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IN VIVO AND *IN VITRO* IMMUNOFLUORESCENT APPROACH TO THE PHYSIOPATHOLOGY OF ESTRADIOL KINETICS IN TARGET CELLS

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SUMMARY

A fluorescent antibody has been employed for investigating the estradiol intracellular kinetics in target cells.

In vivo observations showed that in the very immature rats (5-day-old) the translocation in the nucleus of the cytoplasmic bound estradiol, seems impaired at the level of the nuclear membrane; while in older animals (30-day-old) a normal, predominantly nuclear localization of the estradiol was observed.

In vitro studies allowed the demonstration of the specific binding of the estradiol to the cytoplasm, nuclear chromatin, chromosomes and nucleolus, in various experimental conditions.

Some defects of the cytoplasmic uptake, translocation and nuclear binding of the estradiol, which might be relevant to the hormone-dependence, have been demonstrated in cells from human breast cancers.

INTRODUCTION

Immunocytochemistry has attained today the molecular level of resolution which makes it possible to detect and enumerate the single molecule sites [1, 2]. Furthermore, labeled antibodies appear to be able to trace its antigenic reactant, even when this is incorporated in macromolecular complexes [3]. Therefore, immunocytochemical methods can represent a useful tool for gaining insight into the physiopathology of steroid action on target tissues. This technique, indeed, allows a dynamic monitoring of steroids within the whole cell and would provide additional and more defined information on steroid intracellular kinetics.

We have recently set up an assay system, in which intracellular estradiol is detected by fluorescein-conjugated antibody [4–6]. Suitable control tests have confidently shown that, in our experimental conditions, the observed fluorescence comes from the estradiol bound to high-affinity, receptor-like binding sites, with little or no interference from other, non specific binders.

The present paper extends the observations resulting from such an immunocytochemical approach, undertaken in order to achieve a detailed survey of the normal features and changes of the estradiol intracellular kinetics in target cells (Fig. 1). These results come mainly from an *in vitro* system, with vital, isolated cells from human breast cancers [5, 6]. Also some data, obtained in a preliminary way from *in vivo* animal experiments, will be briefly quoted.

RESULTS AND DISCUSSION

Morphologic time-course of estradiol uptake, retention and release in target cells

As previously reported [5, 6], when exposed to estradiol at 4°C, responsive cells displayed a homogeneous, bright fluorescence throughout the cytoplasm, while only some faint positivity, or none at all, was recognizable with difficulty within the nucleus (Fig. 1a, 2a). This fluorescent pattern fits in well with the well-known cold immobilization of estradiol–receptor complexes in the cytoplasmic compartment [7–9].

When the temperature of incubation medium was raised up to 37°C, a nuclear fluorescence progressively increased (Fig. 1b, c). When first spotted, the fluorescence appeared homogeneous and bright throughout the nuclear area, while cytoplasmic positivity dropped off slightly. In the end (Fig. 1d, 2b), nuclear fluorescence was too bright and evenly spread to demonstrate a preferential antibody binding to any of the nuclear structures.

The direct relationship between the observed fluorescent patterns and the temperature is consistent with the known thermo-dependence of the entry rates of steroids into the nucleus [7–12].

While monitoring the events which follow the maximal estradiol overloading of the nucleus, some interesting observations were noted (Fig. 1e, f).

The nuclear fluorescence became gradually less homogeneous, but persisted a long time as numerous,

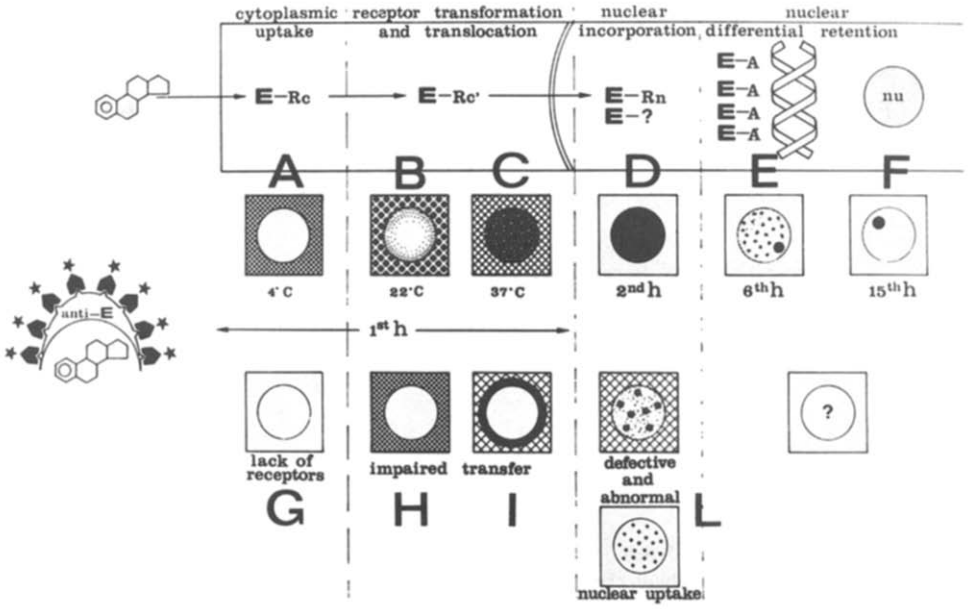

Fig. 1. General scheme of the fluorescent patterns of the intracellular estradiol kinetics in physiologic (A–F) and pathologic conditions (G–L). (See text.)

small, fluorescent spots linked to the chromatin network (Fig. 2c). Moreover, at the mitosis, a selective estradiol-dependent fluorescence of some 20 chromosomes per cell, was appreciable both *in vivo* and *in vitro* (Fig. 3a, b). This observation strongly supports the suggestion that the major acceptor sites lie chiefly in the nuclear chromatin [10–16]. The preferential estradiol binding to only some chromosomes might be a chance of restricting the area where the major nuclear binding sites have to be looked for.

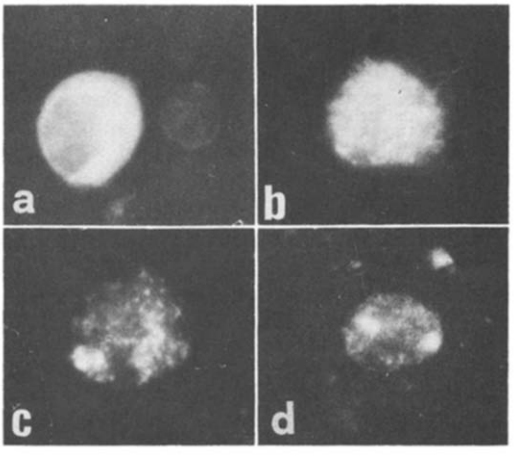

Fig. 2. Immunofluorescent monitoring of the nuclear estradiol binding and its differential retention. Accordingly to the general method [5], isolated cells from human breast cancer were incubated with estradiol 10^{-8} M for 1 h at 4°C, then thoroughly washed and post-incubated in simple tissue culture medium at 37°C. (a) at the 1st h, estradiol is traceable both in the cytoplasm and in the nucleus. On the right side, a "negative" lymphocyte. (b) at the 2nd h, only the nucleus displays a bright and spread fluorescence. (c) a definite fluorescent dotting of the nuclear chromatin was appreciable at the 5th h. (d) estradiol is predominantly localized in nucleoli at the 12th h.

As the nuclear fluorescence was decreased, a nucleolar (or nucleolar-associated chromatin) positivity emerged more and more distinctly (Fig. 2d), until the large round nucleolus present in the interphase nuclei, looked sharply outlined.

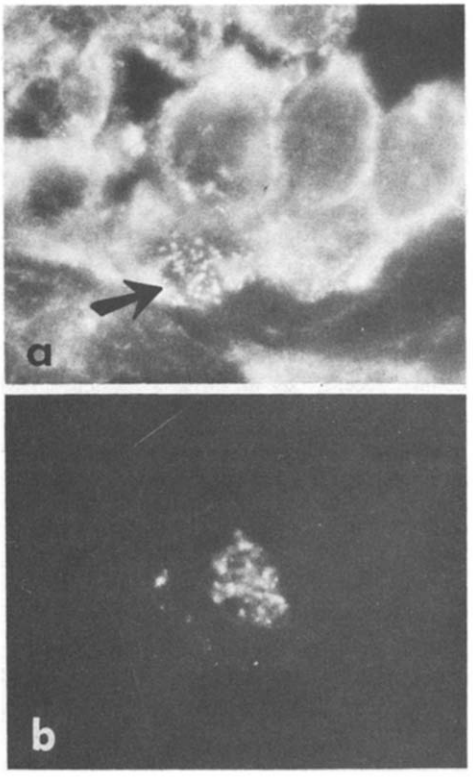

Fig. 3. Selective binding of anti-estradiol antibodies to some chromosomes of mitotic nuclei, *in vivo* (a) and *in vitro* (b).

These dynamic pictures, which indicate the presence of kinetically distinct nuclear binding sites, are consistent with the suggestion that different classes of steroid binders exist in the nucleus of target cells [10–16]. Moreover, the time-course of estradiol distribution, as detected by immunofluorescence in target cells, agrees with the temporal behaviour of the hormone–receptor complexes, investigated by biochemical approaches in responsive tissues [17, 18].

Kinetics of estradiol binding by target tissues during post-natal development

Some preliminary *in vivo* experiments have raised interesting, but still incomplete, observations.

In vivo experiments were carried out on two groups of 5- and 30-day-old female rats, which were killed 1 or 2 h after an intraperitoneal injection of estradiol, 100 ng/g body wt. Pictures of estradiol tracing by specific antibodies on cryostatic sections, showed important differences. The pictures obtained from 30-day-old animals, fully reflected the kinetic aspects of the two-step mechanism [7–9]. Indeed, a bright nuclear fluorescence indicated that a great deal of the estradiol taken up, had been relocated into the nucleus. This predominantly nuclear estradiol uptake was observed in every examined target tissue, such as endometrium, myometrium, granulosa cells, cervical and vaginal epithelium (Fig. 4).

On the contrary, in the 5-day-old rats, only a bright cytoplasmic fluorescence was appreciable, and this feature was typical of the same target tissues (Fig. 5). Moreover, the boundaries of nuclei were marked off by a sharp concentration of the antibody-traced estradiol in the perinuclear region (Fig. 6). Only some fluorescent dots (perhaps nucleoli) were identifiable within nuclei, as if the penetration across the nuclear membrane were difficult and/or delayed. This very apparent impaired redistribution of estradiol in target cells during the early post-natal period, seems to be in agreement with the suggested failure of very immature rats to respond to estradiol, in term of uterine wet-weight increase, general protein and RNA synthesis, thymidine incorporation and DNA synthesis [19–23]. It appears that, while investigating the rate-limiting factors which regulate the highly selective and concentrative process, whereby steroids are transferred into the nucleus [15, 16], one should consider also the permeability properties of the nuclear envelope [24, 25]. In this matter, we could put forward the suggestion that such a rate-limiting membrane mechanism is working during the ontogenic and early post-natal development, in order to give a protection against maternal hormones.

A detailed time-course of the fetal and postnatal development of the estradiol-binding by target cells, is currently under way in our laboratory.

Changes of estradiol intracellular kinetics in tumoural cells

Extensive studies have recently brought about a strong evidence of the relationship between the presence of estrogen cytoplasmic receptors in breast cancers and their responsiveness to the hormonal treatment [26–30]. The opportunity afforded by the immunocytochemical technique of overlooking the dynamics of estradiol-cell interactions, allows the investigation of many potentially regulatory steps of

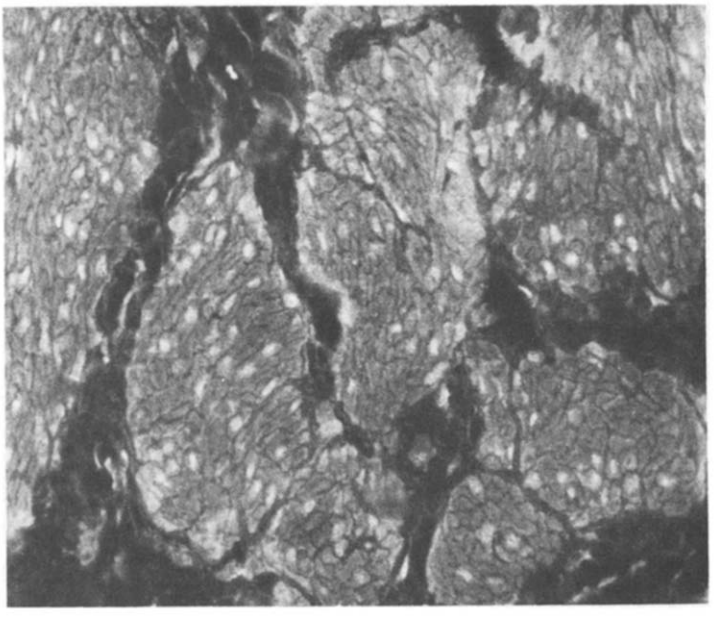

Fig. 4. Cryostatic section of myometrium from a 30-day-old female rat, 1 h after an intraperitoneal injection of estradiol (100 ng/g body weight). A very apparent nuclear estradiol binding is traced by fluorescent antibody.

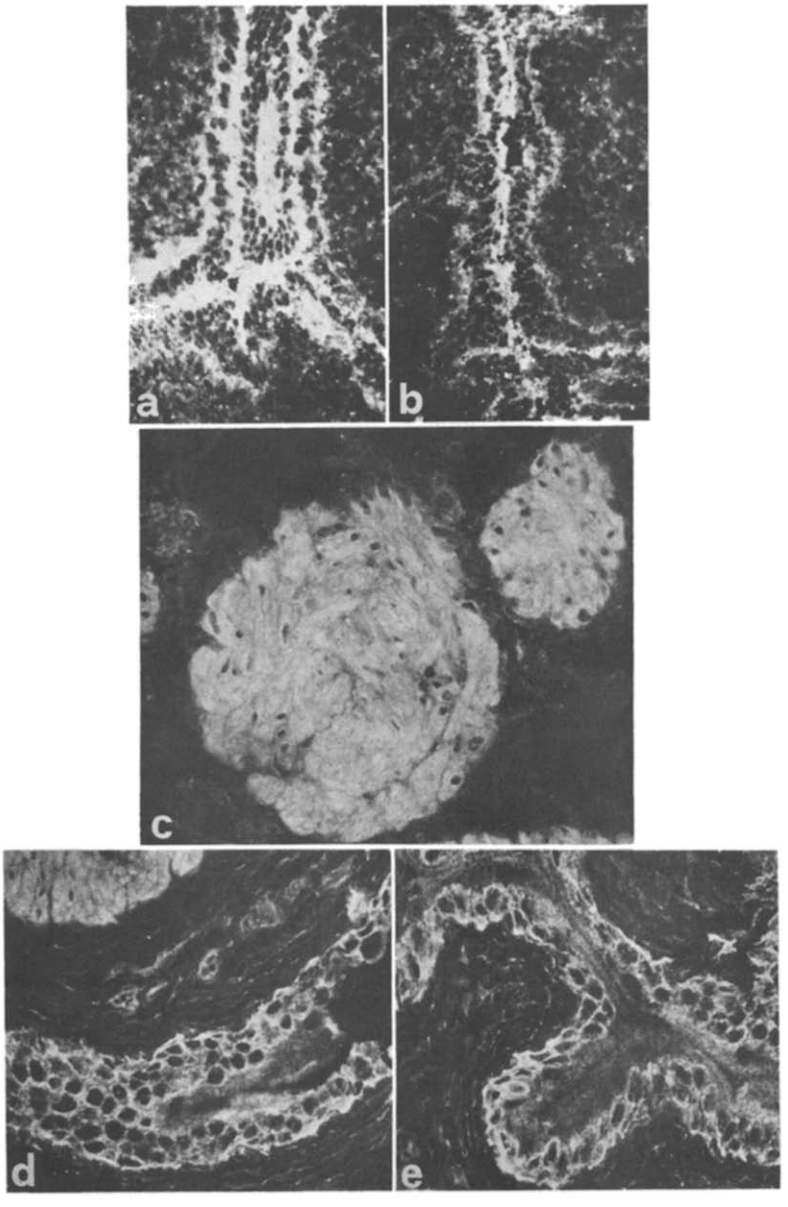

Fig. 5. Cryostatic sections from a 5-day-old female rat, treated as in Fig. 4. A bright cytoplasmic fluorescence is evident, while nuclei did not show any estradiol binding: in endometrium-1 h (a) and 2 h (b) after the injection of estradiol-, myometrium (c), cervical epithelium (d) and vaginal squamous epithelium (e).

steroid mechanism, which might be relevant to the tumoural estrogen-dependence [5, 6].

First, a markedly different cytoplasmic uptake may occur among cells from the same breast cancer (Fig. 7). In only a few of the 55 cases already studied, was an absolute lack of estradiol-binding found. More frequently, two different cell populations were observed in the same tumour, where cells devoid of (Fig. 1g) cytoplasmic binding capacity lay close to the positive cells (Fig. 9a). Negative cells looked well-characterized and often displayed more atypical and/or anaplastic cytological markers than the positive ones. Moreover, the presence of some functional properties, as the casein production—that we are also investigating by immunofluorescence—seems to go along with the preservation of estradiol binding.

Other changes, that can be traced by immuno fluorescence, are concerned with the impaired translocation to the nucleus of the estradiol specifically bound, as a rule, to the cytoplasmic level. In this respect, several situations have to be distinguished.

First, some tumours appeared to be made up by two distinct cell types, one of which exhibited the usual strong nuclear fluorescence after warm post-incubation; the other one, however, did not show any nuclear estradiol incorporation, in spite of the normal

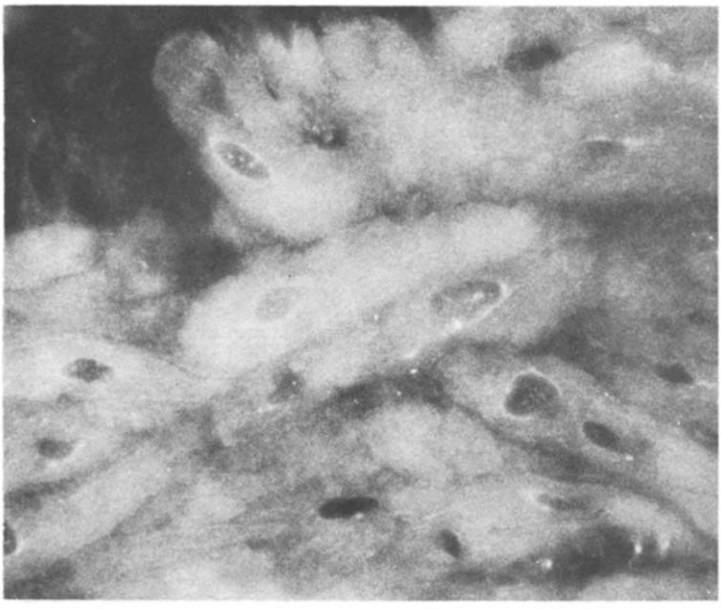

Fig. 6. Myometrium from a rat as in Fig. 5. Besides the diffuse cytoplasmic uptake, an additional concentration of estradiol, just outside the nuclear membrane, were demonstrable by fluorescent antibody; nuclei display only some fluorescent dots.

cytoplasmic uptake (Fig. 1h, 8). That is, estradiol seemed fixed in the cytoplasm, without showing any spread into the nucleus. This observation may be correlated to the blocking of the receptor mechanism found in some experimental tumours [31].

Moreover, besides this immobilization of the cytoplasmic estradiol–receptor complexes, it has been possible to observe cells which displayed a peculiar perinuclear crowding of anti-estradiol antibodies; which seemed concentrated on the cytoplasmic side of the nuclear membrane. This perinuclear concentration (Fig. 1c, 9b, c) looks like that observed in the very immature animals, even in a more evident way. Also in this case, the picture might suggest that the afferent passage into the nucleus is prevented by the nuclear envelope. In this respect, one should remember that tumoural cells and embryonic or fetal cells from the same source, often share some common features, as isoenzymes [32] and antigenic determinants [33]. The reappearing of this handicapped behaviour of the receptor mechanism during malignant transformation, might be allocated to these onco-fetal markers.

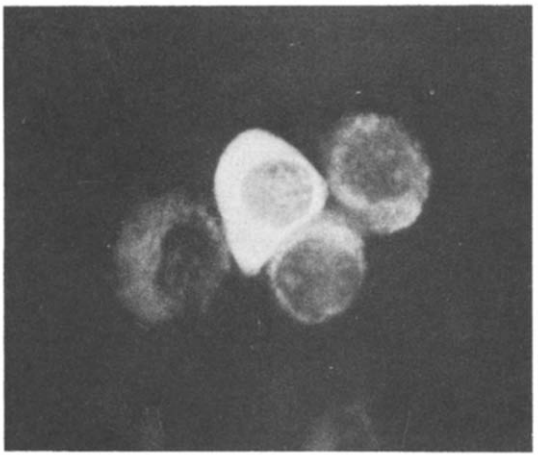

Fig. 7. Cells from human breast cancer, treated as in Fig. 2, at 4°C. A markedly variable cytoplasmic estradiol binding is displayed by cells from the same tumour.

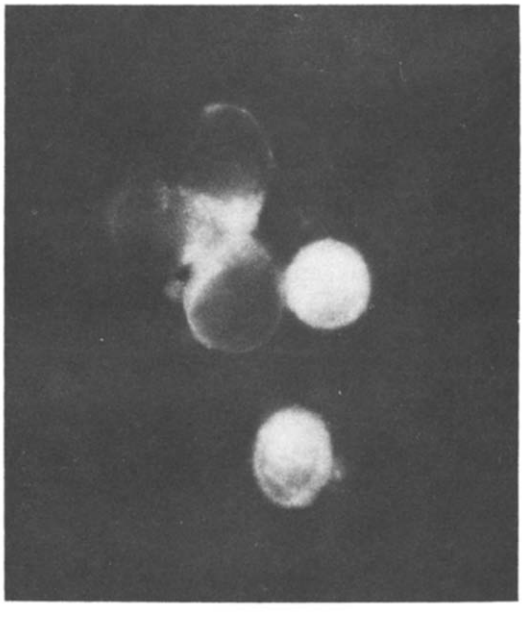

Fig. 8. Cells from human breast cancer, treated as in Fig. 2, at 37°C. An impaired estradiol translocation in the nucleus is displayed by some cells, while others exhibit a normal nuclear uptake.

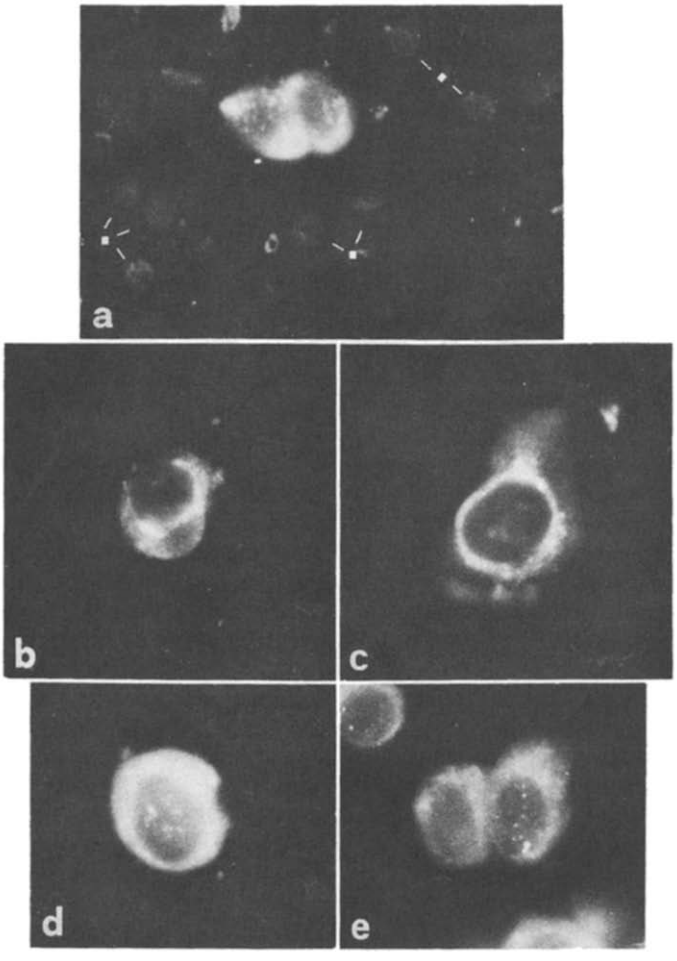

Fig. 9. (a) Double cell population from the same breast cancer (treated as in fig. 2, at 22°C). The small undifferentiated cells (--) did not show any estradiol binding, while binding looks very strong in differentiated, larger cells. (b, c) Some aspects of the perinuclear concentration of estradiol traced by fluorescent antibody in tumoural cells at 37°C. (d, e) Defective translocation in the nucleus of the estradiol bound to the cytoplasmic level, in tumoural cells at 37°C. Only few estradiol binding sites are localizable on the chromatin.

Some changes of the nuclear estradiol incorporation have also been observed (Fig. 1h). In some cells, the homogeneous, bright fluorescence of the total nuclear content was never reached. Instead, a scattered distribution of the antibody binding gives rise to an evident fluorescent dotting of the nuclear chromatin (Fig. 9d, e). Moreover, the cytoplasmic positivity faded out more slowly. We would interpret these pictures as an only partial redistribution of the complexes formed in the cytoplasm, the remaining part being blocked by some damage. These remarks seem to point to the possibility that (in the cytoplasm), multiple estradiol binders might also exist, from the beginning designed for different fates.

It is plain that, in all these cases of abnormal uptake and redistribution, an assay of the hormone-binding of the cytosol does not suffice as a measure of hormone responsiveness.

Drug-induced changes of estradiol intracellular kinetics

Nafoxidine, Tamoxifen and Diethylstylbestrol, in 1000-fold excess concentration, were able—as expected [34–37]—to prevent the cytoplasmic estradiol binding and, consequently, all the fluorescent specific patterns too.

Adriamycin, an almost new antitumoural drug, [38–39] which is incorporated in nuclear structures and fluoresces orange-red in U.V. light, displayed a different effect. The cell pretreatment by Adriamycin (0,5 μg/ml) before the exposition to estradiol, appears to inhibit—in our system—the nuclear incorporation of estradiol, which never accumulates into the nucleus. Adriamycin is known to interact with DNA through intercalation between two pairs of bases [40]. Intercalating drugs, such as ethidium bromide, have already been shown to prevent nuclear binding of estradiol-receptor complexes [41]. We would suggest

that, intercalating drugs and especially Adriamycin which binds to preferential sites of DNA [40], might be usefully employed in studying the nuclear events involved in steroid action.

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DISCUSSIONS

O'Malley. These are very striking demonstrations of what's reported to be the sequence of events in steroid hormone action. I wonder if your pictures should not be accompanied by quantitation. In other words, I would think you could easily perform statistical counting to accompany the pictures.

Piffanelli. Yes, it's possible. We have already done a piece of work with cyto-microfluorophotometry, but we have not yet an exact quantitation like you are suggesting.

O'Malley. Could you monitor translocation of the fluorescent material from the cytoplasm to nucleus?

Piffanelli. Not one cell, not in the same cell, because we perform time-course studies on different samples of the same cellular suspension under different time and temperature conditions.

O'Malley. What is the time course?

Piffanelli. We have looked at the vital cells from tumours until 18–24 h, step by step, hour by hour.

King. Could you give us some information about the method of antibody production and its specificity? The reason I ask this question is that I do not understand how your antibody recognizes the estrogen that is presumably within its receptor protein.

Piffanelli. We have employed a very specific antibody raised to estradiol-17 β-6-carboxymethyloxime-BSA, which sensitivity is greater than 10^{-9} M. As we already suggested [6], the immunohistochemical cell processing, though suitable in preserving antigens intact and localized, can induce some conformational changes in the receptor protein which make steroid molecules accessible even to specific antibodies.

Jungblut. We tried some six years ago to produce a receptor–steroid–antibody sandwich and never succeeded, because, as Roger King just said, the attachment of the steroid to the receptor is of the clathrate type. Therefore, your excellent technique can work only by a very artful artefact. First, the steroid has to be dissociated from the receptor by denaturation of the binder and only then can it be picked up by the antibody. Since you arrive at essentially the same distribution as Walter Stumpf does, the diffusion of the liberated steroid is obviously kept at a minimum.

Piffanelli. We know very well the Liao approach to the receptor quantitation by specific steroid antibodies. Instead, we are working under the peculiar experimental conditions of immunofluorescence. We have carried out

several control tests of binding and immune reactions (see Table 1.) and we are very confident of demonstrating estradiol bound to its cell receptor.

Jungblut. I do not doubt your results, but I am sure that they require the release of the steroid by receptor denaturation. If one saturates all receptor molecules present in an extract and then adds steroid-binding antibodies, no triple complex receptor–steroid–antibody is formed. There is also no change-over of steroid from the receptor to the antibody or *vice versa*, since the K_A's are in the same order of magnitude and the rate of dissociation at 0°C is virtually zero.

Stumpf. There are a few differences between your data and the results of our autoradiographic approach. For instance, you observed a prevailing cytoplasmic localization of fluorescent antibodies in many of your samples. This we have not seen in our *in vivo* experiments.

Piffanelli. How many hours after the injection have you measured the presence of estradiol in nuclei?

Stumpf. Our studies are based mainly on a 1 h time interval after the injection, but include 1 min, 30 min, 3 h and 7 1/2 h.

Piffanelli. We could recognize in our system that estradiol is present in nucleoli only after many hours.

Stumpf. This is very surprising since the half life of estradiol in nuclei is relatively short.

Martini. Dr Jungblut, I want to make a comment on your comment. What about, if the antibody has a better affinity for estrogens than the receptor complex? Is this a possibility?

Jungblut. That is possible, but it won't be in order of magnitude.

Spelsberg. We have detected steroids in the cells and in the nuclei of even non-target cells over quite a long period of time. The question is, are your observations concerned with non-target cells? Are you dealing with free steroids that associate with membranes which are being adsorbed to a lipid region of the cell? Have you studied non-target cells with labelled steroids and observed any type of phenomenon related to what you described to the target tissue?

Piffanelli. We have already done a piece of work with non-target cells but we did not see any specific estradiol localization [5, 6].

Jungblut. I'd like to say again that I do not doubt Dr. Nenci's results, but I disagree with his interpretation of the method. In my opinion, the drying of the cell suspension smears in the cold denatures the receptor and liberates the steroid without translocating it on the microscopic scale. It can then be picked up by the steroid-binding antibody, which is precipitated, virtually *in-situ*, by the anti IgG.

Table 1.

Binding specificity controls	
Non-target cells (mouse liver, human spleen and lymphnode, lung and gastric tumours)	No fluorescence
Binding competition (Nafoxidine, Tamoxifen, Diethylstilbestrol in 10^3-fold excess)	No fluorescence
Binding prevention (N-ethylmaleimide in 10^4-fold excess)	No fluorescence
Immune specificity controls	
Pre-immune serum	No fluorescence
Anti-E_2 antiserum previously absorbed with E_2 in excess	No fluorescence
Unlabelled anti-Ig antiserum before the labelled one	No fluorescence