Journal of Chromatography, 359 (1986) 475–487
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 794

INTERACTION OF ESTROGEN RECEPTOR ISOFORMS WITH IMMOBILIZED MONOCLONAL ANTIBODIES

NOBUKO SATO, SALMAN M. HYDER, LEE CHANG, AMY THAIS and JAMES L. WITTLIFF*
Hormone Receptor Laboratory, Department of Biochemistry and James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40292 (U.S.A.)

SUMMARY

High-performance liquid chromatography was performed to separate the various isoforms of estrogen receptor from human breast cancer, based on size (high-performance size-exclusion chromatography) and surface charge (high-performance ion-exchange chromatography) properties. The ability of these isoforms to interact with the monoclonal antibodies was assessed. All isoforms exhibited similar immunodeterminant sites, but when they are bound to [^{125}I]iodoestradiol-17β (IE), only 30% binding of the radioactive complex to the immobilized monoclonal antibodies was observed. However, the mass of the receptor recognized by the antibody bead, via the estrogen receptor-enzyme immunoassay (ER-EIA), was always significantly higher. This was true for both fractionated and non-fractionated cytosols, suggesting that (1) non-ligand binding forms, such as precursors and products of the estrogen receptor, were also recognized; or (2) the ligand was only selecting for a particular conformer(s); or (3) the monoclonal antibody on the bead recognized other proteins associated with estrogen receptor. Ion-exchange fractionation of unlabeled receptor showed loss of immunodeterminant sites. However, size-exclusion fractionation did not show this effect. Diethylstilbestrol, a competitor of IE binding, showed marked stability of receptor recognized by ER-EIA during both size-exclusion and ion-exchange chromatography. Limited trypsin treatment of the receptor caused the loss of immunodeterminant sites without altering the ligand binding sites. Thus, proteolysis of estrogen receptors in cytosols of human breast cancer could easily lead to underestimation by ER-EIA. Although the components with immunodeterminant sites recognized by ER-EIA were always eluted with the ligand-binding isoforms of the estrogen receptor, our data suggest that the concentration of the protein having the epitope associated with the monoclonal antibody is unequal to that recognized by the steroid ligand. We conclude that application of ER-EIA to clinical assays of estrogen receptors clearly needs further clarification.

INTRODUCTION

The presence of specific estrogen receptors in breast cancer tissues is required for response to endocrine manipulation. Clinical determinations of estrogen receptor

0021-9673/86/$03.50

concentration are usually performed by dextran coated charcoal assay and sucrose-density gradient centrifugation. Some patients with estrogen receptor-positive breast cancer do not respond to the endocrine therapy. One of the reasons may be a defect in the synthesis and turn-over of the receptors, leading to heterogeneity[1–4]. Therefore, it will be necessary to elucidate more precisely those breast cancer patients responsive to endocrine therapy.

Recent research with the use of a specific monoclonal antibody for estrogen receptors[5] may provide information useful in determining certain physicochemical properties of these receptor molecules. It has been suggested that there is a correlation between the monoclonal antibody-based assay and the ligand-binding assay for estrogen receptor measurements[6]. Studies reported in this paper will document that the two procedures are not equivalent when the estrogen receptors are first fractionated into their isoforms by high-performance liquid chromatography (HPLC).

We are particularly interested in the natural history of estrogen receptors from human breast cancer tissues, since clinical usefulness of certain molecular forms has been suggested by our earlier work[1,2]. Previously, our laboratory has demonstrated by HPLC that estrogen receptors from human breast cancer and other tissues exhibit polymorphism (isoforms), based on size, shape, and surface charge properties[2–4]. To ascertain the interrelationships of various species of such isoforms, it is essential either to purify the individual proteins or to utilize a probe directed toward specific composition properties of estrogen receptors. Recently, using monoclonal antibodies directed against different immunodeterminants on the estrogen receptor protein, Lorincz *et al.*[7] confirmed our proposal that estrogen receptor complexes exist in multiple forms. Investigating ovarian carcinomas, they demonstrated for the first time that this tumor exhibits receptor heterogeneity. In addition, the monoclonal antibody, which was raised against human breast cancer, recognized a non-steroid binding component.

In the present study, an estrogen receptor-enzyme immunoassay (ER-EIA) method, based on direct antigenic recognition was used for analyses of estrogen receptor isoforms from human breast cancer tissues. We report here that the various estrogen receptor isoforms, as identified by high-performance ion-exchange chromatography (HPIEC) and high-performance size-exclusion chromatography (HPSEC) possessed similar immunodeterminant sites, measured by immobilized monoclonal antibodies. However, the recognition of liganded receptor species was suppressed. In addition, evidence suggests that at least some components containing antibody-recognized sites (epitopes) do not possess ligand binding sites. Furthermore, our investigation describes the influence of trypsin treatment of receptors on their HPSEC profile, as determined by both a radioligand binding assay and by a monoclonal antibody assay. The latter data suggest that the application of ER-EIA in routine clinical assays needs further investigation.

EXPERIMENTAL

Materials

The ligand, [16α-^{125}I]iodoestradiol-17β (2000–2200 Ci/mmol) (IE) was purchased from Du Pont/NEN Products (Boston, MA, U.S.A.). Unlabeled diethylstilbesterol (DES) was used as an estrogen competitor. Norit A, Dextran T-70, sodium

molybdate, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO, U.S.A.). All reagents were of analytical grade. Trypsin (Type V) and trypsin inhibitor were also obtained from Sigma. ER-EIA Monoclonal kits were purchased from Abbott Labs. (Chicago, IL, U.S.A.).

Preparation of the cytosol

Human breast cancer tissue was obtained through pathologists at local hospitals. Human tissues were homogenized at 1:10 (w/v) in P_{10}EDG buffer [10 m*M* potassium phosphate (pH 7.4), containing 1.5 m*M* Na_2EDTA, 1 m*M* DTT, 10% (v/v) glycerol] with a Brinkmann (Westbury, NY, U.S.A.) Polytron by two 10-sec bursts. To study the influence of buffers on receptor monoclonal antibody interactions, other buffer compositions were used. These are indicated in the appropriate figure legends. For HPLC analysis, the homogenization ratio was increased to 1:2.5 (w/v). The homogenate was then centrifuged at 105 000 *g* in a Beckman (Palo Alto, CA, U.S.A.) rotor (Ti 70.1) for 30 min. These procedures were performed at 0–4°C. The supernatant obtained was referred to as cytosol. The cytosol was used immediately in binding reactions for estrogen receptors[1–2]. Protein concentration of cytosol was determined by the methods of Waddell[8] or Lowry[9], using bovine serum albumin (BSA) as standard.

Radiolabeling of estrogen receptors from human breast cancer

The freshly prepared cytosol from human breast cancers was incubated with 3 n*M* IE for 2–4 h or overnight at 0°C, depending upon the experiment. This incubation was conducted in the presence (non-specific binding) or absence (total binding) of a 200-fold molar excess of a non-radioactive competitor, DES[1,2]. After the incubation, the mixture was mixed in a Vortex mixer with a precipitate of the same volume of dextran-coated charcoal suspension (DCC) (1% of Norit A and 0.1% Dextran T-70) in P_{10}EDG buffer to remove unbound steroid. After centrifugation at 2000 *g* for 5–10 min at 0°C, the labeled protein-bound fraction (supernatant) obtained was used to measure radioactivity. Then it was used for determination of the estrogen receptor concentration by the ER-EIA method and for analyses of estrogen receptor isoforms by HPSEC[10] or HPIEC[3,11].

Limited tryptic digestion of estrogen receptors from human breast cancer

The cytosol from cancer tissues was labeled with 3 n*M* IE, in either the absence or the presence of a 200-fold excess of DES at 4°C. After 2–4 h of incubation, one reaction mixture of labeled cytosol was adjusted to 40 μg trypsin/mg cytosol protein, while the other one was kept as a control. Mixtures were incubated further for 1 h at 4°C and then the tryptic digestion was stopped by adding soybean trypsin inhibitor (2.5 × trypsin concentration). Reaction mixtures were kept at 4°C for an additional 30 min, then treated with a DCC pellet, derived from equal volume of 1% (w/v) DCC, to remove the free from the protein-bound steroid. The bound fraction (*i.e.* IE-receptor) was used for HPSEC analyses[10] and ER-EIA.

High-performance ion-exchange chromatography

Aliquots of unlabeled (steroid-free) or IE-labeled cytosol were applied to a polyamine-coated silica-based AX-1000 anion-exchange column (SynChrom, Lin-

den, IN, U.S.A.), previously equilibrated with P_{10}EDG buffer[3]. Chromatography was performed in a cold-box at 0–4°C. The column was washed with 10 ml of P_{10}EDG buffer, and the receptor protein was eluted with a linear gradient from 10 to 500 m*M* potassium phosphate, generated by mixing P_{500}EDG with P_{10}EDG by using a two-pump solvent delivery system controller (Beckman, Model 421)[4,11]. A flow-rate of 1.0 ml/min was used for all experiments. Fractions of *ca.* 3 ml were collected, using an ISCO (ISCO, Lincoln, NE, U.S.A.) fraction collector. IE radioactivity in the fractions was counted in a Micrometics (Rohm and Hass, Cleveland, OH, U.S.A.) gamma counter with 60% counting efficiency or with a Model 170 flow-through detector (Beckman)[11]. Concentration of antigenic determinants in the HPLC fractions was measured by ER-EIA. Another aliquot was kept unfractionated to determine the concentration of immunodeterminant sites in the cytosol prior to HPLC analysis.

High-performance size-exclusion column chromatography

Chromatography was performed in the cold room at 0–4°C. IE-labeled cytosol, which had been incubated in either the presence or the absence of DES, as well as unlabeled aliquots were analyzed by HPSEC, using Spherogel TSK-3000 SW (Toyo-Soda, Japan) columns, equilibrated with P_{50}EDG buffer containing 0.5% propanol (pH 7.4)[4,10]. Elution was carried out at a flow-rate of 0.7 ml/min. Fractions (1.75 ml) were collected, and both the radioactivity and antigenic immunodeterminants in each fraction were determined as described in the previous section.

Estrogen receptor-enzyme immunoassay

The enzyme-linked immunological assay was used to quantify estrogen receptors in unfractionated cytosol or in samples fractionated by HPIEC and HPSEC. The EIA, which is purported to measure receptor content (mass) (Fig. 1), is based on a sandwich technique, which involves two monoclonal antibodies (MAb) recognizing different sites on the estrogen receptor protein[5,6]. It should be noted that steroid binding sites on receptors associated with the immobilized MAb (D-547) on the polystyrene bead may be assessed by placing the bead in a gamma counter and measuring bound radioactivity.

Either unfractionated or fractionated cytosol (100 μl) was added to 100 μl of specimen diluent buffer (Abbott Labs). Beads containing the immobilized monoclonal antibody were then placed into each mixture and incubated for 18 h at 2–4°C. After incubation, each bead was washed twice with 4–6 ml of deionized water. When IE-labeled samples were used, radioactivity on each bead was determined directly. The second antibody (200 μl), which was H-222 linked to horseradish-peroxidase as the marker in ER-EIA, was then added to the bead and incubated for 1 h at 37°C (as recommended by the manufacturer, Abbott Labs.). After washing each bead twice with deionized water, 300 μl of *o*-phenylenediamine substrate solution was added to each bead and incubated for 30 min at room temperature. The reaction was stopped by the addition of 1 *N* sulfuric acid, and the intensity of color was read at 492 nm in a Quantum spectrophotometer provided by the manufacturer. A standard curve was also constructed in a similar manner with the standard solutions provided. Results were calculated on the basis of the standard curve and expressed as femtomoles of estrogen bound per milligram of cytosol protein or per fraction.

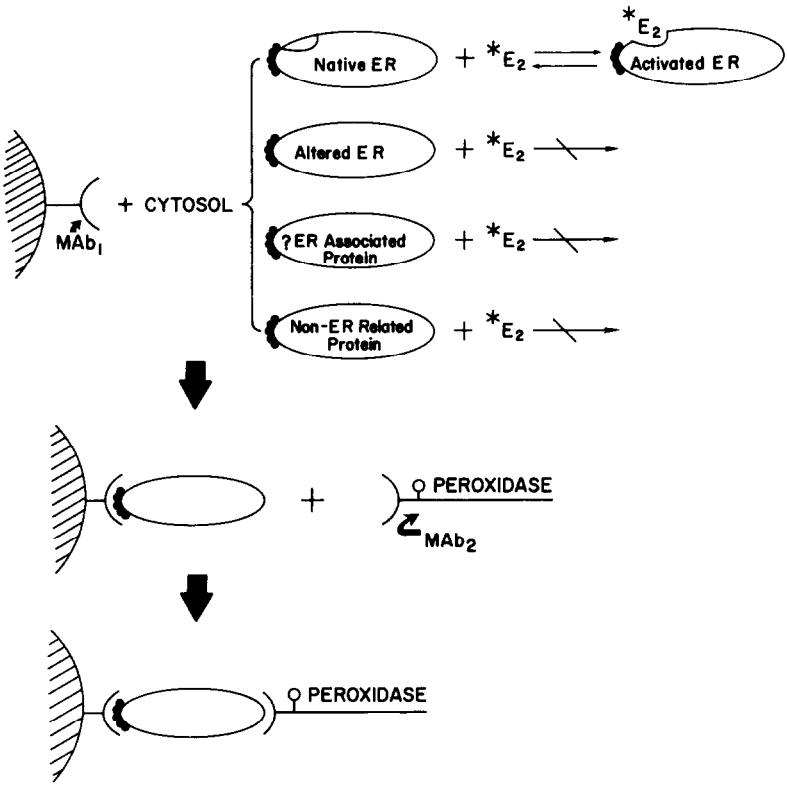

Fig. 1. Schematic representation of enzyme-linked immunoassay for estrogen receptors using monoclonal antibodies. MAb_1 = monoclonal antibody 1; MAb_2 = monoclonal antibody 2, linked to peroxidase; $*E_2$ = radiolabeled estradiol-17β; ER = estrogen receptor. Note: components containing the epitope recognized by the MAb may or may not contain the steroid-binding site.

RESULTS AND DISCUSSION

The important studies of Greene and Jensen[5] have been directed towards the analysis of estrogen receptor molecules with monoclonal antibodies. The availability of such antibodies has opened a new means of recognition of steroid hormone receptors in hormone-sensitive tumors. Earlier, we demonstrated that estrogen receptors exhibit heterogeneity[1–4]. In the course of raising antibodies to an estrogen receptor and its subsequent use in the analysis of steroid-sensitive tumors, one must demonstrate that the monoclonal antibody recognizes all of the receptor isoforms selectively in the presence of numerous other protein molecules which are present in cytosol. Furthermore, this must be accomplished in a quantifiable fashion that agrees with established ligand binding procedures used in assays of breast tumor biopsies. To approach the problem systematically, we have performed HPLC separations of estrogen receptors to demonstrate receptor heterogeneity and then assessed the ability of the various isoforms to interact with the monoclonal antibodies. Fig. 2 describes the experimental set-up for analyzing both receptor content (ER-EIA) and receptor ligand binding capacity.

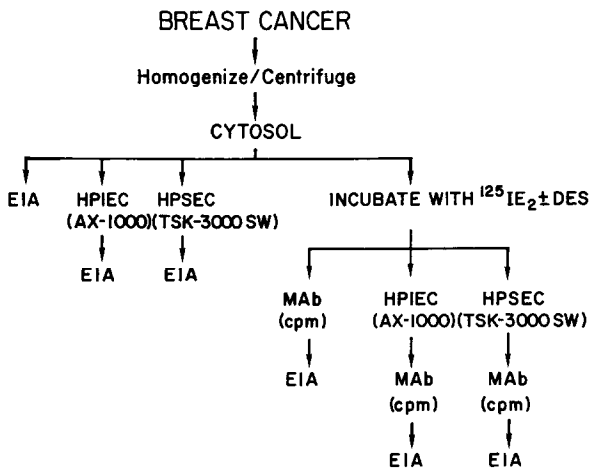

Fig. 2. Experimental protocol for isolation of estrogen receptor isoforms by HPLC and assessment of their content by the monoclonal antibody assay (EIA). Two types of reaction were evaluated: those which had not been associated with [^{125}I]iodoestradiol-17β ± DES (shown on the left) and those first associated with the labeled steroid and then analyzed (shown on the right). Labeled steroid receptor complexes, associated with the MAb-coated beads, were evaluated for radioactivity by gamma counting in a detector, labeled MAb (cpm).

Interaction of estrogen receptor isoforms with monoclonal antibodies, following HPIEC analyses

Under the conditions established in our laboratory, we routinely use phosphate buffer for the separation and analysis of estrogen receptors by means of HPLC. However, the buffer recommended by the manufacturers of the ER-EIA kit (Abbott Labs.) includes 10 m*M* Tris, containing 1.5 m*M* EDTA, 5 m*M* Na_2MoO_4, and 1 m*M* monothioglycerol. Therefore, we have compared the two buffer systems prior to our studies involving measurement of receptors either prior to or following separation by HPLC. Our results showed that the recognition of the receptor by the MAb-coated bead was higher (130%) in the Abbott buffer than in the P_{10}EDG buffer. However, the assumption is made at this stage that the MAb is recognizing only estrogen receptors. The presence of molybdate in the Abbott buffer may be the source of this difference, since the molybdate ion has been known to preserve estrogen receptor quantity and quality over long incubation periods[12,13]. Molybdate may also extract receptor owing to the increased ionic strength which it introduces into the buffer. Nevertheless, when IE-labeled receptor, prepared with either buffer, was allowed to interact with the MAb-coated bead, only 30% was found to be associated with the MAb, irrespective of buffer type. Thus, both buffer systems may be employed for ER-EIA experiments.

Previously, we have demonstrated by HPIEC and HPSEC analyses that estrogen receptors in breast cancer exhibit heterogeneity[1–4]. In the present investigation, we assessed the interaction of these isoforms with monoclonal antibodies, immobilized on polystyrene beads. Isoforms of the cytosolic estrogen receptor in human breast cancer tissues were fractionated by HPIEC. To identify estrogen receptors, IE was used as the ligand, because its high specific radioactivity can be either monitored during HPLC[11] or measured efficiently after HPLC by manual counting.

The data in Fig. 3A show the HPIEC profile of ionic forms of IE-receptor complexes from human breast cancer, obtained by anion-exchange chromatography with a gradient of 10–500 m*M* of potassium phosphate. Three different isoforms of estrogen receptor were observed: (1) a minor component (referred to as peak I), which was eluted by a low salt concentration of *ca.* 50 m*M* potassium phosphate (fractions 7–9); (2) a major component (referred to as peak II), which was eluted with *ca.* 115 m*M* phosphate (fractions 10–12); and (3) an additional component (referred to as peak III) which was eluted with *ca.* 200 m*M* phosphate (fractions 13–15). IE binding to an identical sample of cytosol was inhibited with a 200-fold excess of the estrogenic compound, DES (Fig. 3A). This result indicates that these three ionic species were specific isoforms of the estrogen receptor.

To investigate the interaction between these isoforms and monoclonal antibodies, aliquots from each of the HPIEC fractions were allowed to react with immobilized monoclonal antibodies. Following overnight incubation, the radioactivity on MAb-coated beads was analyzed first and then the same beads were tested by the ER-EIA method to measure the concentration of estrogen receptor (Fig. 3A and B).

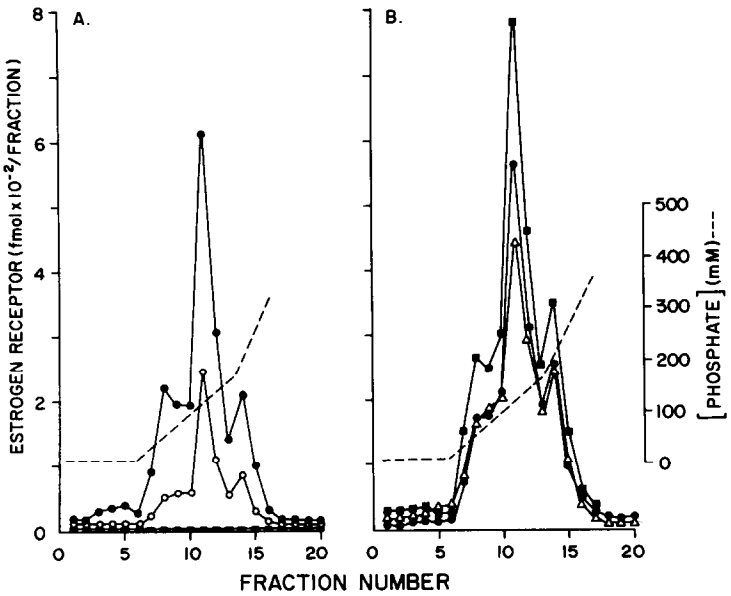

Fig. 3. Interaction of estrogen receptors from human breast cancer with monoclonal antibodies, following HPIEC analysis. The cytosol from breast cancer was incubated with 3 n*M* [^{125}I]iodoestradiol in the presence or absence of a 200-fold excess of DES. Elution of samples from the column was performed with a gradient of potassium phosphate (dashed line). After HPIEC separation, eluates from the column were used for determination of total receptor-bound radioactivity, radioactivity on the MAb-coated bead, and estrogen receptor concentration by ER-EIA. When using the unlabeled sample of an identical cytosol for HPIEC, estrogen receptor concentration in the eluate from the column was determined by ER-EIA. Panel A shows the number of steroid binding sites, determined by radioactivity measurements: (○) [^{125}I]iodoestradiol radioactivity on the MAb-coated bead; (●) radioactivity of [^{125}I]iodoestradiol isoform complexes in solution; (■) radioactivity of DES-containing sample, labeled with [^{125}I]iodoestradiol. Panel B shows the estrogen receptor concentration determined by EIA: (△) measurements of the unlabeled sample; (●) sample previously labeled with [^{125}I]iodoestradiol; (■) the fractions labeled with DES and [^{125}I]iodoestradiol.

In Fig. 3A (o), it may be seen that the bead-bound radioactivity coincided with that separated by HPIEC (ER isoforms). However, only 30% of the total receptor radioactivity was associated with the MAb-coated bead. An obvious conclusion from this result is that the MAb failed to recognize the entire population of estrogen receptors. Nevertheless a consistent proportion of each receptor isoform appeared to possess the same immunodeterminant site. It may be argued that, following HPLC, certain immunodeterminant sites were destroyed or that following MAb association, there was an alteration in the affinity of the receptor for MAb or IE. It is also possible that receptor preparations may contain proteases which could remove antibodies from the bead containing the receptor.

We have ruled out these possibilities[14] and maintain that this reduction in MAb recognition of the receptor is due to the IE ligand associated with the estrogen receptor. Firstly, binding of [^{3}H]estradiol (the native ligand) to the receptor leads to a greater (40%) recognition of the receptor by the bead. The ligand, IE, used in the HPLC studies is a large, hydrophobic molecule and may mask the immunodeterminant site to some extent. Such ligands may also shift the receptor conformation equilibrium towards a form not recognized by MAb. The inhibition by IE of receptor binding to the MAb bead was not due to HPIEC, since this was also observed in the aliquot that was not fractionated (40% binding to the bead in the reported experiment). We have also shown by Sephadex LH-20 (Pharmacia, Piscataway, NJ, U.S.A.) chromatography and HPIEC that IE in the fraction from the bead was bound to a macromolecule.

When the radiolabeled receptor associated with the MAb (Fig. 3A) was compared by EIA with the total receptor mass detected on the same bead (Fig. 3B), it was observed that three times as much receptor was on the bead. However, the ER-EIA profile was the same as that observed for elution from the column of radioactivity bound to macromolecules. Since a large amount of labeled receptor was not precipitated by the MAb bead, it appears the MAb also recognized (1) a non-steroid binding form of estrogen receptor, or (2) precursors and products of the receptor, or (3) non-receptor proteins, showing similar immunodeterminant sites. The possibility remains that a component of the immunodeterminant site is tightly associated with the steroid-binding component of the estrogen receptor. As it binds to the MAb-coated bead, the steroid-binding component is released. In this context, estrogen receptor-negative tissues do not show positive results by ER-EIA. Coffer and coworkers[15,16] have shown that a monoclonal antibody, previously believed to be raised against estrogen receptor from human myometrium, was, in fact, recognizing a component present in estrogen receptor-positive human breast tumors which they believe was regulated in tandem with the estrogen receptor. Interestingly, Joab *et al.*[17] have shown by antibody measurements that a common non-steroid-binding subunit is associated with steroid receptors. Murayama *et al.*[18] have provided evidence for a number of proteins associated with estrogen receptors in the calf uterus. Therefore, the possibility must be ruled out that antibody is produced against any of these receptor-associated proteins. Lorincz *et al.*[7] have also demonstrated the presence of non-steroid-binding components, recognized by the monoclonal antibody prepared against estrogen receptor.

Interestingly, the chromatographic behavior of DES-containing samples, labeled with IE, was similar to that of IE-labeled samples (Fig. 3B) when ER-EIA

measurements were used. This result indicates that DES did not inhibit recognition of the immunodeterminant sites. This appears reasonable since monoclonal antibodies recognize receptors in which steroid-binding sites are occupied by endogenous estrogen (DeSombre *et al.*[19]).

Thus, immunodeterminant sites are distinguishable from steroid-binding sites. As determined by ER-EIA, the estrogen receptor concentration in samples containing DES and IE was higher than that of samples containing only IE. The presence of IE without DES results in an underestimation of immunodeterminant sites indicating that iodine partially interferes with the ER-EIA measurement of the receptor. This underestimation is probably due to the difference in molecular size of DES and IE. This observation is supported by results which show that the estrogen receptor concentration by ER-EIA in [^{3}H]estradiol-labeled cytosol is higher than that with IE-labeled cytosol[14]. The concentration of receptor in cytosol recognized by the MAb was less when either steroid was present than with unlabeled cytosol. Therefore, the steroid-binding site appears close to the immunodeterminant site.

To avoid the interference of ligand, fractionated, unliganded cytosol was also used for interaction between estrogen receptor and monoclonal antibody. The total concentration of estrogen receptor in an unliganded sample by ER-EIA was lower than that of either IE-labeled cytosol or cytosol containing both IE and DES (Fig. 3B).

Interaction of estrogen receptor with monoclonal antibodies, following HPSEC analyses

Our previous paper demonstrated that HPSEC on TSK-3000 SW columns was useful in the detection of receptor heterogeneity[10]. In order to investigate the chromatographic characteristics of estrogen receptor from human breast cancer tissues, aliquots of a receptor preparation identical with that used in HPIEC were also analyzed on an HPSEC column and followed by the ER-EIA method (Fig. 2).

Fig. 4A shows the elution profile of radioactivity in IE-labeled sample following HPSEC. A radioactive peak was observed corresponding to a high-molecular-weight species eluted just after the void volume with a shoulder at fraction 9. By HPSEC, based on size and shape of proteins only one isoform was observed, compared with three isoforms seen on HPIEC of the same cytosol.

Further characterization of the estrogen receptor in HPSEC fractions was accomplished with ER-EIA. As shown in Fig. 4A, chromatographic analyses revealed a single radioactive component associated with MAb-coated beads. As seen by HPIEC, the receptor-bound radioactivity on the MAb-coated beads was one-third of its initial level. No specific steroid binding was observed in the presence of DES.

Fig. 4B gives a representative HPSEC profile of estrogen receptor concentration on MAb-coated beads by ER-EIA in either labeled or unliganded samples (Fig. 2). The estrogen receptor concentrations of unliganded, IE-labeled and DES-containing, IE-labeled samples in the major peak (fraction 6) were 1431, 958 and 1276 fmol/fraction, respectively. In contrast to HPIEC analyses, the estrogen receptor concentration of unliganded cytosol was found to be higher than that of IE-labeled cytosol. This appears to be due to the mild chromatographic conditions (50 m*M* phosphate) during HPSEC and this is supported by the fact that unliganded cytosols could be labeled after fractionation by HPSEC to obtain the same receptor concentrations as obtained by fractionation of previously labeled cytosol.

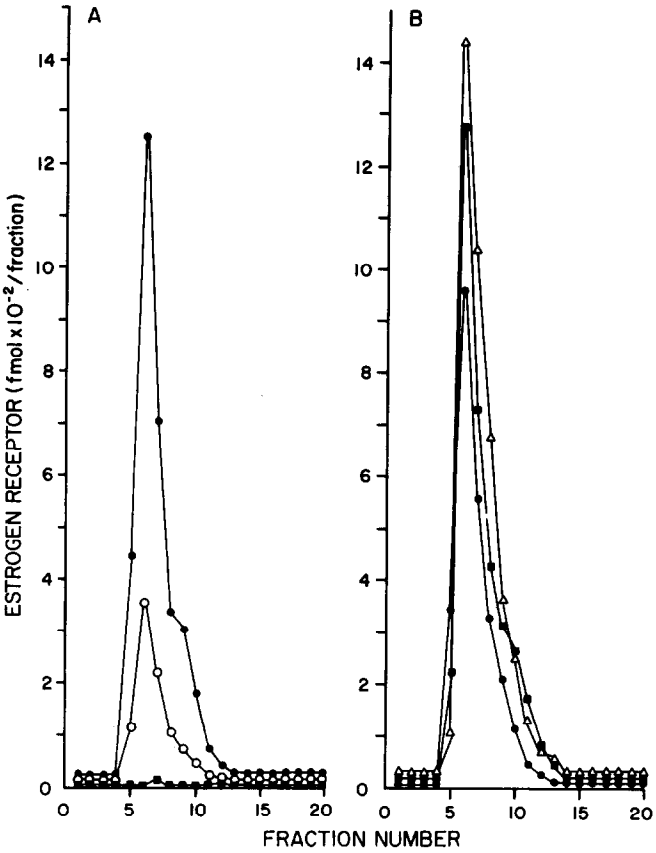

Fig. 4. Interaction of estrogen receptors with monoclonal antibodies, following HPSEC analysis. Aliquots of an identical receptor preparation used for HPIEC analyses were applied to a HPSEC column. HPSEC was performed as described under Experimental. Column eluates of unlabeled and [^{125}I]iodoestradiol-labeled cytosols were treated as described in the legend of Fig. 3. Panel A shows the number of steroid-binding sites, determined by the presence of radioactive IE: (●) [^{125}I]iodoestradiol-isoform complexes in solution; (○) [^{125}I]iodoestradiol-isoform complexes on the MAb-coated bead; (■) fractions labeled with DES and [^{125}I]iodoestradiol indicating non-specific binding. Panel B shows the estrogen receptor concentration determined by ER-EIA: (△) measurements of the unlabeled sample; (●) sample previously labeled with [^{125}I]iodoestradiol; (■) fractions labeled with DES and [^{125}I]iodoestradiol.

The estrogen receptor concentration of DES-containing IE-labeled samples was higher than that of IE-labeled samples. This again suggests that DES, which occupies the steroid-binding sites of the estrogen receptor, did not interfere with the recognition of immunodeterminant sites of the estrogen receptor by the monoclonal antibody. This observation is in accordance with the previously shown HPIEC data (Fig. 3B) regarding the estrogen receptor concentration measured by ER-EIA.

Influence of trypsin treatment on the HPSEC profile of estrogen receptors from human breast cancer tissues

It has been reported that limited proteolysis, either with trypsin or chymotrypsin, results in fragmentation of the glucocorticoid receptor, which consists of

steroid-binding, DNA-binding, and immunological determinant domains[20]. Thus, trypsin was used for limited proteolysis of the estrogen receptor. Trypsin-treated cytosol from human breast cancer was prepared, as described under Experimental. Control or trypsin-treated cytosol, labeled with IE, was applied to a TSK-3000 SW column for HPSEC. Fig. 5A shows the receptor-associated radioactivities of non-treated and trypsin-treated cytosol. Trypsin treatment of IE-labeled cytosol resulted in a shift in the elution of the peak at fraction 6 to a component at fraction 9 without loss of IE-binding sites. Thus, the labeled estrogen receptor was converted into a smaller molecule, retaining the steroid-binding domain. This result indicates that trypsin may have dissociated components containing steroid-binding sites from the intact estrogen receptor molecule. Components with immunodeterminant sites were probably destroyed, since they were not detected after HPSEC. This is in contrast to the experience with the vitamin D receptor, where trypsin treatment separated the immunodeterminant sites from steroid recognition sites[21].

When a trypsin-treated cytosol, containing a 200-fold excess of DES, was separated by HPSEC, no bound radioactivity was detected. Therefore, the components appearing after trypsin treatment contain the steroid-binding sites specific for estro-

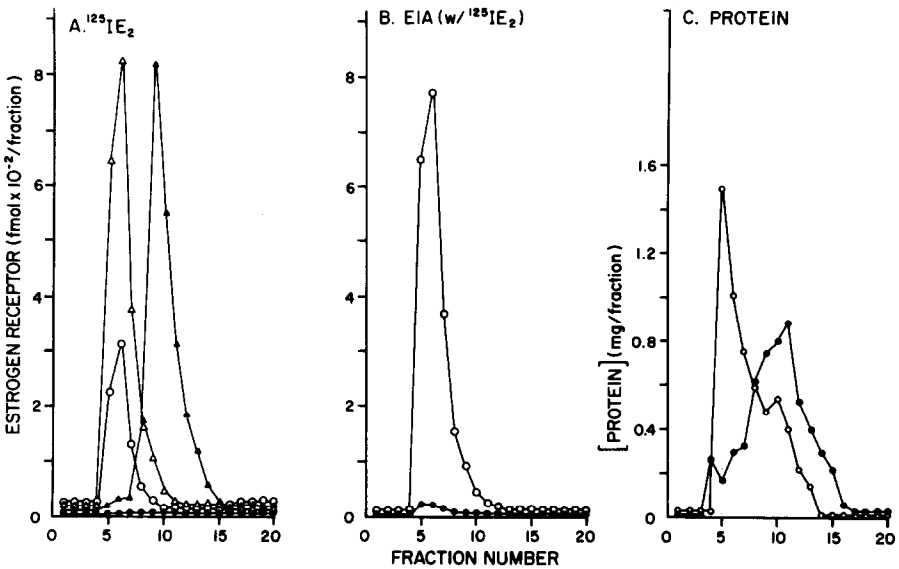

Fig. 5. Influence of limited trypsin digestion of estrogen receptors on the concentration of steroid binding and immunodeterminant sites. Cytosol from breast cancer was labeled with 3 n*M* [^{125}I]iodoestradiol in the presence or absence of a 200-fold excess of DES. After a 2-h incubation, labeled cytosol was treated with trypsin, as described under Experimental. Aliquots of untreated or trypsin-treated samples were applied to a HPSEC column. HPSEC separation was performed as described under Experimental. Eluates from the column were used for determination of radioactivity, ER-EIA, and protein concentration. Panel A shows the number of steroid-binding sites, determined by radioactivity: (△) bound radioactivity in the untreated sample; (▲) bound radioactivity in the trypsin-treated sample; (○) radioactivity on the MAb-coated bead of the untreated sample; (●) radioactivity on MAb-coated bead of the trypsin-treated sample. Panel B shows the estrogen receptor concentration, determined by ER-EIA: (○) untreated sample; (●) trypsin-treated sample. Panel C shows the protein concentration after HPLC of the untreated sample (○) and of the trypsin-treated sample (●).

gen receptor. In the untreated cytosol, a profile of receptor-bound radioactivity on the MAb-coated bead corresponded to that of total receptor-associated radioactivity (Fig. 5A). The amount on the MAb-coated bead was again approximately one-third of the total radioactivity.

Fig. 5B shows the concentration of estrogen receptor by the ER-EIA method in IE-labeled cytosol with or without trypsin treatment. No immunoactive peak was observed by HPSEC after trypsin treatment. However, the immunoactivity of IE-labeled sample without trypsin treatment corresponded quantitatively to the specifically bound radioactivity. This indicates that steroid-binding sites remained after trypsin digestion, whereas immunoactive sites were lost.

The effect of trypsin treatment on the elution profile and protein concentration in the fractions from HPSEC was also studied. As shown in Fig. 5C, two peaks were observed. The first peak shows the protein concentration of untreated cytosol. After trypsin treatment, this peak is shifted to low-molecular-weight components. This corresponded to the movement of the receptor species from high to low molecular weight. This is an important observation, since most human breast tumors are rich in protease activity, which may result in an underestimation of receptor content, as measured by ER-EIA, due to proteolytic activity in the cytosol. Again, MAb-bead incubation time was 18 h, which is significant in terms of proteolysis. Time course data (not shown) show saturation within this time period and argue against proteolysis of immunoglobulin from the MAb-coated bead under our experimental conditions.

CONCLUSION

We have demonstrated that all estrogen receptor isoforms, whether separated on the basis of surface charge or size, recognize the MAb-coated bead to a similar extent. However, the absolute concentration recognized by the MAb appears to be ligand-dependent. It was maximal when the competitor, DES, was used (MAb bead-binding capacity showed the following relationship: unliganded > DES > [^{3}H]estradiol-17β > IE). Furthermore, after HPIEC, the presence of ligand on the receptor provided a greater stability to the complex than when unliganded forms were separated. The results indicate a close relationship between the ligand-binding site and the immunodeterminant site. However, the results do not confirm that the immunodeterminant site and the ligand-binding site reside on the same protein. Monoclonal antibody assay of the ligand-bound receptor suggests that non-steroid binding components are recognized by the MAb bead. It is not known whether these represent receptor precursors or products or other proteins with similar immunodeterminant sites as those of the estrogen receptors.

Regardless, we have now described the presence of intrinsic protein kinase (autophosphorylating) activity of the components associated with the immobilized MAb D-547[22]. Using [γ-^{32}P]ATP as substrate, three protein components separating at 57, 47 and 43 kD by SDS-PAGE were phosphorylated[22]. The significance to the results reported in this study is under investigation.

Finally, limited proteolysis with trypsin removed the immunodeterminant domain without interfering with the ligand-binding domain. This suggests that the estrogen receptor concentration, measured by ER-EIA, may be incorrectly estimated

in tumors possessing a high proteolytic activity. Therefore, the latter data and the ligand-dependent variation in immunorecognition clearly suggests the use of immobilized monoclonal antibodies for the detection of estrogen receptors for diagnostic purposes.

ACKNOWLEDGEMENTS

We thank Drs. J. G. Kuhns, S. E. Wolfson, R. S. Howell, L. L. Ogden, II and G. H. Barrows for their cooperation in the collection of human breast cancer biopsies. This research was supported in part by USPHS Grant CA-42154 from the National Cancer Institute, by Marie Overbey Memorial Award from the American Cancer Society and the Phi Beta Psi Sorority. N.S. is a visiting investigator from Iwate Medical University, Morioka, Japan. S.M.H. is a recipient of a research award from the Graduate School, University of Louisville. L.C. is a visiting investigator from Wannan Medical College, Wuhu, City, PROC. A.T. is a research fellow from Centre College, Danville, KY.

REFERENCES

1 J. L. Wittliff, *Cancer*, 53 (1984) 630.
2 J. L. Wittliff, P. A. Feldhoff, A. Fuchs and R. D. Wiehle, in R. Soto, A. F. DeNicola and J. A. Blaquier (Editors), *Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action*, Alan R. Liss, New York, 1981, p. 375.
3 R. D. Wiehle and J. L. Wittliff, *J. Chromatogr.*, 297 (1984) 313.
4 J. L. Wittliff, N. A. Shahabi, S. M. Hyder, L. A. van der Walt, L. Myatt, D. M. Boyle and Y.-J. He, in J. L'Italien (Editor), *Modern Methods in Protein Chemistry*, Plenum, New York, in press.
5 G. L. Greene, C. Nolan, J. P. Engler and E. V. Jensen, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 5115.
6 D. M. Mirecki and V. C. Jordan, *Lab. Med.*, 16 (1985) 287.
7 M. A. Lorincz, J. A. Holt and G. L. Greene, *J. Clin. Endocrinol. Metab.*, 61 (1985) 412.
8 W. J. Waddell, *J. Lab. Clin. Med.*, 48 (1956) 311.
9 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 R. D. Wiehle, G. E. Hofmann, A. Fuchs and J. L. Wittliff, *J. Chromatogr.*, 307 (1984) 39.
11 D. M. Boyle, R. D. Wiehle, N. A. Shahabi and J. L. Wittliff, *J. Chromatogr.*, 327 (1985) 369.
12 M. K. Dahmer, P-R. Housley and W. B. Pratt, *Ann. Rev. Physiol.*, 46 (1984) 67.
13 W. B. Mujaji, N. A. Shahabi, L. A. van der Walt, S. M. Hyder and J. L. Wittliff, presented at the *Fifth International Symposium on HPLC of Proteins, Peptides and Polynucleotides*, 1985, Abstract 1018.
14 S. M. Hyder, N. Sato, L. Chang and J. L. Wittliff, *Endocrinology*, in preparation.
15 A. I. Coffer, K. M. Lewis, A. J. Brockas and R. J. B. King, *Cancer Res.*, 45 (1985) 3686.
16 A. I. Coffer, G. H. Spiller, K. M. Lewis and R. J. B. King, *Cancer Res.*, 45 (1985) 3694.
17 I. Joab, C. Radanyi, M. Renoir, T. Buchou, M-G. Catelli, N. Binart, J. Mester and E.-E. Baulieu, *Nature (London)*, 308 (1984) 850.
18 A. Murayama, F. Fukai, T. Hazato and T. Yamamoto, *J. Biochem.*, 88 (1980) 955.
19 E. R. DeSombre, G. L. Greene, W. J. King and E. V. Jensen, *Prog. Clin. Biol. Res.*, 142 (1984) 1.
20 J. Carlsted-Duke, Ö. Wrange, S. Dkret and J.-Å. Gustafsson, *Biochem. Pharmacol.*, 33 (1984) 913.
21 E. A. Allegretto and J. W. Pike, *J. Biol. Chem.*, 260 (1985) 10 139.
22 A. Baldi, S. M. Hyder, N. Sato, D. M. Boyle and J. L. Wittliff, in D. Puett *et al.* (Editors), *Advances in Gene Technology: Molecular Biology of the Endocrine System, ICSU, Short Reports, Vol. 4*, Cambridge Univ. Press, Cambridge, 1986, p. 264.