Construction of an Immunochromatographic Determination System for N^1,N^{12} -diacetylspermine

Shun-suke Moriya,¹ Kyoko Hiramatsu,¹ Emi Kimura,² Kyoichi Matsumoto,² and Masao Kawakita^{1*}

¹Translational Medical Research Center, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

²Mikuri Immunological Laboratories, Osaka, Japan

Background: N^1,N^{12} -diacetylspermine (DiAcSpm) is a recently identified tumor marker. Its concentration increases in the urine of cancer patients at early clinical stages. To utilize this characteristic feature and thus contribute to the early detection of cancer, we developed an immunochromatographic determination system for DiAcSpm. **Methods:** We examined the factors that affect the performance and stability of our determination system, including antibody selection and the conditions for the formation of stably dispersed antibody-coated gold nanoparticles. We then tested the performance of the system by determining the DiAcSpm concentration in human urine samples. **Results:** We constructed an immunochromatographic strip using anti-DiAcSpm antibody-coated gold nanoparticles in the conjugate pad and an

acetylspermine–protein conjugate (a DiAcSpm mimic) immobilized on the analyzing membrane. The use of the immunochromatographic strip and an immunochromatoreader allowed for the quantitative determination of DiAcSpm in the range of 20 to 700 nM. The analytical values obtained by this method were well correlated with those determined by a colloidal gold aggregation procedure using an automatic biochemical analyzer. The immunochromatographic strip was stable for at least 8 weeks at 50°C. **Conclusions:** A competitive immunochromatographic device for DiAcSpm determination was developed in this study. This simple device will contribute to increasing the opportunities for early cancer detection and timely care. J. Clin. Lab. Anal. 28:452–460, 2014. © 2014 Wiley Periodicals, Inc.

Key words: biological tumor markers; biomarkers; clinical chemical tests; clinical laboratory techniques; gold nanoparticles; neoplasms; urinalysis; urine

Abbreviations

AcSpm	=	N -acetylspermine
BSA	=	bovine serum albumin
DiAcSpm	=	N^1,N^{12} -diacetylspermine
N^1 -AcSpd	=	N^1 -acetylspermidine
PBS	=	phosphate buffered saline
PEG	=	polyethylene glycol
PVA	=	polyvinyl alcohol
PVP	=	polyvinyl pyrrolidone

INTRODUCTION

N^1,N^{12} -diacetylspermine (DiAcSpm) is a recently identified tumor marker (for review, see ref. 1). It is a minor component of polyamines in human urine, amounting to less than 0.5% of human urinary polyamines. It had

long escaped notice until it was detected in human urine by HPLC separation followed by an in-line enzymatic detection system, which allowed for the sensitive detection of diacetylpolyamines, namely DiAcSpm and N^1,N^8 -diacetylspermidine (2). Subsequent analyses revealed that DiAcSpm, among other polyamine species, was specifically and frequently elevated in the urine of patients with

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*Correspondence to: Masao Kawakita, Translational Medical Research Center, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kami-kitazawa, Setagaya-ku, Tokyo 156-8506, Japan. E-mail: kawakita-ms@igakuken.or.jp

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various cancers, such as prostate cancer, colon cancer, and breast cancer (3–5). One of the features that make urinary DiAcSpm a particularly promising candidate for a novel tumor marker is that the urinary DiAcSpm level is more frequently elevated in patients in the early stages of colorectal and breast cancer compared to other tumor markers. For instance, DiAcSpm in the urine is elevated above the normal level in 60% of early colorectal cancers that remain in the mucosal layers, while carcinoembryonic antigen is elevated in only 10% of such patients (4). A tumor marker that enables us to identify cancer patients at early clinical stages is valuable because it provides us with good opportunities for timely treatment.

In recent years, we have been developing sensitive, convenient, and accurate determination systems for DiAcSpm to provide useful analytical means that aid in promoting its clinical application as a novel tumor marker. For this purpose, we needed an antibody that was highly selective for DiAcSpm, as human urine contains various polyamine species that are very similar in structure to DiAcSpm. In particular, the cross-reactivity with *N*¹-acetylspermidine (*N*¹-AcSpd) had to be minimized because *N*¹-AcSpd has a molecular structure that overlaps with approximately two thirds of that of DiAcSpm. Moreover, it is one of the major constituents of human urinary polyamines, and human urine usually contains up to 30 times more *N*¹-AcSpd than DiAcSpm (2). Therefore, we developed a polyclonal but highly selective anti-DiAcSpm antibody whose cross-reactivity with *N*¹-AcSpd was less than 0.1%. Using this anti-DiAcSpm antibody, we established an ELISA system that was successfully used to accurately determine the DiAcSpm level in human urine (6). More recently, a monoclonal anti-DiAcSpm antibody that shows selectivity between DiAcSpm and *N*¹-AcSpd and is comparable to the antibody mentioned above was obtained, and a reagent kit based on the colloidal gold aggregation procedure was developed (7). The reagent kit, Auto DiAcSpm[®] (Alfresa Pharma Co., Osaka, Japan), is compatible with most of the automatic biochemical analyzers used in clinical laboratories and allows for the rapid and precise determination of the DiAcSpm level in urine samples collected for clinical examination.

The DiAcSpm determination procedures developed thus far, such as those based on HPLC, ELISA, and colloidal gold aggregation, are either time-consuming or require expensive and specialized laboratory equipment and are not convenient for personal use by the general public. The development of a simple procedure for DiAcSpm measurement that is intended for personal use would promote the use of DiAcSpm as a tumor marker and enable us to make the best use of its attractive feature of being more sensitive than conventional tumor markers in detecting early cancers. This simple procedure would prompt apparently healthy individuals to examine their

own urine samples for DiAcSpm, encourage early visits to clinics when they are not conscious of other symptomatic signs of abnormalities, and would eventually contribute to improving the efficiency of the early detection of cancer. This is important in reducing the number of fatal cases of cancer because cancer can often be cured if detected early enough due to the recent progress in cancer therapeutics. These considerations prompted us to develop an immunochromatographic determination system for simple and convenient DiAcSpm measurement.

Immunochromatographic procedures have been developed for a number of substances, including toxins and biomarkers, and have provided us with simple, rapid, and low-cost analytical measures (8–10). In the present study, we developed an immunochromatographic determination system, examined various conditions that affected its performance, and constructed a device that allowed for the determination of the DiAcSpm level in the urine at concentrations ranging from 20 to 700 nM, which encompasses the concentrations that are usually encountered in human urine.

MATERIALS AND METHODS

Reagents, Samples, and Instruments

The mouse anti-DiAcSpm monoclonal antibody CN647 was obtained from TransGenic Inc. (Kumamoto, Japan). The monoclonal antibodies #535, #2009, #2043, #8028, #10040, and #10051 were prepared at Mikuri Immunological Laboratory, Inc. (Osaka, Japan). The antimouse IgG antibody (H+L) was obtained from Life Technologies (Carlsbad, CA). The gold nanoparticle suspension WRGH1 (50 nm- ϕ , $A_{530} = 12$, [Au] = 550 ppm) was purchased from Wine Red Chemicals, Co. (Tokyo, Japan). Hi-Flow Plus HF180 nitrocellulose membrane cards (cat no. HF180MC100), glass-fiber paper (cat no. GFCP103000), and cellulose sample pads (cat no. CFSP223000) were purchased from Millipore (Billerica, MA). *N*-(4-maleimidobutyryloxy) succinimide was obtained from Dojindo (Kumamoto, Japan). The AcSpm-GMB-BSA (BSA is bovine serum albumin) conjugate was prepared as described previously (6).

DiAcSpm was a kind gift from Dr. M. Bakke of Kikkoman Co. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan) and were of the purest grade available.

DiAcSpm was dissolved in artificial urine and used as the standard in the immunochromatographic measurements. The artificial urine contained 420 mM urea, 150 mM NaCl, 27 mM KCl, 4 mM CaCl₂, 9 mM MgSO₄, 0.05% NH₄OH, 60 mM ascorbic acid, 8.8 mM (100 mg/dl) creatinine, 0.15 mg/ml BSA, and 0.09%

Fig. 1. Schematic illustration of the immunochromatographic strip system for the determination of DiAcSpm.

NaN_3 dissolved in H_2O , and the pH was adjusted to 6.0 with phosphoric acid (11).

Urine samples were obtained from apparently healthy volunteers with informed consent, immediately supplemented with 3 mM NaN_3 and stored at -20°C until use. We received prior approval to use these samples from the ethical committees of the Tokyo Metropolitan Institute of Medical Science.

The materials for the test line and control line (see below and Fig. 1 for definition) were applied to nitrocellulose membranes using a ZX1000 Dispense Workstation equipped with AirJet Quanti (BioDot Inc., Irvine, CA). The intensity of the colored lines developed on the immunochromatographic strips was quantified using a C10066 immunochromato-reader (Hamamatsu Photonics Co., Hamamatsu, Japan).

Characterization of Anti-DiAcSpm Monoclonal Antibodies by Competitive ELISA

An ELISA was carried out essentially as described previously (6). Briefly, the wells of a NUNC Maxisorp 96-well plate (Thermo Fisher Scientific Inc., Waltham, MA) were coated with the AcSpm-GMB-peptide conjugate and then blocked with 5% skim milk. After washing, serial dilutions of DiAcSpm and the anti-DiAcSpm antibody at appropriate concentrations were added to each well, and the plate was incubated at room temperature for 1 h with constant shaking. After thorough washing with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), a horseradish peroxidase labeled anti-mouse IgG (H+L) antibody (1:6,000; Cappel Laboratories, Westchester, PA) was added to each well, and the plate was incubated for 1 h at room temperature. After thorough washing with PBS-T, a 0.33 mM tetramethylbenzidine and 0.04% H_2O_2 solution was added to each well, and the plate was incubated for 20 min at 37°C . The peroxidase reaction was halted by the addition of 1 N H_2SO_4 , and the color development was determined at 450 nm in a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA).

Assessment of the Stabilization of Gold Nanoparticles Induced by Antibody Binding

One microgram of antibody was dissolved in 100 μl of either 2 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.0 or 8.0), 2 mM Tris buffer (pH 8.0 or 9.0), or 2 mM CHES ([N-cyclohexyl-2-aminoethanesulfonic acid] buffer (pH 9.0). The gold nanoparticle suspension WRGH1 (10 μl) was added to the antibody solution, and the mixture was incubated for 30 min at 37°C . A 10% NaCl solution (10 μl) was added to each antibody-gold nanoparticle mixture, and after 15 min at room temperature, the $A_{530-580}$ value was determined (12). An aliquot of this reaction mixture was centrifuged at $20,000 \times g$ for 15 min at 20°C , and the amount of free antibody in the supernatant was determined using the Easy-Titer Mouse IgG Assay Kit (Thermo Fisher Scientific Inc.) according to the instructions provided by the manufacturer.

Construction of the Immunochromatographic Strips

A schematic description of the system is provided in Figure 1. The conjugate pads were prepared as follows: the gold nanoparticle suspension (100 μl , $A_{530} = 12$) was added to 1 ml of 10 $\mu\text{g}/\text{ml}$ antibody in 2 mM HEPES (pH 7.0), and the mixture was incubated at 37°C for 30 min. A blocking reagent (1% BSA, 0.1% PEG [polyethylene glycol] in 2 mM HEPES [pH 7.0], 120 μl) was added, and the mixture was further incubated at 37°C for 30 min. The antibody-coated gold nanoparticles were recovered by centrifugation at $20,000 \times g$ for 15 min at 20°C , re-suspended in the blocking reagent solution to a volume of 1.2 ml and sonicated for 30 sec. The gold nanoparticles were then washed three times using the blocking reagent as described above and were finally suspended in $3 \times$ Liquid Plate Sealer[®] (CANDOR Bioscience GmbH, Düsseldorf, Germany) at a concentration that gave an $A_{530} = 3$. The suspension was then applied to a glass-fiber paper strip of 55 mm in width (GFCP103000; Millipore, Billerica, MA) at a concentration of 60 $\mu\text{l}/\text{cm}$ strip. The wet glass-fiber paper strip was then dried overnight under reduced pressure in a vacuum desiccator.

The analyzing membrane was prepared using Hi-Flow Plus HF180 membrane cards as follows: the AcSpm-GMB-BSA conjugate, a protein-bound DiAcSpm mimic, was dissolved in 10 mM phosphate buffer (pH 7.0) and was used as the test line material. The test line was drawn at 8 mm from the bottom of a membrane card with a height of 60 mm and an appropriate width using a ZX1000 Dispense Workstation equipped with a BioJet Quanti[™] 3000 (BioDot Inc., Irvine, CA) at a setting of 1 $\mu\text{l}/\text{cm}$ (drop volume 31.25 nl, drop pitch 0.31 mm, on time

0.25 msec). The antimouse IgG (H+L) antibody (0.1 mg/ml in 10 mM phosphate buffer [pH 7.0]) was used to draw the control line in a similar manner at 16 mm from the bottom of the membrane card. The membrane was air-dried, immersed for 20 min at room temperature in N101 blocking reagent (NOF Co., Tokyo, Japan), washed twice with distilled water, and immersed in 3% sucrose for 10 min at room temperature (13). Any liquid remaining on the filter was wiped off, and the filter was dried at 40°C for 2 h and stored in a vacuum desiccator.

The conjugate pad and analyzing membrane were combined with a sample pad and an absorbent pad and assembled in the appropriate order, as shown in Figure 1. Finally, the assembled membrane was longitudinally cut into strips of 5 mm in width, and these strips served as the immunochromatographic strips.

Determination of the DiAcSpm Concentration

A 200 μ l aliquot of sample solution was applied to the sample pad of an immunochromatographic strip. After 15 min at room temperature, the color development along the test line and control line, respectively, was determined using a C10066 immunochromato-reader.

The DiAcSpm concentration was also determined by the colloidal gold aggregation procedure with a JCA-BM6010 Biomajesty automatic biochemical analyzer (JEOL Ltd., Tokyo, Japan) using the AutoDiAcSpm® Reagent Kit (Alfresa Pharma, Osaka, Japan) (7).

RESULTS

Outline of the Immunochromatographic Determination System for DiAcSpm

A competitive format was adopted (see Fig. 1) because DiAcSpm is a small molecule (MW 286) with a single antigenic determinant and thus cannot be sandwiched by two antibodies (14). Specifically, anti-DiAcSpm antibody-coated gold nanoparticles were reacted with DiAcSpm in a liquid test sample in the conjugate pad and then migrated into the analyzing nitrocellulose membrane where the DiAcSpm mimic were immobilized on the test line. In this format, the presence of DiAcSpm is indicated by the diminished intensity of the test line.

Characterization of Monoclonal anti-DiAcSpm Antibodies

Several monoclonal anti-DiAcSpm antibodies were available when we started this study, and we first examined the sensitivity and specificity of these antibodies by ELISA. These antibodies are the monoclonal antibody CN647 (TransGenic Inc.) and six monoclonal antibodies

TABLE 1. Properties of the Anti-DiAcSpm Monoclonal Antibodies

Antibody	K_i values from the competitive ELISA (nM)		Cross-reactivity (%)
	DiAcSpm	N^l -AcSpd	
CN647	0.32	337.8	0.095
#535	15.63	4,706.2	0.33
#2009	1.07	436.0	0.25
#2043	1.06	461.4	0.23
#8028	2.35	2,580.0	0.091
#10040	0.72	492.1	0.15
#10051	0.82	525.1	0.16

ies developed at Mikuri Immunological Laboratory, Inc. (designated below as #535, #2009, #2043, #8028, #10040, and #10051). The results are summarized in Table 1. CN647, #10040, and #10051 were the most sensitive, as they were able to detect 0.01 to 10 nM DiAcSpm.

We also determined the cross-reactivity of these anti-DiAcSpm antibodies against N^l -AcSpd. The antibodies used for determining the DiAcSpm level in the urine must be highly selective for DiAcSpm, as it is very similar in structure to N^l -AcSpd and healthy human urine contains approximately 30 times more N^l -AcSpd than DiAcSpm (2). Of the seven antibodies tested, #8028, CN647, #10040, and #10051 had sufficiently high selectivity for DiAcSpm over N^l -AcSpd (cross-reactivity of less than 0.16%).

With these results in mind, we chose CN647, #10051, and #8028 to define the appropriate conditions for constructing an immunochromatographic DiAcSpm determination system. CN647 and #10051 were among the most sensitive with sufficient selectivity, and #8028 showed the highest selectivity for DiAcSpm among the antibodies tested.

Stabilization of Gold Nanoparticle Suspension by Coating With Anti-DiAcSpm Antibodies

We examined the stabilizing effect of these antibodies on gold nanoparticle suspensions in several buffer solutions with different pHs to identify the optimal conditions for preparing the antibody-coated gold nanoparticles. The experiments were carried out at neutral and alkaline pHs because naked WRGH1 gold nanoparticles tend to aggregate when kept at an acidic pH. The gold nanoparticle suspensions were incubated with antibodies, and the stabilizing effect of the antibodies was then assessed by measuring the $A_{530-580}$ value of the suspension after the addition of NaCl. In the absence of the anti-DiAcSpm antibody, the color of the gold nanoparticle suspension turned from wine-red to grey upon the addition of NaCl

Fig. 2. Stability of the antibody-coated gold nanoparticle suspensions against electrolytes in different buffer solutions at different pHs. (A) Stability of gold nanoparticle suspensions pretreated with different amounts of antibodies in HEPES (pH 7.0, $\blacklozenge$), HEPES (pH 8.0, $\blacksquare$), Tris (pH 8.0, $\blacktriangle$), Tris (pH 9.0, $\times$), or CHES (pH 9.0, $*$), as assessed by the $A_{530-580}$ values after the addition of NaCl. (B) The amount of antibody bound to the gold nanoparticles after preincubation with different amounts of antibodies in the buffer solutions as indicated in (A).

due to aggregation and the $A_{530-580}$ value was low. The added antibodies protected the nanoparticle suspensions from aggregation by binding to the surface of the particles, and the suspension remained wine-red with a high $A_{530-580}$ value after the addition of NaCl. It may be added that binding of antibodies protected the nanoparticle suspensions also from acid-induced aggregation as noted in the next section. Antibodies #8028 and #10051 were increasingly effective in protecting the gold nanoparticles from NaCl-induced aggregation in a range between 0.2 and 0.5 μg protein in a 110 μl incubation mixture containing 10 μl of the WRGH1 gold nanoparticle suspension, and maximal protection was obtained when more than 1 μg protein was present (Fig. 2A). Antibody #8028 was effective at every pH in every buffer tested, while protection by antibody #10051 varied considerably with the pH and buffer used. Antibody CN647 was poorly effective in protecting the gold nanoparticles under the conditions tested in this study.

To see whether the differential effectiveness of the antibody species in protecting the gold nanoparticles from NaCl-induced aggregation is related to their differential efficiency in binding to the nanoparticles, the binding of the antibodies to the gold nanoparticles was assessed by determining the amount of free antibody in the supernatant after the antibody-treated gold nanoparticles were recovered by centrifugation. The amount of antibody bound to the gold nanoparticles was very similar with each other under the conditions tested and tended to increase linearly in the concentration range tested (Fig. 2B). Of the three antibodies tested, we chose #8028 for further experiments in which we attempted to optimize the experimental conditions for preparing the immunochromatographic strips for DiAcSpm measurement because this antibody is not only highly specific for DiAcSpm but is also able to effectively stabilize the gold nanoparticles against NaCl-induced aggregation under a variety of incubation conditions.

Fig. 3. Performance of the antibody-coated gold nanoparticles prepared under different conditions in immunochromatography. The antibody-coated gold nanoparticles were prepared using 1 μ g of antibody #8028 and 10 μ l of the WRGH1 gold nanoparticle suspension in 100 μ l of HEPES (pH 7.0, $\blacklozenge$), HEPES (pH 8.0, $\blacksquare$), Tris (pH 8.0, $\blacktriangle$), Tris (pH 9.0, $\times$), or CHES (pH 9.0, $*$). Immunochromatographic strips were constructed using these antibody-coated gold nanoparticles, and their performance was examined with respect to the maximum test line intensity and its DiAcSpm-dependent decrease.

Immunochromatography Using Antibody-Coated Gold Nanoparticles Prepared Under Various Conditions

Antibody-coated gold nanoparticles were prepared using 1 μ g of antibody per 10 μ l of the WRGH1 gold nanoparticle suspension in 100 μ l of different buffers with different pH values, and their performance on immunochromatographic strips was examined. We noted that the test line signal intensity varied considerably depending not only on the pH but also on the buffer substance of the solution in which antibody-gold nanoparticle conjugates were prepared (Fig. 3). The highest intensity was obtained using the nanoparticles prepared in HEPES at pH 7.0. Moreover, the resulting gold nanoparticles were stably kept in suspension at pH 4.0 (data not shown). The antibody-coated gold nanoparticles prepared at pH values higher than 7.0 tended to give weaker test line signals.

Effects of Various Blocking Reagents on the Performance of the Antibody-Coated Gold Nanoparticles in Immunochromatography

The antibody-coated gold nanoparticles were treated with several proteins and synthetic polymers to test for their effects on the test line intensity and background coloring. Treatment with proteins was effective in giving sufficient test line intensity with reasonably low background coloring. In contrast, the use of synthetic polymers alone resulted in a very weak test line signal (Fig. 4), but 0.1% PEG was found to be very effective in suppressing the

background coloring (data not shown). The combined use of 1% BSA and 0.1% PEG was then tested and found to give both high test line intensity and low background coloring. The addition of 0.1% polyvinyl alcohol (PVA) to BSA and casein inhibited the binding of the antibody-coated gold nanoparticles to the test line antigen.

Based on these results, the antibody-coated gold nanoparticles were suspended in a BSA/PEG mixture for blocking and then finally suspended in Liquid Plate Sealer[®] for long-term stabilization. The immunochromatographic strips constructed were stable under the condition of accelerated aging test. The test line intensity remained stable after a slight initial decrease after 1 week, and more than 90% of the initial test line intensity was retained after 8 weeks at 50°C.

Immunochromatographic Determination of DiAcSpm

We constructed immunochromatographic strips using the antibody-coated gold nanoparticles prepared in 2 mM HEPES at pH 7.0 and tested their performance. The test line intensity at a given concentration of DiAcSpm varied depending on the composition of the solvent in which DiAcSpm was dissolved, and the slope of the competition curve was less steep when DiAcSpm was dissolved in artificial urine compared to PBS (data not shown). Inclusion of urea mainly contributes to the differential behavior of artificial urine and PBS. As shown in Figure 5A, using DiAcSpm dissolved in artificial urine adjusted to pH 6.0, the test line signal intensity decreased gradually from 20 to 700 nM DiAcSpm, providing a calibration curve for determining the DiAcSpm concentration in urine samples.

When more antibodies were adsorbed to the gold nanoparticles at a higher antibody concentration, a higher DiAcSpm concentration was needed to affect the intensity of the test line (data not shown). This implies that the sensitivity of the DiAcSpm detection and the optimal concentration range for the accurate determination of the DiAcSpm level may be managed by manipulating the amount of antibody bound to the gold nanoparticles.

We then determined the DiAcSpm concentration in 55 human urine samples whose pH values ranged from 5.5 to 7.5 by immunochromatography using the calibration curve shown in Figure 5A. A 200 μ l aliquot of urine sample was applied to the sample pad without dilution. The analytical values determined by the immunochromatography and the colloidal gold aggregation procedure were compared and are shown in Figure 5B. The analytical values obtained by the immunochromatography were linearly correlated very well with those obtained by the colloidal gold aggregation procedure [$y = 1.04x + 12$ (nM)], with a correlation coefficient of $r = 0.94$.

Fig. 4. Agents used for blocking the gold nanoparticle surfaces and their effects on the test line intensity. Antibody-coated gold nanoparticles were prepared using 1 μ g of antibody #8028 and 10 μ l of the WRGH1 gold nanoparticle suspension in 100 μ l of HEPES (pH 7.0) and recovered by centrifugation. The precipitated particles were subsequently treated in the blocking reagent solutions specified below. Immunochromatographic strips were constructed using these antibody-coated gold nanoparticles, and the test line intensity was determined in the absence of competing antigen. The blocking reagents used were the following: 1% BSA, ; 1% casein, ; 1% ovalbumine, ; 0.1% PEG, ; 0.1% PVP, ; 0.1% PVA, ; 1% BSA + 0.1% PEG, ; 1% BSA + 0.1% PVP, ; 1% BSA + 0.1% PVA, ; 1% casein + 0.1% PEG, ; 1% casein + 0.1% PVP, ; and 1% casein + 0.1% PVA, .

DISCUSSION

In this study, we developed a competitive immunochromatographic device for DiAcSpm determination. DiAcSpm is a recently identified urinary tumor marker whose level is elevated more frequently than other tumor markers in patients with early-stage cancer, including colorectal and breast cancer (4). Therefore, DiAcSpm serves as a useful tool for early cancer detection, which is critically important for reducing the number of fatal cases of cancer. The development of a simple immunochromatographic device for DiAcSpm detection that is suitable for personal use will definitely contribute to increasing the opportunity for early cancer detection.

In an immunochromatographic assay, it is important that the gold nanoparticles are stably kept in suspension because aggregated particles are less likely to diffuse into the analyzing membrane. Care should be taken to avoid gold nanoparticle aggregation during conjugation with the antibody, application to the conjugate pad, and subsequent desiccation (15). In addition, the adsorbed antibody should remain active throughout the whole process. Noncoated gold nanoparticles are kept in suspension due to the electric repulsion between each particle and tend to aggregate upon the addition of electrolytes, which weakens the repulsive force (15). The binding of proteins, including immunoglobulins, often helps to keep the gold nanoparticles in suspension in the presence of electrolytes

(8,15). However, the present study indicated that the binding of the antibody is not the sole determinant of the stability of the antibody-coated gold nanoparticles. The amount of protein bound to the gold nanoparticles was very similar at every pH and with every antibody tested, but the stability of the antibody-coated gold nanoparticles greatly varied from antibody to antibody. This is consistent with a previous report that the effects of coating with oligopeptides on the electrolyte-induced aggregation of gold nanoparticles differ among distinct groups of peptides (16).

The performance of antibody-gold nanoparticle conjugates has been known to depend on the nature of antibody, gold nanoparticles, and pH at which the conjugate is prepared, but the actual performance of the conjugates is difficult to be predicted. In addition to these known variables, we found that different buffer used for the conjugate formation significantly affected the performance of the antibody-gold nanoparticle conjugates formed even at a fixed pH. The differential effect of buffer substance has not been recognized and described previously. Although we are at present unable to explain where this differential effect comes from, it represents a novel variable that should be taken into consideration in future designing of immunochromatographic systems.

The active surfaces on the gold nanoparticles that remain after incubation with the antibody must be inactivated by incubating the particles in a solution containing

Fig. 5. Immunochromatographic determination of urinary DiAcSpm. (A) A calibration curve for the determination of DiAcSpm in the urine. A 200 μ l aliquot of standard DiAcSpm sample dissolved in artificial urine was applied to an immunochromatographic strip constructed as described in Materials and Methods, and the test line intensity was determined (B) Comparison between the concentrations of urinary DiAcSpm determined by the immunochromatography and colloidal gold aggregation methods. The abscissa indicates the concentration of DiAcSpm in the urine determined by the colloidal gold aggregation method in an automatic biochemical analyzer using the AutoDiAcSpm[®] Reagent Kit, while the ordinate indicates the corresponding values determined by immunochromatography. DiAcSpm concentration was assessed by using the calibration curve shown in (A).

appropriate blocking reagents. This is necessary to suppress background coloring due to nonspecific binding to the nitrocellulose membrane and other components on the one hand, and to avoid inefficient interaction with antigens which would lead to weaker test line intensity on the other. Blocking reagents adsorbed on the surface may diminish nonspecific binding by increasing the surface charge of the particles and/or sterically hindering nonspecific interactions with other components. Dworetzky and his colleagues used RNA-coated gold nanoparticles to study the nuclear-cytoplasmic transport of RNAs (17). In their study, BSA, ovalbumin, polyglutamic acid, and polyvinyl pyrrolidone (PVP) were tested for the ability to suppress nonspecific interactions with nuclear pore components and were found to be equally appropriate for this purpose. In immunochromatography system, however, we found that the proteins and polyols

had different effects on the performance of the system. In our analytical system, PEG and PVP were effective in suppressing the background coloring, but these polyols alone greatly weakened the test line intensity. However, blocking by proteins including BSA resulted in strong test lines, and we found that a combination of protein (BSA) and polyol (PEG) was the most effective at obtaining both high test line intensity and low background coloring. Polyols were added to stabilize the gold nanoparticle suspensions against aggregation in immune electron microscopy, although the blocking effects of these compounds have not been explicitly mentioned (18, 19). The suppression of background coloring by polyols may partly be related to their suspension-stabilizing effect. Blocking of the surface of the analyzing membrane was also necessary to suppress the background coloring, and this was conveniently effected by a commercial blocking reagent, N101.

To assess the long-term storage stability of the immunochromatographic strip, we performed an accelerated aging test. The accelerated aging test is based on the assumption that the rates of the chemical reactions involved in the deterioration of the materials follow the Arrhenius equation, which indicates that storage for 32 days at 50°C is equivalent to 6 months storage on the shelf at 25°C and that storage for 65 days at 50°C is equivalent to 1 year at 25°C. The use of Liquid Plate Sealer[®] as the stabilizing reagent for the antibody-coated gold nanoparticles, therefore, guarantees almost 1 year of shelf life for the immunochromatographic strips.

The system developed here may need further modification before it is accepted as adequate for public use. The values of urinary DiAcSpm obtained by the present immunochromatography system were linearly and very well correlated with those obtained with colloidal gold aggregation procedure with a slope of 1.04. We demonstrated previously that the analytical values obtained by colloidal gold aggregation procedure are very well correlated with those determined by mass spectrometric analysis (20). The accuracy of the immunochromatographic measurement may thus be verified, but the deviations of data points from the regression line are preferably reduced further.

Variations in the analytical values, which may be due to fluctuations in urinary composition, should be further reduced. Such variations may, in part, be obviated by further optimizing the solvent for preparing the DiAcSpm standard solution that is used in the construction of the calibration curve because the use of artificial urine as a solvent for the DiAcSpm standard for system calibration greatly improved the performance of the analytical system.

The determination system described in this report would therefore serve as a useful prototype for a simple DiAcSpm determination device that is intended for

personal use for the early detection of colorectal and other cancers. The simplicity of the procedure is a definite merit in promoting wider use of DiAcSpm testing; by using this device and simply applying one drop of urine, we can assess the amount of urinary DiAcSpm in 15 min by eye, although use of an immunochromato-reader is preferred for more precise analytical values.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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