet, Stockholm).

were positive. Culture medium was negative for Ig production. Some markers disappeared during long-term culture. According to Western blot analysis, the bcl-2 level of the BHL-89 cells was 5.2 times higher than in the HEP-2 laryngeal carcinoma cells used as controls (Figure 3).

The cells were negative for the presence of EBNA. Attempts to induce EBNA by infecting the cells with B-95-8 EBV were unsuccessful.

Cells treated with 50, 100, 200 and 300 μg/mL of DBD for 2 hours were transferred to soft agar medium. The dose-response curve obtained with DBD-treated cells is shown in Figure 4. The shape of the curve is characteristic of alkylating agents or radiation, having a shoulder region. The IC50 value was 45 μg/mL. No cells survived after treatment with 300 μg/mL of DBD. The effect of TPA on cell proliferation (mitotic activity) was studied with doses between 1 ng/mL and 1 μg/mL. Neither the rate of proliferation, nor the mitotic index changed even by the highest TPA dose. The effect of TPA on the expression of several immunological markers is shown in Table 4. We found that a B marker and two other markers gave significantly stronger reactions following treatment with 1 μg/mL of TPA.

Discussion

The cell line reported here is a malignant lymphoma expressing markers characteristic of early B cells. The presence of the chromosome marker +14q+ supports the lymphoma origin of the cells. Since the cell line was easily established without feeder cells and without any other growth-promoting compounds, it is distinctive, similar to the MN-60 cell line,[9] from other EBV-negative B cell lines. The high clonogenicity of BHL-89 cells in soft agar medium is also a unique characteristic with respect to the previously reported EBV-negative lymphoma[8] and lymphoblastoid lines.[2] The cloning efficiency and tumor-producing capacity in immunosuppressed mice suggested the neoplastic nature of these cells. The tumorigenicity of B lymphoid cell lines might depend on the growth potential associated with the +14q+ chromosome,[20] since most B-lymphoma cell lines having the 14q+ marker were heterotransplanted with success.[21] A karyotype with the +14q+ marker and a modal number of 47 was

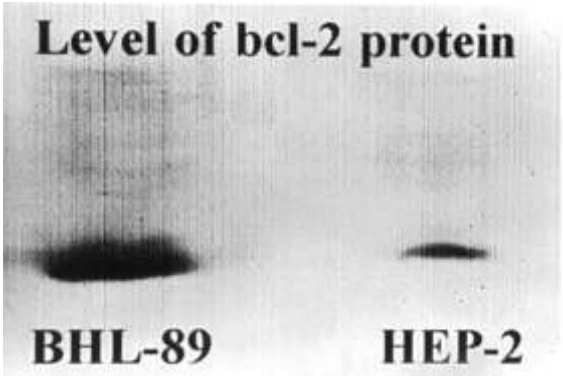

Figure 3. Western blot analysis of the bcl-2 protein of BHL-89 lymphoma and HEP-2 larynx carcinoma cells.

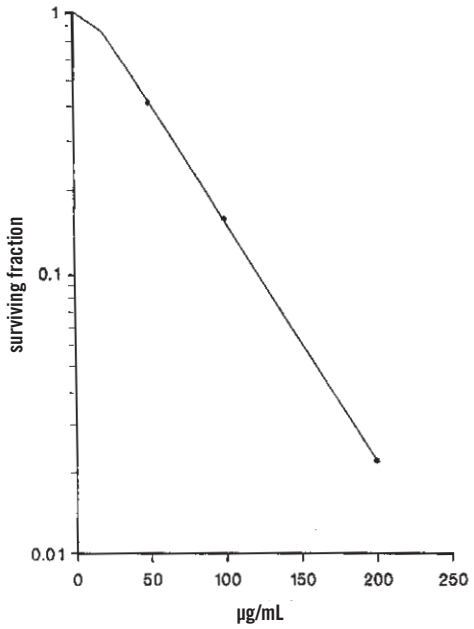

Figure 4. Dose-response curve of BHL-89 cells treated with dibromodulcitol (NSC-104800) for 2 hours. Note the small shoulder regions of the curve.

shown in a previously treated patient with B-CLL.[22] Trisomy 1 is also typical of lymphoproliferative disorders.[23] The immunological markers and the high level of the bcl-2 protein support the view that BHL-89 cells belong to the B lineage.

According to Tarella *et al.*[24], the cell lines PC53 and PC53A established by them were quite unique because of the very immature phenotype and their origin from an adult patient. Our BHL-89 line represents an early, immature phenotype of adult origin, too. The unsuccessful infection of cells with B95-8 EBV is probably due to the absence of EBV receptors.[25]

B cell lines showed sensitivity to TPA at or below 1 ng/mL.[9,26] Conversion of the EBV-negative phenotype to an EBV-positive one, however, resulted in resistance to either the growth-inhibitory or toxic effects of TPA.[26] TPA induced plasmacytoid differentiation of B cell lymphoma cell lines.[27] The lack of sensitivity of our BHL-89 line to TPA needs further investigation. The cells were sensitive to DBD, which has been used to treat some types of lymphoma.[28,29] The high

Table 4. Changes in some immunological markers of BHL-89 cells following TPA treatment.

Parameter	*Control*	*TPA-treated (1 µg/mL)*
CD10	0	0
VIB-C5 (CD24)°	34%	55%
UJ 13 A*	48%	75%
UJ 313 ASC*	15%	52%

°B-marker; *Markers for normal and malignant neuroectodermal tissue (from: Coakham et al., Lancet 1984; i:1095-8).

clonogenicity in soft agar made the cells suitable for clonogenic assay studies.

In conclusion, the established EBNA-negative BHL-89 cell line has a few unique characteristics, e.g. rapid establishment without feeder cells, origin from the lymph node of an adult patient, high clonogenicity in soft agar, and resistance to TPA. Lymphomagenesis is a multistep process that starts in bone marrow progenitors and involves oncogene abnormalities and, possibly, antigen stimulation as well.[30] The cell line is suitable for studying the nature of B lymphomas and testing compounds against lymphoproliferative disorders.

References

1. Nilsson K, Pontén J. Classification and biological nature of established human hematopoietic cell lines. Int J Cancer 1975; 15:321-41.
2. Nilsson K. The nature of lymphoid cell lines and their relationship to the virus. In: Epstein MA, Achong BG, eds. The Epstein-Barr Virus. Berlin:Springer Verlag, 1979:225-81.
3. Klein G, Lindahl T, Jondal M, et al. Continuous lymphoid cell lines with characteristics of B cells (bone marrow-derived), lacking the Epstein-Barr virus genome and derived from three human lymphomas. Proc Natl Acad Sci USA 1974; 71:3283-6.
4. Epstein AL, Kaplan HS. Biology of the human malignant lymphomas. I. Establishment in continuous cell culture and heterotransplantation of diffuse histiocytic lymphomas. Cancer 1974; 34:1851-72.
5. Dillman RO, Handley HH, Royston I. Establishment and characterization of an Epstein-Barr virus-negative lymphoma B-cell line from a patient with a diffuse large cell lymphoma. Cancer Res 1982; 42:1368-73.
6. Nilsson K, Klareskog L, Ralph P, Sundström C, Zech L. Phenotypic and cytogenetics of a new Epstein-Barr virus negative cell line (SKW4) derived from a B cell lymphoma. Hematol Oncol 1983; 1:277-95.
7. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J

Cancer 1976; 17:565-77.
8. Steinitz M, Klein G. Further studies on the differences in serum dependence in EBV negative lymphoma lines and their in vitro EBV-converted, virus-genome carrying sublines. Eur J Cancer 1977; 13:1269-75.
9. Roos G, Adams A, Giovanella B, et al. Establishment and characterization of a human EBV-negative B cell line (MN 60). Leuk Res 1982; 5:685-93.
10. Ichinose I, Nakano S, Ohshima K, et al. Establishment of Epstein-Barr virus-negative diffuse large cell lymphoma cell line with an 8;22 chromosomal translocation. Cancer Res 1991; 51:5392-7.
11. Pályi I, Sugár J, Fleischmann T. Characteristics of human lymphoblastoid cell lines established in culture. Pathologica (Genova) 1981; 73:142-4.
12. Pályi I. Survival responses to new cytostatic hexitols of P388 mouse and K562 human leukemia cells in vitro. Cancer Treat Rep 1986; 70:279-84.
13. Vincze B, Pályi I, Daubner D, et al. Influence of luteinizing hormone-releasing hormone agonists on human mammary carcinoma cell lines and their xenografts. J Steroid Biochem Molec Biol 1991; 38:119-26.
14. Sundström C, Nilsson K. Cytochemical profile of human haematopoietic biopsy cells and derived cell lines. Br J Hematol 1977; 37:489-501.
15. Pócsik É, Mihalik R, Gyódi É, et al. Activation of lymphocytes after platelet allotransfusion possessing only class I MHC product. Clin Exp Immunol 1990; 82:102-7.
16. Jánossy G, Campana D, Coustin-Smith E, Timm A. Leukocyte methods. In: Cawley JC, ed. Laboratory Annual. Immunofluorescence. Edinburgh:Churchill Livingstone 1986: 1-24.
17. Miyashita T, Krajewski S, Krajewska M, et al. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 1994; 9:1799-805.
18. Reedman BM, Klein G. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int J Cancer 1973; 11:499-520.
19. Gergely L, Czeglédy J, Váczi L, Szalka A, Berényi E. Cells containing Epstein-Barr nuclear antigen (EBNA) in peripheral blood. Acta Microbiol Acad Sci Hung 1979; 26:41-5.
20. Abe M, Nozawa Y, Wakasa H, Ohno H, Fukuhara S. Characterization and comparison of two newly established Epstein-Barr virus-negative lymphoma B-cell lines. Cancer 1988; 61: 483-90.
21. Kaplan HS, Goodenow RS, Gartner S, Bieber MM. Biology and virology of the human malignant lymphomas. Cancer 1979; 43:1-24.
22. Sadamori N, Han T, Minowada J, Sandberg AA. Chromosomes and causation of human cancer and leukemia. LII. Chromosome findings in treated patients with B-cell chronic lymphocytic leukemia. Cancer Genet Cytogenet 1984; 4:69-88.
23. Berger R. The chromosomes in hematology. Cancer Genet Cytogenet 1981; 4:69-88.
24. Tarella C, Pregno P, Gallo E, et al. Establishment from an adult leukemic patient of two novel precursor B cell lines with different growth modality. Leuk Res 1990; 14:177-84.
25. Daibata M, Kubonishi I, Eguchi T, Yano S, Ohtsuki Y, Miyoshi I. The establishment of Epstein-Barr virus nuclear antigen-positive (SP-50B) and Epstein-Barr virus nuclear antigen-negative (SP-53) cell lines with t(11;14) (q13;q32) chromosome abnormality from an intermediate lymphocytic lymphoma. Cancer 1989; 64:1248-53.
26. Béchet J-M, Guétard D. Differential cytotoxicity of tumour promoter TPA for EBV-negative human lymphoma cell lines and their EBV-converted sublines. Int J Cancer 1983; 82:61-5.
27. Benjamin D, Magrath IT, Triche TJ, Schroff RW, Jensen JP, Korsmeyer SJ. Induction of plasmacytoid differentiation by phorbol ester in B-cell lymphoma cell lines bearing 8;14 translocations. Proc Natl Acad Sci USA 1984; 81:3547-51.
28. Andrews NC, Weiss AJ, Wilson W, Nealon T. Phase II study of dibromodulcitol (NSC-104800). Cancer Chemother Rep 1974; 58:653-60.
29. Sitarz AL, Albo V, Movassaghi N, et al. Dibromodulcitol (NSC-104800) compared with cyclophosphamide (NSC-26271) as remission maintenance therapy in previously treated children with acute lymphoblastic leukemia or acute undifferentiated leukemia: possible effectiveness in reducing the incidence of central nervous system leukemia. Cancer Chemother Rep 1975; 59:989-94.
30. Caligaris-Cappio F, Bertero MT, Bergui L. Autoimmunity, autoimmune diseases and lymphoproliferative disorders. Haematologica 1994; 79:487-92.