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Effect of Bridge Heterologous Combination on Sensitivity in Enzyme Immunoassay for Cortisol[1)]

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The effect of the bridge heterologous combination of antiserum and enzyme-labeled steroid on sensitivity in heterogeneous enzyme immunoassay of cortisol has been investigated. The enzyme labeling of cortisol was carried out by the N-succinimidyl ester method. Four cortisol derivatives possessing different bridges at C-4 were covalently linked to β-galactosidase at various molar ratios of steroid to enzyme. The anti-cortisol antisera 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 specificity was dependent upon the length of the bridge. It was found that the heterologous system using the steroid–enzyme conjugate obtained from a hapten having a shorter bridge than that used for antibody production resulted in an increase in sensitivity of the assay, whereas the use of a longer bridge was ineffective. This phenomenon can be explained in terms of the steric interaction between antibody and labeled enzyme.

Keywords——enzyme immunoassay; cortisol; N-succinimidyl ester method; cortisol-4-β-galactosidase conjugate; bridge heterology; bridge length; sensitivity

In recent years a number of papers on heterogeneous enzyme immunoassay of steroid hormones have been reported. In general, however, development of a practical enzyme immunoassay is not always easy owing to various factors affecting the sensitivity and specificity. It has been shown that the sensitivity is markedly influenced by the number of steroid molecules incorporated into the enzyme-labeled antigen[2)] and by the combination of antibody and enzyme-labeled antigen.[3)] The latter problem arises from the method of preparing antiserum and enzyme-labeled antigen. Anti-steroid antibody elicited with an immunogen whose hapten is linked to a carrier protein *via* a "chemical bridge," recognizes the structure of the bridge between the enzyme and steroid in the labeled antigen. The use of the same hapten for the preparation of enzyme-labeled antigen as well as immunogen does not always 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 enzyme-labeled antigen whose hapten differs slightly from that of the immunogen used for the preparation of antiserum is employed. This is termed heterologous enzyme immunoassay.[3)] Although the effectiveness of heterology on the sensitivity has been discussed,[3,4)] no report has yet dealt with the basis for selecting the heterology. We described previously that when a heterologous combination is employed, "bridge" heterology rather than "site" heterology is preferred, since the latter is unfavorable with regard to specificity.[5)] This paper deals with the effect of bridge heterologous combination on the sensitivity in enzyme immunoassay for cortisol.

Materials and Methods

Materials——β-Galactosidase (EC 3.2.1.23) from *E. coli* (grade VI, 360 units per mg protein) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and *o*-nitrophenyl β-D-galactopyranoside from Nakarai Chemical, Ltd. (Kyoto). 4-(Carboxymethylthio)cortisol (CMT), 4-(2-carboxyethylthio)cortisol (CET), 4-(2-hemisuccinoyloxyethylthio)cortisol (HST), and 4-hemisuccinoyloxycortisol (HS) were prepared by the methods

previously established in these laboratories.[6] The abbreviations are also used for antisera and enzyme-labeled antigens, which were prepared by using the corresponding carboxylated derivatives. Anti-cortisol antisera used were those reported in the previous paper.[7] 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). Goat anti-rabbit IgG antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs, Ltd. (Tokyo).

Preparation of Cortisol-β-Galactosidase Conjugate——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (3 mg) and N-hydroxysuccinimide (2 mg) were added to a solution of the carboxylated cortisol (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) in a capillary pipet, and the filtrate was evaporated down under an N_2 gas stream to give the N-succinimidyl ester of cortisol. 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 for CMT, CET, HST 11000 and for HS 13000. Calculated volumes corresponding to steroid: enzyme molar ratios of 2, 4, 6 and 8 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 days, 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 cortisol–enzyme conjugate (0.1 μg, 0.1 ml) in the buffer and buffer B (0.1 ml) were added to antiserum (0.1 ml) diluted 1: 100 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. The supernatant was removed, and the immune precipitate was used for measurement of the enzymic activity. At the same time, the procedure without addition of first antibody was carried out to provide a blank value. An experiment using only the enzyme conjugate was also carried out to obtain 100% enzymic activity.

Inhibition of Binding by Addition of Cortisol——A solution of cortisol (0.2 and 1 ng, 0.1 ml) in buffer B and diluted antiserum (0.1 ml) were added to cortisol–enzyme conjugate (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 cortisol–enzyme conjugates were carried out in the manner described above. Simultaneously, the procedure without addition of cortisol or first antibody was carried out to provide B_0 and blank values, respectively.

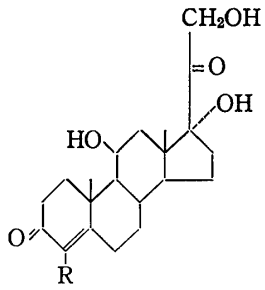
Measurement of β-Galactosidase Activity——The immune precipitate was diluted with buffer A (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and preincubated at 37°C for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in PB was added to the resulting solution, and the mixture was incubated for 40—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 conjugate described above, this procedure was applied to the enzyme solution, and the reaction was terminated after a 20 min incubation. The optical density 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 combination of antiserum and enzyme-labeled steroid on sensitivity in enzyme immunoassay by using cortisol as a model substance. Such a study requires control of the molar ratio of steroid to enzyme in a labeled antigen, since this influences assay sensitivity. The preparation of enzyme-labeled steroids has usually been carried out by coupling the carboxyl group of a steroid derivative with the amino groups of an enzyme by the use of mixed anhydride or carbodiimide reaction. However, these two techniques are not always satisfactory with respect to reproducibility. From this point of view, we previously developed the enzyme labeling of steroids by the N-succinimidyl ester method.[2a] This method was found to be excellent for controlling the molar ratio of steroid to enzyme.

In order to investigate the bridge heterology, that is the effect of length and nature of a bridge, carboxylated cortisol 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)cortisol (CMT), 4-(2-carboxyethylthio)cortisol (CET), 4-(2-hemisuccinoyloxyethylthio)cortisol (HST), and another type of hapten, 4-hemisuccinoyloxycortisol (HS).[6] The N-succinimidyl esters prepared from the carboxylated derivatives by condensation with N-hydroxysuccinimide in the presence of a water-soluble carbodiimide were covalently linked to β-galactosidase. With the aim of obtaining an adequate cortisol-enzyme conjugate,[2a,5] various molar ratios (2, 4, 6 and 8) of steroid to enzyme were used. The enzyme labeling was accomplished by mixing the activated esters with the enzyme 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.



CMT: R=SCH_2COOH
CET: R=SCH_2CH_2COOH
HST: R=$SCH_2CH_2OCOCH_2CH_2COOH$
HS : R=$OCOCH_2CH_2COOH$

Chart 1

Immunoassay was carried out with four homologous and twelve heterologous combinations, in which two preparations of antisera (1 and 2) derived from the same hapten–BSA conjugate and four cortisol-enzyme conjugates obtained at different molar ratios were used for each combination. The bound and free cortisol–enzyme conjugates 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. The binding abilities of a definite amount (0.1 μg) of the steroid–enzyme conjugates were investigated at 1: 100 dilution of anti-cortisol antisera, and some of the results obtained with molar ratios of 4, 6 and 8 are shown in Fig. 1. The immunoreactivity increased with increasing molar ratio. The results obtained with the antiserum CET-2 (Fig. 1A) showed that the immunoreactivity of the homologous label CET was not always higher than those of the heterologous systems HS, HST and CMT. Although higher immunoreactivity is desirable, a steroid–enzyme conjugate showing reactivity on a plateau is often inadequate for use, because of loss of sensitivity.[2a,5] For the purpose of comparing the sensitivities obtainable with all the combination systems, the enzyme-labeled steroids obtained at molar ratios of 4 and 6 were expected to be suitable. The use of conjugate prepared at a molar ratio of 2 is also valuable, when a sufficient optical density of B_0 can be obtained.

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 HST-2 by using the enzyme-labeled HST and CET are shown in Fig. 2. The binding ability was expressed for convenience as a percentage of the ability obtained with 1: 100 dilution. The dilution showing 50% binding can be defined as a titer. Therefore, the use of the antiserum diluted approximately 1: 1500 for the labeled CET and 1: 2500 for the labeled HST may be suitable for obtaining dose–response curves. In practice, however, various dilutions (20—60% binding) were employed in the comparative study of sensitivity, particularly when an assay was less sensitive.

Sensitivity obtainable with these assay systems was then tested by examining the inhibition of enzymic activity caused by the addition of 0.2 and 1 ng of cortisol. The assays were assessed in terms of the optical density 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 optical density obtained upon 1 h incubation should be at least 0.2 was employed

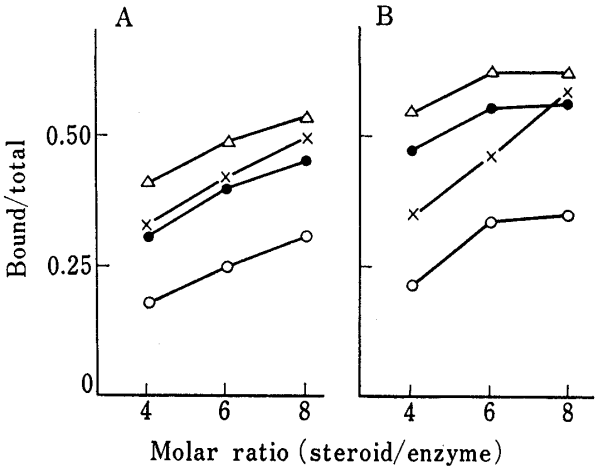


Fig. 1. Binding Abilities of Cortisol-β-galactosidase Conjugates prepared at Various Molar Ratios of the Activated Steroid Ester to Enzyme

A: antiserum CET-2, B: antiserum HST-2, —△—: HS, —×—: HST, —●—: CMT, —○—: CET.

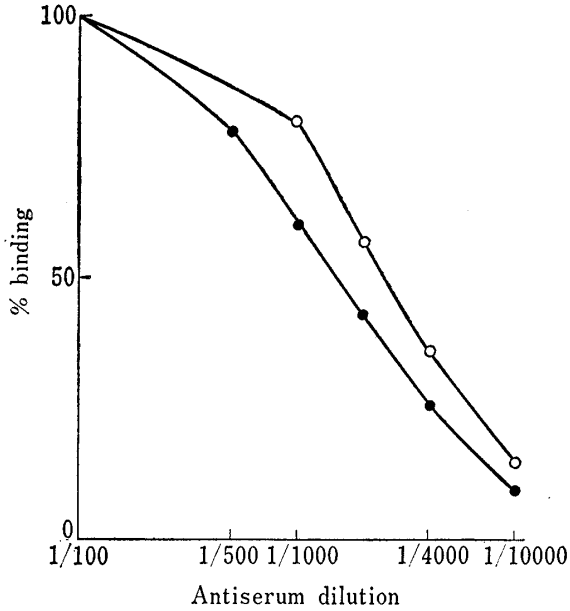


Fig. 2. Antibody Dilution Curves obtained with Antiserum HST-2 by using β-Galactosidase-labeled HST (—○—) and CET (—●—) prepared at a Molar Ratio of 6

in this work. With all the systems, good parallelism of inhibition was obtained between the experiments using 0.2 and 1 ng of cortisol. The fact that the inhibition by 0.2 ng of cortisol was over 50% of that obtained with 1 ng revealed that there was a reasonable dose–response relationship. The results obtained by the addition of 1 ng of cortisol with the antisera CET-2 and HST-2 are listed in Table I. The data appeared to be suitable for the present purpose, because the cortisol-enzyme conjugates prepared at various molar ratios gave nearly equal values besides the desired optical density at a given dilution of antiserum. Two antisera prepared with the same hapten–BSA conjugate gave different sensitivities in the case of homology: for example, inhibition values of 60—70% and 38—52%, respectively, for CET-1 and CET-2

Table I. Inhibition of Bound Enzymic Activity of Various Cortisol-β-galactosidase Conjugates by 1 ng of Cortisol in the Assay using Antisera CET-2 and HST-2

Cortisol–enzyme conjugate	Molar ratio	Antiserum CET-2		Antiserum HST-2	
		Dilution	Inhibition (%)	Dilution	Inhibition (%)
CMT	2	1 : 1000	75(0.26)[a]	1 : 2000	79(0.23)
	4	1 : 2000	74(0.27)	1 : 3000	72(0.27)
	6	1 : 2000	69(0.28)	1 : 3000	71(0.27)
CET	4	1 : 500	38(0.46)	1 : 2000	61(0.40)
		1 : 1000	40(0.34)	1 : 4000	64(0.24)
	6	1 : 2000	47(0.40)	1 : 2000	63(0.57)
		1 : 4000	46(0.20)	1 : 4000	68(0.34)
	8	1 : 4000	52(0.20)	1 : 4000	64(0.35)
HST	4	1 : 2000	53(0.26)	1 : 4000	47(0.31)
				1 : 6000	46(0.19)
	6	1 : 2000	52(0.33)	1 : 4000	42(0.30)
		1 : 3000	50(0.24)	1 : 6000	40(0.18)
HS	4	1 : 3000	71(0.22)	1 : 4000	74(0.31)
	6	1 : 3000	69(0.28)	1 : 4000	72(0.34)

a) Figures in parentheses indicate the optical density of B_0 obtained by enzymic reaction for 60 min.

with an optical density of over 0.2. This shows that the assay sensitivity depends upon the nature of the antibody due to the animal-dependence, namely the difference in the ability of the antibody to recognize a bridge in the enzyme-labeled antigen. Comparison of the cases of homology and heterology showed that sensitivity increased in some heterologous systems, but not in other cases.

For inspection of the effect of bridge heterology on the sensitivity, the inhibition values which were selected by assessing the optical density for B_0 are listed in Table II. When one applies an assay to biological samples, the desirable inhibition is over 60%, which is essential for a sensitivity of 20 pg and a range of measurement of 20—1000 pg. One of two antisera in each homologous system appeared satisfactory except for the antisera HST. Furthermore, the use of a heterologous system provided sufficient sensitivity with most of the antisera. The assay method using a smaller amount of enzyme-labeled steroid and a fluorogenic substrate, 4-methylumbelliferyl β-D-galactopyranoside, may be available as an alternative.

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

Antiserum	Inhibition (%)[a] β-Galactosidase-labeled cortisol			
	CMT	CET	HST	HS
CMT-1	51	43	47	68
CMT-2	64	66	59	71
CET-1	79	69	62	82
CET-2	74	47	53	69
HST-1	28	20	17	43
HST-2	72	68	47	74
HS-1	59	48	36	40
HS-2	81	76	64	69

a) Inhibition of bound enzymic activity of various conjugates by 1 ng of cortisol.

Interesting results were obtained upon comparison of the combinations among CMT, CET, and HST. In the case of heterology as compared with homology, a significant increase was noted with the antisera HST and no increase was observed with the antisera CMT. With the antisera CET, the use of CMT for enzyme labeling resulted in an increase in sensitivity, whereas the sensitivity obtained with HST was almost the same as that in the case of homology. These findings indicate that when a shorter bridge than that used for the antiserum production is employed for enzyme labeling, the assay sensitivity can be improved, and that the use of a longer bridge is ineffective. The effectiveness of alternation in bond nature between the thioether (CMT, CET, and HST) and the ester derivative (HS) is also apparent. The use of HS for enzyme labeling gave sufficient increases in all instances. On the other hand, the effectiveness of a shorter bridge was again observed in heterologous series (CMT and CET), and the use of the longer bridge HST did not result in an increase in the sensitivity. It should be noted that these results were observed with two antisera prepared by use of the same hapten–BSA conjugate without exception.

The effect of bridge length on the sensitivity can be explained in terms of steric interaction. This mechanism is related to the spacer in affinity chromatography, where the chemical groups of the affinant must be sufficiently remote from the surface of the solid matrix to avoid steric hindrance.[8] In the case of enzyme immunoassay, the steric accessibility of enzyme-labeled antigen under the competition of antigen to be measured must influence the sensitivity. Therefore, heterology employing a shorter bridge in enzyme labeling than that in homology can increase the sensitivity, because the steric hindrance between antibody and labeled enzyme

lowers the affinity of antibody for the antigenic determinant in the labeled antigen. On the other hand, the use of a longer bridge is not effective. This phenomenon should not be dependent upon the enzyme used, since the size of the antibody binding site is much smaller than that of enzymes.[9] The ability of antibody to recognize the bridge component may also be important. This may be the case in the heterologous systems using HS as an enzyme label. However, in the case of the thioether derivatives, this factor seems not to be very significant. This argument is based upon the ineffectiveness in the combination of antisera HS and enzyme-labeled HST as compared with other labels, CMT and CET. In addition, it should be noted that the heterologous systems using CMT as a label exerted an effect comparable to that obtained with HS. In separate experiments using 4-(1-carboxyethylthio)cortisol as an enzyme label and the antisera HST-1 and HS-1, the finding that only similar effectiveness to that with CMT was obtained also lent support to this explanation.

The effect observed with different lengths of bridge may be seen between hemisuccinate and hemiglutarate, and between carboxymethyl ether and carboxyethyl ether and so on; these are derivatives widely used for immunoassay of alcoholic and phenolic compounds. Recently, the use of hemimaleate[10] and *m*-maleimidobenzoyl derivatives[11] for enzyme labeling in combination with hemisuccinate has been reported. Considering the present results, it can be concluded that the use of steroid–enzyme conjugate prepared from a hapten having a shorter bridge than that used for antibody production is advantageous for obtaining increased sensitivity in enzyme immunoassay. These results on the effect of the combination of antiserum and enzyme-labeled antigen may be helpful in the development of more sensitive enzyme immunoassays for steroid hormones and drugs. The specificity obtained with the present assay systems will be reported elsewhere in the future.

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