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Immunohistochemical Localization of Estrogen Receptors in Human Mammary Carcinoma using Antibodies to the Receptor Protien*

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Abstract—*An immunofluorescent method has been developed for localizing estrogen receptors (ER) in frozen sections of human breast cancer biopsies which utilizes the biochemically well-characterized antibodies to the cytoplasmic ER of human breast cancer produced in rabbits. Frozen sections of sixteen breast cancer biopsies, in which the receptor content has been quantified using dextran-coated charcoal assay (DCC technique), were used to study the localization of ER. Two aspects of ER detection, namely "estrogen-binding" and ability to react with the homologous antibodies, have been analyzed and the results compared with those of DCC technique. Fluorescent labelled estrogen method ($F.E_2$) of Pertschuk was utilized to detect the distribution of estrogen binding proteins and the indirect immunofluorescent (IM-AR) method using anti-receptor antibodies to assess the antigenic sites of the receptor protein. In addition to the comparison of the results of three techniques, DCC, IM-AR and $F.E_2$, for ER detection, a procedure for processing the frozen sections, which enables the study of estrogen binding characteristic as well as the antigenic reactivity of the receptor molecules, is described.*

INTRODUCTION

RECEPTORS for steroids such as estrogen and progesterone have been identified in steroid target tissues such as lactating mammary gland, endometrium and myometrium, and in human breast cancer. The presence of cytoplasmic estrogen receptors in the breast cancer cells is predictive of clinical response to hormonal therapy. The most widely used biochemical techniques that measure estrogen receptors in the cytoplasm and currently available histochemical techniques which localize estrogen receptors in the tissue sections are both limited to detecting only the steroid receptors whose binding sites are unoccupied by estrogen. The receptors pre-saturated with endogenously circulating estrogen, as in pre-menopausal women, cannot be identified by these methods. Furthermore, those methods that detect receptors exclusively in terms of estradiol binding activity cannot distinguish phenotypic variants as long as they can bind estradiol. Therefore, a procedure that can identify estrogen receptors based upon the detection of antigenic sites is highly desirable.

This report describes an immunofluorescent method which has been developed for localizing estrogen receptors in frozen sections of human breast cancer biopsies. This method differs from any other published histochemical methods for localizing estrogen receptors. It utilizes anti-receptor antibodies produced in rabbits that were immunized with the receptor protein derived from the cytosol of human breast cancers. The specificity of the antibodies to the cytoplasmic estrogen receptors of human breast carcinoma, their cross-reactivity with the calf uterine cytosol receptors [1] and their ability to recognize nuclear receptors of MCF-7 cells (an established cell line of human mammary carcinoma) (personal communication, Fred Frankel, PhD, University of Pennsylvania

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School of Medicine, Philadelphia, PA, U.S.A.) have been tested biochemically. Using the immunochemically well-characterized anti-receptor antibodies, in combination with a tissue processing methodology that neither destroys the antigenic sites nor the estrogen binding capacity of the receptors, we have studied the receptor localization in 16 human breast carcinomas. The results of the immunofluorescent method using anti-receptor antisera are compared with the results of a histochemical method using fluorescent labelled estradiol and of the biochemical quantification procedure (dextran-coated charcoal assay). The use of FITC-conjugated estradiol to localize estrogen receptors is still in the investigational stage and requires validation [2]. Nevertheless, this method provided a tool for identifying estrogen-binding proteins in the tissue sections. It allowed a direct comparison between the two different parameters of "estrogen receptor measurement", namely estrogen binding capacity and anti-receptor antibody binding in the same tissue section.

MATERIALS AND METHODS

Tissues

Human breast cancer biopsies from both primary and recurrent cancer were studied. Histochemical experiments were conducted in those tumor samples where ample tissue material was available for testing for receptors by all three different procedures. The tissues were stored in a Revco ultra low freezer at −80°C until use. The frozen tumors were shattered into smaller pieces by percussion at liquid nitrogen temperature. Randomly selected pieces (about 3 mm cubes) were then embedded in gelatin. Fifteen grams of gelatin were solubilized in 100 ml of warm physiological saline. The temperature of the gelatin solution was allowed to drop to 30°C and the tissues were embedded in gelatin poured into paraffin-coated paper boats. The block was allowed to gel in the refrigerator and the gelled blocks were stored at −80°C until ready for use. As a routine, small pieces of tissue were also fixed in Bouin's solution for studying the histopathology of the tumors. The rest of the tumor was pulverized for biochemical quantification of receptors by DCC assay.

DCC assay

[^{3}H]-labelled 17β estradiol (New England Nuclear Corporation, Billerica, MA, U.S.A.; specific activity, 100 Ci/millimole) at seven different concentrations, ranging from 0.3 to 4 nmol/l, were added in 50 μl quantities to 200 μl aliquots of cytosol prepared from the powders. After incubating at 4°C for 18 hr the receptor-bound [^{3}H]-estradiol was separated from the unbound steroid using 500 μl of dextran-coated charcoal suspension. Two hundred-fold excess of unlabelled diethylstilbesterol was used to assess non-specific binding. The results were analyzed by Scatchard plot and expressed as fmol of estradiol bound per mg cytosol protein. The buffer composition, preparation of cytosol, DCC solution, procedural details for separation of the receptor-bound estradiol etc. were similar to the methodology outlined by McGuire and Dela Garza [3].

Tissue preparation for histochemical techniques

Frozen sections, 4 μm thick, were prepared from gelatin-embedded tissues. Pilot experiments, conducted to choose a suitable fixative, revealed that the most commonly used fixatives such as acetone, formaldehyde and Bouin's solution destroyed both the antigenicity and estrogen-binding capacity of the receptors to an appreciable degree and, therefore, were found unsuitable. Use of unfixed tissues, as well as tissues fixed by directly immersing them in absolute or 95% ethanol, were also found to be inadequate. The following procedure was found acceptable and was adopted for preparing the tissue sections for histochemical techniques: the sections were first dehydrated gradually by 10-min exposure through increasing concentrations of alcohol solutions containing 30%, 50%, 75% and 90% absolute ethanol and xylene. The alcohol solutions were prepared in physiological saline instead of in distilled water in order to prevent changes in the receptor caused by drastic hypotonicity during the fixation–dehydration procedure. Furthermore, while developing suitable conditions for anti-receptor antibody to react with the receptors, it was found that the inclusion of sodium chloride at 0.1 M concentration in the buffer was conducive to the interaction of antibodies with the estrogen receptors but did not alter the estrogen binding capacity of the receptors [1].

Prior to incubating the tissue sections with the appropriate reagents (anti-receptor antibody or FITC-labelled estradiol, control serum or pre-immune serum), they were rehydrated by reversing the dehydration steps. The sections were immersed in physiological saline and incubated with the appropriate reagents.

The immunohistochemical technique using anti-receptor antiserum

For the immunohistochemical method (IM-AR) tissue sections were treated at room temperature with 30-fold diluted anti-receptor antiserum (primary antiserum) for 30 min, followed by FITC-conjugated anti-rabbit IgG (heavy chain specific, Biorad Laboratories, Orangeburg, NY, U.S.A.) made in swine (second antibody) for the same length of time.

Fluorescein-conjugated estradiol technique

FITC-conjugated estradiol technique ($F.E_2$) was performed according to the directions outlined by Dr. Pertschuk, who also provided this reagent for our use [4]. The sections were incubated with $F.E_2$ at a concentration of 40 pmol 50 μl for 1 hr at 4°C, washed thoroughly in saline and mounted in saline. The edges of the coverslips were sealed with Permount to prevent drying of the tissues and viewed under u.v. light. In this state the tissue fluorescence was stable at least for a few weeks when stored at 0–4°C. A Zeiss fluorescent microscope equipped with a halogen lamp and neofluor objectives was used.

Experimental controls

(A) *Reagent controls.*

IM-AR method.

(1) The tissue sections for IM-AR method were treated with the serum procured from the rabbit prior to immunization (pre-immune serum) and diluted 30-fold in saline and used in place of the anti-receptor antiserum.

(2) Sections were treated with FITC-conjugated anti-rabbit IgG, omitting the primary antiserum in order to assess the non-specific adherence of this reagent.

(3) The anti-receptor antiserum was absorbed with different concentrations of:

(a) Normal human sera pooled from different healthy volunteers (both male and female);

(b) Cytosol from a pool of breast tumors negative for receptors by DCC assay;

(c) Cytosol procured from a pool of breast tumors positive ($\geq$ 500 fmol/mg cytosol protein) for receptors by DCC assay.

$F.E_2$ method.

For the $F.E_2$ method the tissue sections were pre-incubated with 50 μl of unlabelled E_2 solution containing 4000 pmol of E_2/50 μl. This quantity of E_2 is a 100-fold excess of FITC-conjugated E_2 used for the experiment. The incubation was carried out at 4°C for 1 hr, identical to the experimental slides which were incubated with FITC-labelled E_2. This control was used to check if estradiol would inhibit the subsequent binding of FITC-labelled E_2.

(B) *Tissue control.*

(1) *Estrogen receptor-negative tissue.* Human tissues known to lack estrogen receptors and pre-checked in our laboratory by DCC assay to confirm the lack of estrogen receptors were utilized as negative controls. These included non-lactating normal breast obtained at autopsy, human kidney and human breast tumors of post-menopausal women lacking estrogen receptor.

(2) *Estrogen receptor-positive tissue.* In addition to human breast tumors rich in estrogen receptors, as ascertained by DCC assay, calf and cow tissues were also tested. Prior studies have established that the anti-receptor antibodies do cross-react (biochemically) with estrogen receptors of bovine uterus [1].

Scoring of results

(A) *DCC technique.* Absolute values are reported. Values less than 5 fmol/mg cytosol protein are considered as receptor-negative; a level equal to or greater than 5 but less than 10 fmol is classified as equivocal (+/−); the amount of receptors equal to 10 fmol or greater are considered distinctly positive for clinical evaluation.

(B) *Tissue sections.* Each of the tissue sections was independently examined by at least three investigators (with no prior knowledge of results of DCC assay). The tumors were classified as negative (−), or +1, +2 or +3. The pathologists, in addition to reading the test results, also recorded their comments on cellularity, histology of the tumor, percentage of tumor cells in the sections stained with hemotoxylin and Eosin and percentage of tumor cells showing fluorescence in all the experimental slides. The independent fluorescence assessments (without results of DCC assay) were given to the two pathologists who arrived at a consensus and the net results are reported in Table 1.

RESULTS

Suitability of gradual dehydration using ethanol–xylene for localizing estrogen receptors

When the tissue sections of calf uteri and human breast tumors that were rich in cytoplasmic estrogen receptors (pre-determined by DCC technique) were "fixed" by the method of

Table 1. *Comparison of results of three different methods of detecting estrogen receptors in breast cancer tissue*

Patient*	IM-AR†	DCC‡ (fmol/mg)	F.E_2§
M.L.	NEG	NEG	Not Done
A.H.	NEG	NEG	+1 (N&C)‖
H.H.	NEG	NEG	+1 (N&C)
M.F.	NEG	NEG	+1 (N&C)
V.R.¶	±	NEG	+2
K.L.**	+1	NEG	±
S.P.††	+2	NEG	NEG
B.Pa.	+1	9	±
R.C.	+1	25	+3 (N&C)
A.J.	±	34	Not Done
E.E.	+3	113	Not Done
L.O.	+1 ~ +2	198	Not Done
D.H.	+2 (N&C)	304	+ (N&C)
G.H.	+2	329	+2
B.Pe.	+3	587	+2
V.P.	+2	613	+2

*Both primary and recurrent breast cancer biopsies.
†Anti-receptor antibody method; + intensity of cytoplasmic fluorescence graded as NEG, ±, +1, +2, +3.
‡fmol of receptor per mg of cytosol protein.
§FITC-conjugated E_2 method (intensity of fluorescence graded similar to IM-AR method.
‖Both nuclear (N) and cytoplasmic (C) fluorescence were evident.
¶V.R. 95% of the tumor cells were negative; 5% distinctly positive by IM-AR technique (+1).
**K.L. Paucity of tumor cells; 95% stroma, 5% tumor cells. All tumor cells were +1 (IM-AR).
††S.P. Pre-menopausal; contained progesterone receptors (9 fmol/mg) by DCC assay.

gradual dehydration and incubated with reagents for IM-AR or F.E_2 technique, cytoplasmic fluorescence was observed in all the sections. The other receptor-negative (by DCC assay) tissues, such as human kidney or normal breasts (non-lactating), obtained at autopsy were negative by both the experimental procedures. Thus the anti-receptor antibodies and FITC-labelled E_2 were able to react with the receptor protein. The dehydration followed by rehydration through series of alcohol appeared to be gentle enough that the antigenicity and the estrogen binding capacity were retained to an appreciable degree, as opposed to the other commonly used fixation methods or unfixed tissues.

The specificity of the antiserum

By all the standard criteria to define the specificity of any anti-serum, the anti-receptor antiserum proved to be specific for a protein present in receptor-rich tissues. The negative controls, namely kidney and normal non-lactating breast, did not exhibit fluorescence when incubated with the reagents of IM-AR technique. The pre-immune serum used in place of anti-receptor antiserum yielded negative results in both the receptor-rich and receptor-negative tissues. Furthermore, omission of the primary antiserum and staining the section with FITC-conjugated second antibody produced negative results in all the tissue sections examined.

Absorption of anti-receptor antiserum (Fig. 4A) with pooled normal human serum (Fig. 4B) or cytosol of receptor-negative tumors (Fig. 4C) did not quench the fluorescence demonstrated by unabsorbed antiserum in the tumor cells. On the other hand, antiserum absorbed with receptor-rich cytosol completely obliterated the fluorescence of tumor cells seen in the sections of receptor-positive (by DCC assay) breast tumors (Fig. 4D). The serum obtained from the rabbit *prior* to immunization, when used in place of the antiserum, did not yield specific fluorescence, as demonstrated in Fig. 4E.

The results obtained on 16 *breast carcinomas* (*Table* 1)

In all the tumors in which receptors are detected by DCC assay ($\geq$ 5 fmol/mg cytosol protein) the IM-AR technique also identified the presence of cytoplasmic estrogen receptors.

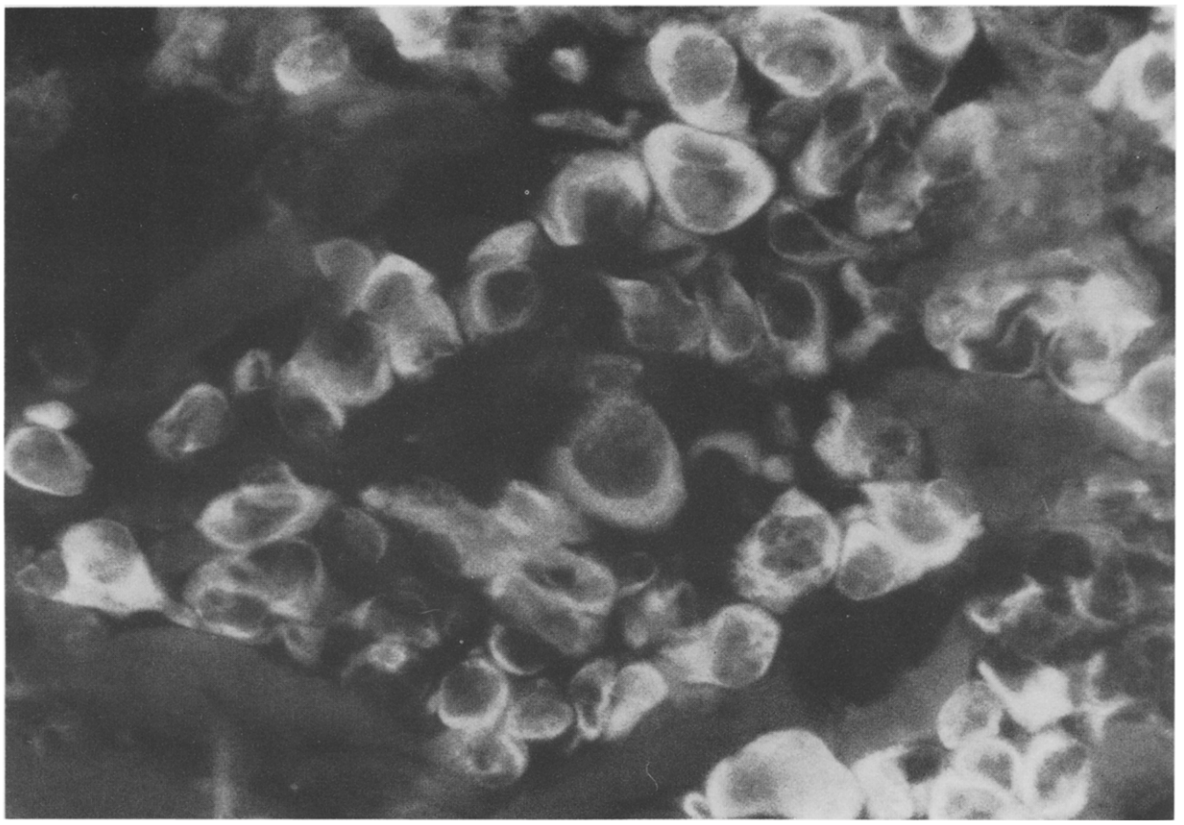

Fig. 1. *Estrogen receptors demonstrated by indirect immunofluorescence (IM-AR technique) in a breast carcinoma which was highly estrogen receptor-positive by dextran-coated charcoal (DCC) assay* (1630 fmol/mg). *There is strong cytoplasmic fluorescence in the tumor cells. Faint nuclear fluorescence in some of these cells may correspond to nucleoli (Magnification,* ×480; *Kodak Tri-X-PAN film; ASA* 400; *for black and white prints; exposure time,* 4 min.)

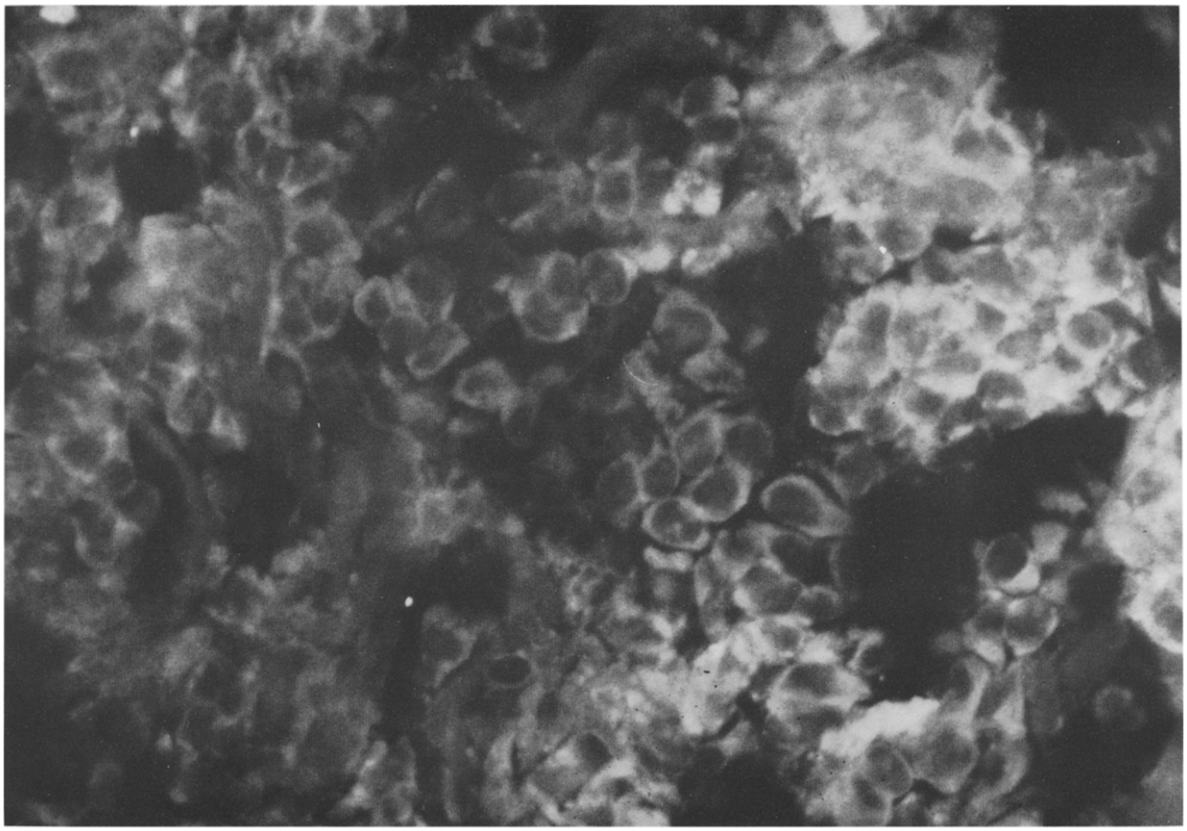

Fig. 2. *Heterogeneity of estrogen receptor among tumor cells in a highly receptor-positive breast carcinoma* (113 fmol *by DCC assay*) (*E. E., Table* 1) *is demonstrated by adjacent areas where the majority of tumor cells exhibit strong* (*right*) *or moderate* (*left*) *fluorescence.* (*Indirect immunofluorescence technique with anti-estrogen receptor antibody; magnification,* ×480; *Kodak Ektachrome; ASA* 400; *for color prints; exposure time,* 4 min; *black and white prints were prepared from color prints.*)

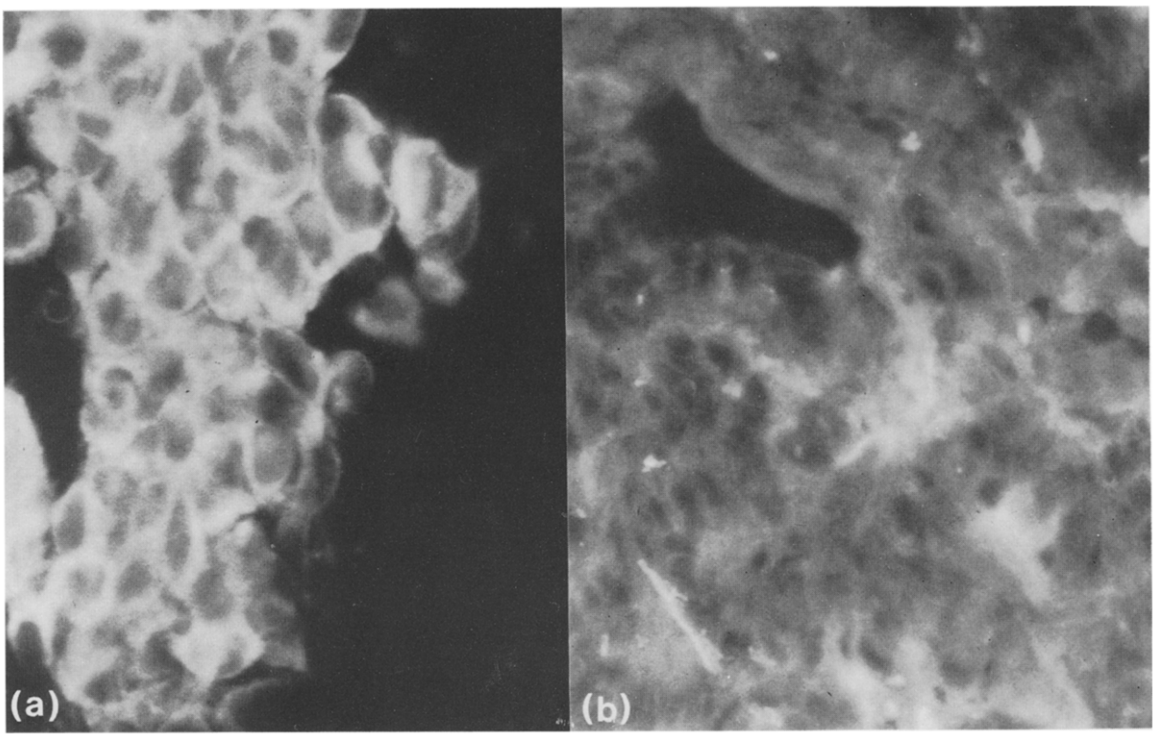

Fig. 3(*A*). *Intense cytoplasmic fluorescence indicates estrogen receptor antibody reacting with most cells of this breast tumor* (*B. Pe., Table* 1) *which was strongly positive by DCC assay* (≥ 587 fmol). (*B*) *Negative cytoplasmic fluorescence indicates absence of estrogen receptor antibody reactive cells in this breast carcinoma which was receptor-negative by DCC* (*H. H., Table* 1). *A non-specific auto-fluorescence is present in the stroma surrounding the negative tumor cells.* (*A & B indirect immunofluorescence; magnification,* ×425; *for both* 3(*a*) *and* 3(*b*), *Kodak Ektachrome color slides; exposure time,* 4 min; *black and white prints were made from the color slides.*)

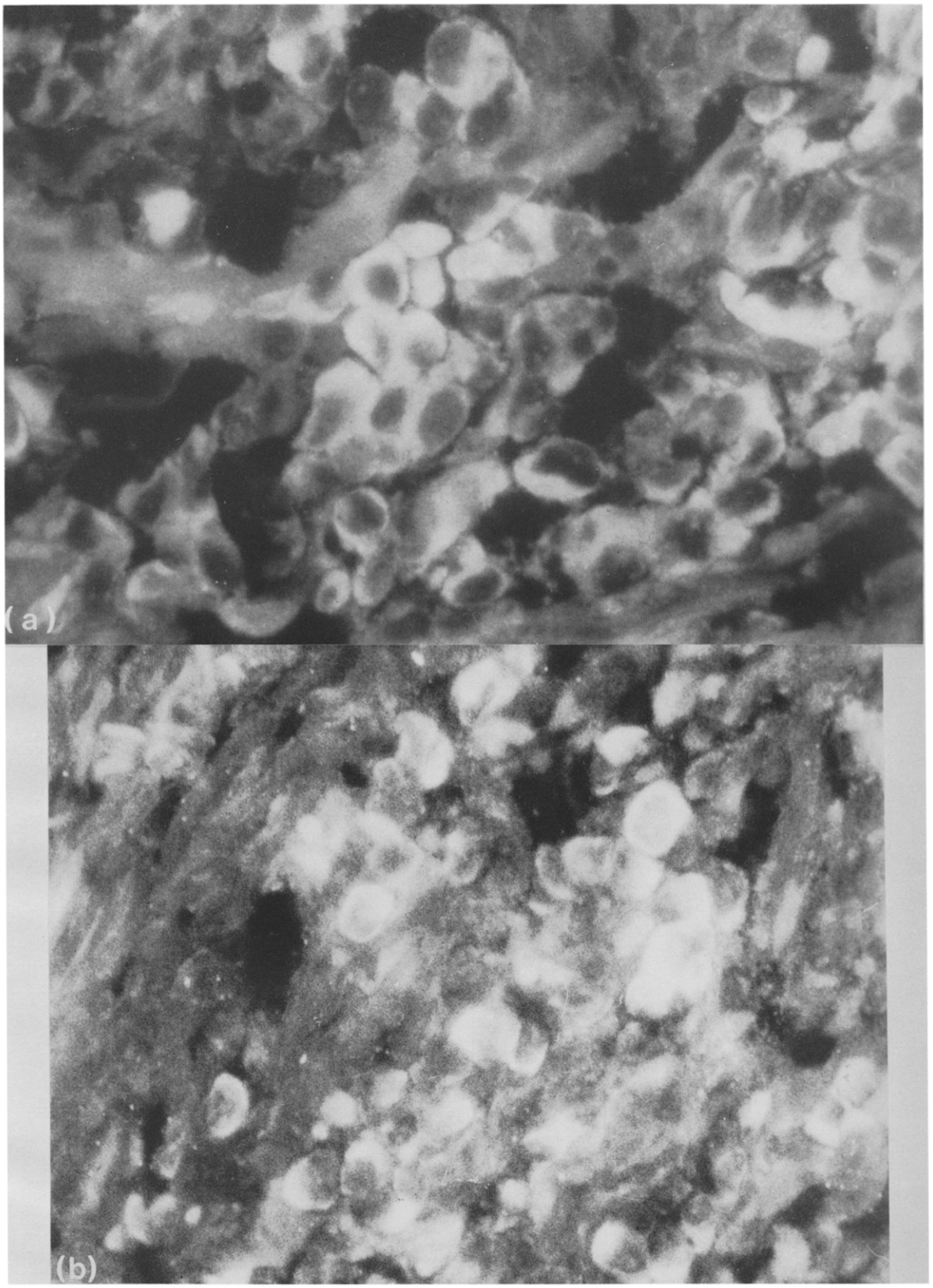

Fig. 4(*A*, *B*).

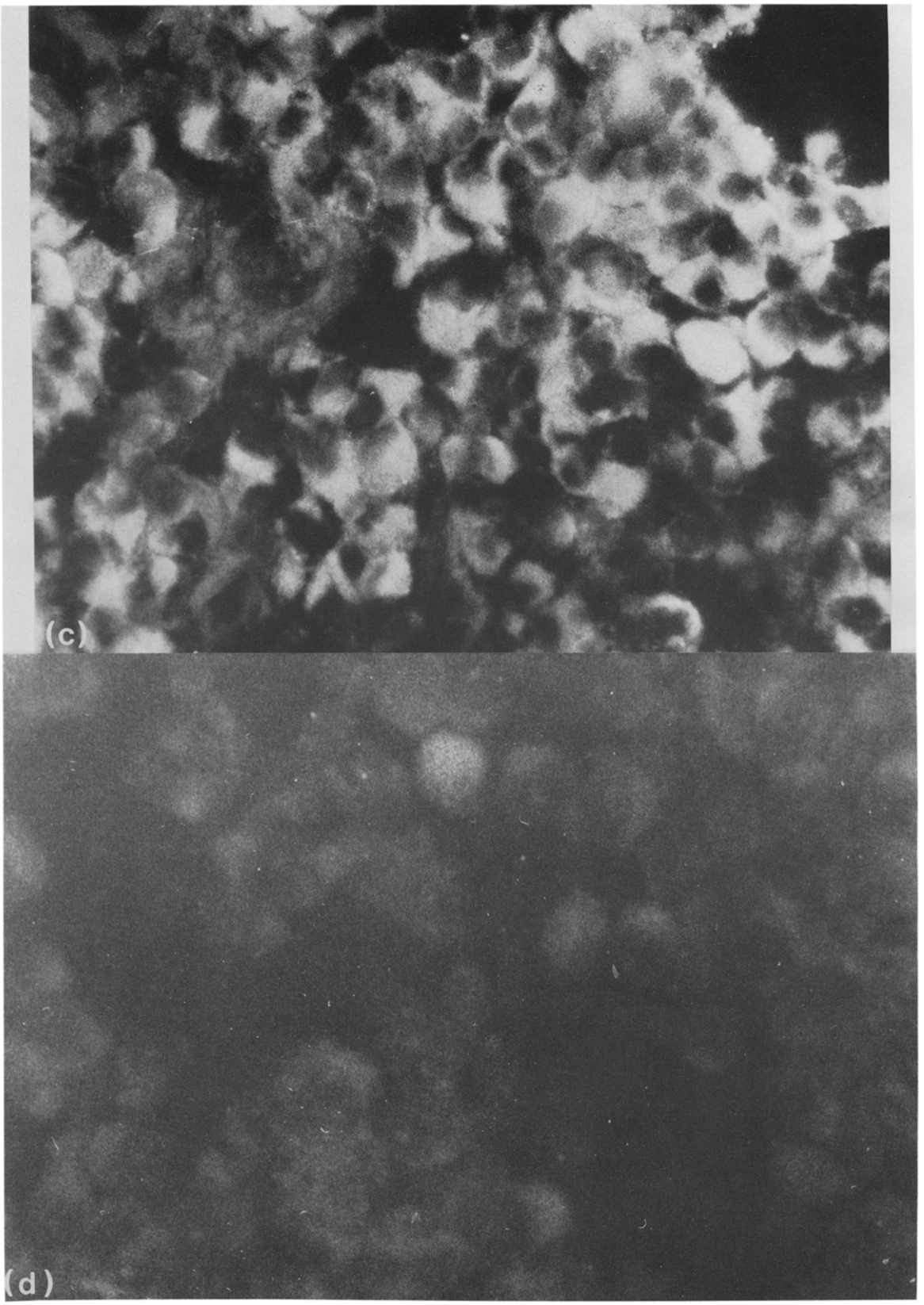

Fig. 4(*C, D*).

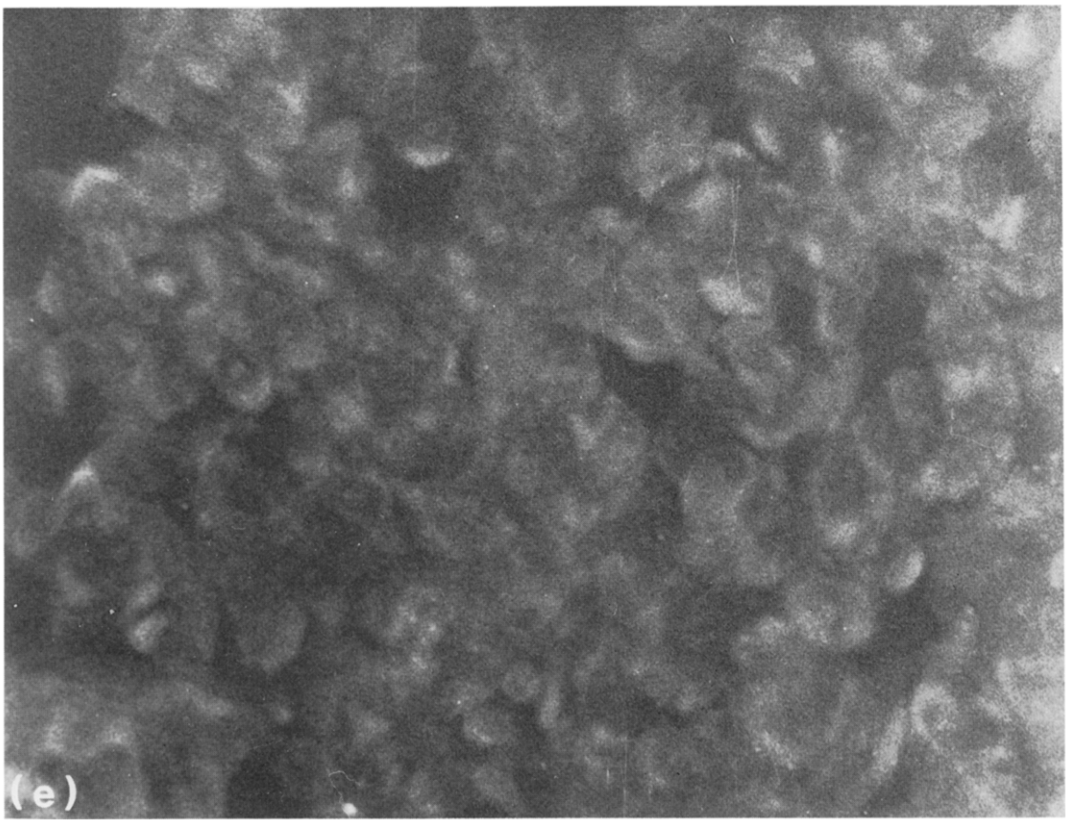

Fig. 4. General comments: for Fig. 4A–E frozen sections of the tumor of patient H.D. (Table 1) was utilized (DCC positive, 304 fmol/mg). *The photographs were taken using Kodak Tri-X-PAN film for black and white prints. All sections were exposed for precisely* 4 min. (*Magnification,* ×475.) (*A*) *Tissue sections incubated with unabsorbed anti-receptor antiserum. Positive cytoplasmic fluorescence is seen in majority of the cells with very light intra-nuclear staining in this area.* (*B*) *Primary antiserum absorbed with pooled normal human serum was used for ER localization. The intensity of the cytoplasmic fluorescence is comparable to that seen in Fig. 4A. Faint intra-nuclear fluorescence is also maintained.* (*C*) *The anti-receptor antiserum absorbed with a pool of cytosols (protein content* 4.5 mg/ml) *prepared from DCC-negative tumors of post-menopausal women was utilized to localize ER. The absorption does not affect the cellular fluorescence obtained with the unabsorbed antiserum (compare with 4A).* (*D*) *The tissue sections were incubated with the anti-receptor antiserum absorbed with the cytosols prepared from DCC-positive human breast tumors (protein content* 4.3 mg/ml). *The fluorescence demonstrated in Figs. 4A, B and C is totally obliterated by this absorption procedure.* (*E*) *The tissue section was incubated with the serum obtained from the rabbit prior to immunization. Very faint non-specific fluorescence is seen in the tumor cells.*

Out of the seven tumors classified by the DCC technique as receptor-negative, four tumors showed no fluorescence in the tumor cells. One case, K.L., was characterized by a paucity of tumor cells. Ninety-five per cent of the tumor was composed of stroma and only 5% of tumor cells. These tumor cells were positive by IM-AR technique. In the case of V.R. the tumor had islets of tumor cells (80%) with 20% stroma. A majority of the tumor cells was negative, with only 5% of the tumor cells demonstrating weak fluorescence. The case S.P. was a pre-menopausal patient (36 years old) and the cytosol contained progesterone receptors (9 fmol/mg). Thus, three out of seven DCC-negative tumors showed distinctly positive fluorescence by IM-AR method.

The cellular localization of fluorescence in all the tumors except for D.H. was exclusively cytoplasmic by IM-AR method. Nuclear fluorescence, additional to cytoplasmic fluorescence, was observed in the tumor cells of this patient.

Using the $F.E_2$ procedure, the fluorescence was observed in 11 out of 12 cases. Five cases out of six DCC-negative tumors also demonstrated fluorescence of varying intensities in both the nuclei and the cytoplasm. The pre-menopausal case, S.P., was the only specimen which was classified as negative by $F.E_2$ method. Pre-incubation with unlabelled estradiol did not inhibit the binding of $F.E_2$ in the majority of cases. This lack of inhibition was observed for both DCC-negative and DCC-positive tissues. Heterogeneity in the distribution of ER-positive cells within a single tumor was frequently observed by both IM-AR and $F.E_2$ methods (Fig. 2).

DISCUSSION

The dextran-coated charcoal and surcrose-density gradient centrifugation techniques are the only clinically validated methods for identifying and quantifying estrogen receptors. The clinical response to hormonal therapy has been correlated with the presence in breast cancers of Type I receptors possessing high affinity for estradiol that are measured by these two biochemical procedures. Questions have been raised about the validity of several published histological localization techniques [4–9] for detecting estrogen receptors. The NCI Consensus Committee for Steroid Receptors recommended that the results of all the histochemical localization techniques be correlated with those of the validated biochemical techniques [2].

Three different procedures were applied to identify estrogen receptors in 16 breast tumor specimens. The following conclusions can be drawn from the results: the tissue-processing technique that involves dehydrating the sections gradually through increasing concentrations of alcohol and xylene followed by rehydration appears to preserve both the antigenicity, as revealed by IM-AR procedure, and estrogen-binding capacity, as demonstrated by $F.E_2$ technique.

The results of the experiments using the antiserum absorbed with the pooled human serum (Fig. 4B) or cytosols pooled from DCC-negative tumors (Fig. 4C) of post-menopausal women or of DCC-positive tumors (Fig. 4D) lend support to the specificity of the antiserum at the histochemical level. It is interesting to note that the absorption of the antiserum with the cytosols of DCC-negative tumors does not affect the anti-receptor activity of the antiserum (Figs. 4A and C). These results can be interpreted to mean that the tumors of post-menopausal women classified as ER-negative by ligand binding assays may not contain receptor molecules that are "non-functional" and cannot, therefore, bind estradiol. In other words, these ER-negative tumors do not contain ER protein that share antigenicity with their "functional" ER molecules present in the DCC-positive tumors. Thus, the post-menopausal DCC-negative tumors are perhaps truly "ER-negative". A greater number of DCC-negative tumors from post-menopausal women need to be analyzed to test the validity of this interpretation.

We are uncertain as to why the $F.E_2$ procedure showed positive fluorescence in five out of six DCC-negative tumors. One of the possible explanations is that $F.E_2$ may combine with Type II receptors which have much lower affinity for estradiol than the Type I forms. The $F.E_2$ procedure utilizes 40 pmol/50 μl or 0.8 mm solution of estradiol for localizing the receptors. At this concentration Type II receptors of both the cytoplasm and the nuclei are known to bind estradiol [10]. The lack of inhibition of $F.E_2$ binding by the unlabelled estradiol is also enigmatic. Therefore, it is crucial to check which proteins, in addition to ER, bind $F.E_2$. The only way this can be categorically tested is by using radiolabelled BSA to conjugate the estradiol and biochemically analyze the binding characteristics of this conjugate and identify the serum and/or cytosol proteins that form complexes with the conjugate.

We intend to explore the possibility that the IM-AR technique may detect ER in the tumors

of pre-menopausal women while the DCC technique and the F.E_2 method cannot identify the receptors blocked by endogenously circulating estradiol. For example, with the IM-AR technique ER is demonstrated in the tumor of the patient S.P. (Table 1). Preliminary observations in ten DCC-negative tumors from pre-menopausal women using IM-AR technique revealed that the anti-receptor antibodies can be used to detect estrogen-occupied ER in the tumors of pre-menopausal women (data to be published elsewhere). Additional data, along with the details of clinical response, are required to ascertain the value of this technique for demonstrating ER in the tumors of pre-menopausal women.

Heterogeneity of tumor cell populations with regard to the presence of estrogen receptors was evident in this pilot study with both the receptor localization techniques (Fig. 2). Furthermore, almost without exception those tumor cells with a large amount of cytoplasm were usually observed to contain ER by both IM-AR (Figs. 1 and 3A) and F.E_2 procedures. However, among the small malignant cells occurrence of both receptor-positive and receptor-negative populations was evident by these localization methods.

In conclusion, the IM-AR method for localizing estrogen receptors in human breast carcinoma shows a good correlation with the results of DCC assay for those of post-menopausal women. It offers the additional possibility of detecting receptor-positive cells in some tumors which are negative by DCC assay. It will be important to identify and follow this group of patients to determine if they correspond to the group of ER-negative patients who respond to hormonal therapy.

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