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Preparation of a Monoclonal Antibody Specific for 1-Methyladenosine and Its Application for the Detection of Elevated Levels of 1-Methyladenosine in Urines from Cancer Patients

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A monoclonal antibody specific for a modified nucleoside, 1-methyladenosine, was prepared and characterized. This antibody, termed AMA-2, reacts with 1-methyladenosine and 1-methyladenine but not with other nucleosides, particularly methylated adenosines other than 1-methyladenosine and methylated guanosines, tested in this investigation. In our experiments, AMA-2 was used in an enzyme-linked immunosorbent assay (ELISA) system for the quantitation of the levels of 1-methyladenosine in urine. Sensitivity was in the picomole range and accuracy was nearly equal to that of the high-performance liquid chromatography (HPLC) assay system. Urinary levels of 1-methyladenosine in healthy donors and patients with various advanced cancers were determined by the inhibition ELISA. The amount of 1-methyladenosine in urine of 33 healthy donors was 1.91±0.66 nmol/μmol creatinine. In 54% (51/94) of patients, urinary 1-methyladenosine was elevated above the mean plus 2 standard deviations for the healthy donors (3.23 nmol/μmol creatinine). In patients with leukemia, esophageal cancer, stomach cancer, colon cancer, and bladder cancer, urinary levels of 1-methyladenosine were significantly elevated. In patients with leukemia, urinary 1-methyladenosine levels changed almost in parallel with the change in the clinical response during chemotherapy. These results suggest that urinary 1-methyladenosine might be useful in monitoring the effectiveness of therapy.

Key words: Modified nucleosides — Anti-1-methyladenosine monoclonal antibody — Inhibition ELISA — Urines from cancer patients

Ribonucleic acids (RNAs), particularly transfer RNAs (tRNAs), contain a wide variety of modified nucleosides which are formed after transcription of the macromolecules.[1] When these RNAs are degraded, the majority of modified nucleosides are excreted in the urine as intact forms because these modified nucleosides have no salvage pathway.[2] Biochemical studies and HPLC studies have shown that the levels of certain modified nucleosides in the urine are elevated in a wide variety of cancer patients.[3,4] The elevation of modified nucleosides in the urine of cancer patients has been suggested to be caused by the higher turnover of tRNA in tumor tissue than in normal tissue, rather than by the destruction of the tissue.[5] HPLC analysis has been widely used in the determination of urinary modified nucleosides[6] and it has been demonstrated that specific modified nucleosides are valuable cancer markers, with potential for early detection of cancer, for monitoring the effectiveness of therapy[7,8] and for diagnosis of populations at risk, such as asbestos workers,[9] but the techniques are complicated and a long time is required for the determination of many samples.

The amount of 1-methyladenosine, which has a potent immunosuppressive effect, impairing the resistance to *Listeria* infection in mice,[10] increases in the urine of cancer patients. Cancer patients are known to reach a state of immunosuppression in the later stage of the disease. These observations suggest that the determination of urinary 1-methyladenosine might be very useful for detection of cancer, monitoring of patients after operation and determination of the effectiveness of chemotherapy.

It this study, we attempted to prepare monoclonal antibodies to develop a sensitive, rapid and specific ELISA system for determination of the urinary 1-methyladenosine. Finally, we obtained a monoclonal antibody

highly specific for 1-methyladenosine. This antibody was designated AMA-2. The specificity of AMA-2 for various nucleosides and bases, and application of this monoclonal antibody for the determination of urinary 1-methyladenosine in cancer patients to elucidate its usefulness as a tumor marker are described here.

Materials and Methods

Chemicals 1-Methyladenosine and other nucleosides and bases were purchased from Sigma (St. Louis, Mo.). Keyhole lympet hemocyanine (KLH) and bovine serum albumin (BSA) were also purchased from Sigma.

Urine Samples Urine samples were collected from healthy donors and cancer patients in the early morning and aliquots were stored at $-20°$ until analysis without any preservative. Randomly collected urine samples were used as sources for the determination as described by Gehrke *et al.*[11] In the cancer detection study, all patients selected were in an active or metastatic disease state and were not receiving systematic therapy at the time of sampling. The amount of creatinine in each sample was determined by using Creatinine Test Wako (Wako Pure Chemical Co. Ltd., Osaka).

Immunogen and Immunization The immunogen was prepared by conjugating 1-methyladenosine and KLH according to the method of Erlanger and Beiser.[12] Female BALB/c mice (6 to 8 weeks old) were immunized ip and sc with 1-methyladenosine-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. Seven days after the third immunization, the mouse received a booster iv injection of 50 μg protein of 1-methyladenosine-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 hyperimmunized mouse were fused with SP2/0-Ag14-K13 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 m*M* glutamine, 0.2% glucose, 1m*M* pyruvic acid, penicillin at 100 U/ml, streptomycin at 100 μg/ml and 15% heat-inactivated fetal calf serum (FCS) (standard medium) supplemented with 100μM hypoxanthine, 0.4μM aminopterin and 16μM thymidine). Cultures were maintained in a 5% CO_2 incubator at 37°. When hybridoma colonies appeared, they were expanded and maintained in HT medium (HAT medium free from aminopterin) and finally in the standard medium. Hybridoma cells were screened for production of anti-1-methyladenosine antibodies by direct ELISA. A 1-methyladenosine-BSA conjugate was used as the antigen to avoid detection of antibodies reactive with KLH. Hybridomas secreting antibodies reactive with 1-methyladenosine were cloned by means of a limiting dilution method using mouse thymocytes as feeder cells.

Inhibition ELISA A 1-methyladenosine-BSA conjugate dissolved in PBS (1 μg protein/ml) was fixed to wells of Sumitomo MS 3496 F polystyrene plates by overnight incubation at 4°. The wells were washed once with PBS and filled with 100 μl of 1% BSA in PBS. After a 1 hr incubation at 37°, the solution was discarded and 50 μl aliquots of serially diluted 1-methyladenosine solutions or urine samples were applied to each well. An equal volume of AMA-2 antibody solution (1 μg/ml) was then added to each well and the plates were incubated at 4° for 1 hr. The wells were washed 5 times with PBS and filled with 100 μl of 1:3000 diluted alkaline phosphatase (ALP) conjugated-goat anti-mouse IgG (Tago Inc., USA) followed by incubation at 4° for 45 min. Following 5 washings with PBS, 100 μl of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) dissolved in 1*M* diethanolamine buffer (pH 9.8) (1 mg/ml) was added and incubated at 37° for 30 min.

The absorbance of the developed yellow color in each well was measured at 405 nm using an EIA reader (Model 2550, Bio-Rad, USA).

HPLC Assay Urinary 1-methyladenosine was fractionated and determined as described by Gehrke *et al.*[13] Briefly, 1 ml of urine sample was buffered with 0.3 ml of 2.5*M* ammonium acetate (pH 9.5) and then applied to a column (5×40 mm) of phenylboronate affinity gel (Mātrex Gel PBA 60, Amicon, USA) equilibrated with 0.25*M* ammonium acetate (pH 8.8). 1-Methyladenosine and other ribonucleosides were eluted with 5 ml of 0.01*M* formic acid (pH 3.1), then lyophilized and resolved in 1 ml of doubly distilled water. Twenty μl of each solution was injected into the reversed-phase C_{18} column (YMC packed column, Yamamura Chemical Lab. Co. Ltd., Kyoto) of a liquid chromatography system (Model 635 A, Hitachi Co. Ltd., Tokyo) equipped with an absorbance detector (UVLOG 5 III, Oyo-Bunko Kiki Co. Ltd., Tokyo). Quantitation of 1-methyladenosine and other ribonucleosides was performed by the internal standard method using 2′-deoxyuridine as the internal standard.

Results

Specificity of Monoclonal Antibody The specificity of AMA-2 monoclonal antibody (IgG_{2b}, κ) was determined by the inhibition

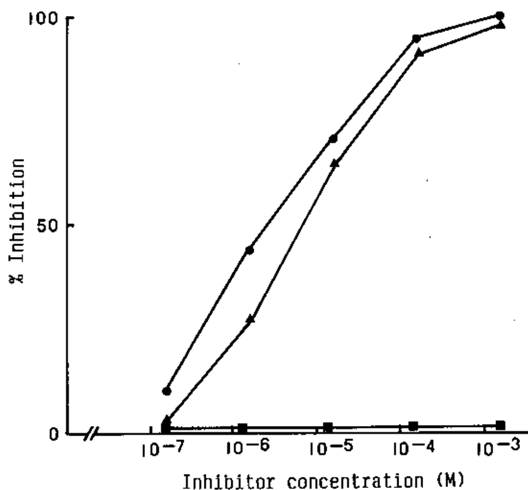

Fig. 1. Specificity of AMA-2 monoclonal antibody. Specificity of the monoclonal antibody to 1-methyladenosine was determined in the inhibition ELISA as described in "Materials and Methods." Inhibitors used were 1-methyladenosine (●), 1-methyladenine (▲), adenosine, guanosine, cytidine, thymidine, uridine and ribose (■). Points: mean values of triplicate determinations. Absorbancy for no added inhibitor (B_0) was 1.352±0.018. The percentage of inhibition was calculated as $[B_0-B)/B_0]\times 100$.

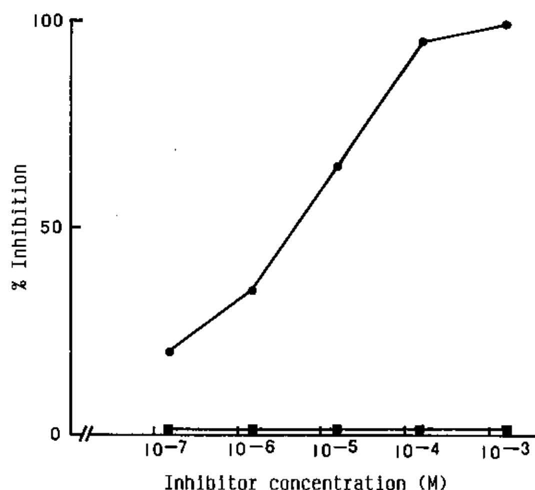

Fig. 2. Specificity of AMA-2 monoclonal antibody. Reactivity of monoclonal antibody to methylated nucleosides was determined in the inhibition ELISA. Inhibitors used were 1-methyladenosine (●), 6-methyladenosine, 1-methylguanosine, 2-methylguanosine, 7-methylguanosine, and 1-methylinosine (■). Points: mean values of triplicate determinations. Absorbancy for no added inhibitor (B_0) was 1.089±0.035. Details were described in the legend to Fig. 1.

ELISA described above. Inhibitors used in this experiment were 1-methyladenosine, 1-methyladenine, adenosine, and other methylated or unmethylated nucleosides. As shown in Fig. 1, 1-methyladenosine and 1-methyladenine inhibited the binding of AMA-2 to 1-methyladenosine-BSA in a dose-dependent manner. However, other nucleosides commonly present in DNA and RNA (adenosine, guanosine, cytidine, thymidine, and uridine) did not inhibit the binding of AMA-2 to 1-methyladenosine-BSA.

Next, reactivity of AMA-2 to a variety of methylated nucleosides was determined to confirm the recognition site of AMA-2. The results are shown in Fig. 2. Only 1-methyladenosine inhibited the binding of AMA-2 to 1-methyladenosine-BSA. 6-Methyladenosine, 1-methylinosine, 1-methylguanosine, 2-methylguanosine and 7-methylguanosine showed no inhibitory effect.

These results suggest that AMA-2 recognizes both the methyl group at position 1 and the amino group at position 6 of 1-methyladenosine (Fig. 3). This structure is characteristic for 1-methyladenosine, accounting for the fact that AMA-2 is highly specific for 1-methyladenosine.

Establishment of Inhibition ELISA System To quantitate the amount of 1-methyladenosine in urine samples, we have established an inhibition assay system and determined the accuracy of this assay system in comparison with the HPLC assay system.

Before establishing the standard curve, the optimal concentration of AMA-2 and 1-methyladenosine-BSA was determined by antigen-antibody dual titration. Figure 4 shows a standard curve for the estimation of 1-methyladenosine based on the inhibition of binding of AMA-2 to 1-methyladenosine-BSA by 1-methyladenosine. In this assay system, the minimum detection limit was approximately 2 pmol of 1-methyladenosine. When 1 μg/ml of 1-methyladenosine was added to urine samples and then determined, 97–112% of the added 1-methyladenosine was re-

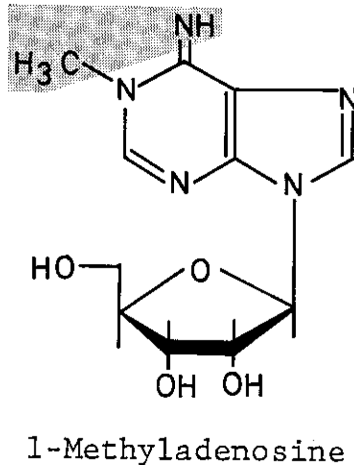

Fig. 3. Epitope of AMA-2 monoclonal antibody. The shaded area (methyl group at position 1 and amino group at position 6) denotes the epitope of AMA-2 monoclonal antibody determined by the inhibiton ELISA.

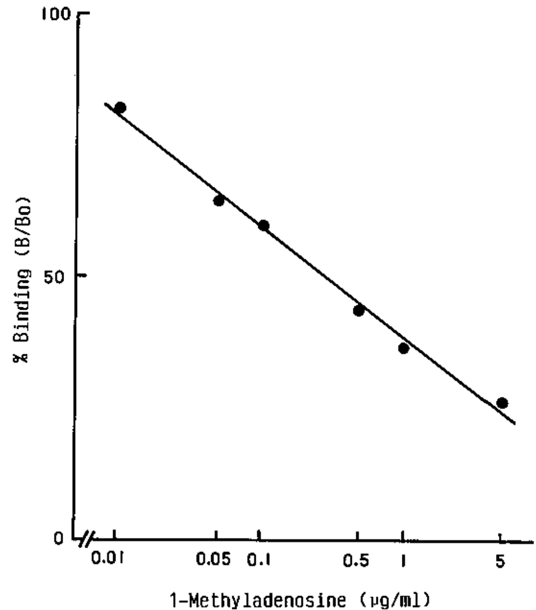

Fig. 4. Standard curve for the quantitation of urinary 1-methyladenosine. Details were described in "Materials and Methods." Data are given in the form of a logit plot. Points: mean values of triplicate determinations.

covered. The coefficients of variation of simultaneous assays and repeated (daily) assays were 7.5 and 9.3%, respectively.

So far HPLC systems have been widely used in the determination of urinary 1-methyladenosine. The accuracy of the ELISA system using AMA-2 was compared to that of the HPLC system. The results are shown in Table I. Values for 1-methyladenosine obtained by ELISA were higher than those obtained by HPLC. In the HPLC system, the samples were applied to a phenylboronate affinity gel to purify and concentrate the nucleosides in urine before the assay. On the other hand, in the ELISA system, non-treated urine samples were used for the assay. This is probably the reason why a greater amount of 1-methyladenosine was detected in the ELISA.

From these results, we consider that the ELISA system using AMA-2 monoclonal antibody is superior to the HPLC system with respect to accuracy, sensitivity, rapidity and simplicity.

Table I. Urinary Levels of 1-Methyladenosine as Determined by ELISA or HPLC[a]

Diagnosis	1-Methyladenosine (nmol/μmol creatinine)	
	ELISA	HPLC
Healthy	2.16±0.03[b]	1.63±0.06
Healthy	2.40±0.13	1.98±0.11
Healthy	2.44±0.02	2.06±0.17
Stomach cancer	5.70±0.28	3.33±0.05
Hepatocellular carcinoma	2.60±0.15	2.67±0.11
Hepatocellular carcinoma	4.17±0.32	2.52±0.20

a) Details of the ELISA and HPLC are given in "Materials and Methods."
b) Values are means±SD.

Form of the Antigen in Urine Recognized by AMA-2 Monoclonal Antibody AMA-2 monoclonal antibody is specific for 1-methyladenosine, and this antibody also reacts with tRNA which contains 1-methyladenosine residues (data not shown). Whole urine samples were used in the ELISA system so the materials in urine recognized by AMA-2 monoclonal antibody could have been in nucleoside form or a constituent of native tRNA. Urine from a patient with acute lymphoblastic leukemia

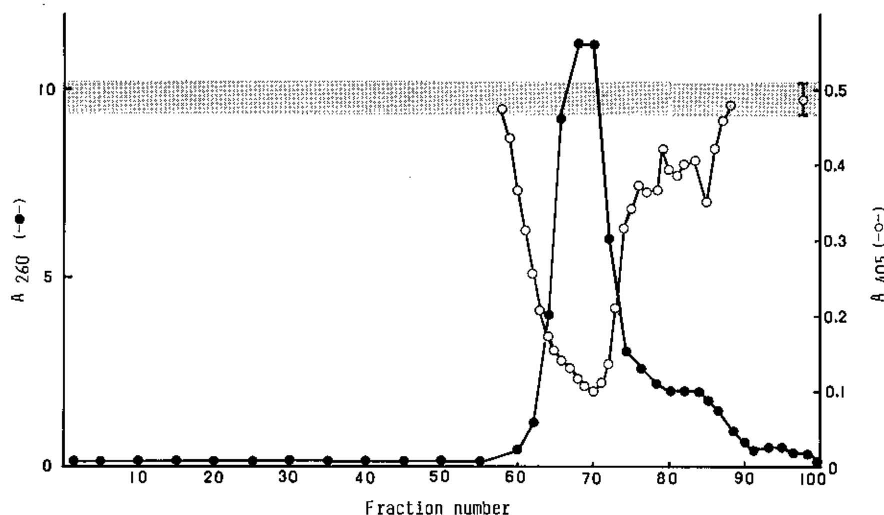

Fig. 5. Elution profile of urine from patients with ALL from Sephacryl S-200. Two ml of urine from patients with ALL was applied to a column (2.6×50 cm) of Sephacryl S-200 equilibrated with 50m*M* potassium phosphate buffer (pH 7.4). Absorbance at 260 nm of each fraction (●); inhibitory activity of each fraction for the binding of AMA-2 to 1-methyladenosine-BSA determined by the inhibition ELISA (○). Shaded area denotes the absorbance (mean±SD) for no added inhibitor in the inhibition ELISA.

(ALL) was fractionated by gel filtration using Sephacryl S-200 (Pharmacia Fine Chemicals, Sweden). Profiles of absorbance at 260 nm and inhibitory activity on binding of AMA-2 to 1-methyladenosine-BSA determined by the inhibition ELISA in each fraction are shown in Fig. 5. In inhibition ELISA, a different lot of 1-methyladenosine-BSA conjugate was used as the antigen, and this is why the absorbance for no added inhibitor was lower than that shown in Fig. 1 or 2. The fractions which give the highest absorbance at 260 nm and those which give the maximum inhibitory activity (the lowest absorbance at 405 nm) coincided well. These fractions were equivalent to the total elution volume of the column, so the immunoreactive materials in urine should be mainly nucleosides having molecular weights of less than 1000 daltons.

Urine Levels of 1-Methyladenosine from Healthy Donors and Patients with Advanced Cancer Urine samples were diluted 8, 16, 32 times with PBS and then used for the assay. These concentrations were chosen so that urine from healthy donors would give appropriate inhibition values in this assay. Each of the diluted samples were assayed in triplicate. The concentration of inhibitor (1-methyladenosine) present in each urine sample was interpolated from the standard curve as shown in Fig. 4. The concentration was then expressed in μg per ml and normalized to the amount of creatinine. The value was finally expressed as nmol per μmol creatinine.[11)]

The amount of urinary 1-methyladenosine in 33 healthy donors was 1.91±0.66 nmol/μmol creatinine. The cut-off level was set at the mean value for the normal subjects plus 2 standard deviations (3.23 nmol/μmol creatinine). Levels of 1-methyladenosine in urines from various cancer patients were determined. The results are shown in Fig. 6. The positive rate ranged from 86 to 38% and significantly elevated levels of 1-methyladenosine were detected in patients with leukemia, esophageal cancer, stomach cancer, colon cancer, bladder cancer, and other cancers. As for the patients with other cancers, in two cases of cholangioma, two cases of pancreas cancer, one case of thyroid carcinoma, and one case of lung cancer, elevated levels of 1-methyladenosine were detected. In patients

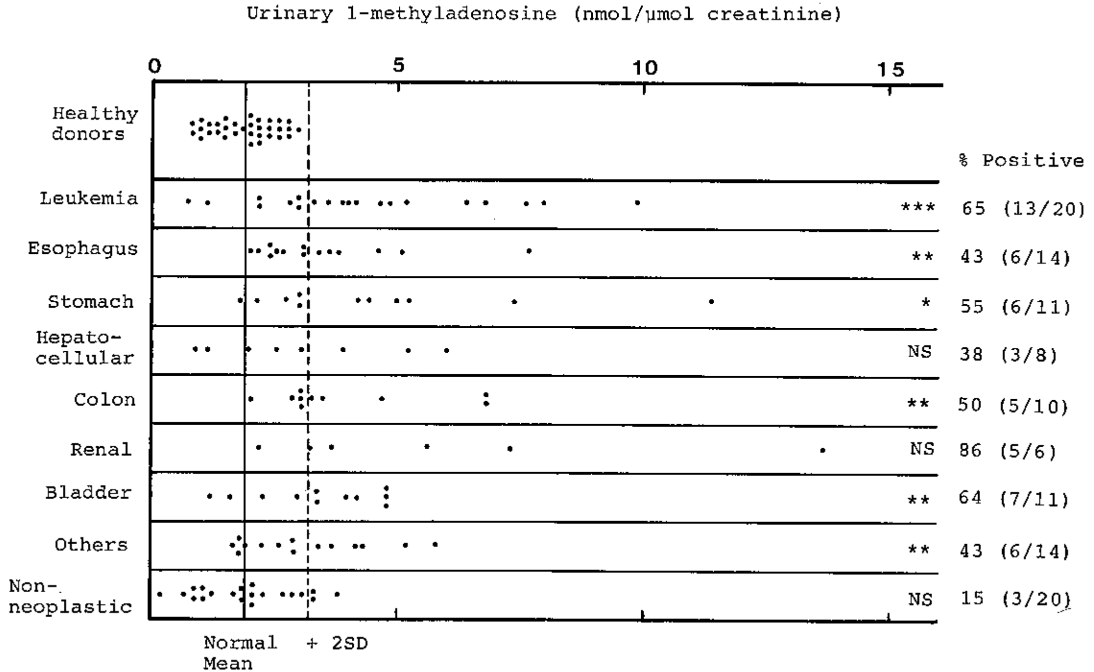

Fig. 6. Levels of 1-methyladenosine in urine from healthy donors and patients with advanced cancer. "Others" included six cases of cervix cancer, two cases of cholangioma, two cases of pancreas cancer, three cases of thyroid carcinoma, and one case of lung cancer. The significance of differences was established by using a *t*-test, comparing each group with the healthy donors. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; NS, not significant.

with non-neoplastic diseases, 15% were false-positive (3/20). They were patients with hydronephrosis, kidney stone, and hypertension. But the mean concentration of 1-methyladenosine in patients with non-neoplastic diseases was slightly higher (2.01±0.98 nmol/μmol creatinine) than that in healthy donors.

Next, we determined the usefulness of urinary 1-methyladenosine in the management of cancer. The levels of excretion of 1-methyladenosine were followed before and after therapy in 8 patients with leukemia which responded well to chemotherapy. The results are shown in Fig. 7. In 87% (7/8) of patients, urinary 1-methyladenosine levels changed in parallel with the change in the clinical response during chemotherapy.

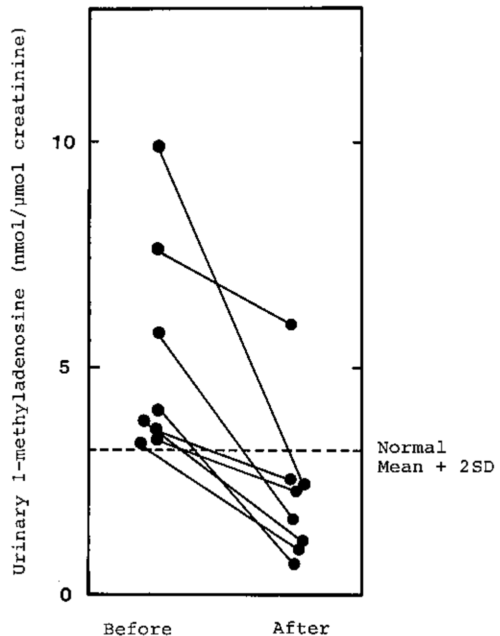

Fig. 7. Changes in urinary levels of 1-methyladenosine during chemotherapy in patients with leukemia.

Thus, elevated levels of urinary 1-methyladenosine were detected in a variety of cancers, but not in non-neoplastic diseases, and urinary 1-methyladenosine might be a useful marker for monitoring the effectiveness of chemotherapy.

The feasibility of early detection of cancer, and the relation to the stage and malignancy of cancer are being investigated.

DISCUSSION

1-Methyladenosine, which is one of the modified nucleosides mainly present in tRNA[14] is present at abnormally high levels in the urines of cancer patients and tumor-bearing animals. This increment may be due to the higher turnover rates of tRNA in tumor tissues than in normal tissues, rather than cell death.[5] It is well known that cancer patients reach a state of immunosuppression in the later stage of the disease. 1-Methyladenosine has been isolated from mice with Ehrlich tumor ascites as a potent immunosuppressive factor which impaired the resistance to *Listeria* infection.[10] The abnormal increment of 1-methyladenosine in the body fluids of cancer patients might lead them to a state of immunosuppression in the later stage of the disease.

From these observations, measurement of urinary 1-methyladenosine might be useful in the detection and management of cancer, so we prepared a monoclonal antibody highly specific for 1-methyladenosine, and established an accurate, sensitive, rapid, and simple ELISA system in this study.

We selected a hybridoma clone secreting monoclonal antibody (AMA-2) reactive only with 1-methyladenosine and 1-methyladenine but not with other nucleosides (Fig. 1). AMA-2 did not react with adenosine (Fig. 1) and did not react with other methylated nucleosides (Fig. 2). These results suggest that AMA-2 recognizes a structure characteristic of 1-methyladenosine. We speculate that the epitope of AMA-2 involves both the methyl and the amino groups at positions 1 and 6 of 1-methyladenosine, respectively (Fig. 3).

Preparation of polyclonal antibodies against methylated nucleosides (1-methylguanosine, 2-methylguanosine, 2,2-dimethylguanosine, 7-methylguanosine, and 5-methylcytidine) was reported previously.[15] These antibodies recognized the position of the methyl group(s) on each base, but minor population of the antibodies in several of the sera showed cross-reactivity to related compounds. Monoclonal antibody to methylated adenosine, AMA-2, which we have prepared, reacts only with 1-methyladenosine and shows no cross-reactivity to other nucleosides. Consequently, AMA-2 monoclonal antibody permitted the specific and accurate determination of 1-methyladenosine in the presence of other modified and unmodified nucleosides in urine samples.

Rapid and simple radioimmunoassay (RIA) systems for the determination of modified nucleosides, N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine,[16,17] N^6-(Δ^2-isopentenyl)adenosine,[16] 2′-O-methylguanosine[16] and N^6-succinyl adenosine,[18] in urine were reported previously. Determination of these modified nucleosides with HPLC was so difficult that RIA systems were used instead. Although urinary 1-methyladenosine may be measurable by HPLC, we have established an ELISA system which has the advantages that the nucleoside can be quantified without any prior treatment of urine samples and that the procedure is more rapid and simple than that of HPLC. The sensitivity of this ELISA system is in the picomole range and accuracy is sufficient for the determination of urinary 1-methyladenosine.

In our experiments, elevated levels of urinary 1-methyladenosine were detected in 54% (51/94) of cancer patients (Fig. 6). As regards organ specificity, it was revealed that urinary 1-methyladenosine had a broad specificity (Fig. 6). This result might support the hypothesis that the increase of modified nucleosides in the urines of cancer patients was due to the high turnover rate of tRNA in tumor tissue.[5] Borek *et al.* reported that elevated levels of urinary 1-methyladenosine were detected in a variety of cancers, i.e. small cell lung carcinoma,[19] mesothelioma,[20] and trophoblastic diseases.[21] Urinary 1-methyladenosine might be also useful in the determination of the effectiveness of chemotherapy (Fig. 7). It has been reported previously that urinary modified nucleosides were more useful than other tumor markers in the de-

termination of the effectiveness of chemotherapy.[7,8,22]

On the other hand, serum levels of modified nucleosides determined by HPLC have been reported previously. The levels of modified nucleosides in serum were much lower than those in urine, but in spite of the lower levels, it might be preferable to estimate serum modified nucleosides because they would be less affected by exogenous factors than those of urine. Elevated levels of 1-methylinosine and 2-methylguanosine were detected in patients with breast cancer,[23] and elevated levels of pseudouridine were detected in patients with small cell lung cancer.[24] We are now investigating the serum 1-methyladenosine levels in healthy donors and patients with advanced cancer.

In urines of patients with various cancers, levels of other modified nucleosides were also elevated. It would be more reliable to determine several modified nucleosides in combination. Among various modified nucleosides, the concentration of pseudouridine is elevated the most frequently and significantly.[25,26] Elevated pseudouridine is detected in patients with hepatocellular carcinoma,[27] small cell lung cancer[28] and acute lymphoblastic leukemia.[29] Preparation of monoclonal antibodies specific for pseudouridine has also been completed in our laboratory and the results will be presented shortly.

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