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Preparation of Antibodies Highly Specific to N^1,N^8-Diacetylspermidine, and Development of an Enzyme-Linked Immunosorbent Assay (ELISA) System for Its Sensitive and Specific Detection[1]

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N^8-Acetylspermidine was coupled to mercaptosuccinylated BSA using a bifunctional cross-linker, N-(4-maleimidobutyryloxy)succinimide, and the resulting conjugate was used to raise N^1,N^8-diacetylspermidine (DiAcSpd)-specific antibodies in rabbits. DiAcSpd-specific antibodies were enriched from crude sera through a series of affinity-based fractionations using ligands with structures mimicking those of DiAcSpd and monoacetylspermidines. With the N^8-acetylspermidine-BSA conjugate as a solid phase antigen in a competitive ELISA system, the selectivity for DiAcSpd over other polyamine species was high, but competition by DiAcSpd added to the fluid phase was too weak for the system to be applicable to measurement of the concentration of DiAcSpd in human urine. In contrast, with the N^1-acetylspermidine-BSA conjugate adsorbed on the ELISA plate, DiAcSpd efficiently competed for the same antibody, thus yielding a sensitive competitive ELISA system for measuring DiAcSpd. The K_i value for DiAcSpd with the latter competitive ELISA system was 54 nM, and the cross-reactivity with DiAcSpd, N^1,N^{12}-diacetylspermine, N^8-acetylspermidine, N^1-acetylspermidine, and acetylputrescine was 100, 1.2, 0.74, 0.12, and 0.08%, respectively. The DiAcSpd-specific antibodies and the competitive ELISA system developed in this study will prove to be useful for analyzing the urinary level of DiAcSpd, that was recently shown to be a promising diagnostic and prognostic indicator of malignant disorders.

Key words: anti-polyamine antibody, diacetylspermidine, ELISA, polyamine, tumor marker.

In one of our recent publications we described a procedure for the comprehensive analysis of polyamines in human urine, which is based on fractionation by HPLC, followed by enzymatic oxidation of polyamines and detection of H_2O_2 quantitatively produced in this reaction (*1*). Using this procedure, we demonstrated that N^1,N^8-diacetylspermidine (DiAcSpd) and N^1,N^{12}-diacetylspermine (DiAcSpm) are present as relatively minor but regular constituents of urine from healthy persons (*2*). Further analysis of urine samples for diacetylpolyamines revealed that they were markedly increased in cases of malignant diseases, and we proposed that diacetylpolyamines may be useful as novel tumor markers in the diagnosis (*3*), and also in the assessment of the conditions of cancer patients under treatment as well as in remission (Hiramatsu, K. *et al.*, to be published elsewhere).

Although our analysis has so far been limited to benign and malignant urogenital disorders, it is an obvious possibility that DiAcSpd and DiAcSpm may be generally applicable tumor markers that are also useful in other malignant disorders, since activation of polyamine metabolism has long been noted to be associated with various types of cell proliferation (*4*). However, the possibility remains to be examined by determining the amounts of DiAcSpd and DiAcSpm in urine samples collected from a large number of patients with a wide variety of diseases. In this context our procedure for the HPLC analysis of polyamines is rather time-consuming and a little inconvenient for handling a large number of samples, although it is sensitive and versatile enough to allow the accurate and simultaneous determination of 11 polyamine species in human urine,

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Abbreviations: Ac-Put, acetylputrescine; AcSpm, acetylspermine; BSA, bovine serum albumin; DiAcSpd, N^1,N^8-diacetylspermidine; DiAcSpm, N^1,N^{12}-diacetylspermine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; GMBS, N-(4-maleimidobutyryloxy)succinimide; HMCS, N-(8-maleimidocapryloxy)succinimide; N^1-AcSpd, N^1-acetylspermidine; N^8-AcSpd, N^8-acetylspermidine; PBS, phosphate-buffered saline; Spd, spermidine; Spm, spermine.

including free, monoacetylated, and diacetylated components.

This prompted us to develop an alternative procedure that allow specific and more convenient determination of diacetylpolyamines in urine. An enzyme-linked immunosorbent assay (ELISA) involving DiAcSpd- or DiAcSpm-specific antibodies, though such antibodies have not been obtained so far, would be a promising alternative to the HPLC-based procedure. Antibodies specific to spermidine (Spd) and spermine (Spm) have been described (*5–11*), and furthermore some of the antipolyamine antibody preparations reported by Fujiwara *et al.* (*8, 9, 12–14*) actually showed a preference for DiAcSpm over acetylspermine (AcSpm) and N^1-acetylspermidine (N^1-AcSpd) (*15*). However, the preference (approximately 10 times) did not seem to be enough to allow the quantification of DiAcSpm in urine, since the DiAcSpm in human urine usually amounts to only about 1/30 the content of N^1-AcSpd (*2*). In fact, they applied one of these antibodies to an ELISA system intending to estimate the amount of N^1-AcSpd, not that of DiAcSpm, in urine (*16*).

In this article we report the preparation of an antibody that is highly specific to DiAcSpd, and describe ELISA conditions that allow the sensitive detection of DiAcSpd present in solutions based on its competition for the antibody with solid-phase antigens.

MATERIALS AND METHODS

Chemicals—Monoacetyl polyamines, *S*-acetylmercaptosuccinic anhydride, and bovine serum albumin (BSA) were purchased from Sigma, St. Louis, MO, USA. DiAcSpd and DiAcSpm were kind gifts from Dr. A. Shirahata of Josai University. *N*-(4-Maleimidobutyryloxy)succinimide (GMBS) and *N*-(8-maleimidocapryloxy)succinimide (HMCS) were from Dojindo Laboratories, Kumamoto, and AF-Carboxy-Toyopearl 650 from Tosoh, Tokyo, respectively.

Preparation of Monoacetylspermidine-BSA Conjugates—N^1-AcSpd and N^8-AcSpd were conjugated to mercaptosuccinylated BSA *via* GMBS or HMCS, as described by Fujiwara *et al.* (*8*), the resulting conjugates being designated as N^1- and N^8-AcSpd-GMB-BSA or N^1- and N^8-AcSpd-HMC-BSA, respectively.

Preparation of Spermidine Derivatives of AF-Carboxy-Toyopearl 650 with Acylamide Linkages—AF-Carboxy-Toyopearl 650 (2 g of swollen gel) was washed once with 0.5 M NaCl, mixed with 2 mM Spd or N^8-AcSpd (7 ml), and then reacted with 40 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at room temperature for 48 h at pH 5–6. The polyamine-conjugated resin was washed with 1 M NaCl, followed with 1 M NaCl in 10 mM HCl, and then finally stored in 10 mM HCl at 4°C.

Detection of DiAcSpd-Specific Antibodies with an ELISA System—The wells of microtiter plates (Corning #25801) were coated with 0.05 ml of N^1- or N^8-AcSpd-HMCS-BSA conjugate (0.2 μg/ml) in 0.1 M phosphate-buffered saline (PBS) for 1 h at room temperature, and then washed 3 times with 0.2 ml of a washing solution consisting of 0.14 M NaCl, 2.7 mM KCl, 25 mM Tris-HCl (pH 8.0), and 0.05% Tween 20. The wells were then blocked with 0.2 ml of 5% skimmed milk in the washing solution overnight at 37°C, and washed 3 times with 0.2 ml of the washing solution. An appropriate dilution of anti-DiAcSpd antibodies in PBS (0.05 ml) was added to the wells, and then the plate was incubated at room temperature for 1 h with constant shaking. The antibodies were removed and the wells were washed 3 times with 0.2 ml of the washing solution. To each well was added horseradish peroxidase-labeled anti-rabbit IgG (Zymed, South San Francisco, CA, USA) at 1:5,000 dilution (0.1 ml), and then the plate was kept for 1 h at room temperature. The wells were washed 3 times with 0.2 ml of the washing solution, and then 0.1 ml of a H_2O_2-2,2′-azino-di[3-ethyl-benzothiazoline sulfonate(6)] (ABTS) solution (KPL, Gaithersburg, MD, USA) was added to each well. After keeping the plate for 30 min at room temperature, the absorbance at 405 nm was measured.

In experiments carried out in the earlier phase of this study, N^8-AcSpd was directly conjugated to the functional groups of maleic anhydride-activated polystyrene plates (Pierce, Rockford, IL, USA). For this purpose, the wells were filled with 10 μM N^8-AcSpd and the plate was kept at room temperature for 1 h. Blocking of the remaining active surface and subsequent steps were carried out exactly as described above.

Determination of the Relative Preference of Anti-DiAcSpd Antibodies for Polyamine Species—A competitive ELISA was carried out to compare the relative affinities of the antibodies for various polyamine species. For the competitive ELISA, the standard procedure described above was slightly modified, that is, at the step of the addition of the anti-DiAcSpd antibodies, an appropriate concentration of competing polyamines (0.05 ml) was added to the wells prior to the addition of the antibody solution (0.05 ml). Other steps of the procedure were carried out exactly as described above.

Preparation of Antisera—Two New Zealand White rabbits for each immunogen were injected subcutaneously with 2 ml of N^1-AcSpd-GMB-BSA conjugate or N^8-AcSpd-GMB-BSA conjugate (1 mg protein) emulsified in complete Freund's adjuvant (Difco, Detroit, MI, USA) at a ratio of 1 : 1 (v/v). A series of five booster injections was given at 2-week intervals in a similar manner to the first injection except that incomplete Freund's adjuvant (Difco) was used. Each animal was finally bled from the heart, and the sera were separately stored at −80°C.

Affinity Purification of Antibodies—The IgG fraction was prepared from crude antiserum using an immobilized Protein A column (Ampure PA; Amersham, Buckinghamshire, UK). One milliliter of Anti-DiAcSpd IgG thus obtained (0.5 ml crude serum equivalent) was diluted with 8 ml of 10 mM Tris-HCl buffer (pH 7.5), and then applied to a column (0.5 ml) of either N^8-AcSpd-conjugated AF Carboxy-Toyopearl 650 when N^8-AcSpd-GMB-BSA was used as the immunogen, or N^1-AcSpd-conjugated AF Carboxy-Toyopearl 650 when N^1-AcSpd-GMB-BSA was used. The column was washed successively with 5 ml each of 10 mM Tris-HCl buffer (pH 7.5) and 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, and then the adsorbed proteins were eluted with 0.1 M glycine-HCl buffer (pH 2.5). The eluate was immediately neutralized by adding a 1/10 volume of 1 M Tris-HCl buffer (pH 8.0), and the fractions containing partially purified anti-DiAcSpd antibodies were combined (Fr. 1) and saved. For further purification, 2 ml of the Fr. 1 antibody (1 ml crude serum equivalent) was diluted to 10 ml with 10 mM Tris-HCl buffer (pH 7.5), and then applied to a column (0.5 ml) of

Spd-conjugated AF Carboxy-Toyopearl 650 equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The flow-through fraction was collected (Fr. 2) and saved as the purified anti-DiAcSpd antibody.

RESULTS

Preparation of Anti-DiAcSpd Antiserum—N^1-AcSpd and N^8-AcSpd were conjugated separately to BSA according to the procedure described by Fujiwara *et al.* (*8*), and the resulting conjugates were used to raise anti-DiAcSpd antibodies in rabbits. As depicted in Fig. 1A, on the crosslinking of monoacetylspermidines to BSA *via* GMBS, an acylamide linkage was formed at the linker-Spd junction through the primary amino group of monoacetylspermidine, giving a structure which closely resembled that of DiAcSpd in the polyamine portion. Thus, the conjugates seemed to serve as haptens for raising DiAcSpd-specific antibodies.

In fact, with either the N^1-AcSpd or N^8-AcSpd conjugate as an antigen, antiserum with a preference for DiAcSpd over other polyamine species was obtained. However, only the properties of the latter were examined in detail and are described below, because preliminary experiments indicated that it is definitely more specific than the former to DiAcSpd. Competitive ELISA experiments involving maleic anhydride-activated polystyrene plates (PIERCE) directly conjugated with N^8-AcSpd as a solid phase antigen revealed that the antiserum raised against the N^8-AcSpd-BSA conjugate showed a definite preference for DiAcSpd over N^8-AcSpd. However, it was also noticed that the serum gave a significant level of binding to the plate that could not be quenched by competing polyamines including mono- and diacetylspermidines (data not shown).

Purification of DiAcSpd-Specific Antibodies by Affinity Chromatography on N^8-AcSpd-Carboxy-Toyopearl—N^8-AcSpd was coupled to Carboxy-Toyopearl through a water-soluble carbodiimide (EDC) to form an affinity ligand that mimics the structure of DiAcSpd (Fig. 1B, 1), and the IgG fraction obtained from anti-DiAcSpd antiserum raised against the N^8-AcSpd-BSA conjugate was fractionated on this affinity resin. Antibodies that could form a complex with DiAcSpd were adsorbed to the resin, and the adsorbed antibodies were then eluted with an acidic elution buffer. The antibodies recovered in this fraction (Fr. 1) did not show any cross-reactivity with BSA, indicating that the N^1- and N^8-AcSpd-BSA conjugates (Fig. 1C) could be employed as solid phase antigens in the subsequent ELISA experiments to detect DiAcSpd-specific antibodies. The monoacetylspermidine-BSA conjugates attached to conventional ELISA plates were highly efficient in detecting DiAcSpd-specific antibodies.

As shown in Fig. 2, the competitive ELISA involving the N^8-AcSpd-BSA conjugate as a solid phase antigen (Fig. 1C, 1) in combination with the Fr. 1 antibody indicated that competition by DiAcSpd was the most prominent. Among other polyamine species only DiAcSpm and N^8-AcSpd exhibited weak competition, but only at concentrations of

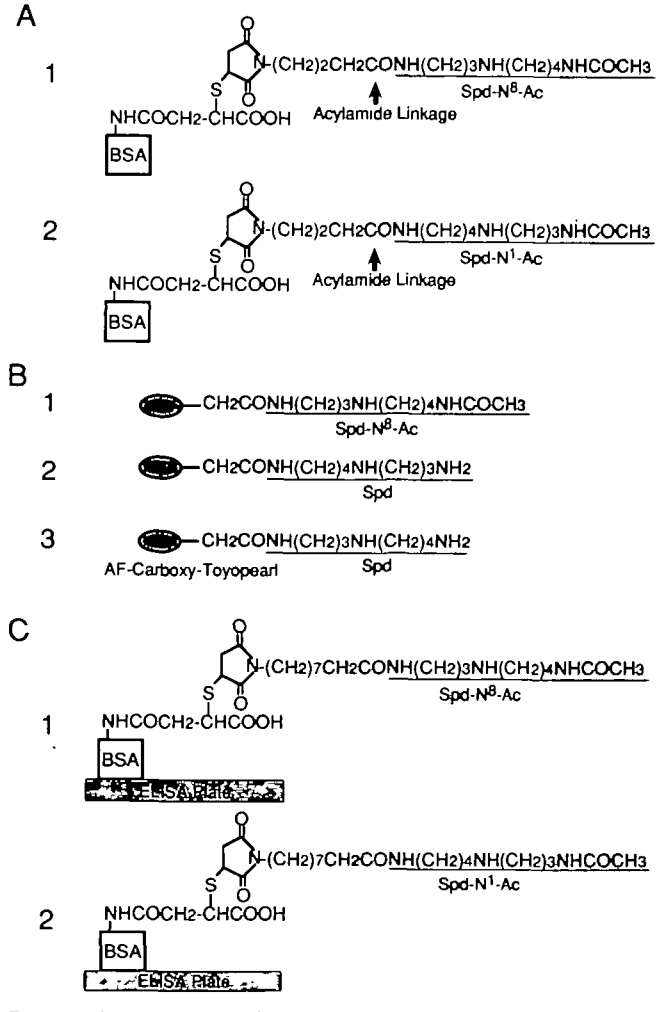

Fig. 1. **Structures of the spermidine conjugates used in the present study as antigens to raise anti-DiAcSpd antisera (A), as affinity-adsorbents for the purification of DiAcSpd-specific antibodies (B), and as solid-phase antigens in the ELISA system to detect DiAcSpd-specific antibodies (C).**

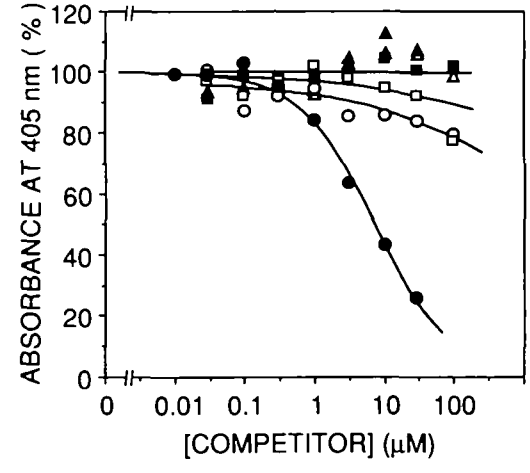

Fig. 2. **Specificity of the Fr. 1 antibody to DiAcSpd, as assessed by competitive ELISA with the N^8-AcSpd-HMCS-BSA conjugate as a solid-phase antigen.** Competitive ELISA was carried out as described under "MATERIALS AND METHODS" with the N^8-AcSpd-HMCS-BSA conjugate as a solid-phase antigen using the Fr. 1 antibody at 1:32,000 dilution. DiAcSpd (●), DiAcSpm (○), N^1-AcSpd (■), N^8-AcSpd (□), AcSpm (▲), and AcPut (△), at the concentrations indicated on the abscissa, were used as competitors.

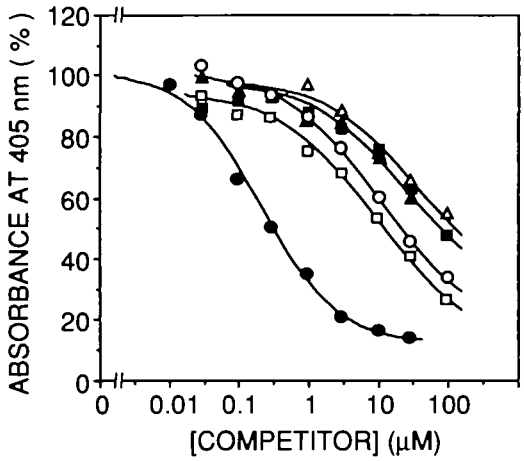

Fig. 3. **Specificity of the Fr. 1 antibody to DiAcSpd, as assessed by competitive ELISA with the N^1-AcSpd-HMCS-BSA conjugate as a solid-phase antigen.** Competitive ELISA was carried out as in Fig. 2 except that the N^1-AcSpd-HMCS-BSA conjugate was used as a solid-phase antigen and that the Fr. 1 antibody was used at 1:30,000 dilution. DiAcSpd (●), DiAcSpm (○), N^1-AcSpd (■), N^8-AcSpd (□), AcSpm (▲), and AcPut (△), at the concentrations indicated on the abscissa, were used as competitors.

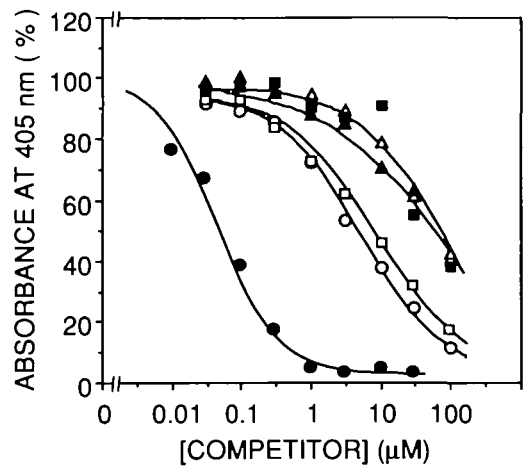

Fig. 4. **Specificity of the Fr. 2 antibody to DiAcSpd, as assessed by competitive ELISA with the N^1-AcSpd-HMCS-BSA conjugate as a solid-phase antigen.** Competitive ELISA was carried out as in Fig. 3 except that the Fr. 2 antibody at 1:5,000 dilution was used. DiAcSpd (●), DiAcSpm (○), N^1-AcSpd (■), N^8-AcSpd (□), AcSpm (▲), and AcPut (△), at the concentrations indicated on the abscissa were used as competitors.

more than 100 times higher than those of DiAcSpd. Other acetylpolyamines did not affect the interaction between the antibody and DiAcSpd practically at all, neither did unacetylated polyamines (data not shown).

Although the Fr. 1 antibody was very specific to DiAcSpd, the concentration of DiAcSpd needed to give 50% inhibition of antibody binding to the solid phase antigen was 8 μM, which is about 25 times higher than that of DiAcSpd usually present in healthy human urine (*2*). This implies that the conditions for the competitive ELISA in Fig. 2 as such are still inadequate for determining the amount of DiAcSpd in human urine.

Use of the N^1-AcSpd-BSA Conjugate as an Alternative Solid-Phase Antigen for Competitive ELISA—To increase the sensitivity of the competitive ELISA, the use of N^1-AcSpd-BSA (Fig. 1C, 2) as a solid phase antigen was examined. As shown in Fig. 3, the efficiency of competition with DiAcSpd for binding to the antibody was markedly increased, 0.3 μM DiAcSpd causing 50% inhibition of the binding of the Fr. 1 antibody to the solid-phase antigen. This implies that DiAcSpd present normally in human urine (0.3 μM) would significantly affect the binding of the Fr. 1 antibody, thereby enabling the determination of its level in the urine.

Use of the N^1-AcSpd-BSA conjugate, however, resulted in poorer selectivity for DiAcSpd over other polyamine species, as compared to the N^8-AcSpd-BSA conjugate. When the reactivity with DiAcSpd was taken as 100%, the cross-reactivity with N^8-AcSpd, DiAcSpm, N^1-AcSpd, and AcPut was 2.3, 1.2, 0.38, and 0.24%, respectively. This is in contrast with the almost negligible cross-reactivity with irrelevant polyamines, as described in the preceding section. We therefore tried to further purify the Fr. 1 antibody to improve the selectivity for DiAcSpd.

Further Purification of DiAcSpd-Specific Antibodies by Affinity Chromatography on Spd-Carboxy-Toyopearl—Spd was coupled to Carboxy-Toyopearl through a water-soluble carbodiimide (EDC) to form a mixture of affinity ligands that mimic the structure of either N^1-AcSpd or N^8-AcSpd (Fig. 1B, 2 and 3), intending to remove antibodies that could form a complex with N^1- and N^8-AcSpd. The Fr. 1 antibody was fractionated on this affinity resin, and the antibodies that were *not* adsorbed to the resin were saved (Fr. 2). The properties of the Fr. 2 antibody were examined by competitive ELISA, as shown in Fig. 4, using the N^1-AcSpd-BSA conjugate as a solid phase antigen. The efficiency of the competition by DiAcSpd for the binding of Fr. 2 antibody to the solid-phase antigen was further increased, a K_i value for DiAcSpd of 54 nM being obtained. At the same time, the cross-reactivity with N^8-AcSpd, DiAcSpm, N^1-AcSpd, and AcPut decreased to 0.74, 1.2, 0.12, and 0.1%, respectively, the reactivity with DiAcSpd being taken as 100%. Since the relative contents of DiAcSpd, N^8-AcSpd, DiAcSpm, N^1-AcSpd, and AcPut in healthy human urine are 1, 8, 0.3, 9, and 30, respectively (*2*), extraneous polyamine species would not significantly interfere with the determination of urinary DiAcSpd with the Fr. 2 antibody.

DISCUSSION

An antibody preparation highly specific to DiAcSpd was obtained, and a sensitive competitive ELISA system for determining DiAcSpd in fluid samples was developed.

The molecular design of the hapten-carrier conjugates seemed to contribute much to the production of specific antibodies. The presence of acylamide linkage(s) in hapten-carrier conjugates seems to be indispensable for raising antibodies that cross-react preferentially with acetylpolyamines, since antibodies against glutaraldehyde-conjugated spermine as a hapten were slightly more reactive with Spm and Spd than with acetylpolyamines on competitive ELISA (*14*). In this regard, monoacetylspermidine-GMBS conjugates must ideally mimic diacetylspermidine, thus yielding serum preparations containing a subpopulation of highly DiAcSpd-specific antibodies.

Similarly, the molecular design of the ligands for affinity

chromatography was important for the purification of the specific antibodies. Thus, the acylamide linkage formed on the derivatization of Carboxy-Toyopearl with N^8-AcSpd and Spd closely mimics the acetyl groups in DiAcSpd and N^1- or N^8-AcSpd, respectively. These affinity ligands thus enabled us to fractionate the antibodies based on the differential affinity to mono- and diacetylspermidine to obtain a subpopulation that was highly specific to DiAcSpd. Fractionation of the antibodies to isolate a subpopulation of anti-DiAcSpd antibodies with a specificity necessary for the determination of urinary DiAcSpd naturally led to a considerable decrease in the titer of the antibodies. However, it did not matter practically in the present case, since the titer of the purified antibodies was still reasonably high, so that the antibodies could be used in our standard competitive ELISA system at a dilution of 1:5,000 as normalized to the volume of the crude antiserum.

The choice of solid-phase antigens profoundly affected the performance of the competitive ELISA involving DiAcSpd-specific antibodies isolated from antiserum raised against the N^8-AcSpd-GMBS conjugate as a hapten. When the N^8-AcSpd-BSA conjugate was used as the solid-phase antigen, the selectivity for DiAcSpd over other polyamine species was higher than that observed when the N^1-AcSpd-BSA conjugate was used instead. However, competition for the antibody by DiAcSpd added to the fluid phase was very weak with N^8-AcSpd-BSA conjugate as the solid-phase antigen. In fact, the K_i value for DiAcSpd in this system was too high practically if the system were to be used to measure the amount of DiAcSpd naturally found in human urine. In contrast, with the N^1-AcSpd-BSA conjugate adsorbed on the ELISA plate, DiAcSpd efficiently competed for the same antibody. A K_i value of 54 nM would make a competitive ELISA system involving this plate sensitive enough to measure urinary DiAcSpd, whose mean concentration in healthy human urine is 0.3 μM.

The efficiency of the competition between DiAcSpd in solution and solid-phase antigens for the antibody depends on their relative affinities to the antibody. The difference in the efficiency of competition by DiAcSpd in the competitive ELISA involving the two kinds of conjugates therefore implies that the antibody binds more weakly to the N^1-AcSpd-BSA conjugate than to the N^8-AcSpd-BSA conjugate. This is quite reasonable in view of the fact that the antibody was raised against the N^8-AcSpd-GMBS conjugate as a hapten, and that N^1-acetyl-N^8-acylamidospermidine and N^1-acylamido-N^8-acetylspermidine are closely similar but definitely distinct in structure. It is also consistent with the observation that the selectivity for DiAcSpd of the Fr. 1 antibody over other polyamine species was higher with the N^8-AcSpd-BSA conjugate as the solid-phase antigen.

Use of the N^1-AcSpd-BSA conjugate thus led to marked improvement in the sensitivity of the competitive ELISA, but at some expense of the specificity to DiAcSpd. The slight loss of specificity inherent to this choice could be successfully compensated for by including an additional step of purification of the antibody on an acylamidospermidine affinity column. The resulting purified anti-DiAcSpd antibody, Fr. 2, showed only 0.74, 0.12, and 0.1% cross-reactivity with N^8-AcSpd, N^1-AcSpd, and AcPut, respectively. This implies that the Fr. 2 antibody is specific enough to be used for determining the amount of urinary DiAcSpd accurately in the presence of excess N^8-AcSpd, N^1-AcSpd, and AcPut, which are on average 8, 9, and 30 times the amount of DiAcSpd, respectively, in healthy human urine (*2*). Attempts to establish a reliable method for the immunochemical determination of urinary DiAcSpd based on these results are now in progress in this laboratory.

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