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DISPLACEMENT BY TAMOXIFEN OF THE ESTRADIOL–ESTROGEN RECEPTOR BINDING: A FUNCTIONAL ASSAY FOR BREAST CANCER STUDIES

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Summary—Displacement curves with estradiol (E_2) and Tamoxifen (Tam) of the [^{3}H]E_2-ER binding in 49 ER+ mammary neoplasias showed a great heterogeneity suggesting the existence of more than one population of ER+ tumors when the relative binding affinity of both ligands for the ER was considered. The ($D_{50}E_2/D_{50}$Tam) × 100 ratio was called Displacement Index (DI) with values assimetrically distributed from 0.05 to 2.90. The range from 0.18 to 0.54 was adopted as central interval given by the median ± 2 SE (median: 0.36; SE: 0.09). DI values below 0.18 (24% of the tumors in our series) were considered as "lower", indicating that higher Tam doses would be necessary to displace the E_2-ER binding. The potency of Tam as displacer is dependent not only of its own affinity for the ER, but also of that of E_2 for the same receptor. The DI expresses their relative binding "strength". DI values were not correlated with ER and progesterone receptor content nor with the D_{50}Tam and $D_{50}E_2$ taken separately.

Antiestrogen binding sites (AEBS) were determined in the cytosol ($AEBS_c$) and in the microsomal fraction of 10 ER+ tumors from our series. The $AEBS_c$/ER ratio was inversely correlated with the DI, that is, displacement of $^3HE_2$ from the E_2-ER complex by Tam would be lower in tumors with higher $AEBS_c$/ER ratio. The DI is another parameter to be considered in the study of the sensitivity of breast neoplasias to antiestrogen treatments.

INTRODUCTION

Hormone dependency of mammary cancer is indicated mainly by the presence of steroid receptors in the tumor. It is accepted that response to endocrine therapy (antiestrogens, aromatase inhibitors, antagonic hormones, ablative therapy) as well as favorable prognosis is correlated with the presence of estrogen receptors (ER) and progesterone receptors (PgR) in the tumoral tissue in approx. 75% of the cases [1]. When only ER are considered, the proportion of responders is around 60% [2, 3].

PgR is one of the proteins induced by estradiol (E_2) and it is considered as an expression of a functional active E_2-ER complex in the stimulation of the codifying gene [4]. Studies of some of the functional manifestations of receptor activity are currently being used to better characterize the hormonal dependency of mammary tumors besides their quantitative measurement [5–7].

Tamoxifen (Tam) is the antiestrogen (AntiE) most used in mammary oncology, both in precautional and active treatments. It is accepted that the molecular basis of its action is given by the binding of the AntiE to the ER resulting in the displacement of the natural hormone E_2 from the complex E_2-ER [8]. The grade of displacement is a function of the relative binding affinity (RBA) of both ligands for the ER.

In this article we describe a displacement assay based on the inhibition of E_2-ER binding by Tam and we propose a Displacement Index (DI) as a functional parameter of the ER directly related to the potency of the antiestrogen as displacer and consequently to its antitumoral effect. We observed heterogeneity in the DI from specimen to specimen that may reflect alterations in the ER dynamics or the existence of several types of ER populations with respect to their RBA.

MATERIALS AND METHODS

The displacement assay was performed in surgical mammary tumor samples of 72 patients

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from four hospitals. The samples were immediately placed in dry ice and sent to the laboratory, where they were stored at −70°C until processed within 30 days after arrival. For ER determinations a tissue homogenate, 1:5 (w/v) in buffer (Tris, 10 mM; EDTA, 1.5 mM; sodium molybdate, 10 mM; glycerol, 10% w/v and 2-mercaptoethanol, 2 mM; pH 7.4) was prepared with an Ultraturrax homogenizer. Homogenates were centrifuged at 100,000 *g* for 30 min and aliquots of the supernatant (cytosol) were incubated for 18 h at 0–4°C with 10 nM saturating concentration of $^3HE_2$ without and with 200 × excess of unlabeled E_2 for total and nonspecific binding. The saturating [^{3}H]E_2 concentration was adopted after several multipoint trials. The carbon–dextran method (C–D) was used throughout to separate the hormone-receptor complex from the free ligand [9]. For the displacement assay, three additional E_2 concentrations and five Tam concentrations were included in the [^{3}H]E_2 incubations to calculate the D_{50} of each ligand. D_{50} is defined as the ligand concentration necessary to inhibit or to displace 50% of the [^{3}H]E_2 specific binding. Ligand concentrations for the displacement assay were: 5, 10 and 50 nM for E_2 and 0.5, 1, 5, 10 and 50 μM for Tam. Incubation, separation by C–D and radioactivity measurements were the same as for determination of ER. All tubes included 2 μM dihydrotestosterone to prevent the binding of E_2 to plasma sex hormone binding globulin. Data were processed by the EBDA program [10]. PgR was determined in another cytosol aliquot by incubation with 10 nM [^{3}H]R5020 in the presence of absence of 200 × excess of unlabeled analogue for 3 h at 0–4°C. The remaining procedures were the same as for ER. ER, PgR determinations and the displacement assay were performed in the same cytosolic fraction by duplicate for each one of the competitor concentrations. For total binding triplicate aliquots were used.

AEBS were determined in the cytosol and in the microsomal fraction of 10 ER+ tumors. Nuclear and mitochondrial fractions were separated by a 12,000 *g* 15 min centrifugation. The supernatant was then submitted to a 100,000 *g* 30 min centrifugation to obtain the microsomal pellet. After washing twice, the microsomal residue was extracted with buffer TE (Tris, 10 mM; EDTA, 1.5 mM and KCl, 0.5 M, pH 7.4) for 30 min at 0°C and centrifuged at 17,000 *g* 30 min. The supernatant and the cytosol were incubated for 18 h at 0–4°C with 10 nM [^{3}H]Tam without and with 2 μM Tam to obtain total and nonspecific binding, in the prersence of 2 μM E_2 to prevent Tam binding to the ER. Separation of the free ligand was accomplished with C–D for 10 min in the cold.

RESULTS

From the 72 tumors processed for this study, 49 were ER+ (68%) and 23 were ER− considering a cut off level of 10 fmol E_2/mg cytosol protein. The displacement studies were initiated by drawing competition curves to calculate the D_{50} for E_2 and for Tam and the RBA of Tam in relation to E_2: $D_{50}E_2/D_{50}$Tam (Table 1). Figure 1 shows the curves for tumors C and D. RBA is 13 times higher in tumor D than in tumor C, that is, E_2-ER binding in tumor D is much more displaceable by Tam than in tumor C. After several orientation trials the concentration referred in the section "Materials and Methods" were selected to reduce the points to calculate the D_{50} for both ligands. The ratio ($D_{50}E_2/D_{50}$Tam) × 100 was called "Displacement Index" (DI).

Figure 2 shows the distribution of the 49 ER+ tumors according to their DI and ER content. We did not find correlation between these parameters. DI values were heterogeneous and ranged from 0.0005 to 2.97. For one additional tumor DI could not be calculated because none of the Tam concentrations used in the displacement assay did significantly inhibit the [^{3}H]E_2-ER binding ($D_{50}E_2$: 22.9 nM; D_{50}Tam: ∞). Another tumor had a DI extremely high (18.5). These two cases were not considered for the calculation of the DI distribution. The data were assymetrically distributed (median: 0.36; mean: 0.55; SE: 0.09).

The reciprocal value of the D_{50} for both ligands is an expression of their affinity for the ER. The frequency representation of D_{50} values is shown in Fig. 3. We found an assymetrical

Table 1. $D_{50}E_2$, D_{50}Tam and relative binding affinity (RBA) in 6 ER+ mammary tumors

Tumor	RE (fmol/mg protein)	RPg	$D_{50}E_2$ (nM)	D_{50}Tam (μM)	RBA ($D_{50}E_2/D_{50}$Tam)
A	51	43	0.37	78	0.000005
B	344	18	7.0	178	0.00004
C	17	10	5.3	10	0.00053
D	228	71	33	4.7	0.007
E	121	—	20.2	1.27	0.016
F	21	4	111	0.6	0.185

Horizontal columns arranged A to F by their increasing RBA. The wide range of RBA suggested the existence of more than one ER+ population.

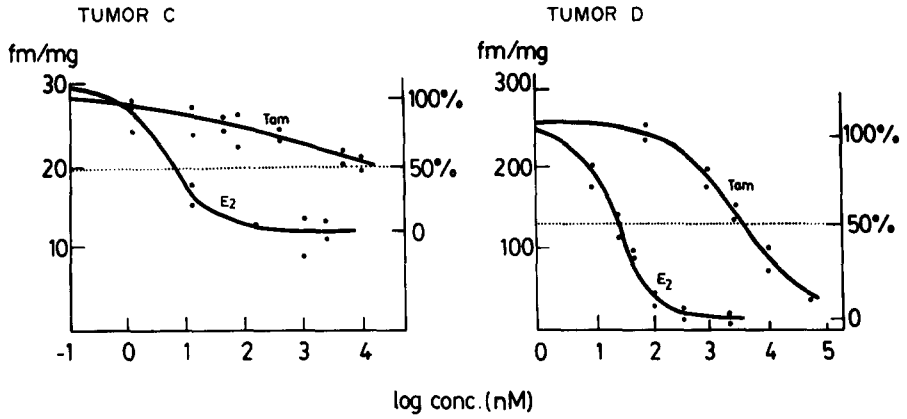

Fig. 1. Displacement curves of [^{3}H]E_2-ER binding by E_2 and Tam in two ER+ tumors. Left ordinates: total binding (specific + nonspecific binding) expressed as fmol [^{3}H]E_2 bound/mg cytosol protein. Right ordinates: specific binding expressed as percentage. D_{50} is the ligand concentration for 50% inhibition of the specific binding. Relative binding affinity (RBA) = $D_{50}E_2/D_{50}$Tam. Tumor C: ER: 17 fmol/mg cytosol protein; RBA = 0.0005. Tumor D: ER: 228 fmol/mg cytosol protein; RBA = 0.007.

distribution of the data for both ligands which reflects the heterogeneity of the affinity values.

AEBS were determined in 10 ER+ tumors of at least 1 g size necessary to perform all the measurements (Table 2). All data were processed by a multivariable computer program and correlations among variables were established by the r coefficient. From the 49 ER+ tumors, $D_{50}E_2$ and D_{50}Tam taken separately, were not correlated with the DI (r: 0.38 and -0.13 respectively), (data not shown). From the data of Table 2, an inverse correlation was found between the $AEBS_c$/ER ratio and DI (Fig. 4). (r: -0.72; $P < 0.01$). No correlation was found between PgR and DI values (r: $-$ $P < 0.09$) indicating that both parameters are independent.

DISCUSSION

The basis for the use of Tam in the treatment of mammary cancer is the binding of the antiestrogen to the ER in competition with E_2. It should result in the inhibition or decrease in the synthesis of proteins or factors related to the replication of tumoral cells. However, approx. 40% of ER+ mammary tumors do not respond to such treatments. One explanation is that the cell proliferation becomes independent of the estrogen induction in spite of the presence of the receptors, probably due to alterations in some

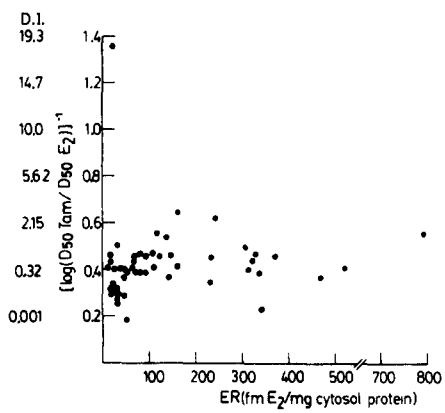

Fig. 2. Representation of 48 ER+ mammary tumors according to the DI and ER content. From the 49 ER+ tumors of our series, one is omitted because its DI could not be calculated: none of the Tam concentrations used in the displacement assay did significantly inhibit the [^{3}H]E_2-ER binding ($D_{50}E_2$: 22.9 nM; D_{50}Tam: ∞). For the graphic representation $D_{50}E_2/D_{50}$Tam is expressed as $[\log (D_{50}\text{Tam}/D_{50}E_2)]^{-i}$. Corresponding DI values are in the parallel scale.

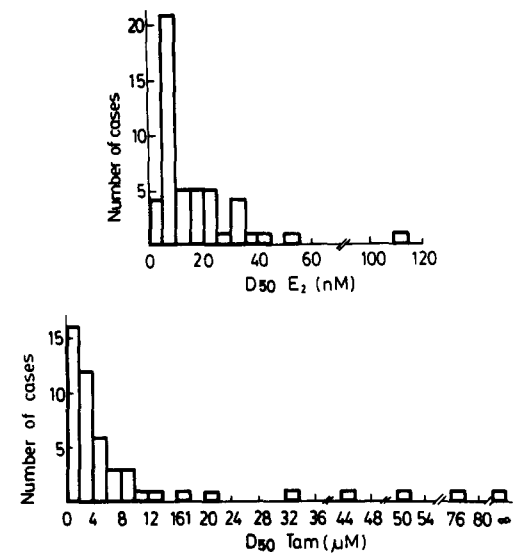

Fig. 3. Frequency distribution of $D_{50}E_2$ (Fig. 3A) and D_{50}Tam (Fig. 3B) for [^{3}H]E_2-ER binding in 49 ER+ mammary tumors.

Table 2. ER, PGR, AEBS, DI and related parameters in 10 ER+ mammary tumors

ER	PgR	AEBS Cytosol	AEBS microsomes	$D_{50}E_2$	D_{50}Tam	$AEBS_c$	
		fmol/mg protein		nM	μM	ER	DI
32	30	39	368	7.8	50.0	1.2	0.01
31	14	64	0	2.1	5.5	2.0	0.04
464	244	84	95	12.9	6.1	0.2	0.20
335	138	132	0	13.0	4.8	0.4	0.27
26	20	24	37	8.2	2.7	0.9	0.30
160	307	103	—	15.9	3.6	0.6	0.68
146	49	34	330	11.4	1.7	0.2	0.74
111	42	32	0	13.9	1.9	0.3	0.74
325	424	105	210	11.3	1.4	0.3	0.78
29	220	0	80	15.9	1.5	0	1.08

Horizontal columns are arranged by increasing DI values.
DI = Displacement Index = $(D_{50}E_2/D_{50}\text{Tam}) \times 100$.

of their functional properties; thus, the cells would replicate as if they were ER−. The presence of several cellular clones in a tumor [11], each with a possible different functional characteristic for their ER, is another factor that may be responsible of the unexpected results in the antiestrogen therapy of ER+ tumors. Among these functional variables, the relative ER affinities for E_2 and Tam expressed by the DI, could not favor the displacement of the hormone by the AntiE. This does not preclude the possible action of Tam at another step of the DNA activation process. Recently, Kumar and Chambon [12] observed an altered mobility of the ER–DNA complex by a gel retardation assay in the presence of Tam. The binding of steroid hormones is produced in the C terminal domain of the receptor molecule. There are at least two other domains, one related to binding with the DNA and the other containing the main antigenic determinants [13, 14]. One defective domain can coexist with "normality" of the others. The complex interaction among domains and their regulation by cellular factors is still unknown and certainly cannot be ascertained by a single determination. Therefore, the content of ER in a mammary tumor is not sufficient by itself to define the grade of hormonal dependency. In a recent study, four ER isoforms were described with different functional characteristics related to the hormonal sensitivity of the tumor [15].

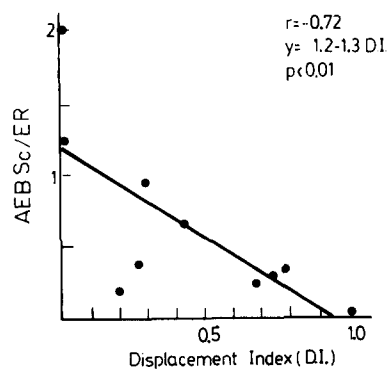

Fig. 4. Correlation between $AEBS_c$/ER and DI in 10 ER+ mammary tumors. Data from Table 2.

When PgR are also considered, around 25% of the ER+, PgR+ tumors do not respond to the antiestrogen therapy. The presence of PgR is indicative of a proper functionality of the ER, at least for this protein and again, the failure of the antiestrogen treatment can be attributed to the lack of displacement due to the unfavorable relation of E_2 and Tam affinities in the binding to the ER. The displacement assay we are proposing takes into account this RBA expressed by the DI, as an additional parameter to orientate the antiestrogen treatments together with the ER and the PgR content. A displacement assay with one Tam concentration was proposed some years ago [3] to classify mammary tumors into Tam sensitive or Tam insensitive, but correlation with the tumor E_2 affinity for the ER was not established. ER+ tumors sensitive to Tam responded better to the antiestrogen therapy and those insensitive corresponded to treatment failures.

The heterogeneity found in the DI values and the asymmetrical distribution of the data may reflect alterations in the ER binding dynamics or the existence of different receptor populations with respect to their affinities among other functional variables. Due to this assymetry, the median value was adopted as the central distribution parameter for a better grouping of the data. The interval defined by the median ± 2 SE (0.36 ± 0.18) gives a range for DI between 0.18 and 0.54. Below 0.18 the DI could be considered as low and includes 12 tumors (24%) of our series. The $D_{50}\text{Tam}/D_{50}E_2$ ratio would be over 555 $(100/0.18 = 555)$. It means that when a DI is low, due to a lower affinity of Tam or to a higher affinity of E_2 for the same receptor, a Tam concentration at least 555 times that of E_2 will be necessary to displace the hormone from the ER. The projection to an antitumoral treatment would be to increase the therapeutic dosage of Tam for a more efficient displacement of the E_2 from the receptor. A higher DI would correlate with a better response to the AntiE therapy. This can only be evaluated by the clinic under a careful protocol and would be a contribution to dilucidate the correspondence between the accepted mechanism of Tam action and the therapeutic response to the antiestrogen.

In 28 patients from one of the hospitals (Hospital Alvarez) the histological examination showed that from 16 ER+ tumors, 5 presented DI < 0.22 with a nuclear differentiation grade II or III (less differentiated) [16] similar to the 12 ER− patients of the group. This finding supports the suggestion that ER+ tumors with lower DI should behave as ER− tumors. The data presented in this article are based on *in vitro* determinations that for the time being cannot be correlated with the evolution of the patients due to their short post-operation interval. The value of the DI as a prognostic indicator will be established in studies on a larger population of patients with a longer evolution. The clinical aspects of the cases and their response to treatment are currently being carried out and will be the subject of a future publication.

AEBS have been found in ER+ and ER− mammary tumors, both in the cytosolic and in the microsomal fractions, predominantly in the latter [17, 18]. In 10 ER+ tumors of our series, where they were determined, their distribution was aleatory and not related to the ER or PgR content (Table 2). There is no correlation of the DI with the $D_{50}E_2$ and D_{50}Tam taken separately. Therefore, the dispersion of DI values shown in Fig. 2 can be attributed to the variability of the D_{50} of both ligands. Besides, the presence of AEBS can exert influence on the DI: although AEBS are not directly related to the antitumoral effect of Tam [19], their concomitant presence with ER might capture part of the ligand, leaving less Tam for its binding with the ER [20]. Its ability to interfere with the E_2-ER complex will be lower in ER+ tumors if AEBS are present simultaneously. This is reflected in Fig. 4 as an inverse correlation between the $AEBS_c$/ER ratio and the DI (r: −0.72; $P < 0.01$). This concept was also proposed to explain the estrogenic agonist effect of antiestrogens in tissues with high levels of AEBS [19].

Other parameters besides the ER content have been reported to "screen" the events involved in the ER dynamics, such as PgR measurement [1], a nuclear binding assay [6], receptor potency [5]. The DI obtained from the displacement assay is a further contribution to evaluate the functionality of the ER related to the AntiE binding in one of the first steps leading to the gene transcription. Besides, the presence of AEBS in the tumor must be born in mind as a collateral factor usually not considered in the evaluation of the AntiE therapy.

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