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Effect of Bridge Heterologous Combination on Sensitivity in Enzyme Immunoassay for 11-Deoxycortisol[1)]

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The effect of the bridge heterologous combination between antiserum and enzyme-labeled steroid on sensitivity in heterogeneous enzyme immunoassay for 11-deoxycortisol has been investigated. Four 11-deoxycortisol derivatives possessing different bridges at C-4 were employed for the preparation of both antisera and enzyme-labeled antigens. They were 4-(carboxymethylthio)-11-deoxycortisol, 4-(2-carboxyethylthio)-11-deoxycortisol, 4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol, and 4-hemisuccinoyloxy-11-deoxycortisol. The *N*-succinimidyl esters of the carboxylated derivatives were reacted with β-galactosidase to give enzyme-labeled antigens. The antisera to 11-deoxycortisol used were those raised against the conjugates of these haptenic derivatives with bovine serum albumin. The sensitivities obtainable with four homologous and twelve heterologous systems were tested. When thioether derivatives were used for enzyme labeling, the effectiveness of heterology on assay sensitivity was dependent upon the length of the bridge. It was found that the heterologous system using the enzyme-labeled steroid obtained from a hapten having a bridge shorter than that used for antibody production resulted in an increase in sensitivity of the assay, whereas the use of a longer bridge was not effective. This finding is in good agreement with that obtained in our previous study on enzyme immunoassay for cortisol.

Keywords——enzyme immunoassay; 11-deoxycortisol; *N*-succinimidyl ester method; β-galactosidase-labeled 11-deoxycortisol; bridge heterology: bridge length: sensitivity

A number of papers on heterogeneous enzyme immunoassay of steroid hormones have been reported. In general, however, development of a specific and sensitive assay system is not always easy owing to various factors. It has been shown that the combination of antibody and enzyme-labeled antigen is an important factor determining the sensitivity.[2)] Anti-steroid antibody elicited with an immunogen haptenized *via* a "chemical bridge" usually recognizes the structure of the bridge between the enzyme and steroid in the labeled antigen. It is often found that the use of the same hapten for both preparation of hapten immunogen followed by immunization (antibody production) and enzyme labeling, *i.e.* homologous combination, does not provide satisfactory sensitivity, because the binding affinity of the labeled antigen to antibody is higher than that of the antigen to be measured. For the purpose of improving the sensitivity, an assay system using different haptenic derivatives is designed; this is termed heterologous enzyme immunoassay.

We reported previously that a "bridge" heterologous system rather than "site" heterology is preferable as regards assay specificity[3)] and the use of enzyme-labeled steroid prepared from a hapten having a bridge shorter than that used for antibody production is advantageous for obtaining increased sensitivity.[4)] The latter proposal is based on the results obtained with various enzyme immunoassay systems for cortisol. However, before it can be concluded that a set of bridges of varying length presents generally a higher success rate for the establishment of enzyme immunoassays, it is necessary to obtain additional evidence. The present paper deals with the effect of bridge heterologous combination on sensitivity in enzyme immunoassay for 11-deoxycortisol.

Materials and Methods

Materials——All organic solvents and reagents were of analytical-reagent grade. β-Galactosidase (EC 3.2.1.23) from *E. coli* (grade VI, 360 units per mg protein) and *o*-nitrophenyl β-D-galactopyranoside were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Nakarai Chemical, Ltd. (Kyoto), respectively. Goat anti-rabbit IgG antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs., Ltd. (Tokyo). 4-(Carboxymethylthio)-11-deoxycortisol (CMT), 4-(2-carboxyethylthio)-11-deoxycortisol (CET), 4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol (HST), and 4-hemisuccinoyloxy-11-deoxycortisol (HS) were prepared by the methods previously established in these laboratories.[5] The abbreviations are also used for antisera and enzyme-labeled antigens, which were prepared by using the corresponding carboxylated derivatives. Anti-11-deoxycortisol antisera used were those reported in the previous paper.[5b] The antisera were diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (buffer A).

Preparation of β-Galactosidase-labeled 11-Deoxycortisol——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (3 mg) and *N*-hydroxysuccinimide (2 mg) were added to a solution of the carboxylated 11-deoxycortisol (CMT, CET, HST and HS) (2 mg) in 95% dioxane (50 μl), and the resulting solution was allowed to stand at room temperature for 2 h. The reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with water and dried over anhydrous Na_2SO_4. The solution was passed quickly through Al_2O_3 (100 mg) packed in a capillary pipet, and the filtrate was evaporated down with the aid of a nitrogen gas stream to give the *N*-succinimidyl ester of 11-deoxycortisol. The residue was dissolved in methylene chloride (1 ml), and the concentration of the activated ester was determined by measuring the ultraviolet absorption at 246 nm in ethanol; ε values for CMT, CET and HST were 11000, and for HS, 13000. Calculated volumes corresponding to steroid: enzyme molar ratios of 4, 6 and 8 (molecular weight of β-galactosidase, 540000)[6] were transferred to a test tube with a micro syringe, and the methylene chloride was evaporated off. A solution of β-galactosidase (1 mg) in PB (0.2 ml) was added to the residue at 0°C, and the reaction mixture was immediately vortex-mixed, then allowed to stand overnight at 4°C with occasional shaking. After dialysis against cold PB (1 l) for 2 d, the resulting solutions were stored at 4°C at a concentration of 500 μg per ml, adjusted with PB containing 0.1% gelatin and 0.1% NaN_3 (buffer B). For the immunoassay procedure, this was diluted with the buffer solution containing 0.5% normal rabbit serum.

Immunoreactivity and Antibody Dilution Curve——The enzyme-labeled 11-deoxycortisol (0.1 μg, 0.1 ml) in the buffer and buffer B (0.1 ml) were added to antiserum (0.1 ml) diluted 1: 400 or more, and the mixture was incubated at 4°C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1: 10 with buffer A containing 0.3% EDTA was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4°C for 16 h. After addition of buffer B (1.5 ml) the resulting solution was centrifuged at 3000 rpm for 15 min, and the supernatant was removed. The immune precipitate was washed once with buffer B and used for measurement of the enzymic activity. At the same time, the procedure without addition of the first antibody was carried out to provide a blank value. An experiment using only the enzyme-labeled steroid was also carried out to obtain 100% enzymic activity.

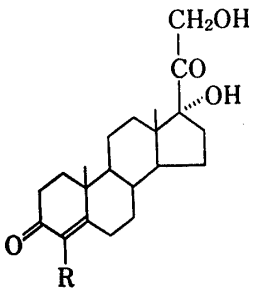
Inhibition of Binding by Addition of 11-Deoxycortisol——A solution of 11-deoxycortisol (0.2 or 1 ng, 0.1 ml) in buffer B and diluted antiserum (0.1 ml) were added to enzyme-labeled 11-deoxycortisol (0.1 μg, 0.1 ml) in the buffer, and the mixture was incubated at 4°C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1: 20 with buffer A containing 0.3% EDTA was added to the incubation mixture. Incubation and separation of free and bound enzyme-labeled antigens were carried out just as described above. Simultaneously, the procedure without addition of 11-deoxycortisol or the first antibody was carried out to provide B_0 and blank values, respectively.

Measurement of β-Galactosidase Activity——The immune precipitate was diluted with buffer A (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and preincubated at 37°C for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in PB was added to the resulting solution, and the mixture was incubated for 60—90 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml). The absorbance was then measured at 420 nm with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. In the case of the use of only enzyme-labeled steroid as described above, this procedure was applied to the enzyme solution, and the reaction was terminated after a 20-min incubation. The absorbance obtained was corrected for the incubation time, and this was defined as 100% enzymic activity.

Results and Discussion

The purpose of this work was to clarify the effect of the bridge heterologous combination between antiserum and enzyme-labeled antigen on sensitivity in heterogeneous enzyme immunoassay for 11-deoxycortisol. Four 11-deoxycortisol derivatives possessing different bridges at the same C-4 position were employed for the preparation of both antisera and enzyme-

labeled antigens. These were the thioether derivatives, 4-(carboxymethylthio)-11-deoxycortisol (CMT), 4-(2-carboxyethylthio)-11-deoxycortisol (CET),4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol (HST), and another type of hapten, 4-hemisuccinoyloxy-11-deoxycortisol (HS).[5]



CMT: R=SCH_2COOH
CET: R=$S(CH_2)_2COOH$
HST: R=$S(CH_2)_2OCO(CH_2)_2COOH$
HS: R=$OCO(CH_2)_2COOH$

Chart 1

In order to control the molar ratio of steroid to enzyme in a labeled antigen, since this influences assay sensitivity, enzyme labeling was carried out by the *N*-succinimidyl ester method.[3,7] The *N*-succinimidyl esters prepared from the carboxylated derivatives by condensation with *N*-hydroxysuccinimide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were covalently linked to β-galactosidase. With the aim of obtaining an adequate 11-deoxycortisol-enzyme conjugate, molar ratios of 4, 6 and 8 were used. The enzyme labeling was accomplished by mixing the activated esters with β-galactosidase in phosphate buffer (pH 7.3), where no significant loss of enzymic activity occurred. The reaction mixtures were dialyzed against the buffer to remove the unreacted steroids and *N*-hydroxysuccinimide. Determination of the number of steroid molecules incorporated per enzyme molecule was not essential for the present purpose, and hence, was not carried out.

Immunoassay was done with four homologous and twelve heterologous combinations, in which two preparations of antisera (1 and 2) derived from the same hapten-bovine serum albumin conjugate and three 11-deoxycortisol-enzyme conjugates obtained at different molar ratios were used for each combination. The bound and free enzyme-labeled antigens were separated by a double antibody method, and the enzymic activity of the immune precipitate was determined colorimetrically with *o*-nitrophenyl β-D-galactopyranoside as a substrate.

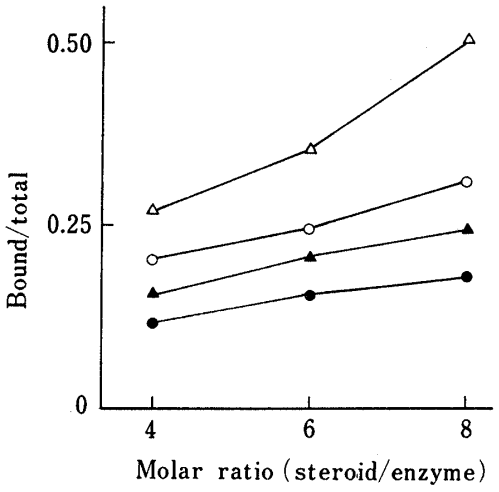


Fig. 1. Binding Abilities of 11-Deoxycortisol-β-galactosidase Conjugates to the Antiserum CET-1

●, CET; ▲, HS; ○, CMT; △, HST.

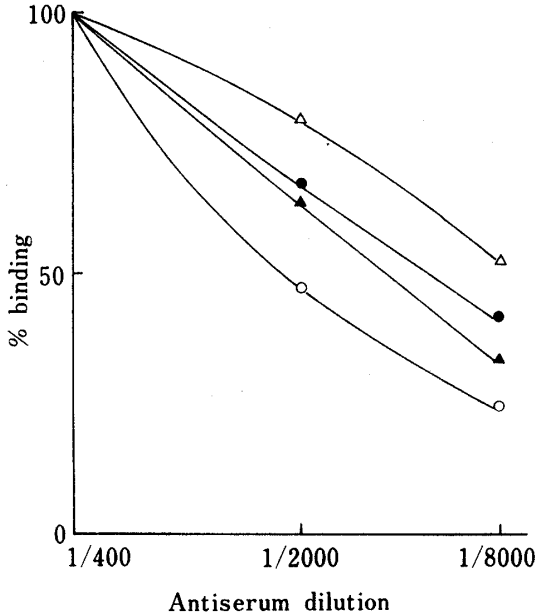


Fig. 2. Antibody Dilution Curves obtained with the Antiserum CET-1 by using the Enzyme-labeled CMT (○), HS (▲), CET (●), and HST (△) prepared at a Molar Ratio of 6

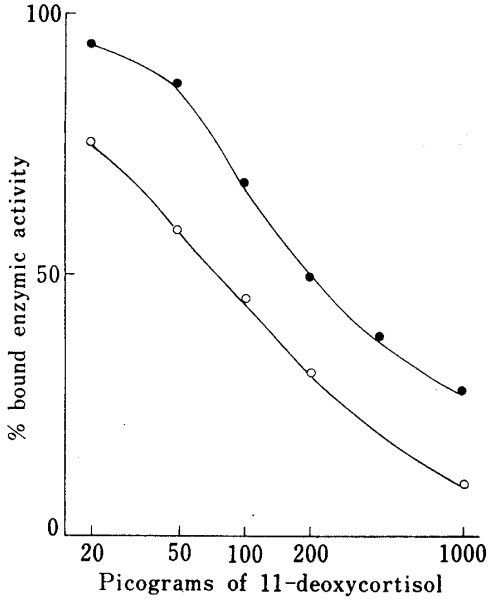


Fig. 3. Dose–Response Curves for 11-Deoxycortisol Enzyme Immunoassay using the Antiserum CET-2 in Combination with Enzyme-labeled CMT (○) and CET (●)

The binding abilities of a definite amount (0.1 μg) of the steroid-enzyme conjugates were investigated at 1:400 dilution of anti-11-deoxycortisol antisera, and the results obtained with the antiserum CET-1 are shown in Fig. 1. The immunoreactivity increased with increasing molar ratio, and similar results were obtained in the cases of other antisera. Although higher immunoreactivity is desirable, a steroid-enzyme conjugate showing reactivity on a plateau is often unsuitable for use, because of loss of sensitivity.[3,7] Thus, the enzyme-labeled steroids obtained at molar ratios of 4 and 6 were used for the purpose of comparing the sensitivities obtainable with all the combination systems.

In order to determine an appropriate dilution of antiserum for use in enzyme immunoassay, antibody dilution curves were then constructed with each system, and the results obtained with the antiserum CET-1 are shown in Fig. 2. The binding ability was expressed for convenience as a percentage of the ability obtained with 1:400 dilution. The dilution showing 50% binding may be most suitable for obtaining the dose–response curve. In practice, however, various dilutions (20—60% binding) were employed in the comparative study of sensitivity, particularly when an assay was less sensitive.

Table I. Inhibition of Bound Enzymic Activity of Various 11-Deoxycortisol-β-galactosidase Conjugates by 1 ng of 11-Deoxycortisol in the Assay using Antisera CMT-1 and CET-1

11-Deoxycortisol-enzyme conjugate	Molar ratio	Antiserum CMT-1		Antiserum CET-1	
		Dilution	Inhibition (%)	Dilution	Inhibition (%)
CMT	4	1:4000	77[a] (0.22)[b]	1:4000	71 (0.26)
		1:6000	76 (0.16)	1:6000	74 (0.22)
	6	1:4000	74 (0.28)	1:4000	68 (0.34)
		1:6000	73 (0.19)	1:6000	72 (0.27)
CET	4	1:2500	77 (0.13)	1:4000	42 (0.13)
		1:5000	82 (0.09)	1:6000	43 (0.12)
	6	1:5000	75 (0.18)	1:4000	40 (0.16)
HST	4	1:4500	83 (0.19)	1:10000	55 (0.22)
	6	1:4500	85 (0.22)	1:15000	50 (0.19)
HS	4	1:2500	96 (0.19)	1:3500	93 (0.17)
	6	1:2500	95 (0.29)	1:5000	92 (0.18)

a) Coefficients of variation ($n=3$) were less than 5%.
b) Figures in parentheses indicate the absorbance of B_0 obtained by enzymic reaction for 60 min.

Typical dose–response curves obtained with the antiserum CET-2 by using the enzyme-labeled CMT and CET are shown in Fig. 3. It can be seen that the heterologous assay is more sensitive than homologous assay. Sensitivity obtainable with all the assay systems was tested by examining the extent of inhibition at the points of 0.2 and 1 ng of 11-deoxycortisol. The assays were assessed in terms of the absorbance for B_0 during a limited period of enzymic

reaction, since higher dilution of antiserum tended to cause an increase in the sensitivity. The criterion that the absorbance obtained upon 1 h incubation should be at least 0.1 was employed in this work. With all the systems, good parallelism of inhibition was obtained between the experiments using 0.2 and 1 ng of 11-deoxycortisol. The results obtained by the addition of 1 ng of 11-deoxycortisol with the antisera CMT-1 and CET-1 are listed in Table I. Comparison of the cases of homology and heterology showed that sensitivity was increased in some heterologous systems, but not in others. It should be noted that the 11-deoxycortisol-enzyme conjugates prepared at molar ratios of 4 and 6 gave nearly equal inhibition values, which did not significantly vary with the dilutions of antiserum, showing that the data were suitable for the present purpose.

Table II. Effect of Combination between Antiserum and β-Galactosidase-labeled 11-Deoxycortisol on Sensitivity in Enzyme Immunoassay

Antiserum		Inhibition (%)[a] β-Galactosidase-labeled 11-deoxycortisol			
		CMT	CET	HST	HS
CMT	1	77	75	85	95
	2	79	78	90	96
CET	1	74	43	55	93
	2	90	73	82	93
HST	1	94	82	53	95
	2	99	83	74	97
HS	1	99	95	96	94
	2	99	96	98	97

a) Inhibition of bound enzymic activity of various conjugates by 1 ng of 11-deoxycortisol. Coefficients of variation ($n=3$) were less than 5%.

For inspection of the effect of bridge heterology on the sensitivity, the inhibition values which were selected by assessing the absorbance for B_0 are listed in Table II. The antisera HS showed inhibition values of over 90% even in the homologous system; the sensitivities were comparable to that in the radioimmunoassay using tritium-labeled 11-deoxycortisol.[5b] This indicates that the antisera HS have weak, if any, binding affinity for the bridge between the enzyme and steroid in the labeled antigen. With the antisera CMT, CET and HST, the effectiveness of heterology on assay sensitivity was found to be dependent upon the length and/or nature of the bridge. A comparison of four combinations between CMT and CET gave interesting results. In the heterologous system using the antisera CET and enzyme-labeled CMT as compared with the homologous CET, a significant increase in sensitivity was noted. In contrast, the combination with the antisera CMT and enzyme-labeled CET resulted in a sensitivity equal to that of the homologous CMT. The findings indicate that when a bridge shorter than that used for the antiserum production is employed for enzyme labeling, the assay sensitivity can be improved, whereas the use of a longer analogous bridge is ineffective. The fact that the use of CMT rather than CET as a label in combination with the antisera HST gives a significant increase can also be explained as the effect of bridge length. The effectiveness of alteration in bond nature was also apparent. The use of enzyme-labeled HS gave significant increases in all instances. On the other hand, the use of HST as a label in combination with the antisera CMT and CET was not very effective. This seems to be ascribable to a close analogy between their bridges and to the fact that the bridge used for enzyme labeling is longer than that used for antibody production.

The findings obtained in the present work are in good agreement with those previously reported for cortisol enzyme immunoassay.[4] We have explained the effect of bridge length on the sensitivity as being the result of the steric interaction between antibody and labeled enzyme.

If this is so, a similar phenomenon may be observed in various immunoassay systems using haptenic antigens covalently linked to a macromolecule, such as protein, antibody or immunobead. Further studies on the bridging phenomena are being conducted in these laboratories. The specificity obtained with the present assay systems will be reported elsewhere in the future.

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