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Fluorophotometric Enzyme Immunoassay of Neocarzinostatin using Peroxidase as a Label

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A sensitive enzyme immunoassay for the determination of neocarzinostatin (NCS), a proteinaceous antitumor antibiotic, has been developed. Horseradish peroxidase was used as the labeling enzyme and was conjugated with NCS according to Nakane's method. Separation of bound and free fractions was achieved by a double antibody solid phase method using Sepharose 4B gel coupled with purified IgG of goat anti-rabbit IgG serum. Thyramine and hydrogen peroxide were used as substrates for the fluorophotometric assay of peroxidase activity. A satisfactory standard curve for NCS was obtained in the range of 10 to 1000 pg/assay tube, corresponding to the range of 0.5 to 50 ng/ml of sample solution. This enzyme immunoassay could be applied to the determination of NCS in serum after its administration to rabbits. NCS in various tissue homogenates could also be assayed by this method.

Keywords——fluorophotometry; enzyme immunoassay; neocarzinostatin; peroxidase; tyramine; fluorescence enzyme immunoassay

Radioimmunoassays (RIAs) are widely used in clinical studies for the quantitative determination of many drugs in physiological fluids of humans or experimental animals. However, for each assay, a chemically stable and serologically active radiolabeled drug or drug derivative is required. Recently, the use of enzyme labels in place of radioisotopes for the measurement of antigens, antibodies, and haptens has stimulated the new and expanding field of enzyme immunoassay (EIA).[2] In many cases, EIA can match RIA in terms of sensitivity and selectivity, yet has advantages of speed, convenience, and reduced cost. In this study, we attempted to develop a highly sensitive EIA procedure using horseradish peroxidase (HRP) as a label enzyme for the determination of neocarzinostatin (NCS) in serum and tissues.

NCS is an antitumor protein agent used in the treatment of leukemia, gastric and pancreatic cancer, and especially bladder cancer.[3–5] Maeda *et al.*[6,7] showed that NCS was extremely rapidly cleared into the urine and rapidly inactivated by proteolisis in the serum. Its biological half-life *in vivo* was only a few minntes when given intravenously.[8] NCS is usually assayed by bioassay,[9,10] which is not very sensitive and is time-consuming. A radioimmunoassay[11] using ^{125}I-labeled derivatives of NCS and a fluorescence polarization method[8] using NCS labeled with fluorescein isothiocyanate were developed. The former is more sensi-

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tive than the latter, and the latter is simple and rapid. However, the sensitivities of these methods are not sufficient to determine NCS levels below 10 nmol/l. Therefore, we sought to develop a more sensitive, simple EIA procedure for the determination of NCS in biological fluids.

Materials and Methods

Reagents——NCS was a gift from Kayaku Antibiotics Research Laboratories. HRP (Type VI, 320 U/mg) was purchased from Sigma Chemical Co., Bio-gel P 60 and Sepharose 4B from Seikagaku Koggyo Co., and dinitrofluorobenzene, ethylene glycol, sodium periodate, and other chemicals from Tokyo Kasei Co. Tyramine was purified by recrystallization from ethanol.

Buffer: The buffer used in this study was 0.05 M phosphate buffer containing 0.15 M sodium chloride and 0.1% BSA, pH 7.0.

Anti-NCS Serum——Anti-serum against NCS was produced in a rabbit immunized with NCS emulsified in Freund's complete adjuvant. The immunization schedule was similar to that used in the preparation of anti-insulin serum.[12]

Preparation of HRP-NCS Conjugate——The procedure for the preparation of HRP-NCS conjugate was similar to that used to prepare HRP-insulin conjugate according to Nakane's method.[13] Finally, the HRP-NCS conjugate was purified by chromatography on a wet-packed column of Bio-gel P 60 (1.0 × 50 cm), eluting with 0.05 M phosphate buffer containing 0.15 M sodium chloride, pH 7.0.

Preparation of Double Antibody Solid Phase (DASP)——The insolubilized second antibody was prepared by the method of Axén *et al.*[14] Sepharose 4B (50 ml) was activated at pH 10.5 using 50 ml of cyanogen bromide solution (50 mg/ml aqueous solution) for 7 minutes. After washing with water and 0.1 M sodium hydrogen carbonate solution, the activated product was mixed with 0.1 g of purified anti-rabbit IgG goat IgG fraction in 10 ml of 0.1 M sodium hydrogen carbonate solution. The reaction was run at 4° overnight with slow stirring, then the coupled product was washed by the method of Wide.[15] The washed DASP was finally suspended in buffer and stored at 4°.

Enzyme Immunoassay Procedure——Twenty μl of the NCS standard solution (0.5—64 ng/ml) or the sample solution, 100 μl of diluted anti-NCS serum (rabbit) (× 30000) and 500 μl of the assay buffer were mixed. After incubation for 1 hr at room temperature, 100 μl of HRP-NCS conjugate solution (× 5000—10000) and 500 μl of DASP solution were added and the whole was incubated overnight at 4° using an immunorotor. The reaction mixture was centrifuged at 1000 g for 5 minutes at 4°. The supernatant was aspirated off and the precipitated DASP was washed 3 times with 2 ml each of saline. After washing, the HRP activity was measured by the fluorophotometric method as described below.

Assay of HRP Activity[12]——The HRP activity was determined by fluorophotometry using tyramine and hydrogen peroxide as substrates. To each assay tube containing the washed DASP, 800 μl of buffer, 50 μl of 1% purified tyramine solution and 50 μl of 0.007% hydrogen peroxide solution were added serially and mixed well. After incubation of the mixture for 1 hr at room temperature, the reaction was stopped by the addition of 50 μl of 1.25% potassium cyanide solution and 50 μl of 0.5 N sodium hydroxide solution. After centrifuging for 5 minutes, the fluorescence intensity was measured at an excitation wavelength of 320 nm and an emission wavelength of 405 nm.

Results and Discussion

In preliminary studies, conjugation methods using glutaraldehyde and soluble carbodiimide were examined, but the yields of conjugates were less than that obtained by Nakane's method.[13] In order to separate HRP-NCS conjugate from uncoupled enzyme and NCS, gel chromatography using a Bio-gel P 60 column was used. Fractions of 1 ml were collected and their absorbance, enzyme activity and immune reactivity were determined by spectrophotometry, fluorophotometry and enzyme immunoassay, respectively. A typical chromatogram is shown in Fig. 1. A sharp separation between conjugated and unconjugated HRP was not obtained. Fraction Nos. 7 and 8 could be successfully used for this assay. They contained 21% of the total activity of HRP used for conjugation. whereas the total yield in all fractions was 53%.

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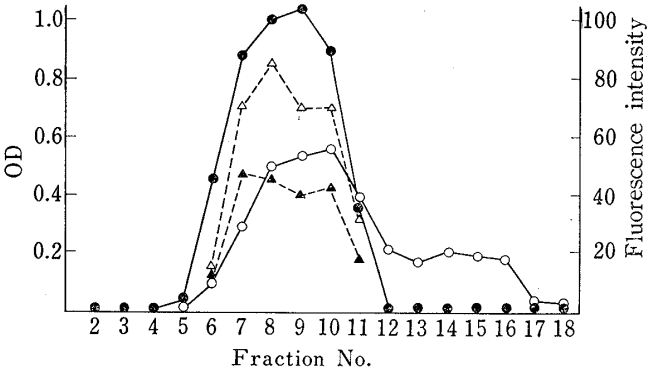

Fig. 1. Column Chromatography of HRP-NCS Conjugate in 0.05 M Phosphate Buffer (pH 7.0); Bio-gel P 60, 1.0×50 cm

—●—: OD at 280 nm, —○—: OD at 405 nm, —▲— fluorescence intensity obtained by EIA (no addition of NCS), ---△--- fluorescence intensity obtained by EIA (addition of NCS, 20 ng/ml).

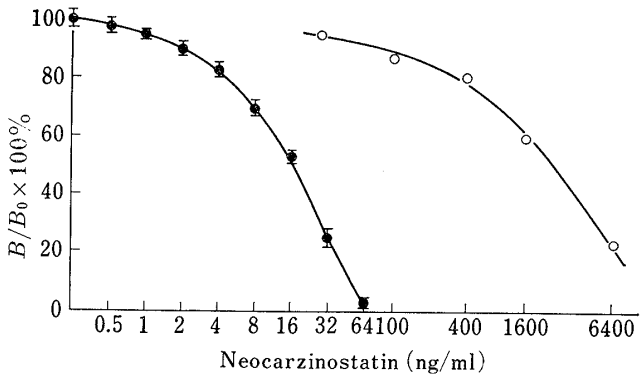

Fig. 2. Standard Curves for Neocarzinostatin obtained by the Double Antibody Solid Phase Method (—●—) and the First Antibody Solid Phase Method (—○—)

represents $\pm 2\sigma$.

The sensitivity of EIA depends on various factors, such as the quality of the anti-serum, the immunoreactivity of the enzyme-antigen conjugate, the order of addition of reagents and the incubation time.[2] Therefore, the optimal concentrations of anti-NCS antiserum and HRP-NCS conjugate were examined; those most suitable for enzyme immunoassay were a dilution of $1:3\times10^4\sim1:5\times10^4$, and a dilution of $1:5\times10^3\sim1\times10^4$, respectively. The procedure used to separate bound and free fractions is also important in relation to the sensitivity of EIA. Van Weemen *et al.*[16] and Avrameas *et al.*[17] showed that the DASP method gave higher sensitivity, greater precision, and more rapid separation than the insolubilized first antibody method in EIAs for human chorionic gonadotropin and human IgG. Similar results were obtained with EIAs for insulin[12] and thyroid-stimulating hormone[18] in previous papers. In this paper, the first antibody solid phase method and DASP method were both examined. As shown in Fig. 2, the DASP method was superior to the insolubilized first antibody method. NCS could be determined in the range of 0.5—64 ng/ml by the DASP method, but only in the range of 25 ng—6.4 μg/ml by the first antibody solid phase method. The lower limits of detection by RIA[11] and the fluorescence polarization method[8] were 0.1 pmol and 4 pmol, respectively. The lowest amount of NCS that could be detected under the optimal conditions using the present method was 10 pg/assay tube, corresponding to 1 fmol. This method is thus much more sensitive than the other methods. The coefficients of variation at each concentration of NCS in the standard curve were 0.9—9.7%.

It is well known, particularly from RIA experience, that serum and plasma at low dilutions may interfere with the immune reaction. An additional problem in EIA is the possibility of influence on the enzymatic reaction. To examine whether serum and various tissue homogenates interfere with EIA, various amounts of serum and tissue homogenates were added to the standard NCS solutions. The effects of normal serum and carcinoma serum are shown in Table I. The greater the amount of serum added, the lower was the difference of fluorescence intensity between each level and the 0 level of NCS, but there was no significant difference between normal and carcinoma sera. It was possible to obtain a useful standard curve even in the presence of 100 μl of serum. Homogenate extracts of spleen. pancreas, lung and muscle increased the fluorescence intensity with increasing amount of extract added, whereas kidney

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Table I. Influence of Serum on the Fluorescence Intensity in EIA of Neocarzinostatin

	Serum added μl/tube	Neocarzinostatin, pg/tube			
		0	12.8	128	1280
Normal	0	91	87 (4)	65 (26)	12 (79)
	20	91	87 (4)	65 (26)	12 (79)
	50	85	82 (3)	69 (16)	10 (75)
	100	75	72 (3)	62 (13)	8 (67)
Carcinoma	0	91	87 (4)	69 (24)	11 (80)
	20	91	87 (4)	69 (24)	11 (80)
	50	86	80 (6)	65 (21)	10 (76)
	100	78	74 (4)	65 (13)	6 (72)

The fluorescence intensity was measured by the EIA procedure presented in the text. The values in parentheses are the differences of fluorescence intensity between 0 and each concentration of NCS.

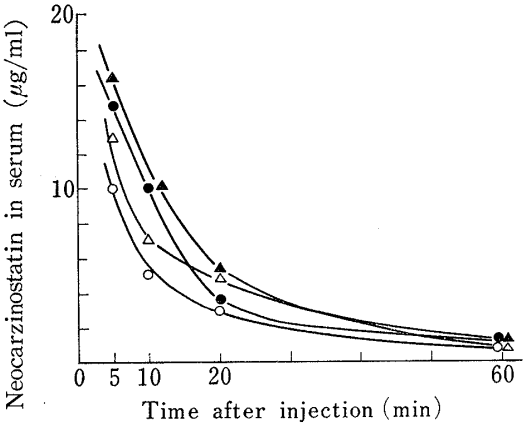

Fig. 3. Time Course of Neocarzinostatin Serum Level after Injection in Rabbit (2.0 mg/kg. *i.v.*)

homogenate extract did not affect the fluorescence intensity, and liver homogenate extract decreased the fluorescence intensity. However, these effects could be corrected by addition of the same amount of blank homogenate extract. Based on these results, 20 μl of NCS-free serum or homogenate extract was added to the standard NCS solution to obtain sufficient sensitivity with reasonable tolerance to interference.

The time-course of the concentration of NCS in serum after a single intravenous dose was assayed in rabbits. Four male albino domestic rabbits weighing 2.2 to 2.6 kg were used. NCS was dissolved in isotonic saline and a dose of 2.0 mg/kg body weight was injected *via* the auricular vein. Blood was sampled from the other auricular vein. NCS in serum was determined by the EIA presented here and by bioassay. The results are illustrated Fig. 3. The concentrations of NCS in the serum at zero time was found to be 20 mg/l, by least-squares analysis and extrapolation. From this result, the apparent distribution volume (V_{app}) of NCS was obtained as follows:

$$V_{app} = \frac{2.0 \text{ mg/kg}}{20.0 \text{ mg/l}} = 0.10 \text{ l/kg}$$

The time required for 50% reduction of the concentration of the drug in the serum from time zero can be obtained from Fig. 3; it is about 8.5 minutes, which is slightly longer than the value (7.0 min) reported by Maeda.[8]

Maeda[6,7] found that the inactivation of NCS occurred *in vivo* or during incubation with serum in a test tube. In order to determine the inactivation of NCS by serum, urine and tissues homogenates, NCS standard solution was mixed with each of them and assayed by the EIA method after incubation for 1 hr at 37°. The inactivation percentages were calculated by comparing the fluorescence intensity at zero time with that after incubation for 1 hr. As shown in Table II, the addition of serum and urine did not affect the determination of NCS, but some tissue homogenates inactivated NCS in the range of 5% to 40%. Therefore, proteolytic degradation of NCS during the assay should be considered in the assay of NCS levels in homogenates of liver and muscle. Although NCS was inactivated by incubation with bovine serum,[6] 10% addition of rat serum to NCS standard solution did not inactivate NCS, as shown in Table II. The reason for this difference between the results obtained by Maeda[6] and us is based on the difference of the assay methods. The bioassay[10] used in Maeda's paper is very specific for NCS, whereas the EIA presented here may be less specific, because the anti-NCS serum might react with some degradation products in the serum.

NCS is usually administered to patients with carcinoma with bleomycin and 5-fluorouracil. Therefore, the effects of these drugs on the assay of NCS by this method were examined.

Table II. Inactivation of Neocarzinostatin by Incubation with Various Rat Tissue Homogenates

Tissue	Inactivated %	Tissue	Inactivated %
Liver (margin)	40.4	Intestine	8.1
Liver (center)	38.0	Uterus	6.0
Muscle	36.0	Kidney	5.0
Stomach	32.0	Skin	0
Thymus	30.0	Brain	0
Spleen	27.0	Lung	0
Pancreas	23.0	Urine	0
Heart	20.0	Serum	0

Each tissue (1 g), serum (1 ml) or urine (1 ml) was homozinized with 9 ml of normal saline, and then the concentration of NCS was adjusted to 64 ng/ml by addition of NCS. The reference is NCS solution (64 ng/ml) in 0.05 M phosphate buffer, pH 7.0, containing 0.1% BSA and saline. Samples and reference solution were incubated at 37° for 1 hr, and then centrifuged at 3×10^3 rpm for 5 min. Aliquots of 20 μl of the supernatants were assayed by the EIA method described in the text.

Twenty μl each of bleomycin solution (50 ng/ml and 5 μg/ml) and 5-fluorouracil solution (15 μg/ml and 1.5 mg/ml) was added to the NCS standard solution and the mixture was assayed by the standard procedure. These compounds had no effect on the assay of NCS in the mixture.

Styrenemaleate derivatives of NCS, which are used as a suppository, could also be determined successfully by this EIA method. The results were compared with the data obtained by bioassay and by the biuret method, which is a chemical assay method for protein. As shown in Table III, the values of NCS obtained by the EIA coincided with those obtained by the biuret method, whereas they did not coincide with those obtained by the bioassay. This result may be due to the partial loss of biological activity of NCS in Lot Nos. 3003 and 3004 during preparation. The time course of NCS levels in the serum after administration of 10 mg of styrenemaleate derivatives by suppository could also be determined in rats. The half-life ($T_{1/2}$) was longer than that of NCS because styrenemaleate derivatives are stable to proteolytic degradation.

Table III. Comparison of Three Methods for the Determination of Neocarzinostatin in Styrenemaleate Derivatives

Lot. No. of styrenemaleate derivative	Neocarzinostatin (%)		
	EIA	Biuret method	Bioassay
3001	19.6	25.0	21.3
3002	25.0	33.0	38.3
3003	60.0	56.0	40.0
3004	80.0	71.8	49.0

The proposed EIA method is more sensitive than other assay systems available at present. The availability of this method should make it possible to obtain more precise data on the pharmacokinetics of NCS and its derivatives. This will be the subject of a future comunication.