Prolactin Binding to Mammary Gland, 7,12-Dimethylbenz(a)-anthracene-induced Mammary Tumors, and Liver in Rats¹

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SUMMARY

Specific binding of radioactively labeled prolactin was determined in membrane preparations from mammary glands and livers of rats during pregnancy and lactation. Prolactin binding to mammary gland increased throughout late pregnancy and early lactation, reached a maximum on Day 11 of lactation, and then declined. Maximum prolactin binding to liver membrane preparations was observed during late pregnancy and declined throughout lactation. Estradiol benzoate (20 μg/day), administered on Days 5 to 10 of lactation, reduced prolactin binding to mammary gland by 55%, increased binding to liver 2-fold, and reduced litter weight gain by 25%.

Prolactin binding to 7,12-dimethylbenz(a)anthracene-induced mammary tumors was 3 times higher than that observed in lactating mammary gland. Administration of prolactin enhanced tumor growth but decreased specific prolactin binding to tumors. Lergotrile mesylate and estradiol benzoate (2 μg/day) enhanced tumor growth, but neither treatment affected prolactin binding to tumor membrane preparations. In contrast, higher doses of estradiol benzoate (20 μg/day) inhibited tumor growth and reduced prolactin binding. Prolactin binding varied widely within all groups of mammary tumors and was not clearly related to growth response or to altered circulating estradiol and/or prolactin levels. Hormone dependence in this animal tumor model is complex and may not be predicted on the basis of prolactin-binding capacity alone.

INTRODUCTION

There is abundant evidence that PRL² is necessary for the initiation and maintenance of lactation in the rat (20, 36). It is thought that PRL interacts with specific receptors on the external surface of the cell membrane of its target cells (11, 24). Various studies have demonstrated specific PRL binding to rat mammary tissue slices (3), subcellular particles (12, 37), and to other rat tissues including ovary, kidney, adrenal gland, ventral prostate, and liver (4, 6, 8, 13–15, 17, 26, 27, 29). PRL injected in vivo is concentrated by mammary tissues and accumulates at the basal surface of the mammary epithelial cell (28). Kelly et al. (13) reported that PRL may regulate synthesis of its own receptor in rat liver. PRL is also important in the induction and growth of DMBA-induced mammary tumors in rats (18, 19). A majority of these tumors grow faster in the presence of elevated circulating PRL and regress or become static when plasma PRL levels are reduced (9, 19, 21, 35). Endocrine ablations and other treatments which suppress PRL levels have been reported to be beneficial in the treatment of human mammary carcinoma (18, 33). Since tumor dependence upon PRL presumably requires the presence of a PRL receptor, it is possible that the quantitation of PRL-binding capacity will be of value in predicting the growth response of a given tumor to altered circulating PRL levels.

Specific PRL binding to subcellular particles from DMBA-induced tumor tissue has been demonstrated (5, 12, 37), and Kelly et al. (12) reported a positive correlation between PRL binding and growth response to exogenous PRL in these tumors. Curiously, in spite of their ability to increase circulating PRL, pharmacological doses of estrogen inhibit lactation in the rat (23) and in women (38), inhibit the growth of DMBA-induced mammary tumors (19, 21), and are clearly effective in the treatment of advanced breast cancer (18, 34). Meites (19) postulated that the inhibitory effect of estrogen was due to its ability to interfere with the action of PRL at the target cell level. In contrast, estrogen administered in vivo has been shown to increase PRL-binding capacity in rat liver (25). In vitro, estrogen did not affect PRL binding to rabbit mammary gland membrane particles (32); however, an effect on binding to rat liver has been reported (1).

In this paper we describe experiments designed to study the changes in PRL binding to rat mammary tissue and liver during pregnancy and lactation; to study the relationship between PRL binding and growth in DMBA-induced mammary tumors; and to measure PRL binding in DMBA tumors, lactating mammary gland, and liver following administration of pharmacological doses of estrogen.

MATERIALS AND METHODS

Hormones and Drugs. The following compounds were used: ovine PRL (NIH-S11; 26.4 IU/mg), bovine somatotropin (or growth hormone; NIH-B18; 0.81 IU/mg), bovine thyrotropin (NIH-B7; 3.58 USP units/mg), bovine folliculotropin (NIH-B1; 0.49 NIH-FSH-S1 units/mg), bovine lutropin (NIH-B9; 0.7 NIH-LH-S1 units/mg), porcine insulin (Eli Lilly Co., Indianapolis, Ind.; Lot 615-D63-10), EB, (Schering, AG, Berlin, Germany), LM (Eli Lilly Co.; Lot A19-A33-69A2), and DMBA (Sigma Chemical Co., St. Louis, Mo.).

Animals and Hormonal and Drug Treatments. Pregnant

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²The abbreviations used are: PRL, prolactin; DMBA, 7,12-dimethylbenz(a)anthracene; EB, estradiol benzoate; LM, lergotrile mesylate.
Sprague-Dawley rats were purchased from The Holtzman Co. (Madison, Wis.) and housed under controlled conditions (23°C; 14 hr light and 10 hr darkness) until they were killed by cervical dislocation or decapitation on Days 15 or 19 of pregnancy or Days 2, 11, or 15 of lactation. Abdominal and inguinal mammary glands and livers were excised, frozen in dry ice-acetone, and stored at −20°C.

When the effect of EB or LM was to be studied, litters were reduced to 6 pups on Day 5 of lactation (day of parturition is Day 1). The mothers were assigned to 1 of the following treatments: control, EB-20 (20 µg EB per day), or LM (25 µg, twice daily). The compounds were injected s.c. in 0.1 ml sesame oil on Days 5 through 10 of lactation. Controls received sesame oil. Mothers and litters were weighed on Days 5, 7, 9, and 11 and were sacrificed by decapitation on Day 11 of lactation. Mammary glands and livers were collected and stored as described above.

Mammary tumors were induced by the weekly administration (gastric intubation) of 5 mg DMBA in sesame oil (1.0 ml) to female Sprague-Dawley rats (Holtzman) beginning at 50 days of age and continuing for 5 weeks. Tumors began to appear approximately 5 weeks after the last dose of DMBA. The tumors were measured with calipers 3 times weekly, and their size was expressed as the product of 2 perpendicular diameters. Rats bearing tumors of sufficient size (2 to 3 sq cm) were randomly assigned to 1 of the following treatments: control, PRL (1 mg, twice daily), LM (25 µg, twice daily), EB-2 (2 µg EB/day), or EB-20. PRL was dissolved in 0.9% NaCl solution, and EB and LM were dissolved in sesame oil. All injections were made s.c. Controls received 0.9% NaCl solution. After 13 to 19 days of treatment, the rats were decapitated, and tumors and livers were excised, frozen, and stored at −20°C. The last PRL or LM injection was made 16 to 20 hr before decapitation. The last dose of EB was 24 to 28 hr before decapitation.

Preparation and Purification of Labeled PRL. 125I-labeled PRL was prepared electrophoretically by a modification of the method of Sammon et al. (30). PRL (50 µg) was added to 10 µCi of Na125I (carrier free; New England Nuclear, Boston, Mass.) and 0.05 mmol KI in 0.33 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 0.1% bovine serum albumin. Current was passed through the stirred solution at 0°C for 10 min while the voltage was maintained at 750 mV. The 125I-labeled PRL was separated from radioactive iodide by passage through Sephadex G-25 (0.9 cm inside diameter x 25 cm) in 25 mM Tris-Cl, pH 7.6, containing 0.1% bovine serum albumin (Fraction V; Sigma Chemical Co.). The labeled hormone (estimated specific activity, 90 to 100 µCi/µg) was stored in 1.0-ml aliquots at −20°C. It was further purified before use in the PRL binding assays by passage through Sephadex G-100 (1.5 cm inside diameter x 90 cm) as described by Shiu and Friesen (32).

Specific binding (defined below) of electrophoretically labeled PRL to membrane particles from rabbit mammary gland and pregnant rat liver was 3.8 and 13.2%, respectively, of the total radioactivity added. In simultaneous assays using 125I-labeled PRL prepared by the lactoperoxidase method (7), these respective membrane preparations bound, specifically, 3.0 and 11.0% of the total radioactivity added to the assay. In addition, the biological activity of the electrophoretically prepared 125I-labeled PRL was similar to that of the unlabeled hormone in a mouse mammary gland explant assay which measures PRL-stimulated [14C]acetate incorporation into medium chain-length fatty acids (D. Borst and H. Bern, personal communication).

PRL Binding Assay. Membrane particles were prepared as described by Posner et al. (27), diluted in 25 mM Tris-Cl, pH 7.6, containing 10 mM CaCl2, and stored at −20°C until assayed for PRL binding activity. Binding assays were performed in duplicate using 100 to 200 µg of membrane protein, 60,000 to 80,000 cpm of 125I-labeled PRL in a total of 0.5 ml of 25 mM Tris-Cl buffer, pH 7.6, containing 25 mM CaCl2 and 1% bovine serum albumin. For determination of "nonspecific binding" of 125I-labeled PRL, a 2nd set of duplicate assay tubes was run as described above, with the addition of 3 µg of unlabeled PRL. "Specific binding" was defined as the difference between the radioactivity bound in the presence and absence of unlabeled PRL. Binding was allowed to reach steady state at 4°C (30 hr for mammary gland and 48 hr for liver and DMBA tumors), after which membrane-bound and free 125I-labeled PRL were separated by centrifugation for 10 min at 1,800 × g. Radioactivity in the membrane pellet was measured in a Packard autogamma counter with counting efficiency of 45%.

Specific 125I-labeled PRL binding determined by this method increased linearly with protein added up to 250 µg for mammary gland, 150 µg for liver, and 300 µg for DMBA tumors, and was shown to approach saturation at high 125I-labeled PRL concentrations. Specific binding of 125I-labeled PRL to membrane particles from mammary gland, DMBA tumor, and liver was found to remain constant over periods of several months when the membrane preparations or whole tissues were stored at −20°C. Nonspecific binding of 125I-labeled PRL (as percentage of total radioactivity added) to mammary gland, liver, and DMBA tumor membranes ranged from 2.6 to 6.6%, 2.1 to 3.6%, and 1.0 to 3.6%, respectively. Specific 125I-labeled PRL binding to all membrane preparations was expressed as cpm/100 µg protein. Protein was measured by the method of Lowry et al. (16) using bovine serum albumin as the standard.

Statistical Methods. The data are presented as the mean ± S.E. Significance of differences was calculated using Student's t test.

RESULTS

Specificity of Binding Assays. When membrane particles of mammary glands from lactating rats, liver from pregnant rats, or DMBA-induced mammary tumors were incubated with 125I-labeled PRL and increasing amounts of unlabeled PRL, curves showing inhibition of 125I-labeled PRL binding were obtained (Chart 1). Total binding (percentage of total radioactivity added) in these experiments was 28.9, 12.8, and 7.2% for liver (68 µg protein), DMBA tumor (133 µg protein), and mammary gland (140 µg protein), respectively. Inhibition of 125I-labeled PRL binding was significant in all tissues when unlabeled PRL concentrations were greater than 1 ng/ml in the assay, and, in the presence of amounts greater than 200 ng/ml, inhibition of binding was 89, 80, and 50% in liver, DMBA tumor, and lactating mam-
Binding of PRL to Liver and Mammary Gland. Changes in specific PRL binding to liver and mammary gland membrane particles during pregnancy and lactation are shown in Chart 2. Binding to mammary gland membranes was low during pregnancy (281 ± 52 and 462 ± 36 cpm on Days 15 and 19, respectively) but greater than that observed in non-pregnant animals (Chart 2). PRL binding increased significantly (p < 0.01) during early lactation to a maximum (1,118 ± 132 cpm) on Day 11, then fell to 814 ± 106 cpm on Day 15 of lactation. In contrast, specific 125I-labeled PRL binding to liver was greater than that observed in mammary tissue, increased (p < 0.01) throughout pregnancy to a maximum (13,097 ± 1,311 cpm) on Day 19, and then decreased (p < 0.05) to 7,454 ± 1,106 cpm on Day 2 of lactation. Binding continued to decrease during lactation and reached a minimum on Day 15 (1,915 ± 442 cpm).

The injection of 20 μg of EB on Days 5 to 10 of lactation reduced 125I-labeled PRL binding to mammary tissue (p < 0.05) but stimulated binding to liver in animals killed on Day 11 (Chart 2). LM treatment (50 μg/day) did not alter PRL binding to either mammary gland or liver membrane particles. Litter weight gain was reduced by both LM and EB-20 treatment, although a greater effect was apparent with EB treatment (Table 1).

Binding of PRL to DMBA Tumors. 125I-labeled PRL binding to DMBA-induced mammary tumors is shown in Chart 3 and Table 2. Binding to tumor membranes from control rats was 3 times greater than that observed in lactating rat mammary tissue (3858 ± 370 versus 1118 ± 132 cpm). Considerable variation in binding was observed among tumors within all groups (Chart 3). 125I-labeled PRL binding in tumors from control animals (3858 ± 370 cpm) did not differ from that observed in LM (3508 ± 443 cpm) or EB-2 (3464 ± 284 cpm) groups. In livers from tumor-bearing animals, EB-2 treatment increased specific PRL binding as it did in lactating animals. However, in contrast to the results observed in lactating rats, LM reduced binding to livers from tumor-bearing animals (Table 2). EB-20 treatment significantly decreased binding (p < 0.01) to tumor membrane particles but resulted in a 2-fold increase in the amount of 125I-labeled PRL specifically bound to membranes from liver. PRL treat...
FIGURE 1. Effect of PRL on Tumor Growth. The diagram illustrates the growth responses of tumors following various treatments. The x-axis represents the treatment types, and the y-axis represents the percentage increase in tumor size. The treatments include controls, injections of estradiol, PRL, or LM. The bars indicate the percentage increase in tumor size observed during the last 10 days of treatment. The figure shows that tumors treated with specific hormones had different growth responses compared to the control group.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor Binding (cpm/100 µg protein)</th>
<th>Liver Binding (cpm/100 µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3,858 ± 370a (n=14)</td>
<td>6,821 ± 1,196 (n=14)</td>
</tr>
<tr>
<td>LM</td>
<td>3,508 ± 443 (n=22)</td>
<td>3,498 ± 898c (n=5)</td>
</tr>
<tr>
<td>EB (2 µg/day)</td>
<td>3,464 ± 284 (n=22)</td>
<td>10,075 ± 787d (n=5)</td>
</tr>
<tr>
<td>EB (20 µg/day)</td>
<td>2,148 ± 299 (n=22)</td>
<td>15,110 ± 1,266e (n=5)</td>
</tr>
<tr>
<td>PRL</td>
<td>1,210 ± 179 (n=18)</td>
<td>2,240 ± 566c (n=4)</td>
</tr>
</tbody>
</table>

a Mean ± S.E.
b Number of observations.
c Treatment effects on the amount of non-plasma membrane protein recovered in the microsomal pellet used for binding studies or the masking of available binding sites by increased circulating PRL (see below for further discussion). However, it cannot be said with certainty that these results reflect a decrease in the number of binding sites per cell. Our results may have been influenced by other factors, such as treatment effects on the amount of non-plasma membrane protein recovered in the microsomal pellet used for binding studies or the masking of available binding sites by increased circulating PRL (see below for further discussion).

d p < 0.05 vs. control.
e p < 0.01 vs. control.

PRL Binding to Rat Mammary Tumors

**DISCUSSION**

The data obtained in these experiments confirm previous reports of the presence of specific binding sites for PRL in the mammary gland (3, 12, 37) and DMBA-induced mammary tumor (5, 12, 37), 2 important target tissues for PRL in the rat. In the mammary gland, the increases in PRL binding observed during the first half of lactation coincided with increases in the rate of milk secretion occurring during this period (36). Lactation in the rat requires the presence of PRL (20, 36). This fact and the simultaneous increases in PRL binding and milk secretion strongly suggest that the hormone binding and changes observed therein are physiologically significant. Additional experiments will be required to elucidate the factors underlying the changes in measurable PRL binding to mammary gland membrane particles during early lactation. Epithelial cell proliferation and/or the hormonal milieu in which PRL predominates and estrogen is present at very low concentrations may be important. Recent reports have indicated that, in certain situations, endocrine factors could influence PRL binding to rat liver (1, 4, 8, 13, 14, 25, 26) and prostate (15).

Meites et al. (19, 21, 23) reported that pharmacological doses of estrogen inhibit milk secretion and reduce the growth of DMBA-induced mammary tumors. Since the administration of estrogen increased circulating PRL in rats (19, 22), it was inferred that the inhibitory effects were due to an interference with the action of PRL at the target cell, possibly at the level of hormone binding (19). Our data confirm that doses of EB, which inhibit lactation and DMBA tumor growth, do indeed reduce measurable PRL binding to membrane fractions prepared from mammary glands and tumors. However, it cannot be said with certainty that these results reflect a decrease in the number of binding sites per cell. Our results may have been influenced by other factors, such as treatment effects on the amount of non-plasma membrane protein recovered in the microsomal pellet used for binding studies or the masking of available binding sites by increased circulating PRL (see below for further discussion). However, it is important to note that LM treatment, which also inhibits lactation and tumor growth, presumably by suppressing plasma PRL (2, 31, 35), did not affect PRL binding to either mammary gland or tumor membrane preparations.

In contrast to the findings in mammary tissue, EB treatment increased PRL binding to liver membranes. These results reflect a decrease in the number of binding sites per cell. Our results may have been influenced by other factors, such as treatment effects on the amount of non-plasma membrane protein recovered in the microsomal pellet used for binding studies or the masking of available binding sites by increased circulating PRL (see below for further discussion). However, it is important to note that LM treatment, which also inhibits lactation and tumor growth, presumably by suppressing plasma PRL (2, 31, 35), did not affect PRL binding to either mammary gland or tumor membrane preparations.

**Tumor Growth Rates.** Tumor growth responses to PRL, EB, and LM and the specific [125I]-labeled PRL binding by individual tumors are shown in Chart 3. EB-20 and LM slowed tumor growth and resulted in regression of some tumors. No regressing tumors were observed in control, PRL, or EB-20 groups, and only 15 to 25% of the tumors in these groups were considered to be static (~30 to +30% change in tumor size). In contrast, 28% of the tumors in the EB-20 group and 17% of those in the LM group regressed; 61% in the EB-20 group and 56% in the LM group were classified as static. No obvious relationship was seen between tumor growth and PRL binding within any of the treatment groups.
results suggest that the effect of EB on PRL binding may vary from tissue to tissue and focus attention on the reciprocal changes observed in liver and mammary tissues during pregnancy and lactation, as well as after estrogen treatment. Since the physiological importance of PRL binding to liver is not known, the significance of these findings remains to be determined. It is interesting, however, that Kelly et al. (12) found a negative correlation between PRL binding to liver membranes and DMBA tumor growth rate in the same animals. In that experiment (12), a positive correlation existed between tumor growth response to PRL and tumor PRL binding capacity. It was, therefore, suggested that tumor and liver might compete for available circulating PRL.

We have mentioned above that the level of plasma PRL might affect measurable PRL binding to tissue membrane preparations. The low levels of hormone binding to both tumors and livers from PRL-treated rats suggested that some of the binding sites may be masked when circulating PRL concentrations reach very high levels, such as those observed during proestrus, lactation, or periods of stress, or following estrogen injection (22). To gain insight into this problem, attempts were made to release endogenously bound PRL by exposing the membrane particles to elevated pH (9.5 to 10.5), EDTA (10 mM), or MgCl₂ (1 to 5 mM). These treatments effectively released ¹²⁵I-labeled PRL bound to membrane particles in vitro, but also resulted in the "non-specific" extraction of considerable amounts of protein from the membrane particles (data not shown). It was therefore difficult to determine whether the enhancement of PRL-binding expressed on a protein basis following 1 of these treatments was due to the "unmasking" of binding sites, the extraction of nonreceptor protein, or a combination of these effects.

In any event, it does not appear that the decreased binding observed in lactating mammary gland and DMBA tumor tissue following the administration of pharmacological doses of EB can be fully explained by the masking of binding sites, since, in both groups, binding to liver membranes was enhanced. Moreover, the lower dose of EB, which increased plasma PRL to levels equal to those observed following the higher dose (19), did not reduce measureable PRL binding to DMBA tumor membranes.

In agreement with DeSombre et al. (5), we were unable to show any obvious relationship between PRL binding and tumor growth response to treatments which alter plasma estrogen and/or PRL concentrations. Treatment with LM, a compound which reduces plasma PRL (2, 31, 35) inhibited tumor growth, in agreement with the results of Sweeney et al. (35). Measureable PRL binding to these static or regressing tumors ranged from very low to high levels and, therefore, was not indicative of "PRL-dependence" as usually defined (18). A similar lack of correspondence between PRL binding and growth response was observed in animals treated with PRL or low doses of EB, both of which promoted tumor growth. Masking of binding sites may have influenced our results and prevented confirmation of the findings of Kelly et al. (12) that PRL binding was greatest in those tumors that were most responsive to PRL. We conclude that, in this animal tumor model, hormone dependency is complex and may not be predicted on the basis of PRL-binding capacity alone. Undoubtedly, hormone dependence is determined not only at the level of the hormone receptors for both PRL and estrogen, but also at other points in the metabolism of the tumor cell.

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Prolactin Binding to Mammary Gland, 7,12-Dimethylbenz(a)-anthracene-induced Mammary Tumors, and Liver in Rats

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