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Development of Enzyme Immunoassay of 2′-Deoxycytidine[1]

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Abstract

In order to study 2′-deoxycytidine (2′-dCyd) as a possible prognostic marker in cancer chemotherapy, an enzyme immunoassay (EIA) was developed. 2′-dCyd as a hapten was succinylated and two *O*-monosuccinyl-2′-dCyd's were purified by high performance liquid chromatography and identified by mass spectrometry and ^{1}H-NMR. Two antigens were prepared by conjugating two *O*-succinyl derivatives with keyhole limpet hemocyanin (KLH) as a carrier. Both antigen produced specific antibodies to 2′-dCyd in BALB/c mice. The spleen cells of one mouse immunized with 5′-*O*-succinyl-2′-dCyd-KLH were hybridized with myeloma cells. One monoclone selected produced a specific antibody. A convenient EIA was attained by using the monoclonal antibody. © 1997 Elsevier Science B.V.

Keywords: 2′-deoxycytidine; Enzyme immunoassay; Monoclonal antibody; Cancer chemotherapy; Antigen preparation

1. Introduction

These days, the number of cancer patients are increasing in many countries and while they recieve high-quality treatment it is desirable to find a therapeutic or prognostic marker for chemotherapy. In our previous paper [1], a previously unknown compound appeared in chromatograms of the plasmas of Egyptian breast cancer patients by high performance liquid chromatography (HPLC) with fluorescent detection for adenine-containing compounds. This compound was identified as 2′-deoxycytidine (2′-dCyd) by various methods. In the further study, 2′-dCyd levels increased in the plasmas of the breast cancer patients treated with cyclophosphamide, methotrexate and 5-fluorouracil (CMF). Its increase was related with the number of treatment courses. Individually, patients with a bad prognosis exhibited the increase in 2′-dCyd, but those with a good prognosis had normal levels. Therefore, it is critical to know whether the 2′-dCyd increase is specific for breast cancer patients in countries other than Egypt, whether the increase occurs in the other

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cancer patients and what is the mechanism of the increase of 2′-dCyd.

In order to solve the above problems, it is essential to develop a sensitive and convenient immunoassay for 2′-dCyd, a test which has not yet been reported. There have been several reports of immunoassays for nucleic acid components such as adenocine [2], cyclic AMP and cyclic GMP [3] as purine bases. These methods have been used for biochemical studies. On the other hand, immunoassays of pyrimidine-base-containing compounds have been developed and applied to cancer researches. Those were immunoassays of 5-methyl-2′-dCyd [4,5], 2′-deoxyuridine [6], 5-fluoro-uridine [6] and pseudouridine [7,8]. In the same way, 1-methyl-adenosine [9] and 8-oxoguanosine [10], metabolites from purine bases, were also measured by immunoassays. These pyrimidine and purine derivatives in urine were expected for diagnostic markers, but their significance was not established.

In the present paper, we attempted to develop an enzyme immunoassay (EIA) of 2′-dCyd, in which two types of antigens were prepared. Both antigens elicited specific antibodies in mice. A monoclonal antibody was also produced from one of the mice.

2. Experimental

2.1. Materials

2′-dCyd, cytidine, cCMP, CMP, CDP, CTP, 2′-dCMP, 5-methyl-2′-deoxycytidine (5ME-dCyd) and other nucleic acid components were obtained from Sigma (St. Louis, MO). Complete Freund's adjuvant and incomplete Freund's adjuvant were obtained from Difco Laboratories (Detroit, MI). Affinity isolated antibody alkaline phosphatase conjugate (Goat anti mouse IgG's $(G+L)$ alkaline phosphatase, Anti IgG-Alp) was obtained from Biosource international (Camarillo, CA). Keyhole limpet hemocyanine (KLH) was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Bovine serum albumin (crystallized) was obtained from Sigma. All the other chemicals were commercially available.

2.2. Succinylation of 2′-dCyd

The hapten was conjugated with a carrier protein after being succinylated according to Sato et al. [2] for preparation of an antigen of adenosine.

Then, 1 ml of 0.1 mM 2′-dCyd was mixed with an equal volume of 0.8 mM succinic anhydride in triethylamine-dioxane (1:9, v/v). The mixture was reacted by shaking at room temperature for 1 h and concentrated to a small volume in vacuo. A 50-μl aliquot of the concentrate was injected into a chromatograph 880 (JASCO, Tokyo) with a MULTI- 340 multi channel detector (JASCO). For analytical HPLC, a column (4.6 mm, I.D. × 250 mm) of Capcell pak C_{18} (Shiseido, Tokyo) was maintained at 40°C. The elution was done under a linear gradient of 0.1% trifluoroacetic acid (TFA) to 0.1% TFA–20% acetonitrile in water for 30 min. The flow rate of the eluent was set at 1.0 ml min^{-1}.

For preparative HPLC, a column (10 mm, I.D × 250 mm) of Capcell pak C_{18} was used. The flow rate of the eluent was set at 3.0 ml min^{-1}. All the other conditions were the same expect 100 μl of the injection volume. This preparative HPLC was repeated several times. Each purified peak was pooled and lyophilized.

2.3. Mass spectrometry (MS) of succinyl-derivatives

Negative and positive ion FAB-MS spectra for the purified sample were measured by a mass spectrometer JMS-DX300 (JEOL, Tokyo) with a glycerin matrix at 3 kV.

2.4. ^1H-NMR spectroscopy of succinyl-derivatives

The purified sample was dissolved in 0.5 ml of deuterium oxide. The solution was added 3-(trimethylsilyl) propionic acid sodium salt-d_4 as an internal standard. ^{1}H-NMR spectra were recorded using a NMR spectrometer 400 GSX (JEOL) at 400 MHz. The probe temperature was maintained at 30°C.

2.5. Spectral data for 2'-dCyd succinyl derivatives

2.5.1. 2'-dCyd

^{1}H-NMR (400 MHz, D_2O) δ:7.84 (*d*, $J = 7.6$ Hz, 1 H), 6.27 (*t*, $J = 6.72$ Hz, 1 H), 6.05 (*d*, $J = 7.3$ Hz, 1 H), 4.45 (*br dt*, $J = 6.4$ Hz, 4.0 Hz, 1 H), 4.08 (*br dt*, $J = 5.2$ Hz, 3.8 Hz, 1 H), 3.86 (*dd*, $J = 12.2$ Hz, 3.7 Hz, 1 H), 3.78 (*dd*, $J = 12.2$ Hz, 5.2 Hz, 1 H), 2.46 (*ddd*, $J = 14.0$ Hz, 6.4 Hz, 4.0 Hz, 1 H), 2.29 (*dt*, $J = 14.0$ Hz, 6.7 Hz, 1 H).

2.5.2. 5'-O-(3-Carboxypropanoyl)-2'-deoxycytidine from peak 2 (5'-sdCyd)

FAB-MS *m/z*: 328(M + 1)$^+$, 326(M-1)$^-$; ^{1}H-NMR (400 MHz, D_2O) δ:7.84 (*d*, $J = 7.6$ Hz, 1 H), 6.23 (*t*, $J = 6.6$ Hz, 1 H), 6.18 (*d*, $J = 7.6$ Hz, 1 H), 4.48 (*dt*, $J = 6.7$ Hz, 4.0 Hz, 1 H), 4.38 (*dd*, $J = 12.0$ Hz, 3.7 Hz, 1 H), 4.35 (*dd*, $J = 12.0$ Hz, 4.0 Hz, 1 H), 4.6 (*q*-like, $J = 4.0$ Hz, 1 H), 2.68–2.57 (M, 4 H), 2.50 (*ddd*, $J = 14.4$ Hz, 6.6 Hz, 4.0 Hz, 1 H), 2.35 (*dt*, $J = 14.4$ Hz, 6.7 Hz, 1 H).

2.5.3. 3'-O-(3-Carboxypropanoyl)-2'-deoxycytidine from peak 3 (3'-sdCyd)

FAB-MS *m/z*: 328(M + 1)$^+$, 326(M-1)$^-$; ^{1}H-NMR (400 MHz, D_2O) δ:7.95 (*d*, $J = 7.9$ Hz, 1 H), 6.25 (*t*, $J = 6.9$ Hz, 1 H), 6.14 (*d*, $J = 7.6$ Hz, 1 H), 5.32 (m, 1 H), 4.25 (*br q*-like, $J = 3.1$ Hz, 1 H), 3.85 (*dd*, $J = 12.5$ Hz, 3.4 Hz, 1 H), 3.80 (*dd*, $J = 12.5$ Hz, 4.6 Hz, 1 H), 2.71–2.62 (m, 4H), 2.57 (*br ddd*, $J = 14.5$ Hz, 6.1 Hz, 2.0 Hz, 1 H) 2.39 (*dt*, $J = 14.5$ Hz, 7.3 Hz, 1 H).

2.5.4. 3'-O,5'-O-Di(3-carboxypropanoyl)-2'-deoxycytidine from peak 4 (3',5'-sdCyd)

FAB-MS *m/z*: 428(M + 1)$^+$, 426(M-1)$^-$; ^{1}H-NMR (400 MHz, D_2O) δ:7.86 (*d*, $J = 7.6$ Hz, 1 H), 6.23 (*dd*, $J = 7.6$ Hz, 6.1 Hz, 1 H), 6.17 (*d*, $J = 7.9$ Hz, 1 H), 5.35 (*dt*, $J = 6.7$ Hz, 2.4 Hz, 1 H), 4.45 (*q*-like, $J = 3.4$ Hz, 1 H), 4.40 (*br d*, $J = 3.7$ Hz, 2 H), 2.73–2.61 (m, 9H), 2.42 (*dt*, $J = 14.6$ Hz, 7.6 Hz, 1 H).

2.6. Conjugation of succinyl-derivative with a carrier

5'-sdCyd and 3'-sdCyd were purified by HPLC and identified by above spectrometries. The derivative (25 mg) was dissolved in 5 ml of 12.5 mM phosphate buffer (pH 5.25). The solution was added to 75 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and its pH was adjusted to 5.0–5.5 with 0.01 M NaOH. The adjusted solution was added 25 mg of KLH for preparation of the antigen for immunization, or 20 mg of BSA for 3'-sdCyd, or 10 mg of BSA for 5'-sdCyd for preparation of the antigen for EIA in 1 mg of 50 mM phosphate buffered 0.1 M saline (pH 7.20) with stirring at 4°C. The mixture was immediately adjusted to pH 6.4 with 0.01 M HCl or 0.01 M NaOH and stood overnight. The reaction mixture was dialyzed against 1 l of water which was replaced with other water at an interval of 3 h. The dialysis was continued for 48 h. The dialysate was lyophilized. Conjugated 2'-dCyd in each antigen was calculated by measuring a UV spectrum from 250 to 280 nm [6].

2.7. Immunization of mice with antigen

The antigen solution (1 mg ml^{-1}) of 5'-sdCyd or 3'-sdCyd in 0.9% NaCl was mixed with an equal volume of complete Freund's adjuvant and stirred to prepare a W/O emulsion. The emulsion (100 μl) was injected into 5 BALB/c mice. After several immunizations at 2 weekly intervals, using the similar emulsion prepared with incomplete Freund's adjuvant, blood was collected 3 days after the booster from the tail vein. The blood was diluted with 0.1 M phosphate buffer (pH 7.0) containing 0.05% tween 20 (PBT) 10-fold and centrifuged at 5000 × *g* for 10 min. The supernatant was stored at −20°C as an antiserum.

2.8. Procedure for EIA

A procedure for EIA of vanilmandelic acid described by Yoshioka et al. [11] was modified so that there was competition between the antigen adsorbed on a wall of a microtiter plate and the hapten for the antibody binding sites.

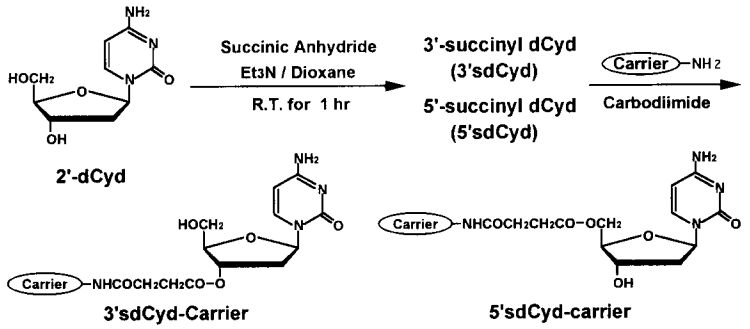

Fig. 1. Preparation of antigens.

A solution (50 μl) of the antigen (5′- or 3′-sdCyd-BSA) or BSA in 0.1 M phosphate buffer (PB, pH 7.0) at 0.6 μg ml^{-1} each was distributed between the wells of the microtiter plate. The plate was incubated at 37°C for 2 h. Each well was washed with PBT 5 times. To the wells were added serial 10-fold diluted antiserum solutions in PBT and incubated at 37°C for 1.5 h, when titer of the antiserum was checked. While cross reactivity of a competitor was checked, 50 μl solution of 10 000-fold diluted antiserum and 50 μl solution of the competitor in PBT were mixed in the well to allow competitive binding between the hapten and antigen to the antibody for 1.5 h. For screening the clone, the culture supernatant of a hybrid described later was used in place of the above diluted antiserum solution.

The well was washed 5 times with PBT and a solution (3.0 μg ml^{-1}) of Anti IgG-Alp was added. The well was incubated at 37°C for 1.5 h and washed with PBT 5 times. To the well was added 50 μl of 22.4 mM *p*-nitrophenylphosphate in 0.1 M sodium bicarbonate buffer (pH 9.80). An increase of the absorbance (A_{405}) of the reaction mixture at 5 min was measured by an EIA reader (BIO-TEK) at 405 nm.

2.9. Preparation of monoclonal antibodies

The preparation was carried out according to Köhler and Milstein [12], after final immunization with an intraperitoneal injection of 50 μg of the antigen in saline, spleen cells from the mouse of highest titer were fused with P3U1 myeloma cells using polyethyleneglycol. Hybridoma were selected in hypoxyanthine-aminopterine-thymidine medium and transferred to hypoxyanthine-thymidine medium. The hybridoma were screened by EIA and cloned by a limited dilution method. At each step, the titer of the culture medium was assayed as described above. The monoclone was injected into BALB/c mice to be grown in vivo. The antibodies were purified by precipitation with ammonium sulfate, a protein A column- and a DEAE-cellulose column chromatography.

3. Results

3.1. Preparation of antigen

The various antigens were prepared by combination of conjugation groups of the hapten with the carrier proteins as shown in Fig. 1. There were two hydroxy groups in 2′-dCyd to which the spacer was introduced. In fact, succinyl residue as the spacer was easily connected to 2′-dCyd. As

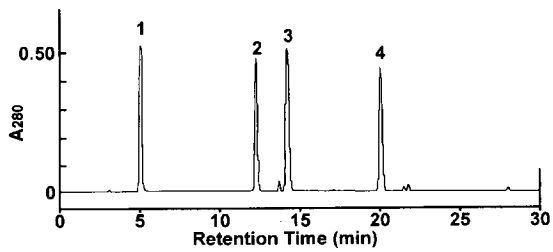

Fig. 2. Chromatogram of succinyl products. The eluate was monitored by the multi channel detector at 280 nm (A_{280}).

shown in Fig. 2, four peaks appeared in the chromatogram as expected. All the peaks showed the same UV spectrum possessing a maximum at 280 nm. The retention time of peak 1 overlapped with that of the starting material. The other peaks were preparatively pooled and the purified compounds were analyzed by MS and ^{1}H-NMR. The compounds of peak 2 and 3 showed the same molecular weight by positive and negative FAB-MS, which meant two monosuccinyl 2′-dCyd isomers. In ^{1}H-NMR, the compound of peak 2 shifted 5′-methylene protons of 2′-dCyd, but did not all the other signals. Thus, 5′-hydroxyl group of 2′-dCyd was to be succinylated in the compound of peak 2. In contrast, the compound of peak 3 shifted 3′-methyne proton downward. Then, the succinylated group was assigned to 3′-hydroxyl group. In the same way, the compound of peak 4 was decided as 3′-*O*,5′-*O*-disuccinyl derivative from the parent ion in FAB-MS and downward shifts of 3′-methyne proton and 5′-methylene protons in ^{1}H-NMR. The disuccinyl derivative was not used for the preparation of the antigens.

Each mono-succinyl derivative was condensed with the carrier protein by the two step carbodiimide method of Henn et al. [6]. KLH was selected as a carrier for its high immunogenecity. It was replaced with BSA to evade the cross reactivity of the carrier influencing the background in EIA.

3.2. Specificity of antibodies

Two groups of 5 mice were immunized with the prepared antigens, 3′-sdCyd and 5′-sdCyd-KLH, respectively. High titer of the antibody was observed in the mice with 3′-sdCyd-KLH even after the first booster. The ranges of the titer designated as dilution of the antiserum were from 100 to 1000-fold in all the antisera. As shown in Fig. 3A, the antiserum to 3′-sdCyd discriminated the hapten moiety against the carrier. After the third booster, the titers attained a plateau of 10 000-folds.

The specificity of the antiserum was crucial to develop EIA. As shown in Table 1, 2′-dCyd was the strongest competitor among Cyd, dAdo,

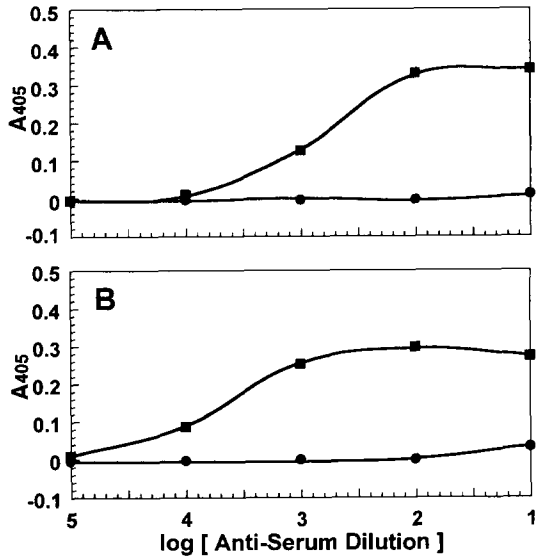

Fig. 3. Titration curves of antisera. A; An antiserum to 3′-sdCyd was diluted and distributed between the wells onto which 3′-sdCyd-BSA (■) and BSA (●) were adsorbed as described in EIA. B; An antiserum to 5′-sdCyd was diluted and distributed between the wells onto which 5′-sdCyd-BSA (■) and BSA (●) were adsorbed.

dGuo and dThy examined, where the concentration of 50% inhibition (IC_{50}) were compared. For the further screening, it was relevant to remark discrimination between 2′-dCyd and Cyd.

Meanwhile, the other group of 5 mice immunized with 5′-sdCyd-KLH slowly produced the antisera of 1000-fold of the titer after the third booster. This antiserum also discriminated the hapten moiety against the carrier as shown in Fig. 3B. Their specificity, however, seemed higher than that of the antisera to 3′-sdCyd-KLH as shown in Table 1.

3.3. Preparation of monoclonal antibody

Thus, the spleen cells from the mouse immunized with 5′-sdCyd-KLH were hybridized with the myeloma cells. The well grown hybridoma were 121 strains. In all the supernatants of the culture media, high titers to 5′-sdCyd-BSA were observed, whereas low titers to BSA were. From the strains of high titer and growth, 17 strains

Table 1
Comparison of cross reactivities of two antisera[a]

Competitor	Antiserum to 3′-sdCyd-KLH		Antiserum to 5′-sdCyd-KLH	
	IC_{50} (μM)	C.R. (%)	IC_{50} (μM)	C.R. (%)
Cyt	>10 000	—	891	1.12
Cyd	398	25.13	473	2.11
2′-dCyd	100	100	10	100
5′-CMP	>10 000	—	5957	0.17
5′-dCMP	>10 000	—	188	5.32
dAdo	>2500	—	>2500	0.40
dGuo	>10 000	—	>10 000	—
dThd	>10 000	—	>10 000	—

[a]Cross Reactivity (C.R. in %) = IC_{50} Value of 2′-dCyd/IC_{50} Value of Competitor.

were cloned by the limited dilution method. Among 163 clones selected, 34 clones produced antibodies of high titer and specificity for 2′-dCyd. Further, 3 clones were cloned again. They produced almost the similar specific monocloned antibodies represented as shown in Table 2.

In conclusion, the antibody was specific to 2′-dCyd. In details, the spacer of succinic ester was recognized with the antibody so that IC_{50}s of 2′-dCyd, 5′-sdCyd, 5′-dCMP, 5′-dCDP and 5′,-dCTP increased in this order. 2′-Deoxy group of 2′-dCyd was also recognized so that IC_{50}s of 3′-dCyd and Cyt were 89 and 422 μM, respectively. Concerning the pyrimdine base, dThd, dUrd, 3Me-dThd had no affinity, while 5Me-dCyd had the same extent in the affinity as 2′-dCyd. The 4-amino group was recognized well, but 5-position was not at all.

For practical use, the clone was grown in the mice in vivo. An appropriate amount of the monoclonal antibody was produced. The purified antibody showed the same specificity as the antibody obtained from the supernatant as shown in Table 2.

A calibration curve was drawn by using the monoclonal antibody as shown in Fig. 4. A linear relationship as logit $Y = -0.862 \log C - 5.23$ ($r^2 = 0.993$) was obtained. The range of concentration from 0.02 to 200 μM was compatible with the blood levels of normal subjects. The constituent of human serum was not affected the calibration curve. Thus, specific EIA for 2′-dCyd was developed in the present paper.

4. Discussion

There have been two methods reported to prepare antigens to nucleosides and nucleotides since the 1960s. In one method, diol groups of ribose in the nucleosides and nucleotides were oxidized to dialdehydes with periodate [13]. The dialdehydes were condensed with amino groups of carrier proteins. This method, however, is not applicable to 2′-dCyd lacking the diol groups. On the other method, anyone of hydroxy groups of the ribose was succinylated and the hemisuccinate was conjugated with the carrier proteins. By this method, two kinds of the antigens to 2′-dCyd were obtained. In the mean derivatives, it was necessary to completely purify the succinic esters of 2′-dCyd to produce the specific antibodies. The derivatives purified by HPLC were precisely identified by MS and ^{1}H-NMR. Both antigens synthesized had high immunogenecity. Any symptomatic effect was not seen in the immunized animals, although dCTP pool in lymphocytes was present as described by Spasokukotskaja et al. [14]. The process to obtain the monoclonal antibody proceeded without any trouble. The specificity of the monoclonal antibody was almost satisfactory, except the cross reactivity of 5Me-dCyd. It was considered that the succinyl residue of the antigen could be closed to 5-position of pyrimidine base. On the other hand, the monoclonal antibody to the antigen prepared from 5Me-Cyd via dialdehydes had higher specificity to 5Me-dCyd than 5Me-Cyd.

Table 2
Cross reactivity of a monoclonal antibody to 5′-sdCyd-KLH

Competitor	IC_{50} (μM)	C.R. (%)	Competitor	IC_{50} (μM)	C.R. (%)
5Me-Cyt	355	0.37	5-FU	>2500	—
Cyt	422	0.31	Urd	>10 000	—
5Me-Cyd	244	0.53	3′,5′-cUMP	>10 000	—
Cyd	150	0.87	5′-UMP	>10 000	—
3Me-dCyd	211	0.62	5′-UDP	>10 000	—
5Me-dCyd	2	65.0	5′-UTP	>10 000	—
3′-dCyd	89	1.46	dUrd	>10 000	—
2′-dCyd	1.3	100	Ade	>1000	—
5′sdCyd	1.7	76.5	Ado	>1000	—
3′sdCyd	168	0.77	3′,5′-cAMP	>10 000	—
3′,5′sdCyd	398	0.33	ADP	>10 000	—
3′,5′-cCMP	355	0.37	ATP	>10 000	—
5′-CMP	7499	0.02	dAdo	>2500	—
5′-CDP	>10 000	—	5′-dAMP	>10 000	—
5′-CTP	>10 000	—	5′-dADP	>10 000	—
3′dCMP	7499	0.02	Guo	>1000	—
5′dCMP	158	0.82	3′,5′-cGMP	>10 000	—
5′dCDP	1995	0.07	5′-GMP	>10 000	—
5′dCTP	6683	0.02	5′-GDP	>10 000	—
5′sdCyd-KLH	0.71 (μg/ml)	—	5′-GTP	>10 000	—
KLH	>100 (μg/ml)	—	dGuo	>10 000	—
5′sdCyd-BSA	0.47 (μg/ml)	—	Ino	>10 000	—
BSA	>100 (μg/ml)	—	dIno	>10 000	—
Thy	>1000	—	Hyp	>1000	—
3Me-dThd	>10 000	—	Xan	>10 000	—
dThd	>10 000	—	Rib	>10 000	—
5′-dTMP	>10 000	—	dRib	>10 000	—
Pyr	>10 000	—			

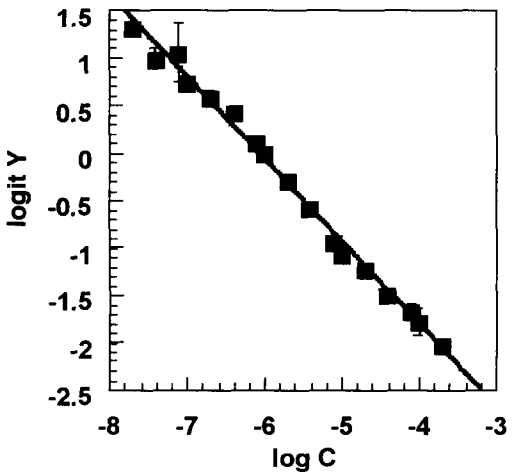

Fig. 4. Calibration curve of EIA for 2′-dCyd

logit $Y = \log IC - IC_0/IC_{100} - IC$

where C is molar concentration of 2′-dCyd.

Further cloning of the hybridoma to 2′-dCyd is under investigation. 5Me-dCyd is a modified metabolite from DNA and supposed to be a prognostic marker for leukemia. Its significance, however, is not established. It increased in human urine with leukemia, but not in plasma. In our HPLC, a peak of 5Me-dCyd did not appear in plasmas from the breast cancer patients. It is possible to study clinical samples using the present EIA.

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