Aneuploidy in Cutaneous T-cell Lymphoma

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A new method is described which makes it possible using skin specimens to perform flow cytometric analysis of DNA content. DNA content analysis was performed on 28 skin specimens and 9 blood samples from 18 patients with mycosis fungoides and Sézary syndrome. The reproducibility was fair, with almost identical results in 6 cases (mycosis fungoides and Sézary syndrome) where two samples (skin specimens or blood samples) were taken 6 hours to 10 days apart. Hyperdiploidy was found in 7 of 11 skin specimens from patients with mycosis fungoides stage I with negative histology. In 13 skin specimens and 3 blood samples from patients with mycosis fungoides stages II and IV, abnormalities including hyperdiploidy, near-tetraploidy and near-hexaploidy were found in 8 of 13 skin specimens and in 2 of 3 blood samples. Four patients with Sézary syndrome were studied: 2 patients in remission showed normal DNA histograms (2 skin specimens, 3 blood samples) and 2 patients with active disease showed aneuploidy in the 2 skin specimens examined and in 1 of 3 blood samples. These studies demonstrate: 1) the importance of flow cytometry as a diagnostic tool for use on skin specimens in the early stages of mycosis fungoides where routine histology is non-diagnostic; 2) the diagnostic and prognostic aid of flow cytometry during the course of mycosis fungoides and Sézary syndrome in addition to the probability of measuring the effect of treatment. (Received November 16, 1982.)

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Mycosis fungoides and Sézary syndrome are at present believed to be T-cell lymphomas that first involve the skin and then may become widely disseminated (5, 6, 7). The skin lesions of cutaneous T-cell lymphomas are non-specific in the early stages (3) and the lesions are classified as parapsoriasis en plaque, poikiloderma, psoriasiform dermatitis, eczematous plaques, or neurodermatitis (10). Histology is usually non-diagnostic in the early patch stage.

In order to characterize these cutaneous lymphomas better, van Vloten used microspectrophotometry to detect abnormal patterns of DNA content in malignant T-cells in situ (12) and found that those patients suspected of having malignant reticulosis yet also having normal DNA histograms usually had a benign course (13); also DNA-cytophotometry was considered an objective aid in the diagnosis of lymph node involvement (14). Later, flow cytometry measurements have been used to characterize haematologic malignancies (1, 11) and other malignant tumours (11, 2, 15) and this clinical application of flow cytometry has recently been reviewed (9). Bunn et al. (4) examined by flow cytometry blood and lymph nodes from patients with cutaneous T-cell lymphomas and demonstrated aneuploidy in 32 out of 46 patients (4). However, only in a few cases have flow cytometric measurements been performed on skin biopsies because of the technical difficulties of cell preparation (17).

[1,2] Züricher Beuteltuchfabrik AG, Scrynel monofilament polyester fabric PE 100 HD and nylon fabric NY 30 HC.

The aim of the present study was to demonstrate the presence of aneuploidy on skin specimens by flow cytometric DNA analysis in the various stages of mycosis fungoides and Sézary syndrome, with special emphasis on the early patch stage where histological criteria for the diagnosis are lacking. We present here a new method by which it is possible, using skin specimens, to perform flow cytometry analyses on dermal cell infiltrates.

MATERIAL AND METHODS

Analysis of DNA distribution was performed on 18 patients with cutaneous T-cell lymphoma (mycosis fungoides (MF) and Sézary syndrome) and on 16 controls.

The control specimens comprised 32 peripheral blood samples (2 from each control person) from 16 normal volunteers, 8 males and 8 females, with a median age of 36 (range: 26–53 yrs). The 18 patients included 6 patients with MF stage I (11 skin specimens), 7 patients with MF stage II (12 skin specimens and 2 blood samples), 1 patient with MF stage IV (1 skin specimen and 1 blood sample) and 4 patients with Sézary syndrome (4 skin specimens and 6 blood samples). The median patient age was 70 years, with a range of 29–93 years.

Classification of mycosis fungoides

The patients were distributed according to the classification used by the Scandinavian Mycosis Fungoides Group (8). Stage I: erythematous stage with negative histology; stage II: plaque stage, histologically showing a mixed dermal cell infiltrate with atypical lymphocytes and/or Sézary cells and with epidermal infiltration; stage III: tumour stage; stage IV: cutaneous tumours and lymph node involvement; stage V: cutaneous tumours and involvement of lymph nodes and viscera.

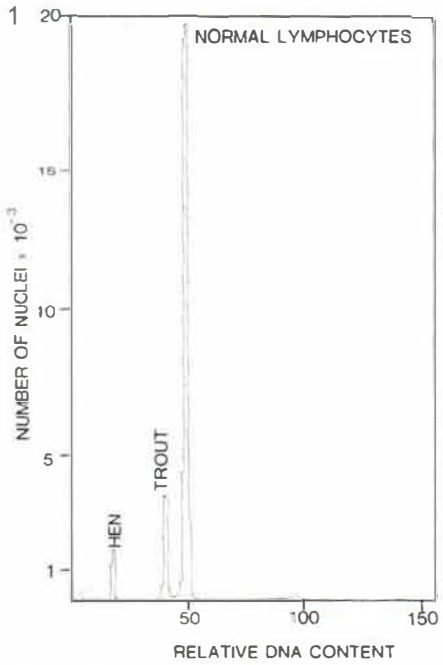
The patient with MF stages I and II were untreated for at least 2 months except for topical steroid which was discontinued one week before the examination. The patient with MF stage IV was treated with 8-methoxsalen plus UVA and systemic corticosteroids. Of the 4 patients with Sézary syndrome, 2 (H. H., K. A.) were studied when they were in remission, one (N. K. J.) was treated with weekly i.m. methotrexate and one (E. L.) was treated with oral cyclophosphamide and systemic corticosteroids. Three 4-mm skin punch biopsies were taken and stored in liquid N_2. Before DNA analysis the epidermis was separated by a tangential cut through the frozen biopsy. The remaining dermal biopsy samples were immediately placed in 2 ml calcium- and magnesium-free phosphate-buffered saline with 1% fetal calf serum. Then the biopsies were cut into pieces smaller than 1 mm and shaken vigorously for 20 sec. The sample was filtered through a 100-μm filter[1] and centrifuged. The sediment was carefully resuspended with a hypotonic buffer solution (Tris 10 mM, EGTA 1 mM, HCl to pH 7.4) containing detergent (Nonidet P40, BDH Chemicals, 1% v/v), propidium iodide (Sigma, 50 mg/l) and ribonuclease (Sigma Type 1A, 100 mg/l). The resulting nuclear suspension was allowed to stain for 15 min and filtered through a 30-μm filter.[2] Finally a similarly stained preparation of trout and hen erythrocyte nuclei, which are kept in liquid N_2, was added to provide internal DNA standards (16). In each sample the DNA content of at least 50000 nuclei was measured without and with internal standards in a Becton Dickinson FACS IV flow cytometer. Measurement with two internal standards allows zero-point adjustment.

The DNA peak values were estimated by maximum likelihood, assuming normally distributed measurement error. The DNA index of patients was calculated as follows. The patient sample peak was divided by the simultaneously measured trout peak, and this result was divided by the mean value of normal male or female lymphocytes relative to trout. When the DNA index was calculated on a basis of hen erythrocytes, almost identical results were obtained.

When skin biopsies were taken for flow cytometric analysis, a biopsy for histological examination was taken simultaneously from the same lesion. Before staining of DNA, a part of the sample was cytocentrifuged and examined cytologically and compared with the histology of the skin biopsy. In 16 patients out of 18, an accordance between the cytocentrifuged sample and the histological examination was found. In addition separated epidermis and dermal fragments were examined histologically to ensure that no major fraction of lymphoid cells was excluded from the preparation for flow cytometry.

Peripheral blood samples were collected in preservative-free heparin and subjected to Ficoll–Hypaque density separation (4). The mononuclear cells from the interface layer were collected and washed with phosphate-buffered saline. Thereafter suspended in ice-cold buffer (sucrose 250 mM, DMSO 5% v/v, sodium citrate 40 mM, pH 7.6) and frozen for storage in liquid N_2 (15). After thawing, the samples were stained as described for the skin specimens.

From 6 male mice and 6 female mice, 2–4 months old, liver specimens were stained and analysed as the skin specimens.



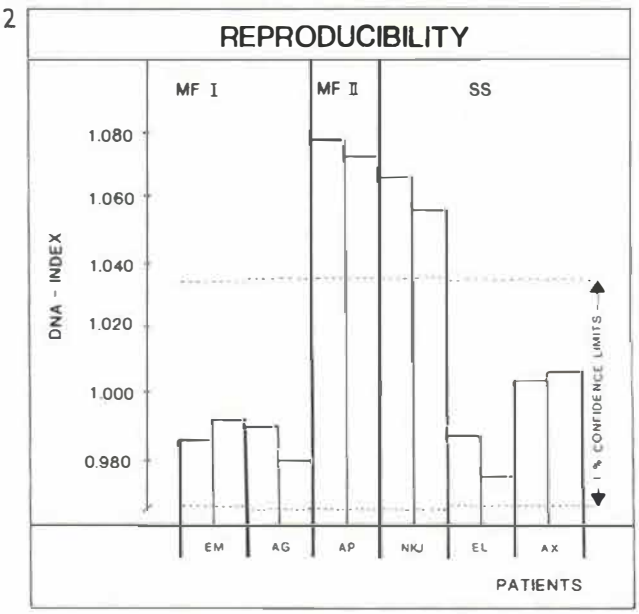


Fig. 1. DNA distribution of peripheral lymphocytes from a control person. First and second peaks indicate hen and trout erythrocyte DNA contents.

Fig. 2. Six cases where skin specimens or blood samples were taken twice with intervals of 6 hours to 10 days. Each column indicates one sample.

RESULTS

Normal controls

The mean ratio between normal diploid human lymphocytes and trout nuclei was calculated separately for females and males, giving the values 1.228±0.017 for females and 1.201±0.017 for males, where SD's include the interindividual variation. As expected, the female mean value was significantly higher ($p<0.001$) than the male. An example of the DNA distribution of peripheral lymphocytes from a control person, with hen and trout erythrocytes as internal standards, is given in Fig. 1. The 1% confidence limit of the DNA index of normal diploid lymphocytes was ±0.036 for both males and females. From rat liver samples the normal position of tetraploid relative to diploid rat nuclei was assessed as 1.979, with a SD of 0.013 including the variation between sexes. The reproducibility of repeated sampling from patients is fair, as in from Fig. 2, which shows 6 cases where two samples (skin specimens or blood samples) were taken 6 hours to 10 days apart. Almost identical results were obtained in all 6 cases.

Six patients with MF stage I had 11 skin specimens examined. In 4 patients, a hyperdiploid G_1 peak was found in 7 skin specimens (Table I). It was necessary to fit two normal distributions to the near-diploid region of the histogram, an example of which is given in Fig. 3. In two skin specimens the means of these two peaks were both significantly different from the normal diploid (Table I: patient B. B. N., patient K. L., sample 3). In the remaining five skin specimens, one of the means was not significantly and the other was significant. The secondary peak varied in the seven skin specimens, between 9% and 25% of the cell population with a median of 17%. In patient K. L. a tetraploid peak was also observed in two skin specimens.

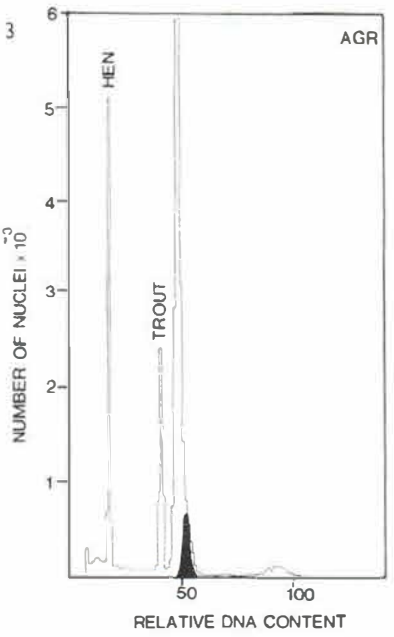
Seven patients with MF stages II had 12 skin specimens and two blood samples examined. In 3 of the patients, aneuploidy was found in six skin specimens and one blood sample (Table I). Patient A. P. showed sequential hyperdiploidy from the same plaque and had only one normal skin specimen. Patient A. G. showed a skin specimen with a hypotetraploid peak which represents 15% of the cell population. A blood sample from the same patient showed a hyperdiploid peak, a tetraploid peak (9%) and a hexaploid peak, which represents 4% of the cell population (Fig. 4).

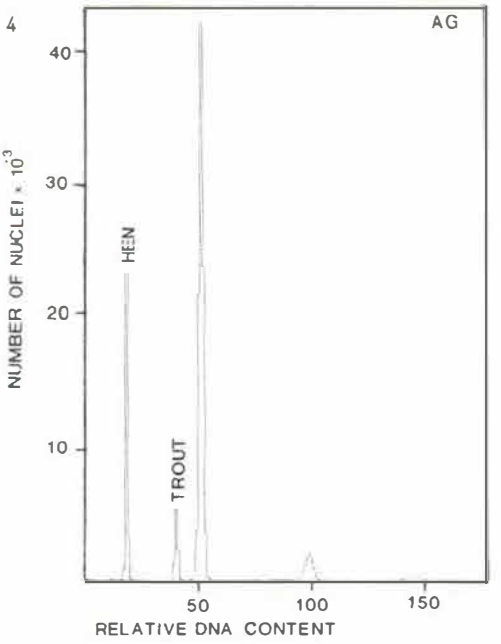
Table I. *DNA-index of skin specimens from patients with mycosis fungoides and Sézary syndrome*

Parentheses indicate % of cell population

Dia-gnosis	Patient	Sex	Age	Sample	DNA indices			
					Regions			
					Diploid		Tetraploid	Hexaploid
MF I	B. B. N.	♂	29	Skin	1.094**	1.181** (25)		
	K. L.	♂	70	Skin	1.025			
				Skin	1.020	1.133** (9)		
				Skin	1.048**	1.126** (22)	1.989 (3)	
				Skin	1.001	1.067** (17)	1.937 (5)	
	E. M.	♀	77	Skin	0.986			
				Skin	0.981			
	V. J.	♂	78	Skin	0.999	1.082** (17)		
	K. S. P.	♀	70	Skin	1.015			
	A. G. R.	♂	80	Skin	0.990	1.070** (10)		
					0.979	1.038** (25)		
MF II	A. G.	♀	69	Skin	1.031*			
				Blood	1.018			
				Blood	1.036**		1.998 (9)	2.923** (4)
				Skin	0.986		1.878** (15)	
	B. J.	♀	73	Skin	0.981			
	A. P.	♂	60	Skin	1.078**			
					1.072**			
					1.083**			
					1.058**			
					0.994			
	M. J.	♂	69	Skin	1.016			
	L. P. J.	♂	88	Skin	1.110**			
	A. K.	♂	93	Skin	1.008			
	F. R.	♂	57	Skin	1.017			
MF IV	E. C.	♀	56	Blood	1.060**			
				Skin	1.064**			
SS	N. K. J.	♂	78	Skin	1.066**		1.970 (62)	
				Skin	1.056**		1.956 (71)	
	E. L.	♀	64	Blood	1.068**		2.028** (33)	
				Blood	0.987			
				Blood	0.975			
	K. A.	♀	62	Skin	1.022			
				Blood	1.004			
				Blood	1.006			
	H. H.	♂	74	Skin	0.983			
				Blood	0.990			

* $p<0.05$, ** $p<0.01$ according to t-distribution.





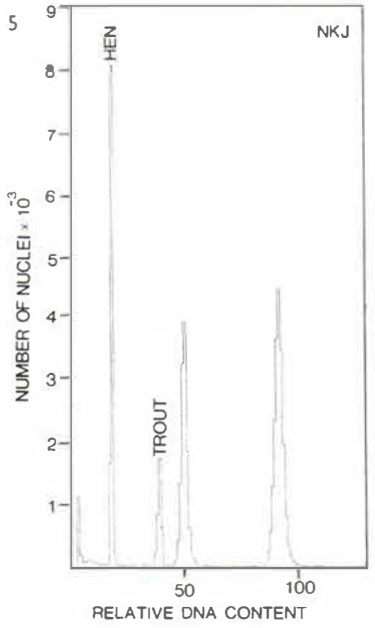


Fig. 3. A patient (A. G. R.) with mycosis fungoides I. Two normal distributions were fitted to the near-diploid peak in the DNA histogram. The first and second peak indicate hen and trout erythrocytes.

Fig. 4. A patient (A. G.) with mycosis fungoides II showing a hyperdiploid, near-tetraploid and a hexaploid peak. The first and second peak indicate hen and trout erythrocytes.

Fig. 5. A patient (N. K. J.) with Sézary syndrome showing a hyperdiploid peak and a tetraploid peak representing ⅔ of the cell population. The first and second peak indicate hen and trout erythrocytes.

One patient, E. C., with MF stage IV, demonstrated a hyperdiploid peak in both a skin and blood sample (Table I). Of the 4 patients with Sézary syndrome, 2 showed abnormal DNA histograms. Patient N. K. J. had hyperdiploid peaks and tetraploid peaks which represented 62% and 71% of the cell population in two skin specimens (one illustrated in Fig. 5). Patient E. L. showed in one out of three blood samples a hyperdiploid peak and a hyper-tetraploid peak which represents 33% of the cell population (Table I).

DISCUSSION

The results obtained with normal peripheral blood lymphocytes showed very good reproducibility of the flow cytometric DNA analyses. That the reproducibility is fair is also seen from Fig. 2, where almost identical values were obtained in 6 cases where samples from patients were taken 6 hours to 10 days apart. Patient A. P. (Table I) had altogether five sequential skin specimens examined from the same plaque over a period of 9 weeks. Based on separate comparison with hen and with trout erythrocytes, four sequential skin specimens showed almost identical hyperdiploid G_1 peaks, whereas the last biopsy was normal, probably due to the histological heterogeneity of the plaque. Thus, although the technique demonstrates reproducible results, the histological heterogeneity must always be taken into consideration. Van Vloten found normal DNA histograms in cases where mycosis fungoides was found histologically and attributed this to sampling error in the imprint preparation and measurements (13). This stresses the necessity of repeated biopsies in all stages of cutaneous T-cell lymphomas. Although the various measurements on skin and blood in patient A. G. (MF II) (Table I) may suggest staining differences between the two samples, no such difference was found in patients E. C., K. A. and H. H. (Table I), thus invalidating this hypothesis.

Patients with MF stage I (Table I) showed aneuploidy in 4 out of 6 cases. In these patients it was necessary to fit two normal distributions to the near-diploid region of the histogram and the second G_1 peak was estimated to be 9–25 % of the cell population. These results are important, since the histology was negative, with no evidence of MF.

Van Vloten found that a normal Feulgen DNA-histogram favoured a benign course, whereas an abnormal DNA-histogram was correlated to subsequent histological features of malignancy (13). Bunn et al. (4) also demonstrated that the presence of aneuploidy at any time in the course of cutaneous T-cell lymphoma implied a poor prognosis. In the present study, however, it has not yet been possible to establish any correlation to the critical course.

In 3 patients out of 7 with MF stage II, aneuploidy was found to be correlated to the abnormal histology (mixed dermal cell infiltrate with atypical lymphocytes infiltrating the epidermis). But as 4 patients with MF stage II showed normal DNA histograms it could be suggested that though aneuploidy is not completely parallel to the histological changes, it might yet have prognostic implications indicating a more malignant course. From these findings it can be concluded that flow cytometric measurements and routine histology have to complement one another and this is also in accordance with the opinion of Bunn et al. (4).

The patient with MF stage IV showed aneuploidy in both the blood sample and skin specimen, a finding in agreement with previously published results (4).

Two of the patients with Sézary syndrome showed normal DNA histograms (K. A., H. H.) and both were in a remission phase without sign of active disease. Two other patients with Sézary syndrome demonstrated aneuploidy (N. K. J., E. L.) which could be expected as their disease was active.

The most characteristic and important type of DNA abnormality was hyperdiploidy. This change was seen in all stages but mostly in the early stages of mycosis fungoides both with and without positive, confirmative histology. Peaks indicating cell clones with pronounced hyperploidy (near-tetraploid and -hexaploid regions) were seen in one patient with MF stage II (A. G.) and in 2 patient with Sézary syndrome. As expected, such peaks were related to advanced disease. The finding of hyperdiploid peaks emphasizes the need for an external diploid standard. Comparison with this was in the present study achieved by the use of two internal standards added to each sample, a principle introduced by Vindeløv et al. (16). Only a few skin specimens from cutaneous T-cell lymphoma have

previously been studied (17). Thus Vonderheid et al. (17) in one case of mycosis fungoides demonstrated two subpopulations of cells in blood and lymph node, but only one small diffuse cluster of cells from the skin specimens.

We want to conclude by saying that this is a new method by which it is possible by using skin specimens to obtain reproducible DNA-index values. The most important result was the finding of hyperdiploidy in mycosis fungoides stage I, where histology is negative. Furthermore, we found aneuploidy in the more advanced stages of MF. In active Sézary syndrome, aneuploidy was present but not when the disease was in remission. These results emphasize the need for flow cytometry as a diagnostic tool for use with skin specimens in the early stages of cutneous T-cell lymphoma where the histological examination is non-diagnostic, and as a complementary diagnostic aid during the course of cutaneous T-cell lymphoma. This new technique might also be used to measure the effects of treatment. The use of flow cytometry in the early stages of cutaneous T-cell lymphoma may in the future prove to have prognostic implications.

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