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Preparation of Monoclonal Antibodies against N-(γ-Maleimidobutyryloxy)succinimide (GMBS)-Conjugated Acetylspermine, and Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for N^1,N^{12}-Diacetylspermine

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We have developed three mouse monoclonal antibodies (mAb) of types IgG_1 and IgG_{2b}, *i.e.* anti-acetylspermine (Ac-Spm)-1 and 2 (ACSPM-1 and 2), and anti-acetylspermine (Ac-Spm)-3 (ACSPM-3), respectively, against Ac-Spm conjugated to bovine serum albumin *via* a heterobifunctional cross-linker, N-(γ-maleimidobutyryloxy)succinimide (GMBS). Among these mAbs, ACSPM-2 was the most useful for the development of an enzyme-linked immunosorbent assay (ELISA) for acetylpolyamines (Ac-PAs) with glutaraldehyde (GA)-conjugated N^1,N^{12}-diacetylspermine (2Ac-Spm) or acetylspermine (Ac-Spm) as the solid phase antigen. However, GMBS-conjugated Ac-Spm did not behave as a solid phase antigen in the competitive ELISA. The ELISA is based on the principle of competition between an analyte and the conjugated antigen for the mAb, followed by immunoreaction with biotinylated anti-mouse immunoglobulin and horseradish peroxidase-streptavidin. The ACSPM-2 mAb reacted with 2Ac-Spm to the highest degree, followed by Ac-Spm, N^1-acetylspermidine (N^1-Ac-Spd), N^1,N^8-diacetylspermidine (2Ac-Spd), and spermine (Spm), the EC_{50} values being 0.06, 0.25, 7.0, 10, and 60 μM, respectively, but exhibited almost no cross-reaction with other polyamine-related compounds or amino acids. The method was used to determine the urinary Ac-PA levels in healthy subjects, the average value of 0.36 μg of 2Ac-Spm/g creatinine ($n=16$) being obtained. The ACSPM-2 ELISA for 2Ac-Spm, which was the PA most relevant to the analysis of human urine among the five PA analogs mentioned above, might have potential for elucidation of the correlation of urinary 2Ac-Spm levels in cancers.

Key words: acetyl polyamines, diacetyl spermine, ELISA, monoclonal antibody, tumor marker.

Polyamines (PAs), namely putrescine (Put), spermidine (Spd), and spermine (Spm), are ubiquitous aliphatic polycations closely associated to many aspects of cell growth (for reviews, see Refs. *1-4*). It is known that PAs excreted in human urine are mainly monoacetylated, and that acetylputrescine, acetylcadaverine, N^1-acetylspermidine, and N^8-acetylspermidine are the most abundant among the PA species. Also, low amounts of free PAs including Put, cadaverine (Cad), Spd, and Spm, and trace amounts of acetylspermine (Ac-Spm), diacetylated Spm [N^1,N^{12}-diacetylspermine (2Ac-Spm)], and Spd [N^1,N^8-diacetylspermidine (2Ac-Spd)] occur (*5-9*). Actually, Hiramatsu *et al.* (*10, 11*) and Sugimoto *et al.* (*12*) recently demonstrated the common occurrence of diacetylated polyamines 2Ac-Spd and 2Ac-Spm in human urine, and pointed out the possibility they could be urogenital tumor markers (*10-12*). The development of a simple, specific and sensitive assay method for the quantification of urinary 2Ac-PAs will lead to a better understanding of their biological significance in cancers. A non-isotopic enzyme-linked immunosorbent assay (ELISA) would be useful for such a purpose, and adequate for the handling of a large number of urine samples from patients with a wide variety of diseases (*13-17*). We recently reported an ELISA for acetylpolyamines in human urine involving a monoclonal antibody (mAb), ASPM-2, raised against Spm conjugated with human serum

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Abbreviations: PA(s), polyamines; ELISA, enzyme-linked immunosorbent assay; mAb(s), monoclonal antibody(ies); GMBS, N-(γ-maleimidobutyryloxy)succinimide; 2Ac-Spm, N^1,N^{12}-diacetylspermine; Ac-Spm, acetylspermine; 2Ac-Spd, N^1,N^8-diacetylspermidine; N^1-Ac-Spd, N^1-acetylspermidine; N^8-Ac-Spd, N^8-acetylspermidine; Ac-Put, acetylputrescine; Spm, spermine; Spd, spermidine; Put, putrescine; Cad, cadaverine; GA, glutaraldehyde; MS-BSA(HSA), mercaptosuccinylated bovine (human) serum albumin.

albumin (HSA) *via* N-(γ-maleimidobutyryloxy)succinimide (GMBS) (*18, 19*). This assay is highly sensitive for 2Ac-Spm, but there is unfavorably almost equal cross-reaction with acetylspermine (Ac-Spm) and N^1-acetylspermidine (N^1-Ac-Spd), the latter of which is present at much higher concentrations than 2Ac-Spm in human urine (*10*). Quite recently, Hiramatsu *et al.* also developed an ELISA system for 2Ac-Spd involving purified rabbit antiserum raised against GMBS-conjugated N^1-Ac-Spd (*11*).

In order to improve the specificity of the ELISA for 2Ac-Spm, we have now prepared and characterized three mAbs raised against Ac-Spm-GMBS conjugated to bovine serum albumin (BSA), and applied the assay to the determination of 2Ac-Spm in healthy human urine.

MATERIALS AND METHODS

Chemicals—Spermine (Spm), spermidine (Spd), putrescine (Put), cadaverine (Cad), 1,3-diaminopropane, acetylspermine (Ac-Spm), N^1-acetylspermidine (N^1-Ac-Spd), N^8-acetylspermidine (N^8-Ac-Spd), acetylputrescine (Ac-Put), and *o*-phenylenediamine (OPD), in the form of hydrochloric salts, were purchased from Sigma Chemical, St. Louis, MO, USA. N^1,N^{12}-Diacetylspermine·2HCl (2Ac-Spm) and N^1,N^8-diacetylspermidine (2Ac-Spd) were prepared according to the methods of Bolkenius and Seiler (*20*).

Hapten-Protein Conjugates—GMBS-conjugated Ac-Spm (Ac-Spm-GMBS-MS·HSA) was synthesized according to the method we previously used for Spm-GMBS-MS·BSA (*15*). Briefly, Ac-Spm was incubated with GMBS at room temperature for 100 min. The maleimide group of GMBS incorporated into Ac-Spm was then conjugated with the thiol groups of a mercaptosuccinylated carrier protein (MS·BSA or MS·HSA) produced from an acetylmercaptosuccinylated carrier by treatment with 0.1 M hydroxylamine. The conjugates purified by Sepharose gel column chromatography were used as the sources of the immunogen or solid-phase antigen. Glutaraldehyde (GA)-conjugated Ac-Spm and 2Ac-Spm (Ac-Spm-GA-HSA and 2Ac-Spm-GA-HSA conjugates, respectively) were prepared according to the method we previously used for Spm-GA-BSA (*19*): Ac-Spm or 2Ac-Spm was allowed to react with HSA with aid of GA in 1 M sodium acetate for 10 min. The coupling mixture was then subjected to reduction with $NaBH_4$, followed by dialysis against 10 mM sodium acetate. The conjugates thus prepared were each used as a solid phase antigen for the 2Ac-Spm ELISA.

Preparation of Anti-Ac-Spm mAbs—BALB/c mice were immunized with four i.p. injections of Ac-Spm-GMBS-MS. BSA conjugate at 2-week intervals; the first injection of 50 μg of the conjugate and complete Freund's adjuvant was followed by three booster injections (25 μg) with incomplete Freund's adjuvant. Following immunization, antisera were collected, and their antibody titers were evaluated determined by means of an enzyme-linked immunosorbent assay (ELISA) as described below. The mouse exhibiting the best immune response was selected for hybridization. This mouse received a fifth i.p. booster injection and was killed 4 days later. The spleen of the immunized mouse was removed, and the spleen cells were fused with NS-1 mouse plasmacytoma cells according to the method of Kato *et al.* (*21*). The hybridoma culture supernatants were assayed by ELISA for anti-2Ac-Spm antibodies. Limiting dilutions of positive cultures were made two or three times to obtain monoclonality, and sub-isotyping of the mAbs was performed with a Mouse Monoclonal Sub-isotyping kit (American Qualex Int., La Mirada, CA, USA).

ELISA Method—This was similar to our previous ELISA method for ASPM-1 and 2 mAbs (*15, 19*). For the screening of clones for the production of antibodies against 2Ac-Spm, the wells of microtiter plates were coated with the Ac-Spm-GMBS-MS·HSA conjugate (10 μg/ml) for 1 h at room temperature. The plates were then incubated overnight at 4°C with antiserum (diluted 1:3,000), a hybridoma culture supernatant, or ascites fluid (diluted 1:100,000), followed by with goat anti-mouse IgG labeled with horseradish peroxidase (HRP, 1:1,000) for 1 h at 25°C. The amount of enzyme conjugate bound to each well was determined with *o*-phenylenediamine as the substrate, and the absorbance at 492 nm was read with an automatic ELISA analyzer.

Competitive ELISA—The wells of microtiter plates were coated with the Ac-Spm-GA-HSA or 2Ac-Spm-GA-HSA conjugate (10 μg/ml). After rinsing with 50 mM Tris-HCl buffer, pH 7.4, the wells were blocked with 1% skimmed milk for 1 h at 37°C. The wells were then filled with 25 μl of ACSPM-2, and 75 μl of urine diluted to various extents with phosphate-buffered saline containing 0.1% Tween (PBST), or 2Ac-Spm in amounts ranging from 100 nM to 100 μM, followed by 3 h incubation at room temperature. After rinsing with PBST, the plates were incubated with 50 μl of biotin-labeled rabbit anti-mouse Ig diluted 2,000 times with PBST for 1 h at room temperature, washed with PBST as above, and then incubated with 50 μl of HRP-streptavidin diluted 3,000 times with PBST for 30 min. Following the final rinse with PBST, the bound enzyme activity was measured as above.

RESULTS

Antibodies prepared against a covalently linked hapten molecule generally recognize, in part, the conjugation sites of a protein carrier in addition to the molecule itself (*22-27*). In our previous study, the Spm-GMBS-MS·HSA conjugate allowed the production of an ASPM-1 mAb more reactive to Ac-Spm than Spm, strongly recognizing the acylamide linkage formed between the Spm and GMBS in the antigen molecule (*18, 19*). Thus, conjugates cross-linking Ac-Spm *via* GMBS seem to behave as antigens for the production of 2Ac-Spm-specific antibodies. Actually, Hiramatsu *et al.* (*10*) prepared an antiserum highly specific to 2Ac-Spd using the N^1-Ac-Spd conjugate as an antigen.

Antibody Response—Antibodies to the Ac-Spm conjugate were produced in each of three mice immunized with the Ac-Spm-GMBS-MS·BSA conjugate. The antibody titers in serum samples were determined by the ELISA method with the Ac-Spm-GMBS-MS·HSA conjugate as the solid-phase antigen, and they were found to peak 2 weeks after the third booster injection. After the fourth immunization, one of the three mice showed a higher antibody titer and thus was killed for hybridization.

Isolation of Stable Cloned Hybrids—Ten to 20 days after fusion, in 603 out of 960 wells (62%), colonies developed. Nine of 590 wells yielded spent tissue culture fluid exhibiting antibody activity toward Ac-Spm-GMBS-MS·HSA and

five exhibited a selectively higher response to Ac-Spm-GMBS-MS·HSA than to HSA alone. In this way three clones of mAbs, ACSPM-1 to 3, were obtained, which continued to secrete antibodies into both the culture supernatant and ascites fluid in mice.

On sub-isotype determination, the ACSPM-1 and 2 mAbs were found to be of the IgG_1 type, and ACSPM-3 to be of the IgG_{2b} type.

Antibody Dilution—Microtiter wells each coated with a fixed amount of Ac-Spm-GMBS-MS·HSA (10 μg/ml) were used to examine antibody binding using serial dilutions of each of the ACSPM-1 to 3 mAbs (hybridoma culture supernatant). As shown in Fig. 1, significant antibody binding activity was observed with a more than 1,000 times dilution of each of the three mAbs. No antibody binding was seen with an anti-penicillin mAb (Cosmobio).

Specificity of the mAbs—The specificities of the ACSPM-1, 2, and 3 mAbs were first analyzed by means of the ELISA using Ac-Spm-GMBS-MS·HSA, MABA-MS·HSA, MS·HSA, or HSA to coat the wells of plates, the latter three of which were either intermediate complexes or carriers of Ac-Spm-GMBS-MS·HSA. Of the solid-phase antigens tested, only Ac-Spm-GMBS-MS·HSA exhibited immunoreactivity with the three mAbs, and no immunoreactivity was observed with other intermediate carrier complexes or HSA itself for any of the mAbs (data not shown).

Competitive ELISA—This was based on the principle of competition between 2Ac-Spm or related analogs (free in solution) and a fixed amount of the bound antigen for a limited number of binding sites on the mAbs. In the ELISA system with an Ac-Spm-GMBS-MS·HSA-coated solid phase, however, no competition for binding to the antibody was seen with any of the Ac-PAs examined regarding the three mAbs. This may be due to the fact that the mAbs bind to the Ac-Spm-GMBS-MS·HSA solid phase much more strongly than the free compounds, recognizing, in part, the conjugation sites (GMBS) for the carrier. Therefore, we searched for alternative solid-phase antigens.

A variety of GA-conjugated Ac-PA analogs, such as N^1-Ac-Spm, N^1-Ac-Spd, N^8-Ac-Spd, 2Ac-Spd, Ac-Put, and 2Ac-Spm were prepared, and examined as to their use as solid phase antigens for the ELISA. Among them, the former five gave almost the same results: for example, in the ELISA system with the Ac-Spm-GA-HSA-coated solid phase, analysis with the mAbs of the relationship between the doses of the analyte and bound enzyme activity produced dose-dependent inhibition curves with 2Ac-Spm, Ac-Spm, N^1-Ac-Spd, and 2Ac-Spd, in decreasing order. The dose of each analyte required for 50% inhibition of binding (EC_{50}) was determined from the curves and used as an index of the degree of inhibition: With the ACSPM-1 mAb, this dose was 0.34 μM with 2Ac-Spm, the strongest inhibitor among the Ac-PAs examine (Table I), this value being more than 10-times greater than the EC_{50} value (29 nM) of 2Ac-Spm determined with our previous ELISA involving the ASPM-2 mAb (*19*). Thus, this solid phase antigen is still not ideal for sensitive measurement of 2Ac-Spm in human urine.

In the ELISA system with the 2Ac-Spm-GA-HSA conjugate-coated solid phase, the efficiency of competition with the above four compounds for binding to the antibody markedly increased with all of three mAbs. With the ACSPM-2 mAb, the EC_{50} values were 0.06, 0.25, 7.0, and 10 μM with 2Ac-Spm, Ac-Spm, N^1-Ac-Spd and 2Ac-Spd, respectively (Table I and Fig. 2). In the case of the ACSPM-1 or 3 mAb, the EC_{50} values for 2Ac-Spm and

TABLE I. **EC_{50} values of acetyl polyamine analogs determined by ELISA using GA-conjugated Ac-Spm or 2Ac-Spm as the solid-phase antigen.**

Analyte	Solid-phase antigen: Ac-Spm-GA-HSA			2Ac-Spm-GA-HSA		
	ACSPM mAb					
	1	2	3	1	2	3
2Ac-Spm	0.34	1.0	6	0.14	0.06	0.2
Ac-Spm	1.1	2.4	14	0.5	0.25	0.4
N^1-Ac-Spd	20	60	N.D.	6.7	7	9
2Ac-Spd	50	100	N.D.	9.0	10	7.5

Each value is of the order of μM. N.D., not determined.

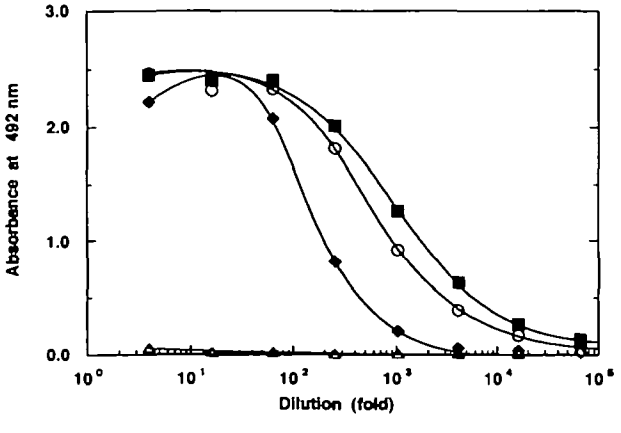

Fig. 1. **Dilution curves for ACSPM-1 to 3 mAbs with wells coated with the Ac-Spm-GMBS-MS·HSA conjugate (10 μg/ml).** Samples of the ACSPM-1 (□), 2 (■), and 3 (◆) mAbs were serially diluted with PBST, followed by assaying the ELISA method described under "MATERIALS AND METHODS" section. Immunoreaction of the ACSPM-1, 2, or 3 mAb, as a control with the solid phase conjugate but omitting Ac-Spm or GMBS (△).

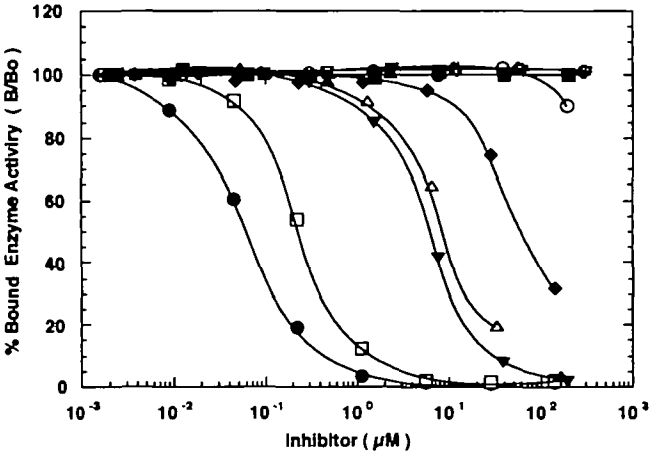

Fig. 2. **Dose-response standard curves for 2Ac-Spm and PA analogs.** The curves show the amounts (percentages) of bound enzyme activity for various doses of 2Ac-Spm or PA analogs (B) as a ratio to that bound with the ACSPM-2 mAb alone (B^*). 2Ac-Spm, (●); Ac-Spm, (□); N^1-Ac-Spd, (▼); 2Ac-Spd, (△); Spm, (◆); N^8-Ac-Spd, (○); Spd, (■); Put, (▲); Cad, (◇); Ac-Put, (▽); L-ornithine, (★).

Ac-Spm were slightly larger than those in the case of the ASPM-2 mAb, but those for N^1-Ac-Spd and 2Ac-Spd were almost the same (Table I).

Optimal Assay Conditions for the ELISA for 2Ac-Spm Using the ACSPM-2 mAb—It was found that a 1:3,000 solution of ACSPM-2 [3.3 ng/ml, this concentration being determined by the conventional "sandwich ELISA" using chromatographically purified mouse IgG (ZYMED Lab., USA)], a 1:2,000 solution of the second antibody, a 1:3,000 solution of horseradish peroxidase (HRP)-streptavidin, 3 h incubation of the ACSPM-2 mAb with the antigen at room temperature, 1 h incubation with the second antibody, and 30 min further incubation with HRP-streptavidin provided an effective assay system at 25°C. A standard calibration curve for 2Ac-Spm quantification is presented in Fig. 2. The lower limit of detection of the assay was 10 nM 2Ac-Spm, and the working range was shown to be 10 nM to 1 μM. For 2Ac-Spm the intraassay coefficient of variation at 3 different levels of 2Ac-Spm (50, 100, and 200 pM, $n=5$) was 3.0-5.9% (4.2% average).

Application of the ELISA—In a preliminary study on the levels of 2Ac-Spm in human urine, the ELISA system with the ASPM-2 mAb and 2Ac-Spm-GA-HSA as the solid-phase was used. Recovery experiments on the ELISA were performed using 2 normal human urine specimens. Each urine sample was assessed with 50, 100, or 200 nM externally added authentic 2Ac-Spm, and the recovery of the added 2Ac-Spm was calculated from the standard curve for the ELISA, almost quantitative amounts of added 2Ac-Spm (96 to 110%) being found, *i.e.*, 54, 108, and 220 nM, respectively, as average values ($n=4$ for each of the three levels). Spot urine samples from 8 male and 8 female human volunteers, all apparently healthy and aged from 21 to 30 years, were collected, and were directly assayed for 2Ac-Spm by means of the ELISA method without pretreatment such as extraction or chromatographic separation of the specimens prior to the analysis, using 2Ac-Spm as a standard. Various amounts of urine samples (1.1 to 75 μl) gave ELISA titration curves similar in shape to that of the standard 2Ac-Spm (Fig. 3). Considering the values on the portions of the curves between 30 to 60% maximal absorbance, the concentration of 2Ac-Spm was determined to be 0.34+0.16 μmol/g creatinine for males and 0.39+0.14 μmol/g creatinine for females (0.36 μmol/g creatinine as the total average). The range of the SE was 0.144.

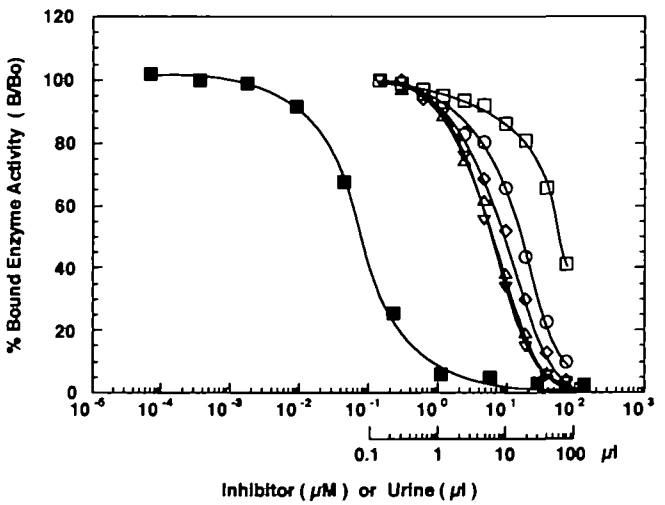

Fig. 3. **Standard curve for 2Ac-Spm, and ELISA titration curves for urine samples.** The curves show the amounts (percentages) of bound enzyme activity for various doses of 2Ac-Spm or for urine samples (B) as a ratio to that bound with the ACSPM-2 mAb alone (B^*). 2Ac-Spm, (■); urine samples 1, ▽; 2, △; 3, ◇; 4, ○; 5, □.

DISCUSSION

The availability of a sensitive and simple assay for acetyl PAs will facilitate not only biochemical studies on PA metabolism, but also the monitoring of pathophysiological states associated with abnormal PA metabolism including cancers (*3, 4, 9, 27, 28*).

Using an Ac-Spm conjugate prepared similarly to the Spm conjugate used for ASPM-1 and 2 production in our previous studies (*18, 19*), we prepared mAbs ACSPM-1 to 3. A large number of hybridomas were obtained (62% of the wells yielded colonies), but only five exhibited selective binding activity for Ac-Spm conjugated to HSA *via* GMBS. Three of the hybridomas, which each showed a prolific growth rate, were cloned, and secreted antibodies of the IgG_1 sub-isotype, the ACSPM-1 and 2 mAbs, and of the IgG_{2b} sub-isotype, the ACSPM-3 mAb, respectively.

The three mAbs exhibited specific immunoreactivity to the Ac-Spm-GMBS-MS·HSA conjugate used as the solid phase for the ELISA (Fig. 1), but no immunoreactivity to the carrier HSA, MS·HSA, and GMBS-HSA, demonstrating lack of a recognition. For the competitive ELISA, however, GMBS-conjugated Ac-Spm, as the immununization antigen, was not useful as a solid phase antigen, possibly due to such strong affinity between the mAbs and the conjugate that no competition for the binding sites of the antibodies would occur with any of the Ac-PA analogs. The finding that among alternative GA-conjugated Ac-PAs, the ELISA system with the 2Ac-Spm-GA-HSA-coated solid phase showed about 2.4 to 20-times higher sensitivity as to the detection of 2Ac-Spm than that with the Ac-Spm-GA-HSA conjugate with each of the three mAbs (Table I), may suggest that the antibodies bind rather weakly to the 2Ac-Spm-GA-HSA conjugate, and is displaced easily by 2Ac-Spm at lower concentrations (Table I). The chemical structure of the 2Ac-Spm-GA-HSA conjugate was not elucidated precisely in the present study, but the secondary amino group(s) of 2Ac-Spm may possibly be cross-linked to one of the two aldehyde groups of the GA molecule, an N-hemi-acetal linkage being formed. Actually, Niitsu *et al.* (*29*) recently reported that the secondary amino group of spermidine reacts with an aldehyde group of malondialdehyde at neutral pH.

An optimal assay procedure was established for the 2Ac-Spm ELISA with the ACSPM-2 mAb, which proved so sensitive that 2Ac-Spm concentrations lower than 10 nM could be measured reproducibly (Fig. 2). The ELISA was specific to 2Ac-Spm, with 24% cross-reaction with Ac-Spm, 0.85% with N^1-Ac-Spd, 0.6% with 2Ac-Spd, and 0.1% with Spm (Fig. 3 and Table I). The high cross-reaction with Ac-Spm may be of almost no significance when assaying 2Ac-Spm in human urine by means of the ELISA, since the level of Ac-Spm was found to be very low in the urine, as mentioned below (*10*).

The ELISA was demonstrated to be applicable to urine samples from healthy humans in preliminary experiments.

The average value of 0.36 μmol of 2Ac-Spm/g creatinine demonstrated in the 16 specimens seems to be about 3.5 times higher than the concentration reported by Hiramatsu *et al.* (*10*), who performed high-performance liquid chromatography (HPLC) with an enzyme reactor and an electrochemical detector. They also described that of the total of N^1-Ac-Spd, 2Ac-Spm, Ac-Spm, and 2Ac-Spd, N^1-Ac-Spd accounted for 86.2%, 2Ac-Spd for 9.58%, and Ac-Spm for only 1.0% in human urine. In the case of the present ELISA, the ACSPM-2 mAb may therefore be supposed to react essentially with 2Ac-Spm in urine, although up to 20% of the total amount of Ac-PAs detected might be N^1-Ac-Spd, Ac-Spm, and 2Ac-Spd, taking their cross-reactivity with the ACSPM-2 mAb into account (Fig. 3 and Table I). This still seems insufficient to explain the discrepancy in the 2Ac-Spm levels determined in the present study and by Hiramatsu *et al.* (*10*), but it might be due, in part, to the fact that the total values determined with the ELISA would be significantly affected by changes in the levels of Ac-PAs other than 2Ac-Spm in a variety of pathophysiological conditions. It would therefore be ideal to develop a more highly specific and sensitive ELISA system than the present one for 2Ac-Spm, which would involve lower cross-reaction with other Ac-PA analogs, especially with N^1-Ac-Spd, for example, a cross-reaction value of less than one-tenth that with the present ELISA using the ACSPM-2 mAb.

In conclusion, it was clearly demonstrated that among the three mAbs produced in this study, the ACSPM-2 mAb is the most useful for an ELISA for the determination of Ac-PAs, especially 2Ac-Spm, in human urine samples. This ELISA has the advantages of simplicity, sensitivity, and specificity, without any pretreatment of urine samples prior to the assay, and thus may be adequate for the handling of a large number of urine samples from clinical laboratories. We are now conducting 2Ac-Spm ELISA studies using the ACSPM-2 mAb to determine if 2Ac-Spm is a possible tumor marker in urine samples from patients with a variety of pathophysiological conditions including urogenital cancers.

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