In Vivo Methylation of DNA in Human Leukocytes[1] (35899)

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Methylated purines and pyrimidines are found as minor components of the nucleic acids of most living organisms (1, 2). Fleissner and Borek (3) first reported nucleic acid methylation *in vivo* by demonstrating the transfer of methyl groups from methionine to methyl-deficient sRNA from *Escherichia coli*. Evidence has accumulated indicating that the methylation of adenine, guanine, cytosine, and uracil of sRNA (tRNA) and rRNA is catalyzed by six distinct enzymes (4), while a single enzyme appears to be responsible for the methylation of cytosine and adenine in DNA (5). The methylation of nucleic acids occurs on the polynucleotide rather than at the level of the mononucleotide precursors (6).

It has been reported that higher levels of methylated bases are present in nucleic acids of tumor tissues (7) and that SV-40 virus induced tumors have a higher incorporation of methyl groups than normal liver tissue (8). Studies on the methylation of nucleic acids of human neoplastic tissue show increased tRNA methylase activities in leukemic leukocytes and in carcinoma of the colon, rectum, and breast (9). Also, Viale *et al.* (10) have reported an increased amount of methylated bases in tRNA molecules isolated from human cerebral tumors. Silber *et al.* (11) have studied the incorporation of ^{14}C-methyl groups from radioactive methionine into DNA and RNA fractions of normal and leukemic human leukocytes. They found an increased rate of methylation in DNA and RNA of leukemic leukocytes, especially in acute leukemia, as compared with that in normal leukocytes; however, the relative abundance of identifiable methylated bases was not determined. This finding is probably not due to differences in either the rate of penetration of the amino acid or the rate of equilibration with preexisting pools since Baldessarini and Carbone (12) observed a very high elevation of *S*-adenosylmethionine in the leukocytes of leukemic subjects. However, recent studies have shown problems with some extraction methods used in previous DNA methylation reports (13). The amount of ^{14}C-methyl groups in hot acid extracts does not constitute a measure of the incorporation of (^{14}C-methyl) methionine into DNA. Hot tricloroacetic acid treatment of acid-insoluble DNA-protein pellets results in the extraction of ^{14}C-labeled oligopeptides and amino acids as well as free purines, pyrimidines, and apurinic oligodeoxynucleotides (13).

In view of these considerations, we have studied the incorporation of ^{3}H-labeled methyl groups into DNA of human leukocytes from several donors as listed in Table I and have identified the methylated bases in DNA hydrolysates. The leukocytes were separated by the dextran method of Skoog and Beck (14) from heparinized venous blood of acute leukemic subjects and from the leukocyte-rich fraction with the aid of a NCI-IBM blood cell separator (15) in normal and chronic lymphocytic leukemic subjects. Freshly prepared cells (3 to 7 $\times$ 10^8) were washed twice in 5 vol of cold saline, then incubated with 10 ml of Hanks' solution containing 500 μCi of L-(methyl-^{3}H) methionine (sp act of 6.2 Ci/mmole, Schwarz Bioresearch, Inc., Orangeburg, New York)

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TABLE I. Incorporation of ^{3}H-Labeled Methyl Groups into DNA of Human Leukocytes.[a]

Leukocyte cell type		Immature cells (%)	Sp act (cpm/mg of DNA)
Normal	(J.G.)	0	328
CLL	(C.J.)	3	853
CLL[b]	(C.R.)	4	640
CLL	(B.S.)	2	763
ALL	(B.S.)	84	2278
ALL	(L.M.)	86	2580
AML[b]	(N.C.)	72	1940

[a] Leukocytes were separated by Skoog and Beck's method (14), and washed twice in five volumes of cold saline. The cells (3 to 7 $\times$ 10^8) were incubated with 10 ml of Hanks' solution containing 500 μC of L-(methyl-^{3}H) methionine (sp act 6.2 Ci/mmole, Schwarz Bioresearch, Inc., Orangeburg, New York) for 60 min at 37°. DNA was extracted according to Kirby and Cook's method (16). CLL = donors with chronic lymphocytic leukemia; ALL = donors with acute lymphocytic leukemia; AML = donors with acute myelocytic leukemia.

[b] The two samples of CLL (C.R.) and AML (N.C.) were incubated with addition of 0.5 m*M* of cold unlabeled thymidine.

for 60 min at 37° in a water bath shaker (90 rpm). One leukocyte sample obtained from a patient with chronic lymphocytic leukemia (C.R.) and one sample from an acute myelocytic leukemia patient (N.C.) were incubated with addition of 0.5 m*M* unlabeled thymidine. DNA was extracted from the incubated cells, washed 3 times with cold saline according to the phenol-*m*-cresol procedure of Kirby and Cook (16). These DNA were dissolved in 0.15 *M* NaCl, 0.015 *M* Na_3 citrate (pH 7.0), and characterized by thermal denaturation profiles (T_M = 83.4 and 84.4°, respectively) and buoyant densities in CsCl (ρ = 1.698 and 1.699 g/cm^3, respectively). TCA-insoluble material was collected on Millipore filters (HAWP 02500, 0.45 μ pore size). The filters were dried and radioactivity was counted by liquid scintillation in a toluene base solvent containing 0.01% 1,4(2(4-methyl-5-phenyloxazolyl))-benzene plus 0.4% 2,5 diphenyloxazole in toluene. DNA concentrations were determined assuming $E^{0.1\%}_{260\,nm} = 20$.

As shown in Table I, the sp act of DNA were higher in leukocyte samples of acute lymphocytic leukemia (ALL) and acute myelocytic leukemia (AML) than in normal and chronic lymphocytic leukemia (CLL). These data are consistent with the results of Silber *et al.* (11).

^{3}H-DNA (1–3 mg) was exhaustively dialyzed against water, lyophylized to dryness, and degraded by formic acid hydrolysis (13). Internal standards of 120 μg each of 7-methylguanine and 5-methylcytosine were added as carrier before lyophilization. The resulting free bases were separated by two-dimensional descending paper chromatography (46 $\times$ 57 cm sheet of Whatman No. 1) with *n*-butanol:0.2 *N* NH_4OH (6:1, v/v) in the first dimension following by isopropanol–conc HCl–water (65:17.2:17.8 by vol) (13). The ultraviolet absorbing spots corresponding to each base were cut out of the chromatogram and placed in glass vials containing 20 ml of toluene–PPO–dimethyl POPOP scintillator and counted for 20 min. As shown in Table II, the highest radioactivity was found in 5-methylcytosine with moderate radioactivity in thymine. As mentioned above, the rate of methylation of DNA in acute leukemic leukocytes was much higher than that of normal and CLL leukocytes even after chromatographic analysis. The addition of cold thymidine depressed the radioactivity in thymine when the cases of ALL (B.S.) and ALL (L.M.) were compared with that of AML (N.C.).

Although the biological functions of methylated bases in nucleic acids are complex and not yet clearly understood, Borek (17, 18) has suggested that enzymic methylation of nucleic acids may play a role in differentiation and/or neoplasia. The previous studies concerning *in vivo* DNA methylation have indicated a temporal dependence of methylation upon DNA synthesis in mammalian cell cultures (13, 19). Scarano *et al.* (20) suggested that the methylation of DNA-cytosine might be one step in a series of reactions involving the interconversion of DNA pyrimidine bases leading to DNA base shifts which might represent points of initiation of RNA transcription. This is a testable

TABLE II. Incorporation of ^{3}H-Labeled Methyl Groups into DNA Bases of Human Leukocytes.[a]

Leukocyte cell type		Bases (net cpm/base/mg of DNA)					
		Guanine	7-Methylguanine	Cytosine	5-Methylcytosine (5-MC)	Adenine	Thymine
Normal	(J.G.)	0	0	0	16	0	2
		0	0	1	14	0	1
CLL	(C.J.)	0	1	2	36	3	12
		0	0	1	31	2	9
	(C.R.)	0	0	0	53	2	4
		0	0	0	42	1	6
	(B.S.)	0	0	0	31	0	6
		0	0	0	35	0	9
ALL	(B.S.)	0	3	8	332	9	107
		0	0	6	285	6	101
		0	0	0	415	6	114
	(L.M.)	0	3	3	468	5	107
AML	(N.C.)	0	0	7	581	0	23
		0	2	5	605	4	26

[a] Isotope counting was carried out for 20 min. 7-Methylguanine and 5-methylcytosine were used as carriers (120 μg of each base). Two analyses on each leukocyte sample are presented.

hypothesis and to our knowledge no reports have appeared comparing RNA polymerase initiation points in undermethylated and methylated DNA. Since methylation occurs at the polynucleotide level, cell proteins must recognize the nucleotide sequences at the site of methylation, leading to the suggestion that methylated bases function by affecting recognition sequences for cell proteins.

The purpose of cytosine methylation in the production and functioning of mature mammalian cell DNA is as yet unknown (21). Since acute leukemic cells have a lower rate of DNA synthesis (22) and a higher rate of DNA methylation (23) than do chronic myelocytic leukemic leukocytes the relationship, if any, between DNA methylation and DNA replication is not obvious. However, a dependence of continued DNA methylation upon DNA synthesis has been found in bacterial (24, 25) and Novikoff hepatoma cells (13).

The observation that a moderate incorporation of methyl groups into thymine occurred in the present short-term incubation is in contrast to the previous studies upon mammalian cell culture systems (19, 21). The scheme of Sneider and Potter (13) suggests this finding may be due to the possibility that deoxythymidine triphosphate can arise from the methylation of deoxyuridylic acid via $N^{5,10}$-methylenetetrahydrofolic acid and/or from the methylation of deoxycytidylic acid with subsequent deamination of 5-methyldeoxycytidylic acid to deoxythymidylic acid. The finding that the addition of cold thymidine depressed the incorporation into DNA-thymidine may support such a view.

The significance of the present data is conjectural since definitive experiments have not been reported on the biological role of methylated bases in DNA. It is quite possible that differences in DNA methylation *in vivo* in several types of human leukocytes may cause an altered conformation of primary and secondary DNA structures.

Summary. The incorporation of ^{3}H-labeled methyl groups into DNA from normal and leukemic human leukocytes has been studied. The sp act of DNA were higher in leukocyte samples of acute lymphocytic leukemia (ALL) and acute myelocytic leukemic

(AML) than in those of normal and chronic lymphocytic leukemia (CLL). The highest radioactivity was found in 5-methylcytosine with moderate radioactivity in thymine and low levels in adenine, 7-methylguanine, and cytosine.

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