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Comparative histological, histochemical, immunohistochemical and biochemical studies on oestrogen receptors, lectin receptors, and Barr bodies in human breast cancer*,**

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Summary. The present study performed on a total of 567 cases of human female breast cancer compares the results of the biochemical assay (dextran-coated charcoal assay = DCC) for oestrogen receptor (ER) with those of several morphological methods developed for the detection of the ER or for the prediction of prognosis by use of other systems (FSA = fluorescent ligand binding assay, ER-ICA = monoclonal antibody assay for ER, LRA = lectin receptor assay using peanut agglutinin, and Barr body estimation). Whereas no correlation at all was observed among the results of the DCC and those of the FSA and Barr body estimation, the ER-ICA and the LRA showed an unanimous tendency towards higher values of ER with increasing intensity of the staining product. The results of the ER-ICA may be expressed by an immuno-reactive score (IRS) calculated from the staining intensity (SI) and the percentage of positive cells (PP). The morphological methods are evaluated with special regard to their correlation with the DCC, their theoretical basis, and their practical application. In summary, the ER-ICA appears to be the sole method directly visualizing the ER protein and – in contrast to the DCC – is therefore completely independent of the content of endogenous or exogenous oestrogens in the tumor tissue. The LRA provides valuable additional information concerning tumour differentiation

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and possible response to endocrine therapy, whereas the FSA and Barr body estimation should be considered as obsolete and should therefore be abandoned.

Key words: Breast cancer – Estrogen receptor (ER) – Monoclonal antibodies for ER – Histochemical methods for ER detection – Barr bodies and ER

Introduction

Since the first reports of Jensen et al. (1960a, b) and of Folca et al. (1961) on the occurrence of oestrogen receptors (ER) in ER-responsive target organs and in human breast cancer, respectively, numerous studies have been performed concerning (1) the biochemical mechanisms of steroid function on target cells, (2) methods for determining the ER content of breast cancer tissue, and (3) the importance of ER assays for the treatment and prediction of prognosis in breast cancer patients. Approximately 60–70% of patients with a positive biochemical assay for ER will respond to ablative or additive endocrine therapy whereas only 7–15% of patients with ER-negative tumours will show the same effect. The prognostic prediction is improved by the additional assay for progesterone since the ER assay only indicates the *binding capacity* of tumour cells for oestrogen whereas progesterone is an oestrogen-induced steroid and the occurrence of PgR therefore indicates a *metabolic response* of the cancer cells to oestrogen.

The present report compares the results of the biochemical assay for ER (dextran-coated charcoal assay = DCC) with those of several morphological methods for detecting the hormone dependency of breast cancer (fluorescein ligand binding assay = FSA, immunohistochemical assay for nuclear ER protein = ER-ICA, lectin receptor assay = LRA, and determination of Barr bodies in cancer cell nuclei). The results will be discussed in detail with special regard to their practical application.

Materials and methods

DCC, FSA, and ER-ICA were performed on unfixed tumour tissue kept on ice immediately after excision and delivered to the pathologist directly from the operation theater. 5 μ thick cryostat sections were prepared for the FSA and ER-ICA, and the residual tissue was frozen at −70° C and later examined by the DCC. If enough tissue was available, different portions of the tumour (center/periphery or radial sectors) were examined by the DCC and ER-ICA. In addition the material used for the frozen section diagnosis then was fixed in formalin and embedded in paraffin. 4 μ thick paraffin sections were either stained with H&E or used for the LRA and the determination of Barr bodies.

The cases examined for the present study were derived either from the material of the Institut für Pathologie, Wiesbaden, or kindly supplied by the following pathologists: Prof. Bäßler (Fulda), Prof. Hieronymi and Dr. Müller (Offenbach/Main), Prof. Höer, Prof. Hübner, Prof. Stutte, Prof. Walther and Prof. Lange (Frankfurt/Main). The authors are indebted to these colleagues for their support of this study.

Biochemical assay for ER (DCC). In the first series (FSA, LRA) 449 cases of breast cancer were examined whereas the second series (ER-ICA) comprised 118 cases with 156 tumour

samples. ER was determined by means of the six-point dextran-coated charcoal assay according to the recommendations of the E.O.R.T.C. (1980). Accordingly, the progesterone receptor (PgR) was also determined in most cases. The present paper, however, only deals with the results of the DCC for ER in order to render the results comparable to those of the other methods. The DCC was performed in all cases by BIOSCIENTIA (L.J.B. and B.H.).

Fluorescein ligand binding assay (FSA). The method originally described be Lee (1978) was used in the modification by Frank et al. (1980) which allows a quantitative estimation of the fluorescent product. Low values as measured by this device indicate a high fluorescence (=high content of binding sites within the cells) while high values indicate a faint fluorescence and therefore a low content of binding sites. The 17β-estradiol-CMO-BSA-FITC conjugate was initially obtained from Dr. Lee, later from Zeus Scientific Corporation (Raritan, New Jersey) and from Travenol (München). 401 cases of female breast cancer were examined with this method. All assays were performed by the same medical technician. For each tumour, the mean value was calculated from 40 to 100 or more (up –300) measurements.

Abbott monoclonal immunocytochemical assay for ER (ER-ICA). We used the ER-ICA kit commercially produced and distributed by Abbott Laboratories, Diagnostic Division (North Chicago, IL, USA; German Branch: Wiesbaden-Delkenheim, FRG) and based upon the preparation of monoclonal antibodies to ER protein by Greene et al. (1980, 1982) and King and Greene (1984). A total of 118 cases with 156 samples was examined by this method. Cryostat sections were stained according to the procedure recommended by Abbott Laboratories. The staining product was graded (a) for its intensity (=SI) into four categories (0=no staining, 1 to 3=faintly, moderately, strongly positive) and (b) for the approximate percentage of positively stained cells (=PP) also into four categories (0=no positive cells, 1=less than 10%, 2=10 to 50%, and 3=more than 50% positive cells). Thus, the ER content of a certain tumour is expressed by two variables each of them characterizing a special aspect of the cellular ER content. By grading each from 0 to 3 an imbalance between them was avoided when calculating the "immuno-reactive scores" I and II (see below).

Due to staining heterogeneity of the tumour cells the grading of the SI was based upon the *predominant* type, smaller subpopulations of cells with a weaker or stronger staining reaction being neglected. The PP was also evaluated by subjective examination of the tissue sections. A more detailled classification (e.g. below 10%, 10 to 33%, 34 to 50%, 51 to 75%, and above 75%) was abandoned because it appeared impossible to distinguish between such steps merely by subjective evaluation.

Two "immuno-reactive scores" were calculated from the SI and the PP. The Immuno-Reactive Score I (=IRS I) was calculated by *summing* SI and PP whereas the Immuno-Reactive Score II (=IRS II) was calculated by *multiplying* SI and PP.

Intra- and inter-observer variation in determining SI and PP were estimated by reexamining the sections of 50 cases after an interval of three weeks by the same author (W.R.) and by two independent investigators one of whom had special experience with the ER-ICA (Dr. Th. Beck, Univ.-Frauenklinik Mainz). The second investigator was Dr. Viera Boublik, first resident, Institut für Pathologie Wiesbaden, who had no former experience with this method. We are indebted to Dr. Beck and Dr. Boublik for their valuable assistance.

All ER-ICA assays were performed by the same medical technician (U.H.) and all sections examined by the same author (W.R.).

Lectin receptor assay (LRA). The lectin-histochemical investigations with labelled peanut agglutinin (PNA) were performed by P.J.K. and M.V. on 122 formalin-fixed primary carcinomas of the female breast. FITC-PNA (Medac, Hamburg) was used in a working concentration of 0.2 mg/ml (Klein et al. 1978, 1979). For evaluation of free as well as neuraminic acid-substituted PNA binding sites, all tissue sections were pretreated with neuraminidase (0.1 U/ml) from Vibrio cholerae (Behringwerke, Marburg, FRG) for 30 min and thereafter incubated with the lectin for further 30 min, followed by investigation of the tissue sections with a Zeiss fluorescence microscope (450–490 nm). According to the lectin-histochemical findings, the carcinomas were graded as follows (Klein et al. 1983): less than 10% positive tumour cells=negative; 10–30% positive cells=weakly positive (+); 31 to 60% positive cells=moder-

ately positive (+ +); and more than 60% positive cells = strongly positive (+ + +). At variance with the other studies in this paper, comparison with the DCC values was based upon a threshold value of 20 fmol/mg cytosol protein (not 10 fmol/mg) in order to render the results comparable with previous investigations by the same authors (P.J.K. and M.V.).

Determination of Barr bodies. In 57 cases from series I and II sex chromatin of the tumour cell nuclei was determined by the same author (H.A.H.). Paraffin sections or semithin sections obtained from the paraffin blocks by re-embedding into Technovit (Kulzer) were stained Feulgen, PAS or toluidine blue, and 100 to 200 cells per case were examined for the presence of nuclear Barr bodies. The tumour was classified as positive if Barr bodies were present in more than 10% of the nuclei.

Determination of cellularity, lymphocytic infiltration, and differentiation of tumour cells. In the FSA studies, *cellularity* (= content of epithelial cells) within a certain tumour was graded into three categories: 1 = predominance of epithelial cells, 2 = equal portions of epithelial cells and connective tissue, 3 = predominance of connective tissue. A similar method was used to grade the epithelial content in the ER-ICA series: 1 = 10 to 20% epithelial cells, 2 = 30 to 50%, 3 = more than 50% epithelial cells. In both series, grading was performed by subjective evaluation. In the ER-ICA series, the DCC values were corrected for cellularity by dividing them by 2 when the tumor belonged to grade 2 (= approximately double content of epithelial cells compared to grade 1) and by 4 if it was graded 3 (= approximately fourfold content of epithelial cells compared to grade 1). The DCC values thus obtained were called ER_{corr}.

In the FSA series, the *lymphocytic infiltration* was graded from 1 (slight) to 2 and 3 (moderate and dense). The *differentiation* of cancer cells was graded according to Bloom and Richardson (1957). All histological classifications were performed by one author (W.R.).

Comparison of results, statistical analysis. When examining a tumour by one of the methods described above, the author was unaware of the results of the other methods performed in the same tumour. Only after the examination had been definitely concluded was its result compared to those of the other methods. If necessary, the results were statistically analyzed (E.Sch.).

Results

1. DCC

138 out of 449 cases of the first series were negative with an ER lower than 10 fmol/mg cytosol protein. Only 19 cases had a value higher than 500 fmol/mg, and only 4 cases had a value of more than 1,000 fmol/mg.

When the ER values were compared to the lymphocytic infiltration in the tumour tissue, lower mean values were found in tumours with an increased content of lymphocytes (Table 1) although no statistical correlation could be observed. The mean ER values decreased with decreasing content of epithelial cells and increasing content of connective tissue and with lower grades of cellular differentiation. Again, these differences were not statistically significant.

In 27 cases of the second series, more than one sample was examined for its ER content (16 × two samples, 11 × three samples). When the results in the first series were considered as 100%, the DCC values of the second sample were 48.2 to 241.7% (in one case 1,200%), and in the third sample 66.7 to 164.7%. The mean values for the first, second and third sample were 260.9 ± 370.7, 215.8 ± 311.5, and 259.7 ± 432.5 fmol/mg. Only in one case was one sample ER-negative (below 10 fmol/mg) and the second sample

Table 1. Mean values of DCC and FSA in relation to lymphocytic content, epithelial content of the tumour tissue and differentiation of tumour cells. Standard deviation is usually very high and not mentioned in the table since only the difference between grade 1 and grade 2 of the lymphocytic infiltration is statistically significant ($P = 0.0003$)

Parameter	DCC			FSA		
	Grade 1	Grade 2	Grade 3	Grade 1	Grade 2	Grade 3
Lymphocytic infiltration	129.5	70.3	91.7	105.8	112.9	116.4
Epithelial content	180.1	120.8	90.2	115.8	208.4	104.0
Differentiation of cancer cells	137.1	110.6	84.3	106.9	106.2	114.6

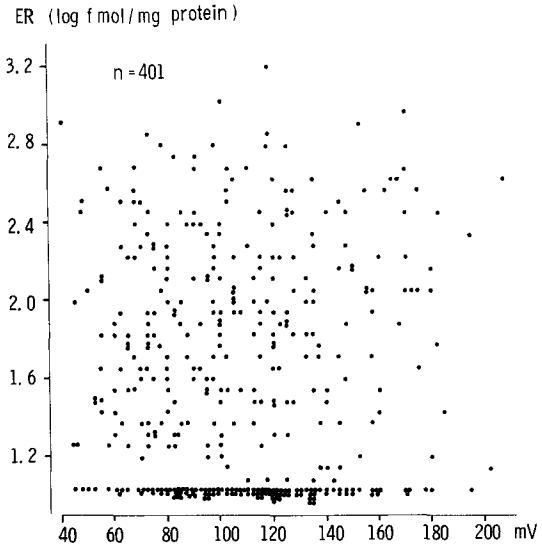

Fig. 1. Comparison of DCC and FSA results in 401 cases of primary human breast cancer (series 1). No correlation between the results of the two methods is observed. Cases with a negative DCC are included with a value of 10 fmol/mg each

ER-positive (120 fmol/mg). In all other cases, all samples of the same tumour were either negative or positive. The correlation coefficient for the first versus the second and for the first versus the third ER values were 0.8472 and 0.9751.

2. FSA

The FSA values did not correlate with the DCC values (Fig. 1). The correlation coefficient was 0.002. Moreover, there was no correlation between the results of the DCC and the grade of lymphocytic infiltration, the content of epithelial cells or the degree of differentiation of the tumour cells (Table 1).

Table 2. Distribution of SI and PP in breast cancer tissue

Grade	Staining intensity (SI)				Percentage of positive cells (PP)			
	All samples[a] $n=156$		All tumours[b] $n=118$		All samples[a] $n=156$		All tumours[b] $n=118$	
	n	%	n	%	n	%	n	%
0	34	21.8	26	22.0	34	21.8	26	22.0
1	20	12.8	19	16.1	12	7.7	9	7.6
2	48	30.8	33	28.0	39	25.0	29	24.6
3	54	34.6	40	33.9	71	45.5	54	45.8
	156	100.0	118	100.0	156	100.0	118	100.0

[a] "All samples" means: All samples examined including double and triple examinations of tissue from the same tumour
[b] "All tumours" means: Only one sample of each tumour examined

Table 3. Different results of DCC and ER-ICA

Sample no.	DCC (fmol/mg)	ER-ICA (IRS I)	ER-ICA (IRS II)
017	<10	4	4
047	<10	5	6
058	<10	5	6
085	<10	6	9
092	<10	6	9
121	<10	2	1
125[a]	<10	4	4
126[a]	<10	2	1
127[a]	<10	3	2
070[b]	17	0	0
071[b]	14	0	0

[a,b] Different samples of same tumour

3. ER-ICA

Of 118 tumours 92 gave a positive staining reaction and of 156 tumour samples 122 were positive (Table 2). The percentages of grade 0 to 3 SI and grade 0 to 3 PP are summarized in Table 2 showing a good agreement between the two groups. In 7 cases (9 samples), the DCC was negative but the ER-ICA positive, and in only one case (2 samples) was a negative ER-ICA observed despite a positive DCC (Table 3). In this case, however, the DCC values were very low (17 and 14 fmol/mg) and would have been classified as negative (below 20 fmol/mg) by many authors.

When the DCC values were corrected for cellularity, they did not correlate better with the ER-ICA than the uncorrected DCC values (Table 4). The correlation coefficients for SI, PP, IRS I and IRS II are summarized in Table 4. They are somewhat higher in IRS I and IRS II than in SI and

Table 4. Correlation coefficients for ER and ER_{corr}, respectively, versus SI, PP, IRS I and IRS II

		SI	PP	IRS I	IRS II
All samples	ER	0.5122	0.5113	0.5372	0.5947
	ER_{corr}	0.4734	0.4802	0.5008	0.5436
All tumours	ER	0.5444	0.5079	0.5517	0.6070
	ER_{corr}	0.4768	0.4568	0.4901	0.5265

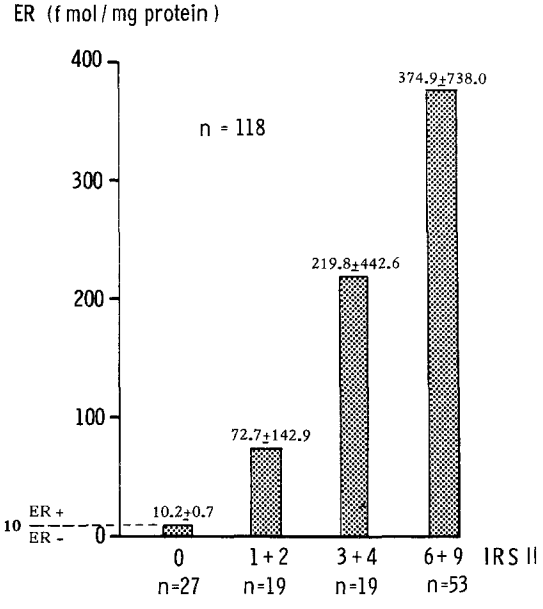

Fig. 2. Correlation of DCC (mean values and standard deviation) and ER-ICA results in 118 cases of primary human breast cancer (series 2). IRS II = Immuno-Reactive Score II (SI × PP). Adjacent scores (1+2, 3+4, 6+9) have been summed in order to increase the number of cases in each column. Cases with a negative DCC are included with a value of 10 fmol/mg each

PP, thus showing that the calculation of an immuno-reactive score is fully justified. IRS II (= SI × PP) gave a better correlation than IRS I (= SI + PP).

Although individual ER-ICA results did not quantitatively correlate with the DCC results in the same case, an unequivocal tendency towards higher mean values of ER was seen with increasing IRS II (Fig. 2). The DCC values for the four SI and PP categories of all 156 tumour samples are listed up in Table 5. All ER-ICA negative tumours (IRS II = 0) were also DCC negative except one case with an extremely low positive DCC value of 14 fmol/mg.

The results of the intra- and inter-observer variation are summarized in Table 6. Intra-observer variation for SI and PP is only 22 and 14%, and 100% agreement is achieved if values differing for only one point below or above the original grading are included. Inter-observer variation is almost the same as intra-observer variation if the sections are evaluated by an experienced second investigator, and it markedly decreases if the second investigator is unexperienced in this field. Generally, PP values agree better

Table 5. DCC results (mean values and standard deviation) in correlation to ER-ICA results (SI and PP). All samples (118 primary tumours and 38 additional samples from 27 of these tumours)

Grade	SI		PP	
	Number of cases	DCC values ER (fmol/mg)	Number of cases	DCC values ER (fmol/mg)
0	34	11.8± 7.8	34	11.8± 7.8
1	20	124.8±163.3	12	42.8± 45.7
2	48	164.6±235.8	39	133.4±143.9
3	54	448.9±379.1	71	407.3±376.5

Table 6. Intra- and interobserver variation of SI and PP in 50 cases of primary human breast cancer

	Difference (points)	Intra-observer-variation A2/A1	Inter-observer variation[a]			
			E/A1	E/A2	U/A1	U/A2
Staining Intensity (SI)	±0	78	58	60	64	52
	±1	100	100	100	98	100
	±2	–	–	–	100	–
Percentage of Positive Cells (PP)	±0	86	78	82	56	56
	±1	100	100	100	100	100

A1 and A2 = first and second investigation by one author (W.R.)
E = investigation by another observer experienced in grading ER-ICA (Th.B.)
U = investigation by another observer unexperienced in grading ER-ICA (V.Bo.)

[a] Percentage values indicate the degree of agreement between the different examinations

than SI values, thus confirming that subjective evaluation of PP is easier than evaluation of SI due to staining heterogeneity of the tumour cells.

Whereas DCC values for different samples of the same tumour varied between 0 and more than 200%, identical ER-ICA results were obtained in 13 out of these 27 cases. In 7 cases either SI or PP varied for one point, in 7 other cases both SI and PP varied for one point. This confirms that different DCC values from the same tumour are primarily due to a different structure in the tissue samples.

4. LRA

The occurrence of PNA binding sites in breast cancer correlated with the results of the DCC in approximately 73% of the tumours (Table 7). In the group of tumours showing different biochemical and histochemical findings, the majority did not possess ER but expressed PNA binding sites whereas ER-positive/PNA-negative tumours were observed only in few cases. There was not only an overall relationship between the ER status

Table 7. Correlation of ER status with LRA (PNA) histochemistry. Breast carcinomas were classified as ER-positive when the ER content was higher than 20 fmol/mg cytosol protein. Tumours were graded as LRA (PNA) positive when more than 10% of the tumour cells showed a positive staining

ER vs. LRA	*n*	%	Correlation (%)
ER+/LRA+	78	(64%)	73%
ER−/LRA−	11	(9%)	
ER+/LRA−	11	(9%)	27%
ER−/LRA+	22	(18%)	
Total	122	(100%)	100%

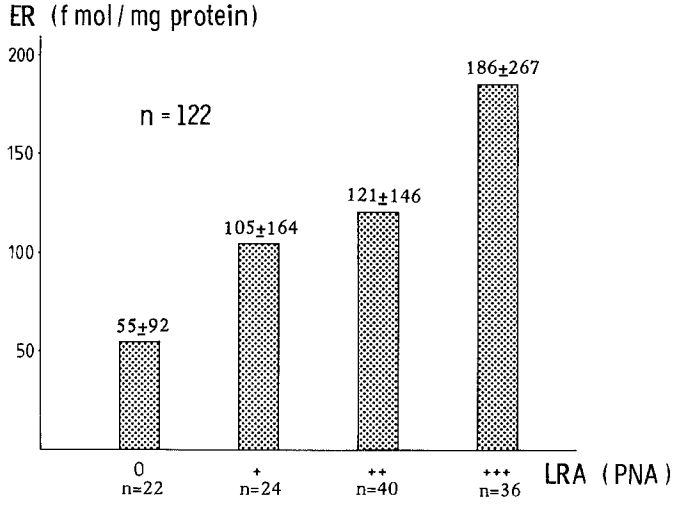

Fig. 3. Correlation of DCC (mean values and standard deviation) and LRA results (semiquantitative evaluation of PNA binding, for further explanation see text)

and lectin histochemistry but also a quantitative correlation between these two variables. As demonstrated in Fig. 3, however, standard deviation of the mean values of ER is high. Therefore, the increasing ER concentrations only represent a trend which appears not to be necessarily significant.

5. Barr body determination

When the results of Barr body determination were compared to those of the DCC (Table 8) by means of a 2×2 contingency table and statistically analyzed with Fisher's exact test, no dependency between the variables "Barr body count" and "DCC" could be found.

In 14 of the 57 cases, ER-ICA had been performed. ER-ICA was positive in 8 out of 9 sex chromatin-negative cases. IRS II varied between 1 (2 cases), 4 (1 case), 6 (1 case) and 9 (4 cases). One Barr-body negative case was also ER-ICA-negative. The five Barr body-positive cases were all ER-ICA-positive with a high IRS II of 6 (1 case) and 9 (4 cases). Although the small

Table 8. Comparison of ER content (DCC) and Barr body estimation in 57 cases of primary breast cancer

	ER-positive	ER-negative
Barr body positive	17	11
Barr body negative	22	7

number of cases does not allow statistical analysis, any significant difference between Barr body-negative and -positive cases appears unlikely.

Discussion

1. Correlation DCC/tumour histology

Although not statistically significant, our results concerning the influence of tumour differentiation, lymphocytic infiltration, and epithelial content upon the DCC values agree with those of previous authors (Feherty et al. 1971; Maynard et al. 1978; Martin et al. 1979; Parl and Wagner 1980; Chabon et al. 1982; Nishimura et al. 1982; Underwood et al. 1983; Horie et al. 1984; Pickartz 1985). It is generally accepted that poor differentiation of cancer cells, dense infiltration of the tissue with lymphocytes, and a low epithelial content are associated with low DCC values.

2. Evaluation of the FSA

Oestrogens may be bound to three different sites within the cell which have been called type I to type III binders (Chamness et al. 1980; McCarty et al. 1981; Daxenbichler 1982). *Type I* binding represents the specific ER itself and is characterized by the highest affinity (K_d in the order of 10^{-10} M) but by a lower capacity for oestrogen molecules than by type II binders. *Type II* binding sites have a K_d of about 10^{-8} to 10^{-7} M. *Type III* binding sites consist of soluble molecules like (pre-)albumin and membrane-associated proteins, their K_d lying between 10^{-7} and 10^{-5} M.

Prior to the development of the ER-ICA (Greene et al. 1980; Greene and Jensen 1982) several authors had described histochemical methods and postulated that ER could be demonstrated by these methods in cryostat sections of frozen tumour tissue. The principle of all these methods consists in binding oestrogen or oestrogen-like substances to the "receptors" and visualizing the reaction product by its fluorescence or by the PAP method (reviews see: Chamness et al. 1980; Daxenbichler et al. 1982; Pertschuk et al. 1980, 1981, 1982; Nenci 1981; McCarty et al. 1981; Underwood et al. 1982; Underwood 1983; van Bogaert 1985; Bosman et al. 1985). One of the most widely used methods is the FSA as described by Lee in 1978. Lee used a 17β-estradiol-CMO-BSA-FITC conjugate with estradiol bound to BSA (bovine serum albumin) and labelled with FITC (fluorescein isothiocyanate). Regardless of the fact that the histotopographical identity between the conjugate and the ER binding sites has never been established, one

major objection to Lee's method and other methods using BSA complexes is the low affinity of the BSA conjugates for ER (only 0.1% of the affinity of the natural steroid). Therefore, high concentations of 10^{-6} to 10^{-5} M are required to saturate the ER sites. This means that their affinity for the ER and for nonspecific binding sites is in the same range and that it is impossible to distinguish between type I, II and III binding sites (Bosman et al. 1985). Moreover, the number of ER molecules per cell is about 100 fold lower than the number of fluorescein molecules which may be identified by the present fluorescence microscopic routine techniques (Bosman et al. 1985).

The present study confirms a preliminary report (Remmele 1981) that Lee's method may not be compared to the DCC because there is no statistical correlation between the results of these two methods. In the meantime, several reports have been published which agree with our results (Danguy et al. 1981; Högel et al. 1982; Penney and Hawkins 1982; Kute et al. 1983; Berns et al. 1984; Manfreda et al. 1985) although some other authors found a 65 to 92% agreement between the FSA and the DCC (Lee 1980; O'Connell and Said 1983; Meijer et al. 1982; Furnival et al. 1983; Pertschuk et al. 1985). A number of other papers lack the biochemical control of the FSA (Fetisoff et al. 1980; Nenci et al. 1981). Reviewing our results and the pertinent literature, we conclude that Lee's method does not measure the ER content of breast cancer tissue. Van Marle et al. (1982) state that the fluorescence "is certainly not due to interaction with type I receptors", but consider it likely "that type II receptors are involved which are probably localized at the same site as type I receptors". We are most doubtful about this statement since the large discrepancy between the results of the DCC and of the FSA in our studies does not favor this opinion. Finally it should be mentioned that BSA-FITC or BSA-tetramethylrhodamin conjugates with other steroids than 17β-estradiol (e.g. hydrocortisone, dexamethasone and even cholesterol) indistinguishably label one and the same tumour specimen. Thus, cytochemical labelling of tumour specimens with steroid-BSA dye conjugates occurs independently of the class of hormone (Manz et al. 1983). In conclusion, Lee's method should definitely be abandoned.

3. Evaluation of the ER-ICA

To date, the ER-ICA represents the only method by which the specific (nuclear) receptor protein (=type I binding sites) may be demonstrated. The other methods only measure the steroid binding capacity of certain other non-specific binding sites. Therefore, the ER-ICA is completely independent of the presence of oestrogen and antioestrogen substances which might block an undefined amount of ER in certain neoplastic cells or in a certain tumour as a whole. The tissue content of identifiable receptors decreases with increasing amounts of endogenous hormones in the cytosol (Caffier and Brandau 1978), while no correlation exists between the plasma hormone level and the ER content (Reiner et al. 1981). Furthermore, the results of the DCC may be negatively influenced by intratumour estradiol

biosynthesis. Tumours having a high capacity to form estradiol from estrone sulphate will show low cytosol receptor values due to extensive blockade of the hormone binding sites (Carlström 1984). These observations substantiate the major advantages of the ER-ICA compared to all other previously described histochemical methods.

There are several questions, however, which have been left unsettled so far and will be discussed shortly:

(a) Our series contains a few cases with a *qualitative* discrepancy (DCC negative/ER-ICA positive) between the results of the DCC and the ER-ICA. This is consistent with the results of Coombes et al. (1984) and Charpin et al. (1984) who also found ER-ICA-positive cases with a negative DCC and vice versa. There are even more cases which lack a *quantitative* correlation between the two methods although a statistical agreement (increasing ER mean range levels with increasing staining intensity) has been described by other authors (Charpin et al. 1984; McCarty et al. 1984; Ozzello et al. 1984; Jonat and Stegner 1984) and confirmed by our present studies (Fig. 2). Such quantitative and even (in cases with a low DCC value) qualitative differences may be due to one or more of the biochemical findings described above or be explained by different histological structures of the section used for the ER-ICA and the tissue block homogenized for the DCC, especially in tumours with a high content of fibrous connective tissue or with large necroses. It is surprising that in our studies the correlation between the IRS and the DCC was worse when the DCC values had been corrected for cellularity. However, this observation may be due to the wider range of the uncorrected values: the scale becomes narrower after a number of DCC values has been divided by a factor from 2 to 4. Another reason may be that the section examined by the ER-ICA is not fully representative of the whole tumour. The structure of a tumour may vary considerably from one portion to another (van Netten et al. 1985). Different DCC values within one and the same tumour have been not only found by previous authors (Kolb et al. 1979; Jakesz et al. 1979; Silfverswärd et al. 1980; Alanko 1985; van Netten et al. 1985) but also in the present study including one case in which one sample was below 10 fmol/mg (=ER-negative) and another one 120 fmol/mg (=ER-positive). This is in agreement with the results of van Netten et al. (1985) who found negative and positive areas within the same tumour in 9 out of 26 carcinomas with 4 samples per tumour examined.

In our opinion, a positive ER-ICA should be used as a basis of endocrine treatment even if the DCC value is negative, at least as long as large comparative studies on the relation between ER-ICA results and the therapeutic response to endocrine treatment are not available. A positive result with the ER-ICA is superior to a negative one with the DCC since the latter may be due to a too small amount of viable cancer tissue.

(b) Secondly, there is no general agreement as to how the result of the ER-ICA should be expressed. Several authors developed an *"Immuno-Reactive Score" (IRS)* which is calculated from SI and PP. Unfortunately, the mode of calculation differs markedly. This is true both for the ques-

Table 9. Expression of ER-ICA results by different authors

Term for IRS	Authors	SI	PP	Calculation of IRS	Threshold value	Hypothetical maximal value
IRS	Beck et al. (1985)	4 categories (0 to3)	5 categories (0 to 4) <20%, 20–50%, 51–80%, above 80%	$(SI \times PP) + C$[a]	none	14
IRS	Gille (1985)	4 categories (0 to 3)	percent per SI category	$\frac{\sum P_{SI} \times SI}{100}$[b]	none	3
HSCORE[d]	McCarty et al. (1985)	5 categories (0 to 4)	percent per SI category	$\sum P_{SI} \times (SI+1)$[e]	75	500
HSCORE[d]	Stegner et al. (1985)	5 categories (0 to 4)	percent (total SI 1–4)	$\sum PP \times (SI+1)$[e]	75	500
None	Poulsen et al. (1985)	4 categories (0 to 3)	4 categories (0 to 3) <10%, 10–50%, >50%	not mentioned	none	–
IRS I	Present study	4 categories (0 to 3)	4 categories (0 to 3) <10%, 10–50%, >50%	SI + PP	none	6
IRS II	Present study	4 categories (0 to 3)	4 categories (0 to 3) <10%, 10–50%, >50%	SI × PP	none	9

[a] C = Correction factor for epithelial content of tumour (epithelial content = stroma: +1; epithelial content larger as stroma content: +2)
[b] P_{SI} = Percentage of stained cells
[c] SI = highest grade observed in the tissue section
[d] HSCORE = Histochemical Score
[e] P_{SI} = percentage of stained cells in each SI category

tion as to how many categories of SI (0 to 3 or 0 to 4) should be used and how the percentage of positive cells should be expressed (either by percentage of positive cells in each SI category or by index number of each category). Table 9 summarizes the present definition of IRS as described by several authors. We found that calculating the IRS by multiplying SI and PP gives a better correlation with the DCC values than calculating the IRS by summing the SI and PP values. A more meticulous mode of calculation has been described by Gille (1985) and by McCarty et al. (1985). Both authors estimate by subjective evaluation the approximate percentage of positive cells in each SI category and sum the different values to form the IRS. We are doubtful as to the usefulness of this mode of calculation, since the determination of SI is much influenced by subjective impression

due to the pronounced staining heterogeneity of the tumour cells within a certain tumour as demonstrated by intra- and inter-observer variation in SI and PP (Table 6).

At present, no definite conclusions may be drawn concerning the value of the different IRS. It is highly desirable, however, that general agreement is reached soon so that future studies might be better comparable.

(c) In this context, the question has not yet been settled if (and where) a *threshold value* should be defined for the separation of "negative" and "positive" tumours. Most authors avoid separating these two groups, obviously in consideration of the fact that large studies concerning the results of endocrine treatment in relation to ER-ICA values are still lacking (Table 9). We also think that it is too early at present to define a threshold value, and in view of the different methodological basis of the DCC and the ER-ICA the search for such a value may even be of no use. From a practical standpoint only such cases which lack any staining reaction at all should be called "negative". Then the clinician may decide even in cases with a low IRS whether he will attempt endocrine therapy or not. In comparison to the DCC results, it should be kept in mind that only 60–70% of DCC positive cases respond to endocrine therapy.

(d) An additional problem concerns the *nature and localization of the ER in the cell.* Until recently, it has been generally accepted that the ER is localized in the cytoplasm and that the steroid-receptor complex is translocated into the nucleus. The DCC measures the ER content of the cytosol which contains both cytoplasmic and nuclear substances. The ER-ICA, however, demonstrates exclusively (Bojar and Ertl 1984; Charpin et al. 1984; McCarty et al. 1984; Greene et al. 1984; present studies) or at least almost exclusively (Jonat and Stegner 1984; Nadji et al. 1985; Poulsen et al. 1985) a *nuclear* ER protein. A predominantly *cytoplasmic* localization of the ER is seen by the ER-D5 monoclonal antibody assay (Lüttges and Zwiorek 1985; Beck et al. 1985) and by a polyclonal antibody for ER developed by Raam et al. (1982). ER-ICA and ER-D5 results do not agree although the correlation between DCC and ER-D5 values is nearly as high as between DCC and ER-ICA values (Beck et al. 1985). Certainly ER-D5 protein is not identical with a specific receptor protein (Heubner 1985). Probably the concept of a primary cytoplasmic localization of the ER must be changed in favor of a primary nuclear localization of both cytosol and nuclear forms of receptor protein (Welshons et al. 1984; Greene et al. 1984). In this context, it is remarkable that high affinity estradiol binding as demonstrated in tissue sections by autoradiography occurs primarily in the nuclei (Martin and Sheridan 1982; Welshons et al. 1981). Also, N-fluoresceine-N′-[17β-(estradiol-hemisuccinamido-)ethyl]-thiourea, a substance readily crossing intact cellular membranes, gives a prominent nuclear staining, and the results of this FSA positively correlate with those of the DCC (Barrows et al. 1980). Biochemical studies have shown that the contents of nuclear and cytoplasmic ER within the same tumour show a positive correlation (O'Connell et al. 1982). This may explain why the results of the DCC and the ER-ICA agree in most cases.

(e) A final question concerns the possible application of the ER-ICA to tumour smears and to paraffin sections. Preliminary studies in our laboratory were not successful. Recently, Eiermann et al. (1985) observed a 66.9% agreement between the ER-ICA and the DCC results in *tumour smears.* In 23.5% of their cases, DCC was positive and ER-ICA negative. They explain this high number of ER-ICA-negative cases by the low cellularity and the lower sensitivity compared to the ER-ICA on frozen sections. In our opinion, it may be explained further by insufficient diffusion of the antibodies into the nuclei because the nuclear membranes are widely left intact in smears compared with histological sections. In any case, there is no practical reason to use smears instead of frozen sections for the ER-ICA. In *paraffin sections,* Poulsen et al. (1985) were unable to find a good correlation between the ER-D5 assay and the DCC, and they discuss one or more of the following factors as a possible explanation: a loss of determinants for the primary antibodies due to the fixation or the embedding process, a too low concentration of ER protein below the limits of detectability by the present immunohistological techniques, an affinity of the ER protein for monoclonal antibodies varying from tumour to tumour, or a different expression of ER from patient to patient. The three latter explanations would be valid, of course, also for the ER-ICA on cryostat sections. Recently, Shimada et al. (1985) described a 88% agreement between ER-ICA studies in frozen and paraffin sections of the same tumours and state "that ER can be demonstrated immunocytochemically by use of paraffin sections as well as frozen sections." This statement disagrees with the results obtained by Poulsen et al. (1985) and with our personal experience in a limited number of cases. Further studies are needed in order to determine the influence of fixation and embedding procedures on the ER content of breast cancer cells.

(f) *In conclusion,* despite all these unsolved questions, the ER-ICA performed on cryostat sections must be considered as a useful and at present as the sole morphological method by which the ER itself may be demonstrated. It should be complemented, however, by the DCC, especially in view of the fact that the question of primary localization of the ER is still open and that an immunocytochemical assay for PgR is not yet available. Recently, Heubner et al. (1985) were able to purify PgR from human uteri and to produce anti-PgR antibodies which reacted with PgR in crude cytosol. Therefore, it appears possible that a PgR-ICA will soon be available.

4. Evaluation of the LRA

As shown previously by Klein et al. (1983), the PNA binding sites are related to the milk fat globule membrane and thus may be used to identify structures in tumour tissue which are not easily detectable by conventional staining methods, especially in undifferentiated carcinoma. Moreover, they reflect the functional status of the tumour cells and in particular allow recognition of hormonal influences on breast cancer cells. In detail, the PNA receptor

represents a carbohydrate structure of a secretion-associated glycoprotein which, as a product of cellular metabolism, is localized at the end of the functional process triggered by hormonal actions. This assumption has been confirmed by experimental (Vierbuchen et al. 1983) and empirical (Klein et al. 1983) investigations. In conclusion, the lectin histochemical method using PNA should not be considered as an alternative for ER and PgR assays but represents a method providing useful additional information about the functional differentiation of the tumour cells. Moreover, PNA histochemistry should be used as an alternate method for the detection of hormone-responsive carcinomas if the tumour has been previously fixed in formalin thus making the DCC and ER-ICA impossible. The response to endocrine therapy is 64.1% in LRA-positive cases and 12.5% in LRA-negative cases. These figures correspond to those of the DCC (Klein 1985).

5. Evaluation of Barr body estimation

The literature concerning the relations between sex chromatin frequency and steroid receptor content in breast cancer is arbitrary. According to some authors, carcinomas with Barr bodies in more than 10–20% of the tumor cells are more likely to have ER than those with a lower proportion of Barr body containing cells (Rosen et al. 1977; Bishun et al. 1979, 1981; Smethhurst et al. 1981). Conversely, Rajeswari et al. (1977) missed a significant correlation between ER and Barr body content in 50 unselected cases of breast cancer. Their results agree with ours in the present series. Consequently, the determination of sex chromatin – originally the first method to predict the possible therapeutic effect of endocrine treatment (Hienz 1959; 1965) – should be generally replaced by the recent biochemical and immunohistochemical methods for the estimation of ER and PgR in breast cancer cells. As in the LRA it should be borne in mind, however, that the lack of agreement between the DCC and the Barr body content of human breast cancer cells does not preclude a prognostic statement with regard to a possible endocrine response of an individual tumour.

Conclusions

At present the *ER-ICA* is the sole morphological method which allows direct determination of the ER protein in breast cancer cells. Moreover, it may be used in cases with too low an amount of tumour tissue (below 0.3 to 0.5 g) for the DCC (Remmele et al. 1982). Prospectively, it would be most useful to combine the ER-ICA with an additional histochemical assay for oestrogen-dependent steroids such as progesterone. Moreover, it would be desirable to arrive at an international agreement for the grading of SI and PP and for the calculation of an IRS from these two variables. It may be useful to exchange reference sections between the different laboratories in order to improve comparability of their results.

However, the methods based on *FSA* should be definitely abandoned for three reasons: (1) Their results lack a positive correlation with those

of the DCC. (2) They certainly do not measure the content of the specific ER in the tumour tissue. (3) They are as expensive and time-consuming as the ER-ICA which undoubtedly is the better method for theoretical and practical reasons.

The *LRA* may not simply be compared to the DCC or the ER-ICA because it does not measure the ER content but the functional differentiation of the tumour cells expressed by their ability to secrete certain glycoproteins.

Generally, *Barr body estimation* in breast cancer cells has been replaced by determination of the ER content by the DCC and/or the ER-ICA. Modern treatment of breast cancer is based upon the results of these methods. The information on Barr body content of a certain tumour would no longer be considered as a sufficient basis for the therapeutic concept.

So far, we have no experience with the Abbott *Estrogen Receptor Enzyme Immunoassay (ER-EIA)* which is a solid phase enzyme immunoassay based on the "sandwich" principle. Beads coated with monoclonal Anti-ER are incubated with tissue cytosols, the appropriate standards and control. A second monoclonal Anti-ER conjugated with horseradish-peroxidase is then reacted with the ER bound to the bead, and the receptor concentration is determined by an enzyme reaction of the horseradish-peroxidase. While some authors report an excellent correlation between the DCC and the ER-EIA (Leclercq et al. 1984), others found a qualitative accordance between the ER-EIA and the DCC in 78.9% (correlation coefficient = 0.70) (Eiermann et al. 1985).

Finally, it must be stressed that the ER-ICA, the ER-EIA and the LRA demand special experience. This is true both for the technical performance and for the subjective evaluation of the tissue sections. Only then may these methods complement or possibly replace the DCC.

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