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Development of Immunological Methods for Analysis of 5'-deoxy-5'-methylthioadenosine

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Abstract : Studies were undertaken to develop a competitive radioimmunoassay (RIA) and indirect antigen capture enzyme-linked immunosorbent assay (ELISA) for the determination of 5'-deoxy-5'-methylthioadenosine (MTA), which is formed from decarboxylated S-adenosylmethionine by spermidine and spermine synthase. Specific antiserum against MTA was raised in rabbits by immunization with MTA-BSA which was prepared by coupling BSA to oxidized MTA with periodate. Since MTA is oxidized easily to the sulfoxide, the sulfhydryl reagent, DTT, was added to the immunogen. For RIA, immunocomplexes were separated from free MTA by using ammonium sulfate precipitation. The antiserum showed almost no cross-reactivity with a variety of other nucleotides and riboses. But, the level of cross-reactivity of 5'-isobutylthioadenosine (SIBA) was high. These results showed the importance of hydrophobicity adjacent to the 5'-OH for determining antigenicity. The lower limit of detection by this assay was 100 fmol of MTA per tube. Using this assay, MTA levels were more easily and precisely determined in biological samples when compared with HPLC analysis. The RIA procedure is less time consuming. More than 24 analyses can be carried out in 2 h and required only a very small amount of sample (20 μl serum). In ELISA, biotin conjugated MTA-BSA was used as the labelled MTA. The sensitivity limit of this assay was lower than 100 pmol.
Key words : 5'-deoxy-5'-methylthioadenosine, RIA, ELISA

5'-deoxy-5-methylthioadenosine (MTA) is a naturally occuring nucleoside formed in mammalian cells during polyamine synthesis (Williams-Ashman *et al.*, 1982). Unlike the polyamines, MTA does not accumulate in cells, but is rapidly degraded by MTA phosphorylase (MTAase, EC 2.4.2.28)) to methylthioribose-1-phosphate and adenine (Pegg and Williams-Ashman, 1969). MTAase is abundant in all normal cells and cell lines derived from normal tissue (Kamatani *et al.*, 1981; Williams-Ashman *et al.*, 1982). In contrast, many malignant cell lines (Toohey, 1977; Kamatani *et al.*, 1981) and a proportion of human leukemias and solid tumors (Kamatani *et al.*, 1982; Fitchen *et al.*, 1986) are MTAase deficient. Several years ago, the locus for MTAP gene encoding this enzyme was mapped to the short arm of chromosome 9, band p21-22 (Carrera *et al.*, 1984), a region that is frequently deleted in mutiple tumor types (Diaz *et al.*, 1988; Miyakoshi *et al.*, 1990; Wang-Peng *et al.*, 1991; Fountain *et al.*, 1992). Recent studies have shown that this map includes the type I IFN gene cluster, the identified candidate tumor suppressor genes CDKN2 (p16) and CDKN2B (p15), and several CpG islands (Olopade *et al.*, 1995). Also, Nobori *et al.* (1996) described that MTAase deficiency in malignancy is due to the codeletion of the MTAP and CDKN2 genes. Depending on the cell type, p16-deficient cancers have all or part of MTAP gene deleted at various frequencies (40% in melanomas, 57% in nonsmall cell lung cancers, 71% in gliomas, and 78% in leukemias). The importance of this enzyme deficiency in malignant cells is unclear, but undetectable MTAase activity was noted in the tumor marker. Moreover, it has been demonstrated that MTAase-deficient cell lines do not accumulate MTA, but rather excrete it into the extracellular medium. Kaneko *et al.* (1984) reported that the amount of MTA in urine from patients with leukemias and malignant lymphomas was higher than in normal subjects, as determined by HPLC. Therefore, measurement of MTA is of clinical interest in view of its possible use as a tumor marker.

The intracellular concentrations of MTA in normal cells were found to be low as compared with those of spermidine, spermine, and S-adenosylmethionine. It is presumed that the concentrations of MTA in serum and urine are very low. To determine the small amount of MTA in biological samples with sensitivity and fa-

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cility, we have attempted to modify the method of antibody preparation and immunoassay method of Ragione *et al.* (1988). This paper describes the measurement of concentrations of MTA in serum by an enzyme-linked immunosorbent assay for the first time.

Materials and Methods

Materials

Bovine serum albumin, 5'-deoxy-5'-methylthioadenosine (MTA), DTT, sodium periodate, sodium borohydride, ethylene glycol, Freund's complete adjuvant, Freund's incomplete adjuvant, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC), and goat anti rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, USA). S-adenosyl-L-[methyl-^{3}H]methionine (91 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). Microtiter plates were obtained from Costar (Cambridge, MA). Avidin-peroxidase, biotin hydrazide, and 2,2'-azine-di[3-ethylbenzthiazoline-sulfonate] (ABTS) were purchased from Pierce (Rockford, USA). RPMI 1640 medium, heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, USA). All other reagents used were obtained from commercial sources and were of analytical grade.

Preparation of the immunogen and antiserum

Conjugates of MTA with proteins were prepared according to Erlanger and Beiser (1964). In this method, MTA (15 mg) was added to sodium periodate (50 mg) dissolved in water (2 ml) and stirred in the dark for 60 min. Excess periodate was destroyed by adding 5 μl distilled ethylene glycol. After 5 min, the reaction mixture was added dropwise to a solution of BSA, Keyhole limpet haemocyanine (KLH), and poly-L-lysine (2.5 mg/ml distilled water) and the pH was adjusted to between 9.0 and 9.5 with K_2CO_3 with constant stirring. After 60 min at room temperature, 2 ml of freshly dissolved sodium borohydride (1.25 mmol) was added and the reaction mixture was left in the dark for 12 h. The reaction mixture was diluted to 10 ml with distilled water and was chromatographed on a Sephadex G-50 column (1.5×24 cm) which had been pre-equilibrated with phosphate buffered saline (PBS), containing 1 mM DTT. Protein fractions were pooled and stored at -20°C. Control proteins were prepared by a similar procedure without nucleoside.

Antibodies against MTA were raised in white rabbits. The initial injection of 1 mg of MTA-protein dissolved in PBS containing 1 mM DTT and emulsified with complete Freund's adjuvant was followed by 2 injections (1 mg MTA-protein in PBS containing 1 mM DTT/incomplete Freund's adjuvant) at 4-week intervals. The rabbits were bled 10 days after the last injection. The antiserum was prepared by centrifugation and stored at -70°C.

Competitive RIA

[methyl-^{3}H]MTA was prepared by acid hydrolysis of S-adenosyl-L-[methyl-^{3}H]methionine (91 Ci/mmol). It was heated for 10 min. The reaction solution was diluted using distilled water (1:200 dilution) and each aliquot was stored at -20°C. Samples were obtained from various tissue. One volume of 1.2 M perchloric acid was added to 3 volumes of samples. Before the assay, the pH was adjusted to 7.0. Alternatively, serum samples were filtered by Centricon with a 10,000 molecular weight cut-off limit. The filtered samples were analyzed.

The antiserum was diluted 10 fold and 20 μl was added to the reaction mixture. The standard solution (100 μl) or samples (50 μl) were added and reaction mixtures (total volume 190 μl) were incubated at 37°C for 30 min followed by addition of 10 μl of [methyl-^{3}H] labeled MTA (100 fmol, 10 nCi/μl). After 30 min, 200 μl of 100% saturated ammonium sulfate solution was added to each sample and left at 4°C for 20 min. The tubes were centrifuged at 13,000×g for 15 min and discarded the supernatant was discarded carefully. The tubes were washed once with 50% saturated ammonium sulfate solution and centrifuged. The precipitate was dispersed into the scintillation fluid and the radioactivity was measured.

Cell cuture

K562, CCRF-CEM, and HeLa cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin, and kept in a 5% CO_2 incubator at 37°C.

Preparation of biotin conjugated MTA-BSA

One hundred microliters of 50 mM biotin hydrazide (in DMSO) was added to 4 ml of prepared BSA-MTA conjugate (7.5 mg/ml) and mixed with 60 μl of EDC (100 mg/ml). The reaction involved an EDC-mediated conjugation between a carboxylic acid of MTA-BSA and biotin hydrazide. The carbodiimide first reacts with the available carboxylic group to form an active O-acylurea intermediate. After overnight incubation at room temperature, the reaction mixture was centrifuged at 13,000×g for 30 min and precipitate was discarded. The supernatants were applied to a Sephadex G-50 column (1.5×24 cm) and the protein fractions were collected (Reisfield *et al.*, 1987).

Indirect antigen capture ELISA

Immunoplates were coated with goat anti rabbit IgG

(1 μg/ml) in 35 mM bicarbonate buffer (pH 9.6) (100 μl/well) and incubated overnight at 4°C. After the coating procedure, the plates were washed three times with PBS and blocked with 1% BSA-PBS (300 μl/well) for 1 h at room temperature. The plates were washed with PBS and aliquots of test samples or standard, purified anti-MTA IgG, and biotin conjugated MTA-BSA were added to each well. The plate was incubated overnight at 4°C and washed three times with PBST. Avidin-peroxidase was added to each well and incubated for 2 h at room temperature. After the reaction, the plate was washed three times with PBST and 100 μl of a peroxidase substrate (ABTS) solution was added to each well. The change in absorbance was measured using a microtiter plate spectrophotometer equipped with a 405 nm filter.

Results and Discussion

Preparation of MTA-Protein

Compounds with molecular weights less than 5,000 often cannot stimulate an immune response. Because of its low MW (297.3), MTA needs to be linked to a carrier protein to generate an antibody response.

Various methods have been reported for covalently coupling nucleosides, nucleotides and oligonucleotides to protein molecules (Sela *et al.*, 1964; Halloran and Parker, 1966; Seaman *et al.*, 1966). However, the periodate procedure has been most extensively used, and conjugates of MTA with protein were prepared according to Erlanger and Beiger (1964). In this method, MTA containing free 2'- and 3'-ribose hydroxyl group was covalently coupled to a protein by oxidizing the ribose with periodate.

The conjugation reaction mixture was applied to a Sephadex G-50 to remove free MTA. The elution profiles showed that MTA conjugated protein in the former peak and free MTA in the latter peak fractions were eluted, respectively. The protein concentration was determined by the Bicinchoninic Acid (BCA) and free MTA was measured by reading the absorbance at 260 nm (Fig. 1). To examine whether MTA was conjugated to the protein or not, each protein fraction was scanned over the wavelength range from 240 to 300 nm. The results indicated that the absorption spectrum of these fractions were distinguished from those of control protein and MTA. A protein has an absorbance peak at 260 nm, while MTA, an analog of nucelosides, has the absorbance peak at 260 nm. The protein fractions after the conjugation reaction showed an increase in the absorbance at 260 nm. The observed changes reflect the formation of linkage between MTA and BSA. The protein fractions 10-15 were chosen for immunization.

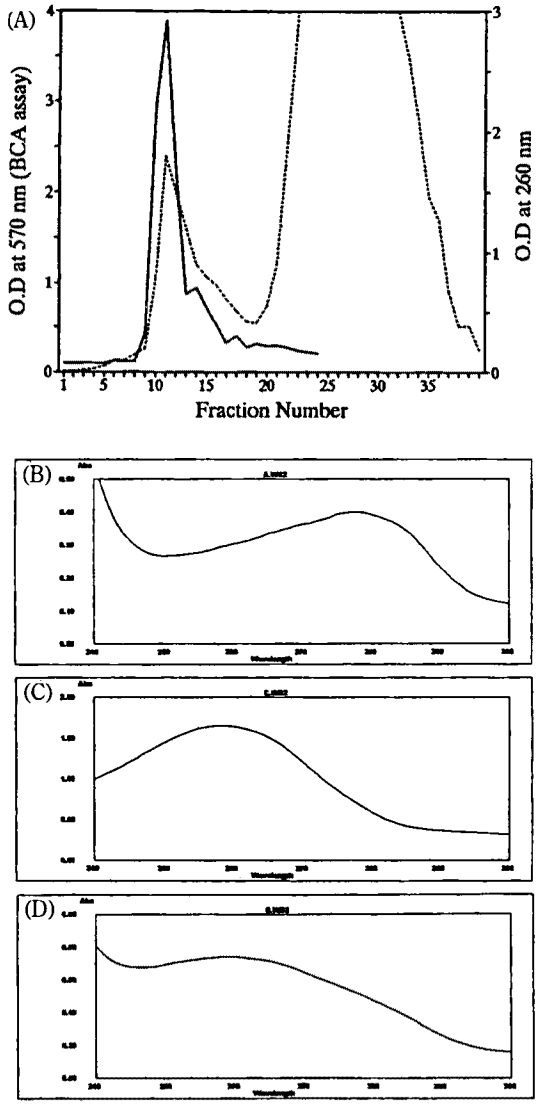


Fig. 1. The conjugation of MTA-BSA was determined by the absorption spectrum. (A) Sephadex G-50 chromatography of MTA-BSA conjugates. Each fraction (1 ml) was collected and estimated by BCA protein determination method (solid line) and UV absorption at 260 nm (dash line). The absorption spectrum at 260 nm of (B) control BSA, (C) MTA, and (D) protein eluted in the former fraction on Sephadex G-50 (1.5 X 24 Cm).

The molecular weight of the conjugates of MTA with BSA was estimated by 10% SDS-PAGE. A smear of protein ranging in molecular mass from 30 kDa to 200 kDa in MTA conjugates of BSA was observed (lane 1, Fig. 2), indicating that the conjugates existed in a heterogenous form of varying molecular weight. Senapathy *et al.* (1985) reported that each of the 2 aldehyde group of a periodate-oxidized nucleoside links to different lysines through Schiff bases, thereby cross-linking different protein molecules. The cross-linked nucleoside-antigen structure is significantly different from that of a native protein, and may be responsible for the increased efficiency

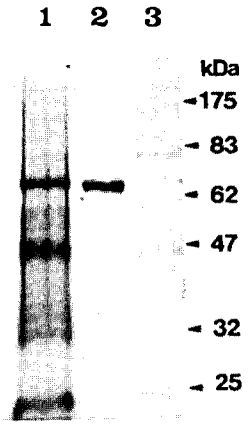


Fig. 2. 10% SDS-PAGE of MTA-BSA conjugates. Lane 1, MTA-BSA conjugates; lane 2, BSA; lane 3, marker protein, MBP-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin A (25 kDa).

of antibody production by these antigens. Also, discrete bands were observed in the lower molecular mass below 66 kDa, indicating that the degradation occurred in a non-random manner.

Antibody response

Antibodies against MTA were produced in each of the 3 rabbits immunized with MTA-BSA, MTA-KLH, and MTA-poly-L-lysine, respectively. These antigens were also emulisified with 1 mM DTT to prevent oxidization of MTA to MTA sulfoxide. The antibody response against MTA-BSA had a higher titer than other conjugates. During the conjugation reaction using KLH, many insoluble particles appeared in solution, leading to results that yields of MTA conjugation and the titer were lower than BSA conjugation procedure.

Competitive RIA

In RIA, a standard calibration curve of MTA quantification in presented in Fig. 3. The lower detection limit was 100 fmol per tube and the working range was shown to be between 0.5 and 100 pmol per tube. Regione *et al.* (1988) reported that the minimum detectable amount of MTA is 10 pmol in RIA. The antiserum against MTA-BSA had both a higher titer and sensitivity compared with an antiserum which the Ragione *et al.* reported (1988). These results indicated that our modified methods, including the addition of DTT and replacement of KLH with BSA, were very efficient.

Specificity of the antibody

The specificity of anti MTA-BSA antiserum was determined by measuring the ability of several compounds of a similar structure to inhibit the [methyl-^{3}H]MTA binding to the antibody. Calibration curves showing the

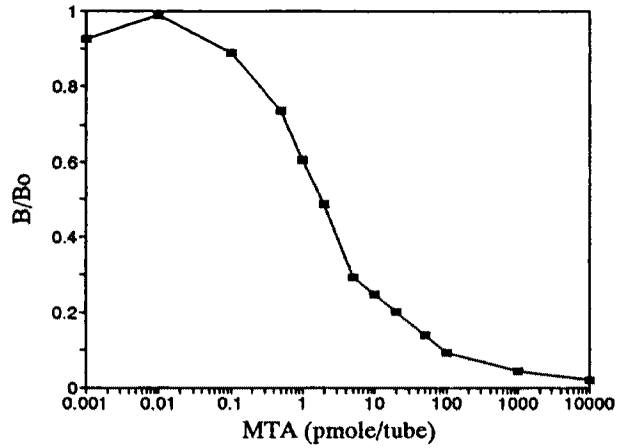


Fig. 3. A standard calibration curve of MTA quantification by RIA. The mixture for the RIA contained PBS, 20 μl antiserum (1:200 dilution), and 100 μl MTA solution with various concentration in a total volume of 190 μl. After incubation for 30 min at 37°C, 10 μl of [methyl-^{3}H] labeled MTA (100 fmol, 10 nCi/μl). The curve showed the amount (ratio) of bound radioactivity for various doses of unlabeled MTA (B) as a ratio of that bound using the [methy-^{3}H]MTA alone (Bo).

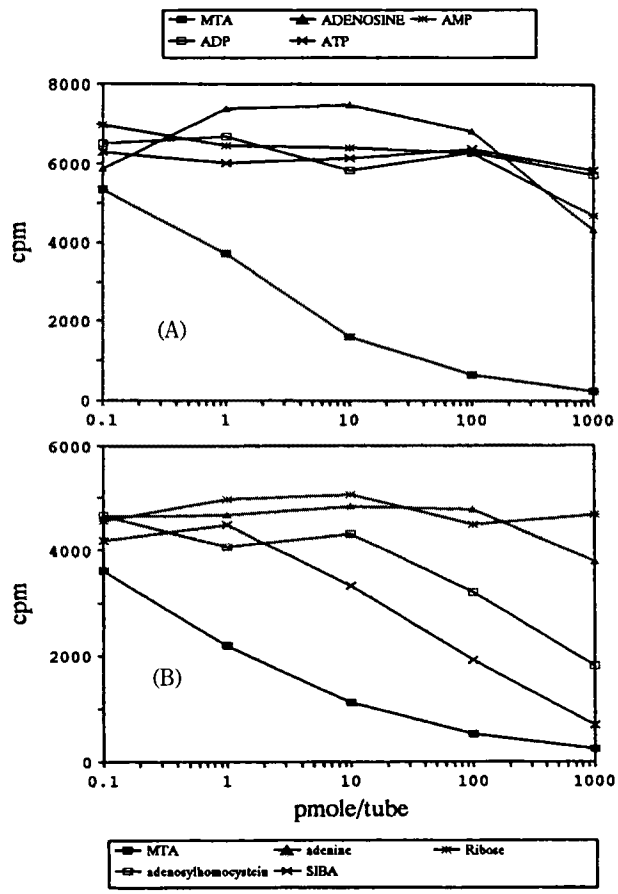
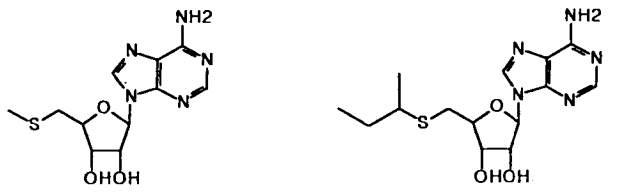


Fig. 4 Affinities of various nucleosides and ribose for anti-MTA antiserum. Calibration curves showing the relationship between concentrations of unlabeled compounds and the amount (cpm) of bound [methyl-^{3}H]MTA were plotted. The cross-reactivities with (A) adenosine, AMP, ADP, ATP and (B) adenine, ribose, adenosylhomocystein, SIBA were estimated.



5'-deoxy-5'-methylthioadenosine (MTA) **5'-isobutylthioadenosine (SIBA)**

Fig. 5. Structures of (A) 5'-deoxy-5'-methylthioadenosine (MTA) and (B) 5'-isobutylthioadenosine (SIBA).

relationship between concentrations of unlabeled compounds and the amount of bound [methyl-^{3}H]MTA are shown (Fig. 4). The anti MTA-BSA antiserum showed a 1% cross-reaction with SIBA and a 0.1% cross reactivity with adenosylhomocystein in terms of the amount of each compound required for a 50% inhibition of binding. Adenosine, AMP, ADP, ATP, adenine, and ribose did not cross-react. The cross-reactivity of SIBA explained the importance of hydrophobicity adjacent to 5'-OH for determining antigenicity (Fig. 5).

The reproducibility of the RIA performed within a day (intraassay) is shown in Table 1. The coefficients of variation for intraassay at 5 different levels of MTA were 3.0-11.3%, respectively. Several investigations, utilizing reverse-phase HPLC, demonstrated that the cellular content of MTA ranges between 0.5 and 7 nmol/g of wet tissue. Our results obtained from RIA analysis were similar (Table 1).

In recovery experiments, soybean homogenate containing 5.8 nmol of endogenous MTA per 1 g tissue were used. Each sample was assessed with and without added MTA (1, 2, 5, 10 nmol) and recovery of the added MTA was calculated from the standard curve. As shown in Table 2, analytical recoveries ranged between 82 and 127%.

Table 1. Reproducibility of Intraassay on MTA determination by RIA

Samples	nmol MTA±SD/tissue 1 g	% CV[a]	n[b]
rat liver	0.606±0.049	8.1	5
soybean axes (1 day)	13.543±0.667	4.9	5
soybean axes (2 day)	6.798±0.482	7.1	5
soybean axes (4 day)	1.012±0.030	3.0	5
soybean callus	0.275±0.031	11.3	5

[a]CV: coefficient of variation
[b]n: number of determination

Table 2. Recovery MTA added to soybean homogenate containing endogenous MTA

Added (pmol/tube)	Estimated (pmole/tube±SD)	Recovery (pmol/%)		n[a]
none	5.80±0.22			5
1	6.62±1.30	0.82	82	5
2	8.87±0.30	3.07	114	5
5	13.73±0.40	7.93	127	5
10	18.80±0.56	13	119	5

[a]n: number of determination

Detection of MTA in serum and culture media

RIA for MTA was used to determine the free MTA level in serum of normal subjects and cancer patients (Fig. 6). The value for the MTA in the sample was expressed as pmol of MTA in 1.0 ml serum. The MTA levels ranged from 1 to 30 pmol/ml for those normal and patients. Differences of the MTA levels found in healthy subjects and cancer patients indicated that rectal and stomach cancers have higher level than normal. MTA levels were more easily determined in biological samples using this assay compared with HPLC analysis. The HPLC procedure is more time consuming than the RIA method as it requires a preliminary purification and enrichment of the sample before the chromatographic analysis.

We also determined the concentration of MTA in the cell culture media. MTAase, which is responsible for the degradation of MTA, is often deficient in leukemia cell lines. Unlike HeLa cells, CRF-CEM and K562 cells derived from leukemia are MTAase deficient. As shown in Fig. 7, MTA was excreted to the culture media in CCRF-CEM and K562 cells. These results indicated that MTAase deficient cell lines do not accumulate MTA, but rather excrete it into the extracellular medium.

Developement of ELISA

An indirect antigen capture ELISA has been develop-

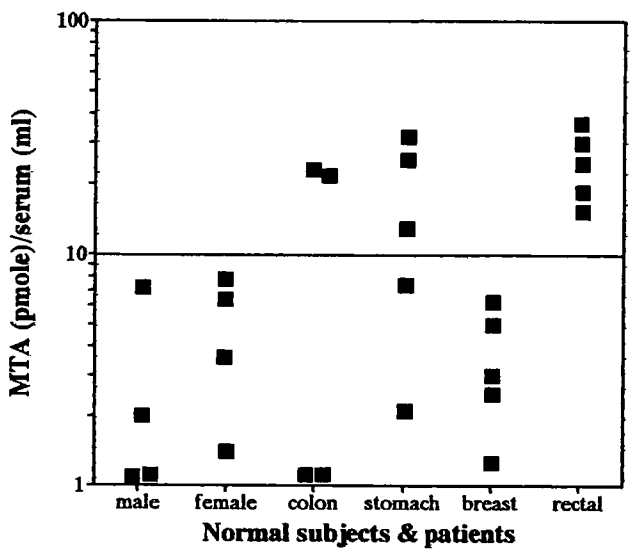


Fig. 6. Concentrations of MTA in serum of normal subjects (male and female) and patients with various malignant diseases (colon, stomach, breast, and rectal cancer).

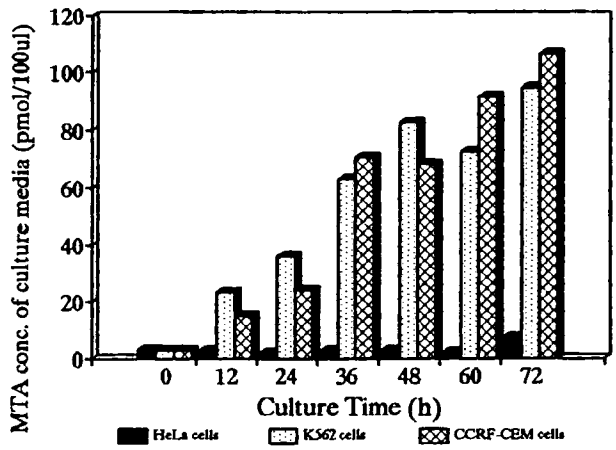


Fig. 7. The determination of excreted MTA in the culture media with times (0-72 h). CCRF-CEM and K562 cells derived from leukemias are MTAase deficient. On the other hand, MTAase activity is abundant in HeLa cells.

ed using biotin-cojugated MTA-BSA and avidin-peroxidase. Biotin-MTA-BSA was used as labeled MTA. Its ability to compete with varying levels of unlabeled free MTA was estimated by using avidin-peroxidase. Avidin provides four active binding sites for biotin. Consequently, avidin-peroxidase bound to biotin-MTA-BSA. Anti-MTA IgG interacting with MTA-biotin-avidin-peroxidase complexes is captured by goat anti-rabbit IgG coated on a microtiter plate. Binding of avidin-peroxidase was monitored by color development using a peroxidase substrate, 2,2'-azine-di[3-ethylbenzthiazolinesulfonate]. Optical densities were measured at 405 nm using a microplate reader.

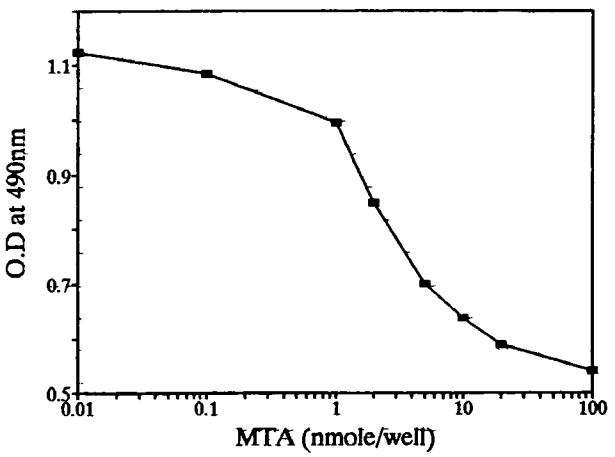


Fig. 8. A standard calibration curve of MTA level by ELISA. The assay was carried out in a final volume of 0.2 ml containing 50 μl of antiserum (dilution 1:10), 50 ng of biotin-BSA-MTA, and 50 μl sample or standard. After incubation and washing with PBS, 100 μl of avidin-peroxidase (dilution 1:1000) was added to each well. The optical density at 405 nm was measured with a Emax microplate reader (Molecular Devices, USA).

Experimental conditions for the ELISA were determined by measuring the optimal concentration of anti MTA-BSA antiserum, biotin-MTA-BSA, avidin-peroxidase, and the optimal incubation time for the immune reaction. A standard calibration curve of MTA quantification is presented in Fig. 8. The lower limit of detection by the assay was 100 pmole per tube. Therefore, the ELISA method is not sensitive enough to allow the determination of MTA in a small volume of biological samples. However, it is the first attempt to develop an enzyme-linked immunosorbent assay for MTA.

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