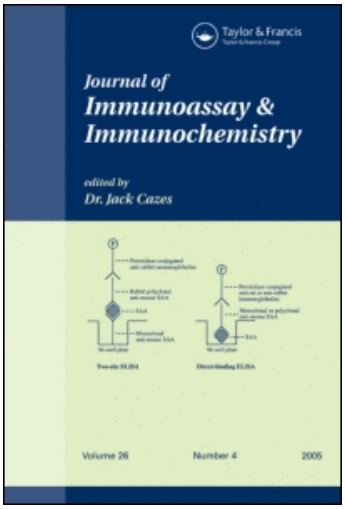

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Use of Calf Uterine Cytosol Estrogen Receptor Coupled to Class Beads as a Stable Internal Control for Estradiol Receptor Assay in Human Breast Cancer

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USE OF CALF UTERINE CYTOSOL ESTROGEN RECEPTOR COUPLED TO GLASS BEADS AS A STABLE INTERNAL CONTROL FOR ESTRADIOL RECEPTOR ASSAY IN HUMAN BREAST CANCER

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ABSTRACT

Calf uterine tissue was homogenized in six volumes (w/v) of 10 mM Tris-HCl buffer of pH 7.4, containing 1 mM monothioglycerol and 10% glycerol (TG) and centrifuged at 800 x g for ten minutes. The supernatant was centrifuged at 105,000 x g for 60 minutes to obtain the cytosol fraction. Sand-blasted uniform glass beads (6 mm diameter) were reacted with 10% gamma-aminopropyl triethoxy-silane in toulene at 60°C for seven hours to generate aminopropyl derivatives. The derivatized beads were activated with 2.5% glutaraldehyde and incubated overnight with the calf uterine cytosol receptor at 4°C. After incubation, the beads were washed with Tris-HCl buffer and stored at -20°C. The cytosol receptor coupled beads were incubated for 18 hours with and without 100 fold excess of diethylstilbesterol. The beads were suspended in scintillation fluid and counted for ten minutes. The binding sites and dissociation constant (K_D) of the calf uterine cytosol receptor coupled to beads were 7.2 fmol/bead or 144 fm/mg protein and 4.3 x 10^{-9} M, respectively. There was little loss of the cytosol

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[3]The abbreviations used are: E_2, estradiol; E_2R, estradiol receptor.

estrogen receptor coupled to glass beads up to a period of three months. Calf uterine cytosol estrogen receptor coupled to beads provided a stable internal control to monitor the inter-assay variations due to the reagents, radioisotopes, and method used in measurement of E_2 receptor in human breast cancer cytosol fraction in pre- and post-menopausal women. The interassay precision was 4.6% ($P < 0.05$). The mean concentration of E_2 and receptors was 19 fm per mg protein in pre-menopausal women; however, no significant difference was observed in the proportion of E_2 receptor positive tumors in pre- and post-menopausal women.

INTRODUCTION

Evaluation of estrogen receptor in mammary carcinoma cell cytosol preparations has been of prognostic value in the treatment of breast cancer in almost every major clinic. Due to the labile nature of the receptor and the radioactive ligand as well as the sophistication of the techniques used in the measurement of estrogen receptor, good precision is always important; due to above factors, it is difficult. We describe here the use of calf uterine cytosol receptor coupled covalently to glass beads as an additional stable internal standard to monitor the inter-assay variation due to the reagents, radioisotopes, and method used in the measurement of estrogen receptors. We have also examined the degree of relationship between menopausal status and the presence of estrogen receptors in human breast cancer tissue.

MATERIALS AND METHODS

Preparation of Cytosol Receptor

Human breast cancer tissues were obtained from surgical pathology, New York Hospital, NY, and calf uteri from the slaughterhouse. The tissues were freed from fat and stored in liquid nitrogen immediately

after removal. For the preparation of the receptor, human breast cancer tissue, calf uterine tissues, and the reference powder were ground separately in a thermovac tissue pulverizer. The ground tissues (approximately 1 g human breast tumor, 10 g calf uterine tissue, and 0.5 g reference powder) were homogenized in six volumes (w/v) of 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM monothioglycerol (Sigma, St. Louis, MO) and 10% glycerol (Fisher Scientific Company, Fairlawn, NJ) designated as TG buffer, in a polytron PT homogenizer (Brinkmann Instruments, Westbury, NY) at speed setting of ten using three 15, 5, and 5 seconds pulses with 45 seconds pause between each pulse. The homogenates were centrifuged at 800 x g for ten minutes to separate the nuclear pellets. The supernatants were centrifuged at 105,000 x g for one hour to obtain the cytosol receptor fractions. The protein content of the cytosol receptor preparations was determined by the method of Lowry (1). The calf uterine cytosol fraction was used for coupling to glass beads as described below. The remaining calf uterine cytosol receptor preparation was stored in suitable aliquots in liquid nitrogen and at -20°C to be examined periodically in the estrogen receptorassay.

Coupling of the Calf Uterine Cytosol Receptor to Glass Beads

Glass beads of 6 mm diameter were obtained from SGA Scientific Incorporated, Bloomfield, NJ, and sandblasted in 60 grit particles at an air pressure of 75 lbs/sq inch for ten minutes in a sandblaster (Ruemelin Manufacturing Company, Milwaukee, WIS). These glass beads (approximately 100 g) were reacted with 100 ml of

10% gamma-aminopropyl triethoxysilane (Silar Laboratories, Incorporated, Scotia, NY) in toulene (J. T. Baker Chemical Company, Phillipsburg, NJ) at 60°C for seven hours to generate reactive alkyl amino groups on the surface (2). The amino propyl glass beads were washed thoroughly with toulene to remove excess and air dried (3). The derivatized beads were activated with 100 ml of 2.5% glutaraldehyde solution (Electron Microscopy Science, Fort Washington, PA) in 10 mM phosphate buffer (sodium phosphate obtained from Mallincknodt Chemical Works, New York, NY) of pH 7.5 for two hours at room temperature, followed by washing with the TG buffer to remove excess reagent. About 50 to 80 activated glass beads were incubated overnight at 4°C with 15 ml of calf uterine cytosol receptor containing 5 mg of protein/ml. After incubation, the receptor coupled glass beads were thoroughly washed with the TG buffer. The estradiol binding assay was performed at different time intervals with cytosol receptor coupled to glass beads stored at -20°C and 4°C as well as cytosol receptor stored at -20°C and in liquid nitrogen.

Protocol for Estradiol Assay (Table 1)

One bead coupled with the cytosol receptor was incubated in 200 µl of 10 mM TG buffer for 18 hours at 4°C in triplicate with 0.5 nM, 1 nM, 2 nM, and 5 nM of [2, 4, 6, 7-^{3}H] estradiol of a specific activity of 92 Ci/mMole (Amersham Corporation, Arlington Heights, IL) in the presence and absence of a 100 fold excess of diethylstilbestrol (DES) obtained from Sigma Chemical Company, St. Louis, MO. At the end of the incubation, the contents of

TABLE 1

Protocol of E_2 Cytosol Receptor Assay

Reagents	Total Count (TC)	Blank (B)	Total Bound (TB)	Displacement (D)
		µl		
Buffer[a]	200	200	100	
Unlabeled Hormone[b]				100
Labeled Hormone[c]	100	100	100	100
Cytosol Receptor or Bead			100 or 1 bead	100 or 1 bead
Total Volume (µl)		300	300	300

Incubate B, TB, and D for 18 hours for E_2 and for P receptors at 4°C. Add 0.5 ml of Dextran coated charcoal (0.5% Norit A, 0.05% Dextran in 10 mM TG buffer, pH 7.4). Incubate 10 min at 4°C. Centrifuge at 2,000 x g for 10 min. Take 0.4 ml aliquot for scintillation counting in 5 ml ACS fluid and count for 10 min.

[a]ER assay, 10 mM Tris-HCl, pH 7.4. P assay, 10 mM sodium phosphate, pH 7.4. The buffers contain 10% glycerol and 1 mM monothioglycerol.

[b]DES for E_2 receptors and R-5020 for P receptors in 100 fold excess of the tracer concentrations.

[c]0.5 nM to 5 nM at ^{3}H-estradiol for E_2 receptors. 0.5 nM - 5 nM of ^{3}H-R5020 for P receptors.

the tubes were aspirated; and the beads were washed twice with TG buffer and counted directly in the Scintillation vials containing 5 ml of ACS fluid (Amersham Corporation). Aliquots of calf uterine cytosol receptor fraction stored in liquid nitrogen and at -20°C and freshly prepared breast cancer cytosol in a final volume of 0.3 ml were also incubated as above. After incubation, 0.5 ml of 0.05% dextran (Pharmacia, Sweden) and 0.5% Norit A (Amend, NY) in 10 mM TG buffer was added to each tube and allowed to stand for ten minutes at 4°C to allow adsorbtion of the free hormone on the dextran coated charcoal (DCC). The DCC was separated by centrifugation at 2,000 x g for ten minutes. An aliquot of 0.4 ml out of the total 0.8 ml was mixed with 5 ml scintillation fluid. Radioactivity was determined in a Packard 3375 liquid scintillation counter with 33% efficiency for tritium. The number of specific binding sites and the dissociation constants were calculated by Scatchard plot (4).

RESULTS

Each glass bead was coated with approximately 50 µg of cytosol protein. The glass beads caused 2 to 3% of quenching. One glass bead was sufficient for the assay. From Scatchard plot analysis, the specific binding sites on one glass bead were calculated to be 7 femtomoles/bead and 144 femtomoles/mg cytosol protein. The dissociation constant was 4.3×10^{-9} M, and the correlation coefficient was 0.92 at P = 0.05 (Figure 1).

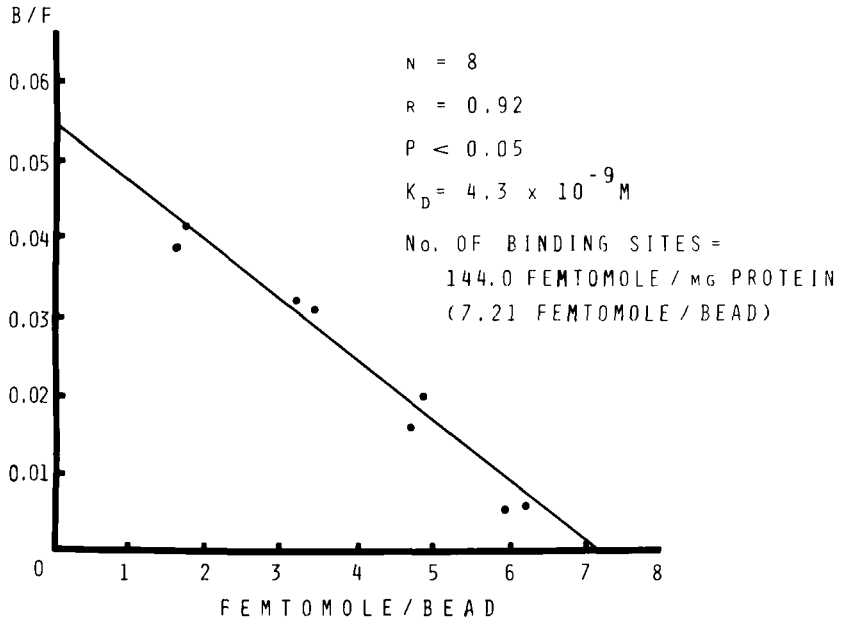

FIGURE 1. Scatchard analysis using one glass bead coupled with calf uterine cytosol receptor. B is the bound hormone; F is the free hormone; N is the number of points plotted (four tracer concentrations in duplicate); R is the correlation coefficient; P is the probability level; K_D is Dissociation constant. The X - axis represents the number of binding sites.

Pulverized calf uterine tissue retained its binding capacity unchanged for three months when stored at -70°C, but began to lose capacity after three weeks at -20°C. By contrast, receptor coupled to glass beads was stable for three months at -20°C and even retained its activity at 4°C for two weeks (Figure 2). During a period of 12 weeks, there was no significant change ($P = 0.05$), providing an interassay precision of 4.6%.

The rate of receptor positive tumors for estrogen was almost similar in pre- and post-menopausal patients, 33% and 44%

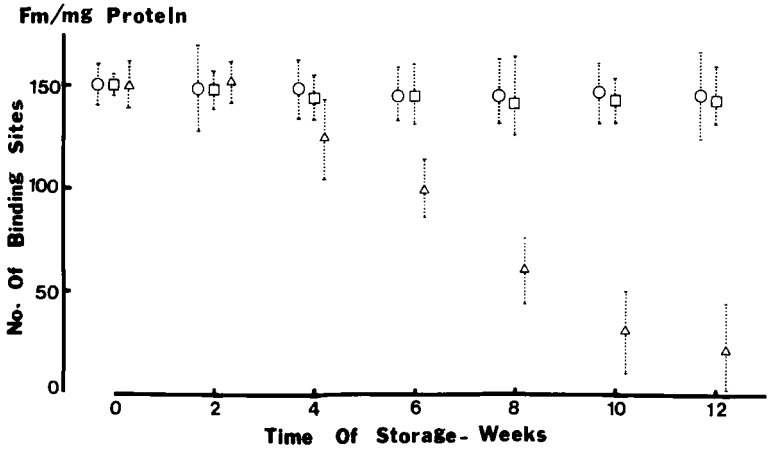

FIGURE 2. Stability of estradiol receptor (E_2R) with time measured at 4°C. ○ Pulverized calf uterine tissue stored in liquid nitrogen; calf uterine cytosol receptor coupled glass beads stored at (i) □ -20°C. (ii) △ 4°C.

respectively. But the mean receptor concentration was less in pre-menopausal (19 fm/mg protein) than in post-menopausal women (38.4 fm/mg protein) (Table 2).

DISCUSSION

The estimation of cytoplasmic estrogen receptor is an established therapeutic strategy for breast cancer treatment (5, 6, 7). Potential uses include prognosis of early recurrence following mastectomy, stratifying patients for adjuvant therapy, and selecting or rejecting endocrine therapy. The greater the estrogen receptor, the higher the rate of response to endocrine therapy. The response rates to endocrine therapy in patients with estrogen receptor negative tumors is less than 10% (8).

TABLE 2

Cytosol Receptors in 149 Patients (Pre- and post-menopausal women; Menopausal age = 45 years).

	Pre-menopausal	Post-menopausal
	# Patients	# Patients
ESTROGEN		
Negative[a]	10	44
Borderline[b]	4	27
Positive[c]	7 (33%)	57 (44%)
Total	21	128
Mean Concentration	19 fm/mg protein	38.4 fm/mg protein

[a] <3 femtomole/mg protein. Remission 0%*.

[b] 3-10 femtomole/mg protein. Remission 50%.

[c] 10 femtomole/mg protein. Remission 66%.

*W. L. McGuire et al. Estrogen Receptors in Human Breast Cancer, Raven Press, 1975: 17-30.

In patients with estrogen positive tumors, the response rate is approximately 55%. There are several controversial reports on the stability of estrogen and progesterone receptors of breast cancer tissue. Some studies report little loss of estrogen receptor activity if stored at -70°C (9, 10); others report up to 75% loss after one month under the same conditions (11, 12). Some qualitative differences between laboratories are explained by differences in thiol reagent content of assay medium and by

the method of homogenization (13). Stable internal standards are essential for monitoring assay performance; and a Reference Powder is available from Dr. J. L. Wittliff, University of Louisville, Kentucky. This material is prepared by powdering calf uteri and does require transportation and storage at -70°C. The glass bead coupled receptor from calf uterine described here has several advantages and provides additional internal standards to monitor the interassay variation due to the reagents, radioisotopes, and methodology used in E_2 receptor assay. The glass bead is uniformly coupled to the receptor and is stable for more than three months at -20°C, and the beads can be prepared easily in any desired quantities. The receptor coupled glass beads are simple and convenient to use.

Our studies with pre- and post-menopausal patients (Table 1) are in agreement with previous reports (6, 14 to 19). It has been shown that the proportion of estrogen receptor positive tumors was almost equal in both pre- and post-menopausal women with breast cancer, but the mean receptor content is less in pre-menopausal than in post-menopausal women.

Current methods in most clinical laboratories employ the DCC or sucrose density gradient procedures for the determination of estrogen receptor. Using DCC, it is not possible to distinguish various forms of cytosol receptors; whereas 3S and 4S receptors are distinguished by sucrose density gradient. Two distinct kinds of high affinity low capacity estrogen receptors designated as Type I and Type II are reported (20, 21). Little

is known on the clinical significance of Type I and Type II receptors, but only the former are measured in the DCC procedure (22). The use of receptor coupled to glass beads provides a stable internal control for the measurement of Type I receptor by DCC assay. Use of these beads for Type II estrogen receptor assay and for progesterone receptor assay is under evaluation.

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