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Production and Specificity of a Monoclonal Anti-11-deoxycortisol Antibody[1)]

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The production of a monoclonal antibody to 11-deoxycortisol is described. Spleen cells from BALB/c mice immunized with 4-(2-carboxyethylthio)-11-deoxycortisol linked to bovine serum albumin were fused with P3-NS1/1-Ag4-1 myeloma cells. A hybridoma clone (S.CET.M8.1.1) was established that secreted immunoglobulin G_1 (IgG_1) with a high affinity for 11-deoxycortisol ($K_a = 2 \times 10^{10}\ M^{-1}$). The specificity of the monoclonal antibody (CET-M8) was assessed by cross-reaction studies with closely related steroids and by measuring the amount of 11-deoxycortisol in human plasma specimens by means of radioimmunoassay, in comparison with that of a conventional polyclonal antiserum. The results showed that the monoclonal antibody is reasonably specific and useful for the determination of plasma 11-deoxycortisol levels in the metyrapone test. Factors influencing the specificity of monoclonal antibodies to steroids are discussed.

Keywords——monoclonal antibody production; 11-deoxycortisol; 4-(2-carboxyethylthio)-11-deoxycortisol; monoclonal anti-11-deoxycortisol antibody; radioimmunoassay; antibody specificity; cross-reaction; metyrapone test; clonal selection theory

Immunoassays of steroid hormones are frequently required in clinical chemistry. Recently, the cell hybridization technique developed by Köhler and Milstein[2)] has been shown to be useful for the preparation of monoclonal antibodies to steroids.[3)] In general, monoclonal antibodies offer many advantages, such as reproducibility, high specificity and availability in large quantities, compared to conventional antisera.

Antigenic stimulation of preexisting clones of lymphocytes is known as clonal selection[4)]: in the production of antibodies to a steroid, the steroid portion of a haptenized immunogen binds to specific surface receptors of B lymphocytes, resulting in the proliferation and differentiation of the cells into plasma cells which secrete anti-steroid antibodies having the same antigen-recognition specificity as that of the receptor. Thus, the available specificities of monoclonal anti-steroid antibodies should be influenced by the position on the steroid molecule used for conjugation to a carrier protein and also by the stereochemistry of the steroid hapten. For the preparation of antibodies to Δ^4-3-ketosteroids, measurements of which are most frequently required, the position C-4 in the steroid molecules appears to be an attractive site for attachment of the carrier.[5)] This paper describes the production of a monoclonal anti-11-deoxycortisol antibody by the use of a carboxylated 11-deoxycortisol derivative possessing a bridge at C-4 as a hapten. The specificity of the monoclonal antibody was tested by cross-reaction studies and by measuring the amount of 11-deoxycortisol in plasma specimens by means of radioimmunoassay, in comparison with that of a conventional rabbit antiserum.

Materials and Methods

Materials——[1,2-^{3}H]-11-Deoxycortisol (50 Ci/mmol) was supplied by Amersham (England). The conjugate of 4-(2-carboxyethylthio)-11-deoxycortisol (CET) with bovine serum albumin (BSA) used as immunogen was prepared by the *N*-succinimidyl ester method previously established in these laboratories.[6] The polyclonal antiserum CMT-2 used was that elicited in a rabbit by immunization with the conjugate of 4-carboxymethylthio-11-deoxycortisol (CMT) with BSA.[6] Fetal calf serum and RPMI-1640 medium were purchased from GIBCO (U.S.A.); incomplete Freund's adjuvant and complete Freund's adjuvant were from Difco Laboratories (U.S.A.) and Iatron Laboratories (Tokyo), respectively. Polyethylene glycol (M_r 4000) was obtained from Sigma Chemical Co. (U.S.A.).

Immunization——Five female BALB/c mice at two months of age (Shizuoka Laboratory Animal Center, Hamamatsu) were immunized by intraperitoneal injections of the immunogen CET–BSA conjugate. Each mouse received 50 μg (50 μl) of the conjugate emulsified in complete Freund's adjuvant. Booster immunizations were done twice by injecting the immunogen (50 μg) emulsified with incomplete or complete (alternately) Freund's adjuvant. The mice were bled ten days after immunization and the titers of the sera were monitored by the 11-deoxycortisol radioimmunoassay method described below. Three mice which showed higher titers were used for cell fusion.

Cell Fusion and Hybridoma Selection——Three days before cell fusion, a final injection of the immunogen (50 μg) in saline (50 μl) was given. Spleen cells (1×10^8 cells) were fused with P3-NSl/l-Ag4-1 mouse myeloma cells (2×10^7 cells) by the use of 45% (w/v) polyethylene glycol, and the washed cells were distributed into 24-well tissue culture plates. The hybridomas were selected under 5% CO_2–95% air in RPMI-1640 medium supplemented with 10% fetal calf serum, HAT constituents (0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), 10 mM HEPES buffer (pH 7.3), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.06% kanamycin sulfate. The culture supernatants were tested for the production of 11-deoxycortisol antibody by the radioimmunoassay method.

Cloning of Hybridomas——This was carried out by limiting dilution in the culture medium without HAT. Hybridoma cells (an average of 0.6 cells per well) were seeded onto 96-well tissue culture plates with a feeder layer of thymocytes (6×10^5 cells/well). The cloned hybridoma cells selected were recloned. One hybridoma clone (S.CET.M8.1.1) secreting a high level of anti-11-deoxycortisol antibody was then propagated in tissue culture flasks.

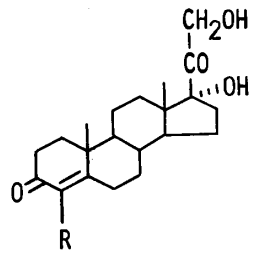
Radioimmunoassay Procedure——All dilutions of the standard, tracer and antibodies were performed in 0.05 M phosphate buffer (pH 7.3) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3. The assay for the determination of 11-deoxycortisol was carried out in duplicate in a glass test tube as follows: [^{3}H]-11-deoxycortisol (*ca.* 18000 dpm, 0.5 ml) and diluted antibody solution (0.1 ml) were added to a series of standard solutions (0, 5, 10, 20, 50, 100, 200, 500 pg of 11-deoxycortisol) or plasma samples in buffer (0.1 ml), and the mixtures were incubated overnight at 4 °C. After addition of dextran (0.06%)–charcoal (1%) (0.5 ml), the suspension was vortex-mixed, allowed to stand at 0 °C for 20 min, and then centrifuged at 4 °C (2000 rev./min for 10 min). The supernatant was transferred by decantation into a vial containing a scintillation cocktail (10 ml), and the radioactivity was measured with a Beckman LS-7000 liquid scintillation spectrometer. Screening of hybridomas for 11-deoxycortisol antibody activity and cross-reaction studies were done without the standard 11-deoxycortisol and with steroids instead of the standard, respectively.

Plasma Sample——Plasma specimens were provided by Drs. Sasaki and Shimizu, Tohoku University School of Medicine. In metyrapone tests,[7] patients received 0.5 g of metyrapone every 4 h for a total of six doses. Blood was withdrawn 24 and 48 h (4 and 8 h also in some cases) after the initial administration, and plasma was immediately obtained. The standard extraction procedure is as follows: H_2O (1 ml) and methylene chloride (2 ml) were added to plasma (50 μl), and the whole was vortex-mixed for 30 s. An aliquot of the organic layer was taken into a test tube by using an Eppendorf-type micropipette with disposable plastic tips. The organic solvent was removed with the aid of a nitrogen gas stream or with a centrifugal evaporator and the residue was redissolved in assay buffer. In the experiments to estimate plasma 11-deoxycortisol levels, the recovery rate was tested by using the tracer in the manner described previously.[6]

Cross-Reaction Study——Seven kinds of steroids related to 11-deoxycortisol were used. The amounts required to reduce the binding of [^{3}H]-11-deoxycortisol by half were calculated from inhibition curves. The per cent cross-reaction was determined according to the method of Abraham[8]

Results and Discussion

The steroid derivative used as a hapten in this study was CET. Female BALB/c mice were immunized by an intraperitoneal injection of the CET–BSA conjugate with complete Freund's adjuvant. Booster immunizations were given at biweekly intervals. Three days before the fusion experiment, the mice were given a final injection of the immunogen in saline. The spleen cells were fused with P3-NSl/1-Ag4-1 myeloma cells by using 45% polyethylene glycol. The hybridomas were cultured in the selection medium and antibody-secreting hybridomas were cloned by limiting dilution.



11-deoxycortisol: R=H
CET: R=SCH_2CH_2COOH
CMT: R=SCH_2COOH

Chart 1

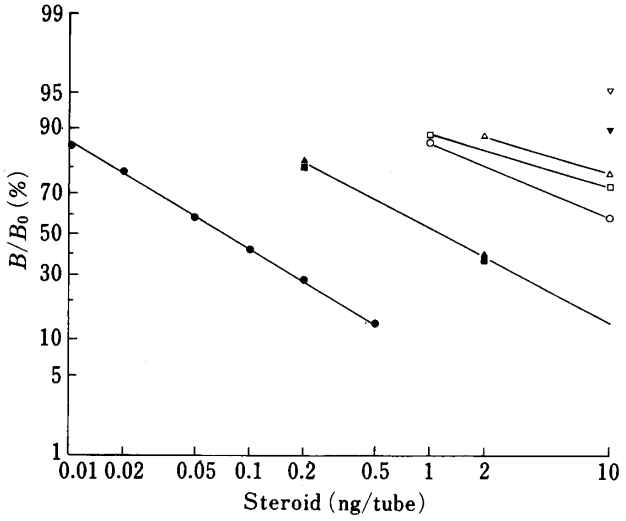


Fig. 1. Dose–Response Curves in the 11-Deoxycortisol Radioimmunoassay Method Using the Monoclonal Antibody CET-M8

●, 11-deoxycortisol; ■, 17α-hydroxyprogesterone; ▲, 11-deoxycorticosterone; ○, cortisone; □, cortisol; △, progesterone; ▼, corticosterone; ▽, 21-deoxycortisol.

The screening of hybridomas for 11-deoxycortisol antibody activity was carried out using the radioimmunoassay procedure: [^{3}H]-11-deoxycortisol was used as a labeled hapten, and the bound and free fractions were separated by a dextran-coated charcoal method. A monoclonal hybridoma cell line was selected that secretes an antibody with a high affinity for 11-deoxycortisol. The monoclonal anti-11-deoxycortisol antibody (CET-M8) was secreted in a satisfactory titer (dilution of 1:400) in the culture supernatants, and was shown to be immunoglobulin G_1(IgG_1) with kappa light chains by an isotyping enzyme immunoassay.[9] The dose–response curve for 11-deoxycortisol radioimmunoassay was obtained by incubating 0—500 pg of unlabeled 11-deoxycortisol and a fixed amount of the labeled steroid, and was linear on a logit-log plot (Fig. 1). The amount of 11-deoxycortisol needed to displace 50% of the bound label was 75 pg. The affinity constant (K_a) of this antibody was $2 \times 10^{10}\,M^{-1}$, as calculated by Scatchard analysis.[10]

The specificity of the antibody CET-M8 was assessed by measuring the ability of various steroids to compete with [^{3}H]-11-deoxycortisol for binding to the antibody. The results with seven kinds of related steroids are shown in Fig. 1 and listed in Table I. The per cent cross-reaction was determined according to the method of Abraham.[8] For comparison, the cross-reactions of the polyclonal antiserum CMT-2, which has been shown to be applicable to the metyrapone test,[6,11] were also tabulated. The monoclonal antibody CET-M8 was reasonably specific for 11-deoxycortisol. The cross-reactivities of cortisol and cortisone show that the antibody CET-M8 is capable of discriminating the functional groups at the 11-position; the values were rather low when compared with those with the polyclonal antiserum. In the case of steroids having a monodeoxygenated side chain, such as 11-deoxycorticosterone and 17α-hydroxyprogesterone, somewhat high cross-reactions were observed. We found 0.2% cross-reaction with progesterone, and 0.02% with corticosterone and 21-deoxycortisol.

These results prompted us to apply the antibody CET-M8 to the determination of 11-deoxycortisol levels in plasma. Taking account of the cross-reactivity of cortisol (0.2%) and its high levels in normal subjects and in patients with Cushing's syndrome, overestimation of 11-deoxycortisol values in these plasma samples may still be inevitable. In the metyrapone test, however, the acceptable base line values are less than 10 μg/l, and hence, this antibody was

TABLE I. Per Cent Cross-Reaction of Antibodies with Selected Steroids

Steroid	Monoclonal CET-M8	Polyclonal CMT-2
11-Deoxycortisol	100	100
Cortisol	0.2	0.6
Cortisone	0.4	0.9
Corticosterone	0.02	<0.001
11-Deoxycorticosterone	7	6
17α-Hydroxyprogesterone	7	4
Progesterone	0.2	0.3
21-Deoxycortisol	0.02	0.04

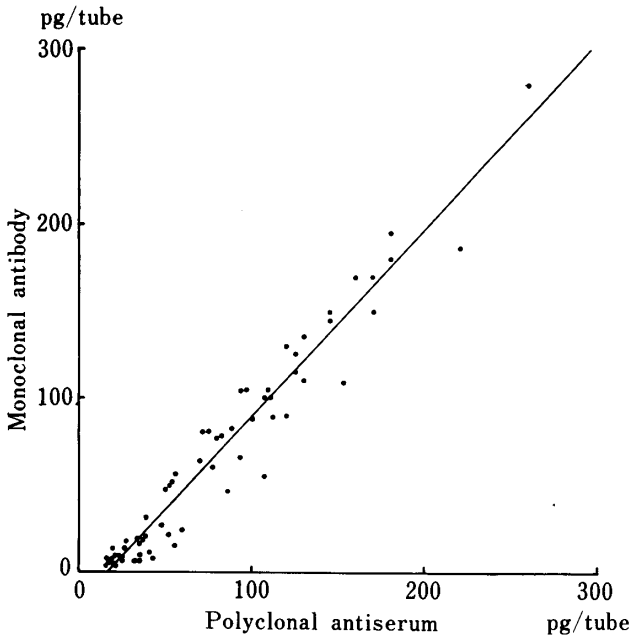


Fig. 2. Correlation between 11-Deoxycortisol Values Determined by Radioimmunoassays Using Monoclonal Antibody CET-M8 and Polyclonal Antiserum CMT-2

expected to be useful. Previously, we have developed a reasonably specific radioimmunoassay of 11-deoxycortisol, using the antiserum CMT-2.[6] The specificity of the polyclonal antiserum was ascertained by several experiments[6,11]: comparisons of various antisera, pretreatment of plasma specimens, and cross-reactions, and consistency of the results with the physiological state of the subject. In the present work, therefore, the specificity of the radioimmunoassay using the monoclonal antibody CET-M8 was examined mainly by comparing the results of measurement of plasma 11-deoxycortisol with those obtained with the polyclonal antiserum.

A comparison of the 11-deoxycortisol values determined by the radioimmunoassays using the antibodies CET-M8 and CMT-2 is shown in Fig. 2. The assay was done on methylene chloride extracts of plasma. The plasma samples used included those obtained from patients with intact pituitary-adrenal reserve and Cushing's syndrome before and after metyrapone administration. The regression equation was $Y=1.07X-18.1$ ($r=0.970$, $n=64$). The results show that the monoclonal antibody is more specific than the polyclonal antiserum in a practical sense. The mean 11-deoxycortisol level in plasma of normal subjects, as determined by using the monoclonal antibody, was $0.90\pm0.37\,\mu g/l$ ($n=9$). In the case of the samples from patients with intact pituitary-adrenal reserve, the pre-metyrapone value was $0.70\pm0.45\,\mu g/l$ ($n=4$), and the samples collected 48 h after metyrapone administration showed much higher values, ranging from 63 to $224\,\mu g/l$, with a mean value of $144\pm58\,\mu g/l$

($n=5$). In the patients with Cushing's syndrome due to adrenal hyperplasia, the plasma 11-deoxycortisol values were 2.0 ± 0.67 μg/l ($n=3$) before metyrapone administration and 81 ± 13 μg/l 48 h after metyrapone administration. Thus, the present radioimmunoassay of 11-deoxycortisol using the monoclonal antibody CET-M8 was found to be useful in the metyrapone test for the evaluation of pituitary-adrenal function and the differential diagnosis of Cushing's syndrome.

In general, antibodies to a hapten can not necessarily recognize the whole structure of the hapten molecule, since the antigen-binding site or antigenic determinant is limited in size, and since the binding of the hapten portion of immunogen to B-cell receptors (surface immunoglobulin) is influenced by a steric interaction between the receptors and the carrier portion of the immunogen. This was likely to be the case in the present monoclonal antibody: the cross-reaction studies, together with separate experiments,[12] indicate that the antibody CET-M8 recognizes the functional groups at C-11 and C-21 in 11-deoxycortisol and, to a lesser extent, the functional groups near the C-4 position. In other words, the antigen-binding site of the antibody is complementary to and covers the steroid portion remote from the position used for attachment to the carrier in the preparation of the immunogen. A clone mono-specific for a steroid, if present, may not be clonally expanded unless the hapten portion in an immunogen does fit the cell surface receptor according to the clonal selection theory. Thus, we can speculate that, for the preparation of hybridoma clones producing monoclonal antibodies specific for a steroid with high frequency, the conformation of hapten molecules on the carrier is very important. In the BALB/c mouse, the CET conjugate was more immunogenic than the CMT conjugate (data not shown). It is hoped that systematic studies will be done to elucidate this problem. The diversity of specificity of antibody against a single hapten may be a factor to be considered even in inbred mice or rats.

The hybridoma clone, S.CET.M8.1.1, producing antibody CET-M8 can be expanded in an ascitic form in mice.[12] The monoclonal antibody should be useful in the standardization of 11-deoxycortisol assay. Development of various immunoassay systems using this antibody is being conducted in these laboratories.

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