Nuclear DNA content of non-endemic Burkitt's lymphoma

T LEHTINEN,* M LEHTINEN,† R AINE,‡ O P KALLIONIEMI,†† T LEINO,*
T HAKALA,* P LEINIKKI,† M ALAVAikko**

*From the Departments of *Oncology, ‡Pathology, and ††Clinical Chemistry, Tampere University Central Hospital, the Department of †Biomedical Sciences, University of Tampere, and the **Department of Pathology, University of Oulu, Finland*

SUMMARY The nuclear DNA content of 26 non-endemic Burkitt's lymphomas was studied by flow cytometry. Eighteen of the tumours showed a pattern characteristic for diploid chromosome distribution, while eight of the tumours were aneuploid. Six of the aneuploid tumours showed an almost diploid, aneuploid DNA index, while two were tetraploid tumours. Patients with aneuploid tumours had a significantly worse prognosis ($p < 0.005$) than those with diploid tumours. One of the aneuploid tumours was positive for Epstein-Barr virus DNA.

Burkitt's lymphoma is characterised by a typical cytogenetic abnormality—the translocation of an oncogene (c-myc) containing fragments of chromosome 8 to the sites of immunoglobulin heavy or light chain genes in chromosomes 14, 2, or 22.¹ Besides the specific chromosomal translocations a proportion of Burkitt type tumours contain secondary chromosomal abnormalities²⁻⁵ that are also detectable by flow cytometric DNA analysis.⁶ In chronic lymphocytic leukaemia (CLL) and immunocytoma comparable chromosomal changes indicate a poor prognosis.⁷

The recently described analysis of nuclear DNA content of formalin fixed, paraffin embedded material⁸ has also been successfully used on non-Hodgkin lymphomas (NHL).⁹ We report the analysis by flow cytometry of 26 archival, non-endemic, Burkitt type tumours. The nuclear DNA content and proliferative activity of the tumours were correlated with histological and clinical follow up data.

Material and methods

Twenty six newly diagnosed cases of NHL classified as small non-cleaved Burkitt type lymphomas were studied.¹⁰ The biopsy material was collected during 1965–85 from the catchment area of Tampere University Central Hospital which had about 410 000 inhabitants before 1980, with an incidence of 15 cases of

NHL.¹¹ After boundary reorganisation the region expanded to about 1 000 000 people and an additional 11 cases were found during 1981–85. The mean age of the patients was 36 (range four to 69 years); nine patients were younger than 15 years; 13 were between 16 and 60 years; and four were over 61 years of age. None of the patients had previously been treated for malignant disease or had received immunosuppressive drugs. Because of the retrospective nature of the study, patients' treatment varied considerably from no treatment to radiotherapy alone, or chemotherapy and radiotherapy with various combinations.

Diagnostic, formalin fixed tumour biopsy specimens were available from 25 patients. Fresh frozen tumour biopsy specimens were available from four patients.

Clinical staging was done according to the Ann Arbor system.¹² Eleven patients were classified as having disease of stages I–II and 10, stages III–IV. Two patients (aged 8 and 19 years) had acute lymphoblastic leukaemia, which was shown by a bone marrow aspirate to have Burkitt type cell infiltration. Three patients had no staging reported in their medical records.

The histopathological analysis of the lymphoma biopsy specimens has already been described thoroughly.¹ The tumours were classified according to their growth patterns into follicular, follicular and diffuse, and diffuse.¹³ A mitotic ratio of the tumours was determined by calculating the lymphoid cells and mitotic activity by using an ocular square and lens

1:32 at a magnification of 100. The presence or absence of aberrant mitoses was also recorded. Six hundred to 1200 cells from randomly selected fields in the diagnostic area of the biopsy specimen were counted. The mitotic activity of the tumour was considered to be high if the mitotic ratio exceeded 19 per thousand cells, a categorisation which has been used previously to divide high grade NHLs into those with low or high mitotic activity.¹⁴

Preparation of the paraffin embedded biopsy material was carried out by the method of Schutte *et al.*,¹⁵ with a few modifications.¹⁶ Briefly, 30 µm sections were cut from the biopsy specimens for flow cytometry in addition to a 5 µm section, which was used for the confirmation of the previous histopathological diagnosis. Usually, one section yielded enough cells for flow cytometry analysis. Predominantly haemorrhagic and necrotic areas were excluded. The preparations were deparaffinised with two changes of xylene and rehydrated in a series of alcohol (100%, 95%, 70%, and 50%) into distilled water. The rehydrated tissues were trypsinised. We used an overnight incubation at room temperature in 0.25% trypsin supplemented with 1 mM edetic acid, 0.3% non-ionic detergent NP-40 (Shell, The Netherlands) in a Tris-hydrochloric acid buffer (pH 7.4). The resulting cell suspension was pelleted at 500g for seven minutes. The pellet was resuspended by vigorous syringing in the Tris-hydrochloric acid buffer containing 50 µg/ml of ethidium bromide (Merck, West Germany). The fresh-frozen samples were minced and stained for 15 minutes in the ice cold Tris-hydrochloric acid buffer supplemented with 0.3% NP-40 and 50 µg/ml of ethidium bromide. Thereafter, ribonuclease A1 (Sigma, USA) was added to a final concentration of 100 µg/ml for 15 minutes at room temperature. Before analysis by flow cytometry the cell suspension was filtered through a nylon net (pore size 55 µm) to remove aggregates.

Analysis by flow cytometry was performed with EPICS-C (Coulter Electronics, Hialeah, USA) flow cytometer, which is equipped with a 5 W argon laser. An excitation wavelength of 488 nm and emission wavelength of over 610 nm (obtained by an absorb-

ance long pass filter) were used. Up to 10 000 cells were scanned for each analysis. The use of internal diploidy standards in the analysis of the formalin fixed material was not possible. A single peak in the histogram, however, was taken to indicate diploid cells and was given a DNA index of 1.0.¹⁷ If two or more peaks appeared the one with the lowest channel number was considered to be diploid (DNA index 1.0). The DNA index of the remaining peaks was calculated as the ratio between the channel numbers of the two peaks (aneuploid:diploid). The proportion of the proliferating cells (S-phase fraction) was calculated according to the method of Baisch *et al.*¹⁸

DNA-DNA hybridisation for the detection of Epstein-Barr virus was kindly performed by Dr Kerstin Falk, Karolinska Institute, Sweden, according to routine methods.

The life table analyses and testing of significance by the log rank test were performed according to the method of Peto *et al.*^{19,20} Fisher's exact test was also used. Only patients followed up for three years or longer were included in the analyses.

Results

ANALYSIS OF THE NUCLEAR DNA CONTENT OF THE TUMOURS

No major differences between the quality of the DNA histograms obtained from the fresh-frozen biopsy specimens and the paraffin embedded material were noticed (fig 1). The nuclear DNA content of the Burkitt type tumours was analysed from the formalin fixed, paraffin embedded material, available from all patients (except for one case of Burkitt type leukaemia, from which only fresh-frozen biopsy specimen was analysed). The coefficient of variation of all the 25 paraffin embedded samples varied between 3.1 and 8.9% (mean 5.7).

We found eight (31%) aneuploid tumours. The mean DNA index was 1.14 (range 1.08–1.24) for the six aneuploid tumours, which were almost diploid (fig 2). The two aneuploid tumours in the tetraploid region had DNA indices of 1.88 and 1.98. Generally, the aneuploid peaks were about as prominent as the diploid peaks (table 1).

Table 1 Flow cytometric variables of DNA histograms of aneuploid small non-cleaved Burkitt type tumours

Case No	Coefficient of variation (%)	DNA index	Per cent of:	
			Diploid cells	Aneuploid cells
1	4.5	1.13	48	31
2	4.9	1.08	41	23
3	4.1	1.24	19	42
4	4.7	1.10	47	53
5	6.7	1.18	29	37
6	7.6	1.13	32	25
7	6.3	1.88	16	50
8	4.6	1.98	36	40

Fig 1 Histograms of fresh frozen (a) and paraffin embedded (b) biopsy specimens of Burkitt's lymphoma. Coefficient of variation of diploid peaks 3.3 (a) and 3.5 (b).

The S-phase fraction could reliably be calculated from 24 tumours. The mean S-phase fraction of these 24 tumours was 15.5. Only two tumours, one aneuploid and one diploid, showed low S-phase fraction of 3.4 and 4.6, respectively. For the aneuploid tumours, the mean S-phase fraction was 14.1 (range 3.4–27.7) and for the diploid tumours it was 15.9 (range 4.6–26.3).

Fig 2 Histograms of two paraffin embedded biopsy specimens of Burkitt's lymphoma. Both tumours show an aneuploid cell population with DNA indices of 1.10 (a) and 1.18 (b). Corresponding coefficient of variation are 4.7 and 4.5, respectively.

BIOLOGICAL PROPERTIES OF THE ANEUPLOID TUMOURS

We compared the survival of the eight patients with aneuploid with the 18 with diploid tumours to see whether the difference in the nuclear DNA content reflected any differences in the biological behaviour of the tumours. The eight cases of aneuploid tumours clearly had a more unfavourable prognosis ($p < 0.005$) than those with diploid tumours (fig 3). An analysis irrespective of staging of survival could not be performed, because of the small number of cases studied. The distribution, however, of different stages within the groups was equal (data not shown).

Neither the S-phase fraction of the aneuploid tumours nor the mitotic activity, as calculated by the

Fig 3 Three year survival of patients with Burkitt's lymphoma bearing diploid or aneuploid tumours. Diploid tumours (—) 18 cases, aneuploid tumours (---) 8 cases.

Table 2 Correlation of DNA analysis and various clinical and histopathological variables in Burkitt's lymphoma

	Mean age	Sex (M/F)	S-phase fraction* mean (SD)	High mitotic ratio (No/%)	Abnormal mitoses (No/%)
Diploid tumours	37	9/9	15.9 (5.4)	9/16 (65)	6/18 (33)
Aneuploid tumours	35	1/7	14.1 (4.8)	1/7 (29)	1/8 (13)

* = Proportion of cells in the S-phase fraction as determined from the DNA histograms of Baisch *et al.*¹⁸

Table 3 Epstein-Barr virus carrier state* and secondary chromosomal abnormalities in Burkitt's lymphoma

Reference	Country	Endemic region	Karyotyping†	DNA analysis‡
28	Japan	No	0/4 (0%)	Not done
4	France	No	3/13 (23%)	Not done
6	USA	No	1/4 (25%)	1/4 (25%)
This paper	Finland	No	Not done	8/26 (31%)
2	Kenya	Yes	5/7 (71%)	Not done
3	Ghana	Yes	7/14 (50%)	Not done

*Endemic > 95%/non-endemic < 25%; †47 chromosomes or more; ‡DNA-aneuploidy.

mitotic ratio correlated well with the aneuploidy. Aberrant mitoses were found in seven tumours, only one of which was aneuploid (not significant, Fisher's exact test, table 2). There was no difference in the age distribution of the two patient groups, either, but men predominated in the group with aneuploid tumours (seven of eight, 88%) compared with the group with diploid tumours (nine of 18, 50%, table 2).

The reported cases of endemic Burkitt's lymphoma are accompanied by secondary chromosomal abnormalities twice as often as the non-endemic cases (table 3). The most noticeable differences between cases of endemic and non-endemic Burkitt's lymphoma are age of the patients (children *v* young adults) and the presence of Epstein-Barr virus genome (95% *v* 10 to 20%) in the tumours. One of our four fresh-frozen biopsy specimens was positive for Epstein-Barr virus (table 4), containing about 15 Epstein-Barr virus genome copies per cell. Two tumour specimens were negative and one could not be evaluated. The Epstein-Barr virus positive case was an 8 year old boy who had an aneuploid tumour by flow cytometry analysis (DNA index 1.13); the remaining three adult patients all had a diploid tumour.

Discussion

The recently described methods for the flow cytometry DNA analysis of formalin fixed, paraffin embedded tumour samples^{8 15} have made it possible to evaluate the validity of the nuclear DNA content as a marker of tumour behaviour. Our retrospective NHL material comprised, overall, more than 300 cases.¹¹ It was especially suitable for evaluation as follow up data of all patients were readily available.¹⁴ Flow cytometry analysis showed that 18 of the non-endemic Burkitt type tumours (lymphomas or leukaemias) consisted of two cell populations: those showing aneuploid or diploid cells.

The results of DNA analysis of NHLs were the same, irrespective of whether formalin fixed or fresh frozen material was used.⁹ The correlation between flow cytometric and cytogenetic analyses of lymphomas has been shown to be good in triploid-tetraploid tumours.^{6 21} In the almost diploid aneuploid tumours the concordance was not as good^{6 21} (because of

differences in DNA in the tumour cells which were in the G₁-phase) as that of the resting normal cells.²² Thus the actual difference between chromosome numbers of the diploid and aneuploid tumours in our material is probably somewhat smaller than what would be expected from the mean DNA index (1.14). According to the findings of cytogenetic studies on endemic and non-endemic Burkitt's lymphoma it is not uncommon to find that these tumours have chromosome numbers of 47-49 resulting from secondary chromosomal changes.⁴ Our results with the almost diploid aneuploid tumours approximate the chromosome numbers within the above mentioned range.

The impact of secondary chromosomal abnormalities on tumour biology has recently been questioned.²³ In our material, however, the patients with aneuploid tumours had a much less favourable prognosis than those with diploid tumours. This agrees with the recent reports on CLL and immunocytoma in which an increased number of secondary chromosomal abnormalities were associated with a poor prognosis.⁷

The aneuploid tumours did not show enhanced proliferative or mitotic activity, the common characteristics of tumours, including NHL, indicating poor prognosis.^{14 17 24} Abnormal mitotic activity has been suggested to be one of the possible mechanisms through which aneuploidy evolves.^{25 26} In our material it was the diploid tumours that showed aberrant mitoses, whereas only one such case was found in the group with aneuploid tumours. Our findings do not exclude the possibility that increased mitotic activity or abnormal mitoses have a role in inducing aneuploidy in Burkitt's lymphoma type tumours but this is a less plausible alternative.

Cases of endemic Burkitt's lymphoma that are consistently positive for Epstein-Barr virus show secondary chromosomal abnormalities twice as often as non-endemic tumours, which show Epstein-Barr virus in less than 25% of the cases.^{4 27} This was also the case for the one patient in this series whose tumour was positive for Epstein-Barr virus and aneuploidy by flow cytometry analysis. In vitro infection of lymphoblastoid cells with Epstein-Barr virus does not cause chromosomal aberrations.²⁸ Whether the virus causes secondary chromosomal changes in the later stages of the clinical disease remains unclear.

Table 4 Epstein-Barr virus carrier state and aneuploidy in non-endemic Burkitt's lymphoma

Case No	Age (years)/sex	Epstein-Barr virus	DNA index*	Abnormal mitoses	Mitotic ratio	S-phase fraction (%)
1	8, male	15 genome copies/cell	1.13	Negative	Not done	10
2	19, male	Not available	1.0	Not done	Not done	8
3	31, male	Negative	1.0	Negative	High	23
4	41, male	Negative	1.0	Negative	High	26

*DNA index 1.0 corresponds to diploid cells.

The financial aid of Finnish Cancer Organisations and Pirkanmaa Cancer Fund is gratefully acknowledged.

References

- Klein G. Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. *Cell* 1983;32:311-5.
- Zech L, Haglund U, Nilsson K, Klein G. Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt and non-Burkitt lymphomas. *Int J Cancer* 1976;17:47-56.
- Biggar RJ, Lee EC, Nkrumah FK, Whang-Peng J. Direct cytogenetic studies by needle aspiration of Burkitt's lymphoma in Ghana, West Africa. *JNCI* 1981;67:769-76.
- Berger R, Bernheim A. Cytogenetic studies on Burkitt's lymphoma-leukaemia. *Cancer Genet Cytogenet* 1982;7:231-44.
- Watmore AE, Murray JA, Potter AM. Nonendemic Burkitt's Lymphoma with complex chromosome abnormalities involving chromosomes 2 and 8. *Cancer Genet Cytogenet* 1985;16:283-8.
- Cabanillas F, Trujillo JM, Barlogie B, et al. Chromosomal abnormalities in lymphoma and their correlations with nucleic acid flow cytometry. *Cancer Genet Cytogenet* 1985;21:99-106.
- Juliussøn G, Robert K-H, Öst Å, et al. Prognostic information from cytogenetic analysis in chronic B-lymphocytic leukemia and leukemic immunocytoma. *Blood* 1985;65:134-41.
- Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content in paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983;31:1333-5.
- Camblejohn RS, Macartney JC. Comparison of DNA flow cytometry from fresh and paraffin embedded samples of non-Hodgkin's lymphoma. *J Clin Pathol* 1985;38:1096-9.
- Lukes RJ, Collins RD. New approach to the classification of the lymphoma. *Br J Cancer* 1985;31 (Suppl II):1-28.
- Alavaikko M, Aine R. The Lukes and Collins classification of non-Hodgkin lymphomas. A histological reappraisal of 301 cases. *Acta Pathol Microbiol Immunol Scand Sect A* 1982;90:241-9.
- Carbone PP, Kaplan HS, Musshof K, Smithers DW, Tubiana M. Report of the committee on Hodgkin's disease staging classification. *Cancer Res* 1971;31:1860-1.
- Butler JJ, Stryker JA, Schullenberger CC. A clinico-pathological study of stages I and II non-Hodgkin's lymphoma using the Lukes and Collins classification. *Br J Cancer* 1975;31 (suppl II):208-69.
- Aine R, Kataja M, Alavaikko M. Prognostic factors for non-cleaved follicular center-cell lymphomas and immunoblastic sarcoma. A Bayesian approach. *Scand J Haematol* 1984;32:475-87.
- Schutte B, Reynders MMJ, Bosman FT, Blijham GH. Flow cytometric determination of DNA-ploidy level in nuclei isolated from paraffin embedded tissue. *Cytometry* 1985;6:26-30.
- Kallioniemi O-P, Hietanen T, Mattila J, Lehtinen M, Lauslahti K, Koivula T. Aneuploid DNA content and high S-phase fraction of tumour cells are related to poor prognosis in patients with primary breast cancer. *Eur J Cancer Clin Oncol* 1987;23:277-82.
- Barlogie B, Raber MN, Schumann J, et al. Flow cytometry in clinical cancer research. *Cancer Res* 1983;43:3982-97.
- Baisch H, Gohde W, Linden WA. Analysis of PCP-data to determine the fraction of cells in the various phases of the cell cycle. *Radiat Environ Biophys* 1975;12:31-9.
- Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient I. Introduction and design. *Br J Cancer* 1976;34:585-611.
- Peto R, Pike MC, Armitage P, et al. Design and analysis of randomised clinical trials requiring prolonged observation of each patient II. Analysis and examples. *Br J Cancer* 1977;35:1-39.
- Tribukait B. Clinical DNA flow cytometry. *Med Oncol Tumor Pharmacother* 1984;1:211-18.
- Darzynkiewicz Z, Traganos F, Kapuscinski J, Staiano-Coico L, Melamed M. Accessibility of DNA in situ to various fluorochromes: Relationship to chromatin changes during erythroid differentiation of friend leukemia cells. *Cytometry* 1984;5:355-63.
- Mittelman F. Clustering of chromosomal breakpoints in neoplasia. *Cancer Genet Cytogenet* 1986;19:67-71.
- Roos G, Dige U, Lenner P, Lindh J, Johansson H. Prognostic significance of DNA-analysis by flow cytometry in non-Hodgkin's lymphoma. *Hematol Oncol* 1985;3:233-42.
- Saksela E. The effect of different human sera on the karyologic picture of HeLa cells. A contribution to the knowledge of environmental influences on cell populations. Helsinki: University of Helsinki, 1962. (Thesis.) 1-73.
- Therman E, Buchler DA, Nieminen U, Timonen S. Mitotic modifications and aberrations in human cervical cancer. *Cancer Genet Cytogenet* 1984;11:185-97.
- Nilsson K, Klein G. Phenotypic and cytogenetic characteristics of human B-lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. *Adv Cancer Res* 1982;37:319-80.
- Miyoshi I, Hamasaki K, Miyamoto K, et al. Chromosome translocations in Burkitt's lymphoma. *N Engl J Med* 1981;304:734.

Requests for reprints to: Dr Tuula Lehtinen, Department of Biomedical Sciences, University of Tampere, POB 607, Tampere SF-33101, Finland.