The Effect of Perphenazine-induced Serum Prolactin Response on Estrogen-primed Mammary Tumor-Host Systems, 13762 and R-35 Mammary Adenocarcinomas

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SUMMARY

The effect of perphenazine, a phenothiazine derivative, on serum prolactin levels, on maturation of the normal mammae, and on growth of mammary adenocarcinomas was studied using two transplantable rat mammary tumor systems, the 13762 mammary tumor (13762 MT) in the syngeneic Fischer 344 strain and the R-35 mammary tumor (R-35 MT) in allogeneic Sprague-Dawley stock. Perphenazine induced a dose-response increase in the serum prolactin levels of both normal and tumor-bearing animals and stimulated maturation of the normal mammae. The resultant responses of the two mammary tumors to perphenazine treatment were in exact contrast. Growth of the 13762 MT was accelerated whereas growth of the R-35 MT was inhibited. There was atrophy of the mammae and a concomitant decrease in serum prolactin levels in young, sexually maturing females with progressively growing 13762 MT tumors. Normal mammae and serum prolactin levels were unaffected in similar females bearing the R-35 MT. Treatment of the hosts with estrogen prior to tumor implantation, as well as treatment of both tumor and host, enhanced the effects of perphenazine on serum prolactin levels, on the normal mammae, and on mammary tumor growth, i.e., stimulation of 13762 MT and inhibition of R-35 MT.

INTRODUCTION

Phenothiazine derivatives have been reported to induce normal mammary growth and initiate milk secretions in humans and in a variety of animals (3, 10, 12, 15). Ben-David (1) has recently shown that the perphenazine-induced mammary development in intact rats is associated with stimulation of pituitary prolactin secretion. Danon et al. (6) demonstrated that both intact females and recently spayed rats respond to perphenazine treatment with lobuloalveolar differentiation of the mammae. On the other hand, when the rats had been ovariectomized 12 days previously, perphenazine was only weakly mammotrophic. In the latter case priming with sufficient amounts of estrogen (8 μg/day) completely restored the mammotrophic response to perphenazine and produced lactation. That estrogen apparently acts on the hypothalamic-pituitary system to stimulate the secretion of prolactin was shown both in vivo by Kanematsu and Sawyer (8) and in vitro by Nicoll and Meites (11) and by Ben-David et al. (2). The results obtained by Danon et al. (6) suggest that prolactin secretion cannot be induced when pituitary secretion of gonadotrophins is high and that inhibition of gonadotrophin secretion by estrogen stimulates or potentiates the secretion of prolactin.

Hilt et al. (7) studied the effect of fluphenazine HCl, a phenothiazine derivative, on growth of the R3230AC mammary adenocarcinoma and mammary glands of the rat and reported that, while administration of this tranquilizer stimulated normal mammary glands, it caused a decrease in the growth of R3230AC tumor. They concluded that creating a hormonal milieu that stimulates normal mammary glands does not necessarily stimulate growth of mammary tumors. The well-recognized variability in the hormone responsiveness of mammary tumors, both in animals and in man, raised the question concerning the sensitivity of the 13762 and R-35 MT's to prolactin. These tumors have been widely used for chemotherapeutic studies (13, 14) as well as for studies designed to evaluate therapy combinations (5). The 13762 MT, originally induced with dimethylbenzanthracene, is an extremely useful experimental mammary tumor model with a predictable (almost 100%) incidence of lung and organ metastases from s.c.-implanted grafts and was found to be very sensitive to the oncolytic effects of the steroidal alkylating agents (13, 14). The R-35 MT, a subline of the Huggins fibroadenoma A, on the other hand, has a relatively low incidence of metastases and, although it is responsive, it lacks the same degree of sensitivity to the steroidal alkylating agents.

This study was designed, therefore, to determine not only the effects of enhanced prolactin secretion, induced by perphenazine, on growth of the 2 mammary adenocarcinomas per se, but also the effect of mammary tumor growth on prolactin-normal mammary tissue interaction. Quantitation of serum prolactin levels by means of radioimmunoassay has permitted the comparison of prolactin levels in the various experimental groups. The experimental design incorporates, therefore, estrogen priming of host prior to tumor implanta-

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2 The abbreviation used is: MT, mammary tumor.
tion as well as estrogen priming of host and tumor. In view of the apparent competition of mammary tumors with normal uteri for endogenous estrogens demonstrated by Bogden et al. (4), it was felt that the administration of the exogenous estrogen would provide the excess of estrogen necessary not only to permit maximal sensitization of both normal and neoplastic tissues to prolactin effects, but also to potentiate the response of the pituitary to perphenazine.

MATERIALS AND METHODS

13762 MT. Forty-five to 50-day-old Fischer 344/CRL females were divided into the various experimental groups as shown in the experimental design outlined in Table 1 and were maintained on Wayne Lab Blox and tap water ad libitum. The designated groups were given s.c. implants on the right side of routine 1- to 2-cu-mm grafts of the syngeneic 13762 MT.

R-35 MT. Forty-five to 50-day-old SCH:ARS(SD) stock females were divided into the various experimental groups as shown in the basic experimental design outlined in Table 1 and were maintained on Wayne Lab Blox and tap water ad libitum. The designated groups were given s.c. implants on the right side of a routine Stadie slice of the allogeneic R-35 MT. Although R-35 MT originated and is transplantable in Sprague-Dawley-derived stock, grafts implanted s.c. are lethal to only 85% of the animals. Therefore, animals with no tumors or with growth followed by regression were deleted from this study.

Drug Preparation and Administration. Estrogen treatment consisted of 8μg estradiol-17β in sesame oil injected s.c. into the nape of the neck daily for 10 days. Perphenazine (NSC 150866, generously donated by the Research Division, Shering Corp., Bloomfield, N. J.) at 3 dose levels, 2, 4, and 8 mg/kg,

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
<th>Perphenazine dose levels (mg/kg/day)</th>
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<tr>
<td>I</td>
<td>Treated with 0.5 ml sesame oil, 1 dose/day for 10 days. Given</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>implant of tumor tissue on Day 11. Treatment with 0.5 ml 0.03 N HCl</td>
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<td>initiated on Day 11, 1 dose/day, continued to survival time.</td>
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<td>II</td>
<td>Treated with 0.5 ml sesame oil, 1 dose/day for 10 days. Given</td>
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<tr>
<td></td>
<td>implant of tumor tissue on Day 11. Treatment with perphenazine</td>
<td>4</td>
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<tr>
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<td>initiated on Day 11, 1 dose/day, to survival time.</td>
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<tr>
<td>III</td>
<td>Treated with 8 μg estradiol-17β, once each day for 10 days. Given</td>
<td>4</td>
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<td>implant of tumor tissue on Day 11. Treatment with perphenazine initiated</td>
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<td>on Day 11, 1 dose/day, to survival time.</td>
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<td>IV</td>
<td>Treated with 8 μg estradiol-17β initiated on Day 9, 1 dose/day for 10</td>
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<td>days. Treatment with perphenazine initiated on Day 19, 1 dose/day, to</td>
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<td>V</td>
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Groups contain 6 to 10 rats at each sacrifice time.
dissolved in 0.03 N HCl in a volume of 0.2 ml, was injected s.c. in the left side. No injections were given in the right side where the tumor was to be implanted. All animals were maintained 2/cage to minimize stress of crowding in a room that had controlled light and dark cycles of 12 hr.

Experimental Design. The overall study was divided into 4 experimental segments, i.e., a Study A and a Study B for each tumor system. Although the experimental protocols and treatment regimens were designed specifically for each tumor system, being based upon the lag time and growth rate characteristic of each tumor, they are, nonetheless, fundamentally identical in design so that valid correlations and comparisons between the 2 mammary tumors can be made. The experimental design for the 13762 MT is summarized in Table 1. In Study A, the hosts were treated with estrogen prior to implantation of tumor grafts. Perphenazine treatment was initiated on Day 1 of tumor implantation and continued daily through survival time. In Study B, both tumor and host were treated with estrogen. Therefore, tumor grafts were implanted on Day 1 and estrogen treatment was not initiated until the grafts showed 1st evidence of palpable growth. Perphenazine treatment was initiated after 8 or 10 days of estrogen administration and when the tumors were in mid-log phase of measurable growth.

Radioimmunoassay of Serum Prolactin. Animals were sacrificed in early morning, solid food having been removed from the cages at 5 p.m. on the previous day leaving only water ad libitum. On the day of sacrifice, animals were removed from their cages, 1 at a time, with minimal noise and agitation and quickly decapitated. Blood was collected in small sterile funnels, allowed to run into 10-ml test tubes, and stored overnight at 5° for clot retraction before being processed for serum. Serum prolactin levels were determined on duplicate serum samples at 2 dilutions, using reagents provided in the National Institute of Arthritis and Metabolic Diseases radioimmunoassay kit for rat prolactin (NIAMD-rat prolactin and antisera were generously provided by the Hormone Distribution Office, National Institute of Arthritis and Metabolic Diseases, NIH), according to the procedure reported elsewhere (9).

Organ Weight End Points. Recording of the initial body weight and the final body weight after tumor excision permitted organ weight determinations based upon 100 g of final body weight for the following organs: ovaries, uterus, liver, spleen, kidney, thymus, adrenals, and pituitary. Tumor weight was determined at sacrifice.

Histology of the Mammæ. At autopsy the left inguinal mammary pad was removed along with the overlying skin, pinned on flat cardboard, fixed in formalin, sectioned, stained with hematoxylin and eosin, and examined for mammary duct growth, growth of the lobuloalveolar system, and for evidence of secretion.

Mammary tumor development was graded histologically on a numerical basis, i.e., 1+, 2+, etc., with the highest number (4+) representing the highest degree of mammary development. Examinations were made of coded slides to aid in objectivity, and the average for each experimental group was reported as a numerical index that is an indication of relative mammary development.

RESULTS

The effect of perphenazine on serum prolactin levels, on organ weights, on the normal mammae, and on mammary tumor growth was studied in an experimental design that permitted evaluation of the effect on mammary tumor growth per se as well as on the interaction of mammary tumor growth and perphenazine on the development of the normal mammae. Since 2 different transplantable mammary tumor systems were used, the results obtained with each tumor system are presented separately. Significant differences and similarities are stressed in "Discussion."

Serum Prolactin Response to Perphenazine Treatment. The dose response of serum prolactin levels to perphenazine in Fischer 344/CRL rats is illustrated in Chart 1. The potentiation of the pituitary for prolactin release by estrogen treatment is also indicated by the dose-response curve, which shows a consistently higher prolactin response at all dose levels in estrogen-treated animals. The serum prolactin response to perphenazine in normal rats pretreated with estrogen indicated that estrogen potentiation of the pituitary declined with time following the termination of estrogen treatment. There was a consistently lower response in serum prolactin levels to perphenazine at all 3 dose levels at 35 days after estrogen treatment than at 21 days (Chart 2).

Serum Prolactin and Normal Mammary Development in Fischer 344 Females. The correlation of serum prolactin levels
Prolactin and Mammary Tumor-Host Interaction

21 DAYS POST ESTROGEN (Means ± S.D.)

Chart 2. Serum prolactin response to perphenazine in normal rats pretreated with estrogen illustrating the decline of the potentiating effect with time.

and development of the normal mammae in perphenazine-treated animals is illustrated in Chart 3. A comparison of the mammary development index and serum prolactin levels of perphenazine-untreated, 13762 MT-bearing with those of the perphenazine-treated groups revealed that perphenazine at the higher dose levels not only increased serum prolactin levels but also counteracted the atrophic effects on the normal mammary glands induced by mammary tumor growth.

Serum Prolactin Response and 13762 MT Growth. The stimulatory effect of perphenazine treatment on the growth of the 13762 MT is shown in Chart 4. In Study A (Chart 4) perphenazine treatment stimulated mammary tumor growth only at the highest dose level. When the host was given estrogen prior to tumor implantation and initiation of perphenazine treatment, tumor growth was enhanced at all dose levels. Stimulation of tumor growth in perphenazine-treated animals was also evident in Study B, although it was not as marked as in Study A. However, all dose levels of perphenazine appeared to be equally stimulatory, primarily when both host and tumor were treated with estrogen. Perphenazine treatment in Study A was initiated in early log growth phase and lasted for 25 days, whereas in Study B perphenazine treatment was initiated in mid-log phase and had a duration of only 18 days.

That the rate of tumor growth in the experimental groups given perphenazine was related to the resultant prolactin levels is shown in Chart 5. Perphenazine treatment was initiated on the day of tumor implantation. The prolactin responses on Day 21, when the tumors were relatively small, were perphenazine-dose related, and the subsequent rates of tumor growth were definitely related to serum prolactin levels. At the 8-mg/kg dose level of perphenazine, which induced the highest serum prolactin levels on Day 21 and, consequently, the largest tumors by Day 35, serum prolactin levels were lower on Day 35 than on Day 21. This is interpreted as possible prolactin “binding” by the tumor occurring during maximal prolactin response with a consequent depletion of serum prolactin levels.

Effect of 13762 MT Growth on the Mammary Glands of the
Sexually Maturing Rat. In view of the effect of prolactin on growth of the 13762 MT and the indication of a possible "prolactin binding" by this tumor, the effect of tumor growth on the normal mammae of the sexually maturing rat was studied in the vehicle-treated control groups. Chart 6 summarizes the atrophic effects of mammary tumor growth obtained in the vehicle-treated controls of Studies A and B and illustrates that the atrophy of the mammae was directly related to progressive tumor growth and to a decrease in serum prolactin levels.

Serum Prolactin and Normal Mammary Development in Sprague-Dawley Stock Females. Chart 7 illustrates a typical dose response of serum prolactin levels induced in normal Sprague-Dawley-derived stock females by perphenazine, and the direct relationship of serum prolactin levels with development of the normal mammae. Potentiation of the pituitary for prolactin release by estrogen was also seen in this stock of animals, and it was evident throughout the study that higher doses of perphenazine not only increased serum prolactin levels but also stimulated development of the normal mammae in tumor-bearing animals.

Serum Prolactin Response and R-35 MT Growth. The effect of perphenazine on growth of the R-35 MT is shown in Chart 8 and is in contrast to the results obtained in the 13762 MT system. Growth of the R-35 MT tended to be inhibited in perphenazine-treated animals. When the host was given estrogen prior to tumor implantation and perphenazine treatment, there was a perphenazine dose-related inhibition of tumor growth. That the tumor inhibitory effect was related to serum prolactin levels is illustrated in Chart 9. The slopes of the prolactin curves indicate that serum prolactin levels continued to increase with time despite progressive growth of the tumors. There was no tendency for the R-35 MT mammary tumor to lower prolactin levels as had been seen in the 13762 MT system.

Effect of R-35 MT Growth on the Mammary Glands of the Sexually Maturing Rat. The absence of an effect on serum prolactin levels by the R-35 MT, as indicated in the previous paragraph, is supported by the uninhibited development of the normal mammae in rats with progressively growing mammary tumors (Chart 10). This is in sharp contrast to the atrophy of the mammae seen in rats bearing the 13762 MT (Chart 6).

Weights. Thymolysis and adrenal hypertrophy resulting from the stress of tumor growth was evident in all tumor-bearing groups and was related to tumor size rather than to treatment.
with perphenazine and/or estrogen. When calculated on a 100-g final body weight basis there was an increase in the kidney weight of tumor-bearing animals that paralleled tumor growth. Estradiol and perphenazine had no effect on kidney weight in the non-tumor-bearing animal.

There was an increase in the ovarian weight of 13762 MT-bearing animals that appears to be associated with tumor growth in perphenazine as well as perphenazine- and estrogen-treated animals. There were no apparent effects of perphenazine or estrogen on the ovaries of non-tumor-bearing animals. In the R-35 MT system there was no significant or consistent effect on ovarian weight resulting either from tumor growth or from treatment with estrogen and/or perphenazine in Study A. However, in the estrogen-treated, tumor-bearing animals of Study B, there was a perphenazine-induced, dose-effect increase in ovarian weight on Sacrifice Day 37.

In the 13762 MT system, uterine atrophy was evident in all tumor-bearing groups, even in the estrogen-treated animals. The somewhat greater atrophy in animals treated only with perphenazine as compared to the vehicle controls can be attributed to the relatively larger tumors. Estrogen- and perphenazine-treated animals had the largest tumors but uterine atrophy on Sacrifice Day 37 was no greater than the other experimental groups. Alleviation of some of the atrophic effects seen in tumor-bearing animals, therefore, can be attributed primarily to the estrogen administration rather than the subsequent treatment with perphenazine. With the R-35 MT system, on the other hand, there was no consistent atrophic effect on the uterus in animals with progressively growing tumors. An estrogen effect on uterus weight was evident in both tumor-bearing and non-tumor-bearing animals.
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In the 13762 MT system there was a direct relationship between increase in pituitary weight and tumor size in all experimental groups except those treated with estrogen. Estrogen administration appeared to increase pituitary weights in both tumor-bearing and non-tumor-bearing animals. The response of the pituitary in Sprague-Dawley stock rats bearing the R-35 MT was similar to the 13762 MT system.

DISCUSSION

The effectiveness of perphenazine to stimulate mammary growth in the young, sexually maturing female rat has been demonstrated in this study, supporting the reports of similar activity in humans and in a variety of other animals (3, 10, 12, 15). Of particular significance was the dose response of serum prolactin levels to perphenazine treatment and the relationship of 13762 MT growth to the serum prolactin response. There is little question that increasing serum prolactin levels stimulated both normal mammary tissue, in terms of functional maturation, and malignant mammary tissue, in terms of proliferation. That normal mammary tissue and malignant tissue can compete for the available endogenous prolactin was indicated by the response of normal mammary and carcinomatous mammary tissue to prolactin, by the atrophy of normal mammary tissue and concomitant decrease in serum prolactin levels occurring in untreated animals bearing progressively growing 13762 MT's, and by the finding that the highest dose of perphenazine was able to abrogate this atrophic effect in tumor-bearing animals.

In sharp contrast to these observations was the inhibition of R-35 MT growth associated with increased serum prolactin levels. Significantly, therefore, there was no detectable impairment of maturation of the mammary or effect on serum prolactin levels in rats bearing progressively growing R-35 MT's.

Inhibition of tumor growth by increased serum prolactin levels was reported by Hilf et al. (7) for the R3230AC mammary tumor. Hilf also reported that uterine weight was significantly reduced in R3230AC tumor-bearing animals receiving fluphenazine HCl. In the present study on both the R-35 and 13762 MT systems, no consistent atrophic effect on the uterus was seen that could be attributed to perphenazine treatment. Uterine atrophy in young, sexually maturing females, bearing progressively growing 13762 MT's has been reported by our group (4) and also was evidenced in this study in vehicle-treated controls bearing the 13762 MT but not the R-35 MT. It would seem important to determine whether the estrogen-binding capacity of mammary tumors is related to the sensitivity and type of response induced by prolactin.

Treatment of the host with estrogen prior to tumor implantation, as well as treatment of both tumor and host, enhanced the effects of perphenazine treatment on both the normal mammary and the mammary tumors. Since the mammotrophic effects appear to be due, primarily, to the resultant increase in serum prolactin levels, the suggestion by Danon et al. (6) that estrogen treatment potentiates the secretion of prolactin by inhibiting gonadotrophin secretion is supported. Action of estrogen on the hypothalamic-pituitary system to “stimulate” the secretion of prolactin was shown in vivo by Kanematsu and Sawyer (8) and in vitro by Nicoll and Meites (11) and by Ben-David et al. (2). The results of the present study provided evidence that the enhanced prolactin response resulting from estrogen treatment persists, although decreasing, with time.

Thus, the primary objectives of this study to determine the effect of enhanced prolactin secretion on mammary tumor growth per se and of mammary tumor growth on prolactin-normal mammary tissue interaction have been achieved. For possible use as contrasting test systems in chemotherapy studies as well as for evaluating combinations of therapy, it is important that, whereas growth of the 13762 MT, which has predictable metastasizing characteristics, was shown to be stimulated by prolactin in these studies, growth of the R-35 MT was found to be inhibited. Both of these tumor systems have stable and predictable transplantation characteristics, as does the R3230AC mammary adenocarcinoma. It is clear that transplantable mammary tumors show the variability of response to prolactin that is reflected clinically and that a realistic preclinical evaluation of chemotherapeutic agents and therapeutic modalities, designed for the treatment of breast cancer, must include a spectrum of well-defined mammary tumor models.

Of particular clinical importance was the finding that mammary tumor growth could be stimulated by prolactin. Since perphenazine treatment increased serum prolactin levels, caution in the use of phenothiazine derivatives for tranquilization in pre- or postoperative breast cancer cases is suggested.

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Prolactin and Mammary Tumor-Host Interaction

The Effect of Perphenazine-induced Serum Prolactin Response on Estrogen-primed Mammary Tumor-Host Systems, 13762 and R-35 Mammary Adenocarcinomas


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