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Flow Cytometric DNA Analysis of Lesions from 18 Children with Langerhans Cell Histiocytosis (Histiocytosis x)

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The DNA content in 26 formalin-fixed, paraffin-embedded histologic specimens from 18 children with Langerhans cell histiocytosis (LCH) was analyzed by flow cytometry. In two cases, the propidium iodide fluorescence histograms showed small (5 and 3% of the analyzed nuclei) but significant aneuploid subpopulations with DNA indices of approximately 1.5. This was confirmed by analysis of unfixed, frozen material in one patient. Both patients had disseminated disease without organ dysfunction and were treated with prednisone. Currently, they are without signs of disease activity after 1 and 10 years. DNA histograms were normal from a patient who died from disseminated disease and from two patients with disseminated disease who experienced several relapses and various chemotherapeutical regimens. The histograms were also normal in lesions from four patients with unifocal bone involvement. Our results show that DNA aneuploidy occurs in LCH lesions in the pediatric age group. Further investigation is necessary to reveal whether DNA aneuploidy is restricted to disseminated LCH or its presence has any value in predicting the course and outcome of the disease. (Am J Pathol 1990, 136:1301–1307)

Langerhans cell histiocytosis (LCH) (Histiocytosis x) is a rare disease characterized by abnormal proliferation of cells phenotypically close to the Langerhans cells of the epidermis.[1] The disease covers a spectrum from unifocal bone lesions to widespread dissemination with multiorgan involvement.[2] Children are primarily affected, and there is general agreement that the disease is proliferative rather than neoplastic.[1]

In spite of extensive analysis of the pathoanatomic lesions, no morphologic clues have been found for predicting the course and outcome in LCH.[3] Recently, DNA aneuploidy was demonstrated by flow cytometry on fresh material from lesions of an adult patient with purely cutaneous LCH,[4] and it was suggested that DNA aneuploidy was indicative of a malignant proliferation. In another recent flow cytometric study, on paraffin-embedded material[5] from 36 pediatric and adult LCH patients, no DNA aneuploidy was detected, and it was concluded that DNA analysis may not be useful in predicting clinical stage and outcome in LCH.[6]

In the present study, we have analyzed lesions from 18 pediatric LCH patients by flow cytometry on paraffin-embedded material. The purpose was to investigate the possibility of DNA content heterogeneity and a possible correlation to the extent, course, and prognosis of the disease.

Material and Methods

Patients

A total of 35 cases diagnosed as Langerhans cell histiocytosis were found for review in the files of the Laboratory of Pediatric Pathology and the Department of Pediatrics, Rigshospitalet, University of Copenhagen, from 1968 to 1988. Seventeen patients were excluded because the histologic material was inadequate for analysis. Twenty-six formalin-fixed paraffin-embedded tissue blocks of sufficient size were available from 18 patients (Table 1). One patient (case 8) had received treatment (prednisone, vinblastine, methotrexate) before the initial biopsy. None of the remaining 17 patients had any treatment before the initial biopsy.

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Table 1. *Clinical Data and Results of Flow Cytometric DNA Analysis of 26 Langerhans Cell Histiocytosis Lesions from 18 Children*

Case	Sex	Age at biopsy	Tissue	DNA heterogeneity*	Organ involvement
1	F	14	Skin	(1)	Skin, lymph node, bone
2	M	14	Lymph node	(1)‡	Ear, skin, bones, lymph node
3	F	11	Temporal bone	1	Bone, lungs
		12	Lung	2 DI 1.4–1.5	
4	M	26	Temporal bone	1‡	Ear, skin, bones
5	F	132	Subcutis	1	Ear, skin, lymph node, lung
		240	Lung	1	
6	F	3	Lymph node	1	Skin, lymph node
7	M	11	Lung	1	Lymph node, lung
		11	Lymph node	1	
8	M	42	Gingiva	1‡	Bones, lymph node, gingival mucosa
		46	Lymph node	1	
		68	Lymph node	1	
9	M	11	Calvary	(1)‡	Skin, bones, bone marrow
10	M	51	Clavicle	1	Unifocal bone
11	M	132	Mandible	1	Unifocal bone
12	F	41	Ulna	1‡	Unifocal bone
13	M	72	Mandible†	2‡ DI 1.3	Multifocal bone
		72	Mandible†	1	
14	F	3	Skin	1	Bones, skin
15	M	5	Ulna	2 DI 1.5	Ear, skin, lymph node, bones
16	M	23	Calvary	1	Multifocal bone
17	M	144	Temporal bone†	1	Unifocal bone
		144	Temporal bone†	1	
18	M	12	Pelvic bone†	(2) DI 1.2	Multifocal bone
		12	Pelvic bone†	1	

* Number of distinct peaks in the propidium iodide histogram indicating a corresponding number of stemlines with different modal DNA content. The flow cytometric data shown are confined to the analysis of paraffin-embedded material.

† Indicating two separate biopsies within one macroscopical lesion. DI = (DNA index) calculated as the propidium iodide fluorescence of the minor population relative to the main population.

‡ Skewed main peak in fluorescence histogram.

( ) Indicated by only one of two or more measurements.

More than one specimen was available in seven of the patients. Three of these (cases 13, 17, 18) had bone lesions, from which two biopsies were taken simultaneously. In the remaining four patients (cases 3, 5, 7, 8), the biopsies were from lesions in different organs and there was an interval ranging from 1 month to 7 years between the initial and the subsequent biopsy. Two of these patients (cases 5, 8) received treatment between the first and the subsequent biopsies. In the remaining two patients (cases 3, 7), no treatment was given during the interval between biopsies.

Evaluation with regard to organ involvement was based on biopsy findings, and clinical information. Organ dysfunction was determined according to the criteria outlined by Lahey.[7]

Histopathology

The available slides were reviewed by one of the authors (KO). Recuts were made from all tissue blocks for hematoxylin and eosin (H & E) staining, and immunohistochemical staining for S-100 protein (DAKOPATTS, Copenhagen, Denmark) and peanut agglutinin (PNA) (DAKOPATTS). In three patients (cases 7, 8, 15), cryostat sections from snap-frozen material (−80°C) or fresh tissue imprints were available for staining of CD1a antigen (OKT-6, Ortho Diagnostics, Raritan, NJ). Ultrastructural examination had been done on lesions from six patients.

Flow Cytometry

The staining method for flow cytometric DNA analysis of paraffin material was a modification of the method of Schutte et al.[8] From each biopsy, two or more paraffin sections were cut with a thickness of 40 μm. The sections were separately processed and analyzed. They were deparaffinized in two changes of xylene for 1 hour at room temperature, and rehydrated in a sequence of 99%, 96%, and 70% ethanol, distilled water, and 0.01% Nonidet P40 (BDH Chemicals Ltd., Poole, United Kingdom) for 30 minutes at room temperature. They then were incubated in 1 ml 0.05% trypsin (Gibco, Grand Island, NY; 1:250, cat. no. 066-7072) in buffer (3.4 mmol/l [millimolar] trisodium citrate, 0.1% Nonidet P40, 1.5 mmol/l spermine tetrahydrochloride, 0.5 mmol/l TRIS, pH 7.6) and whirled slowly at room temperature overnight. The trypsinated sample

was vortexed (1000 rpm for 30 seconds), the remaining tissue was removed, and the sample was centrifuged and resuspended in 750 μl staining solution (50 μg/ml propidium iodide, 0.1 mg/ml RNase [Sigma Chemical Co., St. Louis, MO], R5503), 1 mg/ml trypsin inhibitor (Sigma, T9253), 0.01% Nonidet P40, 0.5 mmol/l ethylenediaminetetraacetic acid (EDTA), in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium, pH 7.2. The stained sample was slowly whirled for 1 hour at room temperature, and finally filtered through 30-μm nylon mesh (Scrynel NY HC 30, Züricher BeuteltuchFabrik AG, Zürich, Switzerland). The DNA fluorescence distribution was measured in a custom-built, microscope-based flow cytometer with a mercury arc lamp (excitation at 546 nm, emission at >590 nm).[9] The number of nuclei measured in LCH biopsies ranged from 2699 to 85,926 nuclei (median, 31,723 nuclei). Samples were excluded because of excessive debris or less than 2000 nuclei, resulting in only one measurement in case 1. Control samples of normal paraffin-embedded breast tissue showed a single, symmetric peak with coefficient of variation (CV) of 3% to 8%. The CV of the main peak in the LCH samples ranged from 3.1% to 9.0% (median, 5.3%). The DNA histograms were deconvoluted by maximum likelihood to give estimates of cell cycle fractions, G_1 peak means, and the CV.[10] This technique made it possible to identify subpopulations by their distinct peaks.

From patient 15, flow cytometric DNA analysis was also performed on unfixed, frozen material (−80°C) from the same biopsy as the paraffin-embedded tissue. Nuclear suspensions were made by use of detergent as well as detergent/trypsin,[11,12] and chicken and trout erythrocytes were used as internal DNA references.[13]

Results

Clinical Data

The clinical features are summarized in Table 1.

The age at which the biopsies were performed ranged from 3 months to 20 years (median, 24 months). Four patients had curettage of localized disease (unifocal bone lesion) requiring no further treatment. Fourteen patients had disseminated disease with either bone and/or soft tissue involvement in two or more sites. These patients all received prednisone, and in some cases, additional chemotherapy. Two patients (cases 2, 9) had organ dysfunction. One of these (case 9) never went into remission and died 11 months after presentation. The remaining 17 patients were all without signs of disease activity after follow-up periods from 9 months to 21 years (median, 6 $^2/_{12}$ years). However, two patients (cases 2, 8) experienced several relapses necessitating combination chemotherapy during the course.

Histopathology

All lesions showed a similar histomorphology: proliferation of large cells with reniform or grooved nuclei, evenly dispersed chromatin, and abundant eosinophilic cytoplasm (Figure 1A, B). Multinucleate cells were present in varying numbers in all lesions, but most prominently in the bone lesions. Macrophages, lymphocytes, and polymorphonuclear leukocytes were intermixed with the abnormal cells. Eosinophils and necrotic foci were noted in the bone lesions and in two lymph node biopsies. Nuclear atypia was absent and only very occasional mitotic figures were noted in the abnormal cells.

Diagnosis was confirmed by positive immunohistochemical staining for S-100 protein showing both nuclear and cytoplasmic positivity. Staining for PNA was also positive in all lesions with a characteristic membrane and paranuclear staining pattern in the proliferating cells. The three cases (cases 7, 8, 15) in which unfixed material was available showed strong anti-CD1a positive staining in the abnormal cells.

Birbeck granules were present in the proliferating cells in lesions from all six examined patients (cases 2, 3, 6, 7, 8, 15).

Flow Cytometry

In 4 of 18 patients (cases 3, 13, 15, 18), the propidium iodide fluorescence histograms measured in preparations of paraffin-embedded tissue showed an additional population with a mean fluorescence intensity 20 to 50% higher than that of the main population, which was defined as DNA diploid (Table 1). In two of these (cases 13 and 18), this was not considered to be significant because the aneuploid peak concerned less than 2% of total population, and furthermore, the additional peak was only found in one of the double measurements in one of the cases (case 18). In the two other patients (cases 3 and 15), the additional population concerned more than 2% of the total number of nuclei. The DNA index was 1.4 to 1.5. This was found in duplicate, in paraffin sections stained and analyzed on different days.

Patient 3, an 11-month-old girl, had patchy bilateral lung infiltrates and an lytic lesion in the right temporal bone. The secondary peak found in the lung lesion (Figure 1A) had a DNA index of 1.4 to 1.5 and concerned approximately 5% of the analyzed nuclei (Figure 2A). The DNA histogram from the temporal bone lesion biopsy a few days before that of the lung showed no DNA aneuploidy.

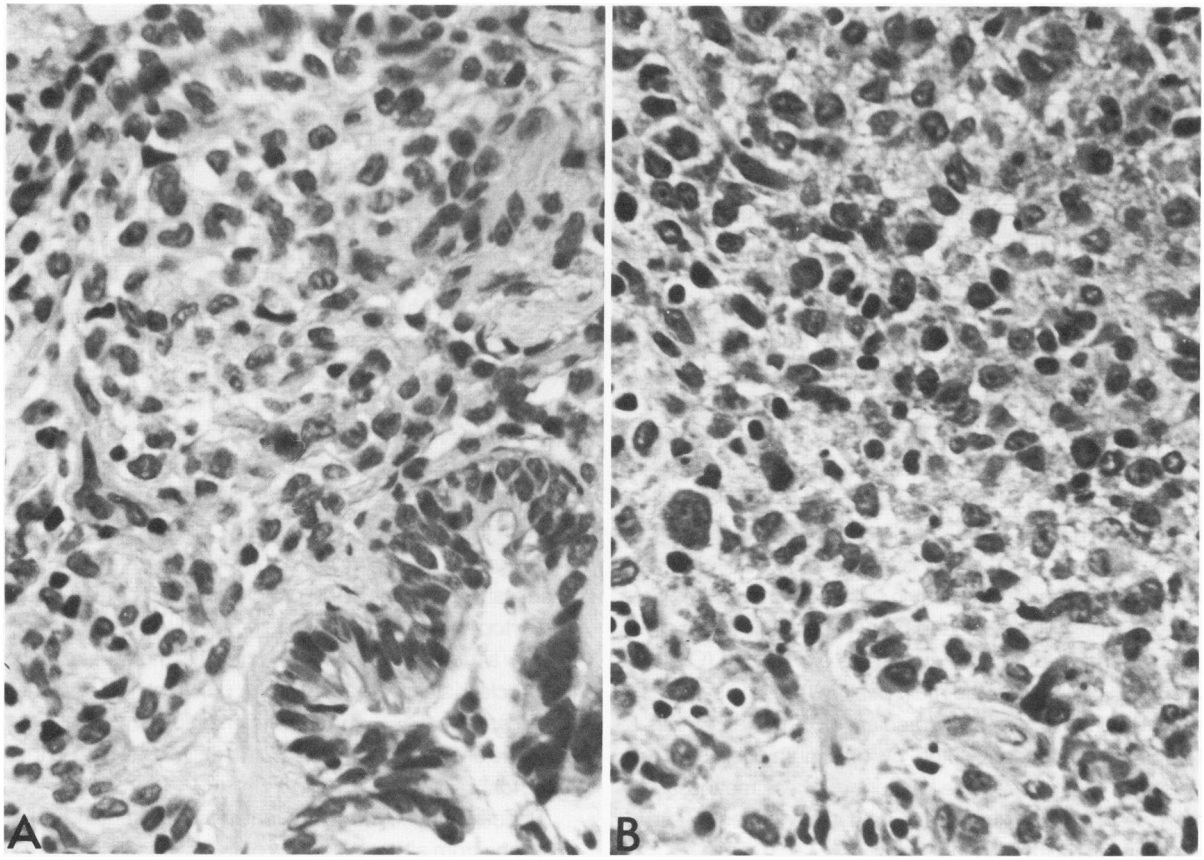

Figure 1. *LCH infiltrates with characteristic reniform or grooved morphology of nuclei, H&E.* **A:** *(case 3) Detail of lung lesion with infiltration around small bronchus (lower right-hand corner).* **B:** *(case 15) Detail of bone lesion from right ulna.*

A semiquantitative assessment of the lung lesion showed that 25 to 50% of the total number of cells in the specimen were either S-100–positive and/or showed the characteristic morphology of Langerhans histiocytosis cells. The patient was treated with prednisone and has shown no signs of disease activity during a follow-up period of 1 year.

Patient 15, a 5-month-old boy, had skin and bone (right ulna and 5. metatarsal bone) involvement, hepatomegaly, unilateral axillar lymph node enlargement, and chronic aural discharge. Electron microscopy of skin lesions (tissue not available for flow cytometry) showed Birbeck granules in the proliferating cells. The DNA fluorescence histogram of the ulnar lesion (Figure 1B) showed a secondary peak with a DNA index of 1.5, concerning approximately 3% of the analyzed nuclei (Figure 2B). Unfixed tissue from the ulnar lesion, stored for 9 years at −80°C, was retrieved, and flow cytometry performed on this material confirmed the presence of a secondary peak (DNA index, 1.6) concerning 11 to 15% of the analyzed nuclei (Figure 2C). A semiquantative assessment with regard to percentage of cells with Langerhans cell histiocytosis characteristics, by morphology and/or S-100–positivity, gave an estimate of 50 to 75% of the total number of cells in the bone lesion. This patient also received prednisone treatment. Follow-up is 10 years and he is currently without evidence of disease activity.

In six patients, the main peak in the fluorescence histogram was heterogenous or asymmetric (Figure 2D).

Lesions from the patient who died (case 9) and from two patients with multiple relapses (cases 2, 8) showed no DNA aneuploidy. Normal diploid histograms with no additional populations were also noted in lesions from the four patients with unifocal bone involvement (cases 10, 11, 12, 17).

Discussion

Goldberg et al[4] recently reported DNA aneuploidy by flow cytometry on fresh tissue from a skin lesion in an adult patient with purely cutaneous LCH. It was suggested that this could indicate a malignant proliferation of Langerhans cells. In another recent flow cytometric study on paraffin-embedded tissue from 36 LCH patients, Rabkin et al[6] found no DNA aneuploidy, implying that DNA content analysis may not be useful in predicting stage and outcome in LCH.

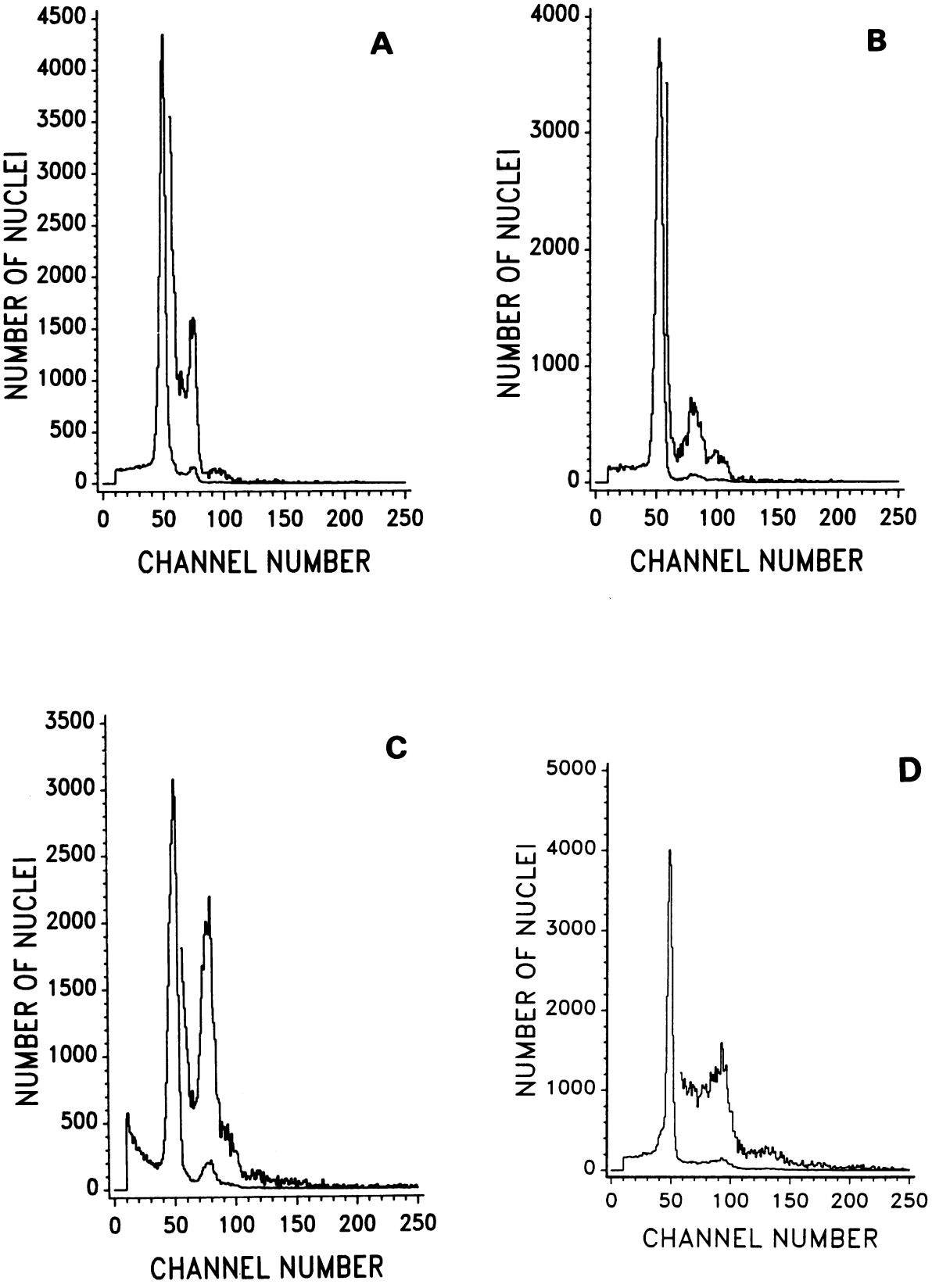

Figure 2. *Fluorescence histograms of propidium iodide-stained nuclei from lesions of Langerhans cell histiocytosis, indicating the presence of DNA aneuploid cells with a DNA-index of approximately 1.5,* A: *in lung lesion from a 11-month-old girl (case 3);* B, C: *in ulnar lesion from a 5-month-old boy (case 15).* D: *In six cases, the G_1 peaks were skewed to the left or right. These were fitted with one additional gaussian distribution. The subpopulations did not exceed 15% of the total number of nuclei and had a DI range from 0.8 to 1.3 (case 2 is shown). Nuclear suspensions were prepared from paraffin-embedded tissue sections* (A, B, D) *or from frozen, unfixed tissue* (C). *The right part of the histograms are also shown with a 10-fold amplification.*

In the present study of 18 LCH cases, we found significant DNA aneuploidy in lesions from two patients. Both had disseminated disease at the time of diagnosis and are without signs of disease activity after prednisone treatment with follow-up periods of 1 and 10 years, respectively.

The lesion from a patient who died with disseminated disease and organ dysfunction, as well as lesions from two patients with multiple relapses of disseminated disease and various chemotherapeutical regimens, showed normal DNA content. The DNA content was also normal in lesions from four patients with unifocal bone involvement. Thus, quantitative DNA analysis by flow cytometry does not appear to predict course or outcome in LCH, but it is possible that the presence of DNA aneuploidy in LCH may be restricted to disseminated disease.

The finding of abnormal DNA content in lesions from LCH is interesting, as there is agreement that the nature of LCH is one of cellular proliferation and/or immunodysregulation and not, neoplasia.

DNA aneuploidy has been shown to occur in some benign tumors, ie, thyroid,[14] parathyroid,[15] pituitary and adrenal adenomas,[16] colonic adenomas,[17] and cutaneous nevi.[18] Little is known about DNA content measured by flow cytometry in cells of inflammatory or reactive lesions. DNA aneuploidy has been demonstrated in Barrett's esophagus[19] and in histologically normal colonic mucosa from patients with ulcerative colitis.[20] Both conditions, however, are known to possess some malignant potential. In a study of 20 patients with inflammatory/reactive conditions,[21] we found DNA aneuploidy in biopsies from a Boeck's sarcoid lesion of the kidney and in nonspecific granulation tissue in relation to inflammatory pelvic disease. None of the patients in question developed neoplasia during follow-up periods of 3½ years.

With regard to the present study, a possible source for abnormal DNA content could be the multinucleate cells present in varying numbers in all lesions. However, if aggregation of diploid nuclei during measurements were responsible for the aneuploidy, polyploid DNA values, rather than the measured values with a DNA index around 1.5, would be expected unless additional artifacts such as fragmentation of the nuclei would occur. Assuming that the observed DNA aneuploidy in the LCH lesions is due to an increased number of randomly occurring mutations as a consequence of increased proliferation and is not due to artifacts, why does the disease process appear non-neoplastic? A possible explanation is, that the clone or group of cells with DNA aneuploidy were unstable, not able to divide, and hence, transient and not immortal. Another possibility is that the surgical or chemotherapeutical treatment was curative. In the present study, the answer to these questions is confounded by the surgical intervention and the prednisone treatment. DNA aneuploidy was found in a lung lesion, but not in an almost simultaneously biopsied temporal bone lesion in one of the patients (case 3). This finding of 'local' DNA aneuploidy is perhaps to be expected if the occurrence of DNA aneuploidy in presumable non-neoplastic, proliferative disease is looked on as an unstable epiphenomenon according to the above. Variability in the nature and degree of interaction between the microenvironment of the individual target organ and the proliferating cells could offer an additional explanation for this local occurrence of DNA aneuploidy. Clearly, multiparameter flow or image cytometric analysis are needed to further characterize the abnormal cell population.

The finding of DNA aneuploidy by flow cytometry in lesions without clinical and histologic evidence of malignancy should be interpreted with caution, as it might suggest a clonal evolution of unstable aneuploid populations.

In solid tumors, DNA aneuploid cell populations are generally reported with highest frequency in the DNA index (DI) regions < 1.2 and >1.4, and rather few are reported in the interval DI 1.2 to 1.4.[22–24] The possible admixture of DNA aneuploid nuclei with DNA diploid nuclei of activated or degenerating $G_{0/1}$ phase cells, which because of an altered chromatin structure may stain differently with propidium iodide, makes the detection of aneuploid populations with peridiploid genotype less efficient. However, relatively small aneuploid cell populations (2 to 5%) can be significantly demonstrated in the region around DI 1.5, especially in slowly proliferating tissues, because of little influence from staining and aggregation artifacts. In the present study, such cell populations were found in paraffin-embedded LCH lesions from the patients 3 and 15. In patient 3, the existence of a subpopulation with a DNA index around 1.5 was confirmed in unfixed, frozen tissue from the same lesion, containing 11 to 15% of these nuclei, ie, a larger fraction than measured in the paraffin-embedded specimen. This leads to the question, how the release of DNA aneuploid nuclei from paraffin-embedded tissue can be selectively increased to improve the detection of their presence and to ensure the comparability between biopsies from different tissues, as for instance skin and bone. It is quite probable that the optimal method for flow cytometric detection of aneuploid populations in paraffin sections has not yet been found, as might be indicated by the lower yield of aneuploid nuclei from paraffin-embedded material when compared with fresh material. Methodologic experiments with sequential sampling of nuclei released according to different enzymatic protocols might be necessary for further development of this technology.

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