Temporal Effects of Estradiol and Diethylstilbestrol on Pituitary and Plasma Prolactin Levels in Ovariectomized Fischer 344 and Holtzman Rats: A Comparison of Radioimmunoassay and Nb_2 Lymphoma Cell Bioassay (43462)

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Abstract. **An Nb_2 lymphoma cell bioassay (Nb_2BA) and a radioimmunoassay (RIA) were used to compare plasma and pituitary levels of prolactin in ovariectomized Fischer 344 (F344) and Holtzman rats treated with either diethylstilbestrol (DES) or estradiol for up to 8 weeks. The objectives were to determine whether there were temporal differences in prolactin responses in strains with different genetic predispositions to estrogen-induced pituitary tumor formation and to determine whether the results of the two assay methods were equivalent. All rats were ovariectomized for 7 days and all except controls received subcutaneous Silastic implants of DES or 17β-estradiol and were sacrificed at intervals from 2 days to 8 weeks later. Pituitary content and plasma levels of prolactin were determined by Nb_2BA and RIA and the ratio of these measurements was calculated. DES induced a significant increase in pituitary prolactin in F344 rats by 2 days of treatment, as measured by RIA. Pituitary content increased to a peak by Day 4, after which a gradual decline occurred until the end of the experiment. Nb_2BA measurements were similar to those obtained by RIA, except at 8 weeks, when the content determined by Nb_2BA was significantly higher than the content determined by RIA. When estradiol was given to F344 rats a pattern of increase and subsequent decrease in pituitary content similar to that seen with DES was observed and levels measured by Nb_2BA and RIA were essentially equivalent. Plasma levels of prolactin in DES-treated F344 rats increased exponentially through the 8 weeks, and the Nb_2BA measurements were significantly greater than levels determined by RIA throughout the treatment period. Estradiol treatment produced a pattern of change in plasma levels of prolactin similar to that observed with DES, except that RIA and Nb_2BA measurements were not different. Comparable results were obtained in Holtzman rats, except plasma levels were not increased to the same degree as seen in F344 rats. From these results, we conclude that DES, but not estradiol, can selectively increase the secretion of prolactin that is more bioactive than immunoreactive and that this effect of DES is observed in F344 and Holtzman rats, although F344 rats released more prolactin in response to estrogens than did Holtzman females.** [P.S.E.B.M. 1992, Vol 200]

The radioimmunoassay (RIA) for prolactin, developed in the late 1960s and early 1970s (1–4), is the most commonly used method for measuring prolactin. This assay has the sensitivity required to measure plasma levels of the hormone that were not possible with the pigeon crop sac bioassay developed in the 1930s (5). In the period since the development of the prolactin RIA, much of the physiology of prolactin

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determined earlier by the pigeon crop sac bioassay has been confirmed and extended substantially. However, there have been reports in which the levels of prolactin measured by pigeon crop sac bioassay and RIA were different (6, 7).

The Nb_2 lymphoma cell bioassay (Nb_2BA), developed in 1980 by Tanaka and co-workers (8), is a method that has the advantages of both the RIA (sensitivity and specificity) and bioassay (direct measurement of a cellular response). Several reports have appeared in which prolactin levels in the rat, determined by RIA and Nb_2BA, have been compared. The results have shown both agreement and divergence between these two assay methods (9–18). RIA measurements have been reported to be both higher and lower than those determined by Nb_2BA. The objectives of this study were to further compare these two assays in the measurement of pituitary and plasma levels of prolactin and to assess the temporal effects of estradiol and diethylstilbestrol (DES) treatment in strains of rats that are resistant or susceptible to pituitary tumor induction by estrogens.

Materials and Methods

Female Fischer 344 (F344) rats, a strain in which chronic estrogen treatment quickly induces pituitary tumors, and Holtzman rats, a strain much more resistant to estrogen-induced pituitary tumors, were purchased from Harlan Industries, Indianapolis, IN, housed in an environmentally controlled room (lights on 0600–2000 hr, temperature 23°C, relative humidity 40–50%), and allowed free access to standard rat chow and tap water. Most females were ovariectomized and, 1 week later (Day 0), were implanted subcutaneously with a Silastic capsule containing 15 mg of 17β-estradiol (E_2) or DES (Sigma Chemical Company, St. Louis, MO). Capsule construction was similar to that described by Legan *et al.* (19). Additional rats were bred and allowed to deliver litters which were adjusted to seven or more pups 3–5 days after parturition. Pups remained with the dams until the dams were sacrificed.

Animals were sacrificed as follows: DES and E_2-treated F344 rats were sacrificed on Days 0, 2, 4, 8, 14, 16, 28, 42, and 56 of estrogen treatment. DES-treated Holtzman rats were sacrificed on Days 0, 16, and 56, whereas E_2-treated Holtzman rats were sacrificed on Day 56 of treatment. Lactating rats were sacrificed between Days 8 and 10 postpartum.

At 1000 hr on the day of sacrifice animals were briefly anesthetized with ether and then decapitated. Trunk blood was collected into heparinized tubes. The plasma obtained by centrifugation of the trunk blood samples was aliquoted into separate tubes and stored at −70°C for subsequent bioassay and RIA determinations of prolactin. The pituitaries were quickly removed and the anterior lobe was snap frozen on dry ice. Each pituitary was weighed frozen and then sonicated in 1 ml of carbonate buffer (0.05 *M* $NaHCO_3$-Na_2CO_3, pH 10) containing 0.1% bovine serum albumin. The sonicates were diluted 1/100 with preservative-free assay buffer (0.01 *M* NaH_2PO_4-Na_2HPO_4 and 0.14 *M* NaCl containing 0.1% bovine serum albumin) and frozen at −70°C in separate aliquots for each assay.

Pituitary sonicates and plasma samples were assayed for prolactin at two dilutions in duplicate by double-antibody radioimmunoassay according to the method of Kuo and Gala (4) using NIDDK-RP-1 (11 IU/mg) as standard and NIDDK-RP-I_5, which was iodinated using the lactoperoxidase method of Tower *et al.* (20), as label. The primary antiserum was prepared by R. R. Gala against rat prolactin purified from pituitary culture medium by preparative gel electrophoresis, and the antiserum was absorbed with hypophysectomized rat serum. The secondary goat anti-rabbit serum and normal rabbit serum were purchased commercially (Antibodies Inc., Davis, CA). The sensitivity of the RIA was 0.25 ng/tube. The coefficients of variation within and between assays was less than 10%.

The Nb_2 lymphoma cells used for the bioassay were originally obtained from Drs. Beer, Gout, and Noble (Vancouver, British Columbia, Canada). These were maintained as a stock culture in RPMI 1640 medium containing 10% horse serum (with ≤1 ng prolactin/ml), 10% fetal calf serum, penicillin, streptomycin, and 2-mercaptoethanol (10^{-4} *M*). The sera were purchased from Hyclone Sterile Systems (Logan, UT) and the other materials were obtained from Sigma. Approximately 24 hr before the assay, the cells were transferred from stock culture to medium similar to stock medium except that it contained 1% fetal calf serum to slow cell replication. On the day of the assay, fetal calf serum was removed altogether by several washes and the cells were diluted to approximately 10,000 cells/ml. The diluted cells were dispersed as 1-ml aliquots to wells of 24-well culture plates. Prolactin standards, identical to those used in the RIA, were prepared in preservative-free assay buffer and were dispensed into duplicate wells in 200-μl aliquots containing 0.05–50 ng. Pituitary sonicates and plasma samples were diluted with assay buffer (no preservatives) such that 25-μl or 50-μl aliquots contained between 0.125 μl and 2.5 μl of original sample. At these dilutions, there was no evidence of potentiating or inhibiting factors which, as we have reported, alter the Nb_2 cell response (21). The sensitivity of the bioassay was 0.1 ng/well. The cells were cultured for 96 hr and then counted in a cell counter (model ZM Coulter Counter; Coulter Electronics, Hialea, FL). In our hands, Nb_2 cells show a much steeper dose response to rat prolactin if cultured in RPMI 1640 instead of Fischer's medium, and, in RPMI 1640, plating the cells at 10,000 cells/well was superior to an initial count of 50,000 or 100,000 cells/well (unpublished).

Statistical significance was determined by Student's paired and unpaired *t* tests and one-way analysis of

variance with Tukey's HSD post hoc test. Differences at $P < 0.05$ were considered statistically significant.

Results

During DES administration, pituitary concentrations of prolactin, as measured by RIA and Nb_2BA, increased sharply until Day 8 in F344 rats (Fig. 1A). This increase was followed by a decline in the concentrations such that, by Day 42, levels approached those seen in untreated ovariectomized rats (Day 0). Between Days 42 and 56, the pituitary concentrations, as measured by Nb_2BA, again increased to the levels seen on Day 8, but a similar increase was not seen when prolactin was measured by RIA. The Nb_2BA to RIA ratio was approximately 1.0 until Day 56, when it increased significantly to 3.0 (Fig. 1B).

In Holtzman rats, pituitary concentrations of prolactin also increased over the course of DES treatment (Fig. 2A). However, since no data were obtained between Days 0 and 16 or between Days 16 and 56, a detailed comparison of the dynamics of estrogen-induced changes in pituitary prolactin with those of F344 rats could not be made. The one difference noted

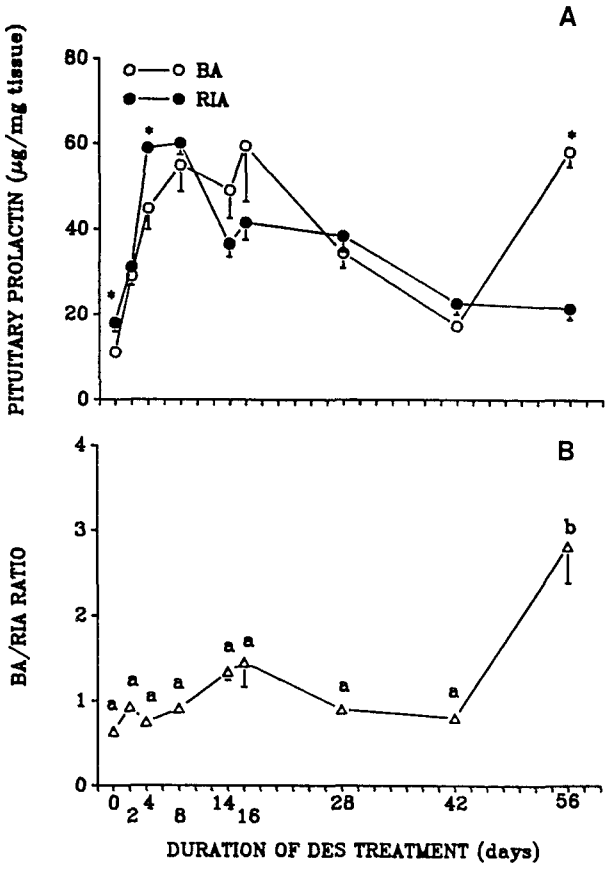

Figure 1. Bioactivity and immunoreactivity of pituitary prolactin after DES capsule implantation on Day 0 in ovariectomized Fischer 344 rats. (A) Absolute levels of pituitary prolactin (mean ± SE; $n = 3$). Statistical differences between assays at each interval of DES treatment are noted by an asterisk ($P < 0.05$; Student's t test). (B) Mean bioassay to RIA ratios ± SE calculated from data in (A). Values with different superscripts are statistically different ($P < 0.05$, Tukey's HSD test).

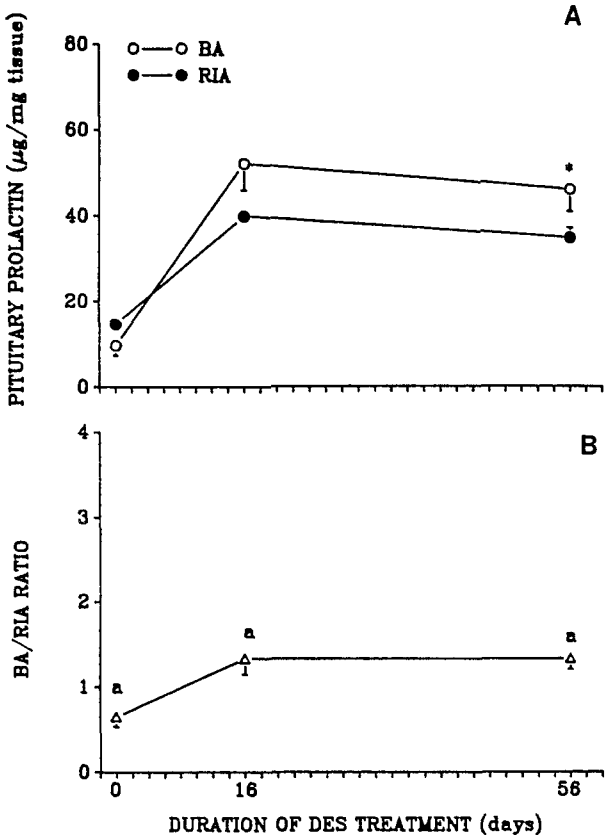

Figure 2. Bioactivity and immunoreactivity of pituitary prolactin after DES capsule implantation on Day 0 in ovariectomized Holtzman rats. (A) Absolute levels of pituitary prolactin (mean ± SE; $n = 5$). Statistical differences between assays at each interval of DES treatment are noted by an asterisk ($P < 0.05$; Student's t test). (B) Mean bioassay to RIA ratios ± SE calculated from data in (A). There were no statistical differences between the ratios on the days examined.

between the two strains was that at Day 56 the Holtzman rats showed a smaller difference between RIA and Nb_2BA measurements than seen in F344 females, and the Nb_2BA to RIA ratio of pituitary prolactin remained at about 1.3 from Days 16 to 56 in the Holtzman rats (Fig. 2B).

Pituitary prolactin concentrations in F344 rats treated with E_2 are shown in Figure 3A. As was observed with DES, there was a rapid increase in pituitary content through Day 8, followed by a gradual decline to levels approximately equal to those of ovariectomized controls. Unlike the response to DES, there was not a second increase in the pituitary concentration measured by Nb_2BA at Day 56, and the bioassay to RIA ratio (Fig. 3A) remained at 1.0 throughout the time course of estradiol treatment. Holtzman rats were only examined at Day 56 (Table I), but the RIA and bioassay measures of pituitary prolactin were not different and the bioassay to RIA ratio was approximately 1.0 at this time.

Plasma levels of prolactin in F344 rats treated with DES are shown in Fig. 4A (inset). Levels measured by both RIA and Nb_2BA increased steadily through Day 42, although those determined by Nb_2BA were signifi-

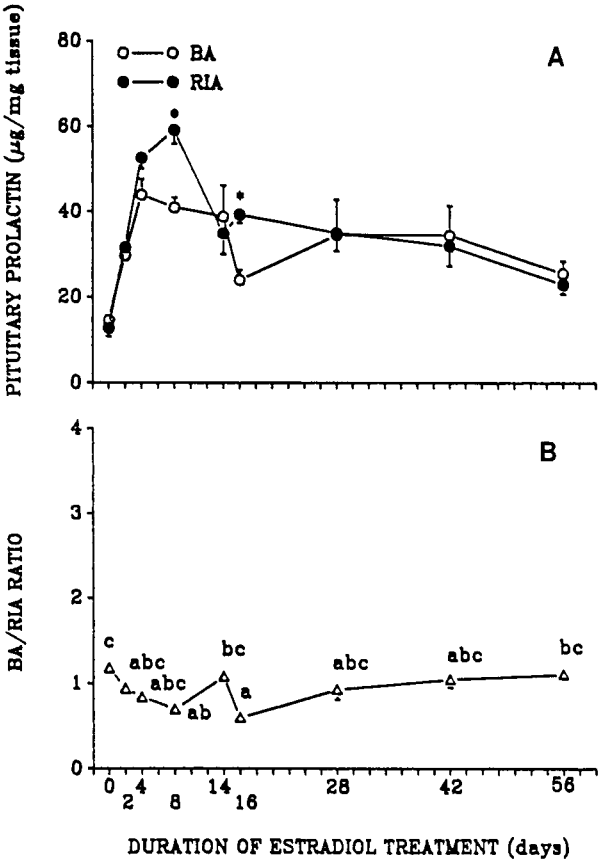

Figure 3. Bioactivity and immunoreactivity of pituitary prolactin after estradiol capsule implantation on Day 0 in ovariectomized Fischer 344 rats. (A) Absolute levels of pituitary prolactin (mean ± SE; n = 5). Statistical differences between assays at each interval of estradiol treatment are noted by an asterisk ($P < 0.05$; Student's t test). (B) Mean bioassay to RIA ratios ± SE calculated from data in (A). Values with different superscripts are statistically different ($P < 0.05$, Tukey's HSD test).

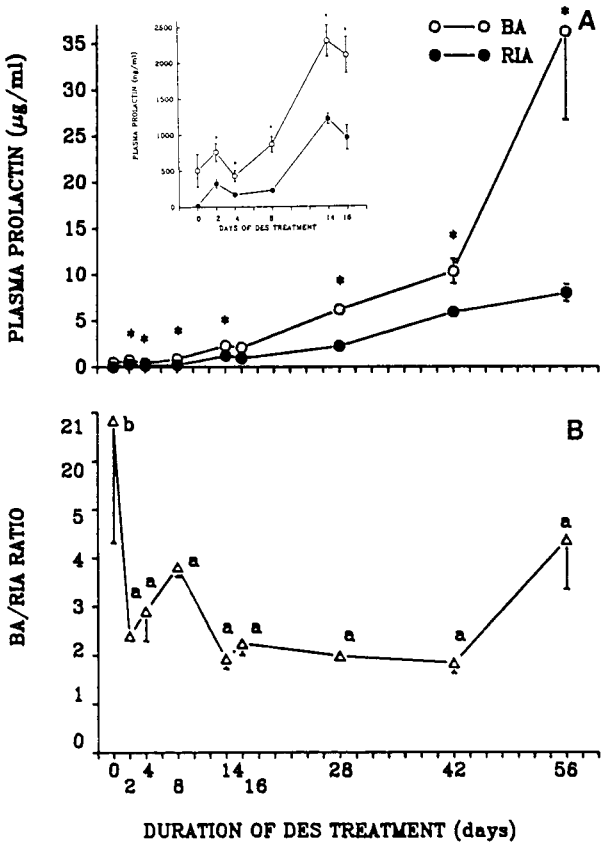

Figure 4. Bioactivity and immunoreactivity of plasma prolactin after DES capsule implantation on Day 0 in ovariectomized Fischer 344 rats. (A) Absolute levels of plasma prolactin (mean ± SE; n = 4). (Inset) Data between Days 0 and 16 are redrawn with an expanded vertical axis. Statistical differences between assays at each interval of DES treatment are noted by an asterisk ($P < 0.05$; Student's t test). (B) Mean bioassay to RIA ratios to SE calculated from data in (A). Values with different superscripts are statistically different ($P < 0.05$, Tukey's HSD test).

Table I. Pituitary and Plasma Prolactin Levels at 56 Days of Estradiol Treatment in Ovariectomized Holtzman Rats[a]

	Nb_2BA	RIA	BA:RIA Ratio
Pituitary (μg/mg tissue)	29.2 ± 4.6	23.5 ± 1.9	1.22 ± 0.12
Plasma (ng/ml)	1039 ± 219	983 ± 146	1.03 ± 0.09

[a] Values represent mean ± SE (n = 4). There were no significant differences between assays.

cantly greater than those by RIA from Day 4 onward. By Day 56, there was a sharp increase in the levels measured by Nb_2BA which was not seen in the levels determined by RIA. The bioassay to RIA ratio (Fig. 4B) was approximately 21 in untreated ovariectomized rats, whereas during most of the course of DES treatment, the ratio was between 2 and 4 and did not differ significantly between Days 2 and 56.

Plasma levels in Holtzman rats also increased over the 8 weeks of DES treatment, but levels at the end of the treatment were much less than those seen in F344 rats (Fig. 5A). As observed in F344 rats, the levels in Holtzman rats measured by Nb_2BA were significantly greater than those measured by RIA at both 16 and 56 days of DES treatment, and the bioassay to RIA ratio was approximately 2.5 during the course of the experiment.

When F344 rats were treated with estradiol, plasma levels of prolactin increased (Fig. 6A) (inset), but not to the same extent as was seen for DES. There were no significant differences between the levels measured by RIA or Nb_2BA at any time during estradiol treatment, and the bioassay to RIA ratio remained unchanged at approximately 1.0 through Day 42. There was a slight and significant increase in the ratio on Day 56, but the ratio increased to only 1.6. Plasma levels in estradiol-treated Holtzman rats on Day 56 (Table I) were elevated above those observed in untreated ovariectomized rats (Fig. 5), but there was no difference between the measurements obtained by RIA and Nb_2BA.

Neither DES or estradiol affected the results of the Nb_2BA when these steroids were included in the me-

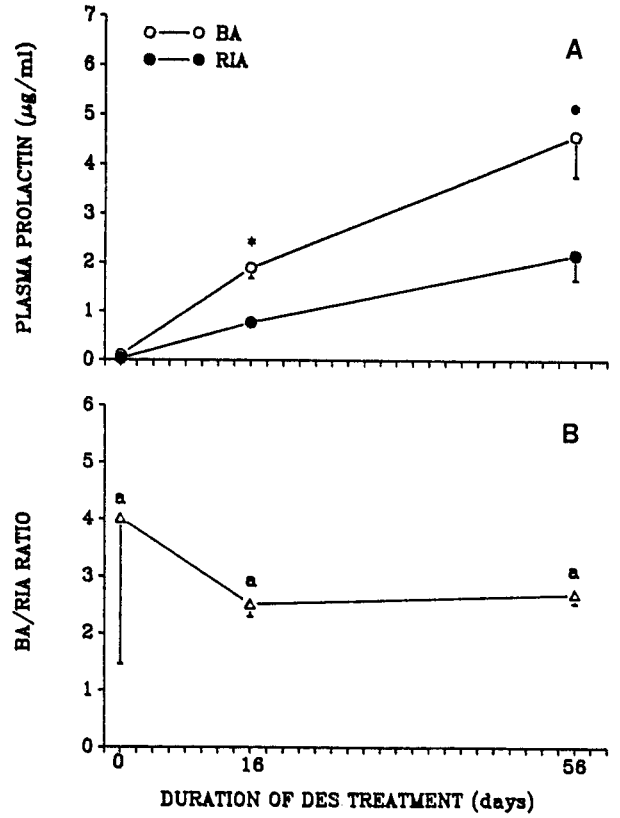

Figure 5. Bioactivity and immunoreactivity of plasma prolactin after DES capsule implantation on Day 0 in ovariectomized Holtzman rats. (A) Absolute levels of plasma prolactin (mean ± SE; $n = 4$). Statistical differences between assays at each interval of DES treatment are noted by an asterisk ($P < 0.05$; Student's t test). (B) Mean bioassay to RIA ratios ± SE calculated from data in (A). There were no statistical differences between the ratios on the days examined.

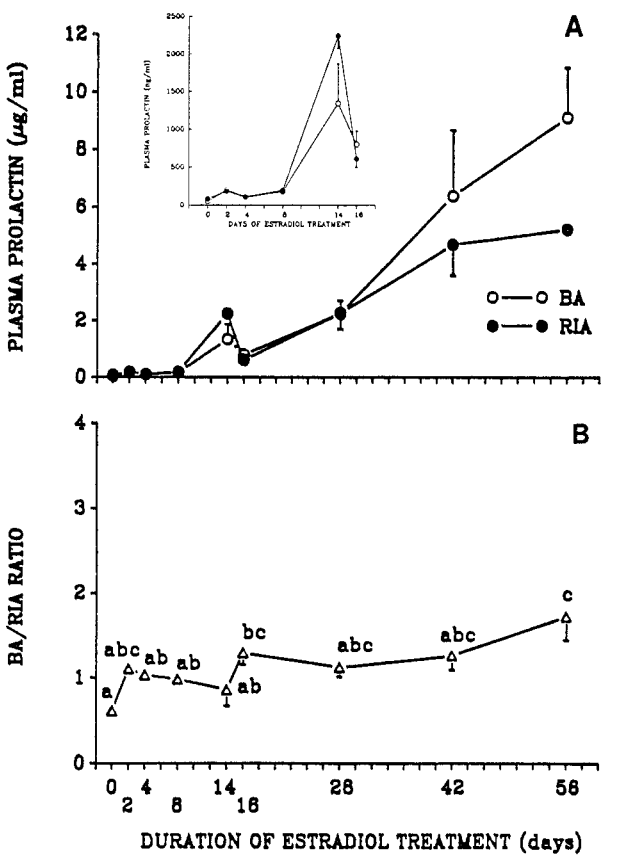

Figure 6. Bioactivity and immunoreactivity of plasma prolactin after estradiol capsule implantation on Day 0 in ovariectomized Fischer 344 rats. (A) Absolute levels of plasma prolactin (mean ± SE; $n = 5$). (Inset) Data from Day 0 to Day 16 are redrawn with an expanded vertical axis. No statistical differences were observed between the assays at any time during estradiol treatment. (B) Mean bioassay RIA ratios ± SE calculated from data in (A). Values with different superscripts are statistically different ($P < 0.05$, Tukey's HSD test).

dium at a concentration of 10 or 100 pg/ml (data not shown).

Pituitary and plasma concentrations of prolactin in continuously suckled lactating rats as determined by both the Nb_2BA and RIA are shown in Table II. Although the levels measured by Nb_2BA were statistically greater than those measured by RIA, the bioassay to RIA ratios were approximately 1.0.

Table II. Pituitary and Plasma Prolactin Levels in Continuously Suckled Lactating F344 Rats on Days 8–10 Postpartum[a]

	Nb_2BA	RIA	BA:RIA Ratio
Pituitary (μg/mg tissue)	30.7 ± 2.7	25.6 ± 2.2[b]	1.24 ± 0.08
Plasma (ng/ml)	624 ± 156	584 ± 139[b]	1.09 ± 0.04

[a] Values represent mean ± SE ($n = 13$).
[b] Significantly less than bioassay values ($P < 0.05$; Student's t test).

Discussion

These results show that DES induced a rapid increase in the concentrations of prolactin in the pituitaries of ovariectomized F344 and Holtzman rats and that these increases were, with one exception, detected equally well by both RIA and Nb_2BA. DES and estradiol also increased plasma levels in both strains, but the time course was different from that seen in the pituitary. In addition, prolactin released into the blood of DES-treated rats was more active in the bioassay than in the RIA, an effect that was not observed in estradiol-treated rats or in untreated, continuously suckled lactating rats.

Several groups have compared pituitary concentrations of prolactin in female rats using Nb_2BA and RIA. Cohen and co-workers (15) found the bioassay to RIA ratio to be 1.6 for rats on diestrus and Blank *et al.* (13) reported a ratio of approximately 2.0 on each day of the estrous cycle, whereas we have observed ratios of 1.0–1.3 lactating rats, ovariectomized rats treated with estradiol, and in proestrous rats during most of the afternoon surge (9, 11, 18). The present results in estradiol-treated rats agree with our previous findings. It is not clear why others have reported higher ratios, because the published methods of all the laboratories appear quite comparable. There may be subtle differences in prolactin between steroid-treated rats and intact rats or between lactating and nonlactating rats which account for these differences. Shah and Hymer

(22) reported that different molecular forms of prolactin extracted from rat pituitary have differing activities in the Nb_2BA and the RIA.

Although there were differences between the pituitary responses to DES and estradiol with respect to Nb_2BA and RIA measurements, the general patterns of changes in pituitary content between the two steroids were very similar. The initial increase (within 4 days) in the F344 rats is interpreted as an increase in synthesis and storage of prolactin without a commensurate increase in release. The decline in content between Days 4 and 42 is believed to be due to the enhanced release that occurred during that period. More work on the rate of synthesis is required to substantiate these interpretations.

That there was a selective increase in bioactivity of plasma prolactin compared with immunoreactivity (Fig. 4) in DES-treated rats is noteworthy. This effect occurred within 2 days of treatment in F344 rats and intensified as treatment continued. Hymer and Motter (23) previously reported that the bioassay to RIA ratio of prolactin secreted *in vitro* from cells obtained from F344 rats treated with DES for 70 to 100 days ranged from 2 to 10 depending on cell of origin and type of culture. We showed recently that the bioassay to RIA ratio of plasma prolactin is approximately 1.5–6.0 after 1 to 4 weeks of DES treatment and that it can increase approximately 2-fold within 2–10 min after thyrotropin-releasing hormone treatment or decrease 3-fold within 60 min after bromocryptine treatment, depending on strain of rat (17). The mechanism by which the bioactivity is selectively increased by DES is not clear. It is possible that alterations in prolactin's structure could produce such changes. We have observed that large molecular weight forms of prolactin present in the blood in DES-treated rats have a bioassay to RIA ratio of 3–5 compared with a ratio of 2.0 for the monomeric form (17). Although Shah and Hymer (22) showed that large forms of pituitary prolactin eluted from sodium dodecyl sulfate acrylamide gels had low bioactivity the method of separation (sodium dodecyl sulfide-polyacrylamide gel electrophoresis) may have affected the bioactivity. Another possibility is that DES induces the formation of prolactin that is cleaved such that it loses considerable immunoreactivity but retains full bioactivity, a property that has been shown by Vick *et al.* (24).

The elevated bioassay to RIA ratio in the ovariectomized rats (Figs. 4 and 5, Day 0) is of interest. We believe that this high ratio may be due to the greater sensitivity of the bioassay (0.1 ng/well) compared with the RIA (0.25 ng/tube). However, another possibility is that prolactin released under basal conditions is more bioactive than immunoreactive. There are reports of high bioassay to RIA ratios (>2.0) in untreated males (12, 15) and in diestrous (12, 13, 15) and ovariectomized females (12, 15), but the bioassay to RIA ratio was <1.0 in other ovariectomized rats in the present study (Fig. 6) and in nonsuckled lactating rats in a previous report of ours (11).

That the bioactivity of plasma prolactin in estradiol-treated rats was not enhanced relative to its immunoreactivity is in general agreement with previous data from our laboratory (9). However, Owens *et al.* (12) observed significantly greater levels of prolactin by Nb_2BA compared with RIA during the estradiol-induced afternoon surge in ovariectomized rats, and Blank and co-workers (13) and Cohen *et al.* (15) made similar observations during the afternoon surge of prolactin in intact, proestrous rats. We, on the other hand, have shown that the bioassay to RIA ratio of plasma prolactin remains near 1.0 through most of the proestrous surge and rises to approximately 2.0 in the late evening (2200–2400 hr) (18). The reasons for these discrepancies in the literature are not apparent. It may be due to the strain of rats used or to some subtle difference in the methods between laboratories. That we observed similar changes in bioassay to RIA ratios in two different strains of rats in the present study suggests that assay differences may account for the discrepancy between laboratories.

Strain differences were noted in the present study. It is clear that the F344 rats were more responsive to DES and estradiol than were Holtzman rats (Figs. 4 and 6 vs Fig. 5 and Table I), which is in agreement with the previous report of Wiklund *et al.* (25), and this enhanced responsiveness may be related to the greater susceptibility of F344 rats to pituitary tumor formation. Although the magnitude of response was different between strains, the patterns of change in both pituitary and plasma prolactin were very similar and differences in the Nb_2BA and RIA were similar in both strains.

In summary, it appears that DES treatment causes a selective increase in the bioactivity of plasma prolactin that is not evident in prolactin extracted from the pituitary until very late in treatment. Estradiol treatment does not produce similar changes in bioactivity of plasma prolactin, although it increases pituitary and plasma prolactin levels measured by RIA in a manner nearly equivalent to DES treatment. The mechanism by which DES selectively increases bioactivity is not known. There are no direct mitogenic effects of DES on Nb_2 cells (unpublished), so it appears that the effect is at the level of processing of prolactin after secretion from the pituitary or on the synthesis and secretion of a very bioactive form of prolactin that comprises only a small percentage of pituitary hormone until late in DES treatment (e.g., 56 days). Perhaps DES produces these effects whereas estradiol does not because of some difference in the effective doses of the two steroids or because of differences in the mechanism of action. Future studies will be required to answer these questions.

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