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OESTROGEN RECEPTOR ASSAY

False positive analysis?

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Abstract

The influence of unlabelled oestradiol, DES, testosterone and R-5020/org 2058 on tritiated oestradiol binding was investigated in 162 ER positive cases of patients with primary breast carcinoma. A dextran-coated charcoal as well as a sucrose gradient method was applied. In 122 cases only unlabelled oestradiol and DES significantly displaced the binding of labelled oestradiol. In the remaining 40 cases, oestradiol, DES, as well as testosterone and R-5020/org 2058 were able to displace the high-affinity, saturable binding of tritiated oestradiol equally. Possible explanations of this new discovery are discussed.

Based on retrospective investigations it is now generally accepted that oestrogen receptor (ER) assays are valuable methods for selection of patients with advanced breast carcinoma for endocrine therapy (9).

One of the major problems, however, is that one-third of the patients with ER rich tumours do not respond to endocrine therapy. Possible explanations have been raised, such as tumour heterogeneity (for review, see 23) and non-functional receptors on either the cytoplasmatic or the nuclear level (13).

According to HORWITZ et coll. (8) determination of the progesterone receptor content (P_GR) in the tumours would probably discover which tumours contained functional oestrogen receptors. The proposal arose from the fact that progesterone receptor synthesis is dependent on an intact oestrogen receptor mechanism in normal target tissue (8), and over the years data have accumulated that to some extent support this hypothesis. It has been shown that patients with both ER and P_GR in their tumours have a significantly better chance of responding to endocrine therapy than those who harbour ER alone (12).

The first step in the oestrogen action on a target cell is a saturable, high-affinity binding to the ER. A third characteristic is that this binding is ligand specific. Therefore, a receptor is expected to display high affinity for a specific hormone or class of hormones. Thus, hormones of the same class should compete effectively for their receptor while not affecting other receptor systems.

Normally, routine analysis of ER in breast tumour tissue only measures its saturability and its affinity for oestrogen, but does not include an investigation of the ligand specificity as regards different classes of hormones.

The present study was therefore carried out in an attempt to evaluate the following problem: Is the saturable, high-affinity oestrogen binding in breast tumours always ligand specific?

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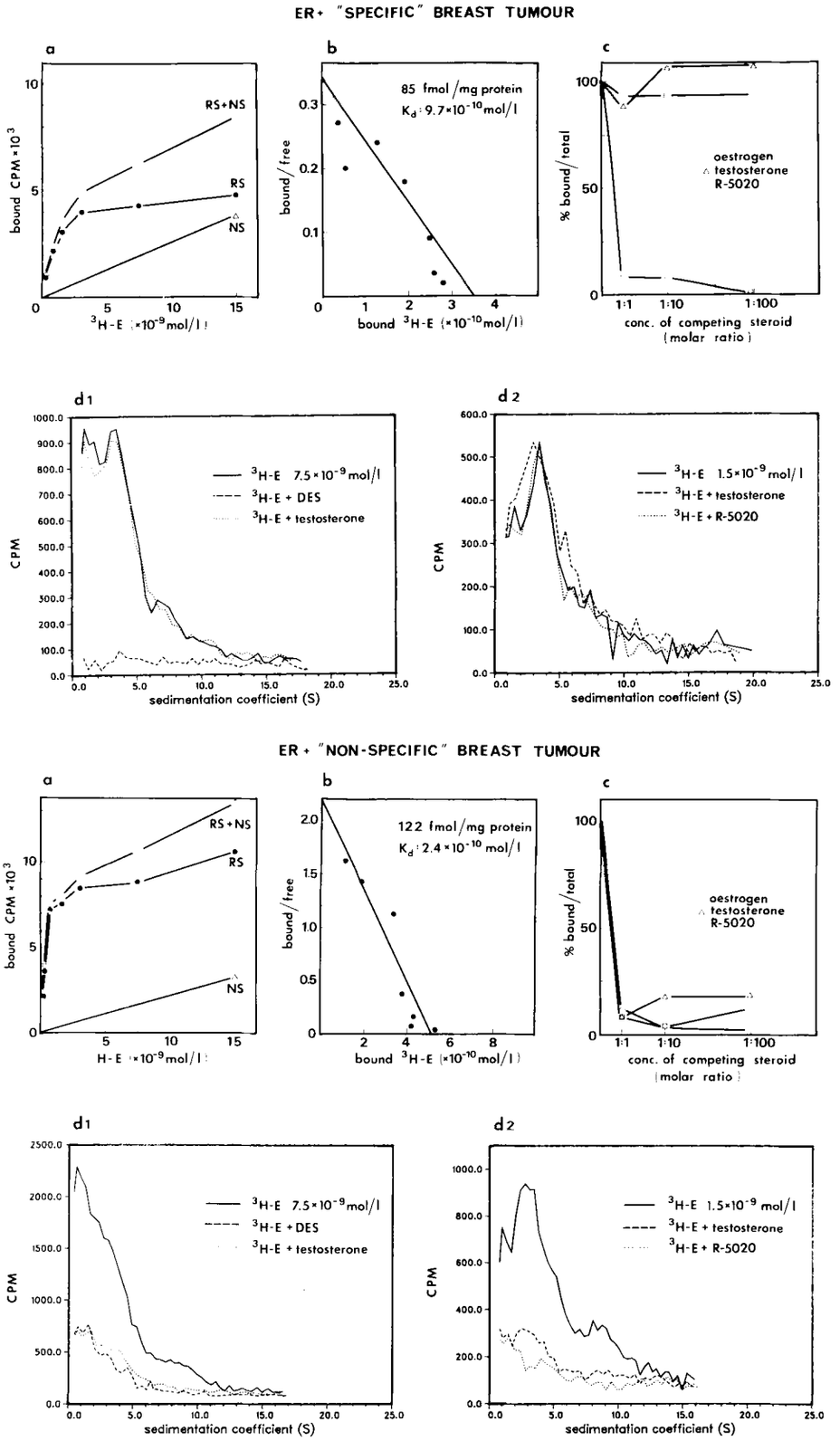

Fig. 1. (For legend see opposite page.)

Materials and Methods

This report is based on a series of 162 selected specimens for oestrogen receptor determination from May 1980 to June 1981, inclusively. The specimens were obtained from patients with primary breast carcinoma. They were included in this study according to the following criteria: 1) the biopsy should be representative of the tumour; 2) the cytosol protein concentration should be 3 mg/ml or more in order to avoid false negative results (21); 3) binding studies should reveal data that demonstrated saturable, high-affinity binding of oestradiol in the isolated cytosol (Figs 1, 2).

Oestrogen receptor assay (DCC)

Tissues were homogenized in Schwingmühle (Retch, Fed. Rep. Ger.) and weighed. The homogenate was suspended in a three-fold volume of H-buffer (5 mmol/l NaH_2PO_4, 5 mmol/l Na_2HPO_4, 1 mmol/l monothioglycerol, 10% (v/v) glycerol, pH 7.5) and centrifuged at 100 000 g at 4°C for 1 h. The cytosol was adjusted to a protein concentration of 3–4 mg/ml with H-buffer. Fifty µl of cytosol was incubated at 2–4°C for 24 h with tritiated 17 β-oestradiol (3 700 GBq (100Ci)/mmol, the Radiochemical Centre, Amersham, U.K.) and at least seven different concentrations of oestradiol (5 µl in ethanol/H-buffer + 25 µl H-buffer—final ethanol concentration <0.5%) ranging from 1×10^{-10} mol/l to 1.5×10^{-8} mol/l were used. All the assays were performed in duplicate. The incubation was terminated by addition of 100 µl dextran-coated charcoal suspension (0.5% charcoal (Norit A), 0.005% dextran (T-70). After adsorption for 15 min at 2–4°C the charcoal was spun down (800 g, 10 min, 4°C), and the bound radioactivity in an aliquot of the supernatant was determined by liquid scintillation counting (Packard Tricarb 3255 spectrophotometer, efficiency for tritium approx. 45%). Quench correction was carried out by the channel ratio method.

In order to correct for low affinity binding, cytosols were run in parallel with 100-fold excess of diethylstilboestrol (DES) (21, 22).

A post-incubation protein measurement was performed (11), and the data are presented of femtomol-bound oestradiol/mg cytosol protein.

The value of the apparent dissociation constant (inverse slope of line) and the concentration (intercept of line with abscissa) of saturable, high-affinity steroid binding sites was calculated from measured bound and unbound ligand by a regression line based on the least square fit from the results in the steepest part of the curve (Fig. 1).

Steroid binding specificity experiments

Aliquots of cytosols were incubated for 24 h at 4°C with 1.5×10^{-9} mol/l tritiated oestradiol in duplicate. Increasingly high concentrations of unlabelled oestradiol, testosterone and synthetic progesterone (R-5020 or org 2058) were added simultaneously in final concentrations of 1.5×10^{-9} mol/l, 15×10^{-9} mol/l and 150×10^{-9} mol/l. Protein-bound radioactivity was separated by charcoal adsorption.

Bound tritiated oestradiol, corrected for low affinity binding (total binding less non-specific binding (bound oestradiol in the presence of a 100-fold excess of DES)) in the absence of unlabelled ligand was taken as 100 per cent (Fig. 1). Rat uterus tissue served as an internal positive control of the assay.

Sucrose density gradient ultracentrifugation (SDGU)

Sucrose density gradient ultracentrifugation was performed as previously described (15, 16, 27). In short, the centrifugation was performed in a SW 41 titanium rotor (Beckman) in a Beckman ultracentrifuge. Linear sucrose gradients (10–18% (w/w)) in H-buffer were prepared in polyallomer tubes and 150 µl samples in protein concentrations of 3 to 4 mg/ml were applied to the gradients. Over-loading was prevented by use of low protein concentrations (25). Centrifugation was carried out at 4°C, at a maximum rotor speed of 40 000 rpm for 26 h. Fractions were collected in pre-weighed tubes from the bottom. After weighing of the tubes with content, the refractive index of each fraction was measured.

Fig. 1. a) Oestrogen binding to specific (RS) and non-specific sites (NS). The quantity of receptor-bound oestrogen (RS) is determined by subtracting non-specific binding (NS) from total binding (RS+NS). b) The specific binding data (RS) from (a) are presented in a Scatchard plot with bound hormone on the abscissa and the ratio of bound to free hormone on the ordinate. The regression curve is calculated by the least square fit from the results in the steepest part of the curve. The intercept of this line with the abscissa gives the binding capacity, whereas the inverse slope of this line gives an approximate value of the dissociation constant of the oestradiol receptor complex. c) The influence of unlabelled oestrogen, testosterone, promegestone (R-5020) on the binding of tritiated oestrogen to tumour cytosols. d1, d2.) Sucrose gradient. Cytosol and 3H-17β-oestradiol (d1: 7.5×10^{-9} mol/l; d2: 1.5×10^{-9} mol/l) were incubated either with or without a 100-fold excess of unlabelled steroids. Following incubation with charcoal, the supernatant was layered on a 10 to 18 per cent sucrose gradient and centrifuged and evaluated as described under Materials and Methods.

Table

Characteristics of 3H-17β-oestradiol binding to various ER-positive tissues (DCC-assay)

		Rat uterus		Calf uterus	Rabbit uterus	BR 10 tumour	T 61 tumour
		No. I	No. II				
Saturation concentration ($\times 10^{-9}$ mol/l)		6	3.5	1.5	3.0	6.0	2.5
Binding capacity, fmol/mg protein		1 558	878	117	618	828	174
Dissociation constant, K_d ($\times 10^{-10}$ mol/l)		1.1	4.5	1.3	3.8	8.7	2.5
Steroid specificity (% of total binding)							
17 β-oestradiol	1.5×10^{-9} mol/l	25	67	18	14	93	25
	15×10^{-9} mol/l	10	9	3	1	13	5
	150×10^{-9} mol/l	5	1	0	0	3	0
Testosterone	1.5×10^{-9} mol/l	97	79	92	95	79	96
	15×10^{-9} mol/l	100	79	89	87	71	102
	150×10^{-9} mol/l	98	76	99	81	58	100
R-5020/org 2058	1.5×10^{-9} mol/l	101/100	85/90	104/98	109/100	67/–	100/100
	15×10^{-9} mol/l	103/98	80/85	103/100	93/98	70/–	97/102
	150×10^{-9} mol/l	98/103	72/80	103/104	82/93	71/–	103/100

Calculation of equivalent sedimentation coefficients ($s_{20,w}$). To allow direct comparison of the data obtained with sucrose density gradient ultracentrifugation (SDGU) with those obtained with conventional binding analyses, the same buffer system (H-buffer) was used for both types of investigation. However, the glycerol content of the H-buffer affects the estimation of the equivalent sedimentation coefficients ($s_{20,w}$) in two ways. First, the viscosity is increased by the glycerol in comparison with the PBS-sucrose gradients normally used. Secondly, the sucrose concentrations of the fractions cannot be calculated from the refractometer readings without correction. We have used well-defined immune complexes (BSA-rabbit anti-BSA IgG complexes) (15) to evaluate the influence of the glycerol on the sedimentation behaviour in the H-buffer. Empirically, it was found that it was possible to correct for the increased viscosity of the H-buffer-sucrose gradient by use of the actual refractometer readings for calculation of the 'sucrose' concentration and hence the viscosity (unpublished observations). By use of these values for the calculation of $s_{20,w}$ for each fraction by a computer program based on stepwise integration (26), it was demonstrated that in the 0 to 22 S range the same zonal profiles were obtained with H-buffer-sucrose gradients as with PBS-sucrose gradients. Therefore, this indirect/empirical approach was used throughout the present investigation. Furthermore, we have run a ^{14}C-BSA standard (Amersham) in parallel with all gradients. In all cases a sedimentation coefficient of 4±0.5 S was found for the BSA standard when using the method described for estimation of $s_{20,w}$. The sedimentation coefficients presented here should properly be termed *apparent* equivalent sedimentation coefficients.

Results

Validation studies. A number of experiments were performed in order to check the experimental conditions.

Cytosols obtained from rat uterus, rabbit uterus, calf uterus, ER-positive tumours grown on nude mice, BR 10 and T 61 (3) were tested. It can be seen from the Table that all tissues contained saturable, high-affinity oestradiol binding sites as measured by DCC analysis. Apparent saturation was reached at a concentration of $3–6\times 10^{-9}$ mol/l. In addition, the binding affinities, K_d, ranged from $1.1–8.7\times 10^{-10}$ mol/l. It appears from the ligand specificity studies that only unlabelled oestradiol significantly reduced this binding.

The mentioned binding features strongly suggest that ER is being measured.

Additional evidence comes from the data shown in Figs 1 and 2. After hormone incubation and charcoal adsorption, cytosols from the mentioned tissues were layered on a 10 to 18 per cent linear sucrose gradient and centrifuged as described under Materials and Methods. Examples are shown in Fig.

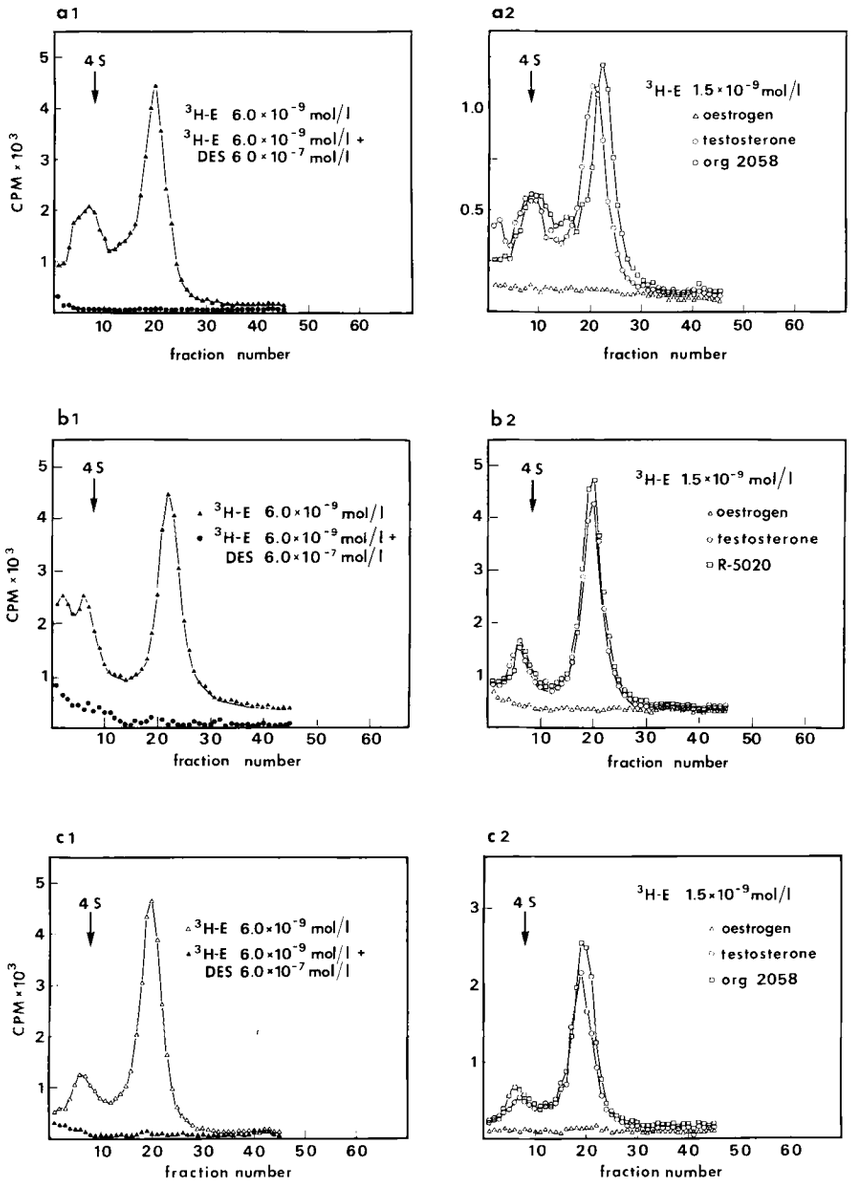

Fig. 2. Sucrose gradient analysis (see legend to Fig. 1, d1, d2). a) Rat uterus, b) BR 10, c) T 61.

2. Using high concentrations of labelled oestradiol (6–7.5×10^{-9} mol/l), a 4 S and 8 S peak were observed indicating oestrogen binding to classical oestrogen receptor macromolecules (5). Some binding, however, was also observed in the region ranging from 1.5 to 3 S. It is unclear to which extent this represents oestradiol binding to ER, or high-affinity binding to something else.

In contrast, by the use of 1.5×10^{-9} mol/l labelled oestradiol which is normally a subsaturating dose, oestradiol became almost entirely bound to the 4 S and the 8 S peaks. It also appears that only unlabelled oestradiol and DES significantly reduced the binding of labelled oestradiol to these peaks.

These observations unequivocally demonstrate that tritiated oestradiol at a concentration of 1.5×10^{-9} mol/l primarily binds to ER, and that a possible reduction of this binding in the presence of other steroids is due to a relatively reduced ER binding.

Oestrogen binding features on cytosols from human breast tumour tissue. Estimated binding ca-

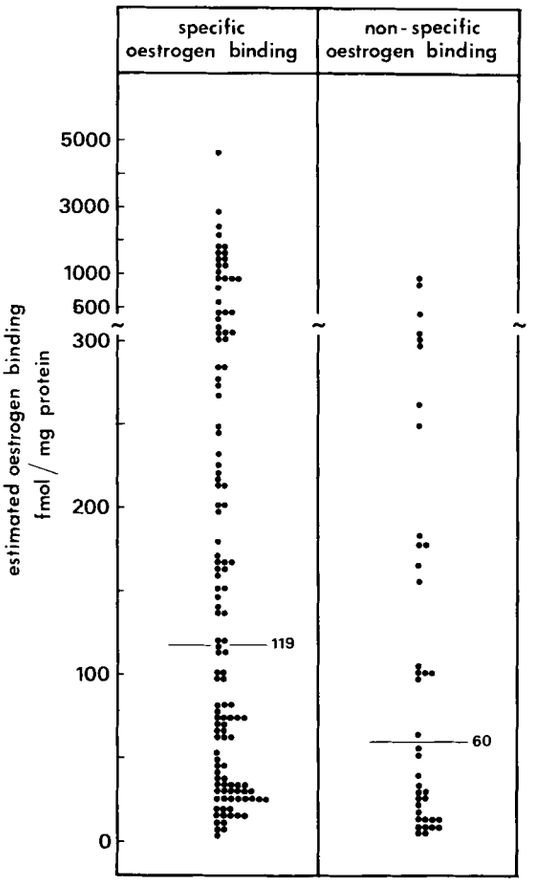

Fig. 3. The relation between binding capacity (fmol/mg proteins) and specific and non-specific ER-positive tumours. Median values indicated by ——.

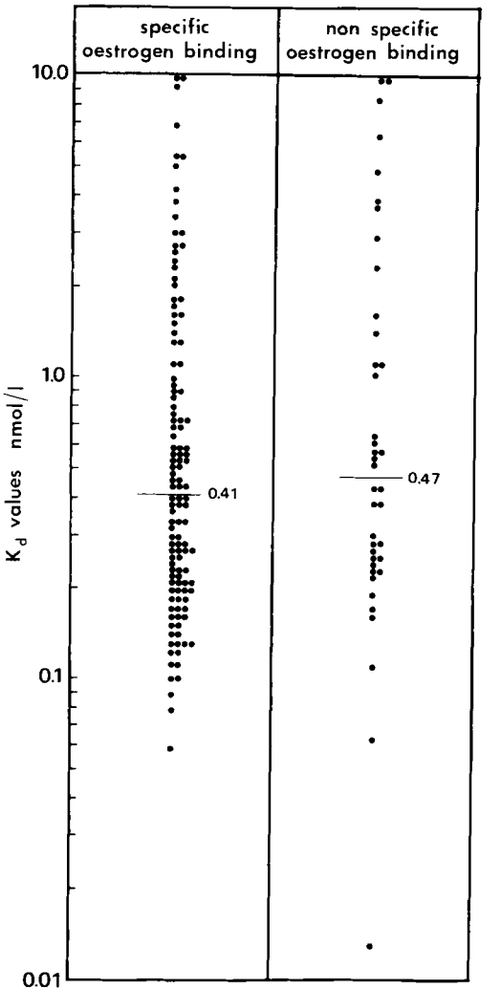

Fig. 4. Comparison of K_d values of specific and non-specific ER. Median values indicated by ——.

pacities of ER ranged from 7–4066 fmol/mg protein. The estimated dissociation constants, K_d, ranged from $0.03–9.5\times10^{-9}$ mol/l (Figs 3, 4).

In 122 cases it was observed that only unlabelled oestradiol significantly reduced the binding of tritiated oestradiol (Fig. 1), thus indicating a ligand-specific oestrogen binding.

In the remaining 40 cases, unlabelled oestradiol as well as testosterone and R-5020/org 2058 were able to displace high-affinity binding of tritiated oestradiol. It was observed that this feature was obtained by the use of equivalent concentrations, which indicates that testosterone and R-5020/org 2058 had the same relative affinity to the binding protein as oestradiol and DES (Fig. 1).

SDGU studies were performed in cases in which tissue was available (52 cases). Two examples are shown (Fig. 1, d1, d2). It is seen that both tumours contained macromolecules which bound oestradiol with high affinity in the region of 4 S, and in one tumour an additional 8 S peak was observed. In addition, the binding of tritiated oestradiol was primarily reduced, corresponding to these two peaks when unlabelled steroids were present. This indicates that both tumour types quantitatively as well as qualitatively contained ER that differed with regard to its ability to distinguish between different classes of steroids.

Other binding characteristics of these two groups are shown in Figs 3 and 4. The median binding capacities did not differ significantly between the two groups. However, the specific group had a sig-

nificantly higher proportion of tumours containing more than 25 fmol bound oestrogen/mg protein (χ^2=6.009, p<0.02). No significant differences in K_d values were observed. In addition there were no differences in patient characteristics between the two groups with regard to age, menopausal status, tumour size, lymph node metastases, TNM classification, tumour type, histologic differentiation of invasive ductal carcinomas, and DNA profile (data not shown).

Discussion

Oestrogen receptor assays on human breast tumour tissue are performed in particle-free extracts from the tissue, the cytosol. The cytosol is an operational identity rather than a well-defined solution. It may contain ER, but at most, it consists of plasma proteins, i.e. albumin and sex-binding globulin (SBG), protein from supporting tissue as well as from tumour cells. Many of these proteins are unknown and may have some affinity for oestrogen. The ER assay must therefore be designed to allow effective discrimination between oestrogen bound to ER and oestrogen bound to non-specific, low-affinity as well as high-affinity binders. It is important to choose the correct hormone concentration range for the binding analysis. The concentration should be within a physiologically relevant concentration range, viz. within $0.1–10\times10^{-9}$ mol/l. It should be demonstrated that ER can be saturated (see RS, Figs 1, 2) as the quantity of ER in a cytosol should be limited to a finite number of binding sites (5). In addition, non-specific binding, viz. oestrogen binding to cytosols containing a 100-fold excess of DES, which is supposed to block all ER binding, should reveal non-saturable binding represented by a straight line (see NS Fig. 1) (2, 4, 5, 6). These criteria have been met in all tissues presented in the present study (Fig. 1). However, the interpretation relies on the assumption that non-specific binding is being measured correctly.

In the present study we used a 100-fold excess of DES in parallel tubes to reduce oestradiol binding. This procedure is widely recommended since DES has an equivalent affinity for ER compared with radioinert oestradiol. It does, however, not bind to SBG.

In contrast, radioinert oestradiol has a high affinity for SBG resembling its binding to ER, and utilizing a 100-fold excess of radioinert oestradiol to estimate non-specific binding may not be as effective as the use of DES as it does not effectively discriminate between oestrogen bound to ER and SBG, respectively. Therefore, non-specific binding of oestradiol as measured in the presence of DES is supposed to originate from non-receptor proteins including SBG.

However, the binding of DES to other proteins is unknown (14). However, it has been shown that DES may possess some weak affinity for α-fetoprotein (AFP) (20, 28). We have demonstrated that the high-affinity, saturable oestradiol binding which can be displaced by DES in some tumours appears to be non-specific, i.e. it can be displaced by other classes of hormones, testosterone and synthetic progesterones. These binding sites do not meet the criteria for ER binding. By the use of SDGU assay, we have demonstrated that tumours and control tissue (Table, Figs 1, 2) contained macromolecules that bound oestrogen with high affinity primarily in the 4 S and 8 S area, indicating that classical ER is being measured (13). However, the lack of specificity demonstrated by both methods may indicate that other proteins or defect ER is being measured.

From published studies that have dealt with steroid binding to AFP, it can be concluded that the peculiar binding mentioned does not represent a binding to AFP since testosterone and progesterone do not bind to AFP (1, 20). In addition, it is unlikely that AFP should influence the data at all as AFP contamination has been shown not to influence ER analysis of human tumours (10). In addition, in tumours in which we have tried to analyse for the presence of AFP, insignificant amounts were recorded (unpublished data).

In a recent paper (17) it has been discussed that the presence of SBG in the cytosols may influence the estimation of ER binding. It was shown that in all ER positive tumours, specific oestradiol binding was lower in the presence of testosterone.

SBG is found in a monomer and dimer form in the plasma with molecular weights of approx. 36 000 and 94 000, respectively (29, 30). Macromolecules of this size would sediment in the 2–5 S area, resembling the 4 S form of ER which has a molecular weight of 70 000 (17, 24). It is possible that some of the apparent binding in this area would account for SBG oestradiol binding. However, the non-specific, high-affinity binding in the 8 S area cannot be due to SBG contamination since these S values represent macromolecules with molecular weights of more than 200 000. Furthermore, R-5020 does not bind to SBG

or ER (18). This very strongly indicates that the observed high-affinity displacement of labelled oestradiol is not due to neither competition about classical ER or SBG binding sites.

It is known that tumours from human breast cancer contain an additional specific binding site for oestradiol, viz. type II oestrogen binding sites (19). They sediment in the 4 S area, bind oestradiol with relatively high affinity (K_d—10^{-8} mol/l). These sites are not supposed to be measured primarily because the hormone concentration ranges are not broad enough in the present assay. However, they do influence the exact evaluation of ER (19, 23). It has been shown, however, that these type II binding sites are ligand specific. Therefore, the observed non-specific, high-affinity binding cannot be due to measurement of type II binding sites even in tumours in which the binding affinities were estimated to 10^{-8} mol/l.

From the considerations discussed it seems reasonable to conclude that in a 100-fold excess DES in some tumours either competes with oestrogen binding sites of unknown origin, which can only be distinguished from ER by its lack of specificity. Alternatively, ER may, in fact, be measured and may have turned into defect receptors losing their capability to distinguish between different classes of hormones during the malignant transformation process.

Our observation is new, and its biologic relevance is yet not known. Clinically, it is consistently being reported that almost 45 to 60 per cent of the patients whose tumours contain ER do not respond to endocrine manipulation irrespective of the assay used for ER evaluation (for review, see 23).

It is possible that the non-specific group discussed in the present investigation might at least in part represent a subclass of breast tumours in which endocrine therapy may have no effect at all.

In routine ER assay, the ligand specificity is not normally measured, and it is possible by including this quite simple check that the predictive value of ER analysis may be improved and that valuable biologic information about the hormonal dependency in human breast carcinoma will result.

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