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Bridging Phenomena in Steroid Immunoassays. The Effect of Bridge Length on Sensitivity in Enzyme Immunoassay[1)]

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The effect of the bridge heterologous combination between antiserum and enzyme-labeled antigen on sensitivity in enzyme immunoassay for 11-deoxycortisol has been investigated. In order to obtain evidence for the bridge length effect, the sensitivity of an assay system using a β-galactosidase-labeled antigen prepared from an 11-deoxycortisol derivative having a carboxymethylthio moiety as a bridge at C-4 was compared with that in the case of a derivative with an *N*-(carboxymethyl)carbamoylmethylthio moiety at C-4. The enzyme labeling of 11-deoxycortisol was carried out by the *N*-succinimidyl ester method. Three anti-11-deoxycortisol antisera elicited in rabbits by immunization with different immunogens were used. It was found that the use of the shorter bridge for enzyme labeling resulted in an increase in sensitivity. This indicates that the bridge length is an important factor influencing the sensitivity of enzyme immunoassay.

Keywords——enzyme immunoassay; 11-deoxycortisol; anti-11-deoxycortisol antiserum; β-galactosidase; β-galactosidase-labeled 11-deoxycortisol; immunoassay sensitivity; bridge heterology; bridge length

Development of a steroid enzyme immunoassay system having high sensitivity and specificity comparable to those of radioimmunoassay using a tritiated steroid as a labeled antigen is not always easy. Antibodies to a steroid, a small nonimmunogenic molecule, are elicited in animals by immunization with the steroid molecule linked *via* a "chemical bridge" to a carrier protein.[2)] It is generally accepted that the hapten portion of the immunogen binds to specific surface receptors of B lymphocytes, triggering proliferation and differentiation of the cells into plasma cells, which secrete humoral antibodies having the same antigen-recognition specificity as the receptor. The binding must be influenced by a steric interaction between the receptor and the carrier portion of the immunogen; a clone ideally specific for a steroid, if present, is of no use when the hapten does not fit the receptor owing to the steric hindrance. This is consistent with the fact that the specificity of the antiserum thus obtained is dependent upon the position on the steroid molecule used for conjugation to the carrier and the stereochemistry of the steroid derivative in the immunogen, including the structural features and length of the bridge.[3)] The anti-steroid antiserum contains various antibodies showing different specificities and at least a portion of the antibody population recognizes not only the steroid molecule but also the bridge structure. It is often found that the use of the same hapten for both preparation of immunogen followed by immunization (antibody production) and enzyme labeling, *i.e.* a homologous combination,[4)] does not provide satisfactory assay sensitivity; similar phenomena are observed in other immunoassay systems. This is ascribable to the fact that the binding affinity of the labeled antigen to antiserum is higher than that of the analyte. Hence, the combination between antiserum and labeled antigen is an important factor determining the assay sensitivity.[4,5)] For the purpose of improving the sensitivity, an assay system using different haptenic derivatives has been

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designed; this is termed heterologous enzyme immunoassay. In a previous report,[6] we showed that a "bridge" heterologous system rather than "site" heterology is preferable as regards assay specificity. This paper deals with the bridging phenomenon in hapten enzyme immunoassay by using 11-deoxycortisol as a model analyte.

Materials and Methods

Materials——β-Galactosidase (EC 3.2.1.23) from *E. coli* (grade VI, 360 units per mg protein) was purchased from Sigma Chemical Co. (St. Louis, Mo.); *o*-nitrophenyl β-D-galactopyranoside was from Nakarai Chemicals, Ltd. (Kyoto). Goat anti-rabbit immunoglobulin G (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),[7] 4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol (HST), and their *N*-succinimidyl esters[8] were prepared by the methods previously established in these laboratories. 4-[*N*-(Carboxymethyl)carbamoylmethylthio]-11-deoxycortisol (CMTG) was synthesized in the manner described below. The abbreviations are also used for antisera and enzyme-labeled antigens, which were prepared by using the corresponding carboxylated derivatives. Anti-11-deoxycortisol antisera were those reported in the previous paper[8]: the antisera CMT, CET and HST were elicited in rabbits by immunization with the haptens linked to bovine serum albumin (BSA). These were diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (assay buffer).

Synthesis of CMTG——Glycine 2,2,2-trichloroethyl ester tosylate[9] (1 g) and triethylamine (0.2 ml) were added to a solution of CMT *N*-succinimidyl ester (**1**) (500 mg) in dioxane (7 ml), and the mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with water and extracted with AcOEt. The organic layer was washed successively with 5% HCl, 5% $NaHCO_3$ and water, then dried over anhydrous Na_2SO_4 and evaporated down under reduced pressure. Purification of the crude product by column chromatography on silica gel with hexane–AcOEt (1:3) as an eluent gave the trichloroethyl ester **2** (220 mg) as colorless semi-crystals. ^{1}H-NMR ($CDCl_3$–CD_3OD (10:1)) δ: 0.68 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 3.32 (2H, s, SCH_2CO), 3.65 (1H, m, 6α-H), 4.1—4.9 (6H, NCH_2CO, 21-H and OCH_2CCl_3). Zinc powder (1.6 g) was added in portions to an ice-cooled stirred solution of **2** (200 mg) in 70% AcOH (5 ml), and the whole was stirred at 0 °C for 2 h. After addition of AcOEt (70 ml) followed by removal of the insoluble material by filtration, the filtrate was washed with water, and evaporated down under reduced pressure. The residue was dissolved in MeOH (5 ml), water (20 ml) and 2 N NaOH (to adjust to pH 11) were added, and the mixture was extracted with AcOEt (50 ml). The aqueous layer was acidified to pH 1 with conc. HCl and extracted with AcOEt (50 ml × 2). The organic extracts were combined, dried over anhydrous Na_2SO_4, and evaporated down under reduced pressure. Recrystallization of the product from EtOH–AcOEt–ether (1:1:4) gave CMTG (80 mg) as colorless leaflets. mp 203—208 °C (dec.). $[\alpha]_D^{22}$ +131° (c=0.12, $CHCl_3$). *Anal.* Calcd for $C_{25}H_{35}NO_7S$: C, 60.83; H, 7.15; N, 2.84. Found: C, 60.62; H, 7.29; N, 2.97. Methylation of CMTG with diazomethane gave its methyl ester, which was identical with an authentic sample prepared from **1** by treatment with glycine methyl ester.

Synthesis of CMTG *N*-Succinimidyl Ester (3)——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (28 mg) and *N*-hydroxysuccinimide (17 mg) were added to a solution of CMTG (50 mg) in 95% dioxane (0.5 ml), and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with water and extracted with AcOEt. The organic layer was washed with water and dried over anhydrous Na_2SO_4. The solution was passed quickly through an Al_2O_3 (500 mg) layer on a sintered-glass funnel, and the filtrate was evaporated down under reduced pressure. Recrystallization of the crude product from methylene chloride–ether gave **3** (35 mg) as colorless leaflets. mp 120—124 °C. $[\alpha]_D^{23}$ +62° (c=0.13, $CHCl_3$). *Anal.* Calcd for $C_{29}H_{38}N_2O_9S \cdot 3/2H_2O$: C, 56.38; H, 6.69; N, 4.54. Found: C, 56.45; H, 6.51; N, 4.68.

Preparation of β-Galactosidase-Labeled Antigen——The *N*-succinimidyl ester of CMT, CMTG, CET or HST (*ca.* 2 mg) was dissolved in methylene chloride (2 ml). Calculated volumes corresponding to steroid/enzyme molar ratios of 4, 6 and 8 (M_r of β-galactosidase, 540000)[10] were transferred to a test tube with a micro syringe and the solvent was removed with the aid of a nitrogen gas stream. 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 48 h, the resulting solutions were stored at 4 °C at a concentration of 500 μg per ml, adjusted with assay buffer. The labeled antigens were stable for several months as regards enzymic activity and immunoreactivity under these storage conditions. For the immunoassay procedure, this was diluted with assay buffer containing 0.5% normal rabbit serum.

Immunoreactivity and Antibody Dilution Curve——The enzyme immunoassay procedures were carried out in duplicate in a glass test tube (10 ml) as follows: the β-galactosidase-labeled antigen (100 ng, 0.1 ml) in the buffer and assay buffer (0.1 ml) were added to anti-11-deoxycortisol 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 assay buffer containing 0.3% ethylenediaminetetraacetic acid (EDTA) was added to the incubation mixture, and the solution was vortex-mixed,

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then allowed to stand at 4 °C for 16 h. After addition of assay buffer (1.5 ml), the resulting solution was centrifuged at 3000 rev./min for 15 min, and the supernatant was discarded by aspiration. The immune precipitate was washed once with assay buffer (1 ml) and used for measurement of the enzymic activity. At the same time, the procedure without addition of the anti-steroid antiserum 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 the Binding of Labeled Antigen to Antibody by Addition of 11-Deoxycortisol——A solution of 11-deoxycortisol (200 pg, 0.1 ml) in assay buffer and diluted anti-11-deoxycortisol antiserum (0.1 ml) were added to enzyme-labeled antigen (100 ng, 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 assay buffer 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 anti-steroid antiserum was carried out to provide B_0 and blank values, respectively.

Measurement of β-Galactosidase Activity——The immune precipitate was diluted with assay buffer (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 assay buffer was added to the resulting solution and the mixture was incubated for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the absorbance of *o*-nitrophenol released was measured at 420 nm. In the case of only the enzyme-labeled steroid, as described above, the 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. In previous papers,[5,11] we reported that the use of enzyme-labeled antigen prepared from a hapten having a bridge shorter than that used for the antibody production increases sensitivity. This proposal is based on the results obtained with various enzyme immunoassay systems employing the antisera and β-galactosidase-labeled antigens prepared by the use of the carboxylated derivatives CMT, CET, and HST or corresponding cortisol derivatives; one example is the fact that the assay using CMT as a label

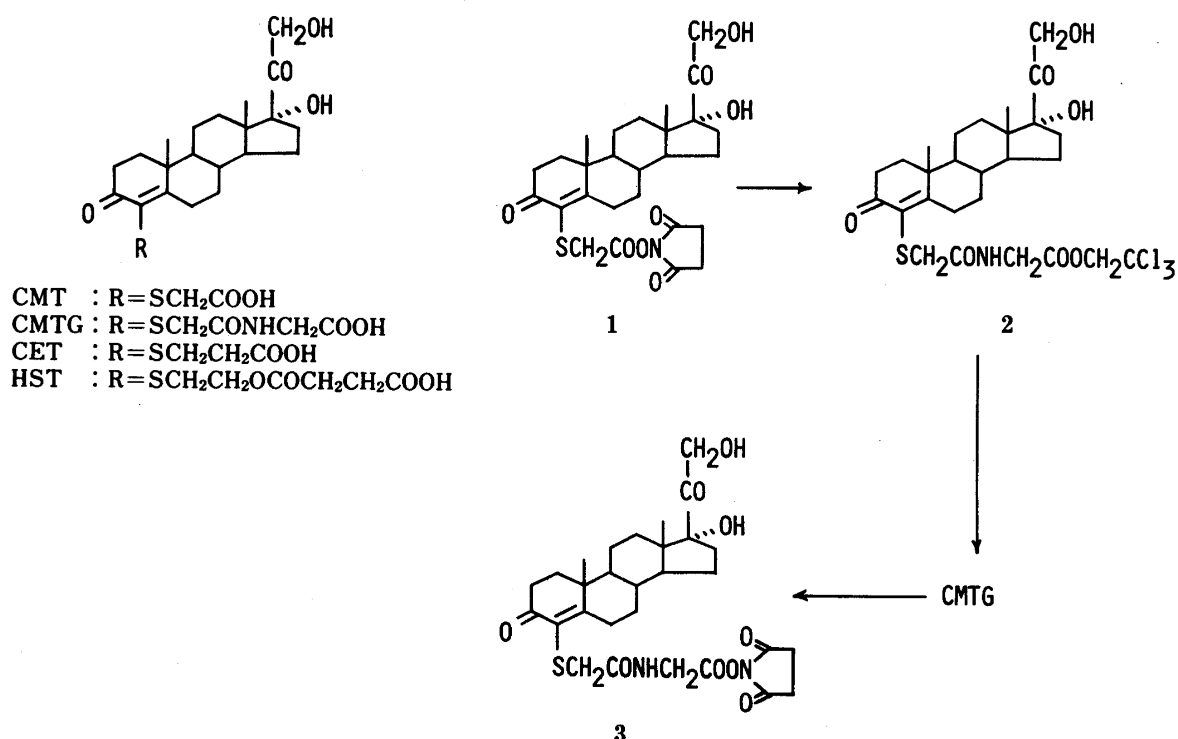

Chart 1

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in combination with the antisera HST is more sensitive than that with CET. However, these previous studies are not necessarily enough to conclude that the bridge length is a factor determining the assay sensitivity, since the possibility that the effect is due to an alteration in bond nature, *i.e.* the change of functional groups, for example, between the methylene unit in CET ($-SCH_2\underline{CH_2}COOH$) and the carbonyl unit in CMT ($-SCH_2\underline{CO}OH$), can not be excluded. In the present work, to obtain evidence for the bridge length effect, sensitivities obtainable with assay systems using enzyme-labeled antigens CMT and CMTG in combination with these antisera were compared. The bridge length of CMTG is longer than that of CMT by the glycine unit, and condensation of the carboxyl groups of these haptenic compounds with the amino group of a lysine residue in β-galactosidase results in a close analogy in their bridge structures, thus yielding a set of bridges of varying length. For comparison, the homologous assay using the labeled antigen CET or HST was also carried out.

The synthetic route to CMTG is outlined in Chart 1. Although a direct method, treatment of **1** with glycine, may be available, we chose the route *via* **2** for convenience of purification of the product. No deoxygenation at C-17 took place under the conditions used in the reductive cleavage of the trichloroethyl ester.

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.[12] The *N*-succinimidyl esters derived from CMT, CMTG, CET and HST were reacted with β-galactosidase to give enzyme-labeled antigens. With the aim of obtaining an appropriate 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. 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 three homologous and five heterologous combinations, in which two preparations of antisera (1 and 2) derived from the same steroid–BSA conjugate and three enzyme-labeled antigens 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. The binding abilities of a fixed amount (100 ng) of the labeled antigens were investigated at 1 : 400 dilution

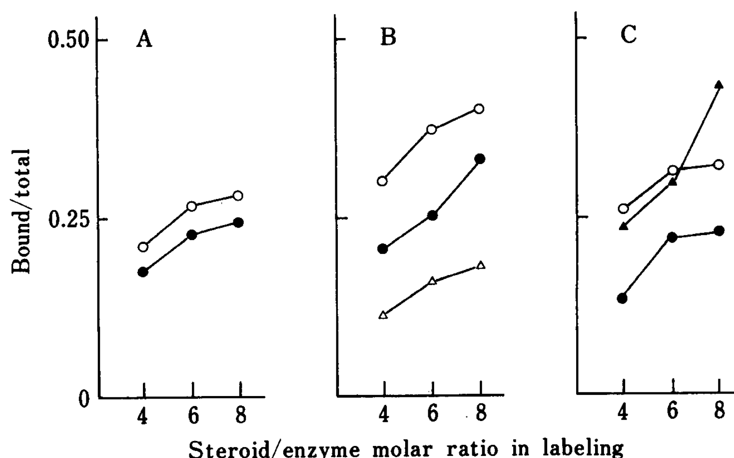

Fig. 1. Binding Abilities of β-Galactosidase-Labeled Antigens Prepared at Various Molar Ratios of the Activated Steroid Ester to Enzyme

A, antiserum CMT-1; B, antiserum CET-2; C, antiserum HST-2; ○, CMTG; ●, CMT; △, CET; ▲, HST.

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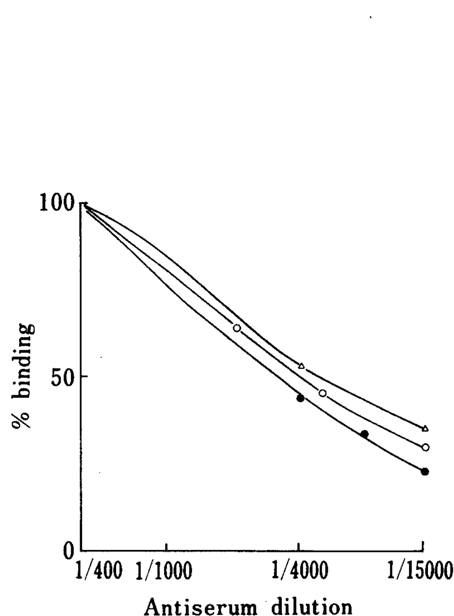

Fig. 2. Antibody Dilution Curves Obtained with the Antiserum CET-2 by Using the Enzyme-Labeled CMTG (○), CMT (●), and CET (△) Prepared at a Molar Ratio of 6

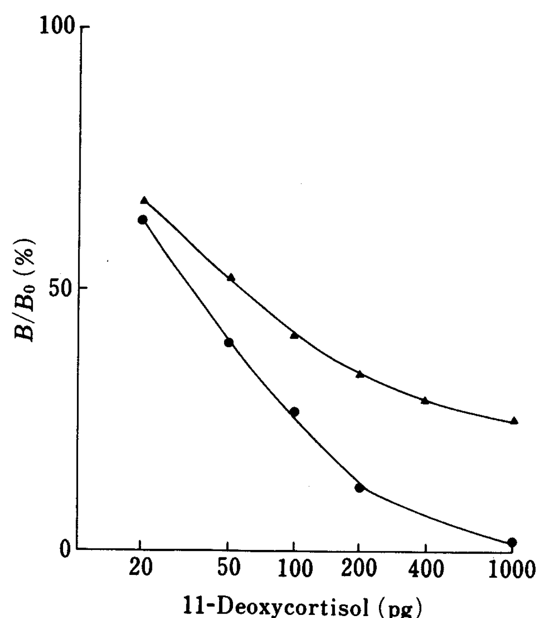

Fig. 3. Dose–Response Curves for 11-Deoxycortisol Enzyme Immunoassay Using the Antiserum HST-2 in Combination with the Enzyme-Labeled HST (▲) and CMT (●) Prepared at a Molar Ratio of 6

of anti-11-deoxycortisol antisera, and the results obtained with the antisera CMT-1, CET-2 and HST-2 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 labeled antigen showing reactivity on a plateau is often unsuitable for use, because of loss of sensitivity.[12] Thus, the labeled antigens 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-2 are shown in Fig. 2. The binding ability was expressed for convenience as a percentage of that with 1:400 dilution. The dilution showing 50% binding may be most suitable for constructing the dose–response curve for 11-deoxycortisol assay. In practice, however, various dilutions were employed in the comparative study of sensitivity, particularly when the assay was less sensitive.

Typical dose–response curves obtained with the antiserum HST-2 in combination with the enzyme-labeled HST and CMT are shown in Fig. 3. These were the "best" standard curves in each assay system. It can be seen that the heterologous assay is more sensitive than the homologous assay. Sensitivity obtainable with all the assay systems was tested by examining the extent of inhibition at the point of 200 pg of 11-deoxycortisol. The assays were assessed in terms of the absorbance of B_0 obtained upon 1 h enzymic reaction; the criterion that the absorbance range should be 0.1—0.4 was employed.

The results obtained with the antisera CET-1 and CET-2 are listed in Table I. Comparison of the cases of homology and heterology showed that sensitivity was increased in the heterologous systems. It is clear that the enzyme-labeled CMT is more effective than the labeled CMTG in increasing the sensitivity. Table II shows the results obtained with the antisera HST-1 and HST-2. The heterologous system using the enzyme-labeled CMTG was more sensitive than the homologous assay, but less sensitive than the heterologous CMT. The

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TABLE I. Inhibition of Bound Enzymic Activity of β-Galactosidase-Labeled Antigens by 200 pg of 11-Deoxycortisol in the Assay Using Antisera CET-1 and CET-2

Labeled antigen (Assay system)	Molar ratio	Antiserum CET-1		Antiserum CET-2	
		Dilution	Inhibition (%)	Dilution	Inhibition (%)
CET (Homologous)	4	1:4000	30	1:6000	47
	4	1:6000	31	1:12000	54
	6	1:4000	30	1:6000	43
	6	1:6000	27	1:12000	55
CMT (Heterologous)	4	1:4000	56	1:8000	63
	4	1:6000	60	1:12000	70
	6	1:4000	56	1:8000	62
	6	1:6000	57	1:12000	69
CMTG (Heterologous)	4	1:8000	35	1:5000	50
	4	1:15000	44	1:7000	49
	6	1:10000	43	1:8000	55
	6	1:20000	38	1:10000	63

TABLE II. Inhibition of Bound Enzymic Activity of β-Galactosidase-Labeled Antigens by 200 pg of 11-Deoxycortisol in the Assay Using Antisera HST-1 and HST-2

Labeled antigen (Assay system)	Molar ratio	Antiserum HST-1		Antiserum HST-2	
		Dilution	Inhibition (%)	Dilution	Inhibition (%)
HST (Homologous)	4	1:8000	35	1:10000	61
	4	1:12000	41	1:15000	67
	6	1:8000	45	1:10000	55
	6	1:12000	40	1:15000	66
CMT (Heterologous)	4	1:2000	74	1:3000	78
	4	1:4000	87	1:6000	87
	6	1:2000	64	1:3000	78
	6	1:4000	82	1:6000	87
CMTG (Heterologous)	4	1:1500	52	1:2500	68
	4	1:2000	57	1:3500	69
	6	1:2000	54	1:4000	72
	6	1:3500	61	1:6000	71

difference in sensitivity between the systems using CMT and CMTG as labels must be due to the alteration in bridge length, but not in bond nature, since the bridge structure of the labeled CMT rather than the labeled CMTG is similar to that of the homologous CET or HST at the site near the steroid molecule. A comparison of two combinations between the antisera CMT and the labeled antigens CMT and CMTG gave results compatible with the above findings (Table III). In the heterologous system using the longer bridge CMTG as compared with the homologous CMT, a rather low sensitivity was noted. This indicates that such a type of heterology is not suitable for the purpose of improving the sensitivity. It should be mentioned that no significant increase in the sensitivity was observed in each assay system even when

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Table III. Inhibition of Bound Enzymic Activity of β-Galactosidase-Labeled Antigens by 200 pg of 11-Deoxycortisol in the Assay Using Antisera CMT-1 and CMT-2

Labeled antigen (Assay system)	Molar ratio	Antiserum CMT-1		Antiserum CMT-2	
		Dilution	Inhibition (%)	Dilution	Inhibition (%)
CMT (Homologous)	4	1:4000	71	1:10000	74
	4	1:6000	70	1:15000	77
	6	1:4000	67	1:10000	71
	6	1:6000	67	1:15000	76
CMTG (Heterologous)	4	1:2000	51	1:5000	59
	4	1:4000	55	1:7000	59
	6	1:3000	55	1:8000	56
	6	1:5000	58	1:10000	58

higher dilutions of the anti-steroid antisera were employed. Thus, it was found, in all cases, that the assay using the labeled CMT was more sensitive than that with the labeled CMTG.

The present results indicate that the bridge length is an important factor influencing the sensitivity of enzyme immunoassay, and the use of a shorter bridge for enzyme labeling results in an increase in sensitivity. We have explained the effect of bridge length on the sensitivity as being the result of the steric interaction between antibody and labeled enzyme;[5] this interaction can be related to that between immunogen and B-cell receptor immunoglobulin in clonal selection, as described above. If this is so, a similar effect can be expected in various hapten immunoassay systems using antigens covalently linked to a macromolecule. Further studies are being conducted to determine whether the bridge length effect depends upon the enzyme used. We are also interested in the bridging phenomena in hapten immunoassays using monoclonal anti-hapten antibodies.

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