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An Investigation of DNA Pyrimidine Isoplythes in Normal Human Lymphocytes and Under Chronic Lymphocytic Leukemia

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Abstract—*The problem of specificity of DNA cluster structure for human lymphocytes of chronic lymphocytic leukemia (CLL) was investigated. The preparations of DNAs from normal human lymphocytes and from the lymphocytes of CLL patients were degraded with diphenylamine in formic acid, and the released isostichs (under the general formula of* Py_nP_{n+1}) *were separated on DEAE-cellulose according to chain length. Each isostich fraction was subfractionated according to base composition on DEAE-cellulose at* pH 3.0. *It was found that the relative content of the first and second isostichs (mono- and dinucleotide DNA fragments) in CLL DNA is significantly lower in comparison with the normal. DNA. The relative content of the fifth and sixth isostichs (penta- and hexanucleotide DNA fragments) are significantly higher in CLL DNA, when compared to the relative content in normal DNA.*

The relative content of T_4, T_5 *and* T_6 *fragments in CLL DNA is sharply increased. The level of CLL DNA cytosine methylation in isostich fractions* I, II, III *and over is higher when compared to the level in normal DNA cytosine methylation in the same isostich fractions.*

INTRODUCTION

ACCORDING to contemporary conceptions it is supposed that the development of malignant tumors and leukemia result from the transformation of cells genome [1–4]. The biological functions of methylated bases in nucleic acids are not yet clearly understood. Borek has suggested that enzymic methylation of nucleic acids may play a role in differentiation and neoplasia [5]. In this respect, studies on DNA methylation under leukemia appear to be of particular theoretical and practical significance. As was previously established in our laboratory, newly-synthesized DNA from the lymphocytes and from the bone marrow of CLL patients is hypermethylated and has a low turnover rate [6, 7]. The above findings have led us to a suggestion that there is a change in DNA methylation specificity in leukemic cells.

The aim of the our work was to elucidate the differences in the relative content, composition and methylation of pyrimidine isostichs in DNA from normal human lymphocytes and from the lymphocytes of CLL patients.

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MATERIALS AND METHODS

1. *DNA degradation and oligonucleotide separation, identification and quantitation*

Lymphocytes from apparently healthy individuals served as the normal controls. Both donor and CLL patient lymphocytes were isolated from the heparinized peripheral blood as already described [8]. CLL patient lymphocytes were obtained at the time when the patients were not subjected to cytostatic therapy. The isolated lymphocytes were collected and the DNA extracted as previously described [9]. This procedure is essentially the same as it was proposed previously by K. S. Kirby [10] but for complete extraction of DNA in our procedure the water-saturated phenol (pH 8.3 instead of pH 7.0) was used. DNA from an RNA admixture was purified with 0.05 M sodium hydroxide. It was found that the alkali treatment of DNA does not change DNA base composition [11].

The method of Burton and Petersen was

generally used for DNA hydrolysis [12]. Twenty milligrams of the DNA from normal human lymphocytes and from the lymphocytes of CLL patients were dissolved in 20 ml of 66% (v/v) formic acid containing 2% (w/v) of recrystallized diphenylamine and incubated in the dark for 20 hr at 37°C. Then the hydrolysis mixture was diluted with an equal volume of distilled water. The diphenylamine and formic acid were removed by extraction with anhydrous diethyl ether. After removing residual ether by passing a stream of air over the surface of the hydrolysate, the solution was evaporated under reduced pressure at a bath temperature not exceeding 30°C. The hydrolysate was dissolved in a small volume of starting eluating solution (usually 2 ml) and loaded onto a column (0.9 × 29 cm) of DEAE-acetate (DE-32, 1 meq/g, dry weight "Whatman", England). The column was washed with 0.05 M lithium acetate (pH 5.6) to remove purines completely. Pyrimidine isostichs were separated with a linear gradient (total volume of 1000 ml) of 0–0.35 M lithium chloride in 0.05 M lithium acetate (pH 5.6) according to the method of Spencer *et al.* [13].

The analysis of the pyrimidine oligonucleotides released following specific depurination and hydrolysis of DNA by formic acid-diphenylamine is often used as the initial investigation of a DNA. There is enough evidence available for complete depurination of DNA under these experimental conditions as well as recovery from DEAE-cellulose column of pyrimidine isostichs to be determined [12–15]. The amount of pyrimidines in given isostich was determined with ultraviolet absorbance [14].

Individual pyrimidine isostich peaks from the pH 5.6 DEAE-column were pooled, diluted with equal volumes of water, and loaded onto columns (0.9 × 29 cm) of DEAE-formate for separation by base composition of the nonisomeric components of each isostich peak [15]. The amount of pyrimidines in each subfraction was determined by ultraviolet absorbance measurements.

2. *Methylation of pyrimidine isostichs of the newly-synthesized DNA from Pha-stimulated normal human lymphocytes and from the lymphocytes of CLL patients*

Both normal human lymphocytes and CLL patient lymphocytes were harvested under sterile conditions at room temperature by low speed centrifugation and washed three times with medium 199. 2.5×10^8 cells were suspended in medium 199 containing inactivated 20% serum of the AB (IVth) blood group, antibiotics (streptomycin, 100 mg/ml and penicillin, 100 units/ml) and phytohaemagglutinin "p" (0.125 ml) (Difco USA). The incubated mixture was saturated with a 5% CO_2 and was kept at 37°C for 72 hr in the case of normal lymphocytes and 96 hr for CLL lymphocytes because in the latter the stimulation peak is on average a day behind.

Twenty-five microcuries of equally labelled ^{14}C-deoxycytidine (specific activity 495 mCi/mole, Amersham, England) was introduced in the incubated medium 2 hr before the end of stimulation, it being kept for 1 hr at 37°C with repeated shaking. The labelled DNA was isolated with the DNA-carrier from normal and CLL patient lymphocytes. The purified DNA was hydrolyzed as described above and the pyrimidine oligonucleotides obtained were separated into isostich fractions. Isostichs I and II were collected separately, and the remainder of the nucleotide material (isostichs III, IV and more) was eluted from the column with 1 M LiCl. Isostich fractions were desalted with chromatography on columns with biogel P-2 (100–200 mesh, Calbiochem USA) and hydrolyzed in 58% $HClO_4$ at 100°C for 1 hr.

Five-methylcytosine (MC), cytosine (C) and thymine (T) were separated by two-dimensional thin-layer chromatography on cellulose as previously described [16]. The spots were eluated with 0.1 N HCl. ^{14}C-Radioactivity was determined in a scintillation counter "Mark-2" (Nuclear Chicago, USA). Counting efficiency was 80–85%. The percentage of isostich DNA cytosine methylation was determined by the formula:

$$\frac{\text{Radioactivity of MC (counts/min)} \times 100\%}{\text{Radioactivity of MC (counts/min)} + \text{radioactivity of C (counts/min)}}$$

The determination of cytosine methylation percentage by the above formula permits revealing the level of methylation of only that DNA fraction which is synthesized in the period of incubation with the radioactive precursor. All the data obtained were treated statistically after Student's method.

RESULTS

The results of our investigation are presented in Fig. 1 and in Tables 1–3.

Figure 1 shows that as a rule 8–9 isostichs

Table 1. Differences in the pyrimidine isostichs content in human normal and CLL lymphocytes (%). Sum total of isostichs I–VI *material is assumed to be* 100%.

	Experiment No.	Isostichs I	II	III	IV	V	VI
Donor lymphocytes	1	29.06	26.06	18.79	12.00	8.68	5.37
	2	27.16	24.07	19.75	16.17	7.77	5.06
	3	30.35	24.57	18.79	14.45	7.48	4.33
	M±m	28.85± ±0.92	24.90± ±0.59	19.11± ±0.31	14.41± ±1.22	7.78± ±0.38	4.92± ±0.35
CLL lymphocytes	1	23.08	21.35	18.39	15.86	11.98	9.32
	2	23.56	21.90	18.76	15.40	11.56	8.79
	3	23.59	21.48	19.08	15.80	11.41	8.60
	M±m	23.41± ±0.10	21.57± ±0.09	18.67± ±0.19	15.68± ±0.13	11.65± ±0.16	8.90± ±0.21
	P	<0.01	<0.01	<0.02	<0.02	<0.001	<0.001
	CLL / normal	0.81	0.87	0.98	1.10	1.50	1.81

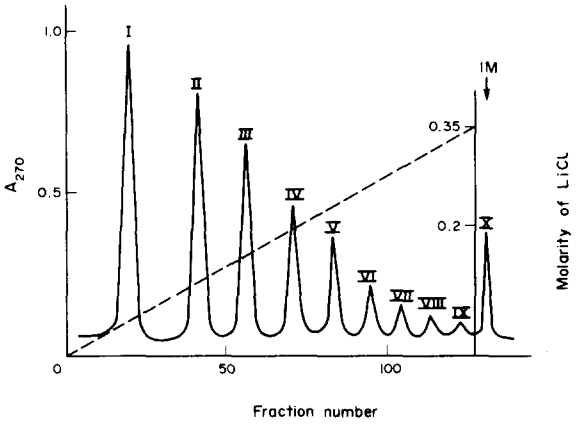

Fig. 1. Chromatography of diphenylamine-formic acid hydrolysates of DNA from normal human lymphocytes on DEAE-cellulose (0.9 × 29 cm, *acetate form*). *The hydrolysate* 20 mg *of DNA* (66% *formic acid*, 2% *diphenylamine and formic acid were removed. The column was washed with* 0.05 M *lithium acetate* (pH 5.6) *to remove purines and orthophosphate completely and then eluted with a linear gradient of lithium chloride from* 0 *to* 0.35 M *in* 0.05 M *lithium acetate* (pH 5.6) (*total volume* 1000 ml). *The absorbance of DNA eluates was recorded with a continuous flow ultraviolet monitor* (*Uvicord* II). *Fractions of* 10 ml *were collected. Chromatography was carried out at room temperature with an elution rate* 0.7 ml/min.

were revealed under conditions of the given analysis.

As is seen from Table 1, the relative content of isostichs I and II in CLL is significantly below normal whereas the relative content of isostichs V and VI is significantly above normal. The relation between the amount of isostich material in CLL and normal isostich material increases with size of isostich.

Table 2 shows the results of isostich chromatographic separation in components by the base composition. It can be seen that CLL isostichs shown the following uniform tendency as compared with normal isostichs: a decrease of the relative content of cytosine components and a rise of thymine components.

Data on pyrimidine isostichs methylation level of newly synthesized DNA from normal and CLL Pha-stimulated lymphocytes are given in Table 3. It can be seen that the greatest percentage of cytosine methylation is associated with mono- and dipyrimidine fragments of DNA from normal and CLL Pha-stimulated lymphocytes. The level of DNA cytosine methylation for CLL is 3.68 times more in isostich I, 2.25 times more in isostich II and 8.05 times more in polypyrimidine fraction (isostich III and over) than in the respective fractions of normal DNA isostichs.

DISCUSSION

Our data (Tables 1 and 2) demonstrate that the T/C ratio is significantly higher in DNA isostichs from CLL lymphocytes when compared with DNA isostichs from normal human lymphocytes. The revealed difference of the primary DNA structure from normal lymphocytes and from the lymphocytes of CLL patients perhaps reflect the changes of the ratio of CLL DNA fractions.

Comparative studies on DNA nucleotide composition in tumor and normal cells have so far failed to reveal in most of the experi-

Table 2. Content of different pyrimidine oligonucleotides of isostichs in the DNA of normal and CLL lymphocytes

Isostich No.	Composition	Content (%) Normal (n)	CLL	CLL 100% n
I	C	42.42	38.00	89.6
	T	57.58	62.00	107.7
II	C_2	27.50	26.90	97.8
	CT	45.60	43.90	96.3
	T_2	26.90	29.20	108.6
III	C_3	24.88	11.73	48.5
	C_2T	23.52	36.17	153.8
	CT_2	37.00	33.47	90.5
	T_3	15.00	18.63	124.2
IV	C_4	9.25	5.97	64.5
	C_3T	16.80	19.00	113.1
	C_2T_2	41.20	34.78	84.4
	CT_3	27.60	28.31	102.6
	T_4	5.15	11.94	231.8
V	C_5	8.80	7.65	86.9
	C_4T	14.95	12.75	85.3
	C_3T_2	29.05	25.25	86.9
	C_2T_3	29.65	31.45	106.1
	CT_4	10.95	14.55	132.9
	T_5	6.60	8.35	126.5
VI	C_6	4.55	2.53	55.6
	C_5T	16.35	10.55	64.5
	C_4T_2	25.82	17.55	68.8
	C_3T_3	29.08	25.45	87.5
	C_2T_4	15.10	24.70	163.6
	CT_5	7.20	13.60	188.9
	T_6	2.70	5.62	208.1

Table 3. The methylation level of pyrimidine isostichs of newly-synthesized DNA from normal and CLL Pha-stimulated lymphocytes (figures designate the percentage of methylation of every isostich)

	No. of experiment	Isostichs I	II	III and over
Donor	1	13.48	8.88	2.86
lymphocytes	2	10.65	7.00	2.80
	3	12.71	8.69	2.98
(A)	M ± m	12.66 ± 0.88	8.19 ± 0.61	2.88 ± 0.19
CLL (B)	1	46.49	16.58	24.58
Patient	2	46.36	20.35	22.67
lymphocytes	3	46.46	17.59	27.52
	4	46.62	17.07	19.35
	5	48.30	20.50	21.82
	M ± m	46.65 ± 0.37	18.42 ± 0.83	23.19 ± 1.36
	P	< 0.0001	< 0.001	< 0.001
	B : A	3.68	2.25	8.05

ments any significant differences. This appears to be explained by the fact that "tumor genes" constitute a small share of the total DNA, nucleotide composition being determined with rather a large error. The pyrimidine cluster analyses of DNA proves to be more sensitive in revealing characteristic distinctions in the primary structure.

By the method DNA–RNA hybridization it was shown that the DNA from human leukemic cells and from lymphomatous tissue contained the sequences that could not be detected in normal DNA [17].

Pero *et al.* have determined that the deoxyadenylate (dA) regions in DNA from both 7,12-dimethylbenzanthracene induced and Rous sarcoma virus induced rat tumors were changed considerably from the (dA) regions in normal rat DNA [18]. They have also reported that CLL DNA has alterations in (dA) sequences when compared to normal human DNA from lymphocytes, breast and placental tissues [19, 20].

With respect to the virus theory of cancer and leukemia origin it is of interest to note that A+T pairs content in the DNA of oncogenic viruses is more than in the non-oncogenic one [21].

The specifically striking differences in the methylation of relatively long pyrimidine isostichs in CLL lymphocytes in relation to normal lymphocytes are in agreement with our early findings [3]. The differences in methylation of pyrimidine isostichs of newly synthesized DNA from human lymphocytes and from CLL lymphocytes indicate possibly on the presence of specific leukemic DNA-methylases.

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