Preparation of a Monoclonal Antibody Specific for 5-Methyl-2'-deoxycytidine and Its Application for the Detection of DNA Methylation Levels in Human Peripheral Blood Cells

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A monoclonal antibody specific for a modified nucleoside, 5-methyl-2'-deoxycytidine (5mdCyd), was prepared using 5-methylcytidine (5mCyd)-keyhole limpet haemocyanin (KLH) conjugate, and was characterized. Termed FMC9, the antibody reacts with 5mdCyd and slightly with 5mCyd and 5-methylcytosine (5mCyt) but not with other nucleosides tested in this investigation. FMC-9 was used in an enzyme-linked immunosorbent assay (ELISA) system for the quantitation of 5mdCyd levels. Sensitivity was in the picomole range. Methylation levels in peripheral blood cells of healthy donors were determined by inhibition ELISA. The percentage of 5mdCyd in peripheral blood cells of 10 healthy donors was 5.08±0.50%. These results suggest that the inhibition ELISA using FMC9 is useful to monitor 5mdCyd levels in the peripheral blood cells.

Key words DNA methylation; modified nucleoside; 5-methyl-2'-deoxycytidine; monoclonal antibody; inhibition ELISA

5-Methylcytosine (5mCyt) is the only modified nucleobase found in DNA of mammals. Modification of residues was performed by enzymatic conversion after DNA synthesis. The methylation patterns are symmetrically distributed in CpG doublets3 and are tissue specific.2) 5mCyt is present in non-coding repetitive sequences, heterochromatins and satellite DNA.3) The ratio of 5mCyt contents to cytosine residue in DNA is believed to be about 4–6% but its biological meaning has not been determined.

The suggested functions are 1) X-chromosome inactivation,4) 2) transition of DNA structure and chromosome,5) 3) regulation of gene expression through modification of DNA-protein interaction6) and 4) genomic imprinting.7) No study, however, has examined the changes systematically or quantitatively. To learn the biological significance of DNA methylation, the development of a determination method of 5-methyl-2'-deoxycytidine (5mdCyd) is important.

The levels of methylation of DNA have been determined by paper chromatography,8) thin layer chromatography (TLC),9) high-performance liquid chromatography (HPLC),10,11) and immunoassay.12–15) Sensitivities of paper chromatography and TLC are very low; HPLC requires a large quantity of sample and a long time for analysis, while the immunoassay using polyclonal antibody (PAb) has low specificity because of the cross-reactivity of PAbs against heterologous antigens. Recently, it has been reported the monoclonal antibody (MAB) to 5-methylcytidine (5mCyd) has cross-reactivity to 5mCyt and 1-methyladenine.16)

In the present study, we prepared a MAb, termed FMC9, highly specific for 5mdCyd. The sensitive, rapid and specific ELISA system using FMC9 was useful to determine the 5mdCyd content in peripheral blood cells. The specificity of FMC9 for various nucleosides and bases, and application of this MAB to determine the 5mdCyd content in peripheral blood cells of healthy donors are described here.

MATERIALS AND METHODS

Reagents 5mCyd, other purified cross-reactants, bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH) as well as complete and incomplete Freund’s adjuvants were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Penicillium citrinum nuclease P1 and calf intestinal alkaline phosphatase were obtained from Boehringer-Mannheim Co. (Mannheim, Germany). Protease K and RNase A were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Other analytical grade reagents were supplied by Wako Co. Ltd. (Osaka, Japan).

Immunogen and Immunization The immunogen was prepared by conjugating 5mCyd and KLH according to the method of Erlanger and Beiser.17) Female BALB/c mice (5 to 6 weeks old) were immunized i.p. and s.c. with 5mCyd–KLH conjugate (50 μg protein/mouse) dissolved in pH 7.4 phosphate-buffered saline (PBS) with Freund’s complete adjuvant. After 2 and 4 weeks, the same immunization procedure was repeated except for use of incomplete adjuvant instead of complete adjuvant. Seven days after the third immunization, the mouse received a booster i.v. injection of 20 μg protein of 5mCyd–KLH solution with no adjuvant.

Preparation of Hybridoma Three days after the last immunization, the mouse was sacrificed and cell fusion was performed. Spleen cells from the hyperimmunized mouse were fused with SP2/O-Ag14 mouse myeloma cells in the presence of 50% (w/v) polyethylene glycol 4000. The fused spleen-myeloma cells were selected by culturing in HAT medium (RPMI 1640 medium containing 2 mm glutamine, 0.2% glucose, 1 mm pyruvic acid, penicillin at 100 U/ml, streptomycin at 100 μg/ml, kanamycin at 60 μg/ml and 10% heat-inactivated fetal calf serum (FCS) (standard medium) supplemented with 100 μm hypoxanthine, 0.4 μm aminopterin and 16 μm thymidine). Cul-

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tures were maintained in a 5% CO₂ incubator at 37°C. When hybridoma colonies appeared, they were expanded and maintained in HT medium (HAT medium without aminopterin) and finally in the standard medium. Hybridoma cells were screened for production of anti-m5CdCyd antibodies by direct ELISA. A m5CdCyd-BSA conjugate was used as the antigen to avoid detection of antibodies reactive with KLH. Hybridomas secreting antibodies reactive with m5CdCyd-BSA were cloned by a limited dilution method using Hybridoma Cloning Factor (Igen, U.S.A.).

Inhibition ELISA A m5Cd–BSA conjugate dissolved in PBS (0.25 μg protein/ml) was fixed to wells of Sumitomo MS 7196F polyvinylchloride plates by overnight incubation at 4°C. Then, the wells were filled with 100 μl of 1% BSA in PBS followed by incubation at 37°C for 1 h. After discarding the blocking solution, 50 μl of serially diluted m5CdCyd solution or sample was applied to each well. An equal volume of FMC9 antibody solution (0.5 μg/ml) was then added to each well. After the wells were washed 3 times with PBS and twice with PBS/Tween 20 (0.05%), 100 μl of 1:4000 diluted alkaline phosphatase (ALP) conjugated goat anti-mouse IgG (Tago Inc., U.S.A.) was added. Following incubation at 4°C for 45 min, washing, and the addition of 100 μl of p-nitrophenyl phosphate (Sigma 104 phosphatase substrate) dissolved in 1M diethanolamine buffer (pH 9.8) (1 mg/ml), the resulting solution and incubated at 37°C for 20 min. The absorbance of the yellow color developed in each well was measured at 405 nm by an EIA reader (UV max, Molecular Devices, U.S.A.).

Quantitation of m5CdCyd Content of Peripheral Blood Cells Peripheral blood cells were prepared from healthy volunteers (5 males and 5 females) with mean ages of 24.2 and 27.2, respectively. The difference between the two groups was not statistically significant (Student’s t-test). DNA was isolated from peripheral blood cells by phenol extraction using RNase A and proteinase K. The purified DNA was digested to nucleosides with nuclease P1 and alkaline phosphatase as previously described. The DNA was dissolved in 100 μl of 30 mM sodium acetate (pH 5.3) and boiled in water for 2 min to inactivate the enzymes. Then, 5 μl of 20 mM zinc sulfate, 7 μl of nuclease P1 (2.1 units/μl), and 3 μl of alkaline phosphatase (3 units/μl) were added followed by incubation at 37°C for 2 h. The pH was raised to 7.0 with 1M Tris–HCl (10 μl) and the mixture was incubated at 37°C for 2 h. The resulting solution was applied to the inhibition ELISA system.

RESULTS

Specificity of MAb The specificity of FMC9 MAb (IgG2a, ramda) was determined by the inhibition ELISA (Table 1). M5CdCyd (IC₅₀: 4 μg/ml), m5CdCy (IC₅₀: 54 μg/ml) and to a slight degree m3Cyt (IC₅₀: 340 μg/ml) inhibited the binding of FMC9 to m5Cyt–BSA conjugate in a dose-dependent manner, while other chemicals did not inhibit it.

This MAb was able to distinguish the unmethylated from methylated forms of cytidine and cytosine. Moreover, since the reactivity to m5CdCyd was higher than those of other methylated forms, this MAb may recognize 2'-hydrogen on the ribose (Fig. 1).

Calibration Curve Figure 2 shows the standard curve for the estimation of m5CdCyd based on the inhibition of binding of FMC9 to m5CdCyd–BSA by m5CdCyd. Good linearity was observed over the range of 1 ng–10 μg/ml of m5CdCyd, and the inhibitory effect of m5CdCyd to FMC9 reactivity was increased in a dose-dependent manner. The coefficients of variations were in a range of

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<tr>
<th>Inhibitor</th>
<th>IC₅₀ (μg/ml)</th>
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<tr>
<td>5-Methyl-2′-deoxycytidine</td>
<td>4¹</td>
</tr>
<tr>
<td>5-Methylcytidine</td>
<td>54</td>
</tr>
<tr>
<td>5-Methylcytosine</td>
<td>340</td>
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<tr>
<td>2′-Deoxycytidine</td>
<td>&gt;1000</td>
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<td>Cytidine</td>
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<tr>
<td>Cytosine</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Others²</td>
<td>&gt;1000</td>
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</table>

¹ The data are represented as concentrations (μg/ml) required for 50% inhibition of the binding of FMC9 to 5-methylcytidine-BSA. ² Others: adenosine, guanosine, uridine, thymidine.

Fig. 1. Epitope of FMC9 Monoclonal Antibody

The shaded area (methyl group at position 5, amino group at position 4 and hydrogen at position 2' on the ribose) denotes the epitope of FMC9 monoclonal antibody determined by the inhibition ELISA.

Fig. 2. Standard Curve for Quantitation of 5-Methyl-2′-deoxycytidine

Data are given in the form of a logit plot.
6.41 and 7.61% with an average value of 7.03%. We were able to determine the methylation levels of DNA in this range. The minimum detection limit was approximately 10 pmol of m5dCyd in this assay system. When m5dCyd (16.5, 33, 66 and 132 ng/ml) was added to the solution of ramda phage DNA and then determined, the recovery of m5dCyd was 101—135% (data not shown).

Determination of the Serum Methylation Level. The DNA fraction was prepared from peripheral blood cells by phenol extraction. After the DNA was digested by nuclease P1 and alkaline phosphatase, we obtained the nucleoside solution, and the content of m5dCyd was determined by inhibition ELISA using FMC9. The recovery of added m5dCyd from the prepared nucleoside solution was 85—120% (data not shown).

The methylation levels of the blood of the ten healthy adults were assayed (Fig. 3). Methylation levels were calculated as the % ratio of m5dCyd to the cytidine content in DNA, and were 4.87 ± 0.27% in male and 5.29 ± 0.62% in female. No sexual difference was recognized. The average methylation level of healthy donors was 5.08 ± 0.50%.

DISCUSSION

The MAbs termed FMC9 was highly specific for m5dCyd and had weak cross-reactivity to m5Cyd and m5Cyt; other chemicals did not react to FMC9. These results suggest that FMC9 recognizes the structure characteristic of 5-methyl-2'-deoxycytidine. We speculate that it not only recognizes both the methyl group at position 5 and the amino group at position 4 of m5dCyd but also the 2'-hydrogen on the ribose (Fig. 1). Since this reactivity was expected to be advantageous for the highly sensitive determination of m5dCyd in DNA, we established an accurate, sensitive, rapid, and simple ELISA system in this study.

The antibody was applied to a competitive enzyme immunoassay, with the antigen immobilized on the solid phase. Good linear response was observed over a range of 1 ng—10 µg/ml of m5dCyd (Fig. 2). The recovery test showed the assay was applicable to the determination of the methylation level in peripheral blood. m5dCyd in peripheral blood cells of healthy human volunteers was 5.08 ± 0.50% of cytidine residues (Fig. 3). This is a primary report on the determination of the methylation levels of human blood using the inhibition ELISA with MAbs. Corveta et al. showed that the methylation levels in peripheral blood mononuclear cells were 5.96 ± 0.22% by HPLC analysis. Another report using HPLC showed the methylation levels in peripheral T cells of 3.81 ± 0.09. There is no obvious explanation for the discrepancy of these data at present, however, difference in sources might be one possible reason.

Previous reports have suggested that the methylation level of blood cells decreased with age but also in patients with autoimmune diseases, for example, rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). It is still unconfirmed whether or not variations are related to the expression of these diseases. Further quantitative studies on the changes of methylation of DNA are required.

The m5dCyd in DNA is generally determined by HPLC but the HPLC technique is a lengthy process. The ELISA system is more rapid and simple, and mass screening is practical. We conclude that this method is useful to determine variations in the methylation levels of DNA and to analyze the physiological significance of DNA methylation.

REFERENCES