Journal of Chromatography, 566 (1991) 481–491
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5812

5-Methylcytosine content of DNA in blood, synovial mononuclear cells and synovial tissue from patients affected by autoimmune rheumatic diseases

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ABSTRACT

The percentage of 5-methylcytosine (m^5Cyt) has been determined in peripheral blood, synovial mononuclear cells and synovial tissue from patients affected by various rheumatic autoimmune diseases. The determination was performed by reversed-phase high-performance liquid chromatography. Fifteen controls were compared to twenty-one patients affected by rheumatoid arthritis and to nine patients affected by systemic lupus erythematosus. The mean percentage of m^5Cyt in normal individuals was significantly higher than in the rheumatoid arthritis and systemic lupus erythematosus patients. In addition, patients with active disease showed lower values than patients in remission. This finding is in agreement with the hypothesis that DNA hypomethylation may play a role in the pathogenesis of the autoimmune diseases, resulting in altered oncogen expression. Therapy with cyclosporin A led to a decrease in the percentage of m^5Cyt in three rheumatoid arthritis patients, but a rebound was observed when the cyclosporin A was suspended. The percentage of m^5Cyt in the DNA of synovial tissue from four rheumatoid arthritis patients and five patients with osteoarthritis was similar; this observation confirms that, in addition to disease-specific and disease activity-specific variations, the percentage of m^5Cyt may also show tissue-specific variations.

INTRODUCTION

The occurrence of methylated cytosine or adenine in cellular DNA is almost universal [1,2]. Both 6-methyladenine and 5-methylcytosine (m^5Cyt) have been detected in prokaryotes; in the DNA of plants and higher animals methylated cytosine seems to have a prominent role among the methylated bases [2]. The concentration of m^5Cyt varies in a tissue-specific manner [1,2] and ranges from 4 to 6% of the total cytosine [1]. More than 90% of the m^5Cyt occurs in CpG sites.

The increasing interest in the m^5Cyt content of DNA has resulted from reports suggesting that it may play a role in: (a) DNA repair, transcription and replication [1–3]; (b) maintaining the chromosome structure [1,2]; and (c) controlling gene expression [4–6].

DNA hypomethylation, *i.e.* a decrease in the percentage of m^5Cyt, has been

0378-4347/91/$03.50

found in carcinogen-treated tissues [7–9], in most tumour cell lines [1,7,9,10] and in tissues exposed to drugs [11–14]. Recent reports suggest that DNA hypomethylation in autoimmune diseases [15,16] may result in altered oncogen expression [17–19]. Indeed, an altered expression of several oncogenes (c-*myc*, c-*fos*, c-*ras*), possibly due to DNA hypomethylation, has been described in systemic lupus erythematosus (SLE) [20,21], rheumatoid arthritis (RA) [20] and Sjögren's syndrome [22].

Several analytical methods have been used to separate, identify and quantify the minor methylated bases, and high-performance liquid chromatography (HPLC) has been extensively employed in the determination of m^5Cyt [23]. The reversed-phase technique used in this study displays consistent advantages over ion-exchange chromatographic methods in separating bases with respect to the elution speed, reproducibility and resolving capacity [24–27]. According to Diala *et al.* [17], the results obtained by HPLC analysis are comparable to those obtained by restriction-enzyme digestion and gel electrophoresis.

In this paper, the use of reversed-phase HPLC to investigate the genome-wide percentage of m^5Cyt in peripheral blood mononuclear cells (PBMC), synovial cells and synovial tissue from patients affected by various rheumatic autoimmune diseases is reported. The effect of immunosuppressive therapy with cyclosporin A (CSA) on the percentage of m^5Cyt is also assessed.

EXPERIMENTAL

Patients

Twenty-one patients affected by classical RA according to the 1989 revised ARA criteria [28] (sixteen females and five males, aged 47 ± 9 years) were studied. Seventeen of the patients were considered to have active disease as all of the following features were fulfilled: morning stiffness of more than 45 min duration; erythrocyte sedimentation rate (ESR) more than 30 mm/h; three or more swollen joints; nine or more tender joints on pressure. Four other patients were observed during a period of clinical remission. Three active RA patients underwent immunosuppressive therapy with CSA and were evaluated throughout this treatment.

Nine patients affected by SLE according to the 1982 revised ARA criteria [29] (seven females and two males, aged 35 ± 10 years) were also studied. Six of these patients were classified as having active disease, *i.e.* as presenting with two or more of the following: rash, fever, arthritis, serositis, alopecia, stomatitis, myositis or glomerulonephritis (as indicated by raised serum creatinine associated with haematuria and/or proteinuria > 500 mg per 24 h). In three cases the disease was in remission.

Fifteen healthy volunteers were used as controls.

Apparatus

A Beckman (San Ramon, CA, U.S.A.) 126 HPLC system equipped with a

Beckman 166 spectrophotometric absorbance detector operating at 280 nm was used for the HPLC analysis. The signal was integrated with a Shimadzu C-R6A Chromatopac integrator (Shimadzu, Tokyo, Japan). The column was a Beckman, 150 mm × 4.6 mm I.D. Ultrasphere ODS (5 μm spherical particles, 80 Å pore).

Reagents

The free standard bases used (adenine, cytosine, 5-methylcytosine, guanine, thymine) were supplied by Sigma (St. Louis, MO, U.S.A.). HPLC-grade water and methanol were from Carlo Erba (Milan, Italy); cellulose acetate syringe filters of 0.2 μm pore size were from Nalge (Rochester, NY, U.S.A.).

The other chemicals were of the highest purity available.

Preparation of standards and eluents

Stock solutions of standard nucleobases were prepared at a concentration of 1 *M* in HPLC-grade water (adenine) or methanol (the other nucleobases); 0.1 and 1 ml of 1 *M* hydrochloric acid were added to each sample, respectively. Working standard solutions were prepared by diluting the stock solutions to 10^{-3} *M* in water. The elution buffer (0.05 *M* KH_2PO_4 at pH 4.5) was prepared daily and filtered under vacuum through a 0.45-μm cellulose filter (Supelco, Meriden, CT, U.S.A.).

Sample preparation procedures

Cells and tissues. PBMC were obtained from heparinized venous blood by standard density gradient centrifugation with Ficoll–Hypaque (Flow Lab., Milan, Italy; density 1077 g/ml). Synovial mononuclear cells were collected after centrifugation of the synovial fluid (300 *g* for 15 min); the pellet was resuspended in saline, loaded onto Ficoll–Hypaque and centrifuged (600 *g* for 25 min at 18°C). The ring obtained from venous blood and synovial fluid was washed twice in RPMI-1640 (Gibco, U.K.), twice in 1% Tris–EDTA and pelletted. Finally, the cells were stored at −20°C until DNA isolation. Synovial tissue, obtained from a therapeutic arthrotomy, was homogenized, washed twice in 1% Tris–EDTA and stored at −20°C until DNA extraction.

DNA isolation and hydrolysis. DNA was isolated using sodium dodecyl sulphate (SDS), proteinase K (Merck, Darmstadt, Germany), RNAse A (Boehringer, Mannheim, Germany), phenol (Merck) and chloroform–isoamyl alcohol (Merck) extraction, and ethanol (Carlo Erba) precipitation, as described by Maniatis [30]. The cells were suspended in 1% Tris–EDTA, and SDS and proteinase K were added to a final concentration of 0.5% and 70 μg/ml, respectively. After incubation for 1 h at 55°C, one volume of phenol (presaturated with 1% Tris–EDTA) was added, gently mixed and centrifuged for 5 min at 700 *g*. Further extractions with phenol and chloroform–isoamyl alcohol (1:1, v/v) were performed. The DNA was precipitated in 99.7% cold ethanol and stored overnight at

−20°C. In order to remove any contaminating RNA, the DNA pellet was suspended in 1% Tris–EDTA and incubated with RNAse A (final concentration 10 μg/ml) for 25 min at 37°C. Following SDS, proteinase K, phenol and chloroform–isoamyl alcohol extractions, ethanol precipation steps were repeated. The DNA purity and quantity were assessed by spectrophotometric analysis (Perkin-Elmer 550/F, Norwalk, CT, U.S.A.). The purity was assessed by using the 260/280 ratio (acceptable values higher than 1.8) and the amount was measured spectrophotometrically at 260 nm. The DNA concentration ranged from 500 to 1200 μg/ml.

Purified DNA was hydrolyzed in polypropylene tubes by adding 200 μl of 72% perchloric acid (Carlo Erba) to each sample according to the method of Wilson and Jones [31] and incubated at 100°C for 1 h. The time and temperature of incubation were carefully controlled in order to avoid the possible deamination of m^5Cyt into thymine [24,31]. After neutralization with 3 *M* potassium hydroxide (460 μl to each 100 μl of perchloric acid), the samples were centrifuged for 5 min at 400 *g* and the clear supernatant was filtered through cellulose acetate syringe filters. The sample pH was finally adjusted to 4.5 by adding a few drops of 1 *M* hydrochloric acid and the sample was then stored at 4°C until HPLC analysis.

High-performance liquid chromatography

Patient samples (60 μl) containing an amount of DNA ranging from 30 to 70 μg were loaded at a constant flow-rate of 0.6 ml/min and eluted in a mixture of methanol–0.05 *M* KH_2PO_4 (1:99, v/v). The detection wavelength was 280 nm with a sensitivity of 0.1 a.u.f.s. The analyses were isocratic at room temperature in about 20 min. The column was re-equilibrated for at least 15 min after each run and washed overnight with a mixture of methanol–water (10:90, v/v). Peak identification was based on the retention times as compared with standard solutions analysed every three runs. The injection volume of the standard solutions was 30 μl, corresponding to about 3 μg of DNA. The purity of DNA (*i.e.* the absence of RNA) was confirmed by the absence of uracil peaks in the base chromatograms.

The percentage of m^5Cyt was calculated from the peak areas according to the formula [12]: $\%m^5Cyt = (m^5Cyt/m^5Cyt + \text{cytosine}) \times 100$.

Statistical analysis

The control group was compared to patient groups using the Kruskall–Wallis statistical analysis; the significance of the difference between each group has been evaluated by the Mann-Whitney test corrected according to Buonferroni's method for multiple comparisons. The coefficient of variation was obtained from the Stat View II calculator program supplied by Apple (Cupertino, CA, U.S.A.). The results represent the average of at least three determinations.

RESULTS

Chromatograms displaying the separation of a standard mixture of the five nucleobases (Cyt, m^5Cyt, Gua, Thy, Ade) and of a standard mixture of cytosine (Cyt) and m^5Cyt are shown in Fig. 1.

A good separation of the five DNA nucleobases was obtained both in the standard mixture and in samples (Fig. 2) in about 20 min. The retention times of Cyt and m^5Cyt were 2.76 and 3.62 min, respectively. The precision of the method is shown in Table I.

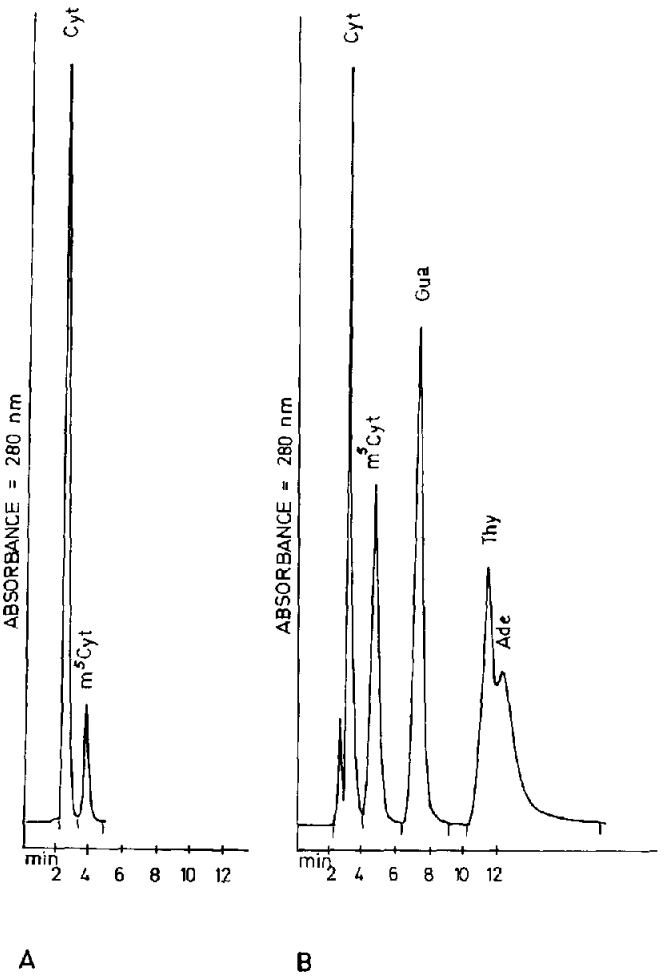

Fig. 1. (A) Separation of a standard mixture of cytosine and 5-methylcytosine. (B) Separation of a standard mixture of five DNA nucleobases (cytosine, 5-methylcytosine, guanine, thymine, adenine). Flow-rate: 0.6 ml/min. Absorbance: 280 nm. A.u.f.s. range: 0.1. Eluent mixture: methanol–0.05 *M* KH_2PO_4 (1:99, v/v).

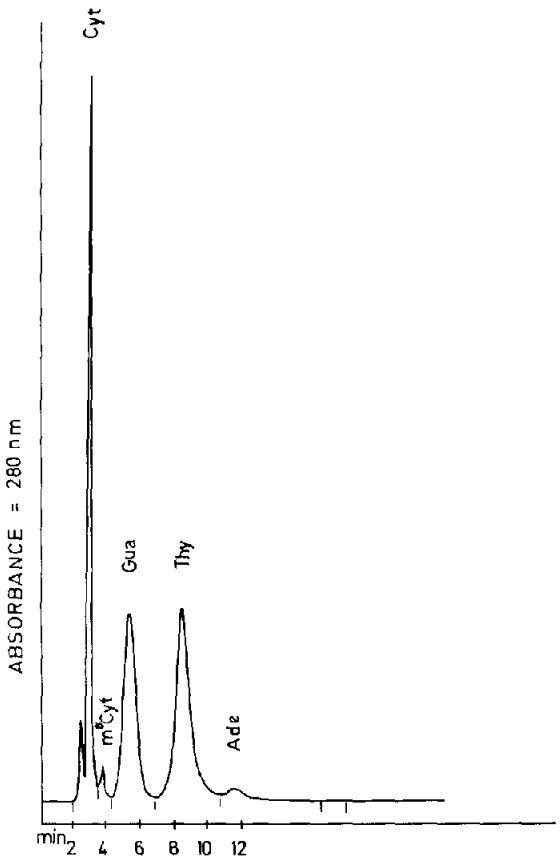

Fig. 2. Separation of the five nucleobases from DNA of peripheral blood mononuclear cells from a control. Flow-rate: 0.6 ml/min. Absorbance: 280 nm. A.u.f.s. range: 0.1. Eluent mixture: methanol 0.05 *M* KH_2PO_4 (1:99, v/v).

TABLE I

INTRA-ASSAY VARIATION

Sample	Percentage of m^5Cyt (mean ± 1 S.D.)	Range (%)	Coefficient of variation (%)
Working standard solution	5.33 ± 0.19	0.44	3.52
Control	5.96 ± 0.25	0.70	3.93
Patient	2.36 ± 0.09	0.30	3.73

The mean percentage of m^5Cyt in normal individuals was 5.96 ± 0.22, *i.e.* significantly higher than in the overall patient population ($p = 0.0001$). Both SLE and RA patients showed statistically significantly lower percentages of m^5Cyt compared to controls (see Table II), whereas no difference emerged when the RA and SLE groups were compared to each other.

TABLE II

PERCENTAGES OF m^5Cyt IN PATIENTS AFFECTED BY RA OR SLE AND CONTROL PATIENTS

Sample	*n*	Percentage of m^5Cyt (mean ± 1 S.D.)	*p versus* control
Controls	15	5.96 ± 0.22	–
RA patients	21	4.18 ± 1.90	0.001
SLE patients	9	2.90 ± 1.47	0.0003

Fig. 3 shows the distribution of the individual results. All the results but one for SLE patients with inactive disease were close to or above the 90th percentile (4.41). The same was true for RA patients with inactive disease (90th percentile, 7.39).

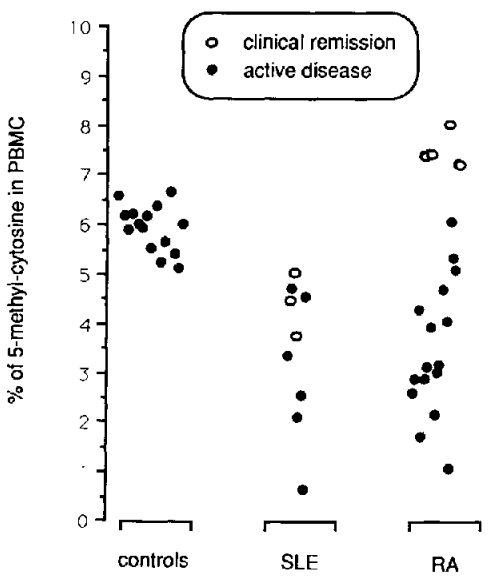

Fig. 3. Individual percentages of the 5-methylcytosine from peripheral blood mononuclear cells of fifteen controls, nine patients affected by systemic lupus erythematosus (SLE) and twenty-one patients affected by rheumatoid arthritis (RA).

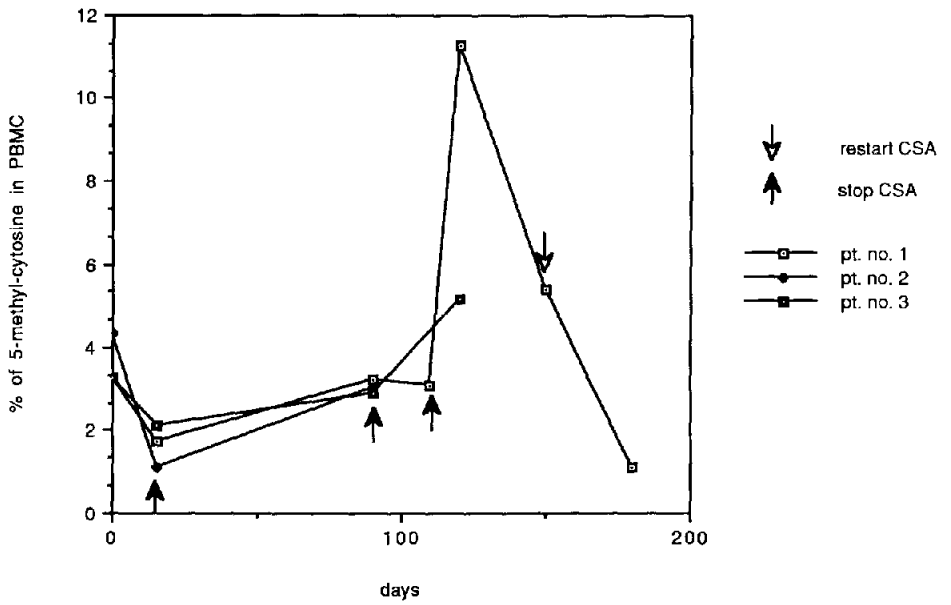

Fig. 4. Behaviour profiles of the 5-methylcytosine percentages in three patients affected by rheumatoid arthritis under therapy with cyclosporine-A.

As shown in Fig. 4, CSA therapy led to a decrease in the m^5Cyt percentages in three RA patients, but a rise was observed when the CSA was suspended.

In one RA patient with active disease, mononuclear cells from the synovial fluid showed a m^5Cyt percentage of 3.08. In another RA patient in clinical remission, the m^5Cyt percentages of the synovial fluid mononuclear cells and of PBMC were 7.22 and 8.11, respectively.

The percentage of m^5Cyt in synovial tissue from four RA patients and five patients with osteoarthritis (OA) was similar; the level of m^5Cyt was 1.90 ± 0.4 in RA and 1.64 ± 0.26 in OA.

DISCUSSION

The reversed-phase HPLC technique used in this study allowed the simultaneous separation and analysis of the principal nucleobases and methylated cytosine. The previously described capacity of this technique to perform a rapid analysis with good resolution and reproducibility was confirmed [24–26].

The PBMC from patients with rheumatic autoimmune diseases present a DNA methylation level significantly lower than controls. This finding may be the consequence of the immune system activation [32–35] which leads to re-differentiation and polyclonal proliferation of autoreactive mononuclear cells [36,37].

The percentage of m^5Cyt is lower in RA and SLE patients with active disease

than in patients in clinical remisson. This finding, if confirmed in a wider number of patients, supports the idea that different phases of rheumatic autoimmune diseases are characterized by different percentages of m^5Cyt, which may lead to different degrees of activation or repression of specific genes [20-37].

Of the rheumatic autoimmune diseases, SLE is characterized by the highest degrees of altered oncogene expression [20,21]. Anti-ds-DNA antibodies, which are a hallmark of the disease, may contribute to the hypomethylation. Indeed, the activity of anti-ds-DNA antibodies as hypomethylating agents *in vitro*, has been described recently [38]. Their role *in vivo*, however, still remains to be clarified. Drugs such as hydralazyne and procainamide, well known as possible inducers of a lupus-like syndrome, inhibit T-cell DNA methylation [36,39,40]. Furthermore, lymphocytes from patients with SLE or other autoimmune diseases seem to be more radiosensitive than those from normal individuals [19] and this difference has been interpreted as the result of DNA damage and its defective repair. Taken together, all these observations suggest the idea that the DNA hypomethylation could be involved in the pathogenesis of the autoimmune diseases as a result of the important role that methylated nucleobases play in the DNA repair, transcription and replication as well as in maintaining the chromosome structure [41,42].

The synovial fluid mononuclear cells from one RA patient studied were characterized by a percentage of m^5Cyt similar to that observed in PBMC. Synovial tissues, either from RA or from OA patients, showed similar percentages of m^5Cyt, which were strikingly lower than those observed in PBMC. This finding suggests that, in addition to disease-specific and disease activity-specific variations, the percentage of m^5Cyt may also show tissue-specific variations [1,2].

It is well known that the percentages of m^5Cyt may vary in tissues exposed to several drugs which directly interact with DNA, such as alkylating agents [13,43,44] and purine analogues [7,11,12,14]. Indeed, 5-aza-cytidine leads to DNA hypomethylation both in normal and neoplastic tissues [12,14], whereas the opposite was observed in tissues exposed to cytosine arabinofuranoside (Ara-C) or hydroxyurea [11]. The variations observed are dose-related.

The effect of CSA on the percentage of the m^5Cyt in PBMC has never been studied. This new immunosuppressive agent could be considered as an anti-proliferative substance specific for lymphocytes [45-47]. This study has shown that CSA leads to a hypomethylation of PBMC and that the percentage of m^5Cyt rebounds after drug suspension. This observation suggests a wide and reversible interaction between CSA and DNA from PBMC.

Additional studies are necessary to clarify the exact nature of the direct or indirect effect of CSA on the genome-wide methylation of DNA.

ACKNOWLEDGEMENT

We thank Dr. Aldo Salvi, M.D., for kind help with the statistical analysis.

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