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An Update on the Immunohistochemical Localization of Estrogen Receptors in Mammary Carcinomas Utilizing Polyclonal Anti-receptor Antibodies*

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Abstract—*An immunohistochemical (IF) method utilizing polyclonal antibodies to cytoplasmic estrogen receptors (ER) and a tissue processing procedure which allows* in situ *precipitation of cytoplasmic ER without denaturation of antigenic sites were first described in this journal. In this report we present data on the immunohistochemical classification of 153 cases of human breast carcinomas (123 frozen sections, 28 cytological specimens) as ER-positive (IF+) or ER-negative (IF−). Results on correlation between the biochemical vs immunohistochemical assessment of the ER status and the relationship between quantity of ER and the proportion of IF+/IF− tumor cells are presented. In addition, a procedure which has been successfully applied to study* in vitro *translocation of cytoplasmic ER in cryostat sections of human breast carcinomas is described. A positive correlation between ER translocatability and status of progesterone receptors (PR) was obvious. All biochemical and immunohistochemical data are collectively reviewed and the question of whether the antibodies are detecting Type I ER is examined.*

INTRODUCTION

AN IMMUNOFLUORESCENT (IF) method utilizing rabbit antibodies to estrogen receptors (ER) of human mammary carcinomas was first described in this journal a year ago [1]. Since then we have examined frozen sections from 123 cases and 28 cytological specimens (cell smears) from pleural effusions or needle-core biopsies for distribution of ER(+) and ER(−) cells. Recently we reported the utility of these antibodies for studying *in vitro* translocation of cytoplasmic ER to the nuclear compartment triggered by estrogens or anti-estrogens and for detecting abnormalities associated with translocation in primary cell cultures [2]. These studies have been extended to human breast cancers. A comparative analysis of ER translocatability in ER(+) tumors that contain progestin receptors (PR) and those which lack PR in their cytoplasm has been examined.

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MATERIALS AND METHODS

Immunofluorescence studies on frozen sections: distribution of ER(+) and ER(−) cells

The details of the source and handling of tumor tissues, procedures for tissue fixation by gradual dehydration/rehydration in ethanol/saline solutions for IF study and biochemical procedure for quantifying hormone-free ER and PR have been described previously [1] and were adopted without any further modifications for all our studies. A 10-min exposure time to each of the ethanol/saline solutions was maintained for optimal results. A more rapid dehydration apparently destroyed the antigenic sites and rapid rehydration resulted in insufficient removal of ethanol and, therefore, a higher background due to non-specific protein precipitation by residual ethanol.

Anti-ER antiserum, pre-immune control serum and fluorescein-labelled anti-rabbit IgG

To remove all the non-specific interactions against human serum proteins all three reagents, the primary antiserum, pre-immune serum and fluorescein-labelled reagent, were routinely absorbed with CNBr–Sepharose-bound normal human serum proteins for all our studies [2]. In order to render these reagents suitable for ER translocation studies, they were all treated with dextran-coated charcoal to effect a removal of free steroids [2].

The fluorescein-labelled goat anti-rabbit immunoglobulin (affinity purified, heavy chain specific) was obtained (since August, 1981) from Kirkgaard & Perry Laboratories, Inc., Gaithersburg, MD, U.S.A. The fluorochrome used in this reagent is dichlorotriazinylaminofluorescein.

Paraffin embedding procedure for ER localization

In order to inquire whether tumor pieces dehydrated through ethanol/saline, 100% ethanol and xylene and embedded in paraffin would still retain their ability to react with the antibodies, the following experiment was performed. Pieces from ER(+) tumors (about 0.5-cm cubes) were dehydrated in ethanol/saline solution and after the xylene step embedded in paraffin at 60°C (after three 5-min changes in paraffin wax kept at 60°C). For comparison, pieces fixed in either formaldehyde or Bouin's fixative and embedded in paraffin were included for this study. Six-micron sections were deparaffinated in xylene, *rehydrated* through decreasing concentrations of ethanol/saline solutions and rinsed in saline to remove all traces of ethanol. The sections were exposed to the anti-ER antiserum or prebleed or the fluorescein-labelled reagent alone for 30 min, washed and re-exposed to fluorochrome-labelled anti-rabbit IgG. Washed sections were then mounted in saline, the edges of the coverslip sealed by Permount and observed under the microscope (Zeiss microscope equipped with a halogen lamp) for immunofluorescence. The intensity of the fluorescence and intracellular distribution of immunofluorescence were compared in sections from tissues that were embedded in paraffin and from sections of frozen tissue processed following the routinely used technique.

A study of heterogeneity of tumor cell population: examination of multiple pieces from tumors for determining the proportion of IF (+) and IF (–) cells

Tumors were classified according to the quantity of hormone-free ER (by DCC assay) into five categories: those with ⩾500 fmol/mg cytosol protein; 200–499 fmol; 50–199 fmol; 5–49 fmol; and those which were normally classified as ER(–) with 0–4 fmol ER/mg cytosol protein. In each group whenever sufficient tumor tissue was available it was divided into different portions (after removal of fat) of equal size and embedded in gelatin. Cryostat sections from each piece were studied for tumor cellularity (proportion of tumor cells), amount of normal or benign components and cells vs stromal components in sections stained with hematoxylin and eosin. Four frozen sections were also processed for ER localization and the proportion of ER(+) vs ER(–) cells was recorded in each piece.

Immunohistochemistry of cytological specimens

Cell smears from needle aspirates or body fluids were made and transported to the laboratory in dry ice. Whenever possible, tumor pieces from the biopsies were saved for cryostat sections for regular immunohistochemical analysis and, in addition, cell smears were also made and transported to the lab. The smears were air-dried for 10 min and processed for IF study after ethanol dehydration/rehydration. Cell morphology was ascertained from smears stained with haematoxylin and eosin. The proportion of IF(+) tumor cells vs IF(–) tumor cells was estimated in all cases.

Anti-ER antibody and nuclear ER of MCF-7 cells

These experiments were performed by Senior *et al.* in Dr. Frankel's laboratory. Ig fractions purified from anti-ER antisera and tested by immunoelectrophoresis to be free from any other rabbit serum proteins were tested for their ability to recognize salt extracted (0.4 M) '4S' or '5S' ER–E_2 and DNA-bound ER–E_2 obtained from nuclei of MCF-7 cells which were labelled for 1 hr with a 5 nM concentration of [^{3}H]-E_2 (with or without molar excess of unlabelled DES). The DNA in these cells was labelled with [^{14}C]-thymidine (76 nmol for 3 days). The anti-ER Ig and irrelevant Ig (anti-cytochrome oxidase Ig, anti-glucocorticoid receptor Ig or normal rabbit Ig) were used in soluble form or in solid phase coupled to CNBr-activated Sepharose (Sepharose–Ig-i). The nuclear salt extracts were incubated with the Ig preparations for 2 hr and analyzed by sucrose density gradient centrifugation. Plain CNBr–Sepharose (deactivated) was also included as another experimental control.

The anti-ER and irrelevant Ig were tested for their ability to recognize ER–E_2 bound to the nuclear acceptor sites. For this purpose the MCF-7 cell nuclei (labelled with [^{3}H]-E^2 and [^{14}C]-thymidine) were subjected to digestion by micrococcal nuclease (details of methodology to be published elsewhere). The Mg^{2+}-soluble fraction of the digests containing ER–E_2 bound to

nucleosomes were reacted with Ig and analyzed by sucrose density centrifugation for immune complex formation.

A study of in vitro *ER translocation in human breast cancer tissues*

Fresh smears from unfrozen biopsies. Cell smears, made from fresh biopsies at the Lemuel Shattuck Hospital, where the Steroid Receptor Lab is also located, were included for this study. After 10 min air drying, a few of the smears were processed for immunohistochemistry or for haematoxylin/eosin stain without any further treatment. A few were directly transferred to a moist chamber kept at 37°C for 1 hr or exposed either to plain saline or saline solutions containing 2.5 nM concentrations of either estradiol, monohydroxytamoxifen (OH-TX), diethylstilbestrol (DES) or the progestogen R5020. The exposure of these smears to the steroid was accomplished as follows. The whole area of the air-dried smears was covered with the appropriate solutions and immediately the excess of the solutions was drained by tilting the slide. The immediate draining of the excess liquid from the air-dried smears was important to prevent the loss of cells which may become detached from the glass if plenty of liquid was left on top. The drained smears were then transferred to a moist chamber kept at 37°C. After 1 hr incubation at that temperature they were *air-dried again*, transferred to a 30% ethanol/saline mixture and subjected to dehydration/rehydration prior to immunofluorescent staining.

Pieces of tumor from fresh biopsies. Whenever the size of the tumor was large, a portion of the tumor was frozen in dry ice for biochemical estimation of ER and PR. The rest of the tumor was divided into pieces of approximately equal size and each piece was introduced into a vial with 1 ml of either plain saline or saline containing 2 pmol of E_2, DES, OH-TX or R5020. One piece was also introduced into an empty vial and tansferred to a 37°C incubator. Incubation was for 0.5, 1 or 2 hr. At the end of the incubation period the liquid was drained from the vial, and the tumor pieces were frozen in dry ice and stored at -80°C until ready for use. Each piece was embedded in gelatin and cryostat sections were processed for IF study. Data related to the proportion of cells with exclusively cytoplasmic IF (C+N-), those showing only intranuclear IF (C-N+), cells with IF distributed in both compartments (C+N+) or those lacking IF (C-N-) were estimated in each section independently by two investigators, one of whom was a pathologist.

Cryostat sections of frozen tumors. Since *in vitro* translocation and DNA binding studies have been successfully performed using the receptor molecules present in the cytosol derived from frozen tumors by several investigators, we decided to examine the feasibility of using the cryostat sections of frozen tumors for *in vitro* translocation studies. Consistent results were obtained if the procedure described below was strictly followed. Frozen sections which were air-dried for 10 min were used. Two slides, each with two sections, were processed without exposure to steroid for determining the native distribution and intensity of IF in the tumor cells unexposed to steroid *in vitro* (unincubated control). Two to four sections were exposed to either plain saline (ligand-free control) or saline containing one of the following ligands: E_2, DES, OH-TX or R5020 at 2.5 nM concentration. To expose the cells in the unfixed frozen sections to steroid solutions without any appreciable loss of cytoplasmic proteins during such incubations with the steroid solutions, the following steps were carefully performed. To the air-dried section, 20 μl of the ligand solution or plain saline was added to cover the sections using a selectapette equipped with glass tips. Without allowing the liquid drop to stay on top of the section, all the liquid was immediately aspirated back into the pipette tip leaving only a thin layer of the ligand solution on top of the frozen section. The slides were transferred to a moist chamber kept at 37°C. The incubations were for either 0.5 or 1 hr. At the end of the incubation period the sections were directly immersed in 30% ethanol/saline solution and processed as usual for IF study. The sections were examined for the intensity and intracellular distribution of IF in cells. Frozen sections from 24 different tumors, 12 of which were ER(+)/PR(+) and 12 of which were ER(+) but PR(-), were included for this study. The level of ER ranged from 21 to $\geqslant$877 fmol/mg cytosol protein and the level of PR from 0 to $\geqslant$1117 fmol/mg.

Absorption of anti-ER antiserum with preparations of purified ER-$_2$ complexes

Anti-ER antiserum was absorbed with two different preparations of ER-E_2 complexes (purification procedure will be described elsewhere) containing 100 fmol equivalent E_2 binding with no detectable protein. No protein could be detected either by Biorad dye technique (microtechnique with detection limit of 1 μg/ml) or by Coomassie blue staining of the polyacrylamide gels which were used for electrophoretic analysis of 50 μl of the purified material. Absorption was accomplished by mixing the antiserum with purified ER-E_2 solution at a ratio of 100 fmol

ER/2 ml of diluted antiserum which had been, as usual, pre-absorbed with solid-phase normal human serum proteins. For comparison, a fraction showing low E_2 binding activity but detectable quantities of protein was used for antiserum absorption. The absorption mixtures were left at 4°C for 18 hr. The unabsorbed antiserum, to which an equal volume of buffer was added, was used as a control. Frozen sections, prepared from different tumors known to be ER(+), were incubated with the unabsorbed antiserum, antiserum absorbed with ER–E_2 solution or a fraction obtained during the purification process containing low E_2 binding activity. The intensity of IF and the percentage of IF(+) vs IF(−) cells were recorded in each case by two investigators, one of whom (a pathologist) scored the data on the coded slides and was, therefore, unaware of which sections received antiserum absorbed with ER–E_2 complexes or the irrelevant protein fraction.

RESULTS

Effect of paraffin embedding on ER detection by immunohistochemistry

All the tumors subjected to paraffin embedding *without* exposure to formaldehyde or Bouin's solution prior to dehydration were found suitable for immunofluorescence study (Figs 1a, b). The antigen appears to retain its capacity to bind the antibodies and no difference in the intensity of IF was observed between tissue which was subjected to the paraffin embedding step and the tumor cells in the cryostat sections that were processed following the routine procedure. A drastic reduction in cellular IF was observed in pieces that were pre-fixed in formaldehyde or Bouin's solution (Fig. 1c). Exposure of frozen tumors to formaldehyde or Bouin's fluid appears to destroy the antigenic sites to a significant degree.

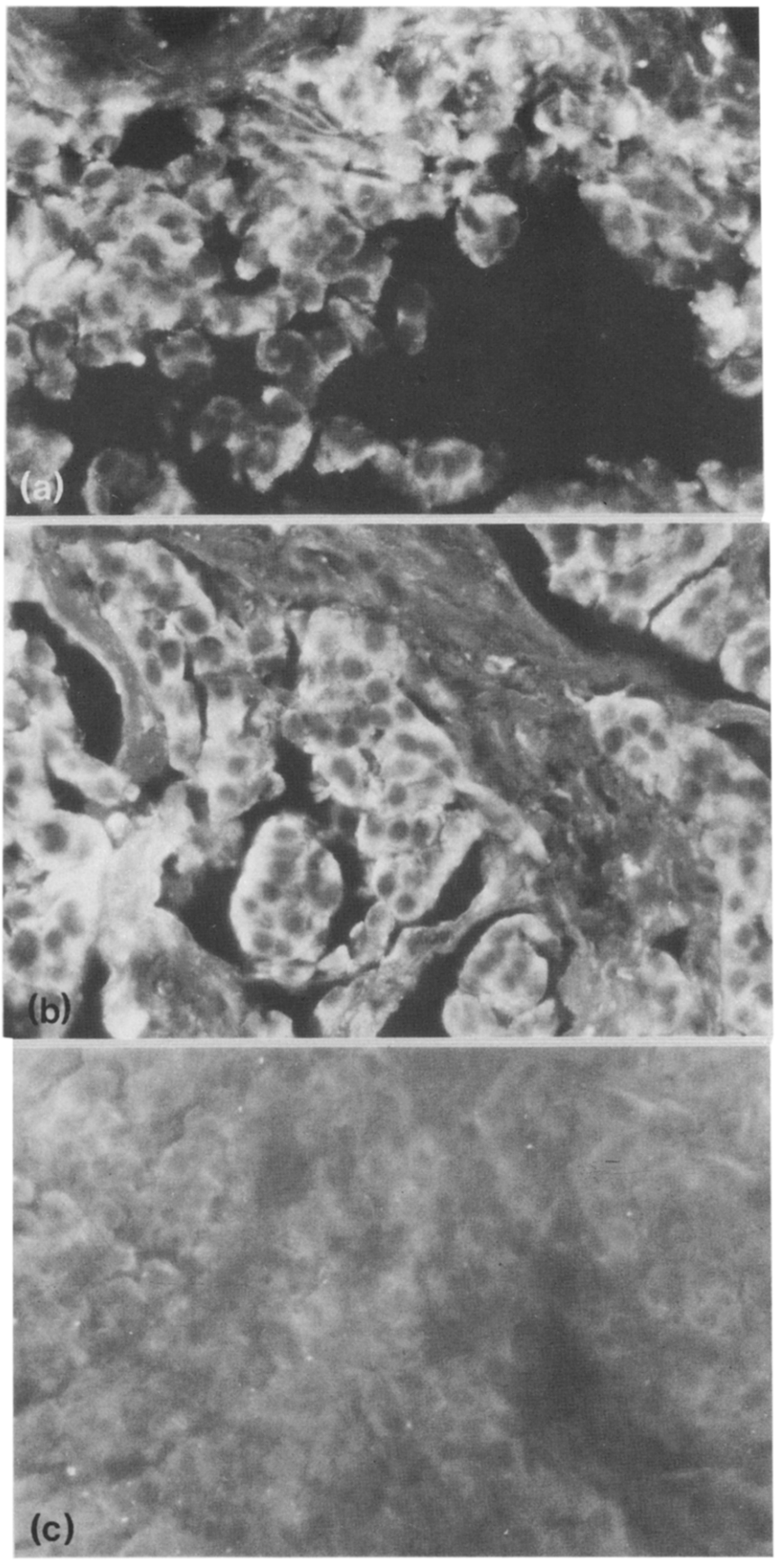
A study of heterogeneity of tumor cell population in relation to intracellular ER distribution

ER(+) tumors (≥5 fmol/mg cytosol protein). The results of 79 ER(+) tumors are given in Tables 1 and 2. Tumors which had an ER content of ≥500 fmol/mg cytosol protein were characterized by high tumor content, high proportion of tumor cells and a distinctly high degree of homogeneity, with 19/24 cases composed exclusively of IF(+) cells in the multiple pieces examined. Tumors with an ER content between 200 and 500 fmol differed from the first category of tumors in exhibiting a greater degree of heterogeneity containing a mixed population of IF(+) and IF(−) cells. Seven out of 20 tumors contained higher than 50% tumor cells without detectable IF. In the next two subclasses of ER(+) tumors (50–199 and 5–49 fmol/mg) only four cases were large enough for a comparative study of multiple pieces. Nevertheless, a significant trend towards tumor heterogeneity was evident. Tumors with either benign or normal areas or a higher amount of stromal and connective tissue components were observed at a higher frequency in this category. In addition, very few tumors were homogeneous with regard to IF positivity. Seven out of 14 cases had ≥50% IF(−) tumor cells. In all these seven cases the ER content was below 20 fmol/mg cytosol protein.

ER(−) tumors(<5 fmol/mg cytosol protein). The results are tabulated in Tables 3–5.

General Information: *All figures are black and white prints taken from Kodak Ektachrome color slides for which ASA 400 film was used. Photographs of fluorescence were taken using a Zeiss Microscope equipped with neofluor objectives, halogen lamp and manually operated camera. Exposure time for all the color slides (immunofluorescence) was* 4 min. *For slides of hemotoxylin/eosin-stained sections the exposure time was* 0.5 sec. *Neofluor oil immersion lens was used to get an original magnification of* ×1000.

Fig. 1. *Paraffin embedding procedures and immunohistochemical localization of ER by IF procedure. For this experiment breast carcinomas which were stored frozen at* −80°C *were utilized. An infiltrating ductal carcinoma of the breast (with lobular features) with cytoplasmic ER content of* 561 fmol/mg *cytosol protein was divided into* 0.5-*cm cubes. One piece was cut into* 4-*μm sections using the cryostat. The sections were processed by gradual ethanol dehydration/rehydration procedure and ER was localized using the IF test (a). The second piece was gradually dehydrated through ethanol/saline mixture as usual followed by* 100% *ethanol, cleared in xylene and embedded in paraffin (kept at a constant temperature of* 60°C). *The paraffin blocks were cut into* 4–6-*μm sections using a microtome, deparaffinated using three changes of xylene and rehydrated by a reversal of the dehydration procedures, and is usually done for frozen sections. After saline rinses to remove all the residual ethanol the ER was localized using IF procedure (b). A third piece was first exposed to formaldehyde (pH adjusted to* 7.0) *for* 30 min *and then processed as described above for the second piece.* Results: *The paraffin-embedding procedure* per se *without prior formaldehyde fixation does not destroy the antigenic sites. While the cells are IF+ in both the cryostat section (a), which was not exposed to paraffin-embedding steps, and the microtome section of the second pioece (b), which was embedded in paraffin but was not exposed to formaldehyde fixation, a drastic reduction in IF is evident in the microtome section of the third piece (c), which was fixed with formaldehyde and then embedded in paraffin. Pre-incubation of the tissue piece in E_2 solution prior to formaldehyde fixation did not prevent the denaturation of antigenic sites by formaldehyde. Bouin's fixative (picric acid/paraformaldehyde) was a denaturing as formaldehyde (results not shown). Similar results were obtained on three other tumors included in this study.* (×380.)


(a)
(b)
(c)

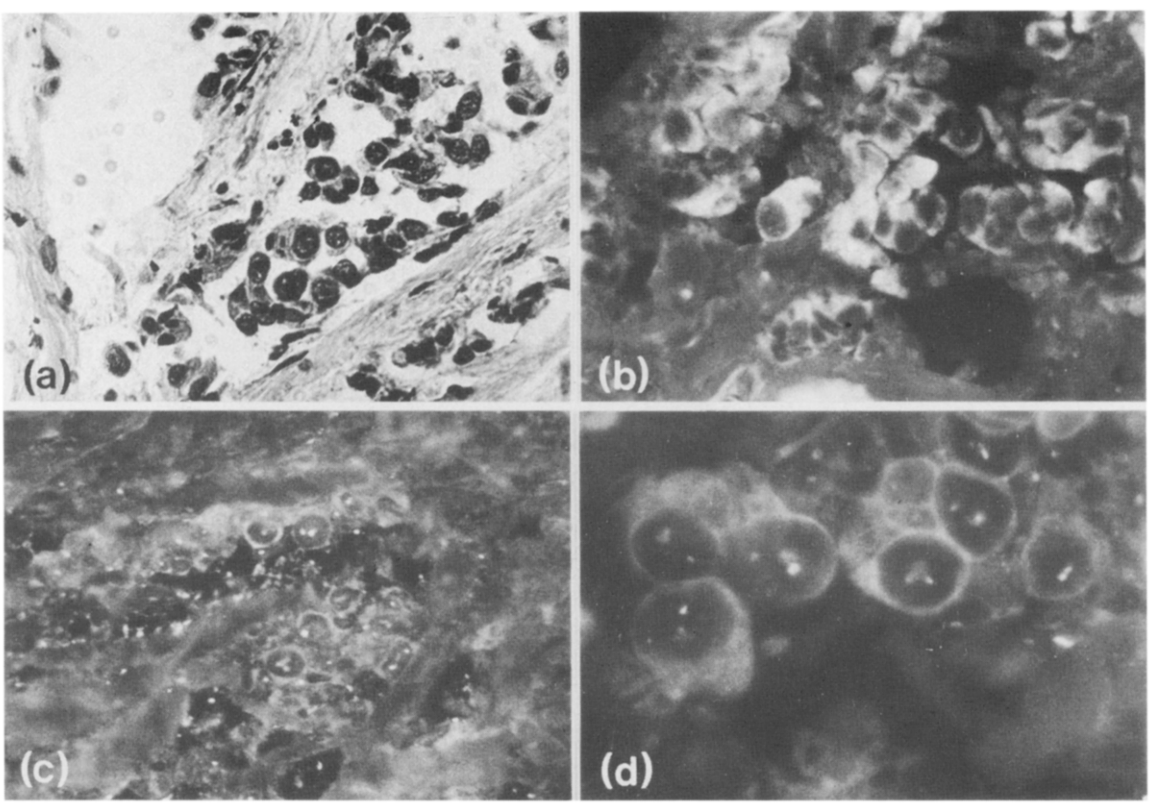


Fig. 3. Estrogen/antiestrogen triggered in vitro *translocation of cytoplasmic ER to the nucleus in ER+/PR+ tumors. The results obtained for infiltrating ductal carcinoma of the breast containing* 134 fmol *ER and* 25 fmol *PR/*mg *cytosol protein are illustrated. (a) Haematoxylin/eosin-stained cryostat section.* (×400.) *(b) Frozen section processed for IF localization of ER without* in vitro *exposure to estrogen or antiestrogens.* (×400.) *The immunofluorescence is localized in the cytoplasm. A mixture of IF(+) and IF(−) cells are seen in this area. (c) Frozen section exposed to nanomolar concentrations of* E_2 for 1 hr *at* 37°C *(as explained in the text) prior to fixation and processing for ER localization by IF. The fluorescence is concentrated as intranuclear spots in all the IF(+) cells.* (×400.) *(d) Experimental conditions are the same as in (b) but the cells are observed using an oil immersion lens.* (×1000.) *The immunofluorescence is clearly localized as distinct spots in the nuclear compartment. Nuclear membrane and/or perinuclear border is also visible. The same results were obtained in sections exposed to DES or* OH-*TX. Incubation with the progestogen R5020 yielded results similar to the experimental control.*

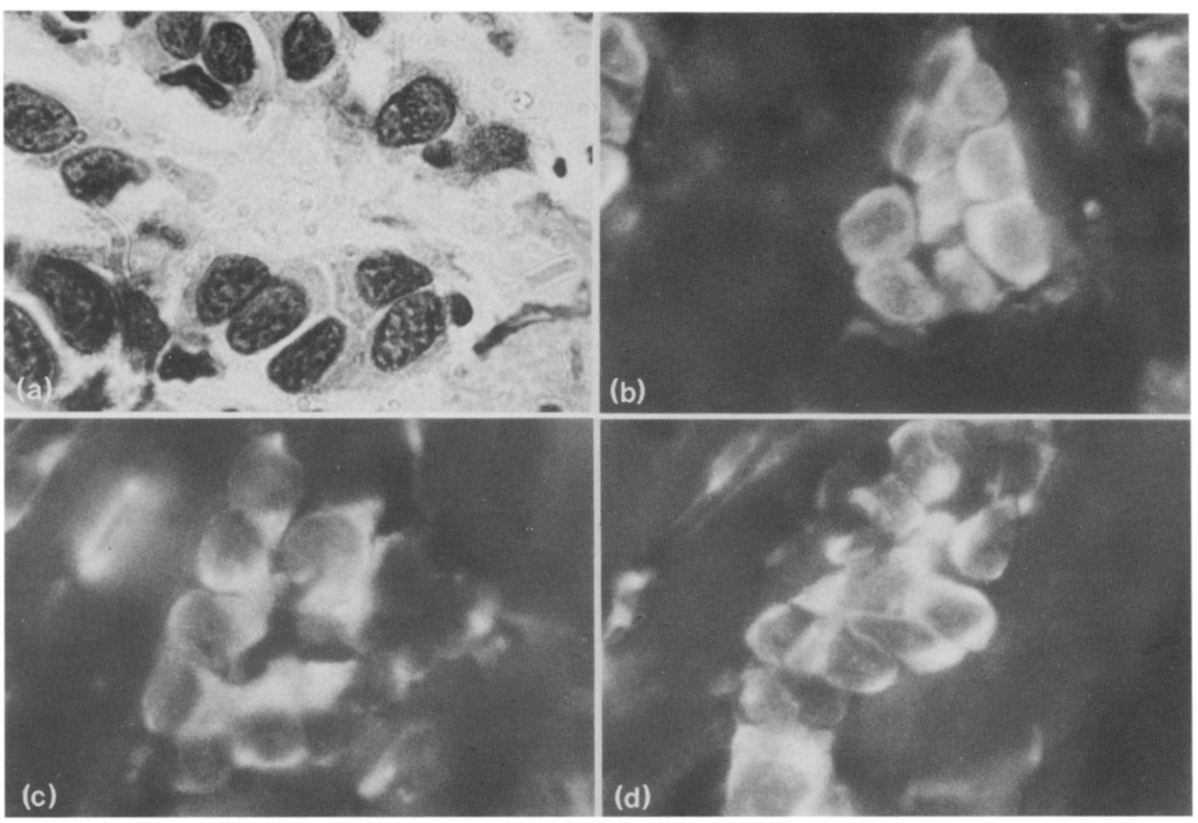


Fig. 4. In vitro *ER translocation studies in an ER(+)/PR(−) tumor. The cytoplasmic ER content of this infiltrating ductal breast carcinoma was* 160 fmol/mg *cytosol protein. The tumor cells are very large, with ample cytoplasm. The tumor cellularity was* 30%. *All cells were IF(+) to a varying degree. (a) Frozen section stained with haemotoxylin/eosin.* (×690.) *(b) Frozen section unexposed to the ligands and processed for IF localization of ER. The immunofluorescence localized in the cytoplasmic compartment in all the tumor cells.* (×690.) *(c) Frozen section exposed to nanomolar concentration of* E_2 *as described in the text prior to processing for IF localization of ER. All the cells retained their IF in the cytoplasm. In this tumor an occasional intranuclear IF was evident in cells exposed to* OH-TX *or DES. (d) Same as (c) but the ligand was progestogen* R5020.

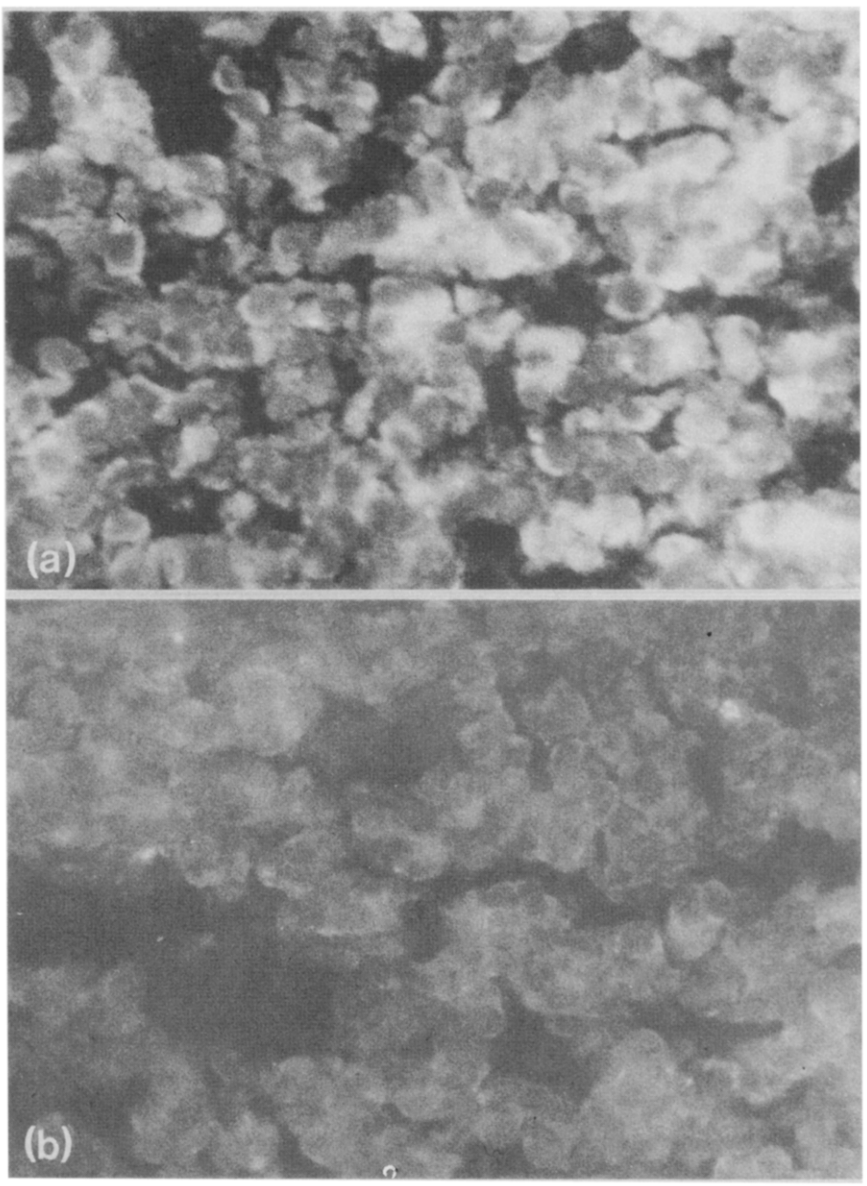


Fig. 5. Absorption of anti-ER antiserum with purified ER–E_2 complexes. For this experiment anti-ER antiserum which had been absorbed with normal human serum proteins coupled to CNBr-activated Sepharose and routinely used for IF studies was used. The final dilution after mixing with appropriate protein solutions was ×28, *a dilution normally used for IF procedure. The antisera were mixed either with plain saline, purified ER–E_2 complexes (at a ratio of* 50 fmol *equivalent* 1 ml *of* ×28*-diluted antisera) or with a protein fraction with irrelevant proteins which did not contain ER. Frozen sections from ER(+) tumors were dehydrated and rehydrated as usual and were incubated with either unabsorbed (saline absorbed) antiserum, antiserum absorbed with ER–E_2 or with the irrelevant protein fractions. The sections were washed to remove the unreacted antibodies and subsequently incubated with flurochrome-labelled anti-rabbit IgG. The resultant immunofluorescence was observed after the sections were washed and mounted in saline. (a) Immunofluorescence obtained in frozen sections exposed to unabsorbed anti-ER antiserum. ER content of this PR(-) tumor was* ⩾756 fmol/mg *cytosol protein. (b) Frozen sections from the same tumor incubated with purified ER–E_2 complexes.* Results: *The antiserum absorbed with ER–E_2 complexes fails to yield immunofluorescence in the tumor cells of ER(+) tumor (b). The immunofluorescence obtained with unabsorbed antiserum (a) is specific for ER. Antiserum absorbed with irrelevant, non-ER proteins yielded the same results as shown in (a). Three other ER(+) tumors (with an ER content of* 94, 121 *and* ⩾426 fmol/mg *cytosol protein respectively) were also examined with absorbed and unabsorbed antisera. In all the cases the absorption of the antiserum with ER–E_2 abolished the antibody-specific immunofluorescence.* (×400.)

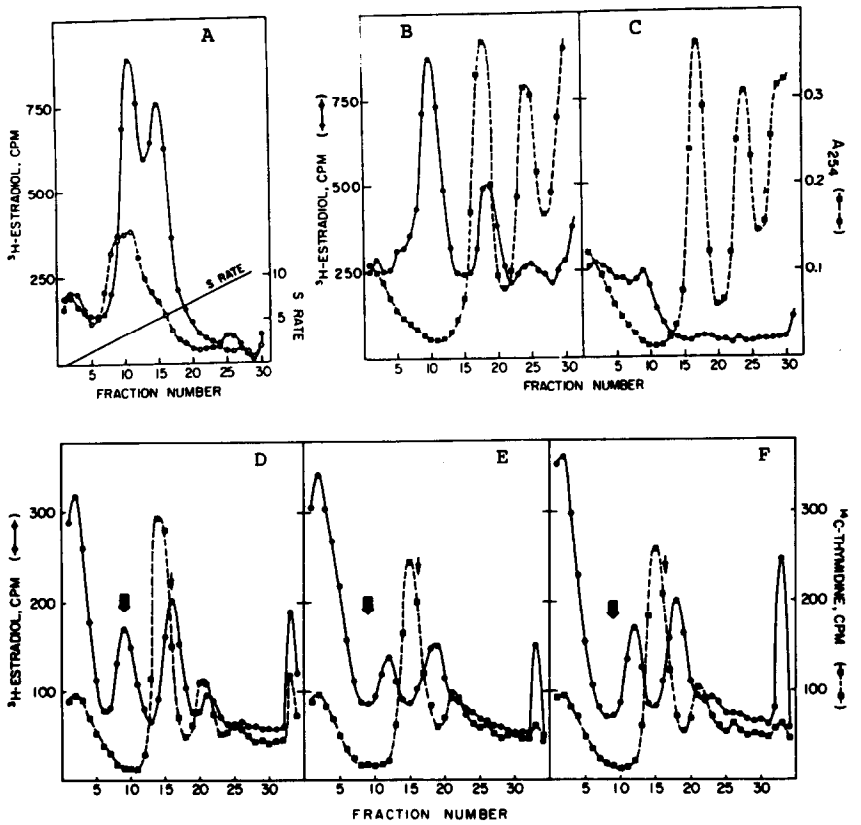


Fig. 2. Demonstration of interaction of anti-ER Ig with nuclear ER-E of MCF-7 cells. ^{3}H-bound radioactivity was saturable by a 200-fold molar excess of unlabelled DES. (a–c) Solid-phase anti-ER Ig and nuclear ER-E. (a) MCF-7 cells maintained in E_2-free growth medium were labelled with 5×10^{-9} M 17β-estradiol for 1 hr at 37°C. ER-E complexes were extracted by exposing the nuclei to a buffer containing 0.4 M NaCl at 4°C for 1 hr. The extract was centrifuged at 100,000 g and the supernatant was incubated for 2 hr at 4°C with 1 vol. of either buffer or control Sepharose (●——●) or Sepharose-bound anti-ER Ig (○---○). The Sepharose was pelleted and the supernatants were then analyzed on a sucrose density gradient (5–20%) using an SW 60 rotor at 52,000 rev/min for 16.2 hr at 2°C. (b, c) The radioactivity profile of the micrococcal nuclease digests of the MCF-7 cell nuclei labelled with [^{3}H]-E_2 for 1 hr. The nuclei, digested with 1400 units of micrococcal nuclease, were centrifuged at 300 g for 10 min and suspended in low salt (10 mM NaCl) buffer. A 150-g supernate was prepared and incubated with 0.25 vols of buffer or control Ig Sepharose (b) or with Sepharose anti-ER (c) for 2 hr at 0°C. Sepharose was pelleted and the reaction products in the supernates were analyzed by sucrose density centrifugation using 5–20% sucrose gradients in an SW 41 rotor at 37,000 rev/min for 17 hr at 2°C. ●——● ^{3}H label; ●-----● absorbance at A254. (d–f) Interaction of soluble anti-ER Ig and [^{3}H]-E_2-bearing nuclear components of Mg^{2+}-soluble fraction of chromatin. MCF-7 cells were labelled for 3 days with [^{14}C]-thymidine (76 nmol) and for 1 hr with [^{3}H]-E_2 (5 nmol) prior to the separation of nuclei from the cytoplasm. Isolated nuclei were digested with micrococcal nuclease. The Mg^{2+}-soluble fraction was incubated for 2 hr with 0.25 vols of buffer (d), soluble anti-ER Ig (e) or soluble anti-ER Ig pre-absorbed overnight with MCF-7 nuclei stripped of 0.4 M KCl-extractable components, including ER-E (f). The incubation mixtures were then analyzed as described for (b) and (c). Ig from unimmunized rabbit, anti-cytochrome-C Ig or anti-glucocorticoid-receptor Ig yielded the same results as shown in (d). In all the three figs (d–f) the heavy arrow indicates the position of 6.9S and the light arrow shows the position of 12.5S. ●——● [^{3}H]-E_2 label; ●-----● [^{14}C]-thymidine label.

Intratumoral variations. Table 3 depicts the consolidated data on 44 ER(−) tumors, while Tables 4 and 5 list the results on individual cases. In addition to ER status, the quantity of cytoplasmic PR was known for 25 cases out of 44 included in this study. Thirteen out of 14 from the postmenopausal group were negative for PR. Seven out of ten from the premenopausal category were PR negative and three had cytoplasmic PR of 9, 131 and ≥436 fmol.

Multiple pieces were examined for cellular heterogeneity in nine tumors (all from postmenopausal women). Surprisingly, the intratumoral variations in tumor morphology, cellularity or proportion of IF(+) vs IF(−) cells were found to be minimal among the ER(−) tumors. In addition, the results scored by the two investigators showed excellent agreement in all but one case (Table 5, case MNL).

Acellularity. The frequency with which acellularity or poorly cellular (≤5% tumor cells) tumors was encountered was about 18% (8/44) and was equal between the pre- and the postmenopausal groups. These eight cases could,

Table 1. A comparison of quantity of ER with the distribution of IF-positive cells and IF-negative cells in human breast carcinomas

	ER (fmol/mg)*			
	⩾500	200–499	50–200	5–49
No. tested	26 (2 acellular)	21 (1 acellular)	16 (2 acellular)	16 (2 benign 1 acellular)
Multiple pieces studied in†	14	15	3	1
100% tumor (no benign or normal tissue)	18/24 (75%)	14/20 (70%)	5/14 (36%)	9/14 (64%)
Cancer cells ⩾50% in	21/24 (88%)	8/20 (40%)	2/14 (14%)	4/14 (29%)
No. of cases with (IF+) cells ⩾95%	20/24 (83%)	10/20 (50%)	4/14 (29%)	4/14 (29%)
No. of cases with (IF−) cells 5–30%	2/24 (8%)	3/20 (15%)	5/14 (36%)	3/14 (21%)
No. of cases with (IF−) cells ⩾50%	0/24 (0%)	7/20 (35%)	3/14 (21%)	7/14‡ (50%)

*Hormone-free cytoplasmic type I ER measured by DCC assay (K_d 1×10^{-11} to 1×10^{-10}M), $r = -0.90$ to 0.99.
†Multiple pieces were analyzed in all tumors with sufficient material. Different areas from the same piece were examined in all cases.
‡In all seven cases the ER value was below 20 fmol/mg.

Table 2. ER-positive tumors with no IF-negative cells

Total No.: 33/72	(46%)	ER-positive tumors*
19/33	(58%)	from ⩾500 fmol/mg group
8/33	(24%)	from 200–499 fmol/mg group
4/33	(17%)	from 50–199 fmol/mg group
2/33	(6%)	from 5–49 fmol/mg group

*When frozen sections from multiple pieces or multiple areas (at least 4) from the same piece were analyzed, no IF-negative cells were detected.

therefore, have been classified biochemically as ER and/or PR(−) because of their acellularity.

Tumor necrosis. A significantly high proportion of necrosis was encountered among the ER(−)/PR(−) tumors from the postmenopausal group. While 10/24 tumors of the postmenopausal ER negatives were heavily necrotic, only 2/20 in the premenopausal category contained necrotic areas. An analysis of immunofluorescence data on the necrotic cells revealed that: (a) autofluorescence in the necrotic cells was common; (b) non-specific fluorescence in the cells exposed only to fluorochrome-labelled Ig was frequent; (c) intensity of specific antigen–antibody interactions varied from a 2+ grade, as was seen in four cases, or a low-grade +/−, seen in a small proportion of cells (5–10%), to no fluorescence in any of the cells, observed in three tumors that were heavily necrotic (tables 4, 5).

Non-necrotic, cellular, ER(−) tumors. As summarized in Table 3, of the 44 ER(−) tumors analyzed by immunohistochemistry, 32 tumors were non-necrotic. Twenty-nine out of these 32 were classified as ER(−) by immunofluorescence procedure. Therefore, among the non-necrotic tumors 90.6% concordance was found between the biochemical and immunohistochemical classification of these tumors as being ER(−). In eight cases of these 29 the ER negativity was attributable to acellularity. The highest discordance between the results of the two techniques was seen only in tumors which were heavily necrotic. Of 12 (12/44 = 27%) heavily necrotic tumors, four (33%) were classified as distinctly positive by IF procedure, five (42%) as equivocal or low positive and only two (16%) as ER(−). Discordancy was also evident in premenopausal cases [ER(−) but PR(+)], with IF procedure classifying these as ER(+) (Table 4).

Immunofluorescent studies on cell smears

A complete concordance of results was obtained in relation to the ER status of the tumor for 27 cases of cell smears in which biochemical data on ER values were available (Table 6). One case, a pleural effusion, lacked biochemical data (Table 6, case 18). Four out of 27 were ER(−) by both IF and biochemical tests. The remaining 23, in which ER values ranged from 12 to 776 fmol/mg protein, were also IF(+). Since in smears the cells were spread over a wide area, it was difficult to estimate the proportion of IF(+) vs IF(−) cells. Evidently no correlation was found between the intensity of IF and quantity of ER (Table 6). The cell morphology was very well preserved and *in vitro* translocation studies yielded excellent interpretable results. Therefore, for cases in which the tissue material is limited, a study of cytological smears by IF procedure may prove to be the best alternative for determining the ER status of the tumor. *In vitro* translocation data

Table 3. IF study on ER-negative tumors: consolidated data*

	Postmenopausal	Peri/premenopausal
Total ($n = 44$)	23 (9 multiple piece analysis)	21
Acellular ($n = 8$)	4	4
Heavy necrosis ($n = 12$) ($\geqslant$50% necrosis)	10	2
	3 IF(+) 10% cells 5 IF(+/-) 50% 2 IF(-) 100%	1 IF(+) 1 IF(-)
Non-necrotic cellular	10 10/10 IF(-)	14 11/14 IF(-) 3/14 IF(+) and PR(+)

*ER value by DCC assay: below 5 fmol/mg cytosol protein.

Table 4. IF study on ER-negative tumors: pre/perimenopausal patients (total No. analyzed = 21; unappraisable = 4)

ID tag		% tumor	% cancer cells	% IF(+)	% IF(-)	Net IF	Comments
MMG		100%	60%	90% 2+	10%	2+	heavy necrosis
PMS*	(9)	100%	60%	50–90 (+/-)	10–50%	(+/-)	heterogeneous
SCA*	(neg)	100%	75%	0%	100%	(-)	
LBT*	(neg)	100%	60%	0%	100%	(-)	
RCR		95%	40%	0%	100%	(-)	
NYJ*	(neg)	100%	50%	0%	100%	(-)	
AGL*	(neg)	100%	50%	0%	100%	(-)	
PILG		100%	40%	0%	100%	(-)	
HENS*	(131)	100%	30%	40% (+/-)	60%	(+/-)	
PG*	(neg)	70%	40%	0%	100%	(-)	
BLD*	(436)	20%	80%	80%	20%	(+)	
BLY*	(neg)	100%	20%	0%	100%	(-)	
CAL†		40%	25%	0%	100%	(-)	
MSSM		10%	100%	0%	100%	(-)	heavy necrosis
GLD		100%	5%	0%	100%	(-)	
SW		10%	5%	0%	100%	(-)	
SHB*	(neg)	(smear only)		0%	100%	(-)	
HSTN		(benign)		0%		(-)	unappraisable
FR		(benign)		0%		(-)	unappraisable
GRYN		(benign)		0%		(-)	unappraisable
MHM		(normal lung)		0%		(-)	unappraisable

Total IF(-)/DCC(-) = 13/18; total IF(+)/DCC(-) = 4/18: 3 PR(+), 1 necrotic.
*PR (values of progesterone receptor content: fmol/mg cytosol protein).
†Recurrent tumor in this patient was IF(+)/DCC(+).

may be valuable for assessing the functional integrity of ER.

Nuclear ER and anti-ER antibodies

Figure 2(a) illustrates the radioactivity profile of the nuclear extracts of MCF-7 cells incubated for 2 hr with or without Sepharose–Ig and analyzed by sucrose density gradient centrifugation. Estimation of the areas under the peak indicated that 78% of radioactivity associated with the 5S species and only 27% of the radiolabel in the 4S region were removed by the antibody treatment.

The interaction of solid phase Ig with the micrococcal nuclease digest of the nuclei of MCF-7 cells labelled with [^{3}H]-E_2 is shown in Fig. 2(b,c). Three peaks of ^{3}H-labelled components are seen in the nuclease digest when analyzed by sucrose density gradient: a major peak in the 6.9S region, another at the 12.5S region and a minor peak at 14.2S. Seventy-nine percent of the radiolabel in the 6.9S region, 88% of the label in the 12S region and all radioactivity in the 14.2S region were precipitated by the Sepharose–Ig, as shown in Fig. 2(c). When soluble Ig instead of Sepharose–Ig was reacted for 2 hr with the nuclease digests and the products analyzed by sucrose density gradient centrifugation, 85% of the [^{3}H]-estradiol associated with the 6.9S peak shifted to the 9.2S region. Essentially all the radiolabel in the 12S peak shifted to the 14.2S region in the presence of Ig, indicating the formation of immune complexes between the ER–E bound to DNA and the Ig (Figs 2d, e). There

Table 5. IF study on ER-negative tumors: post-menopausal patients (total No. analyzed = 23)

ID tag	PR*	% tumor	% cancer cells	% IF(+)	% IF(−)	Net IF	Comments
ARN	UK	100%	40%	100% 1-3+	0%	2+	necrosis‡
CRP	(−)	100%	80%	70-80% 2+	20%	2+	necrosis‡
GHJ	UK	100%	20%	90% 2-3+	10%	2+	necrosis‡
GRN	(−)	100%	25%	5-20% 2+	80-90%	(+/−)	necrosis‡
JSY	(−)	40%	60%	0%	100%	(−)	
RBO	(−)	100%	30%	0%	100%	(−)	
RDF	UK	100%	30%	0%	100%	(−)	
HTH	UK	100%	20%	0%	100%	(−)	
AYX	(+)	100%	20%	0%	100%	(−)	
MCNR	UK	5%	100%	0%	100%	(−)	
LNDM	UK	2%	100%	0%	100%	(−)	
WNE	UK	(no tumor)		0%		(−)	
BNY	UK	(no tumor) (benign)		0%		(−)	
WGN	(−)	(no tumor) (benign)				(−)	
		Multiple pieces analyzed in the following:					
PAM	(−)	100%	60%	5% (+/−)	95%	(−)	
TRH†	(−)	50%	(poor cellularity)	0%	100%	(−)	heterogeneity†
HCK	(−)	100%	95%	5-10% (+/−)	90%	(+/−)	50-60% necrosis
FRY	UK	100%	95%	5% (+/−)	95%	(+/−)	80% necrosis
MRI	(−)	100%	100%	0%	100%	(−)	100% necrosis
WTA	(−)	100%	50%	0%	100%	(−)	60-90% necrosis
SUE	(−)	70-100%	5-20%	0%	100%	(−)	
MZG	(−)	100%	95%	5% (+)	95%	(+/−)	20-50% necrosis
MNL	(−)	100%	40-90%	25-50% (+/−) to (+)	50-75%	(+/−)	95% necrosis

*PR level ≥5 fmol is considered positive.

†Two pieces contained 0-5% tumor; one piece 50% tumor; all cells were IF-negative.

‡Most necrotic cells were IF-positive; frequently both cytoplasm and nuclei were IF-positive.

was also an increase in the radiolabel recovered from the bottom of the gradient in the antibody-treated samples which was equivalent to the radiolabel found in the 14.2S peak of the nuclease extract. Several experimental controls were included in this study to establish the specificity of the ER–antibody interactions. They were: (a) 97% of the protein-bound [^{3}H]-estradiol in the high salt nuclear extracts or in the nuclease digests was saturable by a 200-fold molar excess of unlabelled diethylstilbestrol; (b) Sepharose-bound Ig purified from unimmunized rabbit serum did not bind the nuclear ER–E or nucleosome-bound ER–E; (c) antibodies to cytochrome *c* or glucocorticoid receptors did not form immune complexes with ER–E; (d) anti-ER antibodies pre-incubated for 18 hr with the nuclei of MCF-7 cells from which high salt extractable components have been removed fully retained their capacity to react with ER–E bound to nucleosomes and to form immune complexes (Fig. 2f).

Immunohistochemical demonstration of in vitro *translocation of ER from the cytoplasm to the nucleus: a comparative analysis of PR(+) and PR(−) tumors*

In vitro translocation experiments in which tumor pieces from fresh (unfrozen) biopsies were incubated with the ligands were discontinued due to the following difficulties: (a) unavailability of an adequate number of tumors large enough to accommodate different ligand incubations and controls; and (b) problems associated with inadequate penetration of steroid solutions. Therefore, we present only the data obtained with the cryostat sections of frozen tumors that were exposed to the ligands.

Out of 12 PR(+) cases, ten were appraisable, containing an adequate amount of tumor cells. In nine out of these 10, a complete transfer of cytoplasmic IF to the nuclei was evident in most of the IF(+) cells upon exposure to E_2, DES or OH-TX. The tumor which failed to show translocation was from a male and the cytoplasmic ER was 63 fmol and the PR 259 fmol/mg protein. Preliminary results indicated the following general trends: (a) completeness of intracellular translocation of IF in the presence of ligands was unrelated to the level of cytoplasmic PR in that tumor; and (b) proportion of cells showing translocation was always ≥50% in all the PR(+) tumors showing translocation. However, in tumors in which the PR level exceeded 200 fmol/mg protein, tumor cells exhibiting both cytoplasmic and intranuclear IF were frequently

Table 6. Assessment of ER status from cytological smears

Case No.	CA type	*IF results on frozen section	*IF results on cell smear	DCC value (fmol/mg)
1	medullary CA	(+)	(+)	1026
2	infiltrating ductal CA	(+)	(+)	949
3	*in-situ* and infiltrating ductal CA	(+)	(+/-) very few cells	311
4	infiltrating ductal (Comedo)	2+	2+	296
5	infiltrating ductal CA	(+)	(+)	266
6	infiltrating ductal CA	(+)	2+	265
7	infiltrating ductal CA	2+	(+)	221
8	infiltrating ductal CA	(+)	(+)	207
9	infiltrating ductal CA	(+)	(+)	142
10	infiltrating ductal CA (Scl)	(+)	(+)	117
11	infiltrating ductal CA (Scl)	2+	(+)	76
12	infiltrating ductal CA	2+	(+)	71
13†	infiltrating ductal CA	2+	2+	21
14†	WD ductal CA	3+	2+	12
15†	infiltrating ductal (Comedo)	(+)	2+	12
16	intraductal CA	(-)	(-)	NEG
17	infiltrating lob CA	(-)	(-)	NEG
		*IF intensity		
18	pleural fluid	2+		Not Available
19	needle aspirate	2+		776
20	needle aspirate	2+		415
21	pleural fluid	2+		392
22	needle aspirate	(+)		387
23	QNS for F.S.	(+)		370
24†	needle aspirate	(+)		42
25	needle aspirate	(+/-)		22
26	needle aspirate	poor cellularity		19
27	QNS for F.S.	NEG		NEG
28	pleural fluid	NEG		NEG

*≥25% of the tumor cells IF(+).
†Patients of peri or premenopausal age.

seen, even in the experimental controls unexposed to any ligands *in vitro*. Furthermore, in these cases a partial IF transfer to the nuclei in some cells was also seen in frozen sections exposed to plain saline at 37°C. However, a *complete* translocation of cytoplasmic IF to the nuclei resulted only upon exposure to E_2, DES or OH-TX (Figs 3a–d).

Of the 12 ER(+)/PR(-) tumors examined, two had to be discarded due to poor cellularity (one had 12 fmol and the other 18 fmol ER/mg cytosol protein) and another due to a procedural error. Seven out of nine appraisable cases failed to show nuclear translocation of IF in response to the ligands and IF was retained in the cytoplasm (Fig. 4). In one case partial translocation was seen in some cells only in response to OH-TX; in the other a complete translocation was evident in all the cells in response to E_2, DES or OH-TX.

Absorption of anti-ER antiserum used for immunohistochemical studies with a preparation of purified ER

As illustrated in Fig. 5, while distinct cytoplasmic IF was seen with the unabsorbed anti-ER antiserum (unabsorbed with ER) in the cells of an ER(+) tumor, when the antiserum absorbed with the ER preparation was used at the same dilution, no cytoplasmic IF was evident. Two independent experiments with two different preparations of ER yielded similar results. Absorption of the anti-ER antiserum with another protein fraction with little or no E_2 binding activity but with detectable protein was ineffective in eliminating the antibody-specific immunofluorescence. The details of the ER purification procedure and results of the biochemical analysis of the purified product will be published elsewhere.

DISCUSSION

Radiolabelled steroid hormones, fluorescein conjugated steroids or antibodies to steroid hormones have been used to visualize intracellular distribution of steroid receptors and a detailed account of these methods has been published by Pertschuk *et al.* [3]. In this report we will restrict ourselves to a discussion only of those reports involving the utilization of antibodies to ER protein for the immunohistochemical detection of ER.

An antiserum which is considered to be specific

to Type I ER, therefore, utilized for immunohistochemical localization of this steroid hormone receptor should fulfill the following criteria: (1) the immunoglobulins (Ig) purified from the antisera should react with the different molecular phenotypes of ER (8S, 5S, 4S); (2) it should fail to recognize any cytoplasmic or serum proteins other than ER; (3) the antigen molecules dissociated from the immune complexes should resemble type I ER in molecular size and electrophoretic properties; and (4) in addition to E_2 binding, the antigen recognized by the Ig should resemble type I ER in its functional parameters (namely, transformation and translocation to the nucleus triggered by E_2, DES or OH-TX) [4].

The experimental evidence for interaction of the Ig with different phenotypes of cytoplasmic ER has been discussed in great detail by both us [5] and Tate and Jordan [6]. The evidence for the specificity of interaction with nuclear 5S species either in soluble form or bound to nucleosomes is illustrated in Fig. 2.

Two facts emerged from the immunochemical experiments [5, 6]: (a) the interaction between ER and Ig leads to conformational changes in the 8S phenotype, which results in a significant reduction in the affinity of the bond between the hormone (E_2 or OH-TX) and ER; and (b) some of the epitopes recognized by Ig are probably close to the hormone binding sites [5, 6]. In the hormone-occupied 4S species either most of the antigenic sites present in the 8S phenotype are absent or the antigenic sites which are accessible in the 8S species are inaccessible in the E_2-occupied 4S ER. In the 5S ER–E_2 complexes a significant number of antigenic sites may be accessible to the Ig. It is to be ascertained that detailed studies with purified 8S, 4S and 5S ER are essential for assessment of the relative number of antigenic sites present in different phenotypes.

Additional confirmation for the specificity of the antiserum to type I ER is derived from the *in vitro* experiments [2] in which it was clearly demonstrated that the antigen recognized by the antiserum translocates from the cytoplasmic to the nuclear compartment when the target cells, starved for estrogens, were exposed to the hormones at 37°C [7]. It is now well-established that type II ER neither sediment as 8S species nor translocate from the cytoplasm to the nucleus [8].

Evidence for the absence of non-specific activities with ubiquitous cytosol or serum proteins has been previously discussed [5]. The protein electrophoretically dissociated from the immune complexes under non-reducing conditions has an estimated molecular size expected for 8S phenotype and the electrophoretic properties are identical to that of ER–E_2 complexes [5].

Another parameter by which the specificity of the antiserum employed for immunohistochemical detection of ER can be examined is through an analysis of the degree of correlation between the data obtained for ER positivity of a tissue by biochemical procedures (which usually measure only hormone-unoccupied cytoplasmic type I ER) and by an immunohistochemical method which employs the antiserum that detects type I ER in the tissues. One should be aware of the following factors which may contribute to lack of correlation between the two methods of assessing the ER status (as positive or negative). False negatives in the biochemical methods could be related to: (a) a total lack or paucity of tumor cells in the processed tissue; (b) presence of hormone-occupied ER (as in premenopausal women); (c) extensive tumor necrosis; or (d) denaturation during homogenization of the tissue.

False negatives in the immunohistochemical method would be rare unless the tissue were improperly handled during fixation procedure; however, false positives could occur due to impurities in the reagents (which can easily be checked) or when the tumor contains (e.g. necrotic cells) ER which is incapable of binding E_2 but can react with its homologous antibody. It is very important to realize that the quantity of ER measured by biochemical assay can be directly related to the intensity of IF *only* if the tumors are homogeneously positive with an equal amount of ER in *all* the tumor cells and if the tumors' cellularity is also comparable. An analysis of cellularity of 79 ER(+) and 44 ER(−) (biochemical classification) tumors revealed a lack of linear relationship between quantity of ER and tumor cellularity (Tables 1, 2) leading us to the obvious conclusion that there may be a lack of homogeneity with regard to the ER status of the tumor cells. An analysis of IF data on the ER(+) tumors revealed that 83% of tumors with $\geqslant$500 fmol of ER were homogeneous and highly cellular, containing only IF(+) cells in the multiple areas of the tumors examined (Tables 1, 2). A distinct trend towards greater heterogeneity not only in tumor cellularity but also in antigen status of the tumor cells is observed in tumors containing 50–500 fmol of ER. The majority of these tumors contain a mixture of IF(+) and IF(−) cells. It is also evident that IF(−) cells predominate in more than 50% of the tumors with an ER content less than 50 fmol. Thus, despite a lack of direct relationship between intensity of IF and ER content of the tumor due to heterogeneity of tumors if the total cellularity and the proportion

of IF(+)/IF(−) cells are estimated, a trend towards a quantitative relationship is evident among ER(+) tumors. Both biochemical and IF procedures classified 68 out of 72 ER(+) tumors which had an adequate quantity of tumor cells as ER(+). The four misclassified by IF as ER(−) were characterized by poor cellularity, poor cell preservation or focal necrosis, with necrotic cells showing no IF.

Among the 44 ER(−) tumors a lack of correlation of ER status between the biochemical and IF procedures was related to a high degree of necrosis in the tumors of postmenopausal women and to the possible presence of endogenously hormone-occupied ER in the premenopausal group [three out of 20 tumors which were all PR(+)]. The ER(−) but PR(+) tumors are very rare and an analysis of ER and PR in 528 tumors in our laboratory showed that only 3.7% of all the breast carcinomas were ER(−) but PR(+).

Currently we analyze multiple areas of the tumors by IF and classify the tumors into five groups: (A) homogeneously IF(+); (B) homogeneously IF(−); (C) $\geqslant$50% of the tumor cells IF(+); (D) $\geqslant$50% of tumor cells IF(−); and (E) heavily necrotic tumors. Although data on cellularity, stroma vs cell ratio, presence of benign components, etc. are recorded for each tumor, they are disregarded for classification of ER status. Tumors with at least 5% of tumor cells are considered appraisable. Data on the biological course of these tumors and their clinical response to hormone therapy are being collected. The relevant information may be the proportion of ER(−) cells for predicting early vs late recurrence for primary tumors and disease-free interval in tumors showing clinical response to hormone therapy.

Homogeneous ER(+) tumors (group A) which lack PR may fail to respond to hormone therapy despite the lack of ER(−) cell population. Recently the significance of the PR status of the ER(+) tumors for selecting possible responders to hormone therapy is being emphasized [9, 10]. While 70–80% of the tumors which contain both ER and PR in their cytoplasm show clinical response to hormone therapy, only 25–30% of ER(+) but PR(−) tumors respond to hormone manipulations. It is well-established that the synthesis of PR is dependent on ER-E_2 interaction with the nuclear acceptor sites in normal E_2 responsive cells. Therefore we decided to examine the *in vitro* translocatability of cytoplasmic ER to the nuclear compartments in the ER(+)/PR(+) and ER(+)/PR(−) tumors. Our hypothesis was that if ER-E_2 translocation is aborted in tumor cells, either due to structural receptor abnormality or due to abnormal nuclear acceptor sites, it is highly likely that the tumor cell would be unable to synthesize PR. If there is a correlation between translocation abnormality and lack of PR, then a study of *in vitro* translocatability can be used in lieu of PR assessment. The preliminary data on 24 biopsies appear to be very promising. Binding of the translocated antigen to very distinct spots localized in the nuclear compartment rather than to the general surface of the nuclei may be in support of the specific acceptor sites hypothesis [6] for ER–DNA interactions. The fact that cryostat sections can be utilized for such studies is also very significant. We have examined breast tumors which have been in cold storage (−80°C for 5 yr) and ER translocation analysis in these tumors is found to be feasible. Additionally, biochemical assessment of cytoplasmic PR revealed the stability of PR in these tumors during the 5-yr storage. A positive correlation between presence of PR and *in vitro* translocatability of ER was evident from our preliminary data. These efforts are being pursued in a larger number of tumors. Utilizing biochemical procedure, the formation of 5S ER-E_2, ER-OH-TX or ER-DES phenotypes *in vitro* and their ability to bind the nuclei isolated from the tumors should also be examined. It is important to study the 5S phenotype because the antibodies apparently detect only the 5S ER-E_2 and not 4S ER-E_2 of the nuclear phenotypes of MCF-7 cells (Fig. 2).

Our discussion on the specificity of the antiserum to type I ER can be summarized as follows. The antibodies react with a protein which has all the biochemical characteristics of type I ER; the antibodies recognize different molecular phenotypes of ER (except hormone-occupied 4S species either from cytoplasm or nuclei); the protein recognized by the antibodies resembles type I ER by its *functional* parameter—namely, its ability to 'transform' and translocate to the nucleus; the antiserum has also been found useful for the studies of translocation abnormalities which translate into a lack of PR synthesis [2]. For qualitative assessment of the ER status of the tumor, the IF method offers an alternative to cytological specimens (Table 6). For biopsies, along with the biochemical data, the test provides important information related to the proportionality of the IF(+) and IF(−) cells. Nevertheless, until the information is related to the clinical response data, the IF test should not be used as an alternative for biopsies in which it is feasible to obtain biochemical data on the quantity of ER and PR. For purposes of clinical correlation of the data we are considering all the tumors with heavy necrosis [whether with or without IF(+) cells] as a separate group. Some of

these patients may have been under tamoxifen therapy when the biopsies were taken.

Prior to concluding, we would like to examine two issues relevant to the IF study: (1) the question of intracellular distribution of IF in the ER(+) breast carcinomas in general and (2) the question of difference in the relative intensity of IF in the cytoplasmic vs nuclear compartment in the *in vitro* translocation experiments.

The localization of IF is predominantly cytoplasmic in most of the frozen biopsies that have been analyzed; exceptions are ER(+)/PR(+) tumors in which the PR content is also high, exceeding 200 fmol/mg, and a few tumors from premenopausal women. Even in these cases the intranuclear IF is seen in clusters of cells and is rarely uniform in all the tumor cells. Two interpretations are plausible for such a pattern of ER distribution: (a) the antibodies may detect only the 5S ER-E_2 bound to the nuclear chromatin and, therefore, only in cases where the 5S occurs in sufficient concentration do we detect intranuclear IF; (b) the bondage between 5S ER-E_2 complexes and the nuclear acceptor sites may be labile for the freezing and thawing and, therefore, in the frozen sections processed by ethanol dehydration/rehydration they are found in the cytoplasmic compartment. While we were examining the fresh (unfrozen) smears from breast carcinomas by IF test for ER we observed cytoplasmic and nuclear IF in cells from the ER(+)/PR(+) tumors with a higher frequency than is normally observed among frozen tumors. There were also two cases, one ER(+)/PR(+) and another ER(-), in which some cells with exclusively nuclear IF were encountered. In addition, during our *in vitro* translocation studies we observed a partial translocation of IF into the nuclear compartment in the frozen sections of a few ER(+)/PR(+) tumors when exposed to plain saline at 37°C without any estrogenic ligands. If the 'transformed and translocated' ER-E_2 complexes have dissociated from the nuclei in these tumors, raising the temperature to 37°C may cause them to rebind to the nuclei. A well-structured study designed especially to investigate the validity of these interpretations is, however, essential.

In the *in vitro* translocation experiments [2] a *complete* movement of immunofluorescence from the cytoplasmic to the nuclear compartment is observed in cells exposed to estradiol or non-steroidal antiestrogens. No residual cytoplasmic IF is observed in the cases where there is no translocation abnormality [2] (Fig. 3). When the intensity of cytoplasmic IF in the control cells is compared with the amount of intranuclear IF, the latter appears to be highly localized. The question as to why there is an apparent reduction in the quantity of IF is often asked. Possible explanations are the following: (1) it is very difficult to make a visual assessment of the intensity when IF which is spread over a wide area of cytoplasm is compared with IF concentrated in few loci in the nuclei; or (2) the relative number of epitopes accessible to the antibodies may be greater in number in the 8S ER and 8S ER-E than in 5S ER-E_2 and fewer in nucleosome-bound 5S ER-E than in hormone-free cytoplasmic 4S ER. Quantitative immunochemical studies conducted with purified ER would be essential to establish the number of antigenic sites accessible to the antibodies in different phenotypic forms of ER.

Immunology of ER is a newly emerging field. During this decade we will be gathering a wealth of information of ER either conflicting with or conforming to the presently held theories on the structure, function and cellular distribution of ER, most of which were derived from biochemical ligand-binding experiments without the help of antibody probes. Eventually a widely acceptable pattern may hopefully emerge which may prove to be close to reality.

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