RELATION OF PROLACTIN, ESTROGEN, GH AND PROTEIN DEFICIENCY TO GROWTH OF DMBA-INDUCED MAMMARY TUMORS IN RATS

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ABSTRACT

RELATION OF PROLACTIN, ESTROGEN, GH AND PROTEIN DEFICIENCY TO GROWTH OF DMBA-INDUCED MAMMARY TUMORS IN RATS

By

Carol Joan Bradley

1. The relationship between the growth response of a carcinogen-induced mammary tumor to prolactin and the amount of prolactin binding protein in the tumor was studied. Twelve daily injections of 1 mg ovine prolactin were given to rats bearing 34 DMBA-induced mammary tumors, and tumor response was measured by the increase in the sum of three perpendicular diameters. Dr. Henry Friesen in Montreal, Canada, measured prolactin receptor activity by a radioreceptor assay. Statistical analysis showed a correlation coefficient between tumor growth and amount of receptor protein of $r = 0.69$, $p < .01$. Tumors with the greatest growth response to prolactin exhibited the highest prolactin binding, and vice versa. A negative correlation was noted between the amount of prolactin receptor activity in the liver and the average tumor growth response in individual rats. These results indicate that the amount of prolactin receptors in a mammary tumor of a rat is a good indication of its ability to show a growth response to prolactin.

2. An attempt was made to characterize and quantify estrogen and prolactin dependency of individual rat mammary tumors. The effects of age of tumors on hormone dependency also were studied. Female rats bearing DMBA-induced mammary tumors were subjected to a two-phase treatment regime 2 1/2 or 5 months after DMBA injection. Combinations of ovariectomy with drug or hormone treatments for two weeks caused an estrogen and/or prolactin deficiency in one treatment phase, and corrected the deficiency in the second two-week phase. Tumors were classified as prolactin or estrogen dependent, based upon regression in the absence of estrogen or prolactin and resumption of growth upon replacement with estrogen or prolactin. About 29% of the younger tumors and 33% of the older tumors were classified as prolactin dependent. Estrogen dependency was exhibited by 35% of the younger and 43% of the older tumors as determined by ovariectomy and estrogen replacement treatments. However, estrogen dependency as determined by ovariectomy and replacement with estrogen was drastically reduced in both age groups when high prolactin levels were maintained. Younger tumors had a higher regression rate after estrogen or prolactin reduction than older tumors. More tumors regressed independently of hormone levels in the older rats.

3. The effects of growth hormone (GH) and protein deficiency on mammary tumor number and growth rate were studied in female rats bearing DMBA-induced mammary tumors. Animals were fed diets containing 6%, 12% or 18% casein for three weeks with or without injections of 1 mg GH. Significant increases in tumor number occured in animals treated with GH above that of saline controls. GH also

increased tumor diameter: the greatest increase occurred in rats fed 6% casein, a lesser increase occurred in rats fed 12% casein, and the smallest increase occurred in rats fed 18% casein. However these increases in tumor diameter were not statistically significant. Protein deficiency resulted in a significantly smaller increase in tumor diameter but had no significant effect on tumor number. These results suggest that a protein deficiency results in a decrease in mammary tumor growth and that GH administration can partially overcome this reduced growth.

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TABLE OF CONTENTS

TABLE OF CONTENTS cont'd

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Much of the knowledge of human breast cancer is based on experimental work in rats with carcinogen-induced mammary tumors and in mice with spontaneously developed mammary tumors. Studies using these models have yielded information on drug and hormone treatments that already have been used in the treatment of human patients with some degree of success. It has been well established that estrogen and prolactin can promote growth of some mammary tumors and that removal of these hormones can induce tumor regression. However, not all tumors are equally responsive to hormone treatments. Experiments have revealed that a correlation exists between the effects of estrogen on growth of mammary tumors and estrogen receptor activity in the tumor. Mammary tumors with high estrogen binding activity are responsive to estrogen treatment or ovariectomy, while those with low estrogen binding activity are not. It has not yet been demonstrated whether the same type of correlation exists for prolactin receptors and mammary tumor dependency on prolactin in rats. Do mammary tumors that show the greatest growth response to prolactin also contain the most prolactin receptor activity?

Prolactin and estrogen have been shown to be essential for development and growth of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats. Numerous studies have clearly indicated that altering the levels of these two hormones by drug

treatment or surgical manipulations can profoundly influence the development and growth rates of populations of tumors, but some individual tumors may be relatively independent of these hormonal influences. The response of individual tumors to these two hormones may also vary with the stage of development and the size of the tumor. Therefore, it was of interest to follow the course of individual tumors in order to establish the percentage of tumors responding to estrogen or prolactin at an early and later stage of development, and thus to clarify the dependency of individual tumors on these two hormones.

Previous studies have not yielded definitive results on the effects of protein deficiency on rat mammary tumor growth. Neither is there strong evidence for an effect of GH on rat mammary tumor incidence or growth. However, there are experiments which have shown a relationship between GH and protein in rats. Protein deficiency causes a decrease in pituitary GH (Srebnik et al., 1959), while starvation can cause a decrease in both pituitary and plasma GH in rats (Dickerman et al., 1969). Conversely, GH can promote nitrogen retention in normal and protein deficient rats (Gordon et al., 1947). In view of this interrelationship of GH and dietary protein, it was of interest to examine the effects of these two factors on the growth rate and number of DMBA-induced mammary tumors in rats.

REVIEW OF LITERATURE

DMBA-induced Mammary Tumors in Rats as a Research Model for Human Breast Cancer

The study of human breast cancer has been hindered for many years by lack of a suitable animal model with similar characteristics. The model should (1) occur with relative frequency in a reasonable period of time (2) develop in a manner similar to human breast cancer, with similar gross and histological characteristics (3) respond to endocrine, drug, and other treatments in a manner similar to human breast cancers (4) appear in an animal suitable for laboratory study. Most of the earlier work on mammary cancer was performed in inbred mice, particularly the C_3H strain. However, these mouse mammary cancers appear to be hormone dependent only during the developmental stage and become autonomous and unresponsive to hormones after they appear (Dux and Mühlbock, 1969). In this respect they are unlike many human breast cancers.

The search for a better model led to the development of carcinogen-induced rat mammary tumors. Investigation of the carcinogenic properties of coal dust resulted in the isolation of several carcinogenic substances, including 3-methylcholanthrene and 7,12-dimethylbenz(a)anthracene (DMBA), the latter, one of the most potent carcinogens. These agents are highly carcinogenic upon oral, subcutaneous, or intravenous administration.

DMBA is particularly effective for induction of mammary tumors in Sprague-Dawley rats but will produce other cancers as well. A single intravenous injection of a lipid emulsion containing 5 mg DMBA given to female rats at 50 to 60 days of age results in 95 - 100% incidence of mammary adenocarcinomas which appear in 1 to 3 months (Geyer et al., 1953; Huggins et al., 1965). The DMBA-induced cancers do not readily metastasize (Young and Cowan, 1963), in marked contrast to human breast cancer, which is an important exception to the general similarity between the two cancer types. However, DMBA-induced mammary cancers are particularly suited as a model for human mammary cancer research because of their responsiveness to hormonal, chemical, and immunological factors, which appear to be comparable to many human breast cancers.

Estrogen Effects on Mammary Tumor Induction and Development

Estrogen Requirements

The effect of estrogen on DMBA tumor induction and growth is complex. Dao (1962) observed that DMBA fails to induce tumors in ovariectomized rats. This was confirmed by Talwalker et al., (1964) who also were able to induce DMBA tumors in ovariectomized rats by estrogen replacement. Ovariectomy also caused regression of established mammary tumors, but when exogenous estrogen was used to replace the missing steroid, tumor growth was maintained (Huggins et al., 1959; Sterenthal et al., 1963). Moderate doses of estrogens given to intact rats with established DMBA tumors can increase both

tumor growth rate and the rate of appearance of new tumors. Dao and Sunderland (1959) showed that mammary tumor growth in rats was accelerated during pregnancy and pseudopregnancy, presumably due to increased ovarian hormone secretion.

Large doses of estrogen have been shown to retard rat mammary tumor growth (Dao, 1964; Huggins, 1965; Meites, 1972). A daily dose of 20 ug estradiol benzoate administered at the critical period for tumor induction in Sprague-Dawley rats (55 - 60 days of age) for 20 days before and 20 days after DMBA administration was shown to inhibit the development of tumors, and to delay the time of appearance and the number of tumors appearing (Kledzik, Bradley, and Meites, unpublished data). An attempt to simulate hormonal conditions of pregnancy by administration of various amounts of estrogen and progesterone 15 days after DMBA treatment was reported by McCormick and Moon (1963). This treatment resulted in stimulation of tumor development with progesterone when a constant amount of estrogen was given. However, tumors were inhibited by graded increases in estrogen, whether given alone or in combination with progesterone.

Mammary Tumor Responses to Estrogen at Different Developmental Stages

Griswald and Green (1970) found a greater regression rate upon ovariectomy of rats with recently developed tumors (4 months post-DMBA) than in ovariectomized rats with older and larger tumors (7 months post-DMBA). They found the size of the tumor was a less important determinant of the regression rate in the younger than in the older tumors, as larger tumors were less likely to regress than

smaller tumors following ovariectomy of the older rats. Androgen treatment also caused less regression in large than in small tumors. Thus large mammary tumors are more autonomous and less hormone responsive than small mammary tumors in older rats.

Effects of Anti-estrogens

Anti-estrogens have also proved to be effective for inhibition of mammary tumor growth. MER-25 (Wm. S. Merrell Co., Cincinnati, Ohio), a potent anti-estrogen, was administered about the time of DMBA treatment by Terenius (1971). This delayed tumor induction by DMBA and decreased the number and size of tumors that appeared. Testosterone and progesterone, while possessing anti-estrogenic properties, failed to exert this inhibitory influence on the time of tumor appearance or number of tumors appearing. Terenius (1971) attributed this discrepancy to a different mechanism of estrogen antagonism for steroids than for MER-25. However, many other investigators have reported that early treatment with androgens or progestins can inhibit mammary cancer development in rats. Huggins et al. (1959) found that 1 mg dihydro-testosterone injected intramuscularly for 84 days delayed the mean time of appearance of DMBA-induced mammary tumors from 78.9 days in controls to 177.2 days in androgen-treated rats. MER-25 is believed to act by occupying estrogen receptor sites and reducing the ability of the estrogen to interact with the target tissue. In contrast, Keightley and Okey (1973) have shown, by a charcoal dextran technique, that dihydrotestosterone doesn't show competition for estradiol binding

sites in mammary tissues. Thus, the early effect of the MER-25 to inhibit tumor development appears to depend upon the occupation of the estrogen receptor sites, while the later inhibition by testosterone apparently works through some other mechanism.

In rats bearing DMBA-induced tumors, ovariectomized and immediately treated with testosterone proprionate, the tumor regression rate was less than in untreated ovariectomized rats (Griswald and Green, 1970). This could have resulted from metabolic conversion of the androgen to partially replace the estrogen loss, or from some direct effect of the androgen itself.

Recent work by Quadri, Kledzik and Meites (1974) showed the ability of dromostanolone (a potent androgen) to inhibit growth of DMBA-induced mammary carcinomas. Prolactin injections were able to overcome the androgen-induced regression. The authors attributed these results to peripheral blocking of the effect of prolactin on mammary tumors.

Variability of Mammary Tumor Response to Hormones

Mammary tumors are not as predictable in their responses to hormones as might be inferred from the above discussion. There are some tumors which do not follow the generally observed pattern of regression following ovariectomy. Others may fail to show increased rates of growth with elevated estrogen or prolactin levels. Established tumors are sometimes observed to undergo spontaneous regression, regardless of the prevailing hormonal state (Young and Cowan, 1963). These spontaneous regressions can't be attributed to

any particular factor, but can lead to erroneous conclusions when treatment groups are small.

Young et al., (1963) reported that isolated areas within a single DMBA-induced rat mammary tumor could continue growing while the tumor as a whole was regressing. This confirmed the results of Huggins et al. (1959) who studied the response to ovariectomy of different cell populations within one 3-methylcholanthrene-induced tumor. Thus, even in tumors which had been classified as hormone-dependent or independent, there were groups of cells which did not fall into this classification.

Prior to hormonal treatment, there was no histological indication which allowed the prediction of the response to an endocrine change (Young et al., 1963). Some tumors which failed to respond to ovariectomy were found to regress under massive estrogen doses (Teller et al., 1969). However, this latter was probably due to an interference with prolactin action (Meites et al., 1971). Daniel and Pritchard (1963) concluded that the tissue may be truely independent of estrogen in cases in which tumors fail to regress in response to ovariectomy, or there may be a very small degree of estrogen influence, and other factors which influence tumor growth take precedence.

Since the results of most tumor experiments are presented in terms of average tumor response versus the average response of controls, the response of the individual tumor is often lost in the averaging process. This masks the presence of autonomous and variant tumors which may be relatively frequent because of the heterogeneous nature of the mammary carcinoma. These are important considerations

in evaluating the response of individual tumors to hormone treatments in both animals and humans.

Prolactin Effects on Mammary Tumor Induction and Development

Prolactin Requirements

Prolactin has been shown to play an important role in the induction and growth of mammary tumors. Pearson et al. (1969) and Meites (1972) concluded that the presence of prolactin in the serum was necessary for the development and growth of DMBA-induced mammary tumors. Nagasawa et al. (1973) observed normal serum levels of prolactin in rats with growing DMBA-induced mammary tumors, indicating that tumor growth can occur in the presence of normal prolactin levels. Work by Boyns et al. (1973) indicated that strains of rats with higher prolactin serum levels have a higher tumor incidence following DMBA administration. When prolactin levels were raised above normal, the rate of growth of existing DMBA-induced mammary cancers increased dramatically (Meites, 1972). Welsch et al. (1968) found that increasing serum prolactin levels by grafting 4 pituitaries inside the kidney capsule accelerated the growth of established DMBA-induced mammary tumors. Pituitary stalk section or median eminence lesions, which remove the pituitary from hypothalamic inhibition of prolactin release, also cause mammary tumors to grow more rapidly (Clemens et al., 1968). Drugs that increase prolactin secretion, such as reserpine, methyldopa, and haloperidol, stimulate mammary tumor growth in rats (Welsch and Meites, 1970; Quadri et al., 1973; Lu and Meites, 1971).

A reduction in prolactin secretion inhibits development of DMBA-induced mammary cancers and reduces growth of established cancers. Drugs that lower serum prolactin levels also have been shown to cause tumor regression. L-DOPA, which acts by increasing hypothalamic catecholamines, thus increasing prolactin inhibiting factor (PIF), decreased prolactin secretion and caused regression of DMBA-induced mammary tumors (Quadri et al., 1973). Ergot drugs are believed to increase hypothalamic PIF release (Wuttke et al., 1971) and to inhibit pituitary prolactin release directly (Zeilmacher and Carlson, 1962), thereby reducing prolactin secretion. Ergot drugs have been used to reduce DMBA-induced tumor growth (Nagasawa and Meites, 1970; Heuson et al., 1970). Butler and Pearson (1971) administered rat prolactin antibodies to rats with DMBA-induced tumors and reported reduced tumor growth and tumor regression.

Mammary Tumor Responses to Prolactin at Different Developmental Stages

Clemens et al. (1968) demonstrated that median eminence lesions placed before administration of DMBA raised serum prolactin levels and inhibited the mammary tumor induction process. When prolactin levels were increased by haloperidol injection, administered daily for 20 days before and 20 days after DMBA treatment, a delay of tumor appearance and a reduction in the size and number of tumors resulted (Kledzik, Bradley, and Meites, unpublished data). Stimulation of the normal mammary tissue by increased prolactin levels is believed to render the mammary gland refractory to DMBA.

Evidence for the necessity of a critical level of prolactin at the time of DMBA administration comes from the above study in which

L-DOPA was injected for 20 days before and 20 days after DMBA treatment. This treatment lowered serum prolactin and reduced tumor incidence (Kledzik, Bradley, and Meites, unpublished data). A normal level of prolactin, therefore, seems to be necessary for DMBA to promote mammary tumor development, and either an excess or deficiency of prolactin can inhibit development of DMBA-induced mammary tumors. With existing mammary tumors, an excess of prolactin stimulates growth, whereas a deficiency results in tumor regression.

Interactions of Estrogen and Prolactin

Pearson and Ray (1959) hypothesized that estrogen stimulation of mammary tumors in humans was mediated through the pituitary, rather than only by a direct effect on the tumor. They found estrogen was ineffective in reactivating mammary cancer growth following hypophysectomy in women, although it was successful following öophorectomy. This has been confirmed in DMBA-induced mammary tumors in rats. Estrogen permitted continued growth in ovariectomized-adrenalectomized rats, while it was ineffective after hypophysectomy (Sterental et al., 1963). Talwalker et al. (1964) found that either estrogen or prolactin was able to permit mammary tumor induction by DMBA in ovariectomized rats. This would seem to support a secondary role for estrogen as compared to prolactin. Furth and Clifton (1957) were also strong proponents of prolactin as the primary hormonal stimulator of mammary tumors, with estrogen as a secondary hormone. They postulated that estrogen stimulates prolactin secretion and perhaps synergises or sensitizes the tissue to prolactin. Further

evidence for pituitary mediation of the estrogen effect on tumors is the finding of Nicoll and Meites (1962, 1964) and Meites and Nicoll (1966) that estrogen can promote synthesis and release of prolactin by exerting its actions on both the hypothalamus and pituitary.

However, it must be noted that evidence exists that estrogen as well as prolactin is essential for DMBA-induced mammary tumor growth. A recent report by Sinha et al. (1973) showed that intact rats with growing DMBA tumors produced the expected acceleration of tumor growth rate when median eminence lesions were placed in the hypothalamus, whereas ovariectomized rats with regressing DMBA tumors failed to respond to median eminence lesions with accelerated growth. Reimplantation of the ovaries resulted in the re-establishment of the accelerated growth rate without an increase in serum prolactin levels. This is in agreement with similar earlier work by Clemens et al. (1968). These studies do not completely contradict Pearson and Ray's (1959) belief that estrogen is secondary to prolactin in promoting tumor growth, since the ovarian reimplantation was done in animals with an already elevated prolactin serum level produced by the median eminence lesion. However, both estrogen and prolactin have been shown to be essential for maintenance of established mammary tumor growth.

Significance of Receptor Assays and Their Use in Elucidation of Hormone Mechanisms

Recent development of methods to measure hormone receptors in target tissues has opened new avenues of investigation for determination

of mechanisms of hormone action. Specific tissues can be profoundly affected by a hormone carried in the general circulation because of selective uptake by tissue receptors. Assays reported thus far have made use of the principle of specific displaceable uptake of radio-labeled hormone as an indication of the quantity of receptor protein present for that hormone. These assays were designed for *in vitro* quantification of receptors using tissue slices or selected fractions of tissue protein.

Early workers in the field of estrogen receptors had varying degrees of success with the particular assay technique used. Support for the role of the target organ in selective estrogen uptake was provided by *in vivo* studies which showed that the uterus and vagina, organs exhibiting known responses to estrogen, could concentrate radio-labeled estradiol (Jensen and Jacobson, 1962). Concentration of estrogen also was shown by DMBA-induced mammary tumors in rats (King *et al.*, 1965). Once the ability to concentrate estrogen in tissues was established, researchers worked to establish quantitative assays.

The sucrose density gradient method of separating fractions of tissue cytosol provided a precise method for measuring receptor protein. Jensen *et al.* (1971) used this technique to develop a two-stage binding mechanism theory for estrogen action. He observed that the labeled fraction of the sucrose density band was initially near the 8s region, but with time, an increasing percentage of the label was localized in the 4s band. The sucrose density gradient assay was more precise than other methods (including Sephadex filtration) used to purify the radio-labeled bound estrogen.

More precise assays made possible quantitative detection of receptors in tissues, whereas previous studies had been limited to determination of the presence or absence of receptor protein. Terenius (1973) assayed 23 human breast tumor samples for estrogen receptors by both the tissue slice and cytosol binding techniques. There was good correlation between the two methods for detection of receptors with only one exception, however, the ranking of the tumors by amount of estrogen binding differed by the two techniques.

Researchers working with DMBA tumors in rats or with human mammary cancer biopsies have repeatedly found differing amounts of receptor in the samples. McGuire and Chamness (1973) presented evidence which showed that the range of estrogen receptor protein content in cytosol of 40 human breast cancers was from 612 femtomoles/mg cytosol protein to non-detectable levels. Varying amounts of estrogen receptor in human breast cancers were also measured by Korenman and Dukes (1970), while measurement of estrogen receptor in rat mammary carcinomas was demonstrated by Wittliff et al. (1972).

Attempts to correlate presence of receptor protein with subsequent response to endocrine therapy have proved highly successful. Jensen (1971) found only one of 29 patients whose breast cancer biopsies indicated no estrogen receptors present experienced breast cancer remission after adrenalectomy. However, in human mammary tumors exhibiting estrogen binding, 10 of 13 showed some degree of regression following adrenalectomy. Clinical use of this finding could be of immense value in indicating which patients are likely to benefit from endocrine ablative surgery. It could spare the trauma of surgery to patients unlikely to benefit from adrenalectomy or ovariectomy.

In addition to the findings correlating receptor protein and tumor response to estrogen, Feherty et al. (1971) found that a higher percentage of carcinomas than benign biopsies possessed estrogen receptors. In biopsies from benign breast tumors, only 3 of 41 possessed detectable estrogen receptors. Receptors were present in 37 or 53 mammary carcinomas, indicating that malignant cancers of the breast are more likely to be hormone responsive than the benign tumors.

Prolactin Receptor Assays

Prolactin receptor assays have been developed more recently than the estrogen receptor assays. A particulate tissue prolactin receptor assay was described by Turkington (1974). This method used lactoperoxidase-^{125}I-labeled prolactin to measure the displaceable binding of the hormone. This system was highly specific for prolactin although growth hormone and human placental lactogen cross-reacted to some degree. The receptor was determined to be a protein since pretreatment with trypsin reduced the prolactin binding, while pretreatment with DNase and RNase was without effect on binding. Specific binding was also localized in the plasma membranes with no specific binding found in either the nuclear or ribosomal fractions (Turkington et al., 1973a).

Shiu et al. (1973) reported a radioreceptor assay which is based on tissue membrane uptake of prolactin. This is described as an assay for prolactin using rabbit mammary tissue membranes, but has also been modified to quantify the amount of binding which can be exhibited by membranes prepared from other tissue types (personal

communication). The membrane receptors described (Shiu et al., 1973) have been shown to be specific for lactotropic hormones.

Using a modification of the technique developed by Shiu and coworkers, Costlow et al. (1974) measured the prolactin receptors in tissue slices from the transplantable R3230AC rat mammary carcinoma, a tumor which is not dependent on estrogen or prolactin for growth. High affinity prolactin binding sites were present in the tumor. A calculated dissociation constant for the prolactin binding sites in the tumor was similar to the dissociation constant calculated for receptor sites in normal lactating mammary tissue. Extrapolation of a Scatchard plot of the binding data to the absissa yielded the number of binding sites. This was found to be 0.99 $\pm$ 0.39 femtomoles receptor/ug DNA for the lactating gland, and 0.61 $\pm$ 0.28 femtomoles/ug DNA for the R3230AC carcinoma. This tissue slice determination has the drawback of making the receptor sites less readily available to the labeled hormone in the incubation medium than is true for the purified membrane preparation. It does have the advantage, however, of being closer to the normal physiological condition of the tissue.

Other work on prolactin receptors in tumor cells appeared about the same time in a study comparing mouse C3H tumors with DMBA-induced and R3230AC carcinomas in the rat (Turkington, 1974). He found similar amounts of receptors in lactating mammary tissue and DMBA-induced tumors (15.5×10^{-13} and 14.5×10^{-13} moles/mg protein, respectively). The dissociation constants were the same for these two tissues (7.1×10^{-9}M). The R3230AC carcinoma receptor sites, however, had less affinity (Kd = 6.0×10^{-9}M) and were fewer in number (2.0×10^{-13} moles/mg protein). This agrees with the known

prolactin dependency of the DMBA tumor and the lack of prolactin dependency of the R3230AC carcinoma. Turkington (1974) found that different DMBA carcinomas from the same animal could vary considerably in the amount of receptor present, possessing 30 - 80% of the amount of receptor found in the lactating gland. The R3230AC tumor contained only 15% of the receptors found in the lactating rat mammary gland, while the prolactin independent mouse C3H tumor contained no detectable receptor. It is difficult to compare the quantitative results of this and the previous study (Costlow et al., 1974), even though the R3230AC carcinoma is described in both, because of the difference in technique, as well as the difference in mode of expressing the concentration of the receptor. The trends in both studies are definitely in agreement.

Growth Hormone and Protein Deficiency Effects on Mammary Tumor Induction and Development

Growth hormone (GH) is secreted by the anterior pituitary and has been shown to have numerous metabolic effects. Greenbaum and McClean (1953) followed the time course of the effects of GH on lipid mobilization and showed an increase in lipid in rat liver and plasma 3 to 6 hours after GH injection. These concentrations returned to control values by 24 hours after treatment. GH can induce hypoglycemia and has anti-insulin effects (Young, 1953; Di Bodo and Altszuler, 1957). GH can synergize with steroids and other anterior pituitary hormones in a variety of physiological functions including development of the mammary gland (Li, 1956; Moon, 1961; Lyons et al.,

1958). GH also promotes nitrogen retention. Rats treated with GH while on a protein deficient diet were found to excrete less nitrogen in the urine than rats not given GH injection (Gordon et al., 1947). The effects of GH on nitrogen excretion were also observed in rats fed a normal diet containing adequate protein. This relation of GH to nitrogen retention can be directly correlated with conservation of protein in the animal (Bennett et al., 1948).

Contrary to findings in mice and humans, GH levels in the pituitary are reduced in the protein deficient and starved rats (Srebnik et al., 1959; Dickerman et al., 1969). While experiments in mice and rats have generally shown that establishment of tumors is inhibited by protein deficiency, there is disagreement as to the effects on growth of established tumors (Tannenbaum, 1953). Severe protein deficiency caused transplanted mouse adenocarcinomas to grow at a rate only 74% of that observed in controls after three weeks of observation (White and Belkin, 1945). On the other hand, Green et al. (1950) found no difference in the rate of growth of transplanted Walker 259 (granulosa cell) tumors in rats on low protein diets once the tumors had become established. In a series of experiments on spontaneous mammary tumors and induced skin tumors and sarcomas in mice (Tannenbaum and Silverstone, 1949), no difference in tumor growth rate was found with diets of 9% to 45% protein. None of the studies in rats have attempted to relate the observed effects on tumors to a reduction in GH or the ability of GH to promote nitrogen retention. While a complete listing of all the functions of GH under different physiological conditions in beyond the scope of this review, it is important to consider the metabolic effects of

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GH and the effects of GH on protein retention when assessing the results of experiments involving the relationship of GH to mammary tumor development.

Long-term injections of GH result in increased incidence of neoplasms in rat mammary glands (Evans and Simpson, 1931). This was confirmed by Moon et al. (1950) who also reported increased incidence of lung and lymph tissue neoplasms in rats. However, later Moon et al. (1951) were unable to observe any increase in incidence of tumors in hypophysectomized rats given GH injections for a prolonged period. GH injections were unable to reactivate growth of mammary tumors in rats bearing tumors which had been classified as stable (Young and Cowan, 1963). Nandi et al. (1960) reported that GH in combination with estrogen and progesterone could promote mammary tumor growth in hypophysectomized C3H/Crgl mice to the development seen in control intact mice. Sinha et al. (1974) found higher serum levels of GH in C3H/St mice, a strain which has a high incidence of spontaneous mammary tumors, than in C57Bl/St mice, which have a lower mammary tumor incidence. This same correlation did not hold for prolactin levels in these two strains of mice.

Pearson and Ray (1959) found an increase in urinary calcium excretion in 2 of 5 breast cancer patients who had been hypophysectomized and later were treated with human GH. The increased calcium excretion was considered a sign of progression of the osteolytic metastasized breast cancer. This increase in calcium excretion with GH administration was found only in women who had experienced no regression of breast cancer in response to hypophysectomy. These findings were confirmed by Lipsett and Bergenstal (1960), although

they considered their results paradoxical. They reasoned that if GH promoted breast cancer growth, hypophysectomy should cause a GH dependent tumor to regress, and subsequent GH injections should reactivate growth of the regressing mammary tumor. However, they found GH effective in stimulating calcium excretion only in patients who experienced no mammary tumor regression upon hypophysectomy. Thus, they believed that their results did not support a role for GH in promotion of breast cancer growth.

Inconclusive experiments leave the role of GH in mammary cancer development in doubt. This area of research lacks the substantial body of evidence that exists for the influence of estrogen and prolactin on mammary cancer development. Further studies are necessary to provide a clear understanding of any actions that GH may have on the induction or growth of mammary cancers.

MATERIALS AND METHODS

Research Animals

All animals in these experiments were female Sprague-Dawley rats, 50-55 days of age when obtained from Spartan Research Animals, Haslett, Michigan. They were housed, 4 animals to a cage, in plastic cages in a temperature-controlled (25 ± 1°C) room, with 14 hours of light daily (5:00 AM - 7:00 PM). Animals were fed ad libitum on a diet of tap water and Wayne Lab Blox pellets (Allied Mills, Chicago, Ill.).

Tumor Induction

The rats were given a single injection of 5 mg 7,12-dimethylbenz(a)anthracene (DMBA) in 1 ml of a lipid emulsion via the tail vein at 55 - 60 days of age, according to the procedure of Huggins (1965). Injections were given under light ether anesthesia. Rats were checked for tumor development by palpation once each week from one month after DMBA injection until commencement of treatment. Tumors appeared in 1 to 3 months in all animals injected, with less than a 5% mortality rate occurring the first 60 days after DMBA injection.

Tumor Measurements

Animals were carefully palpated at weekly or more frequent intervals to locate all tumors, and shaved in those areas where tumors were detected. Tumors were measured using calipers while the animal was under light ether anesthesia. Each tumor was pulled up from beneath the skin and held between the thumb and forefinger as measurements of length, width, and depth were taken. These were recorded on a data sheet prepared for each animal which showed the location of the tumor on a diagram (See Fig. 1). Diameters were recorded to the nearest millimeter for each of the 3 dimensions of a tumor, and the sum of these 3 measurements (length + width + depth) was used for data analysis.

Prolactin Receptor Assay

The prolactin receptor assay was performed in Dr. Henry Friesen's laboratory (Royal Victoria Hospital, McGill University, Montreal, Canada) by his personnel according to the procedure described by Shiu *et al.* (1973). This technique requires a lactating mammary membrane preparation obtained from rabbits injected intramuscularly for 4 days with 10 mg human placental lactogen and 5 mg hydrocortisone to induce lactation. The mammary tissue was removed, homogenized, and filtered through cheese cloth. The filtrate was centrifuged at 15,000 g and the resulting supernatent was centrifuged at 15,000 g and then at 100,000 g. The final pellet containing the microsomal membranes was resuspended in 0.025 M tris-HCl buffer at

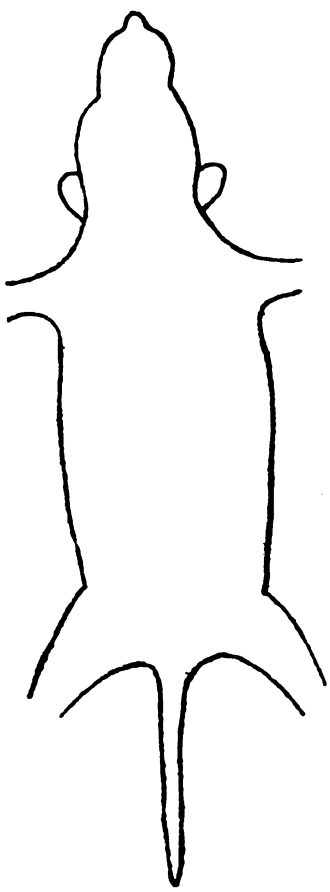
Figure 1

DATA SHEET FOR RECORDING MAMMARY TUMOR LOCATIONS AND MEASUREMENTS

Rat No._ _ _ _ _ _ _ _ _ _ _ _ _ _ _

Treatment_ _ _ _ _ _ _ _ _ _ _ _ _ _

Starting Date_ _ _ _ _ _ _ _ _ _

Wk 3	Wk 2	Wk 1	Pre-tx		Pre-tx	Wk 1	Wk 2	Wk 3
								

Total No. of Tumor_ _ _ _ _ _ _ _ _ _ _ _

Size Average of Tumor _ _ _ _ _ _ _ _ _ _

pH 7.6 containing 10 mm $CaCl_2$ and diluted to a final concentration of 100 - 300 ug of protein per 0.1 ml. This constituted the standard prolactin receptor membrane preparation.

Purified prolactin was labeled with ^{125}I by a lactoperoxidase and hydrogen peroxide method which permitted the prolactin to retain its biological potency. This method has been described by Thorell and Johansson (1971).

One-tenth ml of membrane preparation was incubated with labeled prolactin and brought to a final volume of 0.5 ml with tris buffer. This was incubated at 25°C for 90 minutes with graded amounts of unlabeled hormone in 0.1 ml to provide a standard. At the end of the incubation period, 3 ml ice cold buffer was added and the sample filtered through a millipore filter under suction. The sample was then washed twice with 5 ml cold buffer before counting the filter membrane in a plastic tube in a gamma spectrometer.

Replacing the rabbit mammary tissue preparation with a similarly prepared rat mammary carcinoma preparation allowed assay of the receptor content of this tissue. The carcinoma tissue was incubated with a known amount of labeled and unlabeled prolactin and the amount of displaceable binding per 300 mg of tumor protein was determined.

RELATION OF MAMMARY TUMOR GROWTH RESPONSE TO PROLACTIN TO THE AMOUNT OF PROLACTIN RECEPTOR PROTEIN IN THE TUMOR

Objectives

While estrogen receptor assays are of some value in predicting the response to endocrine ablative therapy (Jensen, 1971), studies seeking to establish a relationship between prolactin receptors and tumor dependency have not been reported. The development of relatively sensitive prolactin receptor assays (Turkington, 1973; Shiu et al., 1973) has provided an opportunity to study the mechanism of prolactin action in promoting mammary tumor growth by measuring the prolactin receptors present in tumor membranes. Some tumors respond to prolactin excess or lack with a greatly accelerated or decreased growth rate, respectively, while others may be indifferent to altered prolactin levels. It was of interest, therefore, to determine whether this growth response to prolactin could be correlated with the amount of prolactin that was specifically bound by the tumor membrane.

Procedure

Female Sprague-Dawley rats, 55 - 60 days of age were given a single intravenous injection of an emulsion containing 5 mg

7,12-dimethylbenz(a)anthracene (DMBA). Two and one half months later when tumors had developed to approximately 2 cm in diameter, 10 rats were given daily subcutaneous injections of 1 mg NIH ovine prolactin (oPRL, 26 IU/mg) dissolved in 0.85% saline made slightly basic with 0.1 N NaOH. Tumors were measured with calipers for length, width, and depth, initially and every 4 days throughout the 12-day treatment period. Following a six-day non-treatment period the animals were sacrificed and a total of 34 tumors were excised, weighed, and frozen on Dry Ice. One to 8 cancers were removed from each animal as well as liver tissue to be included in the prolactin receptor assay.

The difference in the sum of the three diameters of each tumor (length + width + depth) at the beginning and end of the treatment period was calculated and defined as the growth index for each tumor. This was considered to indicate the degree to which the individual tumor responded to prolactin treatment. The 34 tumors present were ranked from 1 to 34 according to their growth response (growth index).

The excised tumors and livers were homogenized in 0.3 M sucrose and the membrane fraction was prepared for assay of specific ^{125}I-labeled prolactin binding for the radio receptor assay. The results of the assays were compiled before the growth index ranking was made known to Dr. Friesen's laboratory in order to rule out possible bias in the assays.

Results

The tumors were arranged in 4 groups according to their rank as shown in Table 1 together with the growth index and the specific binding of ^{125}I-labeled prolactin exhibited by the membrane preparation of each tumor. Those tumors showing the largest growth response to prolactin also showed the greatest amount of specific prolactin binding to membrane preparations (13.4 - 34.1%). Conversely, those tumors showing little or no growth response to prolactin, were found to bind the smallest amount of labeled prolactin (2.0 - 18%). Analysis by linear regression gave a correlation coefficient of $r = 0.69$ ($p < 0.01$) for ^{125}I-prolactin binding and tumor responsiveness to prolactin.

The livers from the 10 animals were also ranked according to the combined growth indices of all tumors from each rat, and separated into 3 groups based on this ranking. An inverse relationship was found, with the rats showing the largest tumor growth responses to prolactin possessing livers with the lowest prolactin binding, and vice versa. These liver groupings, average growth indices, and prolactin binding figures are shown in Table 2. The calculated correlation coefficient from analysis by linear regression was $r = -0.69$ ($p < 0.05$) for ^{125}I-prolactin and average tumor growth index. This is precisely the negative of the tumor prolactin binding correlation with the growth response.

Table 1.

TUMOR GROWTH AND SPECIFIC BINDING OF ^{125}I-LABELED oPRL

RANK	FINAL TUMOR SIZE (cm)	GROWTH INDEX (cm)	SPECIFIC ^{125}I-oPRL BINDING** (%)
1 - 8	6.1 ± 1.1*	2.2 ± 0.2	22.1 ± 2.4
9 - 17	5.0 ± 0.6	1.0 ± 0.6	12.7 ± 2.6
18 - 25	3.3 ± 0.5	0.5 ± 0.0	10.0 ± 2.4
26 - 34	2.8 ± 0.2	0.0 ± 0.0	7.3 ± 1.6

* Average ± Standard Error of the Mean

**Total Counts/Minute Minus Non-Specific Counts Bound to Membrane (Counts in Presence of Excess Unlabeled Prolactin), Expressed as a Percent of Total Counts Added to the Sample.

Table 2.

TUMOR GROWTH AND SPECIFIC BINDING OF ^{125}I-oPRL IN LIVER MEMBRANES OF DMBA-TREATED RATS

RANK	AVERAGE* GROWTH INDEX (cm)	SPECIFIC ^{125}I-oPRL BINDING (%)
1 - 4	1.4 ± 0.1	4.8 ± 1.0
5 - 7	0.6 ± 0.1	10.0 ± 3.5
8 - 10	0.4 ± 0.1	17.2 ± 5.5

*When Rats Possessed More Than One Tumor, the Growth Indices From All the Tumors Were Averaged.

Conclusions

These data indicate that there is a high correlation between the tumor growth response to prolactin and the amount of tumor membrane prolactin binding. There is also a strong negative correlation between the tumor growth rate in response to prolactin and the amount of prolactin binding to rat liver membrane fractions. Thus mammary tumor responsiveness to prolactin appears to depend on the number of prolactin binding sites present in the tumor tissue, and bears a negative correlation to the number of prolactin binding sites found in the liver.

INDIVIDUAL MAMMARY TUMOR RESPONSE TO ESTROGEN AND PROLACTIN AT EARLY AND LATE STAGES OF TUMOR DEVELOPMENT

Objectives

The role of estrogen and prolactin in promoting mammary cancer growth is well established in both rats and mice, but further evidence is necessary to establish the role of prolactin in human breast cancer. Estrogen in small doses and prolactin in any dose can accelerate mammary tumor growth in rats, and a deficiency of either of these hormones results in a general decrease in tumor growth. These observations have been based on the general trend or average response of entire tumor sample populations. However, within a large sample of tumors, varying degrees of response can be observed, with some tumors showing marked growth, others with moderate or slight growth, some with no response, and some that actually regress under estrogen and prolactin treatments. These variations in response to estrogen and prolactin may indicate that some tumors are relatively indifferent to these hormones, or that other factors exist which are exerting a stronger influence on tumor growth at a particular stage of development.

This experiment was designed to examine the response of individual rat mammary tumors to estrogen and prolactin deprivation and replacement, when tested singly or in combination. The effects of

these two hormones at both early and later stages of tumor development also were studied to determine whether age of the tumors at initiation of testing could influence the response to estrogen and prolactin.

Procedure

Virgin female Sprague-Dawley rats, 55 - 60 days of age were given a single intravenous injection of a lipid emulsion containing 5 mg of 7,12-dimethylbenz(a)anthracene (DMBA). The DMBA was obtained from the Upjohn Company, Kalamazoo, Michigan. Two and one half months later, 100 animals were randomly separated into groups of 20 rats each and subjected to 4 weeks of treatment, divided into two phases of two weeks each. The pattern of treatment is shown in Table 3.

Group A received injections of 0.85% NaCl throughout both phases of treatment and served as a control group with normal prolactin and estrogen levels. Group B was ovariectomized (OVX) for the first phase of treatment to remove the primary source of estrogen, and 3.75 ug of estradiol benzoate (EB) was given during the second phase to correct the estrogen deficiency. Group C was given injections of 0.5 mg ergocornine methanesulfonate (EC) obtained from the Sandoz Company, Basel, Switzerland, to reduce prolactin levels during the first phase of treatment, followed by ovariectomy and injections of 120 ug of haloperidol (HAL) obtained from McNeil Laboratories Inc., Fort Washington, Pa., to reduce estrogen and raise prolactin during the second phase. Animals in group D were first

ovariectomized and given injections of 0.5 mg EC to reduce both estrogen and prolactin levels, then 3.75 ug EB and 120 ug HAL were injected to increase the estrogen and prolactin levels during the second phase of treatment. Group E was ovariectomized and given 120 ug HAL during the first phase of treatment to reduce estrogen while maintaining high prolactin levels, while the second treatment of 2.75 ug EB and 0.5 mg EC raised estrogen levels and reduced prolactin.

All injections were given subcutaneously daily between 10 and 11:00 AM in a volume of 0.2 ml. EB and HAL were suspended in corn oil. EC was dissolved in 70% ethanol and diluted with 0.85% NaCl to a final concentration of 14% ethanol.

A pretreatment measurement of tumor size and number and animal body weights were recorded. During the treatment the tumors were measured weekly for length, width, and depth to the nearest mm in each dimension using calipers. The sum of the length, width, and depth of each tumor at the beginning of treatment was compared with the sum of the three diameters at the end of treatment. Each tumor was classified as growing, regressing, or stable at the end of both the first and second treatments. A tumor which had increased by 3 mm or more in the sum of its measurements was classified as growing, while those which had decreased by 3 mm or more were classified as regressing. A tumor which had changed by less than 3 mm in the sum of its diameters was considered stable. The second phase of treatment was started immediately after the first two weeks of treatment in order to determine the effects of changing estrogen and prolactin levels in opposite directions from the first treatment phase. Thus the same tumor could be subjected to both hormone deprival and replacement.

A second experiment with 100 rats was started 5 months after DMBA injection and followed the same treatment schedule as those begun at 2 1/2 months after DMBA injection (see Table 3).

Results

The classification of tumors is presented in Tables 4 and 5 for rats at 2 1/2 and 5 months after DMBA injection. Table 4 shows that the control rats at 2 1/2 months (group A) had 79 tumors at the beginning of treatment and 6.3% were regressing, 17.7% were stable, and 76% were growing. During the second 2 week period there were 87 tumors of which 23% were regressing, 19.6% were stable, and 57.4% were growing. Ovariectomy (group B) caused 89.2% of 65 tumors to regress, while only 6.2% continued to grow. EB injections during the second phase of treatment brought the number of tumors growing up to 41.8% while 27.3% regressed under this treatment. EC injections into intact rats (group C) bearing 56 tumors resulted in 64.3% of the tumors regressing while 14.3% grew. Ovariectomy and HAL injections to these same rats caused 32.1% of the tumors to regress while 41.5% grew. Ovariectomized rats receiving EC injections (group D) had regression of 96.2% of the 52 tumors and none grew. When HAL and EB were given during the second phase of treatment only 31.7% regressed while 61% grew. Ovariectomized and HAL treated rats (group E) had 58.1% of 43 tumors regress and 37.2% grow. EC and EB treatments raised the percentage of regressing tumors to 79.1% during the second 2-week period and only 11.6% of the tumors grew.

Table 3.

TREATMENT SCHEDULE FOR RATS WITH MAMMARY TUMORS

GROUP	FIRST TWO WEEK TREATMENT	SECOND TWO WEEK TREATMENT
A	Intact, .85% NaCl	Intact, 0.85% NaCl
B	OVX, 0.85% NaCl	OVX, 3.75 ug EB
C	Intact, 0.5 mg EC	OVX, 120 ug HAL
D	OVX, 0.5 mg EC	OVX, 3.75 ug EB, 120 ug HAL
E	OVX, 120 ug HAL	OVX, 3.75 ug EB, 0.5 mg EC

OVX = Ovariectomized EC = Ergocornine methanesulfonate HAL = Haloperidol

EB = Estradiol benzoate

Table 4.

MAMMARY TUMOR RESPONSE TO TREATMENTS 2 1/2 MONTHS AFTER DMBA INJECTION

TREATMENT	NUMBER OF RATS	TOTAL TUMORS	REGRESSED	STABLE	GREW
A 1) 0.85% NaCl	13	79	5(6.3%)	14(17.7%)	60(76.0%)
2) 0.85% NaCl		87	20(23.0%)	17(19.6%)	50(57.4%)
B 1) OVX	16	65	58(89.2%)	3(4.6%)	4(6.2%)
2) OVX + EB		55	15(27.3%)	17(30.9%)	23(41.8%)
C 1) EC	14	56	36(64.3%)	12(21.4%)	8(14.3%)
2) OVX + HAL		53	17(32.1%)	14(26.4%)	22(41.5%)
D 1) OVX + EC	15	52	50(96.2%)	2(3.8%)	0(0%)
2) OVX + HAL + EB		41	13(31.7%)	3(7.3%)	25(61.0%)
E 1) OVX + HAL	9	43	25(58.1%)	2(4.7%)	16(37.2%)
2) OVX + EC + EB		43	34(79.1%)	4(9.3%)	5(11.6%)

OVX = Ovariectomized EB = Estradiol benzoate EC = Ergocornine methansulfonate HAL = Haloperidol

1) = 1st 2 weeks of treatment

2) = 2nd 2 weeks of treatment

Table 5.

MAMMARY TUMOR RESPONSE TO TREATMENTS 5 MONTHS AFTER DMBA INJECTION

	TREATMENT	NUMBER OF RATS	TOTAL TUMORS	REGRESSED	STABLE	GREW
A	1) 0.85% NaCl	11	61	14(22.0%)	12(19.6%)	35(57.4%)
	2) 0.85% NaCl		66	24(36.4%)	18(27.3%)	24(36.3%)
B	1) OVX	12	49	37(75.6%)	6(12.2%)	6(12.2%)
	2) OVX + EB		50	9(18.0%)	10(20.0%)	31(62.0%)
C	1) EC	13	61	44(72.2%)	7(11.4%)	10(16.4%)
	2) OVX + HAL		62	14(22.6%)	16(25.8%)	32(56.0%)
D	1) OVX + EC	15	69	61(88.5%)	5(7.2%)	3(4.3%)
	2) OVX + HAL + EB		67	9(13.4%)	18(26.8%)	40(59.7%)
E	1) OVX + HAL	11	67	23(34.4%)	11(16.4%)	33(49.4%)
	2) OVX + EC + EB		69	46(66.7%)	9(13.0%)	14(20.3%)

OVX = Ovariectomized EB = Estradiol benzoate EC = Ergocornine methansulfonate HAL = Haloperidol

1) = 1st 2 weeks of treatment

2) = 2nd 2 weeks of treatment

Table 5 presents the tumor classification when treatment was begun 5 months after DMBA injection. Controls (group A) showed that 22% of the 61 mammary tumors regressed while 57.4% grew during the first treatment. These percentages changed to 36.4% regressing and 36.3% growing during the second 2-week period. Ovariectomy (group B) resulted in 75.6% of the 49 tumors regressing in these older rats, while 12.2% of the tumors grew. After EB injections were given during the second phase, only 18% of the tumors regressed while 62% grew. During EC treatment (group C) 72.2% of 61 tumors regressed while 16.4% grew. In the second phase, ovariectomy and HAL treatment resulted in only 22.6% of the tumors regressing while 56% grew. Ovariectomy and EC treatment (group D) resulted in 88.5% of 69 tumors regressing and only 4.3% growing. Raising the hormone levels during the second phase of treatment caused only 13.4% of the tumors to regress and 59.7% to grow. Ovariectomy and HAL (group E) caused 34.4% of the 67 tumors to regress and 49.4% to grow. EB and EC given the second 2-week period caused 66.7% of the tumors to regress and 20.3% to grow.

Tables 6 and 7 present the percentages of tumors determined to be estrogen or prolactin dependent at each age. The response of each tumor to hormone changes was considered at both the first and second stages of treatment in order for a tumor to be placed in a hormone dependency classification.

At 2 1/2 months post-DMBA (see Table 6) prolactin dependency could be determined from the response of tumors in two of the treatment groups. In group C, 29.8% or 16 or 56 tumors were prolactin dependent. A tumor must have <u>both</u> regressed in the first

Table 6.

CLASSIFICATION OF TUMORS AS PROLACTIN OR ESTROGEN DEPENDENT 2 1/2 MONTHS POST-DMBA

TREATMENT	PROLACTIN DEPENDENT	ESTROGEN DEPENDENT	INDEPENDENT[1]
A 1) SALINE 2) SALINE			0% (0/79)[2] (1. regressed - 2. regressed)[3]
B 1) OVX 2) OVX + EB		35.4% (23/65) (1. regressed - 2. grew)	
C 1) EC 2) OVX + HAL	29.8% (16/56) (1. regressed - 2. grew)		
D 1) OVX + EC 2) OVX + HAL + EB			
E 1) OVX + HAL 2) OVX + EC + EB	28.9% (13/45) (1. grew - 2. regressed)	2.2% (1/45) (1. regressed - 2. grew)	

OVX = Ovariectomized EB = Estradiol benzoate EC = Ergocornine methansulfonate HAL = Haloperidol

[1]Indicated by spontaneous regression in the presence of normal estrogen and prolactin levels.
[2]Number of tumors in this classification/total tumor number.
[3](1. = tumor response to treatment, phase 1; 2 = tumor response to treatment, phase 2). A tumor must exhibit the indicated response in <u>both</u> phases of treatment to be included in the classification.

Table 7.

CLASSIFICATION OF TUMORS AS PROLACTIN OR ESTROGEN DEPENDENT 5 MONTHS POST-DMBA

TREATMENT	PROLACTIN DEPENDENT	ESTROGEN DEPENDENT	INDEPENDENT[1]
A 1) SALINE 2) SALINE			16.4% (10/61)[2] (1. regressed - 2. regressed)[3]
B 1) OVX 2) OVX + EB		42.9% (21/49) (1. regressed - 2. grew)	
C 1) EC 2) OVX + HAL	32.8% (20/61) (1. regressed - 2. grew)		
D 1) OVX + EC 2) OVX + HAL + EB			
E 1) OVX + HAL 2) OVX + EC + EB	34.4% (21/62) (1. grew - 2. regressed)	9.7% (6/62) (1. regressed - 2. grew)	

OVX = Ovariectomized EB = Estradiol benzoate EC = Ergocornine methansulfonate HAL = Haloperidol

[1]Indicated by spontaneous regression in the presence of normal levels of estrogen and prolactin.
[2]Number of tumors in this classification/total tumor number.
[3](1. = tumor response to treatment, phase 1; 2 = tumor response to treatment, phase 2)
A tumor must exhibit the indicated response in both phases of treatment to be included in the classification.

treatment phase when prolactin was reduced by EC injection, and grown in the second phase when prolactin was raised by HAL injections in order to be classified as prolactin dependent. In group E, 28.9% or 13 of 45 tumors were prolactin dependent, based upon tumor growth in response to HAL injections in OVX rats and regression when prolactin levels were depressed by EC injections despite EB replacement. Again, a tumor had to exhibit the proper growth response during both phases of treatment to be classified as prolactin dependent.

Estrogen dependency determinations could also be made in two treatment groups. At 2 1/2 months post-DMBA injection, 35.4% or 23 of 65 tumors were considered estrogen dependent in group B because they both regressed in response to ovariectomy and grew when estrogen replacement was given. In group E, estrogen dependent tumors both regressed in response to ovariectomy when prolactin levels were maintained by HAL injections, and grew during the second phase when EB and EC were given to replace estrogen while lowering prolactin.

Tumors were classified as independent of estrogen or prolactin if they spontaneously regressed in the presence of normal levels of estrogen and prolactin, continuously, throughout both 2-week phases of treatment. No tumors in the control group (group A) regressed during both phases of treatment, although there were tumors which regressed during phase 1 or 2 only, but were stable or grew during the other treatment phase.

The same criteria for hormone dependency were used for tumor classification 5 months after DMBA injection. Again the response of the same tumor was considered in both phases of treatment. Prolactin dependency was found in 32.8% or 20 of 61 tumors in group C (see

Table 7) based upon regression and then growth in response to the first and second treatment phases. In group E, 34.4% or 21 of 62 tumors were prolactin dependent as indicated first by growth, and then by regression in response to the two treatments. Estrogen dependency was found in 42.9% or 21 of 49 tumors in group B. These tumors first regressed and then grew in response to the treatments. In group E, 9.7% or 6 of 62 tumors showed estrogen dependency by regression in response to phase 1 of treatment and growth in response to phase 2.

At 5 months, independent tumors, i.e. those which spontaneously regressed throughout both phases of the 4 weeks of treatment in the presence of normal levels of both hormones, made up 16.4% or 10 of 61 tumors in the controls (group A).

Conclusions

These results show that all rat mammary tumors do not show the same growth response to alterations in estrogen or prolactin levels. At 2 1/2 months after DMBA administration, removal of both hormones by OVX and EC (group D) caused nearly 100% of the tumors to regress. Removal of estrogen by OVX (group B) caused the second highest regression rate, which probably reflected not only estrogen removal, but also the action of OVX in decreasing prolactin secretion. EC suppression of prolactin alone (group C) was slightly more effective in causing tumor regression than reduction of estrogen alone by OVX with HAL injections to prevent concurrent reduction of prolactin (group E).

At 5 months after DMBA injection, the same relative effects of the treatments were seen. Again, reduction of both estrogen and

prolactin (group D) was more effective in causing tumor regression than a decrease of either alone. OVX (group B) caused the second highest regression rate, probably because of loss of estrogen and the secondary decline in prolactin levels. Prolactin reduction by EC (group C) caused slightly less tumor regression than OVX. Again, ovariectomy with prolactin replacement (group E) was the least effective in inducing tumor regression, suggesting that prolactin is more important than estrogen for maintenance of mammary cancer growth.

The percentage of tumor regressions in response to hormone changes is probably more accurately reflected when the percentage of spontaneously regressing tumors in the control rats (group A) is subtracted from the percentage of regressing tumors in each treatment group. This reduces the number of regressing tumors that can be attributed to hormone changes to a greater extent in the older tumors, which had a higher spontaneous regression rate in the controls. Such an adjustment makes the greater hormone responsiveness of the younger tumors more obvious than the percentages of regressing tumors alone indicate.

A larger percentage of DMBA-induced mammary tumors in rats 2 1/2 months after administration of the carcinogen regressed upon withdrawal of estrogen or prolactin than at 5 months post-DMBA. The younger tumors also had a greater number of tumors regressing and fewer tumors growing during the second phase of treatment, when hormone levels were varied in the opposite direction from the levels in the first phase. An exception occurred in group C in which the percentage of regressing tumors was greater during EC treatment at 5 months post-DMBA than at 2 1/2 months post-DMBA. However, this exception

also agrees with the above pattern if the percentages of regressing tumors are adjusted to allow for the higher spontaneous regression rate in the older rats.

The percentage of spontaneously regressing tumors in the control group (A) was greater in the older than the younger rats, indicating a greater independency of tumor growth from the hormonal environment in the older tumors. Also, the percentage of tumors which regressed when the hormones were decreased was generally greater in the younger than in the older rats, another indication of less hormonal dependency and greater autonomy in growth of older tumors.

The hormone dependency classification shows that about the same percentage of tumors was prolactin dependent in both age groups, and a slightly higher percentage of tumors was estrogen dependent in the older rats (Tables 6 & 7). The great difference in estrogen dependency shown between groups B and E in both age groups, is probably due to the dual effects of ovariectomy in removing estrogen and in reducing prolactin secretion. The relatively small percentage of tumors regressing when the ovariectomy reduction of prolactin secretion was prevented by injections of HAL again suggests that prolactin is more important for maintenance of mammary tumor growth than estrogen.

EFFECTS OF PROTEIN DEFICIENCY AND GROWTH HORMONE ADMINISTRATION ON GROWTH OF DMBA-INDUCED MAMMARY TUMORS IN RATS

Objectives

Reduced food intake (Meites and Fiel, 1965; Dickerman et al., 1969) or decreased protein intake (Srebnik et al., 1959) reduces GH in the pituitary and blood, and growth hormone releasing factor (GH-RF) in the hypothalamus. Gordon et al. (1947) demonstrated that GH is effective in causing nitrogen retention within 24 hours after injection into rats on normal or low protein diets. Experiments on protein restricted diets in rats and mice showed a decrease in tumor size and an increase in tumor latency (summarized by Tannenbaum, 1953).

While estrogen and prolactin have been shown to influence mammary tumor growth under a variety of experimental conditions by numerous investigators, the effects of growth hormone are not yet well defined. GH has been reported to have a permissive action on tumor induction in mice and rats (Nandi et al., 1960; Moon et al., 1950). Endogenous GH serum levels are higher in the C3H/St mouse strain, which has a high incidence of spontaneous mammary tumors, than in the C57BL/St strain of mice which has a low spontaneous tumor incidence (Sinha et al., 1974). Short term effects of GH of the growth rate and number of induced mammary tumor in rats have not been

reported. It was of interest, therefore, to investigate the short term effects of GH on DMBA-induced mammary tumor number and growth in rats fed normal and protein limited diets.

Procedure

Female Sprague-Dawley rats 55 to 60 days of age were given a single intravenous injection of a lipid emulsion containing 5 mg of 7,12-dimethylbenz(a)anthracene (DMBA). Animals were palpated weekly to detect tumors. About 2 1/2 months later, after tumors had developed in most animals, 48 tumor-bearing rats were randomly placed in 6 groups of 8 rats each. Body weights were recorded, and tumors were measured with calipers to the nearest mm in length, width, and depth. Special diets modified from that used by McCollum and Davis (1918) and containing 6%, 12% or 18% vitamin-free casein (see Table 8) were prepared fresh twice weekly. NIH-S8-ovine growth hormone (GH) was injected subcutaneously in a solution containing 1 mg GH/0.2 ml in 0.85% NaCl made slightly basic with 0.1 N NaOH. Treatments were as shown in Table 9: Group 1, 6% casein diet + 0.2 ml 0.85% NaCl; Group 2, 6% casein diet + 1 mg GH; Group 3, 12% casein diet + 0.2 ml 0.85% NaCl; Group 4, 12% casein + 1 mg GH; Group 5, 18% casein diet + 0.2 ml 0.85% NaCl; Group 6, 18% casein diet + 1 mg GH. Injections of saline or GH were given daily between 10 and 11:00 AM. The food intake was monitored daily and the feed regulated to maintain approximately 13 g average daily consumption per rat in all treatment groups.

Table 8.

COMPOSITION OF EXPERIMENTAL DIETS

INGREDIENTS	6% CASEIN DIET	12% CASEIN DIET	18% CASEIN DIET
Casein[1]	60*	120	180
Mazola corn oil	150	150	150
Vitamin mixture[1]	22	22	22
Salt mixture (MD No 185)[1]	37	37	37
Sucrose	731	671	611
Total	1000 g	1000 g	1000 g

*All numbers represent grams/kg diet weight

[1]Nutritional Biochemical Company, Cleveland, Ohio

Tumor measurements and body weights were recorded weekly throughout the 3 weeks of treatment. The sum of the 3 dimensions of each tumor (length, width + depth) was totaled for all tumors per rat and was used for the total tumor diameter of that rat. Increases in tumor number and diameter were analyzed by analysis of variance and Student-Newman-Keuls comparisons of the means, with the significance level at $p = .05$. One rat in group 1 and another in group 5 died during the course of the experiment and data obtained from these animals were not included in this study.

Results

The rats on the 6% protein diet without GH (group 1) showed an average tumor number of 4.3 ± 0.8 at the beginning of treatment and 4.9 ± 0.9 (+14.0%) at the end of treatment (Table 9). The sum of their tumor diameters rose slightly from 13.3 ± 2.8 to 14.7 ± 2.8 (+10%), an insignificant increase. In rats on 6% protein and GH (group 2), the average number of tumors increased from 3.1 ± 1.0 to 5.6 ± 1.1 (+80.6%), and the average sum of tumor diameters increased from 7.4 ± 2.7 to 11.3 ± 2.4 cm (+52.7%). In rats in 12% protein (group 3), the average tumor number increased from 3.0 ± 0.5 to 5.0 ± 1.1 (+51.5%), and average tumor diameters increased from 8.2 ± 1.6 to 12.4 ± 3.5 cm (+51.2%). In rats on 12% protein and GH (group 4) the average number of tumors increased from 3.3 ± 1.0 to 6.1 ± 1.2 (+84.8%), and the average sum of tumor diameters increased from 7.9 ± 2.1 to 13.7 ± 3.0 cm (+73.4%). Rats on 18% protein (group 5) showed an increase in average tumor number from 5.0 ± 0.7 to 6.9 ± 0.7 (+38.0%), and a rise in average tumor diameter from

Table 9.

EFFECTS OF GROWTH HORMONE (GH) AND DIETARY PROTEIN CONTENT ON RAT MAMMARY TUMOR SIZE AND NUMBER

TREATMENT GROUP	PARAMETERS	PRETREATMENT	WEEK 1	WEEK 2	WEEK 3	% CHANGE
1. 6% Casein + .85% NaCl (7)	No. tumors/rat	4.3± 0.8*	4.8± 1.0	5.0± 0.9	4.9±0.9	+ 14.0
	TTD/rat (cm)	13.3± 2.8	15.2± 2.8	15.7± 3.0	14.7±2.8	+ 10.5
	Body weight (gm)	268 ±12	257 ±11	250 ±10	253 ±9	- 5.6
2. 6% Casein + GH (8)	No. tumors/rat	3.1± 1.0	3.6± 1.1	5.0± 1.1	5.6±1.1	+ 80.6
	TTD/rat (cm)	7.4± 2.7	8.4± 2.7	11.1± 2.7	11.3±2.4	+ 52.7
	Body weight (gm)	269 ± 6	266 ± 5	266 ± 6	260 ±7	- 3.3
3. 12% Casein + .85 NaCl (8)	No. tumors/rat	3.0± 0.6	3.3± 0.8	4.0± 0.9	5.0±1.1	+ 51.5
	TTD/rat (cm)	8.2± 1.6	9.2± 2.2	10.4± 2.5	12.4±3.5	+ 51.2
	Body weight (gm)	262 ± 4	271 ± 4	282 ± 3	280 ±4	+ 6.9
4. 12% Casein + GH (8)	No. tumors/rat	3.3± 1.0	3.9± 1.1	5.4± 1.0	6.1±1.2	+ 84.8
	TTD/rat (cm)	7.9± 2.1	9.0± 2.2	11.9± 2.6	13.7±3.0	+ 73.4
	Body weight (gm)	265 ± 7	277 ± 5	290 ± 6	288 ±8	+ 8.7
5. 18% Casein + .85 NaCl (7)	No. tumors/rat	5.0± 0.7	5.7± 1.0	5.9± 0.9	6.9±0.7	+ 38.0
	TTD/rat (cm)	11.3± 1.8	15.0± 2.6	17.3± 3.1	20.7±3.2	+ 83.2
	Body weight (gm)	265 ± 5	280 ± 8	288 ± 8	298 ±8	+ 12.4
6. 18% Casein + GH (8)	No. tumors/rat	3.4± 0.3	3.8± 0.8	5.5± 0.7	6.3±0.8	+ 85.3
	TTD/rat (cm)	8.1± 2.0	10.3± 2.4	13.3± 2.4	16.2±2.9	+100.0
	Body weight (gm)	263 ± 3	283 ± 2	299 ± 5	306 ±4	+ 16.3

*MEAN ± SEM

TTD = total sum of tumor diameters

11.3 ± 1.8 to 20.7 ± 3.2 cm (+83.2%). In rats given a diet of 18% protein and GH injections, 3.4 ± 0.3 tumors/rat were present at the beginning of the experiment and 6.3 ± 0.8 at the end (+85.3%); the average sum of tumor diameters rose from 8.1 ± 2.0 to 16.3 ± 2.9 cm (+100%).

The rats fed the 6% casein diet showed a slight decrease in weight (Table 9). The rats fed 12% casein showed a slight gain in weight, and rats on the 18% casein showed the greatest weight gain. Growth hormone treated rats weighed slightly more than the saline injected rats on the same diets. However, the increases in weight due to GH were not significant for any of the groups.

Conclusions

Analysis of variance showed a significant effect of dietary protein content on increase in mammary tumor diameter, but not on increase in tumor number. A protein limitation to 6% or 12% resulted in significantly smaller increases in tumor diameter than in rats fed 18% protein. Tumor growth was smaller in rats fed 6% casein than in rats fed 12% casein, but this difference was not significant. However, this difference suggests a relation between the degree of protein deficiency and the degree of tumor growth retardation. Although the rats fed 6% casein had smaller increases in tumor number than rats on 12% or 18% casein, a progressively greater effect on tumor number was not found with increased dietary protein. Rats fed 12% casein had a slightly greater increase in tumor number than rats fed 18% casein, although this difference was not significant.

Growth hormone increased tumor number significantly but did not increase tumor diameter. Rats fed 6% casein and injected with GH showed a significantly greater increase in number of DMBA-induced mammary tumors during the treatment period than control rats fed 6% casein but not given GH injections. Rats fed 12% and 18% protein diets also showed greater increases in tumor number when treated with GH, but these differences were not as large as the increases in rats fed 6% protein. Although rats given GH injections had greater increases in tumor diameters than rats on comparable diets without GH injections, these increases were not statistically significant. The greatest increase in tumor growth in GH treated rats was seen in the rats fed 6% protein, a smaller increase was found in rats fed 12% protein, and the smallest increase was observed in rats fed 18% protein. Thus, there was a strong indication of a progressively greater influence of GH on growth of tumors in rats with a more severe protein deficiency. These results suggest therefore, that protein deficiency can reduce growth of established mammary tumors in rats, and that GH administration can promote an increase in tumor incidence and perhaps stimulate tumor growth as well.

DISCUSSION

Most DMBA-induced rat mammary cancers respond to prolactin administration with accelerated growth, but the degree of response is not the same for all responsive tumors. The extent of the growth response in those tumors that do respond appears to depend upon the number of prolactin receptors in the mammary tumor cell membranes. However, some tumors did not fit the general tumor growth-receptor correlation pattern. There were a few tumors which grew quickly under prolactin treatment but exhibited only a small amount of prolactin binding to cell membranes, whereas, others had a high percentage of prolactin binding but showed very little growth response to prolactin injections. In these cases, other factors appear to determine the growth pattern of the tumors. These other factors cannot be defined at present, but may include estrogen, other hormones, and non-hormonal agents. Further studies are required to clarify the nature of these factors.

The correlation observed in this study between tumor growth response to prolactin and prolactin receptors provides a suggestion for future research in this area. Intermediate mechanisms between prolactin in the serum and the effect of prolactin on mammary tumor growth can now be studied. It would be of interest to study prolactin binding in the mammary tumor cell membrane to determine how the complex acts to promote mammary cancer growth. There is evidence indicating that prolactin binding to the receptor is the key step

in the initiation of the cellular response to prolactin. Unlike estrogen, which must actually enter the cell, prolactin is able to initiate changes within a target tissue when prevented from entering the cell by binding of the prolactin to sepharose beads (Turkington, 1970). This experiment also indicated that the prolactin receptor must exist in the plasma membrane of the cell, while it did not exclude the possibility of other receptor sites within the cell. This again is contrary to the findings for estrogen, which initially forms a complex with a receptor in the cytosol, sedimenting at 9.5s on a sucrose density gradient. This 9.5s receptor is then transformed to a 5s complex localized in the nuclear fraction of the cell (Jensen et al., 1968).

The two-stage estrogen binding pattern provides an explanation for the observation that the presence of estrogen receptor binding is not exclusive to estrogen dependent tumors. Shyamala (1972) has reported that estrogen binds to the cytoplasmic receptors of an estrogen-independent mouse mammary tumor. However, this type of tumor cell appears unable to transform the cytoplasmic receptor complex into nuclear receptor. In this case, binding of estrogen alone is not necessarily indicative of a cellular response to the hormone.

Turkington et al. (1973b) determined that the mechanism of prolactin stimulation of lactating mouse mammary glands involves RNA directed synthesis of a protein kinase which is then activated by cyclic AMP. This sequence of events could not be initiated by cyclic AMP alone, which indicated that cyclic AMP was not a second messenger for prolactin in stimulating casein synthesis. Further

work is still required to determine the cellular events which occur from the time of prolactin binding to the receptor molecule until the increase in RNA synthesis in the nucleus.

The correlation between the growth response to prolactin and the amount of prolactin receptors established in this experiment also allows examination of the action of various drugs and hormones on this tumor growth response to prolactin. Determination of drug and hormone influence on receptor activity could establish whether agents which influence tumor growth work through an effect on the number of prolactin binding sites, thereby affecting tumor response to prolactin. There are preliminary indications that estrogen in moderate doses can increase the number of prolactin binding sites in the rat liver and, in high doses, can decrease prolactin receptor number in normal rat mammary tissue (Gelato, Marshall, and Meites, unpublished data). However, further experiments are required to determine whether the effects of estrogen in stimulating mammary tumor growth may include an increase in the number of prolactin receptors, and thus an increase in tumor growth response to prolactin. Such a relationship would help explain why estrogen can exert its full effect on mammary gland development and mammary tumor growth only in the presence of the anterior pituitary (Lyons et al., 1958; Sterental et al., 1963).

The negative correlation observed for the growth response to prolactin by the mammary tumors and the amount of prolactin binding in the liver has no immediate explanation. It is possible that there is competition for prolactin between the two types of tissue; however, preliminary data indicate that prolactin has little or no effect on

the development of its own receptors (Dr. Paul Kelly, personal communication). The observation that DMBA-induced mammary tumors can grow in rats with normal serum prolactin levels (Nagasawa et al., 1973) suggests that the tumors do not decrease circulating prolactin levels by receptor uptake of prolactin. This is evidence against the idea of competition between the tumor and liver for prolactin. Since the functions of prolactin in the rat liver and the factors which regulate production of prolactin receptors are not well established, the significance of the negative correlation between prolactin receptors in the liver and growth response of rat mammary tumors to prolactin remains unclear. However, the negative correlation between prolactin receptors in the liver and mammary tumors does suggest a link between liver function and hormonal responsiveness of rat mammary tumors that was previously unsuspected.

Classification of mammary tumors based on their hormone dependency is a complex task because of the difficulty in selecting the criteria for establishing hormone dependency. In the second experiment, the parameter used was that the tumor must grow in the presence of a hormone as well as regress in its absence. It is quite probable that such a test excludes some tumors which possess some degree of growth response to estrogen and prolactin. For example, a tumor that grew in the presence of the hormone but remained stable after hormone withdrawal, would not be classified as hormone dependent by the standards specified above. Since this study did not attempt to measure degrees of response, clear responses to both the presence and absence of the hormone were required to distinguish the tumors which were responding to the hormonal change from those which grew or regressed spontaneously.

A second difficulty in hormone dependency classification arises in the means of depressing endogenous hormone levels. Ovariectomy was performed to reduce estrogen to a level which would not support estrogen-dependent tumor growth, by removal of the primary source of estrogen. However, this procedure also results in a prolactin reduction, and replacement with estradiol benzoate also increases prolactin secretion (Nicoll and Meites, 1962; 1964) unless measures are taken to reduce serum prolactin levels.

Although ovariectomy removes the major source of estrogen, the adrenals also are known to secrete estrogen. While the present study has provided evidence supporting greater tumor regression when prolactin and estrogen levels are lowered concurrently, it does not rule out the possibility that some estrogen was secreted by the adrenals during prolactin reducing treatments. Prolactin stimulation of tumor growth may have been particularly effective in this experiment because of potentiating effects of estrogen secreted by the adrenals. Before assigning prolactin a primary role and estrogen a secondary role in stimulation of tumor growth, similar results obtained in experiments with more complete estrogen reduction would be required.

Estrogen may affect mammary tumor growth indirectly, through the hypothalamus, as well as through direct actions on the tumor. Estrogen binding sites have been demonstrated in the hypothalamus by auto-radiographic techniques (Pfaff, 1968). Hypophysectomy decreased hypothalamic uptake of radio-labeled estrogen, thus suggesting a short-loop feedback of pituitary hormones (presumably gonadotropins) on regulation of hypothalamic estrogen binding sites

(McEwen and Pfaff, 1970). The sex steroids are important for control of the cyclic female pattern of gonadotropin secretion as demonstrated by estrogen and testosterone injections given to neonatal female rats (Barraclough, 1967).

Corticoids are necessary for the development of normal rat mammary glands (Lyons et al., 1958), and Dao and Sinha (1973) have reported that DMBA tumor cells in vitro appear to have much the same requirements for DNA synthesis as the normal mammary tissue. Kitay (1971) reported a decrease in corticosterone levels in female rats following ovariectomy. This resulted from a reduction in ACTH which could be reversed by estrogen injections in the ovariectomized animal. Ovariectomy also appeared to cause an increase in the corticosteroid reducing enzymes. Thus estrogen may affect corticoid levels by more than one mechanism.

Ovarian function is believed to be linked to thyroid activity as the thyroid becomes enlarged during puberty, pregnancy, and lactation (Harris and George, 1969). While exogenous injections of estrogen can decrease thyroid activity, there has been no firm correlation of thyroid activity to female sexual cycles. Ovariectomy doesn't cause a distinct alteration in thyroid function. Brown-Grant et al. (1957) demonstrated that diethylstilbestrol inhibition of thyroid function was absent in pituitary stalk-sectioned rabbits with a wax insert to prevent regeneration of portal vessels. Thus, estrogens appear to influence thyroid function through the hypothalamic releasing factor. Since estrogen has such a wide range of influence on anterior pituitary functions, the effects observed on mammary tumor growth following ovariectomy or estrogen replacement may be through

a variety of indirect actions as well as through a direct effect on the tumor tissue.

Ovariectomy also results in removal of the primary source of progesterone. Progesterone is required for mammary tumor epithelial cell DNA synthesis *in vitro* (Dao and Sinha, 1973). It has been shown to stimulate mammary tumor growth in ovariectomized rats (Huggins *et al.*, 1958). Progesterone binding has been demonstrated in cytosol of human and rat mammary cancers, and the presence of progesterone receptors was found to be independent of the presence of estrogen receptors (Terenius, 1973). Administration of progesterone alone or in combination with estradiol at the time of DMBA injection was able to delay the appearance and reduce the number of induced mammary tumors in rats (Kledzik, Bradley, and Meites, unpublished data). In view of these effects of progesterone on mammary tumor growth, the reduction of progesterone as well as estrogen must be considered in evaluating the effects of ovariectomy on mammary tumor growth.

Reduction of prolactin by hypophysectomy is a more difficult procedure than ovariectomy, and has the complication that all other hormones produced by the pituitary are removed as well. For these reasons, ergocornine, a specific chemical suppressor of prolactin was used. Ergocornine has been shown to reduce prolactin serum levels to the same degree as hypophysectomy without altering gonadotropin secretion, although it is not as effective as hypophysectomy in causing mammary tumor regression in rats (Welsch *et al.*, 1973). The dose of replacement estrogen for ovariectomized rats was based upon the recommended replacement dose (Barnes and Eltherington, 1973), although the replacement dose may vary with the strain of rat.

The dose of haloperidol used to stimulate prolactin secretion in ovariectomized rats may have increased prolactin levels above those found in the intact rat. Serum prolactin levels were not measured in this study. This may have affected the hormone dependency determination, but, if so, the two stage test for hormone dependency should have helped to adjust for any weakness in one phase of treatment. Haloperidol is a competitive antagonist of hypothalamic catecholamines. This may be the mechanism by which it increases pituitary prolactin release, since a decrease in catecholamines results in a decrease in PIF in the hypothalamus. Haloperidol injections result in decreased PIF activity in the hypothalami of female rats (Dickerman et al., 1972). It is also suggested that haloperidol is more effective in raising prolactin levels in female than in male rats because of estrogen sensitization of the hypothalamus to haloperidol.

The consistency of the prolactin dependency determinations in the various treatment groups argues well for the methods chosen to regulate prolactin deficiency and replacement. By contrast, the inconsistency in the determinations of the percentages of estrogen dependent tumors suggests that the methods of regulating estrogen levels were not affecting estrogen alone.

No attempt has been made to determine the percentage of tumors which are dependent on both hormones. The criteria for hormone dependency were chosen to indicate tumors which were influenced primarily by one of the two hormones tested. Tumors which could grow under the influence of either of the hormones would not be detected by the method of reducing and replacing one hormone at a time. The

use of receptor assays for estrogen and prolactin may be of value in determining the relative influence of the two hormones on individual tumors.

Previous experiments to determine estrogen dependency in tumors have generally relied upon regression in response to ovariectomy. Teller et al. (1969) found that 81% of ovariectomized mammary tumor-bearing rats experienced a decrease of 25% in the sum of all the tumor diameters per rat. These results agree remarkably well with the response to ovariectomy in the two age groups represented in this thesis (75% and 89% regressing), which is based on individual tumor response. Also, in a study of mammary tumors regressing after ovariectomy, Young et al. (1963) found 81% (43/53 rats) experienced tumor regression. McGuire and Julian (1971) determined estrogen dependency in individual DMBA-induced rat mammary tumors by regression in response to ovariectomy and subsequent growth upon estrogen replacement. These tumors were called "unequivocally" estrogen dependent. Unfortunately, they did not report the number of tumors that were estrogen dependent. They found that only the "unequivocally" estrogen dependent tumors possessed high affinity estrogen binding sites, but that not all of these dependent tumors possessed the high affinity sites. They acknowledged that their procedure for estrogen determination may have had an effect on prolactin secretion, but discounted its importance. Further studies would be required to determine whether a measure of estrogen dependency which maintains normal prolactin levels would result in a higher percentage of "estrogen dependent" tumors with high affinity receptors.

Rat mammary tumor response to ovariectomy was studied at 4, 5, 6 and 7 months after DMBA injection by Griswold and Green (1970). They found a greater and longer lasting decrease in tumor size after ovariectomy at 4 months post-DMBA than at later periods. This agrees with the data in this thesis which showed that 89% of the tumors regressed in response to ovariectomy at 2 1/2 months, and 75% regressed at 5 months post-DMBA. In the same study, Griswold and Green (1970) determined that large tumors tended to be more responsive to androgen treatment in younger rats but progressively became less hormone responsive with age. Smaller tumors showed about the same response to the androgen regardless of the time they were tested. A study of tumor size and response to estrogen showed little difference in the response of large and small tumors to ovariectomy (Griswold et al., 1966). A plot of the tumor regression curve after ovariectomy showed the same slope for large ($>$ 1 gm) or small ($<$ 500 mg) tumors. However, the time at which the tumors were tested was not stated, and the sample size was small. Samples at different times after exposure to DMBA might reveal differences with time.

Tumor prolactin dependency was tested in rats bearing mammary tumors by administration of prolactin antiserum for 36 days (Butler and Pearson, 1971). Under these conditions, 50% (19/20 tumors) regressed and 35% (7/20 tumors) grew, versus 13% (3/23 tumors) which regressed and 57% (13/23 tumors) which grew in controls without prolactin antiserum. Thus, after adjusting for controls, the percentage of prolactin dependent tumors appears to be about 37% which is only slightly higher than the 29% to 34% prolactin dependency

determined in this thesis. Butler and Pearson (1971) failed to state the elapsed time after DMBA administration, which may be a factor contributing to the differences in prolactin dependency in the two studies.

Spontaneous mammary tumor regressions were reported in 13% of 23 untreated rats in the above study (Butler and Pearson, 1971). The age of the tumors at the time of the study wasn't given, but the percentage falls within the range of 0% at 2 1/2 months and 16% at 5 months post-DMBA determined in this thesis. Young and Cowan (1963) were impressed by the large number of DMBA-induced rat mammary tumors that regressed spontaneously or reached a plateau in growth. They found 27% (49/181 tumors) regressing, 52% (95/181 tumors) stable, and 21% (37/181 tumors) growing when tested at 26 weeks post-DMBA. These figures compare with the 36% regressing, 27% stable, and 36% growing observed at 23-25 weeks post-DMBA in the present study. Because the age of the tumors in these two studies is very close, some inherent difference in the strain of Sprague-Dawley rat may account for the differences in the growth patterns of the tumors in the two studies.

Although earlier studies are generally in agreement that chronic low protein diets can delay the appearance of mammary tumors (Tannenbaum, 1953), the experiment reported in this thesis showed no significant effect of protein deficiency on the number of tumors appearing during the 3 weeks of this study. However, in the present study some tumors aleardy were present at the initiation of the protein deficiency whereas this was not always true of the earlier studies. This study showed a definite decrease in growth of established tumors with a low protein diet. Previous studies in mice

were unable to show differences in growth rates of spontaneous mammary tumors when the animals had been full-fed or placed on a restricted diet months before the appearance of the tumors (Tannenbaum, 1940). However, in agreement with this study, mammary tumor growth was restricted in tumors which arose in full-fed animals which were subsequently placed on limited diets. Thus, there may be some adaptive processes which occur with chronic underfeeding that result in delayed tumor appearance, but have less effect on tumor growth rate once the tumors appear. Following this view further, an animal with established tumors that was suddenly placed on a low protein diet would have no chance to adapt physiologically to the deficiency. This is one possible explanation for the lower tumor growth rate which resulted from protein deficiency in the present experiment.

GH levels are reduced in pituitaries and serum of rats on restricted diets (Srebnik et al., 1959; Dickerman et al., 1969). Dickerman et al. (1969) concluded that a reduction of growth hormone releasing factor (GH-RF) during starvation resulted from a lack of amino acids and energy available for GH-RF synthesis. However, Yamomoto (1974) presented results that suggested that increased release of GH-RF caused the lower hypothalamic GH-RF levels. He found decreased synthesis of GH in the pituitary of starved rats, but an increase in the percentage of GH release. However, the exact relationship of GH-RF synthesis and release during starvation has not yet been firmly established.

The observation that GH injections to rats on 6% and 12% protein diets resulted in increases in tumor number and diameter

over control rats on diets of the same protein content, suggests that part of the effect of protein deficiency may be a result of GH reduction. However, the increases in tumor diameter with GH injections were not significant, even though a progressive increase with more severe protein deficiency was strongly suggested. Further studies with larger sample sizes are required to determine whether the suggested graded response to GH is real. GH did have a significant effect on the number of tumors appearing, while the primary effect of protein deficiency was on mammary tumor growth rate. This suggests that some of the effects of protein deficiency on tumor growth are of a different nature than might be caused by GH deficiency alone.

The decrease in mammary tumor growth during protein deficiency may result from a lack of nitrogen necessary for growth of the tumor tissue. However, White and Belkin (1945) reported that transplanted mammary tumors in mice fed low protein diets were able to concentrate nitrogen and grow despite a negative nitrogen balance in the host. They concluded that the tumor was able to draw on the animal's protein stores at the expense of the host. However the mammary tumors in mice often are highly autonomous and may be less affected by dietary insufficiencies than hormone-responsive rat mammary tumors.

GH may partially compensate for protein deficiency indirectly, through its ability to promote nitrogen retention (Gordon et al., 1947). However, the 6% protein diet probably resulted in a negative nitrogen balance despite increased protein retention with GH injections. The ability of GH to mobilize lipids could possibly

contribute to the increase in tumor number, since an increased incidence of mammary tumors has been reported in rats fed a high fat diet (Gammal et al., 1967; Chan and Cohen, 1974). The wide variety of metabolic events affected by GH make it difficult to select those factors which are most important in producing the effects on tumor growth and number observed in the present study. Further work remains to elucidate these processes.

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LIST OF REFERENCES

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