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An Immunological Comparison Between Mouse α-Fetoprotein and the Uterine Estrogen Receptor (40104)

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Recent observations on the binding of estrogens to α-fetoprotein (AFP) have created considerable interest in the possible functional significance of this interaction. Both rat and mouse AFP bind estrone and estradiol-17β with a high affinity and specificity, suggesting that the interaction may be biologically significant (1–6). One suggestion is that AFP may protect certain tissues from exposure to high estrogen levels during critical stages of their development (7–10).

Another possibility is that AFP may in some way be involved in the action of estrogens in target tissues. A recent report by Uriel *et al.* (11) supports this possibility. These investigators demonstrated an immunological similarity between rat AFP and the uterine estrogen receptor using an affinity resin containing anti-AFP antibody. While the low salt, 8S estrogen receptor did not interact with AFP antibody, the salt-dissociated 4S receptor form was adsorbed to the affinity resin. From these results, it was suggested that AFP is the uterine estrogen binding protein and that AFP associates with another cellular component to form an 8S complex which is resistant to anti-AFP antibody. This is an exciting proposal that could have extensive implications as to the evolutionary origin of these proteins and their functional diversity throughout development of the organism.

We have attempted to confirm these observations in the mouse using similar techniques. A partial adsorption of uterine estrogen receptor to an anti-AFP affinity resin was observed; however, a number of controlled experiments indicate that this binding was not to anti-AFP antibody but appeared to be a nonspecific interaction with the resin.

Materials and methods. [2,4,6,7-^{3}H]Estradiol-17β (91.3 Ci/mmol) was obtained from New England Nuclear. Unlabeled estradiol, ovalbumin, and activated charcoal were from Sigma; Sepharose-4B and dextran T-70 from Pharmacia, Uppsala, Sweden; and Biogel P-20, from Bio-Rad Labs.

Amniotic fluid was obtained from HA/ICR Mice by puncturing the amniotic sacs of animals on the 15–18th day of pregnancy. The fluid had a protein concentration of about 3 mg/ml and was stored at −20°.

Female HA/ICR mice (20–30 days old) were used as a source of estrogen receptor. The uteri were removed immediately after cervical dislocation, trimmed of fat and placed in saline at 4°. They were then transfered to TE buffer (10 m*M* Tris–HCl, 2 m*M* Na_2EDTA, 2 m*M* mercaptoethanol, pH 7.4) (5 uteri/ml) and homogenized using a Polytron PT10 homogenizer (Brinkman). The cytosol fraction was obtained by centrifugation for 1 hr at 150,000*g*. Solid KCl was then added to the cytosol to a concentration of 0.4 *M*.

Antisera to AFP and transferrin were prepared from mouse amniotic fluid (MAF) as follows. MAF was chromatographed on Biogel P-20 to remove low molecular weight components. The void volume was pooled and subjected to polyacrylamide electrophoresis at pH 9.5 in 5% gels (12). The gels were then sliced with a Gilson gel crusher and eluted with phosphate buffered saline, pH 7.2 using 1 mm slices. The locations of the AFP and transferrin in the eluted fractions were tested using specific antisera as well as antisera against anti-whole mouse serum. Fractions showing only AFP or transferrin by Ouchterlony were used to prepare antisera in rabbits. Mouse α-1 antitrypsin (α1AT) was prepared as previously described for the human (13). Anti-AFP showed a single line by

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Ouchterlony gel diffusion and immunoelectrophoresis with MAF and no reactions with normal adult mouse serum. Rabbit anti mouse α1AT and anti transferrin were monospecific as tested by gel diffusion and immunoelectrophoresis. The α-globulins of these antisera were precipitated with ammonium sulfate and linked to Sepharose-4B by cyanogen bromide (14).

Uterine cytosol was mixed with the immunoadsorbant resins in the proportion 0.8 ml cytosol to 0.2 ml packed resin. The mixtures were incubated for 3 hr at 5° with gentle shaking. Similar incubations were performed using mouse amniotic fluid in place of uterine cytosol. The amniotic fluid was first diluted 200-fold in TE buffer plus 0.4 *M* KCl and ovalbumin, 1 mg/ml. One milliliter portions were mixed with 0.2 ml packed resin and incubated for 3 hr at 5° with gentle shaking.

After incubation, the samples were centrifuged (2 min, 800*g*) to remove resin and the supernatant fluids were tested for [^{3}H]-estradiol binding. This was accomplished by mixing duplicate aliquots (0.3 ml of cytosol samples or 0.05 ml of amniotic fluid samples) with TE buffer + 0.4 *M* KCl to a final volume of 0.5 ml. [^{3}H]Estradiol (in TE buffer) was also added to a final concentration of 1×10^{-9} *M*. Additional assay tubes were included which contained a 400-fold excess of unlabeled estradiol for background determinations. The assay tubes were incubated in ice bath for 16 hr. The bound and unbound hormone were then separated by charcoal treatment (15, 16). Five-tenth milliliter of charcoal suspension (0.25% charcoal, 0.025% dextran in TE buffer) was added to each assay tube and mixed rapidly. The samples containing amniotic fluid were centrifuged immediately (1000*g*, 5 min) to minimize dissociation of the estradiol-AFP complex (dissociation was estimated to be less than 20%). The samples containing uterine fluid were incubated with charcoal for 10 min at 4°. The estradiol receptor complex does not dissociate significantly during this treatment (15). After centrifugation, the supernatants containing the bound hormone were decanted into scintillation counting vials, and 5 ml of scintillation fluid (1000 ml toluene plus 42 ml Scintiprep #1, Fisher) were added to each vial. After waiting 3 hr to extract the [^{3}H]estradiol into the toluene phase, the radioactivity was measured with 48% efficiency.

Results. In the studies of Uriel *et al.*, estradiol binding was determined by sedimenting the complex on sucrose gradients (11). We used this technique in our initial experiments with only limited success. There was some loss of estradiol binding when samples were treated with the anti-AFP antibody resin. However, the results were variable and the loss of binding was not complete. In addition, the sucrose gradient analysis is complicated by the fact that, under the ionic conditions of these experiments (0.4 *M* KCl), both the receptor and AFP sediment in the 4S region. Therefore, we decided to use the charcoal adsorption assay which we felt was more quantitative and would readily distinguish receptor binding from binding which was either non-specific or to components with lesser affinity.

Although it is possible to measure accurately estrogen binding to mouse AFP using the charcoal techniques, the charcoal incubation must be brief and precisely timed because of the rapid rate of estradiol dissociation from AFP. This property was used to easily distinguish hormone binding to AFP and to the estrogen receptor. The estradiol-AFP complex dissociates rapidly and is 90% dissociated after 10 min of charcoal incubation (Fig. 1). The half-time for hormone dissociation is between 2 and 3 min. This is somewhat faster than the dissociation rate of the estradiol-AFP complex in the rat (16). That the observed binding was actually to AFP was verified in separate experiments which showed the affinity and specificity of binding to be very consistent with previous studies (1–6). In addition, the binding was selectively removed by antibody to mouse AFP (see below).

Contrary to the results with AFP, the binding in uterine cytosol is almost completely stable during a 10-min period of charcoal

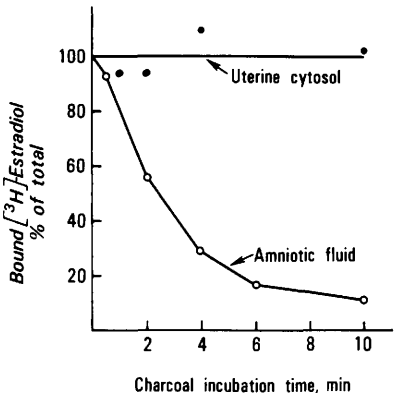

FIG. 1. The effect of charcoal treatment on the binding of [^{3}H]estradiol to receptors in uterine cytosol or to AFP from amniotic fluid. A series of assay tubes (in duplicate) were prepared containing either 0.3 ml cytosol or 0.05 ml amniotic fluid (diluted 200-fold) in a final volume of 0.5 ml. The samples were incubated with 1 × 10^{-9} *M* [^{3}H]estradiol for 16 hr and then treated with charcoal (see Methods and Materials) for various time periods. The bound hormone in the supernatant was measured after centrifugation. The value for 100% binding in the AFP assay was estimated by graphing CPM bound on a log scale versus time and extrapolating to zero time. These values were 2600 cpm for AFP and 25,000 cpm for the uterine cytosol.

treatment (Fig. 1). This agrees with other reports which have shown the dissociation of estradiol-receptor complex to be extremely slow at 0–4° (15, 18). Therefore, the 10-min charcoal treatment would easily distinguish between binding to uterine receptor or to AFP.

Figure 2A illustrates the binding of [^{3}H]-estradiol in uterine cytosol after the cytosol had been treated with Sepharose containing anti-AFP antibody (see legend and Methods section for details). While there was some loss in binding after the treatment, this appeared to be nonspecific since comparable losses occurred after treatment with anti-α1AT antibody, ovalbumin linked to Sepharose, or with Sepharose alone. A much greater loss occurred after treatment with Sepharose containing anti-transferrin antibody. Since this column had been in use for 6 months or more, we subsequently prepared a second antitransferrin antibody resin and this did not show the same results (Fig. 2B) even though the immunoabsorbant was prepared from the same bleeding in both cases. This suggested that the first results may have also been nonspecific.

To verify the effectiveness of the anti-AFP antibody resin, the binding of [^{3}H]-estradiol to AFP from mouse amniotic fluid was tested before and after resin treatment (Fig. 3). The hormone binding was completely removed by the anti-AFP resin, but no effect was observed after treatment with the four control resins. Therefore, the experimental conditions were quite adequate for achieving the selective removal of AFP from solution. Both antitransferrin and the anti-α1AT completely removed their respective antigens from MAF as tested immunologically.

Several experiments of the type illustrated in Fig. 2 were performed and while variable losses of hormone binding occurred in these experiments, in no case was there a selective removal of receptor binding by the anti-AFP antibody resin.

Discussion. The present results do not support the idea of antigenic similarity between

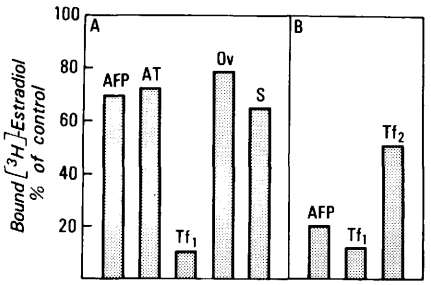

FIG. 2. The binding of [^{3}H]estradiol in uterine cytosol after treatment with Sepharose resins. Samples were incubated with the resins indicated for 3 hr at 5°. The resins were removed by centrifugation and the binding of [^{3}H]estradiol was measured in the remaining supernatant fractions. Values are expressed as percent of control. The control samples had no resin treatment. Experiment A: Samples of uterine cytosol were incubated with the following resins: anti-AFP antibody–Sepharose, AFP; anti-α1AT antibody–Sepharose, AT; antitransferin antibody–Sepharose, Tf_1, (first preparation); ovalbumin–Sepharose, Ov; untreated Sepharose, S. The control binding was 36,500 cpm and the background (plus 500-fold excess unlabeled estradiol) was 2000 cpm. Experiment B: This experiment was performed exactly as experiment A and represents a comparison of two preparations of antitransferin antibody–Sepharose, Tf_1, and Tf_2.

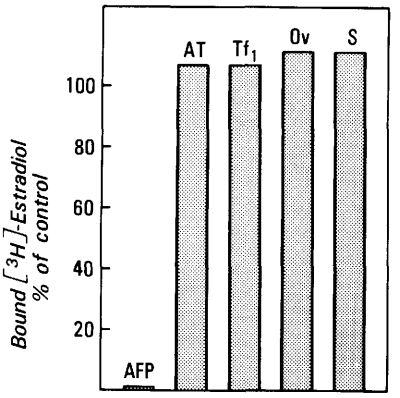

FIG. 3. The binding of [^{3}H]estradiol in amniotic fluid after treatment with Sepharose resins. Samples of amniotic fluid were treated with the indicated resins and [^{3}H]estradiol binding was measured in the remaining supernatant fractions. The control value (without resin treatment) was 27,000 cpm and the background was 1000 cpm.

the estrogen receptor and AFP. In several experiments, we found that the estrogen receptor was adsorbed, in part, to resins of immobilized AFP antibody. However, these results were variable and the receptor adsorption was never complete. In addition, this adsorption appeared to be rather nonspecific since a similar adsorption of receptor was observed using several other control resins which did not contain AFP antibody. These observations are not surprising since several investigators have noted a tendency of estrogen receptor to aggregate and to adsorb to various surfaces (19–21).

The binding analysis used in the present study is different than that of Uriel *et al.* who used sucrose gradient centrifugation (11). However, in our initial studies using sucrose gradient centrifugation (data not shown), the results were similar to those illustrated here with the charcoal adsorption assay.

A recent report by Radanyi *et al.* (17) also indicated that rat AFP and the uterine estrogen receptor were separate proteins. Their conclusions were based on a comparison of the binding specificities and dissociation rates of these components. Another preliminary report by LaBarbera and Linkie (22) claimed a lack of interaction between the rat uterine estrogen receptor and antibody to rat AFP in solution.

It is possible that antigenic similarities occur between AFP and estrogen receptor of the rat, but not the mouse. However, this seems very unlikely since the two species are closely related. Another possibility is that a small region of similarity exists between AFP and estrogen receptor. For example, some similarities may well exist at the steroid binding sites of the two molecules. If the common region represented an antigenic determinant in the system of Uriel *et al.* but not in our system, then our discrepant results could be explained. We have found that our antiserum does not mask the estrogen binding site of mouse AFP since the ability to bind steroid is not diminished after the interaction of AFP with antibody.

While the above possibilities exist, we feel that the simplest and most likely interpretation is that there really is no antigenic similarity between AFP and estrogen receptor. These proteins have very different physiochemical properties even though they both bind estrogen. Both the affinity and specificity of estrogen binding by AFP differ from receptor binding (11, 17, 23). The rate of dissociation of estradiol by AFP is very rapid in comparison to receptor binding. Also we have observed that unlike the estrogen receptor, AFP is a stable protein. Its binding activity is insensitive to sulfhydril agents such as *N*-ethylmaleimide and it can withstand incubations at elevated temperatures (e.g., 10 min, 60°). Finally, estrogen receptors have very similar properties among all mammalian species that have been studied (24), whereas estrogen binding by AFP shows species differences and bovine and human AFP have little or no affinity for estrogens (7; N. Calvanico, D. Toft, and T. Tomasi, unpublished).

Summary. This study was an attempt to identify antigenic similarity between two estrogen binding proteins in the mouse; the uterine estrogen receptor and α-fetoprotein. The adsorption of these proteins onto an affinity resin containing immobilized anti-

body to α-fetoprotein was tested. Alpha-fetoprotein could be selectively bound to this resin. On the other hand, estrogen receptor was only partially bound to the resin and this appeared to represent a nonspecific adsorption to the resin when tested against several control resins. Therefore, no antigenic similarity between these two proteins was observed.

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