Production and Characterization of Anti-estrone Monoclonal Antibody

WANG YONG-CHENG[+], GUO ZHEN-QUAN[#], LI YUAN-ZONG[+], AND CHANG WEN-BAO[+,1]

[+] *College of Chemistry and Molecular Engineering;* [#] *Life Sciences Center, Peking University, Beijing 100871, China*

Objective Determination of estrone (E_1) levels has a significant meaning in evaluating physiological effect and diagnosing some diseases. In order to detect free E_1 in biological fluids, a monoclonal antibody specific for E_1 was prepared after the complete antigen of E_1 was synthesized. The purified monoclonal antibody was fully characterized for later immunoassay. **Methods** 3-O-carboxymethyl ether derivative of E_1 was synthesized and in turn coupled to bovine serum albumin (BSA) to form complete antigen E_1-BSA. A monoclonal antibody (McAb) specific for E_1 was produced both *in vitro* and *in vivo* by a hybridoma anti-E_1. Anti-E_1 was prepared by fusion of SP2/0 murine myeloma cells with spleen cells isolated from immunized BALB/c mouse. The McAb was characterized by enzyme-linked immunosorbent assay (ELISA), SDS-PAGE and Western-blotting. The specificity of the immunoassay was investigated by determining the cross-reactions of E_1 analogs when free E_1 was detected by competitive indirect enzyme-linked immunosorbent assay (CI-ELISA). **Results** Analysis revealed that anti-E_1 McAb (E_1-McAb) was of the IgG1 type, the molecular weight of E_1-McAb was 164 000 daltons. The affinity constant of E_1-McAb with coated complete antigen was 8.2×10^8L/mol. The linear range for free E_1 determined by CI-ELISA was 10pg/mL~10ng/mL. The detection limit was 21.4 pg/mL (defined as twice the standard deviation of the blank). **Conclusion** The CI-ELISA developed with E_1-McAb was both sensitive and specific. The prepared E_1-McAb can be used in some immunoassays.

Key words: Estrone; Monoclonal antibody; Immunoassay

INTRODUCTION

Steroids are very important in the development and differentiation of organs, culminating in a normal functioning individual, for the moment of development in the uterus, the human embryo is exposed to a variety of hormones, especially the estrogens. Estrogens determinations are widely used to monitor pregnancy[1], breast fibroadenoma[2] and breast cancer[3]. Estrone (E_1), one of the three major naturally occuring estrogens, is primarily produced from androstenedione, which originates in premenopausal women in equal

[1]CorrespondeNce should be addressed to: Chang Wen-Bao, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China, Phone: 86-010-627517241，Fax: 86-10-62751708，Internet Address: dxhx@chemms.chem.pku.edu.cn，ychwang@chemms.chem.pku.edu.cn

Biographical note of the first author: Wang Yong-Cheng, majoring in biochemical and immunological analysis, is a Ph. D. student at Peking University in China. His dissertation thesis works on the "Production of Monoclonal Antibodies Specific for Estrogen and Study on Immunoassay Based on Temperature-sensitive Hydrogel".

0895-3988/2002
CN 11-2816

amounts from either the adrenals or the ovaries. Minor portions of E_1 are derived from conversion of androstenedione in peripheral tissue and interconversions of E_1 and 17β-estradiol. During pregnancy large amounts of E_1 are synthesized within the placenta from dehydroepiandrosterone sulfate. Recent research[4] suggested that E_1 could increase neuromuscular transmission. Dogra and his colleagues[5] found that lower concentrations of E_1 increased the carbohydrates contents of leaves from some plants, and estrogens were more effective than plant hormones in some plants treatment[6]. Case-control study of endogenous steroid hormones indicates that high E_1 levels were associated with increased risk of endometrial cancer[7]. Determination of the concentration of E_1 can also help to ascertain some hormone-dependent diseases[2,8]. E_1 concentration detection is helpful for advising a healthy living style[9]. Many methods have been developed to determine the concentration of E_1[10-12]. However, the antibody is the most important ingredient in immunoassay. Its specific and affinity determine not only the specificity and sensitivity of the assay, but also the practicability of many methods. In order to generate antibody with greater specificity to steroid hormones, it is important that the steroids are coupled to carrier protein at the portion of the hapten furthest removed from the unique functional group[13]. The estrogenic hormones estrone (E_1), 17β-estradiol (17β-E_2) and estriol (E_3) all contain a phenolic ring A, but differ from each other in having characteristic functional group at C-17 in the D-ring. Though some antigenic information may lose by the variation in the nature of substituents in the A-ring (C-3-OH), the anti-E_1 antibody with least cross-reactivity can be raised by exposing the unique functional group in the D-ring. Andrieu *et al.*[14] proved that highly specific antisera for E_1 can be obtained by employing E_1-3-hemisuccinyl-BSA conjugate. However, the advantages of using monoclonal antibodies rather than heterogeneous polyclonal antibodies include the ability to produce virtually unlimited amounts of homogenous and highly specific antibodies utilizing a relative impure antigen preparation. This also helps the standardization of immunoassay procedure when applying the same monoclonal antibody in the analysis of E_1 contamination in various conditions.

In this study, 3-O-carboxymethyl ether derivative of E_1 was synthesized, and its bovine serum albumin (BSA) conjugate was prepared. Monoclonal antibody-producing hybridoma was produced by fusion of SP2/0 murine myeloma cells with spleen cell isolated from mouse immunized with a BSA and E_1 conjugate (E_1-BSA). A monoclonal antibody against E_1 was secreted by a hybridoma named anti-E_1 *in vitro* and *in vivo*. The reactivity and affinity of purified McAb with E_1-OVA (E_1 and ovalbumin conjugate) were characterized by ELISA. The specificity of anti-E_1 McAb with E_1 analogs was determined by competitive indirect ELISA. The isotype of McAb was revealed by ELISA, and the molecular weight of McAb was determined by SDS-PAGE electrophoresis. The sensitivity and specificity of the competitive indirect ELISA in the detection of E_1 were demonstrated.

MATERIALS AND METHODS

Reagent

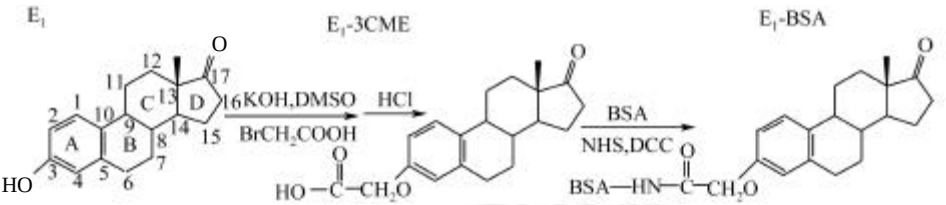
Estrone (E_1), 17β-Estradiol (17β-E_2), Estriol (E_3), Progesterone (P), polyehtylene glycol (PEG, MW 1500), high-glucose Dulbecco' s Modified Eagle' s Medium (DMEM), and goat anti-mouse immunoglobulins for subtyping (heavy chain specific, GAM-Subs) were all purchased from Sigma Chemical Co. (St. Louis Mo. USA). HAT supplement and HT supplement were obtained from Gibco Laboratories (Grand Island, N.Y. USA). Complete and incomplete Freund' s adjuvants were purchased from China Institute for Veterinary Drug Control (Beijing, China). Horseradish Peroxidase-conjugated Goat Anti-Mouse IgG(H+L)

and low molecular weight standards (LMW) were obtained from Beijing Zhong-Shan Biotechnology Co. Ltd. (Beijing, China). The mouse myeloma cell line SP2/0 was preserved in Peking Universiy. Female BALB/c mice (8 weeks old) were obtained from the Center of Laboratory Animals in Peking University. N-hydroxysuccinimide (NHS) and dicyclohexyl carbodiimide (DCC) were obtained from Bailingwei China Chemical Co. Ltd. (Beijing, China). All other chemicals used were of analytical grade.

E_1 and BSA Conjugate Preparation

Synthesis of estrone-3-O-carboxymethyl ether (E_1-3CME): E_1-3CME was prepared by alkylation of estrone with bromoacetic acid. To a solution of estrone (133 mg) in dry dimethylsulfoxide (2.7 mL) was added dry powdered KOH (0.45 mg). After stirring for 5 min., bromoacetic acid (134 mg) was added. Stirring was continued for 2 h, after which the reaction mixture was poured into ice cold water (22.4 mL) and extracted with ethyl acetate to recover unreacted estrone for 3 times. The aqueous solution was cooled and acidified with dilute HCl (2 mol/L) and the separated solids were filtered. It was washed with water till neutral and dried under vacuum. The solid was crystallized from chloroform-methanol to give estrone-3-O-carboxylmethyl ether as a colorless solid. The product recovery was 90%. The product was fully characterized.

Preparation of the complete antigen (E_1-BSA): E_1-3CME was coupled to BSA by the activated ester method employing NHS and DCC as described before[15]. Analysis of the conjugate showed that a coupling ratio of 25 estrone residues per protein molecule. The steroid-protein conjugate was lyophilized and stored at −20℃. E_1-OVA was prepared with the same method. The synthesis procedure of the complete antigen was shown in Scheme 1.



SCHEME 1. The synthesis procedures of complete antigen E_1-BSA.

Immunization Schedule and Cell Fusion

Briefly, 0.2 mg of E_1-BSA was dissolved in 1 mL of 0.9% saline and emulsified with 1 mL of complete Freund adjuvant. Initially 0.5 mL of the mixed emulsion was intraperitoneally injected into each 8-week-old female BALB/c mouse. Three weeks later, the mice were boosted intraperitoneally with the same amount of the E_1-BSA incomplete Freund adjuvant emulsion. Antibody titer was assayed by ELISA after blood samples were collected from the tail of E_1-BSA immunized mouse at intervals. The mice were given a final booster immunization of 100 μg/mL of E_1-BSA in 0.2 mL of saline into the spleen 3-4 days before cell fusion. The day prior to hybridization, normal mouse macrophage cells in the ascites were distributed into each of two 96-well sterile culture trays as feeder layer. The dilution medium used was HAT medium which consisted of 60×10^{-6} mol/L hypoxanthine, 0.5×10^{-6} mol/L aminopterin, 20×10^{-6} mol/L thymidine and DMEM medium and its supplement. Cell line SP2/0 was incubated at 37℃ in 5% CO_2 atomosphere ahead of time.

On the day of hybridization, the myeloma cells (SP2/0) were collected, counted and the viability was determined. Washed and diluted with the HAT medium, approximately 10^7

myeloma cells were mixed with 10^8 spleen cells which were removed from a E_1-BSA immunized mouse. The mixture was pelleted and suspended in 0.5 mL of HAT medium. The fusion procedure began with the addition of 50% warm (37℃) PEG1500, followed by gentle stirring for 1 min. The resulting mixture was kept still at 37℃ for 1.5 min, and 30 mL of HAT medium was slowly added to the fused cells. After the cell mixture was pelleted and diluted to 10^6 cells/mL with HAT medium and distributed (0.1 mL samples) into each well of the feeder cell-laid 96-well microwell culture plates, fresh HAT medium was added on day 5-7, and the plates were examined for cell growth after 10 days. Wells positive for cell growth were screened for the desired antibody production about 14 days postfusion by ELISA. After growing in HT medium for 3-4 days, the positive hybridoma cells were cloned by a limiting dilution technique into the 96-well plates in HT medium with feeder layers till present. Wells were examined for single clones and screened once more for specific antibody activity 14 days later. Clones positive for producing anti-E_1 antibody were expanded stepwise. Parts of the positive hybridoma cells were injected into intraperitoneal cavity of BALB/c mice primed with liquid paraffin for antibody production.

Antibody Production and Characterization

Ascites fluid was obtained after the positive cells were injected into the BALB/c mice for 14 or more days. The anti-E_1 monoclonal antibody was purified from the culture supernatant or mouse ascites according to the method described before[16]. The purified antibody was immediately used for the characterization. The protein content of the antibody was determined by its different absorbances at 260 nm and 280 nm.

The immunoglobulin isotype and subclass of E_1-McAb were determined by ELISA based on the heavy-chain specific goat anti-mouse antibodies (GAM-subs) purchased from Sigma Chemical Co. Briefly, various properly diluted GAM-subs were attached to the solid surface of a 96-well microtiter plate. After the remaining sites on the solid supports blocked, properly diluted E_1-McAb purified from the culture supernatant was added. Then enzyme-labeled third goat anti-mouse antibody was added. The ELISA was finally completed. Different dilution curves of GAM-subs provided information about immunoglobulin isotype and subclass of E_1-McAb. The affinity constant of E_1-McAb with E_1-OVA was measured by non-competitive enzyme immunoassay described by Beatty *et al.*[17], the molecular weight of E_1-McAb was determined by SDS-PAGE electrophoresis using a commercially available LMW and operated as the manufacturer instructed. The epitoped specificity of E_1-McAb was analyzed by Western blot.

Free E_1 Determination by CI-ELISA

A competitive indirect enzyme-linked immunosorbent assay method was developed to detect the free E_1 levels in solutions. E_1-BSA, certain amount in 100 *m*L of carbonate-bicarbonate buffer (pH 9.5, 0.05 mol/L) per well, was adsorbed to 96-well microtitre plates at 4°C overnight. The plates were washed three times with 0.05% Tween-20 in phosphate-buffered saline (PBS-T, PBS, pH7.4, 0.01 mol/L sodium phosphate with 0.15 mol/L sodium chloride). Then each well was blocked by 200 *m*L of 0.7% gelatin in PBS for 2 h at 37°C, and E_1 standards were prepared by dissolving it in 1,4-dioxane (1 mg/mL) with subsequent dilution in PBS. Then 50 *m*L was added to the plates, followed by addition of 50 *m*L/well diluted E_1-McAb. The microtitre plates were incubated for 1 h at 37℃. The goat anti-mouse IgG-HRP conjugate was added and incubated for 1 h at 37℃. After washed with PBS-T, the substrate TMB-H_2O_2 was added. The immunological reaction was stopped by 2 mol/L

H_2SO_4. The optical density (O.D.) of the final mixture was recorded at 450 nm on a Dynatech ELISA Minireader. The same CI-ELISA procedures were used to determine the specificity of the immunoassay, when cross-reactions of E_1 analogs (17β-E_2, E_3 and P) with E_1-McAb in the assay of E_1 were completed, inhibition curves of each analogs against E_1-McAb to E_1-BSA coated were drawn, and the cross-reactions of E_1 analogs with E_1-McAb were calculated.

RESULTS

Hybridoma Preparation

The hybridoma cell clones could be observed about 7 days after the cell fusion. The fusion rate of mouse spleen cells with myeloma cells was about 90%. The positive well rate was 20.8%. After 3 or 4 times of clonings, one clone of hybridoma secreting antibodies reacting with E_1-OVA and E_1 and showing highest growth rate was selected and was named anti-E_1. Anti-E_1 was characterized for its somatic chromosome number and used to produce E_1-McAb *in vitro* and *in vivo*. Anti-E_1 cell had about 50-54 pairs of somatic chromosomes, indicating that anti-E_1 was a hybridoma containing chromosomes both from mouse spleen cell and from SP2/0 myeloma cell, since their chromosome number was 20 and 36 pairs respectively.

Determination for the Immunoglobulin Isotype and Subclass of E_1-McAb

Microtitre plates were coated with different GAM-subs (IgG1, IgG2, IgG3, IgA, IgM), 0.2 g in 100 *m*L carbonate-bicarbonate buffer (pH 9.5, 0.05 mol/L) per well for each GAM-sub. After the microtiter plate was blocked and washed, 100 *m*L of serial diluted E_1-McAb, 100 *m*L of 5000-fold diluted goat anti mouse IgG-HRP conjugate and finally 100 *m*L of TMB-H_2O_2 substrate were stepwise added to it. After reading at 450 nm, the absorbence value of each well was recorded. Different curves of GAM-subs showed that E_1-McAb puried from the cell culture supernatant was of the subclass IgG1 (Fig.1).

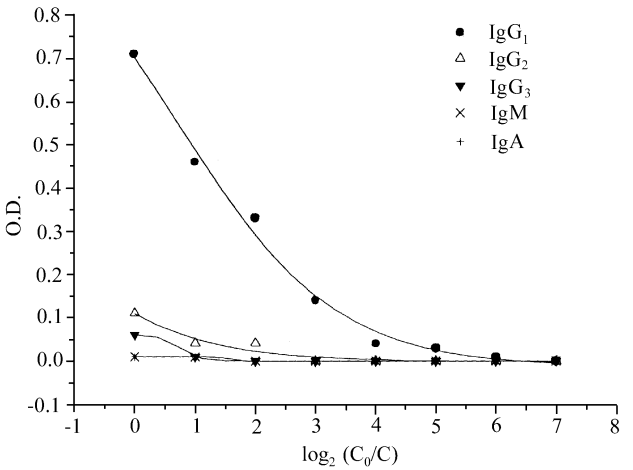


FIG.1. Dilution curves of E_1-McAb for its immunoglobulin isotype and subclass determination. E_1-McAb is 2-time diluted with the primary concentration (C_0) of 2 μg/mL, while C represents the concentrations after diluted. Two curves of coated IgA and IgM are in superposition as shown.

Molecular Weight Determination and Epitope Analysis of E_1-McAb

The molecular weight of E_1-McAb was 164 000 daltons (54 000 daltons for each heavy chain, 28 000 for each light chain) as determined by SDS-PAGE electrophoresis. Two clear bands on SDS-PAGE ascertained that the E_1-McAb was pure enough for later immunoassay. The epitope specificity of E_1-McAb was analyzed by Western blot. The results showed that only the light pieces of E_1-BSA (E_1-containing residue) reacted with E_1-McAb, while those of BSA, 17β-E_2-BSA (17β-E_2 and BSA conjugate) and E_3-BSA (E_3 and BSA conjugate) did not give bands on the membranes from Western blot. This proved that the prepared McAb was truly the desired antibody which could react specifically with E_1-BSA. The titer of McAb in anti-E_1 induced mouse ascites was 4.7×10^{-10} mol/L.

Determination for the Affinity Constant (K_{aff}) of E_1-McAb

According to the method[17], the affinity constant (K_{aff}) could be measured by ELISA using serial dilutions of both coated antigen and E_1-McAb (shown in Fig. 2).

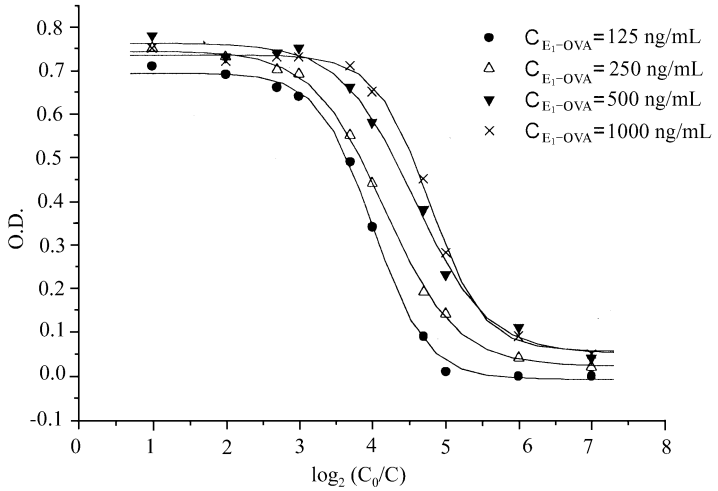


FIG. 2. E_1-McAb dilution curves at different coated antigen concentrations. The antigen concentration is decreased by one-half for each successive curve. The primary concentration of E_1-McAb is 1.0 *m*g/mL.

K_{aff} could be calculated according to the following formula:

$$K_{aff} = (n\text{-}1)/ \{2 (n [Ab']_t - [Ab]_t)\},$$

where $n = [Ag]_t / [Ag']_t$, $[Ag]_t$ and $[Ag']_t$ are the total antigen concentrations in the wells while $[Ab']_t$ and $[Ab]_t$ are the measurable total antibody concentrations in the wells at O.D.-50 (50% of the O.D. maximum value) and O.D.-50' for plates coated with $[Ag]_t$ and $[Ag']_t$. The apparent K_{aff} of E_1-McAb with E_1-OVA is 8.2×10^8 L/mol.

Calibration Curve for Free E_1 and Its Recoveries

Free E_1 was determined by CI-ELISA. Calibration curve for free E_1 determination was shown in Fig. 3. The concentrations of E_1 were linear from 10 ~ 10 000 pg/mL. The linear fit equation was: Y (O.D) = -0.18+0.12X [lg (C_0/C)], R = 0.9997. The sensitivity of the assay

in terms of the lowest detectable dose of E_1 (twice the standard deviation at zero dose) was 21.4 pg/mL.

For recovery measurement, 100 mL of E_1 solution was added to PBS to make the mimetic sample. The sample solution was diluted to get E_1 solutions with concentrations of 3160, 316 and 31.6 pg/mL. The recovery results were summarized in Table 1. The results showed that both the precision and accuracy were satisfactory in the assay of free E_1.

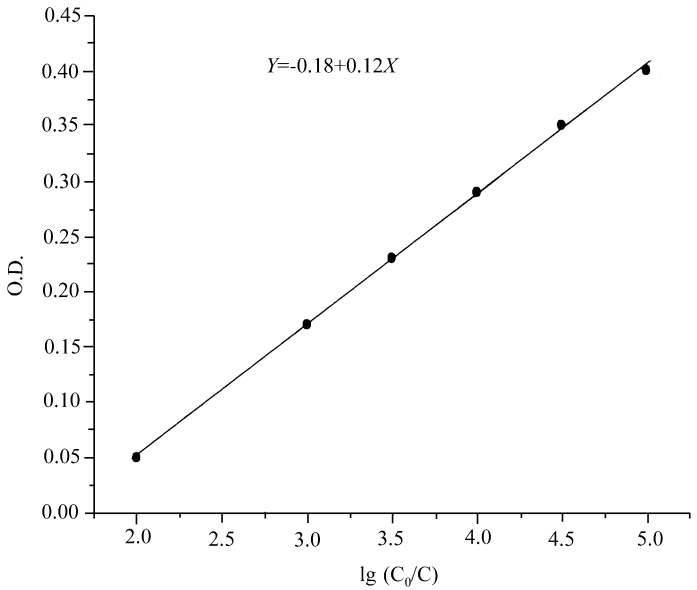


FIG. 3. Calibration curve for free E_1 by CI-ELISA. The coated antigen is E_1-BSA with the primary concentration of 125 ng/ml. The concentration of McAb is 0.5 mg/mL. The concentrations of free E_1 are 10 000, 1 000, 316, 100, 31.6 and 10 pg/mL.

TABLE 1

The Recovery Results of E_1 in Mimetic Samples by CI-ELISA (n=8)

E_1 Added (pg/mL)	E_1 Measured (pg/mL)	Recovery (%)	RSD (%)
3160	3310	104.8	4.8
316	389	126.0	4.4
31.6	47.8	151.6	9.9

Cross-reactions of E_1 Analogs in the Assay of E_1

Cross-reactions with E_1-McAb caused by the E_1 analogs could produce errors in the immunoassay of free E_1. Some typical E_1 analogs (17β-E_2, E_3 and P) were chosen for the investigation of cross-reactions by three different methods[18]. These methods were the 50% displacement method (50%-D), the $CR_{0.5ng}$ Method ($CR_{0.5ng}$) and the 10%-error Method (10%-E). The cross-reactions caused by the E_1 analogs were summarized in Table 2. In the $CR_{0.5ng}$ method, 0.5 ng was an arbitary amount of analogs and E_1.

The results of the $CR_{0.5ng}$ method showed that cross-reactions were relatively high when

the concentration of E_1 was high. Of all the cross-reacting steroids, P had the least cross-reaction, while 17β-E_2 had the relatively highest cross-reaction. The reason might be that 17β-E_2 was more similar with E_1 than E_3 and P.

TABLE 2

The Cross-reactions of 17β-E_2, E_3 and P in the CI-ELISA of E_1

Steroids Assayed	Cross-reacting Steroid	50%-D	Cross-reaction $CR_{0.5ng}$	10%-E
E_1	17β-E_2	2.4%	50%	2.8%
	E_3	0.5%	17%	0.1%
	P	< 0.01%	11%	< 0.01%

DISCUSSION

It is essential to produce highly molecular or structurally specific antibodies to E_1 in the assay of E_1. The antibody's specificity determines the specificity and sensitivity of the immunoassay. For the disadvantages of significant cross-reactions caused by E_1 structurally similar molecules in the biological fluids, conventional heterogeneous antisera have been slowly substituted by monoclonal antibodies since hybridoma technology was presented by Kohler and Milstein[19]. But to a small molecule or hapten such as E_1, it is still a great challenge. A simple hapten can easily react with a monoclonal antibody but can induce weak or no immune responses. While the conjugate of a hapten and a carrier protein can induce much stronger immune responses. So E_1 was bound to BSA to form a complete antigen for animal immune. The three estrogens E_1, 17β-E_2 and E_3 are similar to each other very much, and the only differences from each other are functional groups in ring D. In order to eliminate the chances of cross-reactions caused by the E_1 analogs, the complete antigen (E_1-BSA) was prepared by employing a C-3 coupled steroid-protein conjugate.

Since the immunogen injected to the mice to generate the monoclonal antibody is E_1-BSA, some antibodies may recognize some epitopes in BSA instead of in E_1. In order to select better hybridoma cells to produce monoclonal antibodies only recognizing E_1 (free or conjugated to other biomolecules), the complete antigen, E_1-OVA, was used for the screen and determination of the affinity constant. After proper immunization, fusion and selection procedures, a stable cell clone anti-E_1 was obtained. Then McAb could be produced both *in vitro* and *in vivo*.

The anti-E_1 McAb was characterized after purified from the mouse ascites. The anti-E_1 McAb was of the IgG1 type, with a molecular weight of 164 000 daltons. In determination of the affinity constant of E_1-McAb to the coated antigen, two different antigens, E_1-OVA and E_1-BSA were coated on the microtiter plates. The affinity constants of E_1-McAb with E_1-OVA and E_1-BSA were 8.22×10^8 L/mol and 8.17×10^8 L/mol, respectively. No significant difference was found. This provided the information that no structural formation happened on the small molecule hapten when the hapten-carrier conjugate was coated on the solid support. For the epitopes on the antigen (especially on the hapten) was not influenced in the coating procedure, the affinity constants determined by different complete antigens in a separate assay was the same.

The anti-E_1 McAb was used for the determination of free E_1 by CI-ELISA. The concentrations of free E_1 were linear from 10 pg/mL to 10 ng/mL. The detection limit for the determination of free E_1 was 21.4 pg/mL (defined as twice the standard deviation of the

blank). In order to measure the specificity of the immunoassay of free E_1, the cross-reactions of 17β-E_2, E_3 and P with E_1-McAb were investigated. Three ways, 50%-D, $CR_{0.5ng}$ and 10%-E, were used for the cross-reaction estimation. The often-used 50%-D method took little account of the region of the highest reaction at high antigen concentrations. The 10%-E method gave a realistic estimation of the cross-reactions of the E_1 analogs. These methods basically covered the whole region of different concentrations. The results given by the cross-reaction estimations showed that 17β-E_2, E_3 and P had no influence on the determination of free E_1 by CI-ELISA. The E_1-McAb prepared by hybridoma technology was good enough for immunoassay of E_1 in biological fluids.

CONCLUSIONS

Antigenic complete antigen E_1-BSA was synthesized. After immunization and cell fusion, a stable hybridoma, anti-E_1, producing monoclonal antibody against E_1 (E_1-McAb) was obtained. The specific monoclonal antibody specific for E_1 was produced and purified. E_1-McAb was fully characterized by ELISA, SDS-PAGE and Western-blotting. The CI-ELISA developed with E_1-McAb is both sensitive and specific.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation of China (Grant no.20075001).

REFERENCES

1. Ishida, J., Kai, M., and Ohkura, Y. (1988). Determination of estrogens in pregnancy urine. *J. Chromatogr.* **431,** 249.
2. Pasqualini, J.R., Cortes-Prieto, J., Chetrite, G., Talbi, M., and Ruiz, A. (1997). Concentrations of estrone, estradiol and their sulfates, and evaluation of sulfatase and aromatase activities in patients with fibroadenoma. *Int. J. Cancer* **70,** 639.
3. Selcer, K.W., Hegde, P.V., and Li, P. K. (1997). Inhibition of estrone sulfate and proliferation of human breast cancer cells by nonsteroidal (p-o-sulfamoyl)-N-alkanoyl tyramines. *Cancer Res.* **57,** 702.
4. Jing, H.E., Yang, S.R., and Lu, G.N. (1997). Effects of estrone on transmission at neuromuscular junction of the toad. *China Univ. Med. Sci*. **28,** 26.
5. Dogra, R. and Thukral, A.K. (1995). Effect of steroid pre-treatment on some inorganic constituents of wheat plants. *Acta Botanica Indica* **23,** 33.
6. Dogra, R. and Thukral, A.K. (1995). Effect of steroid pre-treatment on the carbohydrates content of Zea Mays plants. *Acta Botanica Indica* **23,** 115.
7. Potischman, N., Hoover, R.N., Brinton, L.A., Siiteri, P., Dorgen, J.F., Swanson, C.A., Berman, M.L., Mortel, R., Twiggs, L.B., Barrett, R.J., Wilbanks, G.D., Dersky, V. J., and Lurain, R. (1996). Case-control study of steroid hormones and endometrial cancer. *J. Nat. Cancer Inst. (Bethesda).* **88,** 1127.
8. Reginster, J.Y., Deroisy, R., Collette, J., Albert, A., and Zegels, B. (1997). Prediction of bone loss rate in healthy postmenopausal women. *Calcified Tissue International* **60,** 261.
9. Vine, M.F. (1996). Smoking and male reproduction: A review. *Int. J. Androl.* **19,** 323.
10. Peter, L., Sabine, H., Ingrid, S., Christian, B., and Hagan, H. (1995). 6α-Biotinylated estrone: Novel tracer in competitive chemiluminescence immunoassay of estrone in serum. *Clin. Chem. Endocrinol. Metab*. **41,** 564.
11. Bakola-Christianopoulou, M.N., Apazidou, K.K., and Psarros, L. (1997). GC-SIM / MS profiling of urinary steroids as their per-trimethysilyl derivatives. *Appl. Organometal. Chem.* **11,** 205.
12. Lim, S.K., Won, Y.J., Lee, J.H., Kwon, S.H., Lee, E.J., Kim, K.R., Lee, H.C., Huh, K.B., and Chung, B.C. (1997). Altered hydroxylation of estrogen in patients with postmenopausal osteopenia, *J. Clin. Endocrinol. Metab*. **82**, 1001.
13. Gosling, J.P. (1990). A decade of development in immunoassay methology (Review). *Clin. Chem.* **36,** 1408.
14. Andrieu, J.M., Mamas, S., and Dray, F. (1975). Viroimmunoassay of steroids: methods and principles. D. (Cameron, S.G. Hillier, K. Griffiths, Eds.) In *Steroid Immunoassay*, pp.189. Alpha Omega Publishing Ltd.,

Cardiff.
15. Mattox, V.R., Litwiller, R.D., and Nelson, A.N. (1979). A comparison of procedures for attaching steroidal glucosiduric acid to bovine serum albumin. *J. Steroid Biochem.* **10,** 167.
16. Tang, D., Wang, Y.C., Chang, W.B., Ci, Y.X., and Guo, Z.Q. (1999). Time-resolved fluorescence immunoassay of estradiol in serum. *Chin. J. Anal. Chem.* **27,** 899.
17. Beatty, J.D., Beatty, B.G., and Vlahos, W.G. (1987). Measurement of monoclonal antibody affinity by on-competitive enzyme immunoassay. *J. Immunol. Methods* **100,** 173.
18. Pratt, J. J. (1978). Steroid immunoassay in clinical chemistry. *Clin. Chem.* **24,** 1869.
19. Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256,** 119.

(Received October 28, 2001 *Accepted December* 3, 2001*)*