

Differentiation Between Lymphomas and Pseudolymphomas of the Skin by Computerized DNA-Image Cytometry

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The histologic and immunohistologic differential diagnosis between pseudolymphomas (PL) and malignant lymphomas (ML) of the skin can be difficult. Since DNA cytometry has been found to be of both diagnostic and prognostic value in various neoplasms, its ability to discriminate between ML and PL in Feulgen-stained imprints of 17 PL and 49 ML skin biopsies was examined by high-resolution image analysis. The reliability of the following algorithms of DNA distribution was evaluated: 1) 2cDI (2c-deviation index), which reflects the variation of the nuclear DNA values around the diploid DNA peak; 2) percentage of cells having a DNA value $\geq 5c$ (5cER; 5c-exceeding rate); 3) percentage of cells presenting with a DNA value $\geq 4c$ (4cER). A 2cDI of 0.1 was

found to be the most reliable marker for the differentiation between PL and ML. On the basis of this feature, 16 of 17 cases of PL and 46 of 49 cases of ML were correctly classified. The sensitivity, specificity, and efficiency of this feature were 94%. A 5cER $\geq 1\%$ had a specificity of 100%, but the sensitivity was only 43%. For the 4cER, a sensitivity of 61% and a specificity of 94% were found. In conclusion, the calculation of the 2cDI and the 5cER based on high-resolution image analysis provided additional helpful diagnostic features, and therefore should be included as a diagnostic tool. If the 5cER is at least 1%, the diagnosis of a ML can be confirmed with a specificity of 100%. *J Invest Dermatol* 94:254-260, 1990

The histologic differential diagnosis between cutaneous pseudolymphoma (PL) and malignant lymphoma (ML) can be difficult [1,2,3,4]. This may be due to poorly defined histologic criteria. When rules are devised to differentiate among malignant and benign lesions, exceptions to these rules abound [3]. To obtain more precise and more objective diagnostic criteria, investigators have applied several techniques to cutaneous lymphomas, e.g., immunophenotyping [5,6], cytogenetic analysis [7-11], quantitative and morphometric electron microscopy [12-14], flow cytometry [15], and DNA-cytophotometry [16-19]. Of these methods, only immunophenotyping and DNA cytophotometry have been used for the differentiation between ML and PL. Immunophenotyping has been shown to be inconclusive, particularly in T-cell infiltrates, and it

was stated that further experience must be gained with this modality [2,3].

Since the majority of neoplasms is characterized by an increased rate of aneuploid cells [20,21], DNA cytophotometry was found to be of both diagnostic and prognostic significance in various neoplasms of the skin [19-21] and of many other tissues [20-24].

As far as we know, to date only Van Vloten et al [18] have provided DNA-cytophotometry data for the differentiation between PL and ML of the skin. However, in their study only a rough estimation of the DNA histograms was performed, and therefore the sensitivity of the method reported was only 75%.

In the present study a computerized high-resolution TV-image analysis system was used for the differentiation between cutaneous ML and PL. Recent developments in computer technology and digital TV-image processing permitted both rapid measurement of the nuclear DNA content [25] and application of more complex algorithms for data evaluation.

The 2c-deviation index (2cDI) first proposed by Böcking et al [26] proved to be the most reliable parameter for the differential diagnosis between cutaneous ML and PL.

MATERIALS AND METHODS

Patients DNA measurements were performed on imprint preparations of skin lesions of 66 patients with either PL or ML. The diagnosis was established by histologic, immunohistologic, and clinical criteria prior to DNA analysis. Of the 66 cases, 17 were classified as PL (four T-cell PL and 13 B-cell PL, of which seven were follicular and six non-follicular) and 49 as ML. ML were classified according to the Kiel classification. In addition, two cases

Manuscript received March 28, 1989; accepted for publication August 17, 1989.

Supported by the Deutsche Forschungsgemeinschaft (Grant Sto 189/1-1) and the Friedrich-Baur-Foundation, Munich, F.R.G.

The data shown in this paper represent a major part of a M.D. thesis of the Ludwig-Maximilians-Universität München/F.R.G. (40).

Presented in part at the XVI Annual Meeting of the German Society for Dermatological Research, Cologne, November 25-27, 1988.

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Abbreviations:

- PL: pseudolymphoma
- ML: malignant lymphoma
- 2cDI: 2c deviation index
- 4cER: 4c exceeding rate
- 5cER: 5c exceeding rate

of lymphomatoid papulosis were investigated, and, as controls, DNA measurements were also obtained in nine cases of eczema. For detailed data concerning diagnosis, age, and sex, see Table I.

Imprints Imprints were prepared on clean glass slides by touching with the freshly cut surface of a skin biopsy. The imprints were fixed in methanol (100%), formaldehyde (37%), and glacial acetic acid (96%) 85:10:5 (V/V/V), and Feulgen staining was performed according to Böhm [27] with hydrolysis in 5 N HCL for 50 min at 28°C and staining in Schiff's reagent for 60 min.

DNA Measurements DNA analysis was performed on imprints by the high-resolution image analysis system IPS (Kontron, Eching, FRG). The specimens were digitalized with a density of 16 pixels/ μm , with a TV Pasecon camera (Bosch, Stuttgart, FRG) connected with a 100-oil objective of an Axioplan microscope (Zeiss, Oberkochen, FRG).

Automatic Segmentation In a first step, the contrast within the original video image (Fig 1a) was increased by mapping the actual grey levels linearly into the full range between black (grey value 0) and white (grey value 255) (Fig 1b). The contour of the nuclei was

automatically found by use of the function EMPHASE, which performed an addition of the original image with the difference of the original image and the low-passed filtered version of it (Fig 1c). Subsequently, a constant grey value threshold of 130 was used for calculation of the contour of the nuclei automatically (Fig 1d). Inaccurate segmentation was rejected by the contour being given in the overlay of the original image.

Parameters Calculated For each nucleus the optical density (OD) and the area were calculated. The integrated optical density (IOD) was obtained as the product of the OD and the area. In Feulgen-stained nuclei the IOD is linearly correlated with the DNA content [27]. In each slide, 20 chicken erythrocytes were used as an internal reference. In a previous pilot study, a comparison of nuclei of chicken erythrocytes and normal human lymphocytes revealed the following relationship: $\text{IOD}_{\text{ery}} \times 3.26 = 2\text{cDNA}$, where 2cDNA stands for the DNA content of a normal human diploid cell. A euploid cell in G₂-phase contains 4cDNA. In this study, the relative DNA content in each of the 100 randomly selected cells was calculated as follows: relative DNA content = $\text{IOD}_{\text{nucleus}} / \text{IOD}_{1\text{cDNA}}$.

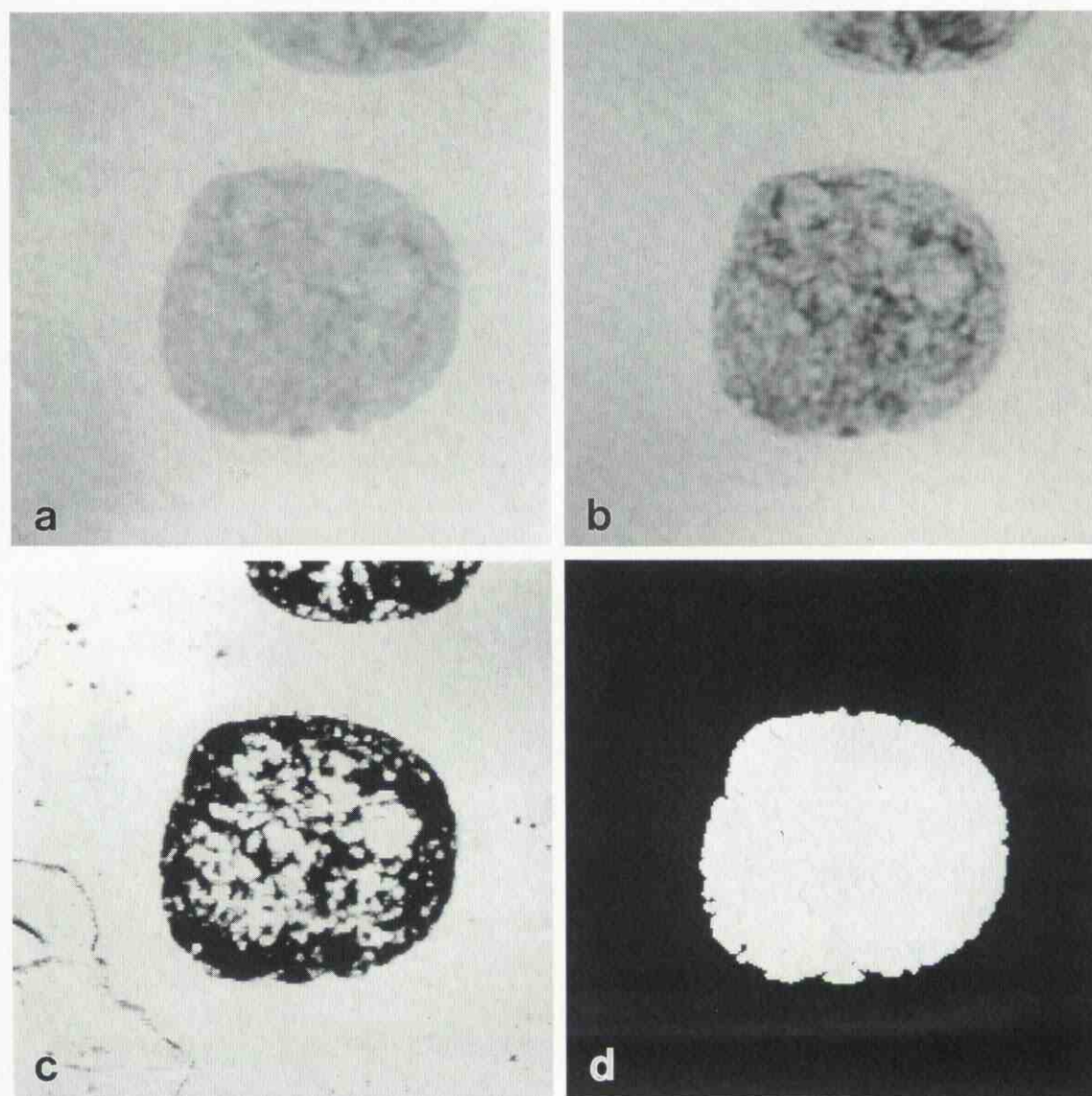


Figure 1. Automatic segmentation: (a) original image; (b) image after mapping the actual grey levels into the full range; (c) image after application of the function EMPHASE; (d) image after using a grey value threshold (for details see *Materials and Methods*).

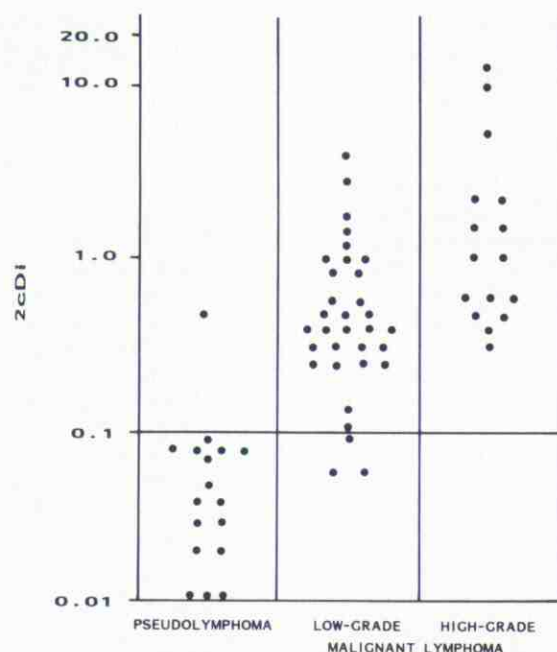


Figure 2. Using a 2cDI > 0.1 as marker for malignant lymphomas, 16 of 17 cases of pseudolymphoma (94%) and 46 of 49 cases of malignant lymphoma (94%) were correctly classified. The three false negative cases were of low-grade malignancy.

Algorithms for Data Evaluation The discriminating power of the following features of the DNA histogram was evaluated:

2c-Deviation Index (2cDI): The 2cDI is defined according to Böcking et al [26] as the ratio between the sum of the squares of the differences between the DNA values of individual cells (c_i) and the 2c value and the number of cells measured (N):

$$2cDI = \frac{1}{N} \sum_{i=1}^N (c_i - 2c)^2.$$

Roughly, the 2cDI reflects the variation of the nuclear DNA values around the 2c value within one case.

5c Exceeding Rate (5cER): The 5cER is defined according to Böcking et al [26] as the percentage of cells with a DNA content equal to or more than 5c. Since euploid-polyploid cells should not contribute to the 5cER, cells with DNA values which are integer-valued exponents of $2c \pm 12.5\%$ are not taken into account. A value of

12.5% was considered as the maximum of the error of measurement [26]. Thus the 5cER reveals only clearly aneuploid cells.

4c Exceeding Rate (4cER): In addition, the criterion for a malignant lesion given by van Vloten et al [16] was tested. Those authors classified a lesion as malignant if at least 5% of its cells contained 4cDNA or more.

For each parameter the diagnostic sensitivity, specificity, and efficiency were determined using formulas as follows: diagnostic sensitivity = $TP / (TP + FN) \times 100$; diagnostic specificity = $TN / (TN + FP) \times 100$; diagnostic efficiency = $(TN + TP) / (TN + TP + FN + FP) \times 100$, whereby TP = true positive, TN = true negative, FP = false positive, and FN = false negative results for malignancy.

The reproducibility of DNA measurements was tested four times during the study by the demonstration of the different ploidy peaks in rat-liver imprints.

For the evaluation of differences between low- and high-grade, as well as between ML of T- or B-cell type, the chi-square test was used. For significant results, $p < 0.05$ was required.

RESULTS

2c-Deviation Index (2cDI) A 2cDI of 0.1 proved to be the most reliable marker for differentiation between PL and ML of the skin. On the basis of this feature, 16 of 17 cases (94%) of cutaneous PL and 46 of 49 cases (94%) of cutaneous ML were correctly classified. Thus the sensitivity and specificity of the 2cDI was 94% (Table II and Fig 2). The sensitivity of this feature was lower for low-grade lymphomas (91%) than for high-grade lymphomas (100%). The efficiency, which is a marker for the usefulness in daily practice, was also 94%. The chi-square test revealed no significant difference in the rate of correctly classified cases between B- and T-cell lymphomas for $p < 0.05$.

The results of the DNA analyses in 66 cases are given in Tables I and II and Figs 2 to 4. The diagnoses of the cases, which were made prior to DNA measurements, are also presented in Table I.

5c-Exceeding Rate (5cER) On the basis of there being at least 1% of cells with a DNA value equal to or higher than 5c, all cases of pseudolymphoma were correctly classified (specificity = 100%). In contrast, only 21 of 49 cases of the malignant lymphomas were diagnosed as malignant (sensitivity = 43%). The efficiency of this criterion was therefore 57%. The rate of false classifications was lower in low-grade lymphomas (52%) than in high-grade lymphomas (69%), but this difference proved to be insignificant in chi-square tests for $p < 0.05$. There was no significant difference in the rate of correctly classified cases between B- and T-cell lymphomas ($p < 0.05$).

Table I. Characteristics and Results of Calculated Parameters of DNA Distribution of 66 Cases of Pseudolymphoma and Malignant Lymphoma

Diagnosis	Number	Mean Age	Sex f:m	Number of cases with a 4cER $\geq 5\%$	Number of cases with a 5cER $\geq 1\%$	Number of cases with a 2cDI > 0.1
Pseudolymphoma						
Pseudolymphoma of B-cell type	13	42	6:7	1	0	1
Pseudolymphoma of T-cell type	4	38	0:4	0	0	0
Malignant lymphoma						
Chronic lymphatic leukemia	2	75	0:2	0	1	1
Sézary syndrome	2	51	0:2	1	0	2
Mycosis fungoides, premalignant lesion	4	57	1:3	2	1	3
Mycosis fungoides, plaque lesion	5	58	3:1	2	3	5
Mycosis fungoides, tumor lesion	7	64	3:4	5	4	7
Immunocytoma	5	63	3:2	2	3	4
Centroblastic-centrocytic lymphoma	8	59	3:5	3	4	8
Centroblastic lymphoma	6	55	2:4	6	3	6
Immunoblastic lymphoma	6	72	2:4	5	1	6
Lymphoblastic lymphoma	4	67	2:2	3	1	4

Table II. Sensitivities, Specificities, and Efficiencies of Calculated Parameters of DNA Distribution in Differentiation between Pseudolymphoma and Malignant Lymphoma

	2cDI (%)	5cER (%)	4cER (%)
Sensitivity	94	43	61
Specificity	94	100	94
Efficiency	94	57	70

4c-Exceeding Rate (4cER) When 5% or more of cells were required to have a DNA value of 4c or more for the diagnosis of malignancy, the sensitivity was 61%, whereas the specificity was 94%. The efficiency was 70% (see Tables I and II).

DNA Histograms Typical patterns of DNA histograms are given in Figs 4a-d. Figure 4a depicts a distribution with one high peak at 2c and a small peak at 4c (less than 5% of the cells) due to cells in the G₂-phase. This type of histogram was most frequently seen in PL. 94% (16 cases) of PL lesions showed this pattern, in contrast to only four cases (8%) of the malignant lymphomas (chronic lymphatic leukemia; mycosis fungoides, stage I; immunocytoma; centroblastic-centrocytic lymphoma). Three of these four cases were also falsely classified using the 2cDI.

Figure 4b demonstrates a bimodal distribution with a high peak (> 10% of the cells) at 4c in addition to the 2c peak. Such types of histograms were found in a plaque stage of mycosis fungoides and in one case of PL. The latter was classified false positively using the 2cDI, but was diagnosed as benign by the 5cER. A bimodal distribution similar to Fig 4c with more than 5% of the cells in the space between 2.25c and 3.5c (S-phase cells and aneuploid cells) was found in 23 (47%) of the malignant lymphomas.

A distribution pattern similar to Fig 4d, which is characterized by the presence of at least 1% aneuploid cells having DNA values > 5c, could be observed in 43% (21 cases) of the malignant lymphomas. The highest DNA values observed were about 10c. None of the cases of pseudolymphoma had a DNA histogram resembling Figs 4c or d.

In one of the two cases of lymphomatoid papulosis, a 2cDI higher

than 0.1 was found, but in both cases the 4cER (respectively 5cER) was below 5% (respectively 1%). All cases of eczema showed normal DNA-distribution patterns (2cDI < 0.1, 5cER = 0, 4cER < 5%).

DISCUSSION

The most striking and important result of this cytometric investigation was that the efficiency, the specificity, and the sensitivity of the 2cDI proved to be 94%. The sensitivity of the 2cDI was higher than in previous investigations, in which cytophotometry was used on skin imprints for both the differentiation between malignant lymphomas and pseudolymphomas (sensitivity = 75%) [18] and for the diagnosis of cutaneous T-cell lymphomas (sensitivity = 65% and 50%, respectively) [12,28]. In those studies, the percentage of cells having a DNA content in excess of 4c (4cER) [18] or a content of more than 4c + 2 S.D. of the mean DNA value [12,28], which is similar to the 5cER, were considered to be the most reliable criteria. Applying the 4cER in our series, we found similar results with a sensitivity of 62% and a specificity of 94%. In contrast to 4cER and 5cER, in our study the 2cDI was more valuable and already increased when cells with a DNA content between 2.25c and 3.5c were present. These cells were either aneuploid cells or normally proliferating cells within the S-phase, and their relevance was not considered in previous studies [12,18,28].

Four typical, different patterns of DNA histograms of ML and PL were found (Figs 4a-d), as also reported in a cytophotometric study of lymph node touch imprints in cutaneous T-cell lymphomas [29]. A pattern similar to Fig 4d was found only in lymph nodes with focal or diffuse infiltration of cutaneous lymphoma.

In our study the 5cER had a specificity of 100%. Therefore, a 5cER > 1% is definitely diagnostic for the presence of cutaneous malignant lymphoma. However, the 5cER can not be used for the exclusion of a malignant lesion.

DNA measurements were also performed in two cases of lymphomatoid papulosis, type A [30]. The results were inconclusive. In one case, the 2cDI was above 0.1, but the 5cER was below 1% in both cases. Our data are consistent with the evaluation of 13 cases of lymphomatoid papulosis using flow cytometry [31], in which greatly abnormal DNA values could be demonstrated in only two of 13 patients, but are in contrast to Willemze et al [30]. They found abnormal DNA histograms in all cases of lymphomatoid papulosis, type A.

Computerization of image analysis has made microscopic DNA measurements faster, and permitted use of more complex algorithms for the analysis of the DNA distribution [25]. Further developments in computerized TV-image analysis systems, such as autofocus, will help to make DNA analysis easier and more rapid.

The DNA content of thousands of cells can be measured by flow cytometry, which was also used for the early diagnosis of cutaneous T-cell lymphomas [32]. It has been suggested that a hyperdiploid peak is diagnostic for malignant lesions, but such findings were also demonstrated in pseudolymphomas and lesions of positive patch tests [33]. Joensuu et al [34] performed DNA flow cytometry combined with fine needle aspiration biopsies for the differentiation between extracutaneous lymphomas and benign lymph nodes. DNA aneuploidy was demonstrated in only 11 of 35 malignant lymphomas. However, if the number of proliferative cells, a feature similar to the 2cDI in this study, was taken into account the diagnostic efficiency increased to 81%. In contrast to image cytometry, DNA flow cytometry is capable of detecting aneuploid cell lines close to 2c. However, in flow cytometry the interpretation of hypertetraploid signals of low frequency is difficult due to the high number of false alarms induced by cell aggregations in this region [35]. DNA-image cytometry permits visual examination of each cell and its segmentation (see Fig 5), thus excluding false alarms due to cell aggregation. Therefore, cells with a DNA value $\geq 5c$ in DNA image cytometry seem to be absolutely indicative for malignancy. However, we recommend that diagnosis should not only be based on such laboratory methods alone, but represent an integration of clinical signs and laboratory results.

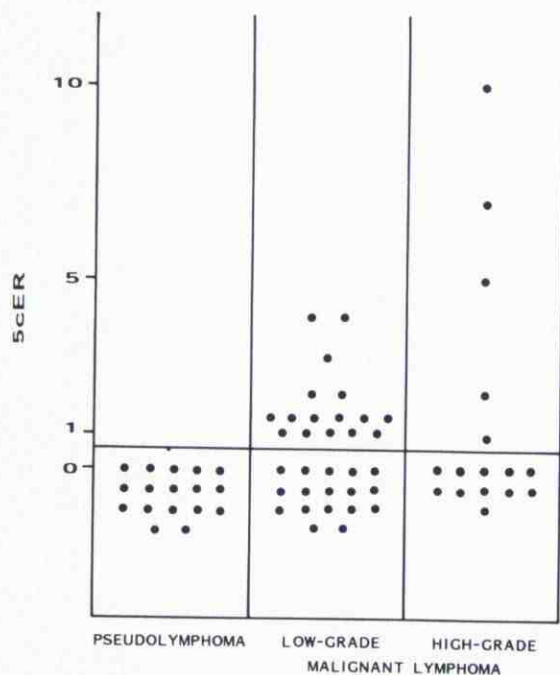


Figure 3. Differentiation between pseudolymphomas and malignant lymphomas of the skin using the 5cER. The 5cER had a specificity of 100% (none of the pseudolymphomas was classified falsely positive), but sensitivity was low (43%).

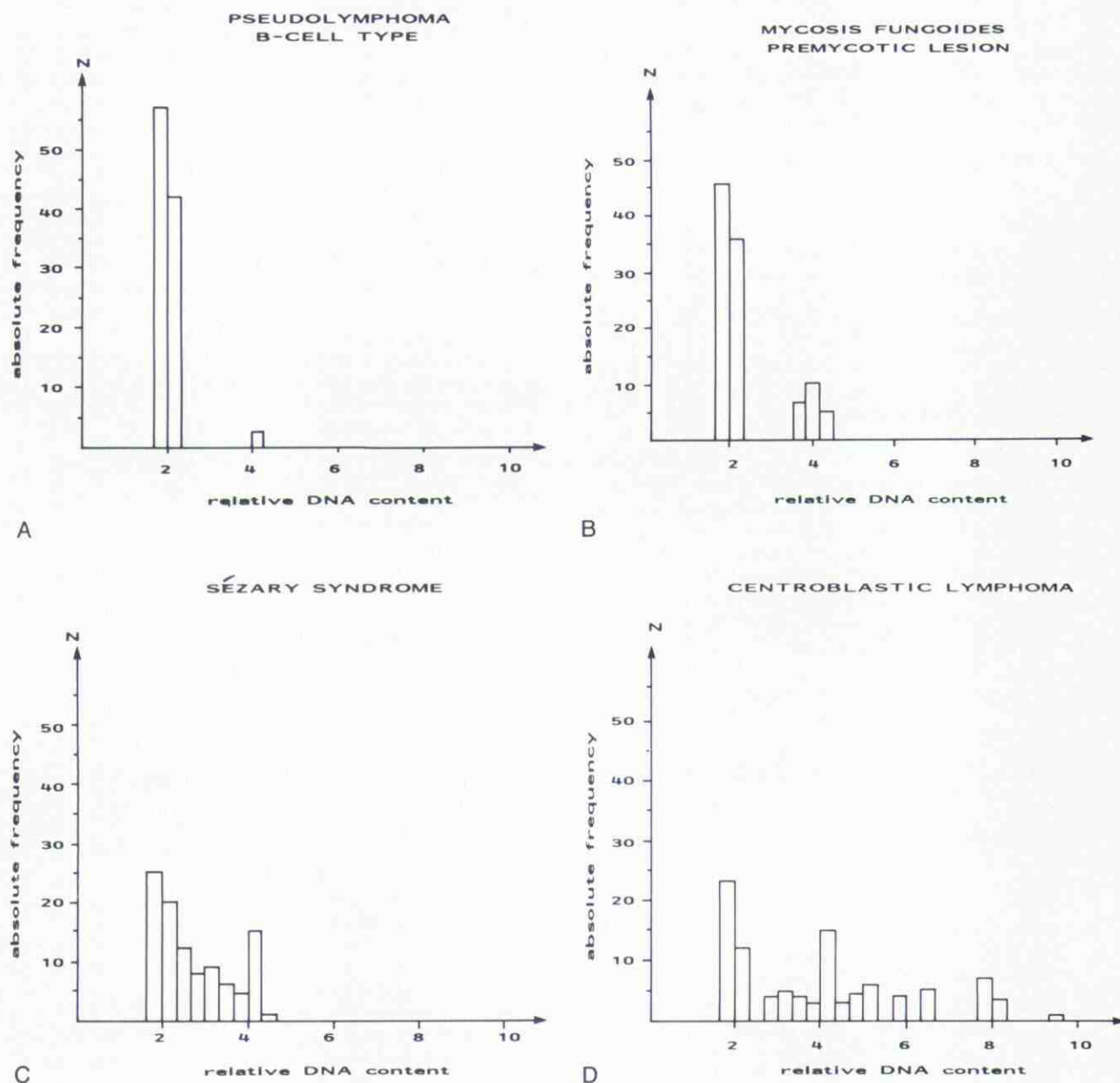


Figure 4. Characteristic patterns of DNA histograms. (A) Pseudolymphoma: cells with DNA content higher than 5c were not detected, and also a gap between 2.25c and 3.5c was present; (B) intermediate type of histogram with a 4c peak higher than 5%; (C,D) histograms of malignant lymphomas. Histograms resembling c lacked a gap between 2.25 and 3.5. Histograms similar to d showed cells with a DNA content of more than 5c.

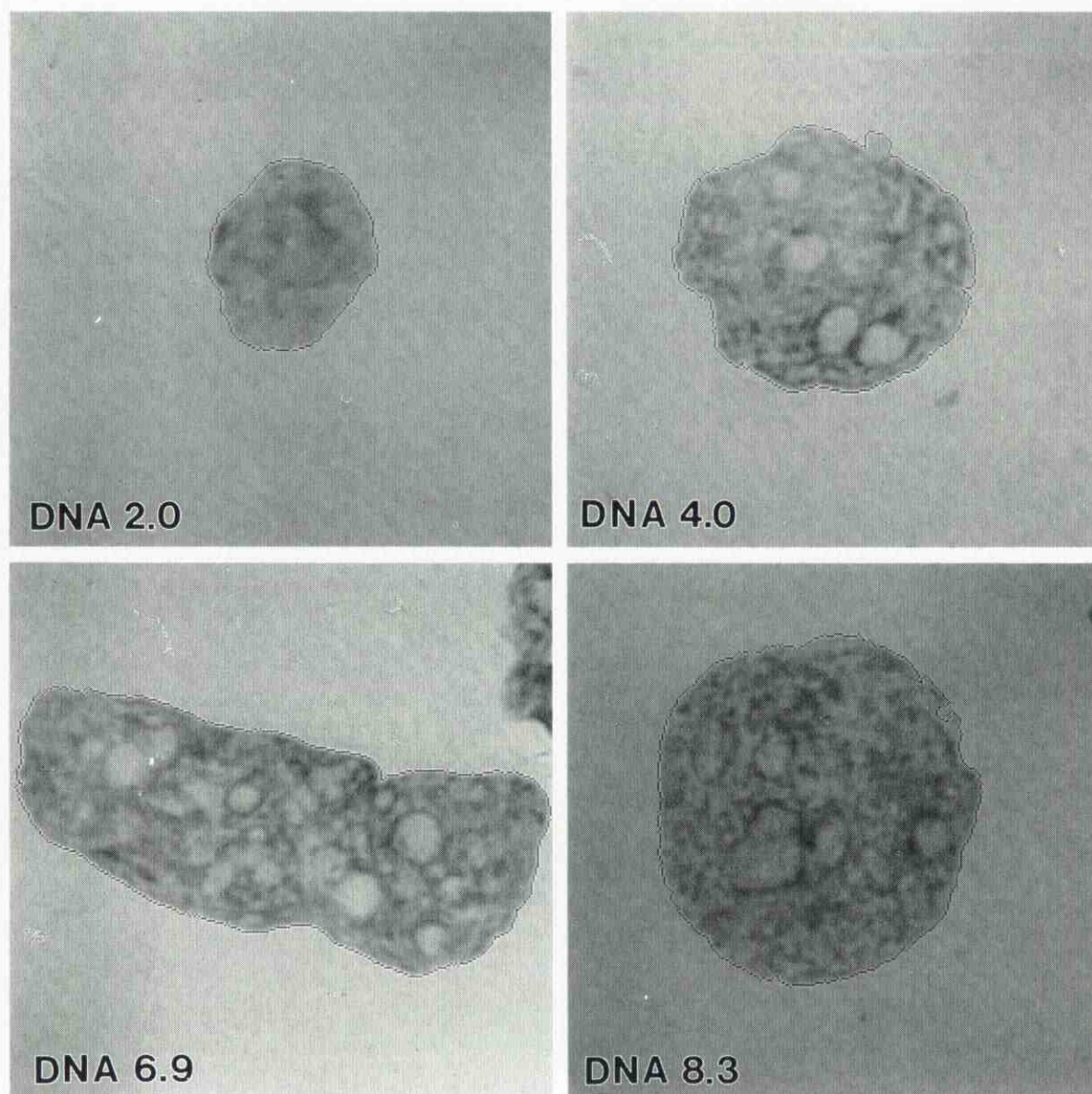


Figure 5. Examples for nuclei with different relative DNA values.

High-resolution image cytometry offers also the opportunity to study simultaneously DNA content and chromatin structure [36,37], as has been reported for lymphoid blood cells of cutaneous T-cell lymphomas [38]. Since abnormalities of chromatin structure may precede quantitative DNA changes [39,40], further insights in the field of ML can be expected from the combined analysis of DNA contents and chromatin structure.

The skillful technical assistance of E. Januschke, E. Ebmeyer, and I. Pfab is gratefully acknowledged.

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