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In Vitro Interaction of Estrogen and Prolactin on Hormone-Dependent Rat Mammary Tumors[1] (38511)

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(Introduced by R. B. Jennings)

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Rat mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) are hormone-dependent, since either ovariectomy or hypophysectomy in the host animal results in tumor regression (1). Estrogen and prolactin appear to be the principal hormones responsible for tumor growth (2). The presence of a high amount of estrogen receptor in the tumor tissue (3, 4), and the demonstration of a direct action of estrogen on leucine incorporation by the tumor (5), support the concept that the tumor is estrogen-responsive. Nevertheless, some evidence suggests that the tumor is prolactin-dependent rather than estrogen-dependent (6–9). As the controversy continues, our knowledge on the understanding of hormone dependence in DMBA-induced rat mammary tumors remains incomplete. The experiment reported in this paper demonstrates that both estrogen and prolactin act directly on the tumor tissue and stimulate the *in vitro* incorporation of ^{3}H-leucine. Moreover, there appears to be a synergistic relationship between the two hormones.

Materials and Methods. Mammary tumors were induced in female Sprague–Dawley rats (ARS/Sprague–Dawley, Madison, WI) by DMBA (Sigma Chemical Co., St. Louis, MO) according to the method of Huggins *et al.* (10). As tumors reached 1.5–2.0 cm in diameter, animals were ovariectomized. Four to 7 days later, they were sacrificed by decapitation. Mammary tumors quickly were dissected and were prepared for incubation with tritiated leucine as described previously (5). Briefly, the tissue was incubated in 4 ml of medium-199 (Grand Island Biological Co., Grand Island, NY) containing 2 μCi of ^{3}H-4,5-L-leucine (New England Nuclear, Boston, MA) at 37° under 95% oxygen and 5% carbon dioxide for 2 hr.

For hormonal treatments, ovine prolactin (NIH-P-S10), at a concentration of 5 μg/ml, and 17β-estradiol (Sigma Chemical Co.), at 0.1 μg/ml (0.04% ethanol), were added into the incubation medium either individually or in combination. The control medium contained alcohol vehicle in place of estrogen and bovine serum albumin in place of prolactin. Thus each tumor was subjected to four different media: (a) control, (b) estrogen added, (c) prolactin added, and (d) combination of the two hormones. This arrangement constitutes a 2 × 2 factorial experiment with a randomized complete block design. We used ovariectomized animals to ensure an hormone deficient environment for the tumor (8). And, only those carcinomas showing microscopic signs of regression (11) were included in this study. Ten tumors from 10 animals were used.

At the end of the 2-hr incubation period, the protein of the tissue was precipitated with 10% trichloroacetic acid (TCA) and was washed with solvents (5). The final product was dried at 70° for 24 hr and weighed to the nearest 0.1 mg. The dried protein fraction was placed in a counting vial and dissolved by 1 ml tissue solubilizer (Soluene 100, Packard Instrument Co., Downers Grove, IL) before the addition of 10 ml of scintillation fluid. The radioactivity was measured by a liquid scintillation spectrometer (Model 3310, Packard Instrument Co.). An internal standardization procedure was used with tritiated toluene as the marker to determine the efficiency of the counting

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TABLE I. *In Vitro* Effect of 17β-Estradiol on the Incorporation of ^{3}H-Leucine into the Protein Fraction by Mammary Tumors Removed from 7-Day-Ovariectomized Rats or from Intact Rats

Conditions of host animals	No. of tumors	^{3}H Incorporation, dpm/mg		% Increase by estradiol
		Control medium	Estradiol 0.1 μg/ml	
Ovariectomized rats	5	11,876 ± 986[a]	12,776 ± 939	7.6[b]
Intact control rats	5	20,058 ± 2390	20,089 ± 2677	1.5

[a] Values are mean ± SE.
[b] Significant at $P < 0.01$ by paired t test.

TABLE II. Effect of 17β-Estradiol and Prolactin on the Incorporation of ^{3}H-Leucine by Regressing Rat Mammary Tumors *In Vitro*.

Treatment of medium	^{3}H Incorporation, dpm/mg protein	% of control
Control	15,580 ± 1,150[a]	100.0
Estradiol, 0.1 μg/ml	16,560 ± 1,120	106.3 ± 1.57
Prolactin, 5.0 μg/ml	16,620 ± 1,070	106.6 ± 2.06
Estradiol & Prolactin	18,660 ± 1,490	119.6 ± 2.30

Analysis of variance table

Source of variation	df	MS	F
Blocks	9	57,702,586	92.0[b]
Treatments	3		
Estradiol (E)	1	22,838,773	36.4[b]
Prolactin (P)	1	24,547,063	39.1[b]
(E) × (P)	1	2,820,134	4.5[c]
Error	27	627,240	

[a] Values are mean ± SE, $N = 10$.
[b] $P < 0.01$.
[c] $P < 0.05$.

for each sample. Results were expressed as dpm/mg TCA insoluble protein in the tissue.

Results and Discussion. A preliminary study (Table I) indicates that the addition of estradiol into the incubation medium stimulated the incorporation of the radioactivity only in tumors removed from ovariectomized animals. Tumors from intact control animals did not respond to estrogen *in vitro*. It appears that the exposure of endogenous estrogen to the tumor in rats during the estrous cycle (12) might render the tumor tissue insensitive to further estrogen *in vitro*. To eliminate the endogenous sources of estrogen and prolactin (8), mammary tumors were therefore collected from animals having been ovariectomized for 4–7 days.

The data in Table II show that there is a small but significant increase in the incorporation of ^{3}H-leucine (6–7%) when the tumor tissue is incubated with estrogen or with prolactin. However, when the tumor tissue is incubated with both hormones, ^{3}H-leucine incorporation is increased to 19%, a figure which is greater than the sum of the effect of each hormone measured independently. To our knowledge, such a synergistic relationship, though suggested (13, 14), has not been demonstrated before.

In previous studies (5) we have shown that the large variation in incorporation of ^{3}H-leucine among different tumors reflected differences in the cellular activity of the tumor at the time it was taken for incubation. However, our experiment was designed so that comparisons for hormonal effects were

made within each tumor (complete block design). The among-tumor-variation (block effect), therefore, did not influence the treatment effect. Results from statistical analysis (Table II) indicated that the individual hormonal effect and the interaction between the two hormones were significant at 1% and 5% level, respectively.

Our system measures the rate of ^{3}H-leucine incorporation into the protein fraction. Results from a preliminary study (not shown here) indicated that the amount of radioactivity incorporated by the tumor tissue during the incubation was linear over a period of 5 hr. (Intervals beyond 5 hr were not studied.) Therefore, the specific activity of the purified protein, observed at any interval during the 5-hr period, represents a measure of the rate of protein synthesis in the tissue. However, since the specific activity of leucine in the intracellular pool was not measured in this study, it is also possible that changes in cellular permeability of ^{3}H-leucine have been the rate limiting factor, which leads to differences in the quantity of incorporation.

The most important feature of this study, anyhow, is the demonstration that the tumor responds directly to the stimulation of both estrogen and prolactin, while the combination of the two hormones creates a synergistic effect. The data support both arguments that the tumor is estrogen-responsive as well as prolactin-responsive. According to recent reports from *in vivo* studies (13, 14), although DMBA-induced mammary tumors in rats can be maintained for a short period by a large amount of endogenous prolactin, for continuous growth of the tumor, the presence of both hormones is necessary. Our findings are consistent with the reports that each hormone is able to stimulate the tumor, but at a small magnitude, perhaps, unable to balance with the rate of catabolism. A marked stimulation is achieved as a result of hormonal synergism.

Summary. Hormone-dependent, regressing mammary tumors in rats were incubated in a chemically defined culture medium containing tritium labeled leucine at 37° for 2 hr. The rate of tritium incorporation into the protein fraction by these tissues was measured with and without the addition of 17β-estradiol and/or ovine prolactin into the medium. Tumor tissue responded to either estrogen or prolactin by a 6–7% increase in the rate of ^{3}H-leucine incorporation. When the incubation medium contained both hormones, the increase in the rate of ^{3}H-leucine incorporation was 19% which was significantly greater than the sum of the effect of each single hormone measured independently. These results demonstrate a synergistic effect between estrogen and prolactin on rat mammary tumors.

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