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STEROID HORMONE RECEPTORS IN HUMAN BREAST CANCER AND THE CLINICAL SIGNIFICANCE

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SUMMARY

Estrogen- and androgen-receptors were determined in human breast cancer tissue by several techniques, mainly by agar gel electrophoresis.

In primary breast cancers estrogen receptors were detected in 50–60% of the cases, in metastatic tissue in 35–40%. The number of binding sites are 90 femtomol/mg tissue protein in average. DHT-receptors were found in 20% of the primaries and 10% of metastases.

There is no correlation to the menopausal status, the histological type of the tumor, or the free interval. The content of spare estrogen receptors decreases with increasing serum estradiol levels.

Regarding clinical results in patients with advanced disease it can be stated that cases lacking steroid hormone receptors in their tumor tissue have a minimal chance to respond to any kind of endocrine treatment.

INTRODUCTION

The evidence for specific binding of estrogens in the tissue of human breast cancers is well established by many investigators since the first report of Jensen *et al.*[1]. The published data were recently reviewed by Wittliff[2]. It could be demonstrated that the cytosol of human breast cancer tissue contains specific estrogen binding proteins mainly sedimenting at 8–9 S on sucrose gradients [2].

The properties of the cytosol receptors are strikingly similar to those of the uterus [3] and of rat mammary tumors [4].

There is also evidence for the presence of androgen [5–8] and progesterone receptors [9] in human breast cancer tissue.

In this paper the experiences with the determination of estrogen and androgen binding in human breast cancer tissue with the especial reference to clinical correlations are reported [10–13].

EXPERIMENTAL

The surgical procedure in the treatment of primary breast cancers and for removing tissue specimens from metastatic lesions is performed at the Department of Gynecology and Obstetrics with only a few exceptions. The histological (and electronmicroscopical) examinations are done at the Department of Gynecology Histopathology (Dr. H. E. Stegner). Immediately after removing the tissue, specimens for receptor determinations are selected by the pathologist and transferred to the laboratory.

Methods for the determination of specific estrogen binding: (*A*) *Uptake-competition technique* (*Jensen* et al.[1]. *Maass* et al.[10])

Uptake control: 14 tissue slices (5–10 mm in dia.) were incubated and stirred magnetically in 200 ml of a 10^{-10} M solution of [6,7-H^3]-estradiol-17β in Krebs–Ringer-$NaHCO_3$-0,1% glucose buffer at 37°C. Uptake competition: same conditions as in uptake control but with the addition of 10^{-5} M nafoxidine.

Two tissue slices each were removed from the incubation flasks at 15 min intervals, washed in cold buffer for 3 min, dried on filter paper, lyophilized, weighed, and 2–3 mg aliquots were lashed in an oxygen atmosphere according to the procedure of Maurer[14]. After cooling of the counting vials in a methanol-dry ice bath, the condensed tritiated water was dissolved in 10 ml of the scintillation fluid according to Hayes. The actual radioactivity of the individual samples was measured in a Packard Tricarb scintillation counter, model 3380 and ranged from 800 to 3000 c.p.m. All counts were corrected to 100% efficiency by external standardization.

Evaluation. The radioactivity measured in the slices which were incubated in the presence of nafoxidine was subtracted from the value obtained in the specimens incubated with [6,7-^{3}H]-estradiol alone and was expressed as Δ(d.p.m./mg dry weight).

Values of Δ above 400 d.p.m./mg of dry weight were called "positive" in regard to the presence of estrogen receptors.

(*B*) *Assay of spare cytoplasmic receptor*

1. *Preparation and incubation of tissue extracts.* The frozen tissue specimen (0·2–2·0 g) was pulverized with

4 vol./weight of Tris–EDTA buffer (0·01 M, pH 7·5, 0·001 M NaN_3) in a porcelain mortar, which was immersed in liquid nitrogen or in a microdismembrator (Braun, Melsungen, Germany). After thawing the sample was centrifuged in a SW 56 rotor (L 2–65 B, Beckman Instruments) at 2°C and 40,000 rev./min (157,000 *g* for 90 min. The supernatant was removed by pipetting and used immediately. One part of the extract was heated at 48°C for 60 min, the other part was kept at 2°C for the same time. [6,7-^{3}H]-estradiol-17β was added to both samples to a final concentration of 4×10^{-9} M, and incubated overnight at 2°C. Recently the control samples have been run with nafoxidine (10^{-5} M) under the same conditions instead of heating the extract.

2. *Agar gel electrophoresis.* The gel electrophoresis was performed according to Wagner[15]. The [^{3}H]-oestradiol receptor complex is shifted towards the anode (Fig. 1). The radioactivity found at the anodic peak which disappears after exposure to heat or nafoxidine represents the amount of bound estradiol. The unspecific estradiol albumin complex is not influenced by heating or nafoxidine (Fig. 2). The free steroid migrates towards the cathode.

Gel layers were prepared with a 1% agar solution (0·05 M Michaelis buffer, pH 8·2). In the centre line of the gel plate wells were punched out and 50 μl aliquots of the labeled tissue extracts were applied. The prepared gel plates were then placed on a Teflon coated brass plate which was cooled to 1°C within an airtight electrophoresis chamber. Electrophoresis was carried out for 90 min at 110 mA/300 V. After the run, the gel was divided lengthwise and then cut into 3-mm wide sections. Radioactivity was eluted from the strips with scintillation fluid according to Hayes for at least 20 h before counting.

3. *Determination of androgen receptors.* Wagner applied the agar gel electrophoresis also to separate androgen receptors. One part of the cytosol is incubated with [^{3}H]-5α-dihydrotestosterone (5×10^{-9} M) instead of [^{3}H]-estradiol. The DHT–receptor complex also gives an anodic peak which disappears after heating the extract or incubation with cyproterone acetate (Fig. 3). The DHT peak is clearly

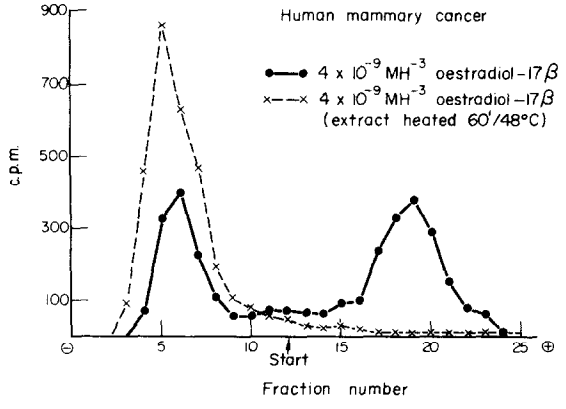

Fig. 1.

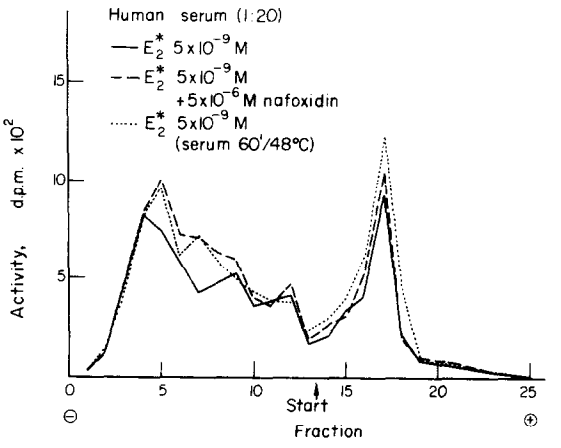

Fig. 2.

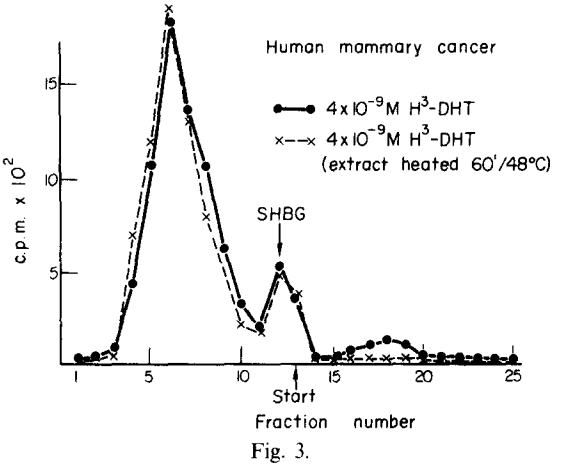

Fig. 3.

separated from the SHBG. The SHBG binding is not heat labile or influenced by cyproterone acetate.

4. *Determination of tissue protein.* The total protein content of the extracts was determined according to Lowry[16]. The amount of serum proteins was estimated by measuring the albumin content by means of a radial immunodiffusion test according to Ouchterlony[17] as modified by Mancini *et al*[18] and Augustin and Hayward[19]. Total tissue protein content was then calculated by the formula used by Wagner[15]: tissue protein = total protein—(serumalbumin × 100)/60.

5. *Charcoal adsorption technique.* This method was employed according to the standards defined by the European Organization on Research for Treatment of Cancer (EORTC) [20]. After incubation of [^{3}H]-oestradiol with an aliquot of cytosol (overnight at 4°C) an equal volume of charcoal suspension was added (0·5% norit A, 0·05% dextran T 70, 0·1% gelatine in Tris–HCl buffer, 0·01 M, pH 7·5). This mixture was agitated for 90 min at 4°C. Charcoal was spun down by centrifugation (15 min at 9500 *g*). Aliquots of the supernatant were pipetted off for scintillation counting.

6. *Hydroxyapatite (HAP) column assay.* HAP-columns (Bio-Gel HTP, Bio-Rad in 10 v/w 0·001 M

$K\text{-}PO_4$ buffer, pH 7·5) were prepared 5 ml plastic syringes according to Erdos *et al.*[21]. A 200 µl aliquot of labeled cytosol was subjected to the column. The column is washed with cold buffer to elute the unbound estradiol. The [^{3}H]-estradiol–receptor complex is adsorbed by the HAP and can be determined by measuring the radioactivity which is included in the column material. A duplicate assay was run with a heated or nafoxidine containing extract.

(C) Evaluation of objective response (EORTC)

1. Only those patients in whom more than 50% of nonosseous lesions decrease measurably in size (more than 50%) although all bone lesions are static, or more than 50% of total lesions improve while the remainder are static, are to be considered as having a favourable effect.

2. No patient showing progression of any lesions is to be classified as having shown a favourable response, regardless of what the response of the other lesions may have been.

3. Patients in whom the lesions are static are considered as failures.

RESULTS

(A) Estrogen receptors

Table 1 gives the results of estrogen receptor studies on breast cancer tissue. In about one half of the primary cancers the receptors were determined by the slice technique. This may be one explanation for the relative low rate of positives in the primaries. The same seems to hold true for the hydroxyapatite column as can be seen in Table 2. Table 2 shows a comparison of the four methods used in our laboratory with regard to the percentage rate of positive assays. The majority of metastatic tissue was determined by electrophoresis. The extracts analysed by the charcoal technique underwent also the electrophoresis procedure. The results were identical. Therefore the differences in the rates of "positives" are not due to the applied technique. The rate of "positives" in primary cancers investigated by electrophoresis is 60–65% in average.

Table 1. Proportion of human breast cancer tissue containing estrogen receptors (primary tumors and metastases)

Primaries	
Premenopause	42/89 = 47%
Postmenopause	109/237 = 46%
Total	151/326 = 46·3%
Metastases	76/219 = 34·7%

In most cases of the 3 last years estrogen receptors were determined by agar gel electrophoresis. The results are expressed as femtomoles bound estradiol per mg tissue protein. The extent of contamination by serum proteins in the cytosol is 33% in average. The degree of contamination varied widely between 5 and 70% (Table 3).

In Fig. 4 the quantitative data with regard to the content of free receptor protein are summarized. Most of the values move within the range of 15–150 fmol/mg tissue protein. There exists no significant difference between post- and premenopausal patients.

A comparison of the electrophoresis and the charcoal technique yielded identical results regarding the distribution of receptor-positive and -negative specimens. This is also true for the quantitative data (Table 4). This was with few exceptions also the case for the comparison of the electrophoresis and the uptake competition technique [10] or the adsorption on hydroxyapatite. Assays run in parallel gave a smaller rate of "positives" with the two latter methods.

Of essential practical relevance for the determination of estrogen receptors in human breast cancer is the possible influence of endogenous estrogens. We therefore correlated the estradiol binding capacity in breast cancer tissue with the plasma levels of free estrogens at the time of mastectomy (Fig. 5). Most patients were postmenopausal and had low estrogen concentrations in blood. In this low range below 100 pg/ml no relationship exists between binding and plasma levels of estrogens. When plasma estrogen values exceed 300 pg/ml no spare receptor could be detected. In order to avoid false negative results therefore we propose to operate premenopausal breast cancer patients only during the beginning of the menstrual cycle and also to exclude all patients with very

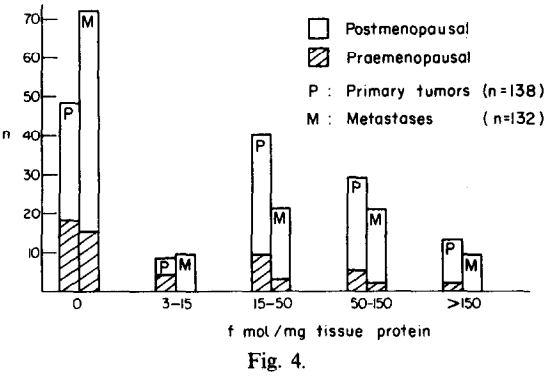

Fig. 4.

Table 2. Determination of oestradiol binding in human breast cancer with different methods. Comparison with regard to the rate of positive assays

Method	Slices	HAP-column	E-Phoresis	Charcoal
Positive assays	40·7% (n = 194)	39·8% (n = 73)	55·3% (n = 270)	70% (n = 37)

Table 3. Contamination of tumor extracts by serum proteins

50 Cases	Serum contamination (% of total protein)	Albumin (mg/ml)
Prim. T.	36	1·4
Metast.	29	0·9
Total	33	1·18

Table 4. Assay of spare estrogen receptors in tumor cytosols simultaneously by the charcoal adsorption technique and agar gel electrophoresis

	Charcoal	Agar E-phor.
E_2-Binding		
Positive cases	26	26
Mean (fmol/mg t.p.)	90	88
Negative cases	11	11

Total 37.

high plasma estrogen concentrations from this study [10, 22].

(B) Androgen receptors

The proportion of cases with primary breast cancers and metastases containing androgen receptors demonstrated by a specific 5α-dihydrotestosterone binding is given in Table 5. The quantity of dihydrotestosterone binding expressed as femtomoles per mg tissue protein is 4 times less in average compared with the estrogen receptor content. There are extracts with higher dihydrotestosterone than estradiol binding.

The rate of tumors containing androgen receptors is about 20%. They are more frequent in postmenopausal women. There are some tumors containing androgen receptors only.

(C) Clinical correlations

1. *Primary breast cancer.* In agreement with other investigators we found no correlation of the presence or absence of estrogen receptors to the stage of the disease or to the axillary involvement in primary breast cancer cases. There is also no correlation to classical histological criteria, but there is some evidence that ultrastructural criteria for differentiation are more frequent in receptor positive tumors (Table 6).

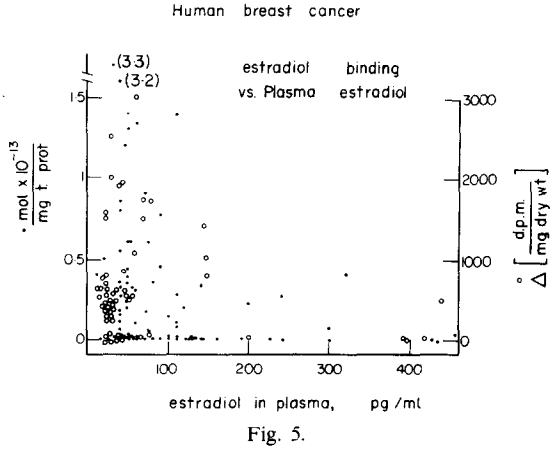

Fig. 5.

Table 5. Proportion of human breast cancer tissue containing 5α-Dihydrotestosterone (5α-DHT) receptors

	Rate
Primaries	
Premenopause	4/30
Postmenopause	13/49
Total	17/79
Metastases	7/57

Table 6. Correlations of the presence of estrogen receptors in primary breast cancer to clinical and morphological data*

	Correlation coefficient	*n*	*P*
T	0·09	100	
N	0·23	100	0·05
N (histol.)	0·18	90	
Number of invaded ly-N	0·13	90	
Differentiated	−0·07	98	
Fibrosis	−0·05	98	
Tight junctions	0·28	93	0·05
Half desmosomes	0·32	54	0·05
Cytoplasmic ductules	0·35	54	0·01

* According to Drs. Stegner and Pape.

2. *Advanced breast cancers.* Table 7 gives the overall results with regard to the correlation of estrogen receptor finding to the outcome after an endocrine treatment of metastases. At the right hand of the table the rates of objective remissions in the groups of receptor positives and negatives for the different treatments are summarized. The best correlations have been observed so far for the outcome after castration which in the majority was performed by ovariectomy.

The hypophysectomy group is very small. Only one patient gained a remission of very short duration (8 weeks), but all have had remissions before, after other kinds of endocrine therapy. This was the presupposition for performing the hypophysectomy.

Compared with the castration the correlations are worse after hormone additive treatments, especially after antiestrogens. The only remissions we observed in the group of receptor negative patients had been treated with Nafoxidine. The durations of remission, however, in these three patients were very short with 4 months in average. Besides there are some patients who were treated with Tamoxifen 20 mg per day, a dose which possibly is not effective in some cases [23].

The duration of remission in the receptor positive group is more than 11 months. Eight patients are still in remission.

Of the 33 evaluable cases treated ethinylestradiol (3 mg/day) in seven patients an induction dose of 6–

Table 7. Correlations of estrogen receptor determinations to the rate of objective remissions in metastatic breast cancer. Comparison with regard to the lesion taken for ER assay

	Tissue assayed: Primary breast objective remissions		Metastatic lesion objective remissions		ϵ	
	ER+	ERϕ	ER+	ERϕ	ER+	ERϕ
Castration	2/2	0/4	3/4	0/18	5/6	0/22
Hypo-ect.			1/4	0/1	1/4	0/1
Ethinyl-			13/22	0/8	14/23	0/10
Estradiol	1/1	0/2	(4/6)*	(0/1)*	(4/6)	(0/1)*
Androgens + Primobolan®	0/1	0/1	3/4	0/3	3/5	0/4
Antiestrogens	0/1	1/4	2/4	2/9	2/5	3/13
ϵ	3/5	1/11	22/38	2/39	25/43	3/50
Free interval					29	25
Duration of remissions					11·2+	4

* Ethinylestradiol-monotherapy after combination with Cytoxan for 8 weeks.
ER = estrogen receptors.

8 g cytoxan had been given in combination with estrogens. Only patients with an adequate long period of ethinylestradiol monotherapy are included in these series.

One of the reasons for the relative high rate of "false positive" cases regarding the correlation to endocrine treatment could be derived from the assumption that in some cases with metastatic disease only tissue from the primary tumor was available. This was the case in one sixth of the patients. On the other hand as shown in Table 7 it is obvious that this distribution has no influence on the remission rates in the receptor positive and negative groups respectively.

The difference in the rates of remissions of the two groups of patients is not explained by differences in the free interval, which is almost identical in the receptor positive and negative group.

The site of the lesion from which specimens for the receptor determination had been taken is compared with the site for the evaluation of response in the next table (Table 8) for the patients with estrogen receptors.

In the upper part the responders and in the lower part the non responders are listed. The free interval in the latter group is somewhat shorter. Three of the six patients denoted as "secondary breast" were patients with inoperable primaries and distant metastases at the time of diagnosis.

In 14 of the 25 responders the dominant lesion for the evaluation of response was different from the assayed lesion. So the receptor determination in a more accessible lesion can prognosticate for distant metastases. On the other hand there are patients with receptor positive lesions where a progression occurred at the same site. This was true for one inoperable breast cancer, four skin and two bone metastases (these two were hypophysectomized).

The next table (Table 9) shows the correlations in reference to the applied methods of estrogen binding determination. Although the number of cases estimated by the slice technique is low the correlations in the positive group seems to be better. "Cytosol" stands for the determination of cytoplasmic receptors, mainly by electrophoresis (S.A.).

Regarding the mean value of quantity of receptors

Table 8. Results of endocrine treatment in ER positive cases. Comparison of the site of lesion for the ER assay and for the evaluation of response

	FJ	ER + tissue for assay		Dominant lesion: Cutan.	Oss	Visc.
Responder	30	Prim. breast	3		2	1
		Sec. breast	4	2	1	1
		Lymph node	5	2	1	2
		Skin	13	7	3	3
		Bone				
Nonresponder*	22·5	Prim. breast	2	1	1	
		Sec. breast	2	1	1	
		Lymph node	2	2		
		Skin	9†	4	3	2
		Bone	3		2	1

* Remissions after previous treatments = 3.
† Regress of the assayed lesion = 2.

Table 9. Rates of objective remissions in ER positive and ER negative cases in reference to the methods of ER determination

	Objective remissions	
	ER+	ERϕ
Slice	7/10	1/14
Cytosol	18/33	2/36
ϵ	25/43	3/50

Table 10. ER content in ER positive cases in responders and non responders

	Responder	Non responder	*n*
Slice*	820 (402–1040)	900 (425–1400)	10
Cytosol†	164 (5–1550)	73 (13–190)	33

* Δ = [d.p.m./mg dry wt]ϕNAF − [d.p.m./mg dry wt] + NAF.

† Femtomoles/mg tissue protein.

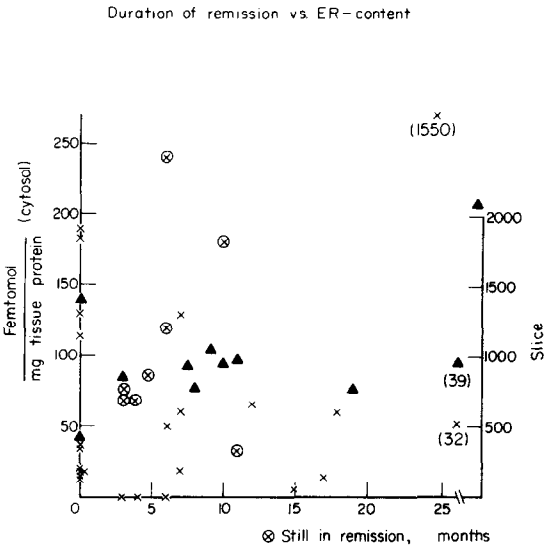

Fig. 6.

there is no real difference whether a response were observed or not (Table 10). The higher value in the responder group is due to one extremely high receptor content.

There is also in our material no direct correlation to the amount of free receptors in the cytosol (Table 11). There are responses in patients who had low amounts of detectable receptors in their tumor tissue.

The same is true for the duration of remission as it is shown in Fig. 6. Nevertheless eight patients are still in remission at the present state.

In 19 patients who are evaluable for the correlation to endocrine treatments 5α-dihydrotestosterone receptors were determined too. Four of five patients with dihydrotestosterone and estrogen receptors gained a remission under ethinylestradiol [3] and primobolan [1]. Two of the patients lacking dihydrotestosterone receptors responded after ethinylestradiol or nafoxidine. These two contained estrogen receptors. So far there are no patients in this group who had been treated with androgens. The number of patients is too small to draw any conclusions regarding the usefulness of dihydrotestrosterone-receptor determinations.

DISCUSSION AND CONCLUSIONS

The results of estrogen receptor determinations on primary human breast cancers demonstrate clearly that no correlation exists to the stage of disease or to the morphological type of the tumor. So there is no evidence that rapid growing tumors or such with light microscopical criteria for low differentiation are characterized by the presence or absence of estrogen receptors. The almost identical free interval between primary treatment and manifestation of metastases in both groups of patients points in the same direction.

The evidence first published by Jensen *et al.* [24] and shortly thereafter by our group [10] and others [25–27] that patients lacking estrogen receptors in their tumor tissue have a very small chance to respond to any kind of endocrine treatment is confirmed by the here presented data.

This trend is now well established on the basis of experiences on 600 cases presented at the "Estrogen Receptor Workshop" at Bethesda, Md., in July 1974.

In receptor negative cases only 19 out of 282 (=7%) patients developed a favourable response compared with 162 of 298 (=54%) receptor positive and 8 of 20 "borderline" cases. This is also in agreement with our data summarized in Table 7.

Table 11. Rates of objective remissions in dependence on the quantitative ER content (femtomoles per mg tissue protein) cytosol assays only

Femtomoles / mg tiss. prot.	Objective remissions					
	Castr.	Hypo-ect.	Ethinyl-estradiol	Androg.	Antiestrog	ϵ
0–0	0/16		0/5	0/3	2/12	2/36
5–10	1/1				1/1	2/2
11–50	2/3	0/2	0/3	2/2	1/2	5/12
51–100			6/6	1/2	0/1	7/9
>100		0/1	4/9			4/10

The possible reasons for the relatively high rate of patients with receptors in their tumor tissue who fail to respond to endocrine measures was already discussed earlier [12] as well as by Jensen *et al*[24] and Wittliff[2]. There is evidence for the possibility that different lesions may be different with regard to the presence of estrogen receptors. Also the investigated specimen may contain receptor positive and negative cells, the latter continue to grow and are responsible for the clinical progress.

All studies so far are based on the determination of the cytoplasmatic receptors. It is conceivable that hormone independence is due to defects in the steps beyond the initial reaction of the estrogen with the 8 resp. 4 S receptor, i.e. the transformation to the 5 S complex, the transport into the nucleus and/or the interaction of the estrogen–receptor complex with chromatin.

Finally, it cannot be ruled out that different endocrine treatments have different mode of actions without any relation to estrogen receptors. Some better correlations in the "positive" group might be achieved by a better quantifaction of the receptor determinations with the especial consideration of the heterogenity of the tissue.

At the present state we may conclude that in receptor-negative patients endocrine ablative procedures, especially the adrenalectomy should be avoided, because the severity of the operation is without any relation to the minimal chance of response. Efforts should be made to achieve a better characterization of receptor positive tumors.

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REFERENCES

1. Jensen E. V., DeSombre E. R. and Jungblut P. W.: In *Endogenous Factors Influencing Host-Tumor Balance* (Edited by R. W. Wissler, T. L. Dao and S. Wood Jr.). The University of Chicago Press (1967) pp. 62–71.
2. Wittliff J. L.: *Seminars in Oncology* **1** (1974) 109–118.
3. Jensen E. V., DeSombre E. R.: *Ann. Rev. Biochem.* **41** (1972) 203–230.
4. McGuire W. L., DeLaGarza M.: *J. clin. Endocr. Metab.* **36** (1973) 548–552.
5. Wagner R. K., Görlich L. and Jungblut P. W.: *Hoppe-Seyler's Z. physiol. Chem.* **353** (1972) 1654.
6. Raith L., Wirtz A. and Karl H. J.: *Klin. Wschr.* **52** (1974) 299–301.
7. Engelsman E.: B.A.C.R meeting, Leeds, 1974.
8. Maass H., Engel B., Nowakowski H., Stolzenbach G. and Trams G.: *Archs Geschwulstforsch.* (in press).
9. Terenius L.: *Europ. J. Canc.* (in press).
10. Maass H., Engel B., Hohmeister H., Lehmann F. and Trams G.: *Am. J. obstet. Gynecol.* **113** (1972) 337–382.
11. Maass H., Bruemmer P., Egert D., Engel B., Hohmeister H., Lehmann F. and Trams G.: *Acta obstet. Gynecol. scand.* **19** (1972) 30–38.
12. Maass H., Trams G. and Nowakowski H.: *Strahlentherapie* **147** (1974) 381–387.
13. Maass H. and Trams G.: International Congress Series No. 321 (ISBN 90 219 0229X) (Edited by W. Davies and C. Maltoni). Characterisation of Human Tumors. Excerpta Medica, Amsterdam, 1973.
14. Maurer H. R.: *Hoppe Seyler's Z. physiol. Chem.* **349** (1968) 115–119.
15. Wagner R. K.: *Hoppe Seyler's Z. physiol. Chem.* **353** (1972) 1235–1246.
16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–273.
17. Ouchterlony Ö.: *Acta Pathol. microbiol. scand.* **26** (1949) 516–529.
18. Mancini G., Carbonara O. A. and Heremans J. F.: *Int. J. Immunochem.* **2** (1965) 235–243.
19. Augustin R. and Hayward B. J.: *Int. Archs Allergy appl. Immunol.* **11** (1957) 192–213.
20. E.O.R.T.C. Breast Cancer Cooperative Group: *Europ. J. Cancer* **9** (1973) 379–388.
21. Erdos T., Best-Belpomme M. and Bessada R.: *Analy. Biochem.* **37** (1970) 244–258.
22. Trams G., Engel B., Lehmann F. and Maass H.: *Acta endocr., Copenh.* **72** (1973) 351–360.
23. Ward H. W. C.: *Brit. Med. J.* **I** (1973) 13–17.
24. Jensen E. V., Block G. E., Smith S., Kyser K. and DeSombre F. R.: *Nat. Canc. Inst. Monogr.* **34** (1971) 55–79.
25. Leung B. S., Fletcher W. S., Lindell T. D., Wood D. C. and Krippaehne W. W.: *Archs Surg.* **106** (1973) 515–519.
26. Engelsman E., Persijn J. P., Korsten C. B. and Cleton F. J.: *Brit. Med. J.* **2** (1973) 750–752.
27. Savlow E. D., Wittlif J. L., Hilf R. and Hall T. C.: *Cancer* **33** (1974) 303–309.