J. Steroid Biochem. Molec. Biol. Vol. 39, No. 4A, pp. 519–527, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

EVOLUTION OF IMMUNOREACTIVITY OF MONOCLONAL ANTIBODIES H222 AND/OR D547 USED IN THE DETECTION OF BREAST CANCER ESTROGEN RECEPTORS. VARYING REACTIVITY OF RECEPTOR ISOFORMS

H. CREN, C. LECHEVREL, G. ROUSSEL and J. GOUSSARD*
Laboratoire d'Analyses Isotopiques, Centre François Baclesse, 14021 Caen, France

(Received 4 July 1990; received for publication 17 June 1991)

Summary—From 1984 to 1990, human breast cancer estrogen receptors have been measured both by a radioligand assay (RLA[^{3}H]estradiol) and by an enzyme immunoassay (Abbott ER-EIA kit). The ratio EIA/RLA results increased continuously from 1.04 (1984) to 1.87 (1990), and this evolution was consistent with the last trial of the E.O.R.T.C. receptor study group (Trial 1989-II, EIA/RLA = 2.5). Dilution studies of cytosols with the current ER-EIA kits showed an important parallelism defect of the standard curve, the final result of cytosols (fmol/mg protein) obtained from the upper part of the curve (between 100 and 500 fmol/ml) being 1.5 to 2 times higher than the results obtained from readings of the lower part of the standard curve (between 0 and 50 fmol/ml). Chromatographic experiments were carried out during 1986 and the measures of binding sites by RLA and of immunoreactive sites by EIA on chromatographic fractions were compared. Identical results were obtained with EIA and RLA, either on polymeric forms of the estrogen receptor, or on monomeric forms obtained after dissociation by 0.4 M KCl. The same experiments performed during 1990 showed that, in the chromatographic fractions, the concentration of immunoreactive sites was twice as large as that of ligand-binding sites, detected by tritiated estradiol. Furthermore, the detection of polymeric and monomeric receptor isoforms by monoclonal antibodies varied, and was increased by the presence of KCl (0.4 M) and/or bovine serum albumin (BSA) (1 mg/ml) in the cytosol. These findings showed that the large differences between enzyme immunoassay and ligand-binding assay results currently observed were due to differential reactivity of monoclonal antibodies for the estrogen receptor standard provided in the ER-EIA kits and for the estrogen receptor present in cytosols from human breast cancers, suggesting modifications of immunoreactivity of the monoclonal antibodies actually provided in the ER-EIA kits.

INTRODUCTION

Although estrogen receptor assays have been studied extensively with radioligand assays (RLA) and more recently with the Abbott enzyme immunoassay (EIA, [1]), a clear agreement has not been obtained between these two techniques, and important variations in the results are observed, especially in international quality control programs. Beside specific difficulties inherent to both techniques, other difficulties arise from the macromolecular composition of estrogen receptors (ER) which exhibit polymorphism [2], with several protein components (isoforms) retaining steroid-binding domains as well as antigenic determinants [3, 4]. The detection of these antigenic determinants by monoclonal antibodies (Mabs) seems to depend, at least, on their spatial orientation in the receptor polymeric structure and is modified during the activation process [5, 6]. Previous studies [7], employing monoclonal antibodies D547 and H222 raised against ER (EIA), compared the assay results of 241 breast cancer cytosols with those obtained with the classical Scatchard method. These experiments showed an high correlation between both methods and the slope of the regression curve was 1.04. Six years after these first experiments, problems have been encountered, variations in the quality of Mabs

*To whom correspondence should be addressed.
Abbreviations: ER, estrogen receptor(s); Mabs, monoclonal antibodies; HPSEC, high performance size exclusion chromatography; hsp, heat shock protein; kDa, kilodalton(s); E.O.R.T.C.: European Organization for Research and Treatment of Cancer.

have been observed by users [8], and important discrepancies exist today, with EIA results being on the whole twice higher than those of RLA. In this report, we present the comparison of results obtained in the period 1984–1991 using both Mabs and radioligand, in addition with high performance size exclusion chromatography experiments, performed to compare the detection of ER isoforms by Mabs with their detection by [^{3}H]estradiol.

EXPERIMENTAL

Reagents

[2,4,6,7-^{3}H]Estradiol (96 Ci/mmol) was obtained from Amersham International Plc. (Amersham, U.K.). Estrogen receptor enzyme immunoassay kits (ER–EIA) were from Abbott Laboratories (Chicago, IL, U.S.A.). All other chemicals were of analytical grade.

Tumors and cytosols

All tissue handling and assay procedures were carried out at 0–2°C with pre-cooled equipment. Tumors from postmenopausal patients were homogenized in phosphate buffer 25 mM, pH 7.4, as described previously [9], according to the E.O.R.T.C. protocol, and cytosols were obtained by centrifugation of the homogenate for 60 min at 40,000 *g*. Cytosols were stored in liquid nitrogen until used.

Radioligand assay

Details concerning ER radioligand assay have been described elsewhere [7]. Briefly, crude ER preparations were incubated for 16–18 h at 0°C with 5 nM [^{3}H]estradiol in the presence or absence of a 500-fold excess of diethylstilbestrol (DES) as competitor. Unbound steroid was removed by dextran-coated charcoal treatment, pelleted by centrifugation, and the supernatant which contained protein-bound [^{3}H]estradiol was counted for radioactivity. The reproducibility of the method was $B = 0.96\,A + 9$ fmol/mg protein (42 cases; $r = 0.99$; A and B: first and second assay). With this technique, 92 of 96 results from the trials of the E.O.R.T.C. receptor study group, obtained between 1984 and 1991, were on the mean $\pm$ 1 standard deviation and 4 of 96 results at ± 1.5 standard deviation. Cytosol protein concentrations were determined by the method of Lowry [10], using bovine serum albumin (BSA) as standard.

ER-EIA

The assay was carried out as described in the instructions provided with the Abbott ER-EIA kit. Aliquots (100 μl) of cytosol or chromatographic fractions were mixed with 100 μl of specimen diluent buffer containing 160 nM estradiol, thus giving a final estradiol concentration of 80 nM, after which the diluted cytosols were incubated with polystyrene beads coated with anti-ER antibody (rat monoclonal D547) at 0–2°C for 18 h, to bind ER to the solid phase. The beads were then washed with distilled H_2O to remove unbound materials, and then incubated at 37°C for 1 h with 200 μl of a second anti-ER antibody (rat monoclonal H222) conjugated with horseradish peroxidase, which binds to ER on the beads. The beads were then washed with distilled H_2O to remove unbound conjugate and incubated for 30 min at room temperature with 300 μl of enzyme substrate solution (H_2O_2 and *ortho*phenylenediamine.2HCl) to develop a colour proportional to the amount of ER conjugate present. The reaction was stopped by the addition of 1 ml of 1 N sulfuric acid and the absorbance was read using a spectrophotometer set at 492 nm. A standard curve was obtained by plotting the ER concentrations of the standards (0–500 fmol/ml) vs the absorbance. The ER concentration of cytosols and of chromatographic fractions were determined from the curve. Each assay was performed with a single bead, and the reproducibility of the method was $B = 1.05\,A - 5.5$ fmol/mg protein (53 cases; $r = 0.976$; A and B: first and second assay).

High performance size exclusion chromatography (HPSEC)

All chromatographic separations were performed at 4°C with an L.K.B. model liquid chromatograph using a model 2150 solvent pump and a Rheodyne 7125 injection valve, as described previously [9]. All buffers and cytosols were filtered through a 0.2 μm filter before use. HPSEC was performed on a Spherogel-TSK-G3000 SW column (7.5 × 600 mm, L.K.B., Sweden), in line fitted with a TSK precolumn. Cytosols labelled in the absence of radioinert DES as competitor (total binding) were cleared of free steroid, and aliquots (200 μl) of these receptor preparations were applied to the column. For some experiments, aliquots of receptor preparations were treated with KCl (0.4 M) before injection to allow dissociation of

receptor isoforms. The contribution of nonspecific binding in the aliquots injected was always lower than 1% of total binding. Isocratic elution was with phosphate buffer alone [PEDG: 25 mM potassium phosphate, pH 7.4, containing 1.5 mM Na_2EDTA, 0.5 mM DTT, 10% (v/v) glycerol, 10 mM sodium molybdate] or with PEDG-K buffer containing 0.4 M KCl. Fractions (0.5 ml) were collected in tubes containing 0.5 ml BSA at 2 mg/ml in PEDG buffer to prevent ligand dissociation. Both the radioactivity of ligand-binding sites, and immunoreactive sites in each fraction were measured.

RESULTS

Evolution of immunoreactivity of the Abbott ER-EIA kit from 1984 to 1990

As part of a multicenter study on Abbott ER-EIA in Europe, we have studied the comparison of Mabs and tritiated ligands for ER assays in 241 breast cancer cytosols, during 1984. Published results [7] showed an excellent correlation between both methods with the regression curve EIA = 1.04 RLA ($r = 0.963$). In this first series, the ratio EIA/RLA was studied and the median of individual ratios was 1.12. New assay series were performed during 1988–1990, and the median of ratios EIA/RLA obtained with three different series were 1.32 ($n = 17$, 1988), 1.73 ($n = 17$, 1989) and 1.87 ($n = 26$, 1990). The medians, 25th and 75th percentiles of these series are presented in Table 1. During this period (1984–1990), pooled cytosols used as controls for long times (3 to 8 months) showed low coefficients of variation from 2.1 to 5.7%, for RLA. Furthermore, our last EIA results were consistent with those of the last trials of the E.O.R.T.C. receptor study group (trial 1990–1 and –2) where ER-EIA results were 2.2–2.5 times higher than RLA results, suggesting an increased immunoreactivity of Mabs provided in the Abbott ER-EIA kit, or/and changes in standard curve sensitivity. During our last experiments with ER-EIA kits (October 1990, batch 42756-M200), 4 lyophilized standards from ER-EIA kits with different expiration dates, March 1989, August 1990, November 1990 and July 1991, respectively, were analysed in the same series of assays. All standards presented similar results, and this showed the great stability of lyophilized standards stored at 4°C, even for standards stored for more than 20 months after their expiration date.

Repeated assays of samples stored in liquid nitrogen for 2 to 6 years

Between 1984 and 1986, ER assays were performed on 63 human mammary tumors stored in liquid nitrogen for more than 18 months, in order to study the stability of ER molecules and/or the stability of ER-EIA kits. In 1984, ER assays were first performed on fresh human mammary tumors, and a second assay was performed 18 to 24 months later, using both EIA and RLA methods. Published results [7] showed that: (1) with fresh tumors, values obtained by EIA were close to those obtained by RLA (slope of the regression curve: 1.04); (2) antigenic sites were not modified by long-term storage in liquid nitrogen (90% of immunoreactive sites recovered after a 18–24 months storage); and (3) ligand-binding sites were lowered during the same storage conditions. Thus, modifications of the ratio EIA/RLA during this long-term storage represented mainly the degradation of binding sites, and not variations of the EIA. Despite the fact that long-term storage was considered to change receptor structure (ligand-binding sites, at least), samples assayed by both methods in 1984–1985, and stored in liquid nitrogen up to the present time, were assayed with the current EIA kit, to provide comparisons with the earliest ER-EIA kits. Five different cytosols were analysed during both periods (Table 2). The mean RLA results were 121 and 103 fmol/mg protein for assays performed during 1984 and 1991, respectively, and showed

Table 1. Evolution of the ratios ER-EIA/ER-RLA from 1984 to 1990

	n	Median	25th Percentile	75th Percentile
1984	80 (Scatchard)	1.12	1.01	1.30
	39 (5 nM)	1.09	0.92	1.32
1988	17 (5 nM)	1.32	1.19	1.52
1989	17 (5 nM)	1.73	1.54	1.87
1990	26 (5 nM)	1.87	1.78	2.10

Four series of breast cancer ERs were measured both by EIA and RLA from 1984 to 1990. Steroid-binding assays were performed either at saturation (Scatchard), or with a "saturating" concentration of tritiated estradiol (5 nM). *n* = Number of cases. Individual ratios EIA/RLA were calculated to obtain the median, 25th and 75th percentiles.

Table 2. EIA and RLA assays performed in 1984 and again in 1991 on five breast cancer cytosols stored in liquid nitrogen

	EIA (fmol/mg/protein)		RLA (fmol/mg/protein)	
Cytosols	1984	1991	1984	1991
1	126	263	99	92
2	111	153	91	96
3	316	457	253	177
4	112	181	75	78
5	110	134	86	71
Total	775	1188	604	514
Mean	155	238	121	103

Due to long-term storage, there is some loss (15%) of binding sites, as measured by tritiated estradiol (103/121 fmol) meanwhile there is a 54% increase of immunoreactive sites measured by the current ER-EIA kits, in comparison with ER-EIA kits used in 1984 (238/155 fmol).

a 15% loss of binding sites during this long-term storage, confirming preceding observations [7]. The mean EIA results obtained with the same cytosols assayed in both periods were 155 fmol/mg protein (1984) and 238 fmol/mg protein (1991). These results showed a 54% "increase" of immunoreactive sites measured by the current ER-EIA kit, in comparison with EIA kits used during 1984–1985. As the same cytosols and as the same RLA method were used for both periods, this increase of immunoreactive sites, in spite of some receptor molecule degradation observed by loss of binding sites, suggested an enhanced immunoreactivity of the current ER-EIA kits used in 1990–1991.

Parallelism defect of the Abbott ER-EIA kit

To further study the quality of the ER-EIA kit, parallelism experiments were performed with diluted cytosols. If one plots the ER assay results of a dilution study against the y axis, using a logarithmic scale, and compares the resulting curve with a standard curve by using the same axes, parallelism of the two lines indicates a fixed ratio between unknown and standard, independent of y values [11]. Such experiments were performed in 1984 and again in 1990. Results presented in Fig. 1 showed that parallelism was observed during the earliest period [Fig. 1(A)] showing that the different dilutions of the standards and of three cytosols presented identical result variation (optical density). Dilution experiments performed in 1990 [Fig. 1(B)] showed that parallelism was not observed, and that consequently optical density increased more rapidly for cytosols than for standards, for increasing receptor concentration of the samples. Nevertheless, parallelism was still observed for ER standards from both

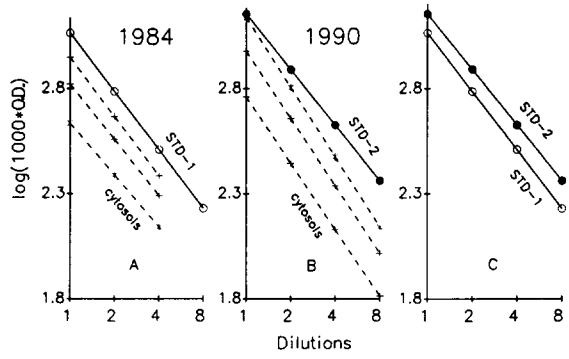

Fig. 1. Parallelism experiments with the Abbott ER-EIA. The results (optical density) of EIA of cytosol dilutions (1, 1/2, 1/4, 1/8th) were compared to results obtained with dilutions of the standard of the ER-EIA kit. (A) Parallelism was observed in 1984 between standard and cytosols. (B) In 1990, parallelism was observed between different cytosols, but it was not observed between the standard of the ER-EIA kit and cytosols. (C) Parallelism was observed between the standards from both periods (1984 and 1990).

periods [Fig. 1(C)] indicating that the actual problem seemed to be related to a different immunoreactivity of standards and of cytosols for monoclonal antibodies of the ER-EIA kit.

As a consequence of non-parallelism, variations of the ratio EIA/RLA with cytosol dilutions were observed in 1990, and this ratio increased from 1.22 ± 0.2 to 1.83 ± 0.3 with

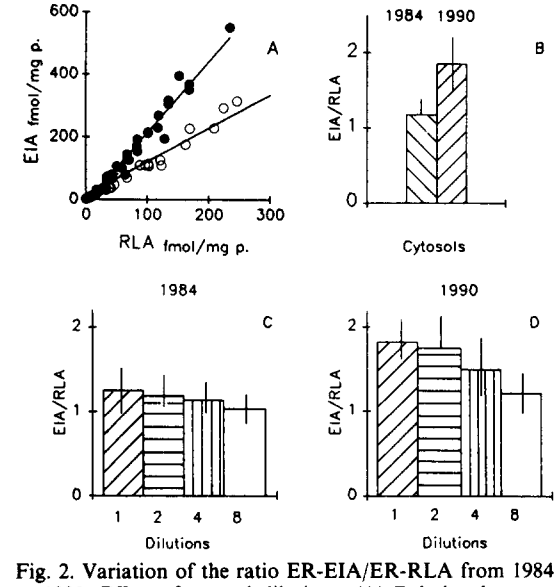

Fig. 2. Variation of the ratio ER-EIA/ER-RLA from 1984 to 1990. Effect of cytosol dilutions. (A) Relation between EIA and RLA with different cytosols; (○) 1984, EIA = 1.1 RLA ($n = 27$); (●) 1990, EIA = 1.87 RLA ($n = 30$). (B) Variation of the ratio r = EIA/RLA: $r = 1.1 \pm 0.2$ in 1984 and $r = 1.87 \pm 0.4$ in 1990. (C) and (D) Variation of the ratio EIA/RLA with cytosol dilutions; no significant variations were observed during 1984–1986 (C, $n = 72$), but an important variation of the ratio EIA/RLA was observed with cytosol dilutions in 1990 (D), the ratio r varying from 1.22 ± 0.19 (dilution 1/8th) to 1.83 ± 0.3 (dilution 1, $n = 16$).

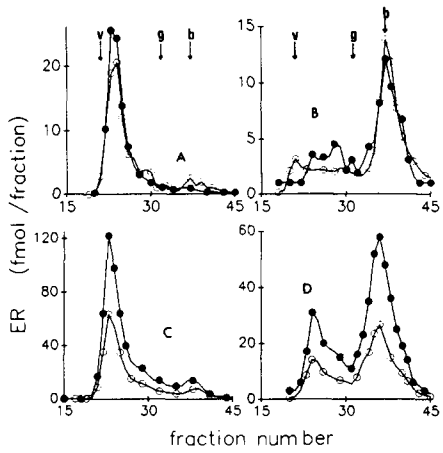

Fig. 3. Chromatographic experiments performed during 1986 and again in 1990 on interaction of human breast cancer ERs with Mabs (Abbott ER-EIA kit). Aliquots of radiolabelled receptor preparation were cleared from free [^{3}H]estradiol and applied to a TSK G3000 SW column. HPSEC was performed with PEDG-K buffer (KCl 0.4 M), fractions were collected, and both the radioactivity (RLA, ○—○) and immunoreactive sites (EIA, ●—●) in each fraction were determined. (A) and (C) non-activated ER directly chromatographed without KCl pretreatment in 1986 (A) and in 1990 (C); (B) and (D) treatment of ER by KCl (0.4 M) for 1 h at 2°C before chromatography, in 1986 (B) and in 1990 (D). Ratio EIA/RLA obtained after integration of EIA and RLA peaks (fmol): 102/104 = 0.98, and 127/124 = 1.02, for A and B, respectively (1986); 629/337 = 1.87, and 523/250 = 2.09, for (C) and (D), respectively (1990). v: void volume (thyroglobulin, 670 kDa); g: γ-globulin (158 kDa); b: BSA (66 kDa).

increasing receptor concentrations. This effect was not observed in 1984 (Fig. 2).

Increase of EIA detection of ER isoforms separated by size exclusion chromatography

As an explanation of the differences observed between ER-EIA and ER-RLA results, it has been suggested that some immunoreactive sites could be hidden in the polymeric structure of the receptor and inaccessible to the Mabs [5, 6, 12]. We have studied this assumption by two series of chromatographic experiments performed at two different periods: first in 1986, and again in 1990. During the earliest period, labelled cytosols were injected into a TSK-3000 SW column, eluted with PEDG-K buffer, and fractions were collected and analysed for ER binding sites by RLA and for ER immunoreactive sites by EIA. Examples of ER chromatographic patterns [Fig. 3(A)] showed that chromatographic fractions of the ER polymeric form contained the same quantities of immunoreactive sites and of binding sites, with only small variations. Furthermore, after proteolysis of 80% of receptor binding sites (ER kept 24 h at 20°C, to allow proteolysis by endogenous proteases), immunoreactive sites were degraded in the same extent as binding sites, and the same degraded receptor forms were detected by both EIA and RLA. On the whole, 6 different chromatographies were performed, with cytosols treated in different conditions before injection in the column (incubation at 0° or 28°C, ±KCl 0.4 M), and after integration of peaks measured by both EIA and RLA, the ratios EIA/RLA varied from 0.73 to 1.06 (median 0.98).

Chromatographic experiments performed in 1990 clearly showed different results: both techniques, EIA and RLA, detected the same receptor isoforms, but the number of immunoreactive sites detected was surprisingly about twice those of binding sites [Fig. 3(C)]. Assay results of chromatographic fractions obtained by both EIA and RLA during 1986 and 1990 are presented in Table 3. These results clearly showed the evolution of the median (and 25th–75th percentiles) of individual ratios ER-EIA/ER-RLA from 1.11 (0.96–1.28) in 1986, to 2.04 (1.77–2.16) in 1990.

To study further the differences observed in the detection of ER molecules by their physiological and immunological binding sites, labelled cytosols were treated by KCl (0.4 M), to dissociate the molybdate-stabilized receptor polymeric form into the monomeric 70 KDa isoforms and to study detection of these by RLA and EIA after chromatographic separation in PEDG-K buffer (+0.4 M KCl). EIA and RLA chromatographic patterns [Fig. 3(B) and (D)] confirmed preceding results and showed that polymeric and monomeric forms of ER were detected in the same extent by RLA and EIA during the first period [Fig. 3(B) 1986], whereas immunoreactive site levels detected during the second period [Fig. 3(D) 1990] were twice as more elevated as binding sites. During the last period (1989–1990), nine different chromatographies were performed and after integration of peaks the ratios EIA/RLA were between 1.78 and 2.27 (median 1.95). Very interesting results were obtained in 1989 with one cytosol chromatographed three times with different experimental conditions over a three month period: the ratios EIA/RLA of chromatographic fractions were between 1.78 and 2.04 for the "8S" polymeric part of the chromatogram, and between 1.04 and 1.13 for the "4S" monomeric part, showing that for this cytosol a difference in the detection of polymeric

Table 3. Results of receptor assay after HPSEC in PEDG-K buffer

	1986			1990		
	EIA	RLA		EIA	RLA	
Fraction no.	(fmol/ml)		EIA/RLA	(fmol/ml)		EIA/RLA
20	1	2	0.5	5	2	2.5
21	8	13	0.65	11	4	2.75
22	13	16	0.81	37	15	2.46
23	19	17	1.12	119	55	2.16
24	26	21	1.23	186	92	2.02
25	24	20	1.2	158	75	2.1
26	23	18	1.34	90	45	2
27	20	15	1.33	53	27	1.96
28	17	14	1.21	34	18	1.88
29	20	16	1.25	32	14	2.28
30	19	17	1.11	26	13	2
31	14	16	0.87	22	11	2
32	15	13	1.15	24	11	2.18
33	11	10	1.1	28	13	2.15
34	12	9	1.33	31	14	2.21
35	12	9	1.33	41	19	2.15
36	10	9	1.11	47	23	2.04
37	11	9	1.22	39	18	2.16
38	14	13	1.07	36	17	2.11
39	17	19	0.89	32	18	1.77
40	15	24	0.62	27	20	1.35
41	11	15	0.73	19	15	1.26
42	10	11	0.9	12	9	1.33
43	10	9	1.11	7	5	1.4
44	9	7	1.28	3	2	1.5
EIA/RLA median		1.11			2.04	
(25th p–75th p)		(0.96–1.28)			(1.77–2.16)	

Two different breast cancer tumors were used. Estradiol labelled cytosols were chromatographed, fractions (1 ml) were collected and both the binding sites (RLA) and immunoreactive sites (EIA) in each fraction were determined. After integration of results of the 25 chromatographic fractions, the ratio EIA/RLA were 361/342 = 1.05, and 1119/555 = 2.01, for experiments performed during 1986 and 1990, respectively. Individual EIA/RLA ratios were calculated for each fraction to obtain the median, 25th, and 75th percentiles, for each series of measures.

and monomeric receptor isoforms by EIA could be observed. These results were confirmed again in 1990–1991 with other cytosols [see Fig. 4(B)]. These observations showed that the actual ratio EIA/RLA ≈ 2 could not be systematically observed in an assay of a series of chromatographic fractions, and consequently, that this increased ratio must rather represent a differential reactivity of Mabs against ER isoforms than a systematical variation of results due to an increased sensitivity of the standard curve.

Modification of the immunoreactivity of Mabs by buffers, BSA and KCl

In our series of chromatographic experiments, we have observed that the number of immunoreactive sites recovered in chromatographic fractions were always higher than the number of immunoreactive sites measured in the aliquot before injection in the column, recoveries varying from 103 to 171%. Aliquots of cytosol to be injected were either in low salt Tris or phosphate buffer, elutions were in PEDG-K buffer (0.4 M KCl), and fractions were collected in tubes containing PEDG buffer and BSA (1 mg/ml) to protect receptor structure. Thus, the main differences between aliquots injected and recovered as the presence of KCl and BSA in collected fractions. To evaluate the possible effect of KCl and BSA on the detection of ER immunoreactive sites, a tumor was homogenized in low salt phosphate buffer without molybdate (to obtain both polymeric and monomeric receptor isoforms) and, after incubation with tritiated estradiol, the cytosol was injected in a TSK G3000 column and eluted with PEDG buffer (without KCl). The 25 collected fractions were separated in 4 aliquots each (100 μl, A, B, C and D) and 100 μl of the following solutions were added: PEDG for A, PEDG + BSA (2 mg/ml) for B, PEDG + KCl (0.8 M) for C and PEDG + BSA and KCl for D. Steroid-binding sites and immunoreactive sites were then measured in each fraction. Results presented in Fig. 4 showed that immunoreactive sites detected in a given fraction depended on the presence or absence of BSA or/and KCl. In low salt buffer [Fig. 4(A)], the receptor polymeric form was detected better by Mabs than the monomeric form, in comparison with tritiated estradiol, and after integration of peaks (fmol) the ratio EIA/RLA was

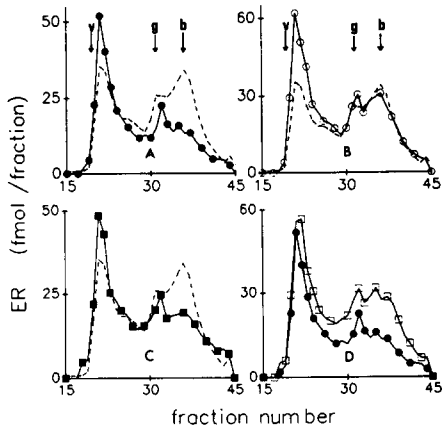

Fig. 4. Effect of phosphate buffer, BSA and KCl on the detection of ER isoforms by Mabs, after separation by HPSEC. A tumor was homogenized in low salt phosphate buffer without molybdate, in order to obtain both polymeric and monomeric ER isoforms. After incubation with tritiated estradiol (5 nM), the cytosol (500 μl) was injected in a TSK G3000 SW column and eluted with PEDG buffer (without KCl). Collected fractions were separated in 4 (100 μl)-aliquots each (A, B, C, D) and 100 μl of the following solutions were added: PEDG for A (●—●), PEDG + BSA (2 mg/ml) for B (○—○), PEDG + KCl (0.8 M) for C (■—■), and PEDG + BSA + KCl for D (□—□). Integration of EIA peaks: A = 406 fmol, B = 597 fmol, C = 482 fmol and D = 645 fmol. Chromatographic differences between EIA peaks measured in low salt buffer (406 fmol, A) and in buffer containing both KCl and BSA (645 fmol) are presented in D. v: void volume (thyroglobulin, 670 kDa); g: γ-globulin (158 kDa); b: BSA (66 kDa). RLA peaks: (- - -).

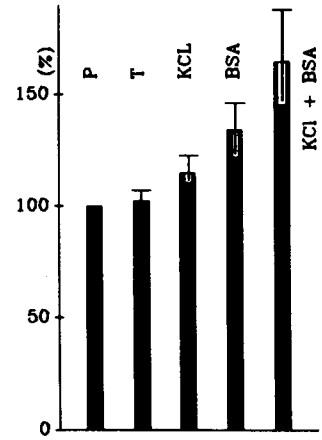

Fig. 5. Effect of Tris and phosphate buffers, KCl, and BSA on ER-EIA detection in breast cancer cytosols. EIA were performed on 48 cytosols in the following conditions: cytosols were diluted in Tris (T) or phosphate buffer (P), in buffer containing either KCl 0.4 M (KCl), or BSA 1 mg/ml (BSA), or in buffer containing both KCl and BSA (KCl + BSA). Results are presented as percent of immunoreactive sites detected in phosphate buffer alone. KCl, BSA, and KCl + BSA increased the number of immunoreactive sites detected in comparison with those detected in phosphate buffer: Tris (103 ± 3%), KCl (115 ± 5%), BSA (134 ± 8%) and KCl + BSA (165 ± 14%).

242/207 = 1.17 for the polymeric form and 164/285 = 0.58 for the monomeric form. In the presence of BSA [Fig. 4(B)], polymeric forms were detected better by Mabs: EIA/RLA = 310/207 = 1.5 and 287/285 = 1 for polymeric and monomeric forms, respectively. Similarly, in the presence of KCl [Fig. 4(C)] antigenic sites were detected better (482 fmol) than in low salt buffer (406 fmol), and in the presence of both KCl + BSA the effect was cumulative [Fig. 4(D)] and integration of EIA peaks varied from 406 fmol for low salt assays [Fig. 4(A)] to 645 fmol for high salt + BSA assays. Thus, in these experiments, the detection of immunoreactive sites could be increased up to 60%, depending on the composition of the incubation medium during the EIA.

Finally, assay series were performed with 48 cytosols diluted either with Tris buffer or with phosphate buffer, and in the presence of KCl, BSA or KCl + BSA, to verify preceding results observed after the chromatography of ERs. The dilution buffer, Tris or phosphate, had no effects on ER-EIA results, but the presence during incubation with beads of KCl (0.4 M), BSA (1 mg/ml) or both KCl + BSA, increased EIA results up to 115, 134 and 165%, respectively, in comparison with results obtained in low salt phosphate buffer (Fig. 5). The presence of BSA both increased results and corrected partly the parallelism defect of the assay.

DISCUSSION

Six years ago, when Abbott ER-EIA kits were used for the first time by numerous laboratories [1], all users obtained an excellent correlation between ER-RLA and ER-EIA assays, and the slope of the regression curves was $S \approx 1$ [13–15]. From these first observations, it was assumed that Mabs D547 and H222 each detected one immunoreactive site per ER molecule [4, 16]. Today, our results, confirmed by recent results of the quality control program of the E.O.R.T.C. group, demonstrate clearly that this correlation no longer exists, the ratio r = EIA/RLA having increased from $r \approx 1$ to $r \approx 2$ or 2.5, from the earliest period (1984) to 1991. These findings suggest either that there has been a progressive shift of the calibration curve, or that properties of Mabs purchased today in the ER-EIA kits have changed, and that Mabs are now able to eventually detect more than one immunoreactive site per receptor molecule. A shift of the calibration curve cannot

be precisely verified by controlling the standard value of the ER-EIA kit, because the presence of 80 nM estradiol in this standard saturates all receptor binding sites and does not allow their direct detection by tritiated estradiol, without pre-treatment, and thus without alteration of the sample. Nevertheless, extraction of ER from MCF-7 cells used as standard, as well as standardization of lyophilized preparations by Scatchard, are well documented [17] and a variation in the standard quality is not likely to occur. This observation is confirmed by the great stability of lyophilized standards stored at 4°C for more than 20 months. Furthermore, the stability of ER antigenic sites is verified by long term storage studies where 90% of immunoreactive sites were recovered when stored in liquid nitrogen for 18 to 24 months. On the other hand, as stated above, the binding assay used in our laboratory for ten years presents high precision and reproducibility and can be taken as a reference method: thus, the evolution of the EIA/RLA ratio from ≈ 1 to ≈ 2 between 1984 and 1991 can be attributed neither to technical variations of the RLA method, nor to a relative unstability of antigenic binding sites, and must rather be attributed to changes of the properties of the current ER-EIA kit. Furthermore, results obtained with cytosols assayed twice, first in 1984 and again in 1991 with both techniques, show mainly an increased reactivity of Mabs used in 1991, and only a small variation of binding sites.

The parallelism defect of the Abbott ER-EIA observed in this study may be the key-problem, indicating that there is no clear identity between the ER from the standard and from crude cytosols, and that recognition by Mabs of ER immunoreactive sites differs for standard and for cytosols. Different cytosol dilutions, as well as modifications of incubation medium (BSA, KCl), can influence the spatial orientation of immunoreactive sites in the ER molecule and, consequently, can modify their detection by the Mabs currently used [6, 18]. The fact that these same experimental conditions had no effect with the earliest ER-EIA kits must be emphasized, and argue for biochemical modifications of the current EIA kit.

High concentrations of KCl (0.3 to 0.8 M) are known to provoke the separation of low molecular weight non-ligand-binding proteins such as the 90 KDa heat shock proteins (hsp) which are associated with steroid receptors [19, 20]. The separation of hsp from the receptor during chromatography in high salt buffer can expose hidden immunoreactive sites [5, 6] and could explain the increased results obtained with EIA. Nevertheless, whatever the chromatographic conditions may be, the presence of KCl (0.4 M) during the EIA is supposed to dissociate ER polymeric forms into the monomeric 70 kDa isoforms, thus allowing the detection of all immunoreactive sites, as well from polymeric forms as from more or less dissociated forms originally studied. Consequently, the increase of immunoreactive sites detected by EIA after chromatography with buffer containing KCl and BSA cannot be attributed exclusively to the unmasking of antigenic sites by high salt dissociation of the receptor molecule during the chromatographic step. Moreover, the fact that polymeric and monomeric ER isoforms present eventually differential reactivity with the Mabs currently used argues for the actual recognition of some receptor structure which was not recognized with the earliest Mabs, which detected polymeric as well as monomeric isoforms, in both high salt and low salt buffer. Furthermore, the actual differential detection of polymeric and monomeric isoforms by Mabs is more in agreement with a differential interaction with the molecular structure than with an increased sensitivity of the standard curve whose effects should be reflected on both isoforms.

According to the principle of sandwich assay, the solid phase (beads) used in the ER-EIA kit is coated with a large excess of high affinity D547 anti-RE antibodies, and during incubation for 18 h in the presence of KCl 0.4 M, nearly all receptor molecules are bound to the beads exclusively as monomeric forms. In these conditions, the washing steps eliminate only very small quantities of unbound receptors. Therefore, the differential detection of polymeric and monomeric forms by Mabs cannot be attributed to the eventual leakage of different isoforms bound to the beads.

It has been shown that treatment of human breast cancer cytosol with tamoxifen enhances the immunoreactivity of the ER toward monoclonal antibody H222 (but not toward monoclonal antibody D547) by producing a conformational change in the receptor protein that exposes an occult antigenic determinant recognized uniquely by H222 [18]. Such conformational changes of the receptor can be induced by different drugs [18] and different physico-chemical conditions, including pH,

ionic strength (KCl), protein levels (BSA), and can explain a modulation of the reactive capacity of the ER protein toward antibodies leading to an increased detection of immunoreactive sites in certain experimental conditions. Again, it must be emphasized that this increased detection was not observed with ER-EIA kits used during the period 1984–1986, and, consequently that current Mabs of the ER-EIA kit present other properties than those used in the earlier kits.

Acknowledgements—This work was supported by the Association pour la Recherche sur le Cancer (A.R.C., Villejuif), the Ligue Nationale Française Contre le Cancer (Paris), and the Ligue du Calvados Contre le Cancer (Caen), France.

REFERENCES

1. Leclercq G., Bojar H., Goussard J., Nicholson R. I., Pichon M. F., Piffanelli A., Pousette A., Thorpe S. and Lonsdorfer M.: Abbott monoclonal enzyme immunoassay measurement of estrogen receptors in human breast cancer: European multicenter study. *Cancer Res.* **46** (1986) 4233–4236.
2. Brandt D. W. and Wittliff J. L.: Assessment of estrogen receptor–monoclonal antibody interaction by high-performance liquid chromatography. *J. Chromat.* **397** (1987) 287–297.
3. Greene G. L. and Press M. F.: Structure and dynamics of the estrogen receptor. *J. Steroid Biochem.* **24** (1986) 1–7.
4. Greene G. L. and Press M. F.: Immunochemical evaluation of estrogen receptor and progesterone receptor in breast cancer. In *Immunochemical Approaches to the Diagnosis and Therapy of Breast Cancer* (Edited by R. Ceriani) Plenum Publishing Corp., New York (1987) pp. 119–136.
5. Linstedt A. D., West N. B. and Brenner R. M.: Analysis of monomeric-dimeric states of the estrogen receptor with monoclonal antiestrophilins. *J. Steroid Biochem.* **24** (1986) 677–686.
6. Giambiagi N. and Pasqualini J. R.: Interaction of three monoclonal antibodies with the nonactivated and activated forms of the estrogen receptor. *Endocrinology* **126** (1990) 1403–1409.
7. Goussard J., Lechevrel C., Martin P. M. and Roussel G.: Comparison of monoclonal antibodies and tritiated ligands for estrogen receptor assays in 241 breast cancer cytosols. *Cancer Res.* **46** (1986) 4282–4287.
8. Molnar L. A., Dhirani N. and McBlain W. A.: Immunoassay for estrogen receptor does not detect inactivated receptor. *Clin. Chem.* **35** (1989) 824–828.
9. Goussard J., Lechevrel C. and Roussel G.: Immunological and physiological analysis of human breast cancer progesterone receptor heterogeneity, following KCl dissociation and size exclusion chromatography. *J. Steroid Biochem.* **33** (1989) 1063–1072.
10. Lowry O. H., Rosebrough N. J., Farr N. J. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
11. Walker W. H. C.: An approach to immunoassay. *Clin. Chem.* **23** (1977) 384–402.
12. Sato N., Hyder S. M., Chang L., Thais A. and Wittliff J. L.: Interaction of estrogen receptor isoforms with immobilized monoclonal antibodies. *J. Chromat.* **359** (1986) 475–487.
13. Goussard J., Lechevrel C., Martin P. M. and Roussel G.: Détermination des recepteurs d'estrogénes par anticorps monoclonaux dans 160 tumeurs mammaires: comparaison de la methode immunoenzymatique Abbott avec la méthode au charbon dextran. *Bull. Cancer (Paris)* **72** (1985) 168–182.
14. Mobbs B. G. and Johnson I. E.: Use of an enzymeimmunoassay (EIA) for quantitation of cytosolic and nuclear estrogen receptor, and correlation with progesterone receptor in human breast cancer. *J. Steroid Biochem.* **28** (1987) 653–662.
15. Thorpe S. M.: Monoclonal antibody technique for detection of estrogen receptors in human breast cancer: greater sensitivity and more accurate classification of receptor status than the dextran-coated charcoal method. *Cancer Res.* **47** (1987) 6572–6575.
16. Press M. F. and Greene G. L.: An immunocytochemical method for demonstrating estrogen receptor in human uterus using monoclonal antibodies to human estrophilin. *Lab. Invest.* **50** (1984) 480–486.
17. Greene G. L. and Press M. F.: Structure and dynamics of the estrogen receptor. *J. Steroid Biochem.* **24** (1986) 1–7.
18. Martin P. M., Berthois Y. and Jensen E. V.: Binding of antiestrogens exposes an occult antigenic determinant in the human estrogen receptor. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 2533–2537.
19. Catelli M. G., Binart N., Jung-Testas I., Renoir J. M., Baulieu E. E., Feramisco J. R. and Welch W. J.: The common 90 kDa protein component of non-transformed 8S steroid receptors is a heat shock protein. *EMBO Jl* **4** (1985) 3131–3135.
20. Sanchez E. R., Faber L. E., Henzel W. J. and Pratt W. B.: The 56–59 KDa protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-KDa heat shock proteins. *Biochemistry* **29** (1990) 5145–5152.