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BINDING OF MONOCLONAL ANTIBODIES TO THE NUCLEAR ESTROGEN RECEPTOR IN INTACT NUCLEI

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SUMMARY. A monoclonal antibody to calf uterus cytoplasmic estrogen receptor shows a specifically displaceable and saturable binding to intact nuclei of mouse uterus after estradiol stimulation. The binding is complete after 3 hr at 0°C. The binding of the antibody correlates with the exchangeable estradiol binding activity of the nuclei over a 4-hr time course following *in vivo* injection of 17β-estradiol.

Since its discovery, the estrogen receptor has been studied using the hormone specific binding activity for its identification (1,2). Although the radiolabeled ligand allows a highly specific and functional identification of the receptor protein (3), the binding activity is labile in certain experimental conditions (heat, detergents, etc.). Furthermore, in some instances the steroid binding site of the receptor is occupied by non-radiolabeled hormone of endogenous or pharmacological origin. In order to circumvent this problem, many assays have been developed that use high temperature (4) or chaotropic salts (5) to cause the exchange of the receptor-bound endogenous hormone with the radiolabeled one. In recent years monoclonal antibodies to the estrogen receptor have become available (6-8); their interaction with the receptor is independent of the occupation of the estradiol binding site. The binding of monoclonal antibodies to the nuclear receptor in intact nuclei is described and possible applications of this finding are discussed.

MATERIALS AND METHODS

Materials. All reagents were of analytical grade. Tris, EDTA disodium salt, dithiothreitol, lactoperoxidase, tyramine·HCl and bovine serum albumin were from Sigma; bovine γ-globulin was from Miles. Na[^{125}I] was purchased

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from Atomic Energy of Canada and [2,4,6,7-^{3}H]-17β-estradiol (91.5 Ci/mmole) was from New England Nuclear.

Animals. Ovariectomized CD-1 mice (weight range 25-30 g) and Sprague-Dawley rats (150-180 g) were from Charles River. Estradiol was administered by i.p. injection and animals were sacrificed at the specified times by cervical dislocation. Uteri were removed and placed in ice cold buffer.

Tissue Homogenization and Fractionation. Uteri were homogenized with a Polytron in 10 volumes of ice-cold TEDS buffer (20 mM Tris·HCl, pH 7.4; 1 mM EDTA; 1 mM dithiothreitol, 10% [w/v] sucrose). The homogenate was filtered through a nylon mesh (200 μm) and centrifuged at 800 x g for 20 min. The nuclear pellet was washed twice with the same buffer and resuspended in TEDS at 0.2-0.5 mg DNA/ml.

Monoclonal Antibodies Preparation. Production and characterization of the monoclonal antibodies to estrogen receptor from calf uterine cytosol have been described elsewhere (8). An aliquot of purified antibody from the clone JS34/32 was radiolabeled by the lactoperoxidase method (9). The reaction mixture (total volume 150 μl) contained 30 μg of antibody, 2 mCi Na[^{125}I], 50 μg of lactoperoxidase and 0.002% H_2O_2 in 0.3 M sodium acetate, pH 5.6. The reaction was started by addition of the hydrogen peroxide and stopped after 2 min at room temperature by addition of 100 μl of tyramine·HCl (2 mg/ml), followed after 1 min by 100 μl of NaI (2 mg/ml). The radiolabeled antibody was purified on a Sephadex G-100 column (5 ml) equilibrated in phosphate buffer (50 mM), pH 7.5, containing bovine serum albumin (4 mg/ml) and the fractions in the void volume were pooled. The specific activity of the radiolabeled antibody was in range of 10-50 μCi/μg.

Estradiol Binding Assay. Aliquots of nuclear suspension were incubated in duplicate with 10 nM 17β-[^{3}H]estradiol in absence or presence of a 500-fold excess of unlabeled hormone in a final volume of 0.5 ml for 30 min at 37°C. The incubation was terminated by addition of 2 ml ice-cold TEDS buffer to each tube, followed by centrifugation at 800 x g for 10 min. The pellet was washed once with the same buffer and extracted for 30 min at 30°C with 1 ml of 100% ethanol. The total ethanolic extract was then counted for radioactivity with 10 ml of Aquasol II. Efficiency of counting was determined by the external standard method.

Antibody Binding Assay. Aliquots of the nuclear suspension were incubated in duplicate for 3 hr (unless otherwise specified) with various dilutions of radio-iodinated antibody in presence or absence of 5 μg/assay of unlabeled JS34/32 antibody in a final volume of 200 μl. Antibodies (either cold or labeled) were diluted in TEDS buffer containing 1 mg/ml of bovine γ-globulin. At the end of the incubation, 4 ml of ice-cold TEDS buffer containing 1 mg/ml bovine serum albumin was added to each tube, followed by centrifugation at 800 x g for 20 min. The nuclear pellet was washed once and counted for radioactivity in a gamma counter.

DNA Assay. Concentration of DNA in the nuclear suspension was determined by the method of Burton (10).

RESULTS

After estradiol injection, the estrogen receptor in the target tissues translocates to the nucleus (11). Incubation of nuclei, prepared from uteri of mice injected with estradiol, with various concentrations of radiolabeled monoclonal antibody to estrogen receptor shows a saturable binding of this antibody (Fig. 1). This binding is displaceable by an excess of unlabeled antibody produced by the same clone. The specific binding is expressed as difference between the total binding and the non-displaceable binding. As can

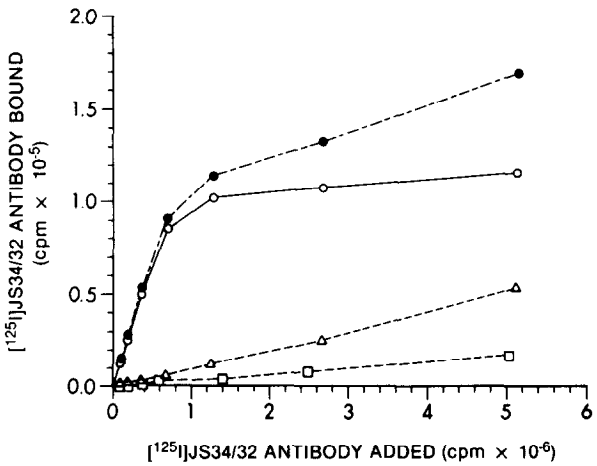

Fig. 1 - Binding of [^{125}I]labeled monoclonal antibody against estrogen receptor to uterine nuclei from ovariectomized mice treated for 1 hr with 17β-estradiol (1 μg/mouse). The experimental procedure is described in the Methods section. Aliquots of 50 μl of nuclear suspension (14 μg of DNA) were incubated 3 hr at 0°C. ○, specific binding; ●, total binding; △, non-displaceable binding; □, background (nuclei omitted).

be seen in the figure, the background (incubation in absence of nuclei) accounts for about 40% of the non-displaceable binding and it is probably due to the binding of antibodies to the glass since more extensive washing does not reduce significantly the blank value. Among the various types of test tubes used for this purpose (polyethylene, polycarbonate, etc), the glass test tubes gave the lowest background levels. The specific estradiol binding level in the intact nuclei used in the above experiment, assayed by exchange method, was 2.6 pmole/mg DNA. Figure 2 shows the time course of the antibody-nuclei

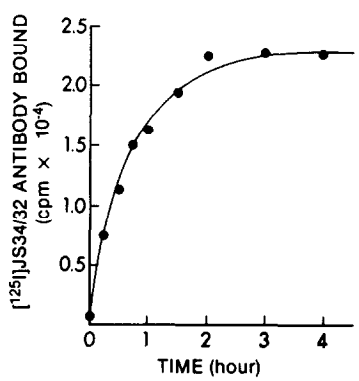

Fig. 2 - Time course of the binding of [^{125}I]labeled monoclonal antibodies to estrogen receptor to uterine nuclei from ovariectomized mice treated for 1 hr with 17β-estradiol (1 μg/mouse).

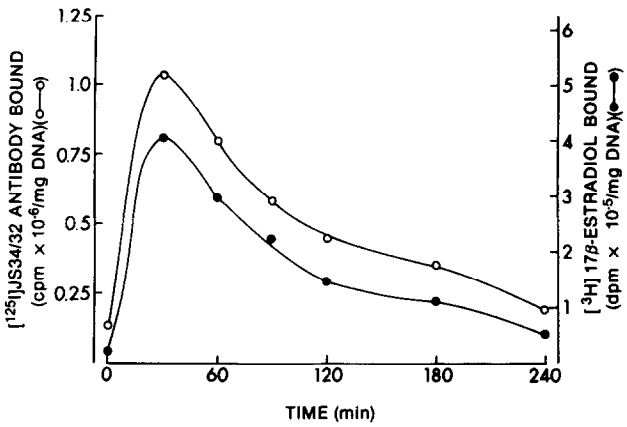

Fig. 3 - Comparative profile of the binding activity of [^{125}I]labeled monoclonal antibodies to estrogen receptor and 17β-[^{3}H]estradiol to nuclei from mouse uteri. The abscissa shows the time periods after which the animals were sacrificed following a single injection of 17β-estradiol (1 μg/mouse).

interaction at 0°C. The interaction reaches a steady-state level at 3 hr. This time was chosen for the other experiments. In order to verify that the binding of the antibody was indeed to the nuclear estrogen receptor, nuclei were prepared from animals treated with 17β-estradiol for various times and binding of the antibody compared with the estradiol binding of the same nuclei. The time course of both binding activities over a 4-hr period is shown in Fig. 3. The estradiol binding activity was measured by an exchange assay at 37°C (4). Figure 3 shows that the binding of both antibody and estradiol to the intact nuclei follows the same pattern. Furthermore, it appears from this figure that the low level of the receptor present in the nuclei in absence of estradiol-induced translocation (0 time) is also experimentally quantifiable.

The monoclonal antibody binds to intact nuclei from target tissues from different species, however not to the nuclei fron non-target tissues in which specific exchangeable estrogen binding is absent. Table I shows a comparison of the steroid as well as antibody binding to intact nuclei from different tissues from ovariectomized rats sacrificed 60 min after an injection of 17β-estradiol.

DISCUSSION

The monoclonal antibody JS34/32 was produced by a hybridoma cell line derived by the fusion between SP2/0-Ag14 mouse myeloma cell line and spleen

Table 1. Specific binding of 17β-[^{3}H]estradiol and [^{125}I]JS34/32 antibody to nuclei from target and non-target rat tissues

	17β-[^{3}H]estradiol bound (DPM/mg DNA)	[^{125}I]JS34/32 bound (CPM/mg DNA)
Uterus	24,450	193,900
Quadriceps	4,940	64,200
Lung	n.d.	950
Ileum	n.d.	n.d.

Tissues were removed from an ovariectomized rat 60 min after a single i.p. injection of 17β-estradiol (10 μg/kg). Nuclei were prepared as described in Methods. n.d. = non-detectable.

cells from a mouse immunized with estrogen receptor purified from calf uterus (8). This antibody recognizes all mammalian estrogen receptor tested (i.e., calf, mouse, rat, human). Furthermore, it has been shown that this antibody does not interfere with the nuclear translocation of the estrogen receptor in vitro (8) In light of the above findings and of the high affinity of this antibody for the receptor protein, it was logical to investigate its interaction not only with the soluble receptor, but also when the latter is linked to the structure(s) that mediate its function(s). The assay described here may be useful for quantitation of estrogen receptors. It appears to offer a certain advantage over conventional methods in that it works at low temperature and does not require the use of chaotropic salts. Although, the described assay permits a relative analysis of the estrogen receptor levels, an absolute quantitation may require a precise determination of the specific activity of the antibody and an understanding of the type of immune complexes formed. A quantitative evaluation of the nuclear receptor by a method alternative to a direct measurement of the steroid binding site, however, may be useful for situations in which the latter is occupied by a pharmacologically administered hormone (as shown in the experiment of Fig. 3) or by endogenous hormone.

The findings presented here also suggest that this monoclonal antibody may be useful to morphologically or biochemically investigate (via the physiologically translocated receptor) the "nuclear acceptor" to which the receptor is linked for the expression of its function(s). The recognition of the receptor by the antibodies in nuclei from tissues other than uterus and the ability to detect receptor levels lower than those detectable with estradiol

binding assay may certainly be useful in achieving further insight in the action of estradiol in "non-conventional" target tissues. It may also be of great value in those cases where low levels of receptors are masked by larger amounts of other estrogen binding proteins (e.g., in liver). Finally, the parallel profiles shown in Fig. 3 indicate that there is a definite correlation between the receptor content of the nucleus accessible to the antibody and the exchangeable estrogen binding activity. The existence of a relatively large amount of estrogen receptor in the nucleus without steroid binding activity therefore appears to be less likely.

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