EFFECTS OF OVARIAN STEROIDS ON BOVINE MAMMARY EPITHELIAL CELLS: IN VITRO AND IN VIVO EVIDENCE OF INDIRECT STIMULATION OF PROLIFERATION

by

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(ABSTRACT)

Three studies were conducted to determine the effects of ovarian steroids on bovine mammary epithelial cell proliferation. In a first study, estrogen (E), progesterone (P), or E + P were administered to prepubertal beef heifers and biopsied mammary parenchyma taken before and following treatment were compared for growth by evaluation of histoautoradiographic incorporation of thymidine. Estrogen increased epithelial cell growth by 24 h, and fibroblasts to a lesser magnitude by 48-96 h. Estrogen and P was less effective and P was ineffective in increasing proliferation in all cell types studied. Proliferation of adipocytes was not altered.

A second study characterized hormone responsive proliferation of Mac-T cells, a recent clonal bovine mammary epithelial cell strain. Mac-T cells responded to all hormones tested as would be expected *in vivo*. Additionally, passage, harvesting, quantification, freezing, and co-culture techniques were modified to facilitate uncomplicated, timely, inexpensive, effective testing of growth responsiveness to hormones or growth factors.

In a third study E and P alone, together, with or without serum were unable to increase Mac-T cell proliferation. Serum from prepubertal Holstein heifers after E

treatment did not increase growth of Mac-T cells over serum before treatment. Conditioned media from Mac-T or Fib-T (mammary bovine fibroblast cell line) with or without steroids were tested for ability to increase Mac-T cell proliferation. Growth of Mac-T cells was greatest in Fib-T + E conditioned media followed by Fib-T, then Mac-T and lastly fresh media. Steroid exposure did not enhance the ability of Mac-T cell conditioned media to increase Mac-T cell proliferation.

In conclusion, E appears to be the primary ovarian steroid involved in initiating bovine mammogenesis. However, estrogen's action is not direct and may be caused by paracrine release of growth factors.

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LITERATURE REVIEW

I. INTRODUCTION

The mammary epithelial cell nourishes an entire class of animals from birth to weaning, a period of growth critical to sustaining life. Man's consumption of milk surpasses his capacity to produce it. In fact, pre-weaning needs may not be met by the majority of mothers, only 19, 36, and 50 percent of small, medium, and large breasted women lactate sufficiently to supply their babies' requirements (Fulkerson, 1979). This need and development of other dairy products has fueled the maturation of the modern dairy industry.

In the early nineteenth century, average annual yields of dairy cattle were in the vicinity of 2000 liters, whereas by 1980 it was not uncommon to regularly exceed 6000 liters/yr averages in commercial herds (Cowie et al., 1980). However, herd averages only represent a fraction of maximal, as peak yields near 25,000 liters/yr have been reported. Nutritional, genetic, managerial, and physiological techniques have been employed to achieve these increases.

Although there appears a multitude of methodologies aimed at increasing milk yield, all techniques are dependent upon number of epithelial cells present in the mammary gland. Sud et al. (1968) confirmed that maximal milk production is highly dependent upon maximal udder development. This study was one of many to reveal high correlations ($>.8$) between mammary deoxyribonucleic acid (DNA) and mammary weight or maximal volume. Such DNA measurements made accurate measurements of mammary growth more feasible. Following discovery of the importance of the ovary in mammary development (Hammond, 1925),

exogenous treatment with estrogen (E) and/or progesterone (P) was the first endocrinological method to stimulate udder development. Unfortunately, the details of the mode of action for both hormones is still poorly understood.

II. METHODS OF MEASURING MAMMARY SIZE AND GROWTH

Early gauges of mammary growth involved gross area, weight, or volume displacement measurements. However, Fulkerson (1979) notes that the size of the mammary gland is not an accurate estimate of the amount of glandular tissue present. Using DNA as a method for quantifying cell number proved more effective (Kirkham and Turner 1954). Unfortunately DNA analysis indiscriminately measures all cells present. This can be of particular concern in early growth of the gland, when proportion of parenchyma is modest while adipose and connective tissue is significant.

Attention was soon focused on other cellular components as reliable indices of cell growth or cell number. Many researchers were interested in determining the non-secretory portion of the mammary gland and subtracting that from the total amount of tissue present. One approach was to take mammary tissue and dissect away excess fat, dry to eliminate water and extract lipids by immersing remaining tissue in hot ethanol and then hot ether. The dried, fat-free tissue could then be prepared for ribonucleic acid (RNA), DNA, and hydroxyproline measurements. Amount of hydroxyproline present gives an indication of connective tissue present since this amino acid is a specific and significant component of collagen, the primary constituent of mammary gland connective tissue (Anderson, 1974). These methods in combination with DNA analyses allows for a reasonable estimate of amount of parenchymal tissue. Direct quantification

of epithelial proliferation would be optimal, however.

Proliferative response of various cell types can be individually quantified through autoradiographic techniques in combination with histology. This process exploits the use of thymidine as a necessary specific precursor for DNA synthesis (Walker, 1959). Thus, tritiated thymidine is incorporated only in growing cells. Traurig and Morgan (1964 a and b) used this technique to evaluate mammary growth mediated by ovarian steroids. Because tritium disperses one micron or less from its incorporation site in cells (Traurig and Morgan 1964a), it enabled these researchers to pinpoint which cells were incorporating thymidine and thus preparing to divide. Several reviews have since confirmed that the best way to estimate mammary growth is to use DNA and quantitative histological measures including autoradiography of thymidine incorporation (Kirkham and Turner, 1955; Tucker, 1981).

The methodologies previously described concern whole mammary gland, or biopsied tissue analyses. Abundant work has been done with tissue and cell culture as well. Quantifying growth is obviously an easier task *in vitro*: care of animals and surgical removal of tissue is eliminated and use of radioisotopes is much easier to manage. However, in tissue culture, quantifying growth is still complex owing to the presence of parenchymal and stromal cells. Studying growth in the whole gland or in tissue cultured from the gland often involved semi-qualitative measures including: scoring of whole mounts, preparation of histological sections to determine proportion of stroma to parenchyma, or estimation of areas covered by ductular branching (Cowie and Tindall, 1971). Methods of quantifying growth mentioned previously were also employed. More recently use of isolated cells, especially stem cell culture (culture of cells from a single cell; Schaeffer, 1990), has

provided investigators with other procedures to quantify cell number and study cell growth, providing the cultured cells do not transform their phenotype with successive passages. Hemacytometer counts, when used with trypan blue or erythrocin B stains, are valuable in estimating cell number and viable cell number (Hay, 1986; Freshney, 1987). Additionally, cell culture methods offer the opportunity to study specific types of mammary cells independently.

Measurement of DNA is an accurate estimate of cell number since the amount of DNA per cell is normally constant (Baserga, 1989). Quantifying cell growth by RNA is faulty, since RNA can double before cell division. RNA is a better index of cell size and biosynthetic capacity (Baserga, 1989). Some attempts have been made to quantify cell number by volume with limited success (Bird and Forrester, 1981). Additionally, wet weight has been used to approximate cell number (Freshney, 1987.), however only very large cell numbers can be measured with accuracy. Also, determining slight differences in growth rates is unreasonable using weight or volume methods.

Incorporation of labeled thymidine is a useful technique in ascertaining growth rates. Moreover, in cell culture, incorporation of the DNA precursor does not require subsequent histological evaluation, assuming cells are clonal in nature. Finally, flow cytometry has made direct counting of large numbers of cells feasible. Differential staining of DNA or proteins within a cell make it possible for the flow cytometer to sort cells as well (Crissman et al., 1979; Van Dilla, 1985; Freshney, 1985). These advances in cell research combined with the capacity to isolate and study single cell types from the mammary gland have made cell culture a puissant tool in the study of lactation physiology.

III. PREPUBERTAL PERIOD

Growth of the mammary gland in relation to body growth has been investigated extensively in several species (Cowie, 1949; Silver, 1953; Flux, 1954; Sinha and Tucker, 1966; Sinha and Tucker, 1969; Sejrsen et al., 1982; Foldager and Sejrsen, 1984). In general, the mammary gland grows isometrically relative to the body from birth to a period preceding puberty. Allometric growth occurs at two times: 1) from just before puberty until well into puberty, and 2) during pregnancy. Normal growth of the mammary gland at other times is isometric to that of the body. Growth of the mammary gland during pregnancy and subsequent differentiation of the epithelial cells and secretion of product at birth has received far more attention than the peri-pubertal period of allometric growth.

The research of mammary gland physiology at parturition was partially spurred by extensive literature citing the artificial induction of cull dry cows or young heifers into lactation (presumably to market for public consumption) by exogenous hormones. Folley, Scott, Watson & Bottomley originally published data in 1940 on induced lactation involving only (synthetic) estrogens. Cowie et al. (1952) added P to E, since earlier studies demonstrated P was needed for extensive alveolar development in many species. Moreover, Cowie et al. noted that milk yields from previous E induced lactation trials never approached normal lactational yields (even in animals that showed the best response to exogenous hormones). The next improvement in induced lactation featured a mimicry of pregnancy by administering hormones (E and P) for periods of several months (Turner et al., 1956; Meites, 1961; Naito et al., 1968). Later studies reduced the time needed for hormonal induction of lactation to seven days (Smith and Schanbacher, 1973; Smith and Schanbacher, 1974; Erb et al., 1976). Furthermore, milk yield increases

were noted with additions of corticosteroids (Fulkerson and McDowell, 1975). Soon after another advancement came when reserpine (an alkaloid used to release Prolactin (PRL) from the anterior pituitary) further increased milk yields of cows induced into lactation (Collier et al., 1976). Notwithstanding, E is a necessary component of induced lactation (while P usually increased yields). Estrogen functions principally in enhancing lactational performance by augmenting mammary growth prior to lactation (See Naito et al., 1968 and Keller et al., 1977 for review). Estrogen may not be needed once lactation is established (Tucker, 1974).

These extensive trials provided the dairy industry with a great deal of knowledge about the endocrinological mechanisms mediating a successful lactation. However, a paucity of information exists in the ruminant concerning the peri-pubertal period of allometric growth. The information available on the subject suggests that this period may be of critical importance in determining capacity of ensuing lactations. Several studies have demonstrated that increasing feed consumption during this peri-pubertal period will substantially reduce milk yields (Sejrsen, 1978; Sejrsen et al., 1982; Johnsson et al., 1986; Sejrsen et al., 1986). The mechanism of this decreased milk yield is believed to be a decrease in circulating growth hormone (GH) during the period of high feeding (Johnsson et al., 1986; Sejrsen et al., 1986). Sejrsen et al. showed that exogenous GH increased udder parenchyma in identical twin heifer pairs. Thus, this period of growth and its hormonal control could be crucial to the commencement of a normal lactation.

IV. ESTROGEN AND DEVELOPMENT OF THE MAMMARY GLAND

A. General

Estrogen has long been known to be a mammogenic hormone released during pregnancy. As early as 1915, researchers had determined properties of a substance that is 'active' only during pregnancy and perhaps responsible for breast growth (Frank et al., 1915). By 1939, Bacsich and Folley reported that E does not cause an increase in mammary growth and may in fact inhibit lactation in intact or ovariectomized animals. Spreet (1940) soon after published an article refuting earlier work; stating that E does increase mammary gland growth in hypophysectomized animals if applied topically. This local application of E was also practiced in a human trial in the early 1940's with a woman who had received a ovariectomy (Baron, 1958). Soon researchers were scurrying to elucidate the role of E and P in mammary growth in male and female mice, rabbits, and cows with varying ablation/replacement therapies (Walker and Stanely, 1940; Trentin and Turner, 1941; Loeb and Suntzeff, 1941; Walker and Stanely, 1941; Lyons and McGinty, 1941; and Scharf and Lyons, 1941). Later Lyons (1944) demonstrated that P was responsible for lobule-alveolar development while E is responsible for ductal development as well as making the lobule-alveolar development 'most regular'.

Quantitative measures were employed by the early 1950's in attempt to determine the optimal dose required for maximal growth of mammary tissue (Silver, 1953). Flux (1954) found that mammary gland growth was increased by low levels of E. Also, branching and proliferation of mammary ducts increased with addition of anterior pituitary suspension and P. Lyons et al. (1958) used adrenalectomized-hypophysectomized-ovariectomized mice to show E, P, growth hormone, PRL, and corticosteroids were all needed for proper development of

mammary alveoli.

Studies of E action in the early 1960's proved E was capable of eliciting mammary gland growth in the fetus (Raynuud, 1961). Quantitative analysis was done on effects of E and interactions with other mammogens in stimulating mammary growth in rodents (MacDonald, 1961; Cole and Hopkins, 1962). Although some studies indicated that E may have no effect, or an inhibitory effect, on epithelial proliferation (Godlewski, 1959; Dierwechter, 1962), most research demonstrated that E is a direct or indirect stimulant of mammary epithelial cell growth. *In vitro* studies by Koziorowaska (1962) showed mixed results with direct E stimulation of murine mammary gland tissue. Data supported previous hypotheses that E may stimulate growth indirectly.

The middle sixties and early seventies were marked by a noticeable increase in literature published on artificial induction of lactation in ruminants. Several important findings were made or confirmed. Estrogen and P stimulate mammary proliferation, but estrogen appears to be responsible for ductular, while P is involved with lobule-alveolar formation. The steroids are most effective at defined levels that can alter significantly with species, sex, age, weight, and estrus or menstrual cycle status. The actual amounts of each steroid administered were more important than percentage of one to another as previously thought. Lastly, interactions with other hormones may augment or reduce responsiveness of mammary epithelium to steroid induced growth.

Several events in the last few decades have allowed more comprehensive study of E and progesterones' actions. Cell culture methodology has rapidly expanded such that numerous well characterized mammary epithelial lines from human (tumor or normal) and murine origin are currently available. Unfortunately,

no normal bovine epithelial cell line has been available. Recently, however, two cell lines have been introduced (Hadsell et al., 1990; Hung et al., in press). Consequently, data accumulated on the actions of E on mammary proliferation have been primarily human or rodent oriented. The large body of data from human research on E dependent mammary cell proliferation is directed toward understanding E dependent breast tumors. Besides having a mammary epithelial cell line available for many years, mice are relatively inexpensive and are easy to house, breed, and feed. Data on E and progesterones' effects on human or rodent models will be discussed, but this does not necessarily indicate that data are applicable to the bovine.

Previously it was established that E mediates mammary epithelial proliferation. However, several researchers have demonstrated conditions where growth is not altered. Direct additions of E with or without phenol red (a pH indicator in many commercially available media which has recently been shown to exhibit weak estrogenic effects) do not increase growth of mammary epithelial cells in primary culture from human, rat, or mouse donors (Richards et al., 1988). Estrogen did, however, increase P receptors 150% in murine cells, indicating that the cells were responsive to added E. A separate study found that lactating murine glands were not stimulated by estradiol, but virgin mice exhibited significant increases in DNA synthesis (Shyamala and Ferenczy, 1982).

Breast tumor research as previously stated has amassed much literature. Breast tumor cells in culture seem to respond to mitogens as do normal mammary epithelial cells. In fact, Sonnenchein and Soto (1980) acknowledged "no evidence has suggested that the initiation of growth is different in normal or malignant cells. As far as we know the difference between normal cells and malignant cells depends

instead on the respective abilities of these cells to respond to signals to stop growing." These scientists suggest E may act indirectly by blocking a growth inhibitor. However, the authors also acknowledge that a mitogen released from the pituitary may be responsible for 20-40% of E dependent growth increase obtained in ovariectomized rat uterus. Shafie (1980), working with MCF-7 human breast cells, also hypothesizes that E could depress an inhibitor and cause cell proliferation.

Beck and Garner (1989) dismiss the idea that E blocks an inhibitor of growth, and offer instead that E indirectly stimulates growth through the release of autocrine or paracrine growth factors. Sirbasku has, perhaps, led researchers suggesting that E indirectly prompts growth factors (estromedins) to be released, which subsequently elicit epithelial growth. Sirbasku and Benson (1979) were unable to obtain a mitogenic response to three separate E dependent tumor cell lines by administering E *in vitro*. However, growth of these cells in response to extracts of various organs in E treated animals was significantly increased over control animals. Sirbasku (1980) and Sirbasku and Leland (1982) subsequently strengthened their estromedin proposal and introduced three models of E responsive tumor growth *in vivo*: autocrine, paracrine, or endocrine.

B. Estrogen Stimulation of Various Mammary Epithelial Mitogens

i. Growth Hormone and Insulin Like Growth Factor

Another mechanism by which E may indirectly stimulate mammary growth is through direct or indirect release of growth hormone (GH). However, GH has not been reported to increase mammary epithelial cell growth directly. Concomitantly, GH receptors are lacking in the mammary gland (Akers, 1985, Keys and Djiane,

1988). A recent study indicated GH receptor (GHR) message RNA (mRNA) is present (Glimm et al., 1990), but the majority of evidence indicates GH to be ineffective in altering mammary growth directly. Additionally, Topper and Freeman (1980) observed, "presence of hormone receptors is no guarantee of hormone-responsiveness." However, growth hormone is a known stimulator of insulin like growth factor I (IGF-I) (Mathews et al., 1986; Phipps, 1988; Davis et al., 1989; and Kleinberg et al., 1990). Insulin like growth factor I is one of the few mammary epithelial mitogens considered to elicit direct effects in most mammals surveyed (cow: Shamay et al., 1988; Baumrucker and Stemberger, 1989; Campbell and Baumrucker, 1989; sheep: Winder et al., 1989; goat: Prosser et al., 1988; mice: Imagawa et al., 1986; rat: Daughaday and Rotwein, 1989; rabbit: Costantino et al., 1991; human: Cullen and Lippman, 1989; human tumor: Freed and Herrington, 1989).

Early studies revealed some controversy on whether E increases GH or not. Estrogen, with or without trenbolone acetate (a synthetic androgen), has been tested as an exogenous hormone to improve rate of weight gain, and feed conversion efficiency in steers. In 1976, Wiedemann et al. reported no change in GH levels following exogenous E injections in adult males. However, IGF-I levels dropped. Other studies by several researchers demonstrated endogenous E correlated well with IGF-I levels (Rosenfield et al., 1983; Harris et al., 1985; Daughaday and Rotwein, 1989). Also, exogenous E may increase IGF-I (Hunt et al., 1991). However, E has also been shown to increase uterine growth and uterine IGF-I mRNA without changing serum IGF-I, indicating autocrine or paracrine regulation of growth by E regulated IGF-I (Murphy et al., 1987). Low dose E increased growth hormone in prepubertal girls or rat anterior pituitary cells (Moll

et al., 1986; Simard et al., 1986; Daughaday and Rotwein, 1989). Precocious pubertal children given a luteinizing hormone releasing hormone (LHRH) agonist decreased their abnormally high (for their age, not pubertal status) IGF-I levels (Harris et al., 1985). Moreover, Simard et al. (1986) postulated that the GH increase was caused by E stimulation of growth hormone releasing hormone (GHRH). Wennink additionally confirmed the hypothesis that E increased GH secretion may be caused by an augmented hypothalamic GHRH secretion in his studies with pubertal girls. Studies with rams, wethers or steers showed GH secretion follows E stimulation in ruminants as well (Davis et al., 1977; Brier et al., 1987). However, Gettys et al. (1988) reported ovariectomized heifers did not have lower GH levels than normal heifers. Exogenous E administered in tests performed by Brier et al. (1988 a and b) was only 0.3% of that commonly used to induce lactation. Thus, E induced release of GH may be dose related (Wennink et al., 1991). Abundant literature is available on E mediated increases in GH in the rat and human, but data in the ruminant appear to be primarily limited to males. High dose E experiments in the female (typical of induced lactation experiments) tend not to increase GH levels in circulation (Gettys et al., 1988), but as previous experiments in rats, rams, and humans indicate only low dose E (particularly in pre or peripubertal subjects) elicits substantial GH increases. Regardless, E in doses used in induced lactation trials have little if any effect on serum GH.

ii. Prolactin

Positive changes in serum PRL are often associated with more successful induced lactations. Estrogen may increase mammary growth by increasing PRL levels. Prolactin has been shown to increase the growth of normal mammary

glands in the absence of steroid hormones in mice (Nagasawa et al., 1986; Topper and Freeman, 1980). Data indicate that E causes substantial increases in PRL levels in mice and rats by increases in synthesis and secretion (Lyons et al., 1958; Lieberman et al., 1978; Frank, 1983; Shull and Gorski, 1985; Pan and Chen, 1990). Bromoergocryptine, a potent PRL secretion inhibitor, decreased overall mammary parenchyma area in non-pregnant ewes treated with E+P (Russe et al., 1984). Additionally, PRL concentration was higher in E+P treated animals when compared to controls. Lastly, Seshadri and Shah (1984) reported that PRL elevates estrogen receptor (ER) levels in rat, rabbit and rat tumors. Thus, E may have dual modes of action by eliciting direct growth effects on mammary epithelium as well as increasing PRL production which in turn would increase growth directly or via increased ER.

iii. Epidermal Growth Factor

Epidermal Growth Factor (EGF) is a protein found in male submaxillary glands in quantities more than ten times that of female mice (Rall et al., 1985); although, the submaxillary glands of female mice are still their primary source of this protein. Quantities in the kidneys (tissue containing the second largest amount of EGF) are approximately .05% and .7% of that in submaxillary glands of male and female mice, respectively (Rall et al., 1985). Turkington (1969) first identified EGF as a mammary gland mitogen in mice in the late 1960's. Subsequent reports have confirmed the mitogenic activity of EGF on mammary epithelium of mice (Taketani and Oka, 1983 a and b), benign human epithelial cells (Stocker et al., 1976), and human breast cancer cells (Imai et al., 1982). Epidermal growth factor has questionable mammogenic effects in the bovine (Haslam and Shyamala,

1979a). Collier and McGrath (1988) have shown an increase in wet weight and DNA content of udders when heifers were treated with recombinant human EGF, but only DNA increases were significant in mammary tissue when using murine EGF. Murine prepro EGF transcription (DiAugustine et al., 1988) and human MCF-7 tumor cell EGF levels (Dickson et al., 1986) appear to increase following treatment with E. Additionally, Vanderboom and Sheffield (1991) recently reported E and EGF may synergize to increase growth in NMuMG murine mammary epithelial cells. A material which cross-reacts with antibodies against mouse EGF has been detected in sheep submaxillary glands (Akers, unpublished observation). However, no data are available concerning EGF regulation in ruminants.

iv. Transforming Growth Factor

Transforming Growth Factors (TGFs) were originally named such because of their ability to induce anchorage independent growth of normal rat kidney fibroblasts in soft agar (Cullen and Lippman, 1989). The transformed phenotype is quickly reversed when TGF is removed. Since this time, several laboratories have experimented with TGF activity on mammary epithelium and demonstrated mitogenic and inhibitory effects.

a. Transforming Growth Factor-α

Transforming growth factor-α is structurally related to EGF and can bind to EGF receptors (Schreiber, et al., 1986). Thus, TGFα probably elicits many of its effects through the EGF receptor. In fact, Schreiber et al. presented evidence that TGFα can bind to EGF receptors and actually create a greater stimulus than EGF. Transforming growth factor-α is often produced by tumor cells (Schreiber et al.,

1986; Zwiebel et al., 1986). However, TGF activity has been demonstrated in normal bovine mammary tissue (Eckert et al., 1985). Estrogen dependent MCF-7 tumor cells show increased TGFα levels following estradiol treatment (Dickson et al., 1986; Cullen and Lippman, 1989). This may account for some, but not all, mitogenic effects of E treatment in E dependent human breast cancer. Estrogen regulated increases in TGFα in the bovine have not been demonstrated.

b. Transforming Growth Factor-β

Silberstein and Daniel have devoted much research to TGFβ and its relation to the mammary gland. DNA synthesis in mammary end buds has been inhibited in a reversible manner by TGFβ1 (Silberstein and Daniel, 1987; Daniel, et al., 1989; Cullen and Lippman, 1989). Ductal elongation has been stopped by TGFβ1 without affecting alveolar DNA synthesis (Daniel et al., 1989). This would affect E regulated duct growth, but not P controlled alveolar proliferation. Daniel et al. suggested that TGFβ acts as a growth regulator, preventing overgrowth since TGFβ is nearly ubiquitous in animal tissues and cells *in vitro*. Moreover, it appears that TGFβ1 synthesis and secretion is inhibited by E and stimulated by anti-estrogens (Daniel et al., 1986). Another report recently indicated that estradiol-17β may stimulate secretion of a TGFβ-like factor by rat granulosa cells (Dickson et al., 1986). Unfortunately, similar studies in ruminants are lacking.

v. Thyroid Stimulating Hormone

Another pituitary hormone that has been indicated to be stimulated in culture by E is thyroid stimulating hormone (TSH) (Miller et al., 1977). Thyroid stimulating hormone is known to cause release of thyroxine (T_4) and

triiodothyronine (T_3) from the thyroid. These thyroid hormones may metabolically regulate mammary growth, but are ineffective alone. Thyroid hormones have been shown to slightly increase mammary DNA in pregnant animals (Anderson, 1972). Thus, the release of T_3 or T_4 by the thyroid does not adequately explain the magnitude of E related mammary growth.

vi. Platelet Derived Growth Factor

Cullen and Lippman (1989) propose that platelet derived growth factor (PDGF) may be an important mitogen in human breast cancer. Platelet derived growth factor fails to stimulate epithelium, but is a potent stimulator of mesenchymal cells. Additionally, PDGF can induce fibroblasts to secrete IGF-I (Clemmons et al., 1981). Moreover, PDGF mRNA expression has been shown to increase after E exposure in E dependent MCF-7 cells. Lack of similar studies in normal tissue suggests that PDGF may only be regulated by E in E dependent tumors.

vii. Progesterone

Dharmarajan et al. (1991) showed that removal of exogenous E in pseudo-pregnant rabbits may significantly decrease ovarian venous P concentrations by 24 h. Additionally these researchers observed that estradiol may significantly raise P secretion in ovaries cultured *in vitro*, but only when the ovaries retain their corpora lutea. Although P is not effective in promoting ductal growth, it seems to elicit growth of mammary alveoli, as formerly noted. Further studies are needed to elucidate the role of E mediated P increases in other species.

C. RECEPTOR MODULATION

Indirect growth does not necessitate endocrine, paracrine, or autocrine release of a growth factor. Mitogens are often present in the blood or mammary gland in quantities sufficient to stimulate growth if receptors are available. Receptors can be too low in number, that is, there is abundant hormone available, but too few receptors. Receptors can also be present, but unable to elicit a response when mitogen attaches. (Topper et al., 1980). Finally, some receptors may only be able to bind their counterpart mitogens at certain times or after hormonal stimuli that alters cell or membrane morphology. Thus, another indirect stimulus would be to increase receptor number, or augment hormone binding to receptors.

i. Insulin Like Growth Factor Receptors

As previously shown IGF-I is an effective stimulator of mammary epithelium. Insulin like growth factor-I and II have separate receptors, but may bind to the other with a lower affinity (Dehoff et al., 1988). Dehoff et al. demonstrated that both Type I and II IGF receptor binding increase during lactation in the bovine. Increases are more dramatic in IGF-I receptors, but total number of IGF-II receptors is greater. Ghahary and Murphy (1989) discounted Sirbasku's circulating estromedin hypothesis. Instead they suggest that estromedins may be paracrine in nature. Although the author's focus is on uterine growth regulation by E, many reports on mammary and uterine stimulation by E refer to each other due to similarities. Ghahary and Murphy and others reported increases in IGF-I mRNA levels in uterine tissue (Murphy et al., 1987). These researchers report an increase in IGF-I receptors by E, though this stimulation may admittedly be indirect: estrogen stimulates IGF-I production and IGF-I autoregulates its receptors.

Unfortunately, similar data in the bovine is lacking.

ii. Epidermal Growth Factor Receptors

A possible role of EGF receptors in mediating E effects in uterine tissue was studied in immature female rats (Mukku and Stancel, 1985) by measuring EGF binding in uterine membranes before and after exogenous E treatment. Binding of EGF increased three fold following treatment, with no change in the affinity of receptors for EGF. The authors report the stimulation of EGF binding was mediated through increase in receptor levels occurring as quickly as 6 h following treatment. Sheffield (1988a) determined E increases EGF binding in mouse mammary glands, while P synergizes with E to increase EGF binding, but is ineffective alone. Spitzer and Grouse (1987) reported the presence of EGF receptors in the bovine mammary gland of pregnant and lactating animals, with glands from pregnant cows having twice the binding capacity for EGF as lactating cows. Higher binding during gestation (compared to lactation) is consistent with increased growth during gestation. However, Leung et al. (1991) have shown presence of an EGF receptor gene in an E dependent human breast cell line (CAMA-1), but curiously, EGF/TGFα had no effect on proliferation of these cells. Collier and McGrath (1988) report exogenous EGF to be active in bovine mammary epithelial development and differentiation, but no report has positively identified EGF in the bovine. Also, as previously shown TGF may attach to 'EGF-receptors', so it is reasonable to assume the bovine may have any number of receptors that could bind EGF, TGF, or like peptides without having the corresponding mitogen in its circulation. The role of EGF in the bovine mammary gland is still unclear. Additionally, since no positive identification of EGF has

been reported, studies on E mediated EGF receptor increases are nonexistent.

iii. Prolactin Receptors

Prolactin seems quite capable of causing mammary gland proliferation in rodents. Furthermore, PRL binding in murine mammary gland appears to be under E control (Sheth et al,. 1978). However, the authors suggest much of the binding increases may be mediated through the enhancement of PRL concentration. Akers and Smith (1986) treated pregnant ewes with enclomiphene citrate (an anti-estrogen) and binding of human GH to mammary membranes was compared with control animals one week postpartum. An earlier study indicated human GH specifically binds PRL receptors in ruminant mammary tissue (Akers and Keys, 1984). Akers and Smith found anti-estrogen treated ewes had significantly lower mean specific binding of human GH to mammary membranes. A separate study by Akers et al., (1988) revealed that anti-estrogens decrease serum PRL levels, but had no effect on PRL receptors during late gestation. Prolactin concentrations did not change by treatment with anti-estrogens, so autoregulation of PRL receptors is not likely (Smith et al., 1989). Thus, increases in PRL receptors or PRL binding are questionable as a mode of E regulated mammary epithelial proliferation in the bovine, though more thorough study should be undertaken.

iv. Progesterone Receptors

Perhaps the greatest attention in E modulation of receptors in mammary tissue have been on progesterone receptors (PgR) (Haslam and Shyamala, 1979 a and b; Haslam, 1988 a,b,c; Skarda et al., 1989; Wang et al., 1990; Shyamala et al.,

1990). Murine studies have shown P to be important in mammary cell proliferation (Bresciani, 1965), and bovine studies indicate its role in alveolar epithelial proliferation. In the late seventies, PgR were found to be under E control in neoplastic mammary tissue (Horowitz and McGuire, 1977). By 1979, Haslam and Shyamala (1979b) described the control of PgR by estradiol in murine mammary gland. It seems the glands of lactating mice contain no detectable PgR nor could PgR be induced with E (Shyamala et al., 1990). However, virgin mice contain PgR and their levels can be substantially augmented with exogenous E. Haslam later extended her work by showing that E modulates PgR in mature adult non-lactating glands, but not immature mice (Haslam, 1988 a,b,c). Wang et al. (1990) reported that 80 percent of PgR in murine mammary glands are E inducible and all these are located in the epithelium. Whereas, the other 20 percent of PgR are E independent and located in the stroma. Research has also demonstrated E sensitive PgR in rat uterine cells (Aronica and Katzenellenbogen, 1991). Parallel studies in the bovine are absent, although it is clear that P receptors increase in the udder during pregnancy (Smith et al. 1989) and are present in mammary tissue from lactating cows (Capuco et al. 1982) in contrast with rodents.

v. Estrogen Receptors

Some studies have shown that E may stimulate its own receptors (Muldoon, 1979; Gaubert et al., 1986). If E is proven to directly increase mammary growth this finding could be very important. Moreover, if E binding to its own receptor causes release of some mitogen or changes in other receptor sensitivity, E autoregulation of its own receptor could magnify this response, assuming sufficient E is available following increases in ER.

D. ENZYMES AND CYCLIC NUCLEOTIDES

Topper and Freeman (1980) discussed the inhibition of casein synthesis and other lactogenic effects by increase in cyclic adenosine monophosphate (cAMP), stimulated by cholera toxin (CT). Cholera toxin apparently inhibits hydrolyzation of GTP to GDP, causing constant production of adenyl cyclase (AC). Topper and Freeman note that P is capable of stimulating AC from pregnant rats, but not lactating rats. Thus, P may be inhibiting milk synthesis through the activation of AC. However, Sheffield and Welsch (1985) found cAMP significantly increases proliferation of three human mammary tumor lines: MCF-7, T47-D, and Hs578t. Moreover, MCF-7 and T47-D showed growth that was higher still when supplemental E was supplied. These two cell lines that responded to E after growth in CT did not respond to E alone. Additionally, Hs578t was E receptor negative and the other two were E receptor positive. Cullen and Lippman (1989) argue that most evidence indicates cAMP is a growth inhibitor in tumor lines. Progesterone and/or E have also been implicated in increasing cAMP in mice in late pregnancy (Rilema, 1976) and decreases during lactation. Recently, Hund and Sheffield (1991) reported CT significantly decreased DNA synthesis in bovine Mac-T cells. Additionally, they reported these results differ from previous work with NMuMG mouse mammary epithelial cells, but mimic results from bovine fibroblasts cells. Since reports contrast so markedly on the role of cAMP in mammary gland growth (Cullen and Lippman, 1985; Sheffield and Welsch, 1985; Dickson and Lippman, 1987; Sheffield et al., 1987; Sheffield, 1989; Waksman et al., 1991), it is unclear whether the growth of mammary epithelium by E (or P) can be explained by changes in cAMP or AC. Other enzymes and cyclic nucleotides have been

implicated as markers or mediators of E induced growth with similar controversy (Cullen and Lippman, 1985; Lippman et al., 1986; Kaye et al., 1986; Waksman et al., 1991).

V. EFFECT OF PROGESTERONE ON MAMMARY GROWTH

A. General

Progesterone's history as a catalyst for mammary growth nearly parallels that of E. Estrogen is generally recognized as the primary ovarian steroid able to elicit mammary mitogensis. In fact, P does not always give mitogenic results. In the early 1950's Cowie et al. (1952) added P to induction of growth and lactation trials that had previously only used E. Progesterone was known to be necessary for normal alveolar development in rabbits, rats, and mice. Cowie and co-workers (1952) arrived at similar conclusions with goats. However, milk production was not different in E + P groups when compared to E alone. Progesterone in the bovine is known to stimulate mammary lobule-alveolar growth (Tucker, 1985). Flux (1954) and Traurig and Morgan (1964b) found P to promote morphogenic differentiation and epithelial proliferation in mice, but not to the same levels as E alone. Freeman and Topper (1978) concluded P was "limited to its effect on the formation of alveolar structures" in mice.

Studies with human tissues have demonstrated a strong correlation between breast growth and estradiol, but P does not appear in the circulation until long after breast development begins (Drife, 1986). Culture of normal human breast tissue has shown inhibition of growth by R5020, a synthetic progestin (Kaye et al., 1986; Malet et al., 1986). In contrast, Haslam (1988a) contends mammary epithelium DNA synthesis in the rodent is more dependent on P than E. The growth obtained

by E alone may be due to increase in PgR (Haslam, 1988a; Wang et al., 1990). Skarda et al. (1989) reported stimulation of ducts and alveoli by P alone and inhibition by E in young mice of either gender. Most evidence, however, contests the claims of E as a growth inhibitor, except with pharmacological doses. In fact, Haslam admits that E can increase epithelial DNA synthesis and end-bud size in immature mice. Thus, in several animal models P acts as a late growth stimulator, mainly of alveoli (which occur after ductal formation). Although P, like E, presumably exhibits species differences with respect to mammary proliferation. Notwithstanding, E and P synergize to provide higher growth than either alone in a number of species (Flux, 1954, Fulkerson, 1979).

B. Receptors

Progesterone may regulate growth by alteration of receptors, as earlier demonstrated by E. Increasing ER could increase growth potential, whether or not Es' effects are direct or indirect. However, P has been shown to reduce ER in rat and human mammary tissue (Seshadri and Shah, 1984; Kaye et al., 1986). In fact, rat ER decreased from 30 fmol/mg to 5.8 fmol/mg with injection of 0.25 mg P/animal. The same study shows drastic increases in PgR following P treatment. More recently, Wei et al. (1988) determined P decreases PgR initially, but later they return to normal in human breast cancer cells. However, the synthetic progestin R5020 chronically decreases PgR, since it is not readily metabolized by the breast cells, unlike normal P. Moreover, P or R5020 significantly decreased IGF-I receptor content and IGF-I mRNA in human breast carcinoma, potentially through stimulation of IGF-II secretion (Papa et al., 1991). The decrease in ER or IGF-I receptor would generally support the growth inhibitory role of P.

VI. RECEPTOR LOCATION

Steroid hormone research has encompassed nearly a century, yet receptor location within the cell is debatable and merits mention, since detailed knowledge will certainly aid our understanding of E related growth. O'Malley probably indicated the difficulty involved in ascertaining detailed analysis of steroid action best in a 1984 review, "I wish to emphasize the complexity of steroid hormone action at the level of the intact cell. I suggest that steroid hormone regulation of gene expression may require a series of coordinate structural interactions that occur at least at four separate levels of cellular organization." O'Malley proposed steroidal binding occurs in the cytoplasm and the activated steroid-receptor complex is translocated to the nucleus where subsequent gene activation occurs. This was in agreement with Shyamala and Nandi (1972) who demonstrated that E did not bind to nuclei of cells obtained from lactating mammary gland in *in vitro* studies, but did *in vivo* and suggested the hormone must first bind to cytoplasmic receptors. Auricchio et al. (1976) continued this work and cited the cytosol as the location of ER in the mammary gland of virgin, pregnant and lactating mice. A few years following, Rotondi and Auricchio (1978) reported the ER was in the cytosol of calf mammary gland as well. However, both studies prepared cytosol fractions by homogenization, filtration, and ultracentrifugation. Whereas a number of recent studies suggested that steroidal receptors are always located on the nuclear membrane (Muller and Traish, 1986; Puca et al., 1986). Onetti-Muda et al. (1991) used snap-frozen, freeze-dried and parrafin-embedded human breast tumors and found ER and PgR were confined to the nucleus with little or no staining of the cytoplasm. Furthermore, Jordan et al. (1985) proposed a model to explain

previously conflicting reports. Estrogen receptors are located in the nucleus *in vivo*, but the receptors 'fall out' of the nucleus when disrupted *in vitro*. Others claim the receptor is released from the nucleus by processing: homogenization or temperature (Muller and Traish, 1986; Puca et al., 1986; Seshadri and Shah, 1984). Receptor location is important to our understanding of E mediated proliferation. Any efforts that could elucidate further what subcellular compartments are involved with binding could be of critical importance to our understanding of E mediated cell proliferation.

VII. PARACRINE AND AUTOCRINE GROWTH BY ESTROGEN AND PROGESTERONE

The actions of E and P appear to be indirect in stimulating mammary growth in most species studied. It is unclear whether the action of ovarian steroids is autocrine, paracrine, endocrine, a combination of these actions or does not involve estromedins whatsoever. Several groups have demonstrated the importance of the mammary stromal tissue in murine mammogenesis (McGrath, 1983; Levine and Stockdale, 1984). In fact, estrogenic regulation of epithelial proliferation may be dependent upon stromal tissue in the mouse (McGrath, 1983; Shyamala and Ferenczy, 1984; Haslam, 1988d). Treatment of mice with exogenous E caused proliferation of mammary stroma within 24 h, with adipocyte growth being highest. Epithelial proliferation was not different from control, however, until 48-72 h elapsed. Thus, the prior division of fibroblasts and adipocytes may be a prerequisite for epithelial growth. It is entirely possible that the stimulation of stromal cells prior to epithelium, causes release of growth factors that stimulate epithelium. Parallel studies in the ruminant are lacking.

VIII. MAMMARY GLAND GROWTH: DIFFERENCES BETWEEN AND WITHIN SPECIES

Extrapolation of data across species has long been sought as a method to defray costs and enable testing (with regard to human research). Much data have been presented demonstrating subtle or distinct differences in mammary growth between species. Cowie, Forsyth, and Hart (1980) review species differences throughout their book, encompassing general growth patterns, mammary gland structure, circulating hormone levels and their actions. Rodent glands have often been considered an attractive alternative to ruminant or human studies. Recently, however, many researchers have questioned the validity of such extrapolation: rodent to human (Etreby and Graf, 1979; Kleinberg and Newman, 1986; Anderson et al., 1988); canine to human (Etreby and Graf, 1979); and rodent to ruminant (Sheffield, 1988a; Akers, 1990). Additionally, differences in mammary gland responsiveness have been noted between species as closely related as monkey and humans (Cowie, Forsyth and Hart, 1980); ovine and bovine (Wallace, 1953); and even between breeds of cattle (Wallace, 1953; Keys et al., 1989). Differences have included endogenous serum hormone levels, mammary gland responses to various mitogens, *in vitro* mammary epithelium responses to mitogens, differences in prepubertal, estrous cycling, or gestational growth, receptor levels and responsiveness of receptors. Steroid related growth differences of mammary epithelium have even been shown to occur on different mammary glands of the same animal (Bolander, 1990). These researchers, in separate experiments, have demonstrated thoracic and abdominal mammary glands of mice differ in their growth responsiveness to E and P. Reports also indicate differences in tumor

versus normal mammary cells (Myal et al., 1984; Cavalie et al., 1986). The details of mammary proliferative differences between species are extreme, and will not be discussed except when particularly relevant to data presented. Caution should be exercised in extrapolating data across species; however, breed and gland differences are most often subtle or negligible.

IX. SUMMARY AND RESEARCH DIRECTION/PROPOSAL

Obviously research on E and P mediated mammary epithelial proliferation has merited much attention. Much data are conflicting with regard to E action. However, researchers utilizing mice are nearly in unity on two crucial points of E mediated mammary epithelium proliferation: 1) estrogen has no direct effect on epithelial proliferation and 2) estrogen does cause substantial growth when administered *in vivo*. Much of the deficiency of information in the bovine can be attributed to lack of a hormone responsive immortal epithelial cell line. The development of such a cell line has made study in our laboratory possible. The objective of this thesis was to ascertain information regarding the action of E and P in the bovine mammary gland. The thesis is composed of three studies with three separate objectives. The first study was designed to determine the effects of ovarian steroids on proliferation of stromal and epithelial cells in the bovine mammary gland in vivo. The objective of the second study was to characterize the Mac-T cell strain with regard to its responsiveness to hormones and growth factors. Finally, the third study was designed to investigate how ovarian steroids cause growth of bovine mammary epithelium in a series of trials.

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CHAPTER 1

CELL INTERACTIONS IN INITIATION OF MAMMARY EPITHELIAL PROLIFERATION BY ESTROGEN AND PROGESTERONE IN PREPUBERTAL HEIFERS

INTRODUCTION

Treatment of multiparous non-pregnant non-lactating cows with estrogen and progesterone induces growth and differentiation of epithelium in the udder such that it resembles the morphology of a lactating gland (Turner et al. 1956). However, detailed cellular study of the initiation of estrogen and progesterone action in pre-lactational mammary glands of nulliparous cattle has received little attention. Many studies have been done with mice (Haslam and Shyamala, 1981; Shyamala and Leland, 1982; McGrath, 1983; Imagawa et al. 1985; Haslam, 1988a; Haslam, 1988b; Skarda et al. 1989), however, researchers argue over whether estrogen (E), progesterone (P), or the combination (B) is responsible for induction of mammary epithelial cell proliferation. Murine studies suggest that the mammary fat pad is a likely site for initiation of E and P action in normal mammary glands; as suggested from both *in vivo* (Hoshino and Martin, 1974; Haslam and Shyamala, 1981; Shyamala and Ferenczy, 1984) and *in vitro* (Enami et al. 1983; McGrath, 1983; Beck and Hosick, 1988; Beck et al. 1989) studies. Some researchers relate this growth to adipocyte (Hoshino and Martin, 1974; Shyamala and Ferenczy, 1984; Beck and Hosick, 1988; Beck et al. 1989), or fibroblast priming of the epithelium (Enami et al. 1983; McGrath, 1983; Shyamala and Ferenczy, 1984) in

response to the actions of E or P. Estrogen and P treatment has shown the greatest increases in mammary DNA content compared with these steroids alone (Traurig and Morgan, 1964). Progesterone treated mice exhibit substantial increases in mammary DNA as well (Haslam, 1988; Wang et al., 1990). However, studies with ovariectomized mice and cells in culture indicate E does not directly affect the mammary epithelium (Shyamala and Ferenczy, 1982); Haslam, 1988; Skarda et al. 1989). Similar results have been obtained in normal human breast cells (Richards et al. 1988) and human breast tumor cell lines (Sirbasku and Benson, 1979; Shafie, 1980). In fact, many human breast tumor cell lines occur spontaneously only when E is available (or administered), that in they are E inducible. Several of these cell lines are not responsive to E in culture, however. Instead proliferation appears to require mitogens induced by E (Sirbasku and Benson, 1979; Sheffield and Welsch, 1985).

Several researchers have hypothesized the abundant growth of epithelium following B treatment is P mediated, since E increases progesterone receptors (PgR) in the fat pad and epithelium (Haslam and Shyamala, 1981; Skarda et al. 1989). Furthermore, it has been suggested that growth factors from the fat pad, cell-to-cell contacts or extracellular matrix components may be responsible for epithelial dependence on stromal or adipose tissue for initiation of proliferation (Sonnenschein and Soto, 1980; Sirbasku and Leland, 1982; Wicha et al. 1982; Dickson and Lippman, 1987; Beck and Hosick, 1988).

Detailed studies on mechanism(s) of E, P, or B action in prepubertal bovine mammary glands are lacking. Additionally, it is unclear whether fibroblasts, adipocytes, or epithelial cells show differential temporal proliferative responses to the aforementioned sex steroids. The present study was undertaken to determine

effects of ovarian steroids on induction of mammary epithelial cell proliferation in prepubertal heifers and specifically to evaluate the acute time course of proliferative response among cell types in the udder.

MATERIALS AND METHODS

Animals

Nine prepubertal Simmental X Hereford X Angus crossbred heifer calves were housed at the VPI & SU Catawba Research Facility. The animals weighed between 136 kg and 161 kg with a mean weight of 152 kg and mean age of 5 months. All were determined to be prepubertal based on measurement of serum progesterone prior to the trial.

Hormone treatment

All hormones were purchased from Sigma Chemical Co. (St. Louis, MO). The heifers were divided at random into three groups: three animals received estradiol 17-β (E, .1 mg/kg/day), three received progesterone (P, 0.25 mg/kg/day) and three animals received both E and P (B). The heifers were given daily subcutaneous injections of these steroids for four consecutive days.

Tissue Recovery

Mammary biopsies were taken from each heifer immediately before, then 24, 48 and 96 h after onset of hormone treatment. Hair was shaved from an area selected for incision and the skin was scrubbed with 70% ethanol. Heifers were given a general anesthetic, Xylazine 100 mg/ml (Rompum, Mobay Corp, Shawnee, Kansas) at 0.2 ml IM (caudal to the scapula) and 0.1 ml IV (via the jugular vein).

An electroscalpel was used to cut the skin and cauterize small blood vessels. An incision approximately 4 cm was made immediately dorsal to the teat on the

lateral aspect of the udder. Subcutaneous connective tissue was separated by blunt dissection and a mass of fascia and attached parenchymal tissue (approximately 50 g) was removed using sterile forceps and placed in sterile medium 199 at 30° C. The skin was sutured and subsequent biopsies on the same animal were performed in an identical manner on previously unsampled glands. No apparent inflammation or udder health problems resulted from the biopsies. The +96 h tissue recovery was done at the Meats Laboratory on VPI & SU campus by slaughtering the animal, excising whole mammary glands and transporting the glands back to the laboratory in an insulated container (approximately 5 min.).

Tissue Incubation

Tissue samples were carefully cut into small explants (1-3 mg each). Approximately 200 mg of explants (or 50 explants) were incubated in 2 ml medium 199 containing 2 μCi/ml of methyl-3 tritated thymidine (ICN Biomedicals Co.) for 1 h in a shaking water bath at 37° C. Radiolabeled media were removed by aspiration and replaced with 3 ml fresh media (without isotope) and incubations continued for 10 min. A sample of explants (approximately 10) was transferred to Karnovsky's fixative for subsequent histological and autoradiographic evaluation of cells incorporating thymidine. Tissue was washed three times at 4° C in 0.1 M phosphate with sucrose (to adjust osmolarity to equal the fixative) and stored until pieces were embedded. Remaining tissue was transferred to preweighed tubes containing 5 ml of phosphate saline buffer (PBS: 0.05 M Na_2HPO_4, 2.0 M NaCl, 0.002 M EDTA, pH 7.4). Tubes with tissue and buffer were weighed, then frozen at -80° C until assay.

DNA Assay

Buffer and tissue were thawed in a cold water bath (5° C) for 1 h. Tissue was

homogenized using a polytron tissue homogenizer (Brinkman Instruments, Westburg, NY) at a intermediate setting for 40 seconds. The homogenizer probe was rinsed with 3 ml of PBS for 10 sec which was then added to the homogenate. Additional PBS was added to reach a final volume of 10 ml. Samples were centrifuged at 800 X g for 10 min (Beckman J-6B centrifuge). An aliquot of homogenate was saved for trichloroacetic acid precipitation. DNA content of supernatant of homogenates was determined using the flurometric method described by Labarca and Paigen (1980). Stock Hoescht Dye #33258 (Polysciences Inc) was diluted to 20 μg/ml with distilled water and stored in a foil covered bottle for use in the assay. Samples were prepared by addition of 20 μl of homogenate to a tube containing 3.780 ml PBS and 200 μl diluted dye. Fluorescence readings were obtained with a Model TKO 100 flurometer, (Hoefer Scientific, San Francisco) designed for use in this assay according to manufactures' instructions. Samples were assayed in duplicate. A standard curve was prepared with calf thymus DNA (Sigma). Useful range of the assay was from 0.08 to 3 μg DNA per tube. All samples were within the linear portion of the standard curve.

Determination TCA Insoluble ^{3}H-Thymidine Incorporation

Homogenate, 100 μl, was added to duplicate scintillation vials along with 2 ml ice cold trichloroacetic acid (TCA) 100 gm/l (Fisher). Vials were refrigerated 20 min and immediately centrifuged at 1900 X g for 20 min at 4° C. Supernatant was removed and 5 ml of Cocktail Ecoscint Scintillation Fluid (National Diagnostics, Manville, NJ) added. Vials were capped and vortexed thoroughly. Counts per minute were obtained using a (Beckman LS 1800) scintillation counter.

Histological Evaluation

The PBS was aspirated and the tissue was dehydrated by progressively

washing in increasing concentrations of ethanol (30% 50% 70% 95% 100% 100%, 10 min each). Tissue was subsequently placed in 1:1 mixture of ethanol (100%) and infiltration solution (JB-4 embedding component A, Polysciences Inc.) for 12 h at 4° C. Solution was removed and replaced with 100% infiltration solution for 2 h at 4° C. For embedding procedure all solutions were kept on ice. Tissue was embedded in molding blocks by suspending tissue pieces in a solution of 25 parts infiltration solution and 1 part hardener (JB-4 embedding component B).

After blocks were fully hardened (about 2 days) sectioning began. Sections were cut at 2.5 μm thickness with a microtome (model 2218 Microtome Historange LKB, Bromma, Sweden). Three independent areas of multiple tissue pieces were obtained from each block by taking sections at 500 μm intervals. Sections were floated in a water bath, put on slides and dried on a hot plate at 75° C. Duplicate slides were made and one stained to determine quality of sectioned material and presence of epithelium. Unstained slides were dipped in emulsion gel (Kodak NTB2), developed, fixed, and stained (in Kodak Developer D-19, Kodak Fixer, Azure II, respectively) as described by Smith et al. 1989. Test slides were developed after 7 and 14 d. Slides were adequately exposed by 14 d.

Individual slides were examined (1000X) to determine the labeling index (proportion of cells with autoradiographic grains over the nucleus). Nine independent areas of epithelium/stroma were evaluated for tissue from each heifer for each biopsy. The following types of cells were counted with minimum number of cells counted per biopsy listed in parenthesis: Enduct epithelium (1000), ductal epithelium (600), adipocytes (200), endothelial (100). In addition, fibroblasts were counted in three individual areas: adjacent to enduct, adjacent to ductular epithelium, and in areas away from epithelium (non-adjacent), 150 for each of

these three groups. Adjacent areas were within 50 μm (via 100 X 100 μm eyepiece grid) of the epithelial structure. Enduct epithelium was classified as epithelium which appeared at the extremity of ducts and ductal epithelium was clearly part of longitudinally sectioned ducts. Examples of such ductal areas are illustrated in a previous paper (Smith et al., 1989).

Statistical Evaluation

The general linear models procedure (PROC GLM) of SAS (SAS, 1988) was used to analyze DNA and labeling index data. The repeated statement was used to analyze the time periods separately for DNA and labeling index data. There were 3 heifers and 3 treatments for a total of 9 observations at each time. For statistical analysis of labeling index data, zero value data were adjusted by the formula: (observation = .25/total number of cells counted -.25). Labeling index data were transformed as the arcsin of the square root to adjust for percentage data and heterogeneity of variance. Additionally, labeling index data were analyzed separately for each cell type. Untransformed means for labeling index data are presented. The model statement used the pretreatment value as a covariant and contained treatment as well. All treatments were contrasted to each other. The critical F value from these contrasts was then replaced by Bonferroni's F test (Zar, 1984).

RESULTS

Data clearly indicated substantial induction of epithelial cell proliferation in E and E + P (B) treated heifers, but not in those given P alone. Fig 1 (A-D) shows histoautoradiographic evaluation of enduct and ductal epithelium including fibroblasts adjacent to each. Increases in epithelial labeling index by E were

evident by 24 h whether measuring enduct or ductal areas. Moreover, epithelial cell labeling index was significantly increased ($P < .01$) by 96 h in E treated heifers. Compared with pretreatment, epithelium of B treated heifers did not respond by 48 h, but only approached statistical significance. Proliferation was also apparently less than that of heifers given E alone in both enduct and ductal areas. Overall, fibroblast labeling index did not respond to any treatment before 48 h and responded with less magnitude than epithelium.

Fig 2A, illustrates a high labeling index for endothelial cells in E heifers by 48h ($P < 0.10$), moderate increases in B heifers by 96h, but no labeling in P treated heifers. These trends mimic epithelial labeling scores; however, large variation exhibited by one animal negated significant differences between E and B heifers. There was no apparent change in labeling of adipocytes (Fig 2B) or fibroblasts not adjacent to epithelium (Fig 3) with time or treatment. Progesterone treated heifers showed no substantial labeling for any time period or cell type (Fig 1A-D, Fig 2A,B and Fig 3). Additionally, labeling responses in E group heifers were greater than those of the B treatment group for most cell types.

Measurement of tritiated thymidine incorporation revealed treatment and time results similar to epithelial autoradiographic evaluation. Table 1 demonstrates 10-fold ($P < 0.05$), 1.3-fold, and 3-fold increases in tritiated thymidine incorporation expressed as dpm/μg DNA in E, P, and B groups respectively by 96 hours or 11-fold ($P < 0.05$), 2-fold, and 5-fold increases when expressed as dpm/mg tissue. Estrogen treated animals increased ^{3}H-thymidine incorporation, measured by dpm/ug DNA, significantly ($P < 0.05$) by 24h. B treated heifers showed increases in dpm/mg tissue and dpm/μg DNA by 48h, although reponse only approached statistical significance.

DISCUSSION

Unlike Shyamala and Ferenczy's study with mice (1984), initiation of epithelial cell proliferation does not appear to be reliant on prior proliferation of surrounding stroma or adipocytes in prepubertal heifers. In fact, adipocyte proliferation (as measured by autoradiographic labeling) is negligible at any time or treatment. Also, increases in fibroblast proliferation occur after abrupt increases in epithelial proliferation and responses of fibroblasts are of a markedly lower magnitude.

The fat pad in mice seems to be crucial to the normal growth and functioning of the mammary gland as noted in experiments where mammary tissue is transplanted into the fat pad compared with other transplantation sites (Hoshino and Martin, 1974; Woodward and Akers, unpublished observations). Moreover, this concept has been substantiated through murine *in vitro* experiments. The use of media conditioned with mammary adipocytes, fat pad homogenate, or fibroblasts has been shown to increase epithelial growth and morphogenesis (Wicha et al., 1982; Enami et al., 1983; Beck and Hosick, 1988; Beck et al., 1989). McGrath (1983) documented an inhibitory role of the fat pad (in the absence of steroids) as well, but only with direct cellular contact. Thus, at least in rodents the fat pad appears to directly or indirectly increase epithelial proliferation, while direct cellular contact may limit growth to the area defined by adipocytes.

The association of adipose to epithelium in the ruminant is much different than in the mouse. The epithelium of the murine mammary gland is situated within an area of adipose tissue, the aforementioned "fat pad," with direct epithelial-adipocyte cellular contacts with epithelial end buds (McGrath, 1983), whereas the ruminant mammary gland, although surrounded by adipose tissue,

shows a distinct separation of the epithelium from adipocytes by stromal cells (Akers, 1990). Thus, there is a lack of direct adipocyte-epithelial contact. Our data suggest that epithelial tissue in the prepubertal bovine mammary gland does not require prior proliferation of adipocytes or fibroblasts, as in the mouse (Shyamala and Ferenczy, 1984), since thymidine incorporation by adipocytes is absent, while epithelium exhibits marked increases in response to steroid treatment.

Additional differences exist between murine and bovine species with respect to response to ovarian steroids. Data accumulated on mice demonstrate that P alone stimulates mammary epithelium growth, while E alone has no effect (or an inhibitory effect) in lactating (Shyamala and Ferenczy, 1982), and prepubertal mice (Skarda et al., 1989), or cultured epithelial cells (Haslam, 1988). Others suggest that E modifies epithelial proliferation through altered synthesis or secretion of growth factors or mammogenic hormones: IGF (Rosenfield et al., 1985; Harris et al., 1985), GH (Moll et al., 1986; Simard et al., 1986), PRL (Shull and Gorski, 1985; Pan and Chen, 1990), EGF (Dickson et al., 1986; DiAugustine et al., 1988), TGFα (Cullen and Lippman, 1989), TGFβ (Dickson et al., 1986; Daniel et al., 1988), and P (Dharmarajan et al., 1991). However, receptor modulation has also been suggested as a mode of E action: IGF (Murphy et al., 1987; Ghahary and Murphy, 1989), EGF (Mukku and Stancel, 1985; Sheffield, 1988a), P (Wang et al., 1990; Shyamala, 1990), PRL (Sheth et al., 1978; Akers and Smith, 1986), and E (Muldoon, 1979; Gaubert, 1986). In the prepubertal heifer, our data suggest E alone may be responsible for initial stimulation of ductal growth in the mammary gland, although admittedly the stimulation of growth may occur indirectly. P not only fails to stimulate epithelial growth it may inhibit it, since labeling of all cell types and incorporation of tritiated thymidine (per unit DNA or tissue weight) was

lower in B treated heifers than in those given E alone. Our findings, relative to the action of E and P in the prepubertal heifer are consistent with studies where exogenous steroids were administered to mature cows, that is E increased udder size while P did not (Erb, 1977).

This study yields results that contradict findings of experiments with mice, namely this experiment demonstrated that E increases epithelial growth while P inhibits E action. Additionally, E increases labeling index for endothelial cells, indicating the mitogenic activity has also increased in vascular tissue. Furthermore, adipocytes and fibroblasts in bovine mammary tissue may not play an equivalent role in the induction of mammary epithelial proliferation. Many studies on induction of mammary gland growth by E or P in the mouse have been done in cell culture, which allows testing of these steroids without other endocrine interactions, eliminating mitogens from sources other than the epithelial portion of the gland. Similar cell culture studies with bovine mammary cells are now under way in our laboratory. These studies should serve to provide better understanding of these presumptive differences in steroid induced epithelial cell proliferation in the ruminant and rodent mammary gland.

SUMMARY I

Estrogen was found to be the primary ovarian steroid involved in initiating *in vivo* mammogenesis in prepubertal heifers. Progesterone appears to be ineffective in promoting mammary gland growth. Additionally, its effect may inhibit E regulated growth. Estrogen also causes early (24 h) stimulation of epithelium while adipocytes and non-adjacent fibroblasts do not increase proliferation at all. Thus, it appears E may have a direct effect on epithelial growth or its actions may be mediated through endocrine or autocrine mitogens. Paracrine regulation of epithelial cell division can not be ignored. However, if growth was caused by paracrine action, the adipose or fibroblasts do not have to divide to facilitate release of a growth factor or inhibit release of a growth inhibitor, unlike murine studies. The data demonstrate that murine or human models for mammogenesis may be inappropriate for extrapolation to ruminants. Additionally, subsequent studies are needed to further elucidate how E mediated bovine mammary epithelial cell proliferation occurs.

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TABLE 1. Tritiated thymidine incorporation by mammary tissue from heifers treated with estrogen, progesterone, or both.

Time	Treatment	^{3}H-Thymidine Incorporation			
		dpm/μg DNA		dpm/mg Tissue	
		mean	S.E.	mean	S.E.
PRE	Estrogen	37	7	58	10
	Progesterone	37	7	58	10
	Both	37	7	58	10
24 hour	Estrogen	267	80	289	49
	Progesterone	35	65	91	42
	Both	119	68	128	47
48 hour	Estrogen	178	23	236	24
	Progesterone	28	19	54	21
	Both	101	20	229	23
96 hour	Estrogen	383	74	639	165
	Progesterone	46	60	128	143
	Both	121	63	264	157

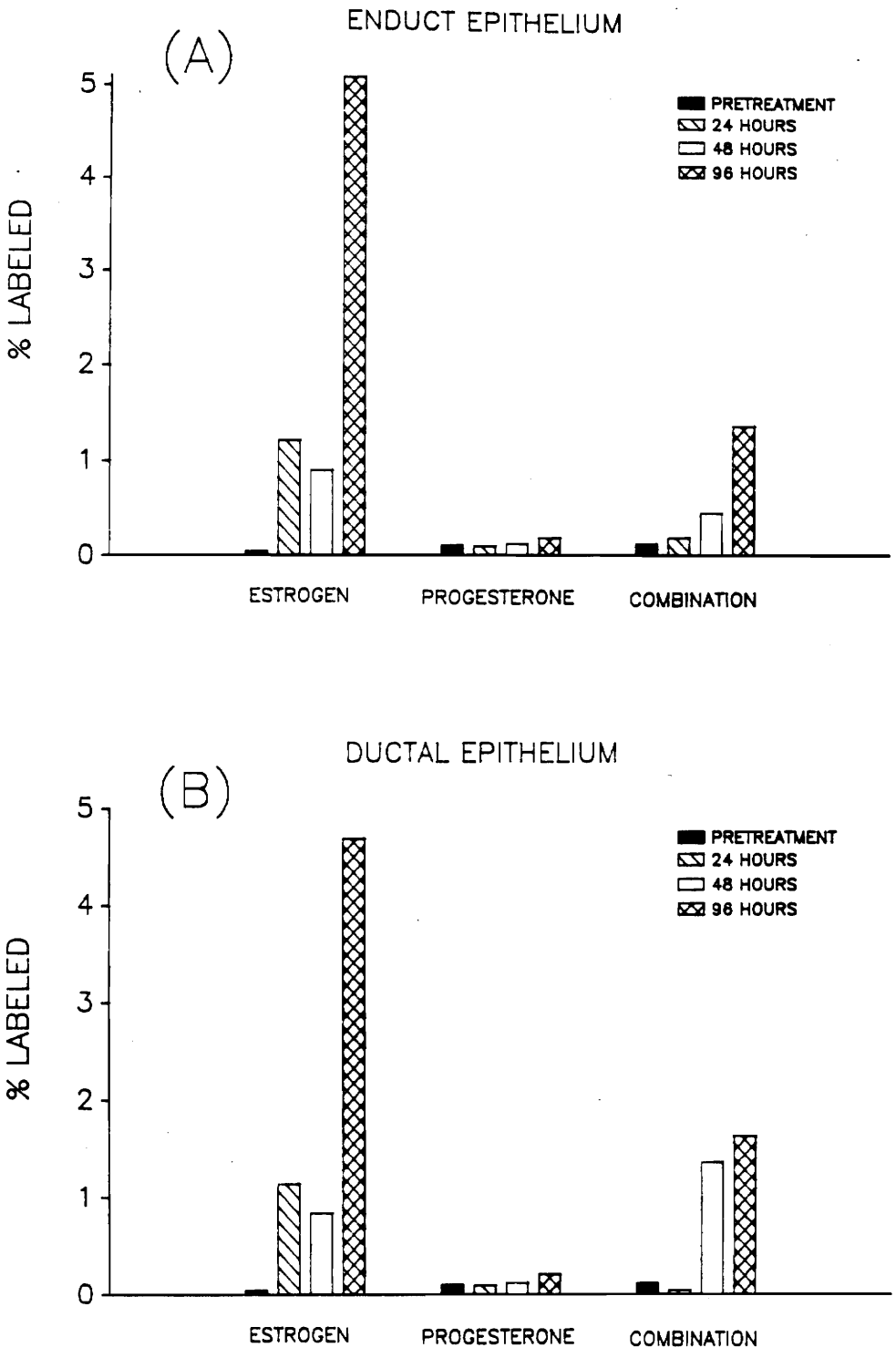
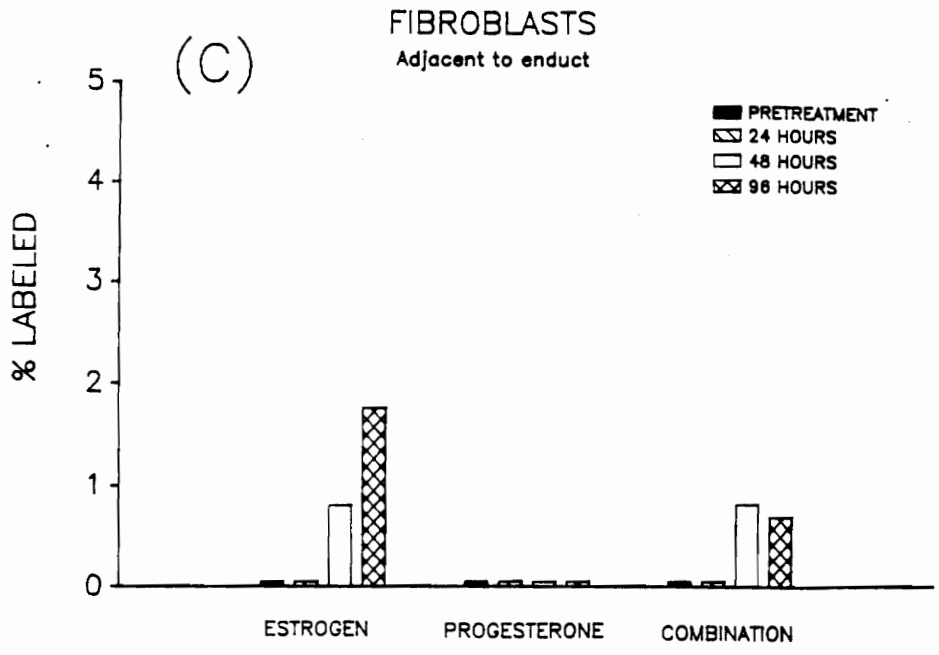


Figure 1 A, B. Effect of time and treatment on labeling index in (A) enduct epithelium (B) ductal epithelium. See text for methodological details.



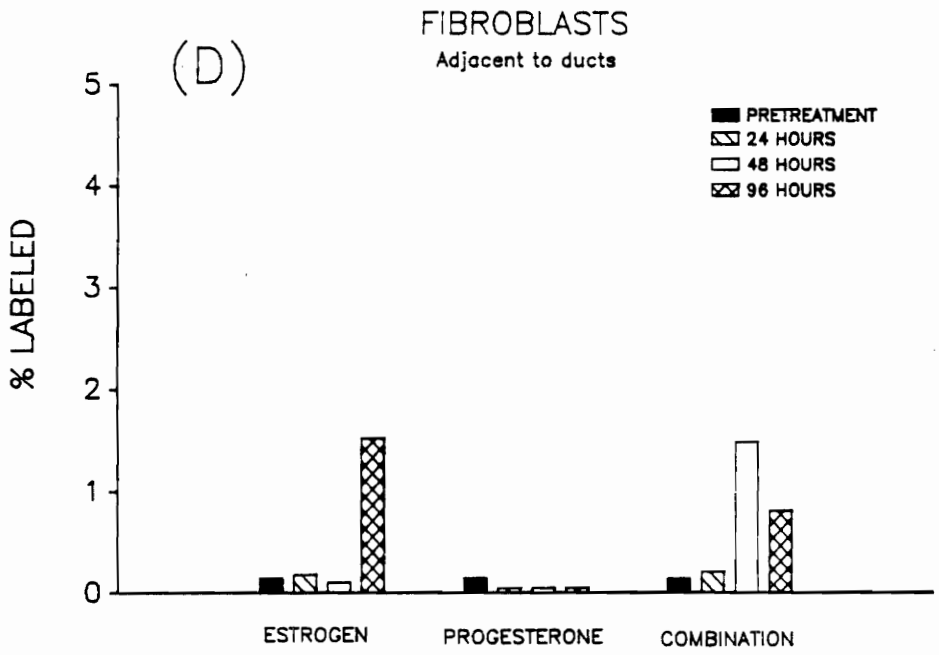


Figure 1 C, D. Effect of time and treatment on labeling index in (C) fibroblasts adjacent to enducts, and (D) fibroblasts adjacent to ductal structures. See text for methodological details.

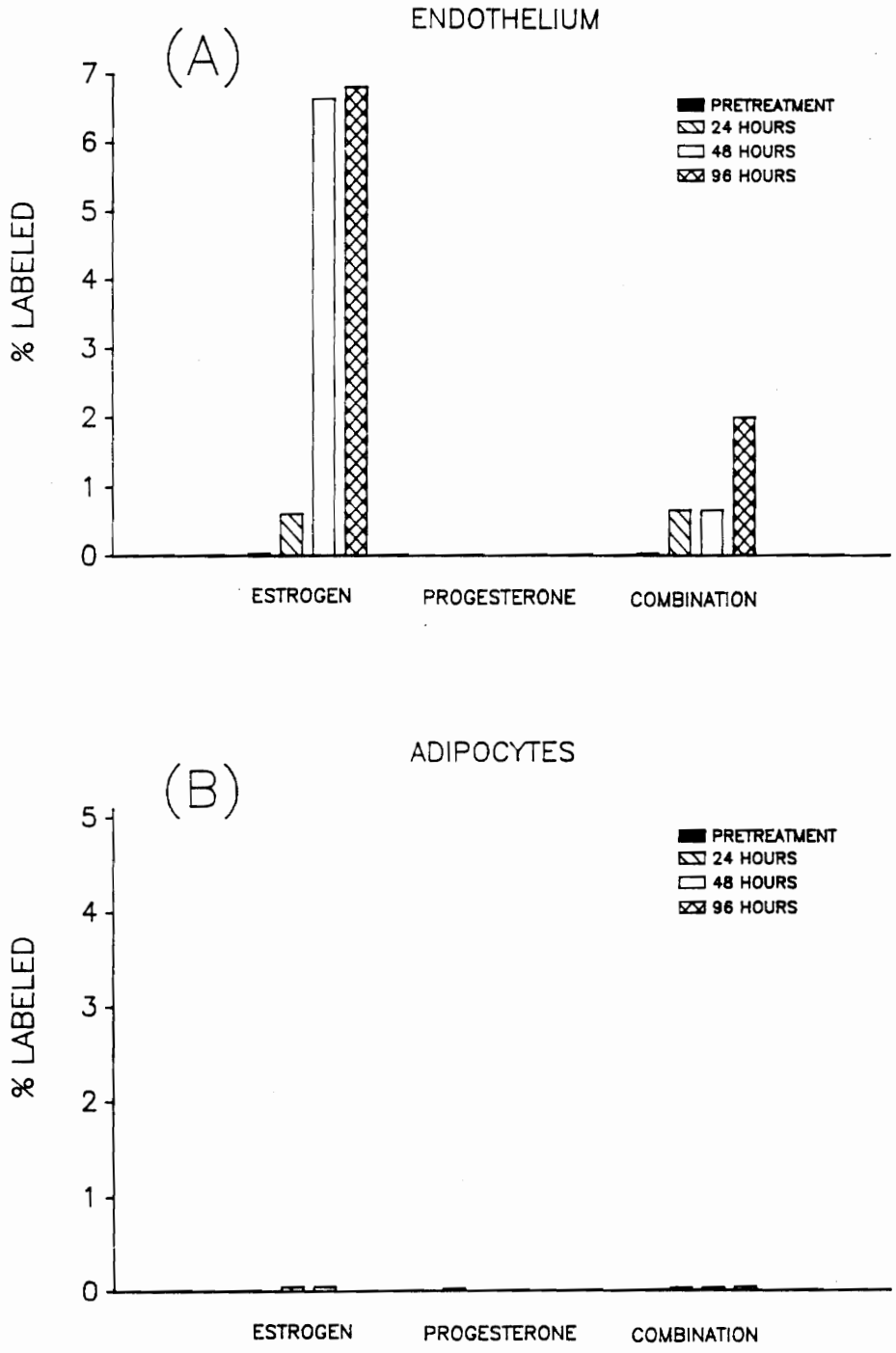


Figure 2 A, B. Effect of time and treatment on labeling index for (A) endothelium and (B) adipocytes.

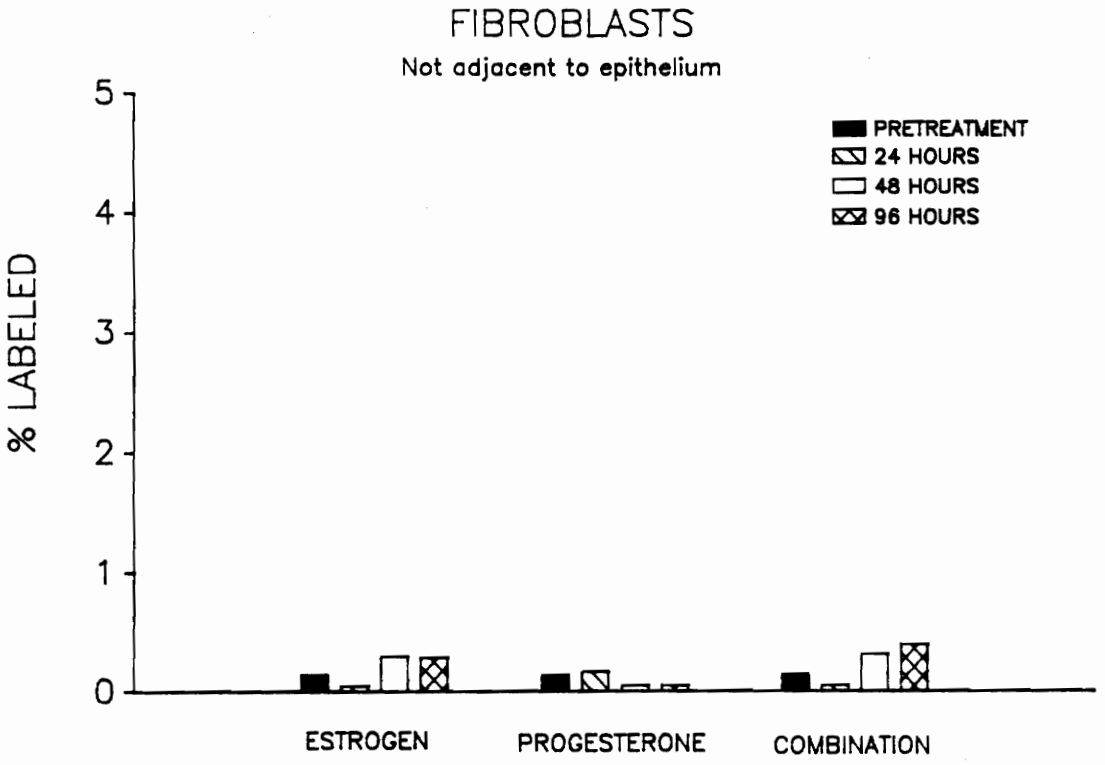


Figure 3. Effect of time and treatment on labeling index for fibroblasts not adjacent to any epithelium.

CHAPTER 2

CHARACTERIZATION OF GROWTH RESPONSIVENESS OF THE MAC-T CELL STRAIN

INTRODUCTION

Discernment of mode of action of specific mammary cell mitogens remains inherently complex when data are obtained solely from *in vivo* research. However, analysis of cells from biopsied tissue following hormone treatment *in vivo* can yield much data. Methods are available to quantify cellular constituents and functions including: hormone receptors, proteins released by hormones, enzymes triggered in the pathway of hormone binding or action, and alteration of nucleic acid structure or content. Despite *in vivo* research advances, isolation of a particular cell type facilitates assessment of various mitogens directly, eliminating endocrine and paracrine growth factors and effects of cell to cell contact between different cell types, which has been shown to modulate growth (Hoshino and Martin, 1974; Enami et al., 1983; Levine and Stockdale, 1984; Kawamura et al., 1986).

Tissue or organ culture of mammary parenchyma has encompassed several decades and species and has increased fundamental understanding of factors which influence mammary cell function. Such cultures isolated mammary cells from hormones in circulation and provided evidence of direct, endocrine or metabolic effects not possible with whole animals. Despite the usefulness of organ culture, specific understanding of secretory cell function is difficult to unambiguously ascertain because explants contain a variety of cell types in addition to the epithelial cells. Thus, effects of hormones or growth factors may depend on

interactions among cells within the mammary parenchyma. Isolation and study of the individual cell types which compose mammary parenchyma offers a possible solution to this problem. Use of such cell cultures should allow identification of growth regulators specific to that cell type.

Lasfargues (1956) published data on a crude collagenase solution that allowed dissociation of mammary cells, paving the way for culture of specific populations of mammary gland cells. Subsequently, he obtained enriched populations of murine mammary epithelial cells and was able to keep them in culture for more than 5 weeks. Ebner and Larson (1958) performed similar work with collagenase digested mammary tissue from the bovine. Many attempts to prepare isolated cells were only partially successful. Many epithelial cell preparations were described as enriched rather than pure, since they were often hampered by the presence of stromal cells. Also, production of specific milk constituents was often lost after several days of culture. These problems limited use of primary culture.

Others worked to develop pure mammary cell lines capable of continuous growth. For example, E inducible mammary tumors in humans provided tissue to isolate cells which gave rise to mammary cell lines, such as the MCF-7 (Soule et al., 1973). Such neoplastic cells are able to achieve immortality because continual growth is the inherent nature of cancerous cells. Normal cells, by contrast undergo crisis and die after successive passages in vitro (Freshney, 1987) as illustrated by work with primary cell cultures. However, normal cell lines can be immortalized by transfection by a subgroup of viral or cellular oncogenes. A murine mammary epithelial cell line was developed in 1977 (Sizemore, 1979), NMuMG (Normal Murine Mammary Gland epithelial cell line), that responded to many hormones as

expected *in vivo*. Several murine lines were subsequently developed with characteristic differentiation when cultured *in vitro*. The RAMA-25 line (Bennett et al., 1978), isolated from an adenocarcinoma differentiates into myoepithelial cells with successive passage. The LA-7 line, a subclone of RAMA-25, differentiates to form domes that mimic *in vivo* mammary epithelial morphology (Dulbecco et al., 1980). The formation of domes and morphological differentiation subsequently received much attention (Visser and Prop, 1974; McGrath, 1975). Differentiation is more commonly defined by the ability of the particular cell type to produce product, (in this case milk constituents) termed functional differentiation. Comma-1D cells, introduced in 1984, exhibit morphological and functional differentiation *in vitro* (Danielson et al.). The production of casein, the primary protein in milk, was the preeminent determinant of its functional differentiation. Many of the previously mentioned cell lines are still popular today as *in vitro* models of mammary growth and differentiation.

A limited number of bovine mammary epithelial cell lines have been reported (Schmid et al., 1983a and b), but until recently none have shown abundant lactose and casein secretion inducible by PRL, as occurs *in vivo*. Recently, Hung et al. (in press) have developed such a cell line. This immortal clonal cell line (Mac-T) was produced from primary bovine mammary epithelial cells (obtained from lactating Holstein cows) transfected with the temperature sensitive Simian Virus 40 (SV-40) large-T-antigen. However, Mac-T cells have been shown to express the large-T-antigen gene at low levels indicated by direct immunofluorescence. Concomitantly, Mac-T cells do not appear to be temperature dependent (Hung et al., in press), unlike other cells transfected with the SV-40 large-T-antigen virus (Jat and Sharp, 1989). Additionally, Mac-T cells do not

exhibit many characteristics common to continuous cell lines. Mac-T cells are not tumorogenic when injected into athymic nude mice and are anchorage dependent, since they do not grow in soft agar.

Additional evidence supports the belief that Mac-T cells function as normal mammary epithelial cells. Presence of epithelial specific cytoskeletal proteins and lack of vimetin filaments indicate epithelial origin. Moreover, the cells proliferate and acquire a duct-like morphology typical of developing mammary gland when cultured in floating collagen gels or on matrigel (EHS matrix; Akers, Boyle, Romagnolo and Woodward, unpublished data).

Mac-T cells are capable of functional differentiation as well. When cultured on floating collagen gels and in the presence of PRL, Mac-T cell production of casein (α, β, and k) and lactose are increased and the cells also produce α-lactalbumin and β-lactoglobulin. Finally, Mac-T cells have been in continuous passage for over 200 doublings, without displaying characteristics of genetic drift i.e. presence of giant cells or stellate fibroblastic morphology.

Before using the Mac-T cells as a bioassay for growth, the cells must demonstrate normal growth responses towards agents previously tested on the bovine mammary gland, in addition to functioning as *in vivo* with respect to differentiation, as previously discussed. Thus media, atmosphere, and temperature optimums need to be ascertained. Typical passage, harvesting, freezing and growth on plastic and collagen need characterization. Quantification of growth must also be accessed in an accurate and timely manner. More importantly, the cells should be studied for growth following treatment with hormones known to be mitogenic to bovine mammary epithelium. Evaluation of appropriate culture conditions, methods of evaluation and response of Mac-T cells to several known mitogens is

the focus of this paper.

MATERIALS AND METHODS

Hormones and Culture Materials

All hormones were obtained through Sigma Chemical Corp (St. Louis, MO)., unless otherwise noted. All culture reagents, vessels, media, serum, and enzymes were obtained from Gibco (Gaithersburg, MD) unless otherwise noted.

Cells

Bovine mammary epithelial (Mac-T) cells (clone LMH17) were obtained from Dr. Turner, McGill Univ., Montreal. The cells were from mid-lactational Holstein cows, clonal in nature, non-tumorogenic in nude mice, anchorage dependent, and transformed with SV-40 large-T-antigen to achieve immortality (Hung et al., in press).

Passage

Cells were routinely maintained for subsequent experiments in 100 X 20mm culture petri dishes (Falcon brand, Becton Dickson, Lincoln Park, NJ) with 15 mls of DMEM (Dulbecco's modified Eagle Medium) supplemented with 10% Fetal Calf Serum, 1% antibiotic-antimycotic solution (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B (Fungizone)), and 0.1% gentamicin reagent solution (10 mg/ml gentamicin sulfate). The cells were cultured at 37 C in an humidified atmosphere of 95% air and 5% carbon dioxide. The pH was maintained at 7.4 by 3.7 g/L tissue grade $NaHCO_3$ and atmospheric carbon dioxide, and monitored visually by phenol red pH indicator. Cells were routinely passed before attainment of monolayer. Cells were first rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) several times to disrupt bonds

between cells. Thereafter, 1 ml .25% trypsin and 1 ml enzyme free cell dissociation solution (Specialty Media, Inc., Lavallette, NJ) were added and cells incubated for 15-20 min. Addition of 5 ml DPBS and subsequent rinsing was used to completely dissociate the cells. An aliquot of the mixture was generally saved for hemacytometer counts. The cell solution was then centrifuged at 200 X G for 10 min and tubes inverted to remove supernatant. Pelleted cells were routinely resuspended in DMEM with 10% FCS at a concentration of approximately 5 X 10^4 cells/ml for plating into fresh culture dishes. Cells were passed approximately every 5 days.

Freezing Cells

First, cells were harvested normally and centrifuged as previously described, but resuspended in 20% FCS, 5% dimethyl sulphoxide (DMSO), and 5% glycerol in DMEM. Subsequently, cells were stored at 4 C for 30 min and transferred to -80 C in a styrofoam rack to insure slow freezing. Cells were then plunged directly into liquid nitrogen (-196 C) 24 hours later for long term storage. Storage for periods of 8 to 12 months had no noticeable effect on cell viability.

Quantification of Cell Number and Cell Growth

Hemacytometer

Harvested cells in trypsin, cell dissociation solution and DPBS were mixed 1:1 in a solution with 0.5% Trypan Blue stain (in .5% NaCl saline) or 0.4 g/L Crystal Violet stain (in 0.1 M citric acid) and were counted in 10 (1 mm) squares of a hemacytometer. Proportion of trypan blue stained cells was calculated to estimate cell viability. Cells stained with crystal violet were counted to determine total cell number. Because cell counts obtained immediately were not different from those after storage, for some experiments cell counts were determined up to two weeks

after cells were harvested.

DNA assay

After incubation of the cells in cell dissociation solution and trypsin for at least 20 minutes, 2X phosphate buffered saline (2X PBS) (.1 M Na_2HPO_4, 4.0 M NaCl, and .004 M EDTA) was added in quantity to double the volume of cell suspension. The suspension was then sonicated for 5 seconds (Artek Systems Model 300 sonic dismembrator) at 35% power using a 3.9 mm diameter microprobe supplied with the power unit. Samples were assayed for DNA concentration with a model TKO 100 DNA fluorometer (Hoefer Scientific Instruments, San Fransisco, CA) according to manufactures' instructions based on the method of Labarca and Paigen (1980).

^{3}H-Thymidine incorporation

Routine testing of methyl ^{3}H thymidine (^{3}H-TdR) incorporation consisted of 1 ml of cell suspension (4 X 10^4 cells) plated into each well of a 24-well culture dish (Nunclon, Denmark) in the presence of 5% FCS. Following incubation for 24 h, media were removed and replaced with 1 ml DMEM and the cells were incubated 72 h. DMEM was removed and replaced with test media and cells incubated 16 h. Thereafter, test media were replaced with DMEM supplemented with 1 μCi/ml ^{3}H-TdR (ICN, Irvin, CA; No. 24039, S.A. 64 Ci/mMol) and incubation continued for 2 h. Subsequently, media were removed and cells were rinsed with 1 ml DPBS. Next, .25 ml of a 1:1 mixture of .25% trypsin and cell dissociation solution were added and incubated at 37 C for 30 min. Finally, .5 ml 2X PBS was added to each well. Contents of the well were then transferred to a test tube, and 1 ml 1X PBS, used to rinsed the well, was added to this. The contents of the tube were sonicated as previously described.

An aliquot (200 μl) of the suspension was transferred to a glass scintillation vial and diluted with 2 ml of ice cold 10% TCA in water. Vials were chilled for 20 min, centrifuged at 1500 X g for 20 min at 4 C and the supernatant discarded. The pellets were dissolved by addition of 100 μl of tissue solubilizer (NCS, Amersham Corp., Lincoln Park, IL) and incubated at 50 C for 30 min. Vials were then cooled to room temperature. Next, 25 μl glacial acetic acid and 7 ml scintillation cocktail (Ecoscint, National Diagnostics, Manville, NJ) were added. TCA insoluble radioactivity was determined using an LKB model 1219 Rackbeta scintillation counter (LKB Corp., Gaithersburg, MD). Other aliquots of harvested cells were assayed for DNA concentration as described previously. Data were expressed as CPM/well and/or CPM/μg DNA and represent the mean +/- standard deviation of at least 3 wells per treatment.

Testing Potential Mitogens

Prior to the testing of any mitogens, Mac-T cells (2 X 10^4/ml/well) were given 24 h to attach to 24 well culture dishes (Nunclon, Denmark). Subsequently, media were removed and replaced with test media. Also, cells from several wells were harvested and stored in 2X PBS at room temperature to obtain an estimate of pre-treatment DNA concentration. Cells were then incubated for 72 h, harvested and assayed for DNA as previously described.

Statistical Analysis

The general linear models procedure (PROC GLM) of SAS (SAS, 1988) was used to analyze dependent variables. Growth of Mac-T cells in hormones was analyzed using Dunnett's test (Zar, 1984), comparing all hormone treatments to a control (no hormone). Comparisons between treatments utilized contrasts to obtain F values. The critical F values obtained from non-orthogonal contrasts were

then replaced by the Bonferroni F test (Zar, 1984). All models contained treatment only.

RESULTS

Harvesting and Assaying

Comparison of DNA recovered by harvesting cells with PBS, trypsin, cell dissociation solution or a combination of trypsin + cell dissociation solution (T + C) is shown in Fig. 1. All treatments were not different from PBS in recovering DNA when cells were cultured after growth in media alone for 72 h (low final cell concentrations). However, when cell densities were higher (as when cultured in media plus 10% FCS), less DNA was obtained when PBS was added compared with use of trypsin, cell dissociation solution, or T + C ($P < .05$). Although trypsin, cell dissociation solution, and T + C were not different in recovering DNA at low or high cell densities, T + C caused cells to dissociate more quickly (data not shown).

After cells were harvested in the usual manner, subsequent lysing of cells with DDH_2O did not significantly alter DNA values of high or low cell density cultures (Fig. 2). However, sonication of cells (at medium setting for 5 s) following harvesting and addition of PBS increased DNA fluorometric readings ($P < .0001$) (Fig. 3). Although both standard curves in Fig. 3 are linear ($P < .001$), sonication decreased fit to a quadratic curve as illustrated by decreased fluorescence with higher cell number in cells not sonicated. Additionally, sonication for 20 s did not change calf thymus DNA standards (Fig. 4). FCS in samples increased DNA readings whether sonicated or not ($P < .05$), by addition of 80 μl media containing 10% FCS (Fig. 5). Volumes of DMEM had no affect on reading of fluorescence in

the DNA assay (not shown).

Growth of Mac-T cells

The effects of various concentrations of ethanol, an alcohol commonly used to dissolve lyophilized steroids, on DNA accumulation after 72 hours exposure are shown in Fig. 6. Ethanol inhibited growth at concentrations as low as .01% ($P < .05$). Higher concentrations (1%) reduced growth of Mac-T cells in 10% FCS to below that of DMEM alone. Growth of Mac-T cells was linear in media containing commercial FCS or filter-sterilized serum from a multiparous dry Jersey cow at concentrations from 0 to 8% ($P < .001$) (Fig. 7). However, Jersey serum also fits a quadratic curve ($P < .05$). Both new born calf serum (NBCS) and FCS were more effective stimulators of DNA accumulation than serum from a Jersey cow ($P < .05$) (Fig. 8). Mac-T cells also exhibited increased proliferation in response to a complex of mitogens (1 ng/ml triiodothyronine, .5 μg/ml hydrocortisone, 1 μg/ml prolactin, and 1 μg/ml insulin (I)) even when cultured in 10% FCS (Fig. 9) ($P < .01$).

Response to Agents Considered Mitogenic to Some Mammary Epithelial Cells

Mac-T cells increased growth in response to I added to DMEM, and presence of a small amount of FCS (1%) seems to augment this response; growth in FCS was increased by addition of .5 ng/ml I ($P < .01$), whereas in the absence of FCS growth was not significantly increased until 5 ng/ml I or more was added ($P < .05$) (Fig. 10). Mac-T cell's rate of proliferation was not altered by estrogen (E), however, whether cells were cultured in media only or media supplemented with 1% FCS (Fig. 11). Similarly, progesterone (P) was unable to elicit growth under identical conditions (Fig. 12). Moreover, various combinations of E or P failed to stimulate growth over control media without (Fig. 13) or with (Fig. 14) 1% FCS added.

Culture of Mac-T cells with IGF-I (Fig. 15), however, markedly increased growth with additions as low as .1 ng/ml in the absence of FCS ($P < .05$). Growth in 1% FCS was not apparent until concentrations of IGF-I were 10 ng/ml or higher ($P < .01$). Concentrations above 30 ng/ml did not further increase growth in the absence or presence of 1% FCS (Fig. 16). Concentrations of EGF up to 250 ng/ml did not increase growth of Mac-T cells alone, with 1% FCS (Fig. 17), with 10 ng/ml IGF (Fig. 18), or with 10 ng/ml IGF and 1% FCS (Fig. 19) above controls.

Data for thymidine incorporation generally showed proliferative responses of Mac-T cells which mimic results obtained from direct DNA assay. As illustrated in Table 1, incorporation was markedly increased ($P < .01$) 8 and 16 h following exposure of serum starved cells to as little as 1% FCS in DMEM. However, depending on treatment, incorporation was approximately 2 to 10 fold lower ($P < .01$) after 8 h compared with 16 h. Relative proliferative effects were also evident whether data were expressed as CPM/well or CPM/μg DNA. Consequently, in subsequent incorporation studies cells were incubated 2 h in the presence of 1 μCi/ml TdR beginning 16 h after addition of test media onto serum starved cells. A second experiment showed that incorporation of TdR was increased 3 fold ($P < .001$) by as little as .05% FCS and that concentrations of FCS of .4 or greater actually reduced the effect of FCS on incorporation (Table 2). A proliferative response as demonstrated by DNA accumulation after 72 h of exposure to IGF-I and insulin was also evident from acute stimulation of TdR incorporation after only 16 h of exposure in serum starved cells ($P < .01$). Like DNA responses, EGF alone or in combination with IGF-I or insulin had no effect on TdR incorporation. IGF-I appeared to increase incorporation with as little as 1 ng/ml in DMEM and consistently stimulated TdR incorporation at 10 ng/ml. Addition of bovine PRL or

bovine GH alone had no effect on TdR nor did these hormones alter incorporation associated with insulin alone (Tables 3 and 4).

DISCUSSION

Utilization of Mac-T cells as a model for bovine mammary epithelial cells mandates characterization sufficient to demonstrate the cells behave as would be expected *in vivo*. Previously, the ability of Mac-T cells to differentiate morphologically and functionally in response to appropriate hormones was shown (Hung et al., in press). The cells are immortalized, but do not express any tumorogenic behavior, making them ideal for culture.

Techniques to measure cell growth were contrasted for accuracy, expense and timeliness, since quantification was to be performed frequently. Incorporation of ^{3}H-thymidine represents an appropriate method to analyze rate of cellular division at specific periods of time. Whereas, quantification of total DNA present is an inexpensive method to quantify growth, particularly accurate in measuring growth over a longer interval of time. Hemacytometer counts, although seemingly accurate, consumed too much time to make extensive testing feasible. DNA assay also represented a cheap alternative to obtaining new equipment or extensive use of radioisotopes. The normal procedure for assay of DNA in tissue involves dividing tissue into small pieces, homogenization, and transfer to high salt buffer (PBS) (Labarca and Paigen, 1980). Free cells do not need mechanical division or homogenization since they can easily be dissociated by quick incubation in any of several dissociation solutions (trypsin, EDTA, commercial cell dissociation solutions, dipase, or pronase). Our data indicate that for PBS to free DNA adequately for binding of dye used for DNA assay, cells must first be dissociated

from the plastic and one another, especially if cell densities are high (Fig. 1). Dissociation solutions such as trypsin or cell dissociation solution may take 30 min or longer, whereas T + C takes only 15 min to fully dissociate cells, providing cells are rinsed briefly following incubation to free remaining cells. Obviously cells should not be exposed to dissociates too long or the cell membrane may weaken or rupture, especially at incubation temperatures (37 C) (Freshney, 1987).

Freshney (1987) suggests sonication of cells, after they have been dissociated, to fully release DNA for assay. This not only appears to increase DNA readings (presumably by allowing for easier attachment of DNA to dye), but makes the curve more linear at higher cell number and reduces variance between samples. Moreover, sonication for periods 20 s (4 X as long as cells are normally sonicated) does not affect DNA fluorometer readings, since purified calf thymus DNA fluorometer readings were not different after sonication. Sonication must be performed before addition of dye for DNA measurement, however. Cell culture often uses FCS to promote growth during normal passage and allow for normal growth during experiments. Care must be taken to ensure residues of FCS are not left behind during assay, since levels as low as 8 μl FCS per 2 ml assay volume can significantly alter DNA readings. This emphasized, that fetal calf serum apparently contains small amounts of DNA (or material detected as DNA).

Caution must also be exercised when preparing solutions for use in cell culture. Ethanol, used as a solvent for many solutes, inhibited growth at concentrations as low as .01% (Fig. 6). However, various oils and other solvents may be inappropriate for use in cell culture since they may modify growth.

Mac-T cells respond to serum in a linear manner up to 8%. The origin of serum determines mitogenic effectiveness even when obtained within the bovine

genus. Serum from fetal or newborn animals apparently possesses a higher or more effective concentration of mitogens than that from a multiparous dry cow. FCS appears to give repeatable results with low variability, making it an ideal growth media for normal passage. Mac-T cells are capable of significantly increasing growth in response to mitogens typically used to induce differentiation, when cultured in 10% FCS. Thus, Mac-T cells are capable of expressing growth differences whether growing slowly or very quickly.

Mac-T cells increase growth in the presence of insulin, especially at higher concentrations, like mammary epithelial cell lines from other species. However, when I is added in low concentration (.5 ng/ml) to Mac-T cells in 1% FCS growth was stimulated. This may be associated with the I in FCS combining with the I added to enhance I concentration, or a permissive effect. Since, when I is present in pharmacologically high doses it may bind to IGF-I receptors to stimulate growth (Massague and Czech, 1982; Flier et al., 1986). Unlike some murine studies, E or P do not stimulate growth of Mac-T cells directly individually, combined, with FCS, or combined with FCS. Controversy exists concerning the ability of E or P to directly stimulate mammary epithelial growth in humans and rodents. Though, most evidence suggests indirect stimulation (Sirbasku, 1980; Richards et al., 1988; Beck and Garner, 1989), as does these experiments with Mac-T cells (Figs. 11-14).

Insulin like growth factor-I is thought to mediate the growth promoting effects of GH in the mammary gland of several species including bovine (Mathews et al., 1986; Phipps, 1988; Davis et al., 1989; and Kleinberg et al., 1990). Additionally, most mammary epithelial cells respond to IGF-I with marked increases in growth (cow: Shamay et al., 1988; Baumrucker and Stemberger, 1989; sheep: Winder et al., 1989; mice: Imagawa et al., 1986; human: Cullen and

Lippman, 1989; human tumor: Freed and Herrington, 1989). Likewise, IGF-I is mitogenic to Mac-T cells at low concentrations in the absence of FCS. The addition of 1% FCS appears to mask the effects of IGF-I at low concentrations; however, Mac-T cells still readily respond to IGF-I at concentrations of 10 ng/ml or higher.

Another hormone considered to be mitogenic to mammary epithelium across several species is EGF. Epidermal growth factor, found primarily in the submaxillary gland (Rall et al., 1985), has been shown to increase growth in rodents (Taketani and Oka, 1983 a and b) and humans (Stocker et al., 1976). Stimulation of growth by EGF in the bovine mammary gland is controversial (Haslam and Shyamala, 1979a; Collier and McGrath, 1988). In fact, isolation and positive identification of EGF in the bovine has yet to be reported. Mac-T cells do not respond to EGF alone or with 1% FCS (Figs. 17-19). However, some studies claim EGF is not a potent mitogen unless IGF is present also (Imagawa, 1985). Mac-T cells, on the other hand, do not increase growth over control cells when cultured in EGF, EGF + FCS, EGF + IGF, or EGF + IGF + FCS. Perhaps, bovine EGF is different than murine EGF or perhaps bovine mammary epithelial cells are unresponsive to any EGF or EGF like peptides. Moreover, mitogenic effects of FCS, I, and IGF-I were also demonstrated by measurement of TdR in serum starved cells (Tables 1-4).

Therefore, Mac-T cells appear to respond to known mitogens as expected. No results obtained in our studies of characterization of Mac-T cells strayed from uncontroversial published data on bovine mammary epithelial cells. Several of our studies demonstrated differences between bovine mammary epithelium and murine mammary epithelium, however. This is not surprising as others have often

found differences between the murine and bovine mammary glands (Sheffield, 1988a; Akers, 1990). Data can not readily be transferred across species. Necessary methods and procedures for normal culture and testing of the cell strain were resolved. The Mac-T cell strain appears to behave like *in vivo* bovine mammary epithelial cells in morphology, in morphological and functional differentiation and in growth characteristics in response to various mitogens.

SUMMARY II

The characterization of Mac-T study provided an invaluable tool in further elucidating the effects of E and P on mammary cell growth. Isolation of the cell responsible for the production of milk allows researchers to determine those hormones which can directly stimulate cellular proliferation. However, such cells should function like normal mammary epithelial cells *in vivo* before results obtained with the cell line can be considered valid. Characterization of the cells structure and differentiation characteristics have formerly been elucidated in part by Dr. Turner and co-workers, McGill Univ. However, this is the first attempt to characterize Mac-T cells as normal with regard to their response to various mitogens. Additionally, procedures were designed or modified to allow testing of mitogens on Mac-T cells to be uncomplicated, timely, and inexpensive. Some results, such as response to E and P, were discussed briefly only to support the normalcy of responsiveness of the Mac-T cell strain. More extensive discussion of E and P effects on bovine mammary tissue is covered in the following chapter.

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Table 1. Effect of incubation time and serum concentration on tritiated thymidine incorporation into Mac-T cells after 72h of serum starvation.[1]

	8 Hours		16 Hours	
TRT	cpm/well	cpm/μg DNA	cpm/well	cpm/μg DNA
DMEM	5.7 ± 1.3[a]	4.6 ± .6[a]	18.3 ± 4.3[a]	12.8 ± 3.4[a]
1% FCS	12.2 ± 2.1	9.8 ± .9	121.2 ± 22.6	98.2 ± 11.2
2% FCS	12.5 ± 1.7	11.2 ± 1.3	110.0 ± 30.1	93.9 ± 14.2
4% FCS	14.6 ± 3.8	12.6 ± 2.3	127.6 ± 38.2	90.8 ± 16.4
8% FCS	9.7 ± 2.2	9.4 ± 1.9	96.0 ± 29.8	74.3 ± 12.5
16% FCS	11.9 ± .5	10.6 ± 1.1	93.9 ± 23.9	61.2 ± 13.5

[1]Cells were plated (~20,000) into each well of two 24-well culture dishes and allowed to incubated for 24h in the presence of 5% FCS in DMEM. Thereafter, medium was removed and replaced with unsupplemented DMEM. After 72h medium in each of 4 wells of each dish was replaced with DMEM alone or as shown above (N=4). After 8 or 16 hours, media were replaced with DMEM supplemented with 1 μCi/ml tritiated thymidine and incubation continued for 2 h, when cells were harvested and assayed to determine TCA insoluble radioactivity. Data are given as mean ($X\ 10^{-3}$) ± S.D.

[a]Indicates value is significantly lower than others in the column ($P \leq .05$).

Table 2. Effects of low concentrations of FCS on tritiated thymidine incorporation into serum starved Mac-T cells.[1]

Treatment	CPM/well
Control (DMEM)	80 ± 7
.05 % FCS	251 ± 18
.1 % FCS	248 ± 7
.2 % FCS	204 ± 10
.4 % FCS	165 ± 6
.8 % FCS	126 ± 3

[1]Cells ~ 40,000/well were tested for TdR incor poration after 52h of serum starvation and 16h incubation with FCS. Data given as mean ± S.D. X 10^{-3} (N=4).

[a]Indicates value is greater than control ($P \leq .05$).

Table 3. Dose response effects of IGF-1 and EGF on tritiated thymidine incorporation into serum starved Mac-T cells.[1]

Growth Factor	Conc (ng/ml)	CPM/well
Control (DMEM)	--	84 ± 10
IGF-1	.01	90 ± 13
IGF-1	.1	93 ± 18
IGF-1	1	149 ± 13[a]
IGF-1	10	201 ± 17[a]
IGF-1	100	234 ± 15[a]
EGF	.01	84 ± 14
EGF	.1	90 ± 14
EGF	1	71 ± 12
EGF	10	79 ± 24
EGF	100	75 ± 22

[1]Cells ~ 30,000 per well were plated onto 24-well culture dishes and serum starved for 52h prior to addition of mitogens and measurement of TdR incorporation. See legend to Table 1 for procedural details.
Data given as mean ± SD X 10^{-3} (N=4).

[a]Indicates value greater than control ($P \leq .05$).

Table 4. Effects of IGF-1, EGF, Insulin, bGH and bPrl tritiated thymidine incorporation into serum starved Mac-T cells.[1]

Factor(s)	Conc[1]	cpm/well
Control (DMEM)	--	84 ± 10
BI	10 ng	147 ± 16[a]
EGF	10 ng	83 ± 8
IGF-1	10 ng	227 ± 9[a]
IGF-1 + EGF	10/10 ng	226 ± 13[a]
BGH	1 μg	73 ± 8
BPrl	1 μg	64 ± 16
BI + IGF-1	10/10 ng	231 ± 11[a]
BI + EGF	10/10 ng	157 ± 12[a]
BI + IGF-1 + EGF	10/10/10 ng	219 ± 3[a]
BI + BPrl	10 ng/1 μg	134 ± 17[a]
BI + BGH	10 ng/1 μg	120 ± 10[a]
BI + BPrl + BGH	10 ng/1 μg/1 μg	83 ± 16

[1]See legend to Table 4. All concentrations per ml. Data given as mean ± S.D. X 10^{-3}. (N=4)

[a]Indicates value is greater than control ($P \leq .05$).

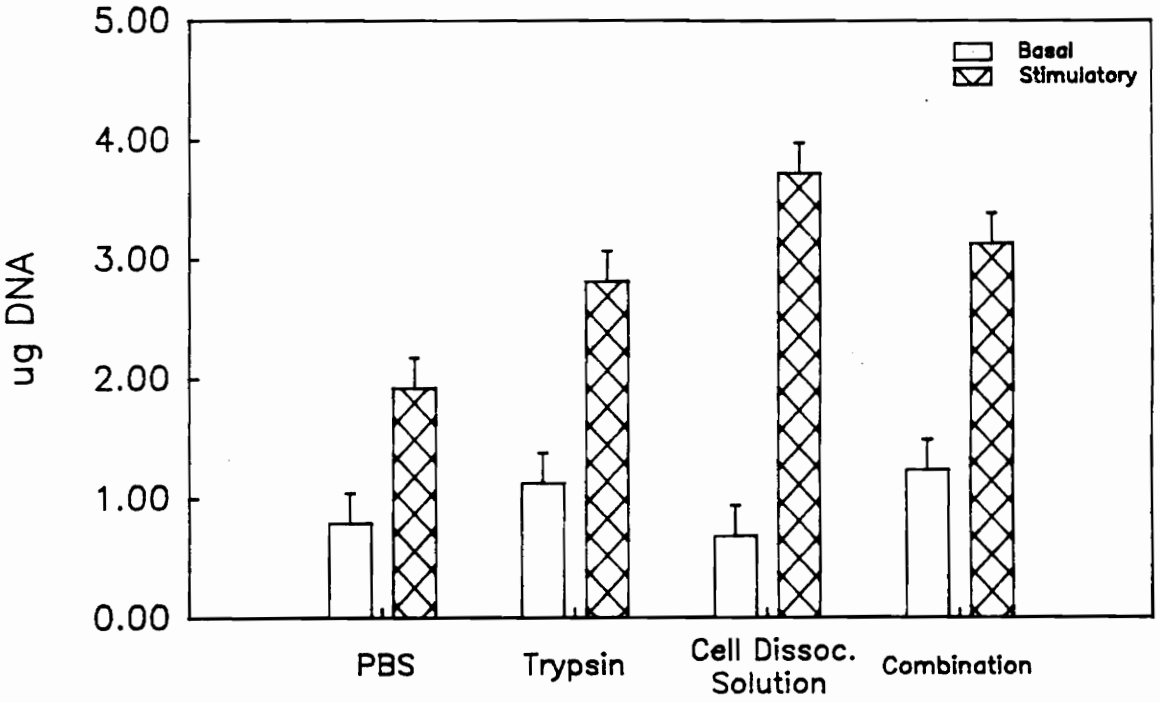

Figure 1. Comparison of methods to harvest Mac-T cells for DNA assay I. Basal media = media alone (sparse cell #); Stimulatory = 10% fetal calf serum (cell at or near monolayer).

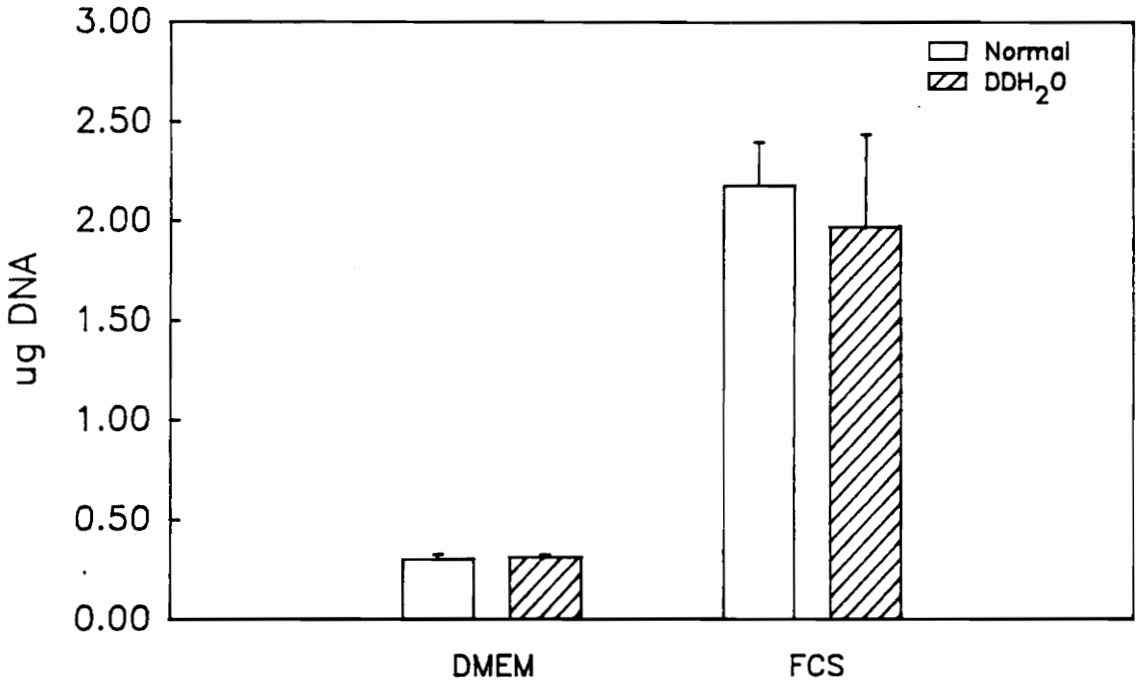

Figure 2. Comparison of methods to harvest Mac-T cells for DNA assay II. Normal = harvest of cells with trypsin + cell dissociation solution and 2X phosphate buffered saline; DDH_2O = harvest normally, add double distilled water then 2X PBS.

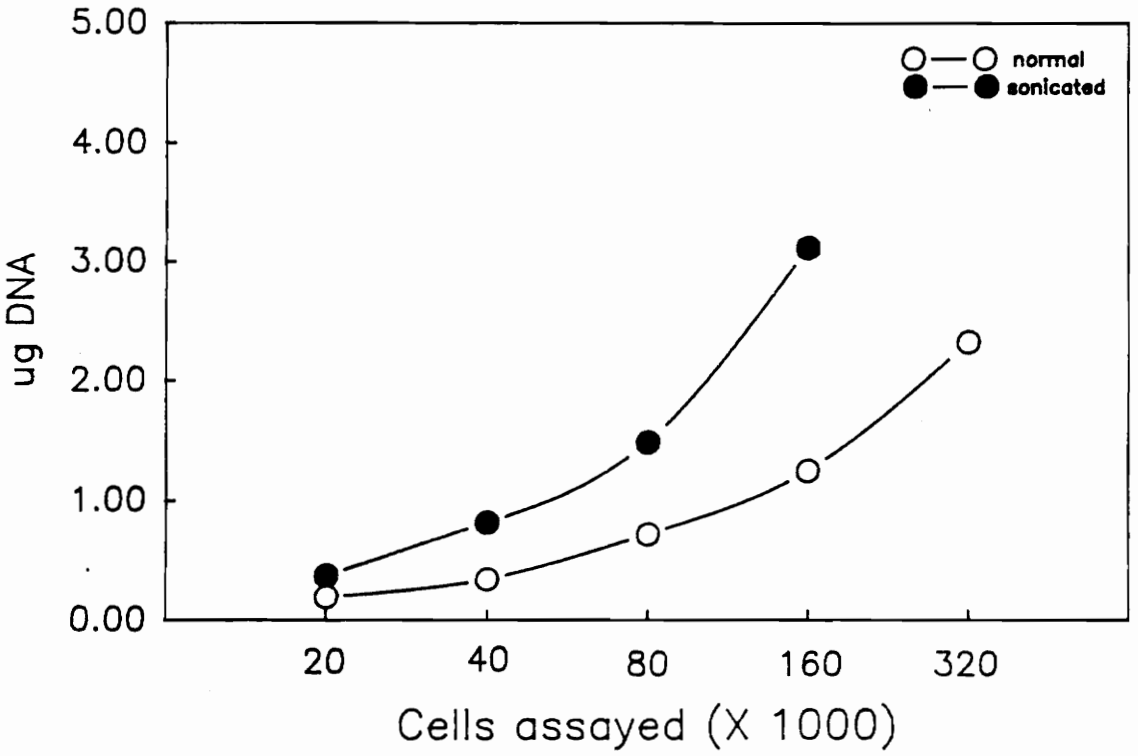

Figure 3. DNA readings of Mac-T cells in high salt buffer with or without sonication of cells for 5 s.

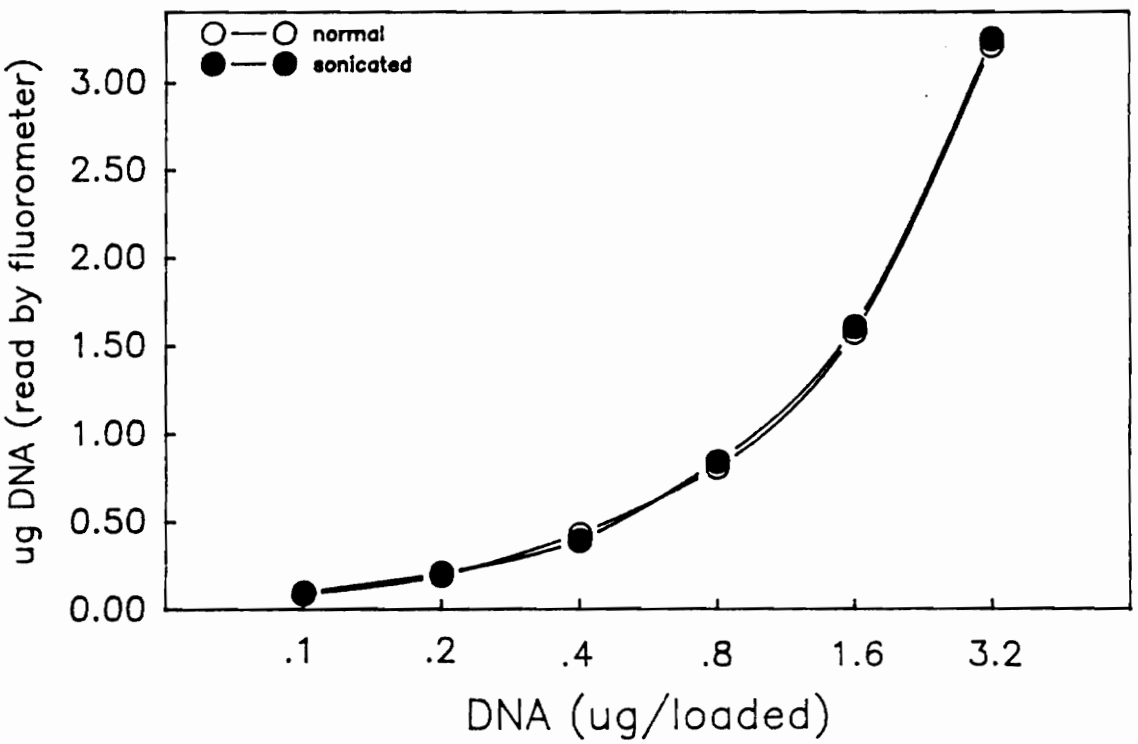

Figure 4. DNA fluorometer readings of calf thymus DNA standard that have been sonication for 20 s or not sonicated.

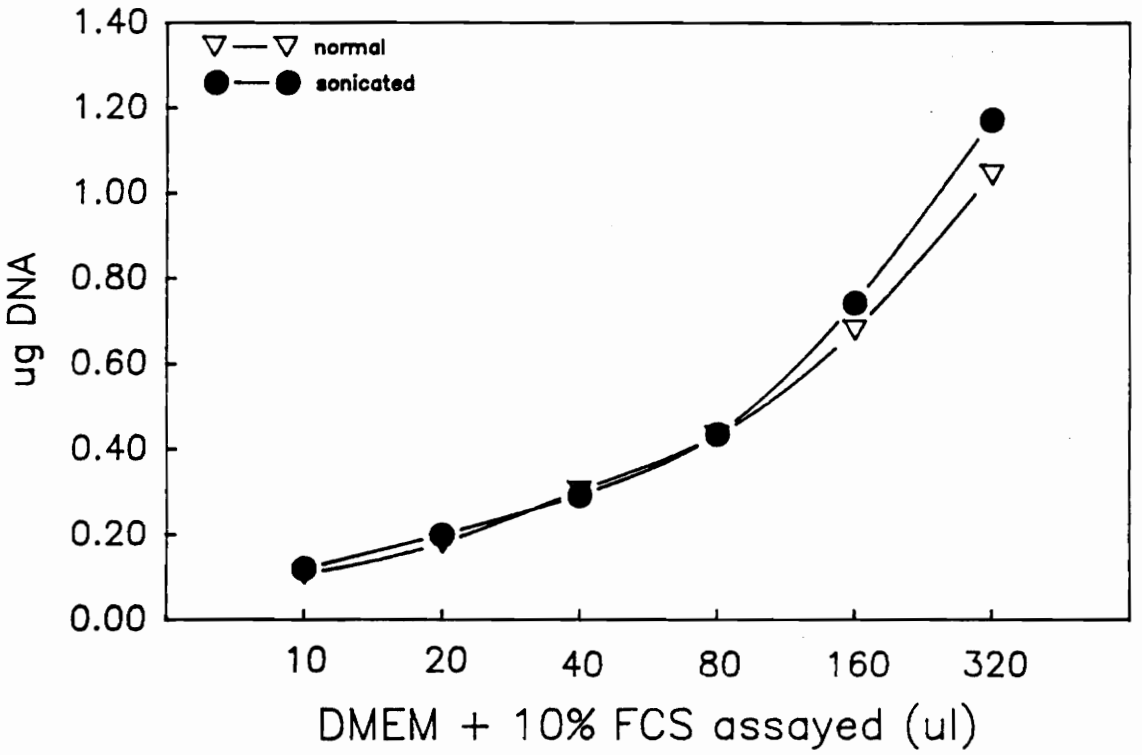

Figure 5. DNA fluorometer readings of fetal calf serum that have been sonicated for 20 s or not sonicated.

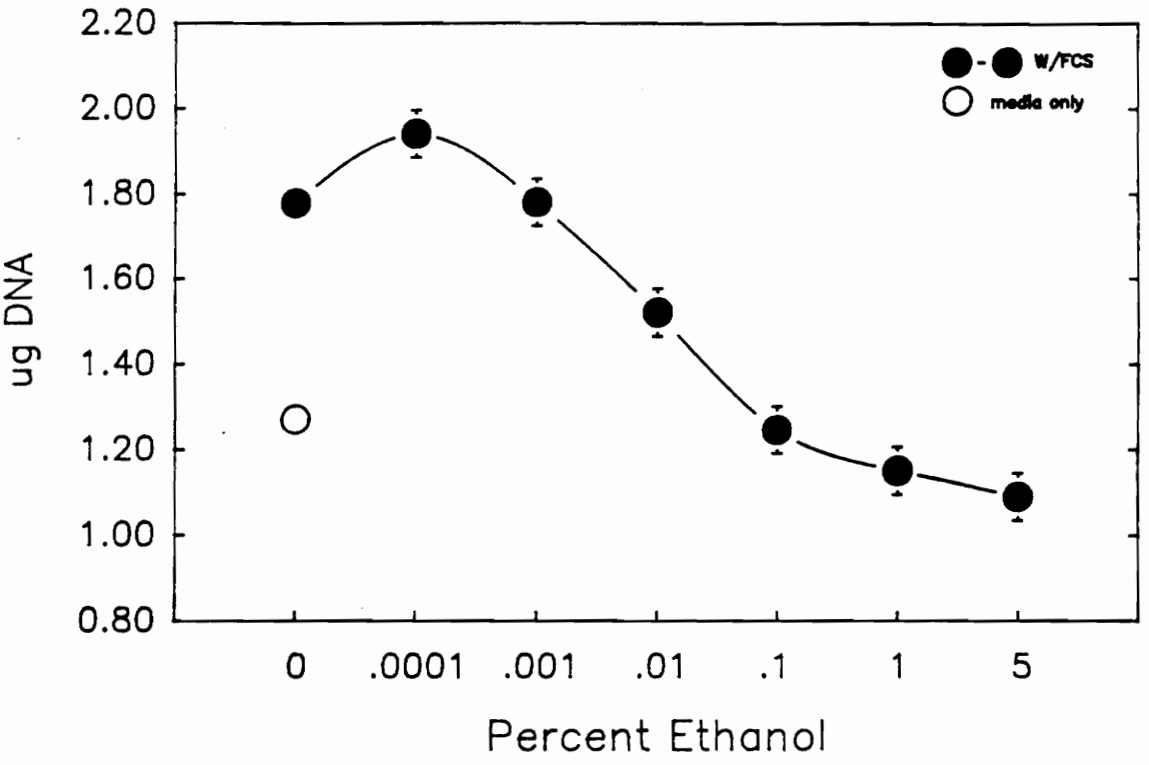

Figure 6. Culture of Mac-T cells in 10% fetal calf serum with increasing amounts of ethanol contrasted with DNA value for Mac-T cells cultured in media alone.

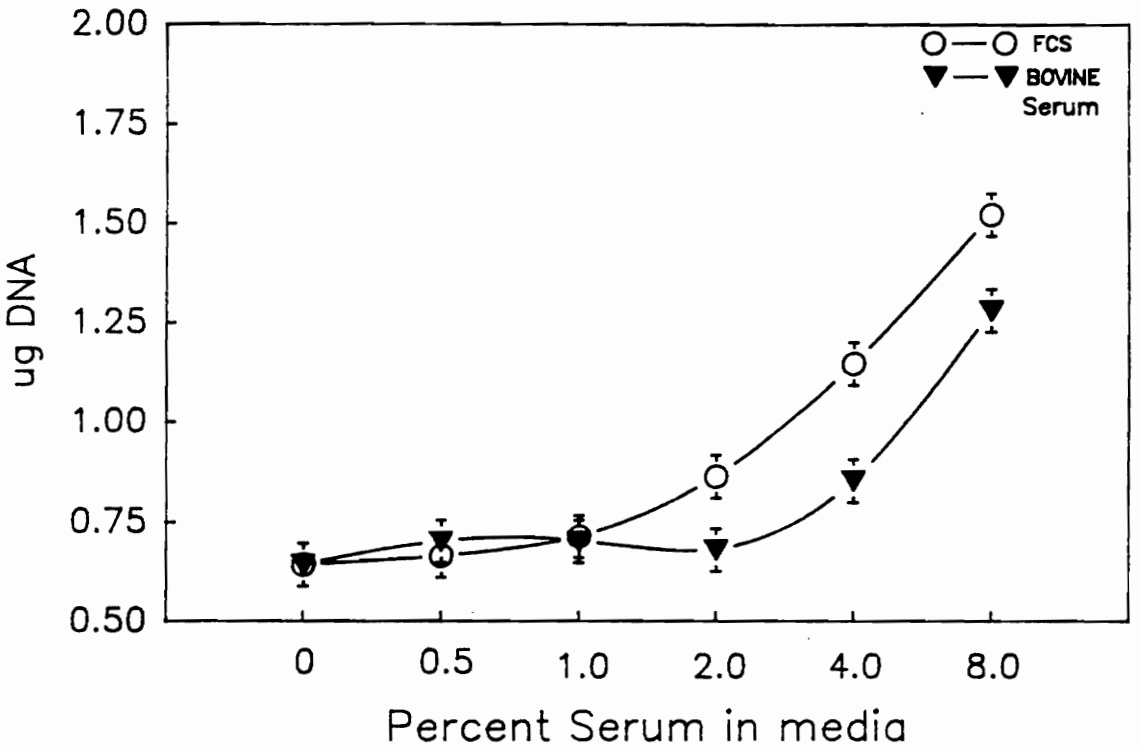

Figure 7. Growth responses of Mac-T cells cultured in increasing levels of fetal calf serum and serum from a Jersey cow.

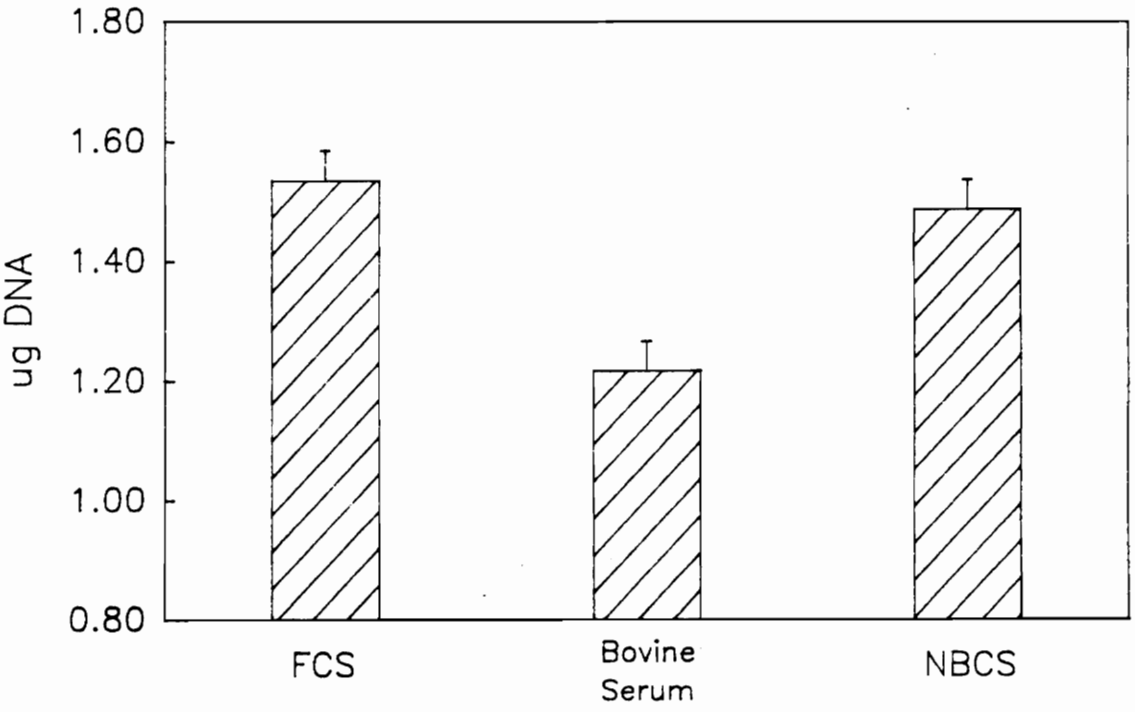

Figure 8. Growth responses of Mac-T cells cultured in 10% serum from bovine animals of different ages.

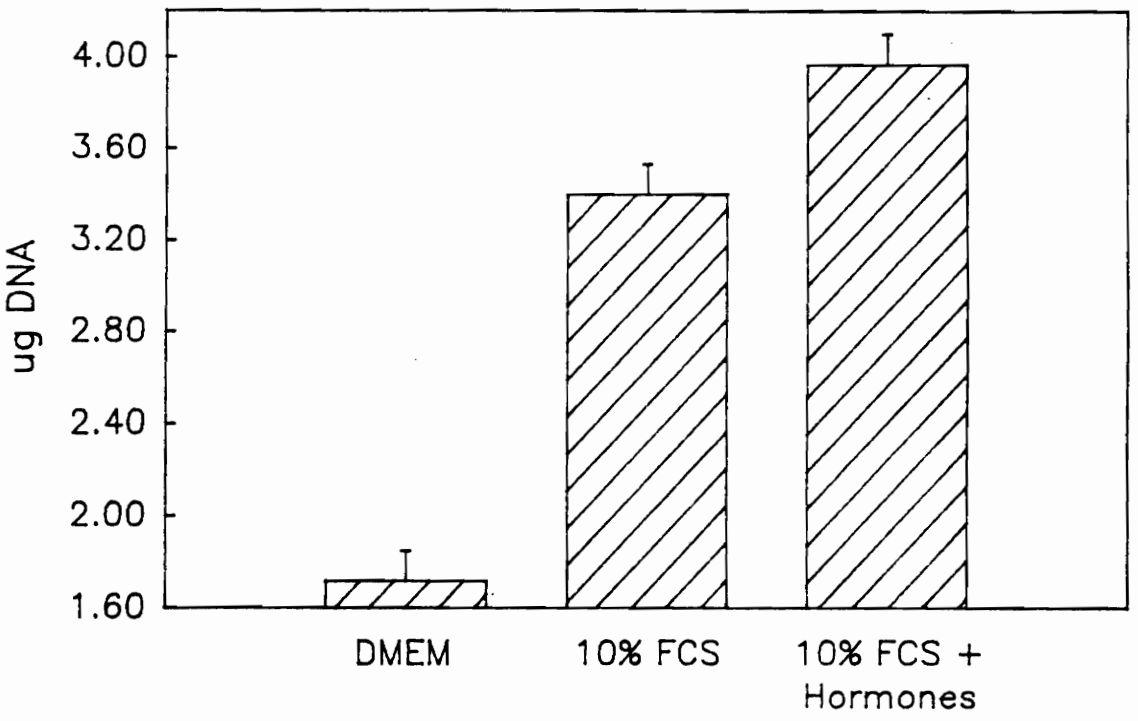

Figure 9. Growth responses of Mac-T cells cultured in 10% fetal calf serum, media only (DMEM), or 10% fetal calf serum + bovine insulin (1 μg/ml), hydrocortisone (.5 μg/ml), triiodothyronine (1 ng/ml), and bovine prolactin (1 μg/ml).

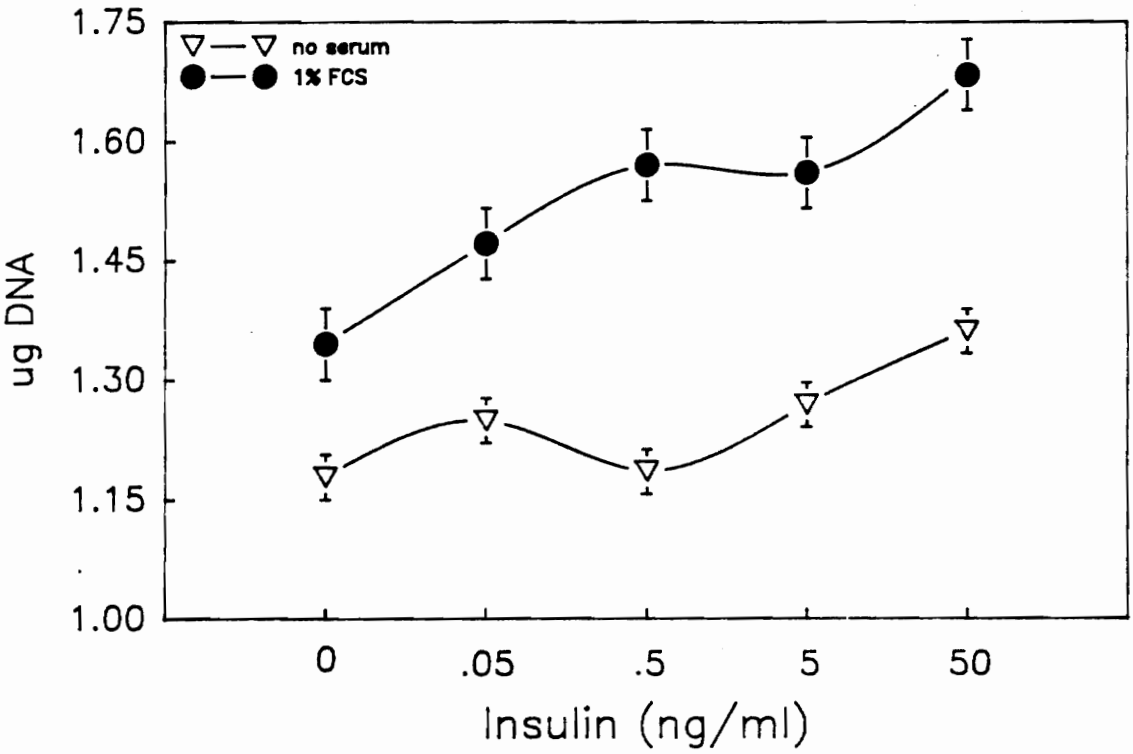

Figure 10. Growth responses of Mac-T cells cultured in bovine insulin treated media with or without fetal calf serum.

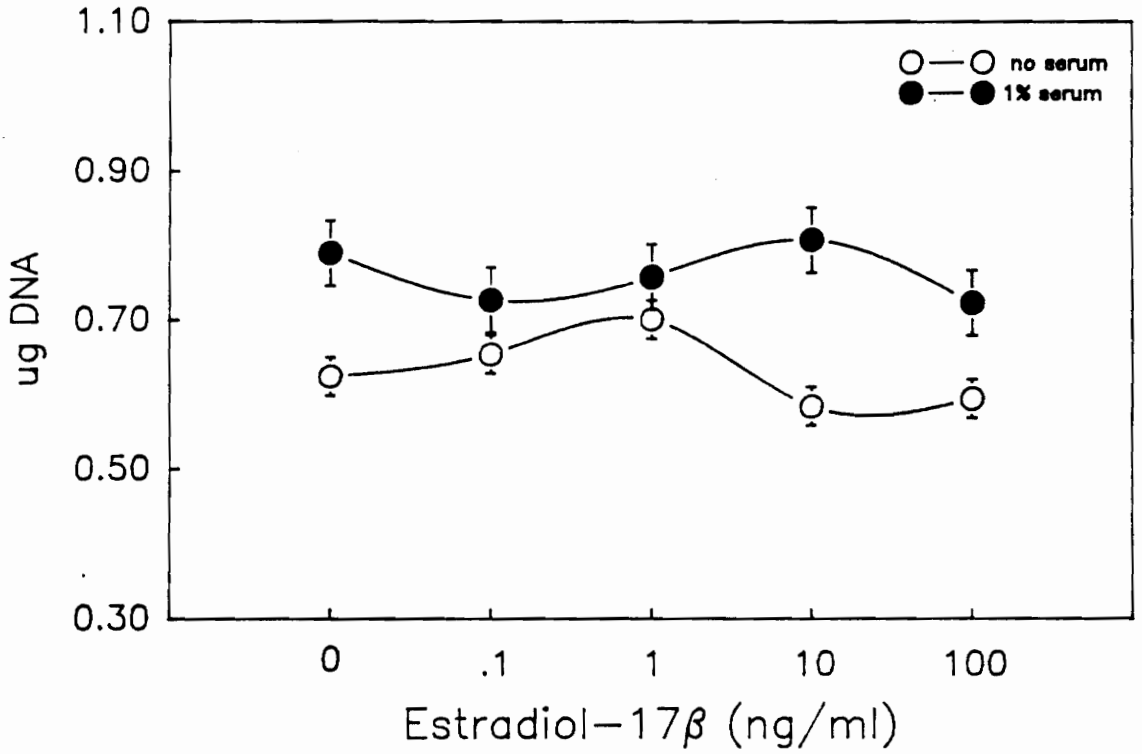

Figure 11. Growth responses of Mac-T cells cultured in estrogen treated media with or without fetal calf serum.

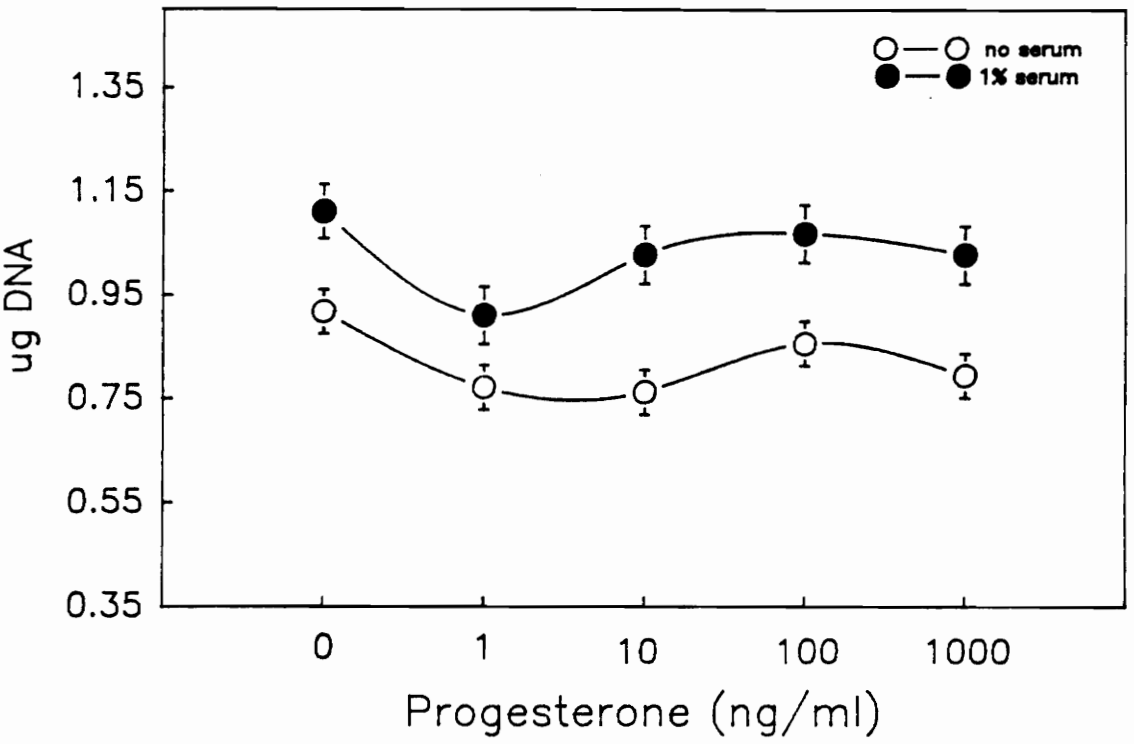

Figure 12. Growth responses of Mac-T cells cultured in progesterone treated media with or without fetal calf serum.

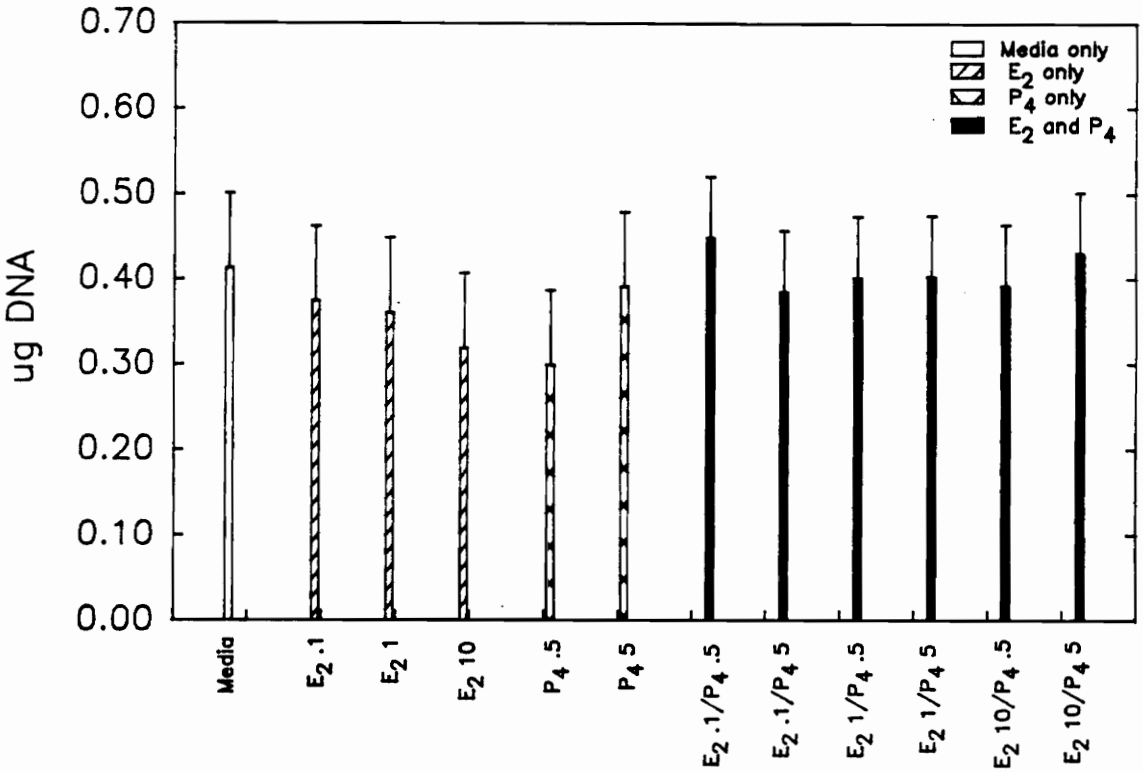

Figure 13. Growth responses of Mac-T cells cultured in combinations of estrogen and progesterone without fetal calf serum.

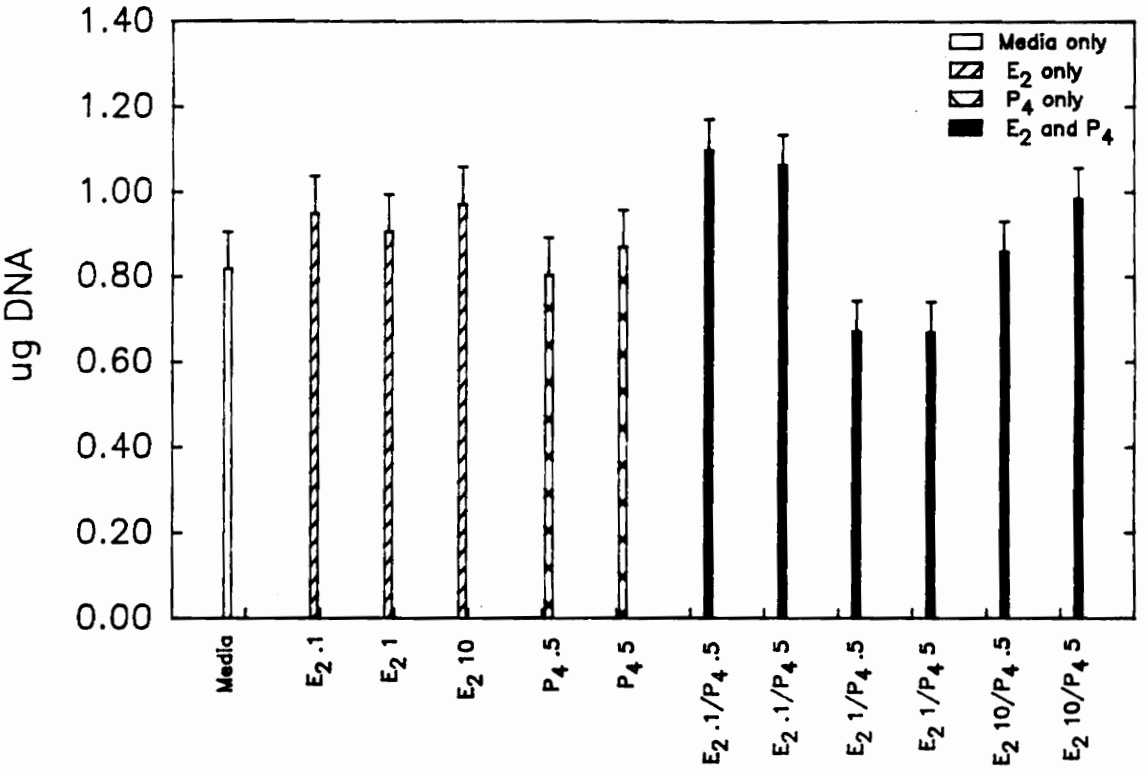

Figure 14. Growth responses of Mac-T cells cultured in combinations of estrogen and progesterone with fetal calf serum.

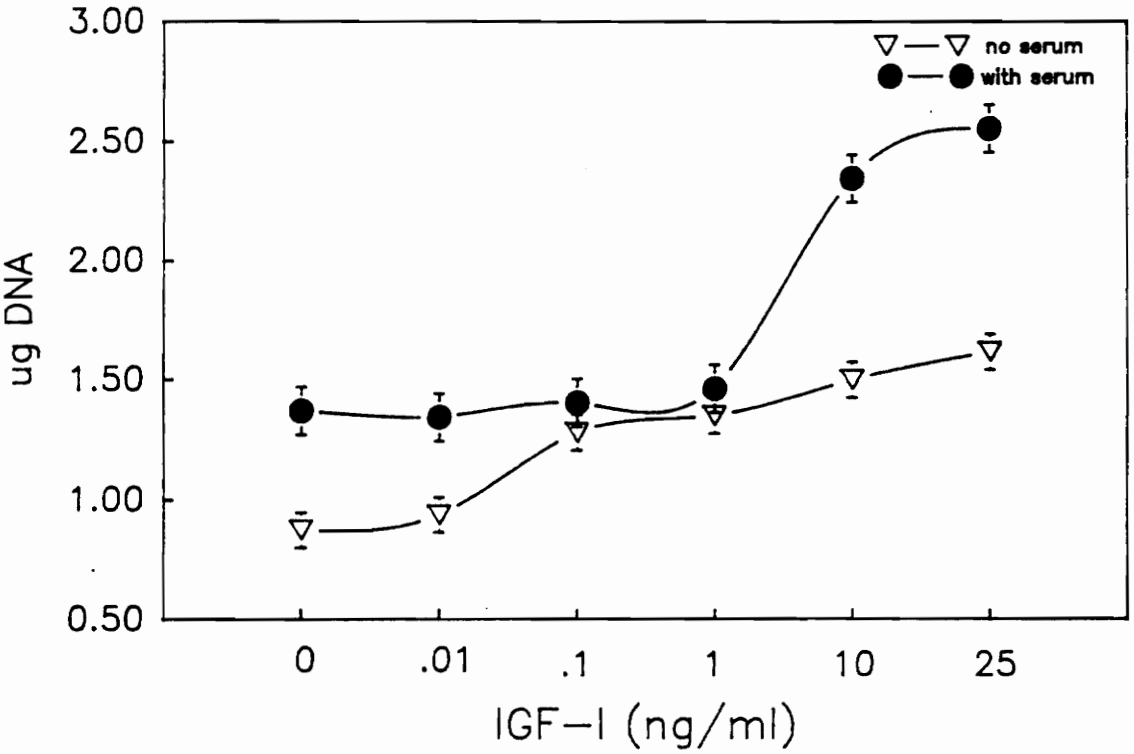

Figure 15. Growth responses of Mac-T cells cultured in human insulin like growth factor-I treated media with or without fetal calf serum. Low insulin like growth factor-I doses (< =25 ng/ml).

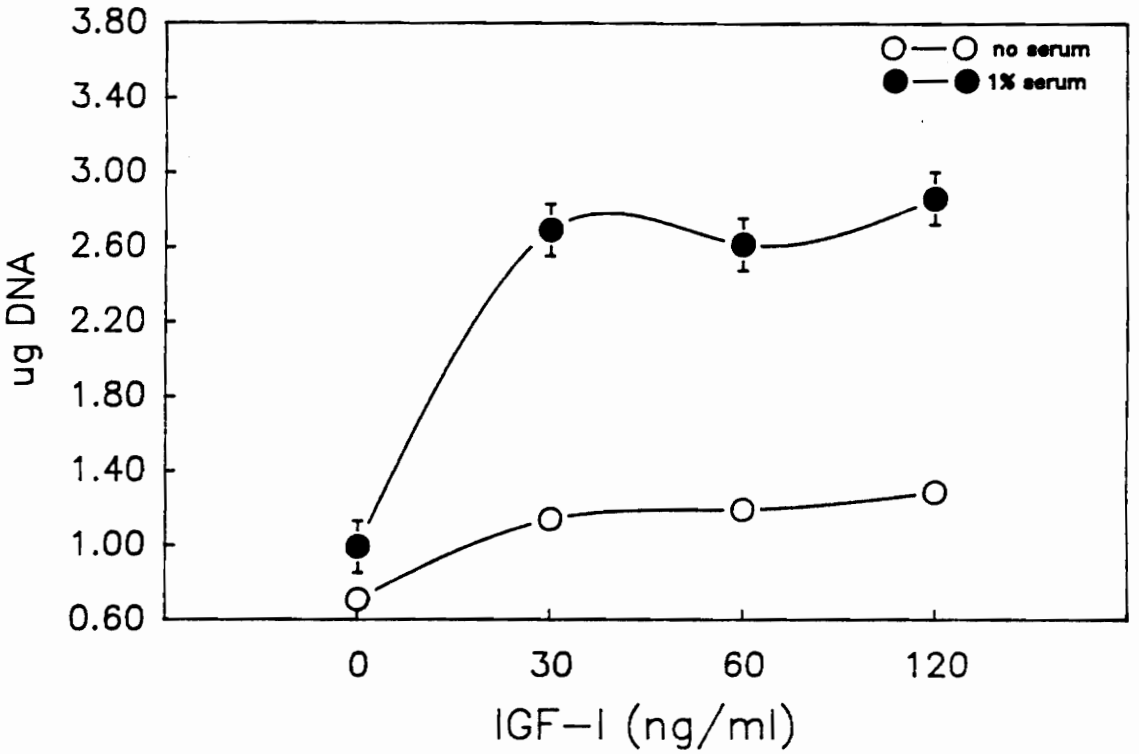

Figure 16. Growth responses of Mac-T cells cultured in human insulin like growth factor-I treated media with or without fetal calf serum. Moderate insulin like growth factor-I doses (30-120 ng/ml).

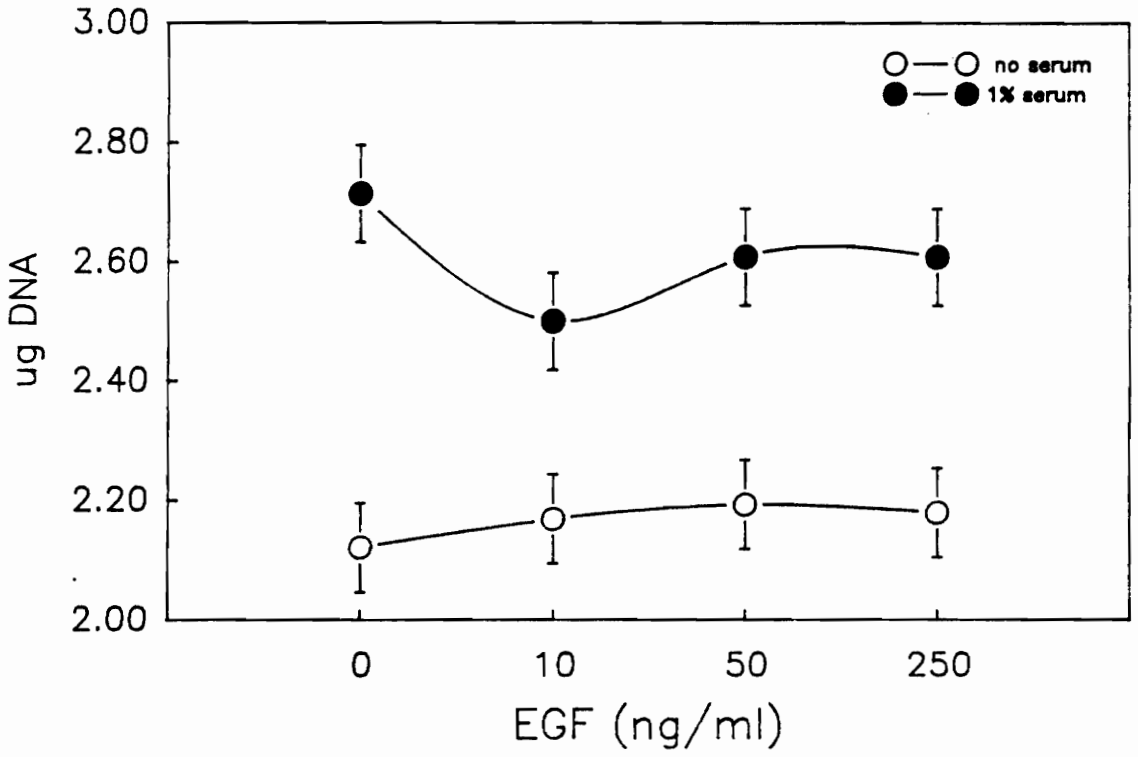

Figure 17. Growth responses of Mac-T cells cultured in murine epidermal growth factor treated media with or without fetal calf serum.

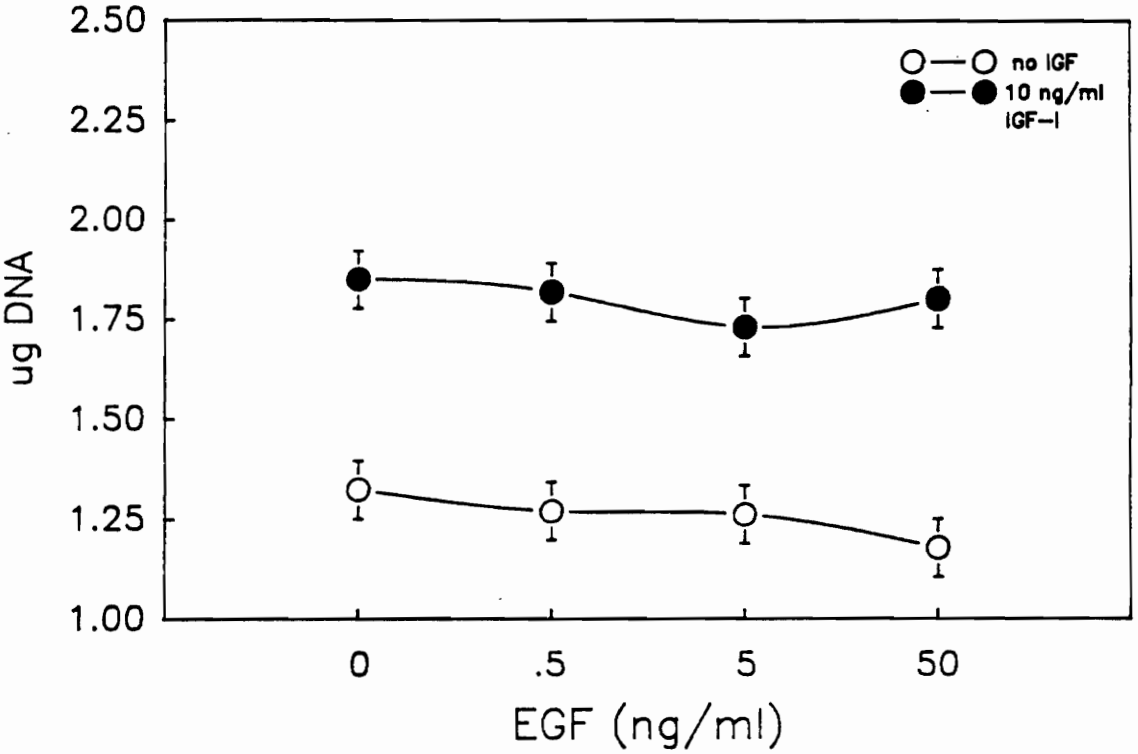

Figure 18. Growth responses of Mac-T cells cultured in murine epidermal growth factor treated media with or without 10 ng/ml human insulin like growth factor-I in the absence of fetal calf serum.

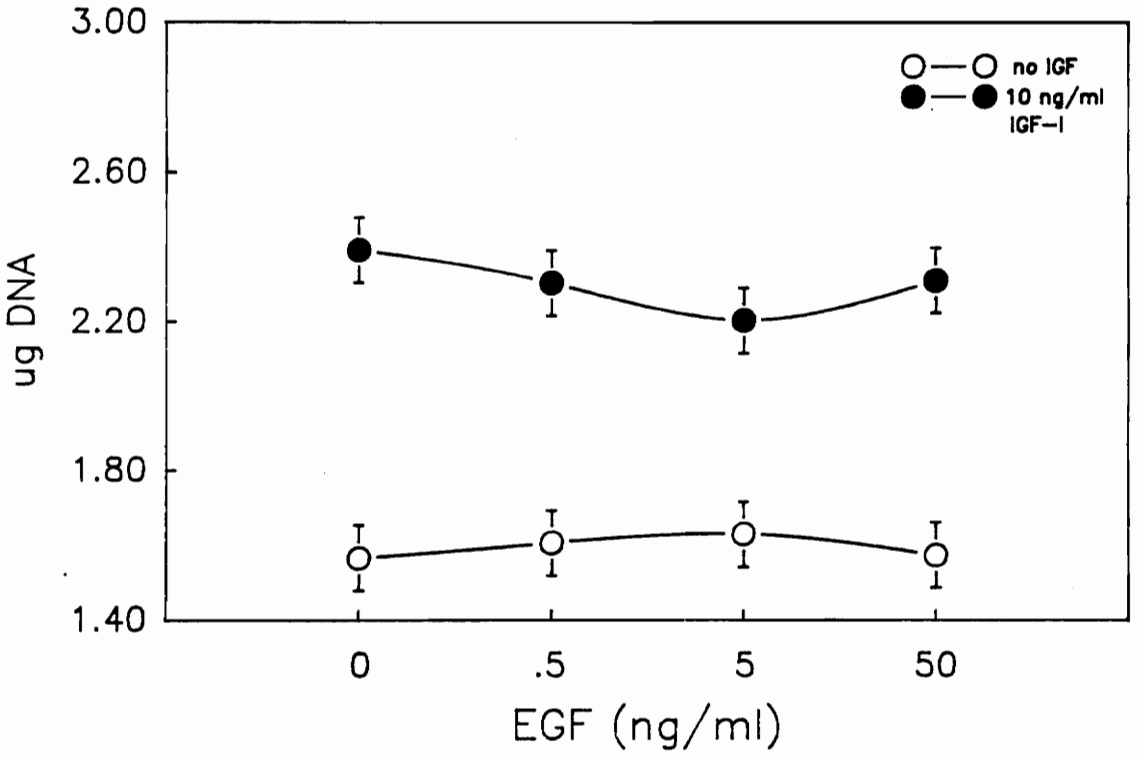

Figure 19. Growth responses of Mac-T cells cultured in murine epidermal growth factor treated media with or without 10 ng/ml human insulin like growth factor-I, in the presence of 1% fetal calf serum.

CHAPTER 3

MODE OF ACTION OF OVARIAN STEROIDS ON PROLIFERATION OF BOVINE MAMMARY EPITHELIAL CELLS AND FIBROBLASTS

INTRODUCTION

Ovarian steroids play a major role in induction of mammogenesis in several species. In fact, estrogen (E) and progesterone (P) are commonly used to induce mammogenesis and lactation in nulliparous heifers and dry cows (Turner et al., 1956; Meites, 1961; Smith and Schanbacher, 1973). Estrogen alone *in vivo* stimulates mammary gland growth in the bovine (Bacsich and Folley, 1939) and rodents (Traurig and Morgan, 1964). We demonstrated earlier that progesterone (P) does not stimulate mammary growth *in vivo* in prepubertal heifers and may inhibit E mediated mammary cell proliferation (Woodward et al., 1991). Detailed analyses of mode of E mediated mammogenesis in the bovine is lacking. However, murine and human studies have provided useful insight into methodology of ascertaining information regarding mode of E action.

In mice, mammary adipocyte and fibroblast proliferation occurs before epithelial proliferation when E is administered *in vivo* (Shyamala and Ferenczy, 1984). It was thus hypothesized that stromal cell division may be a prerequisite to epithelial proliferation in E mediated mammary growth in the murine; perhaps by adipocytes or fibroblasts releasing a growth factor to cause epithelial cells to divide. However, we have previously demonstrated (Chapter 1) E causes early proliferation in mammary epithelium followed by fibroblastic proliferation of a

lesser magnitude in the bovine. Moreover, E and P do not cause proliferation of adipocytes at any time in prepubertal heifers. The failure of mammary stromal cells to divide before epithelial cells in the bovine does not ensure E mediated proliferation is direct or negate the possibility of paracrine release of a growth factor from adipocytes or fibroblasts, however.

Similarly, because treatment of murine or human mammary epithelial cell lines with E has failed to elicit cellular proliferation (Edery et al., 1984; Nandi et al., 1984; Haslam and Levely, 1985; Haslam, 1986; Richards et al., 1988), E has been hypothesized to cause endocrine release of estromedins (Sirbasku, 1980; Sirbasku and Leland, 1982) or cause autocrine or paracrine release of growth factors (Beck and Garner, 1989), or block growth inhibitors (Shafie, 1980). Prior proliferation of adipocytes and fibroblasts in the mouse strengthened the hypothesis that epithelial cell proliferation was paracrine mediated. Several researchers have demonstrated proliferative activity of epithelial cells in response to adipose tissue (Levine and Stockdale, 1984; Beck and Hosick, 1988; Beck et al., 1989), extracellular matrices (Wicha et al., 1982; Kawamura et al., 1986), and conditioned media from fibroblasts (Enami et al., 1983).

A previous study in our laboratory demonstrated E responsiveness is quite different in the bovine. Thus, direct effects of E were still a possibility in the bovine. Attempts to study the effect of ovarian steroids on bovine mammary epithelial cells has been limited to *in vivo* and tissue culture experiments, since no bovine mammary epithelial cell line exhibited normal morphology, differentiation, and growth responsiveness until recently. The Mac-T bovine mammary cell line developed by Hung et al. (in press) has been extensively characterized and shown to mimic *in vivo* bovine mammary epithelial cells in functional and morphological

differentiation, growth, and response to various mitogens (Chapter 2).

This study is composed of three separate experiments, all designed to determine how E mediates its proliferative effects. The first experiment tests E (and P) directly on proliferation of Mac-T cells (direct effects). The second experiment determines if treatment of heifers with E causes release of a substance into the blood that is mitogenic to mammary epithelium (endocrine effects). The last study analyzes the effectiveness of bovine mammary fibroblasts to stimulate proliferation of Mac-T cells by themselves or in response to E (autocrine effects).

MATERIALS AND METHODS

Hormones and Culture Materials

All hormones were obtained through Sigma Chemical Corp. (St. Louis, MO), unless otherwise noted. All culture reagents, media, and enzymes were obtained through Gibco (Gaithersburg, MD) unless otherwise noted.

Animals and Serum

Ten Holstein prepubertal heifers were housed at Virginia Tech Dairy facilities. The heifers' ages ranged from 4.5 to 6.5 months and mean weight was 167 kg. Blood was taken prior to and 24, 48, 72, 96, and 120 h following daily IM injections of .1 mg/kg bovine estradiol-17β by jugular vena puncture. The area from which blood was drawn was scrubbed with 70% ethanol prior to injection and blood was collected in sterile vacutainers (Becton-Dickson, Rutherford NJ). Subsequently, blood remained at room temperature for 1 h and was stored at 4° C overnight to allow coagulation. Blood was centrifuged at 3000 X G for 1 h and serum collected. Subsequently, serum was passed through a graded series of filters (Gelman Sciences, Ann Arbor, MI) including a pre-filter, 3 μm, 1.2 μm, .8 μm and

.2 μm porosity sterilizing filter. Serum was stored at -70 C until testing began. Subsequently, Mac-T cells were plated in serum from heifers before and following treatment and total DNA per well measured.

Cells

A clonal bovine mammary epithelial cell strain (Mac-T) was kindly provided by Dr. Turner, McGill University, Montreal. Cells were obtained from mid-lactational Holstein cows. The cell strain was transfected with Simian virus-40 large-T-antigen. Bovine mammary fibroblasts (Fib-T) obtained and transfected in the same manner were also available.

Use of the Mac-T cell strain as a bioassay for growth

Mac-T cells were cultured in a humid atmosphere containing 95% air, 5% CO_2. Sodium bicarbonate buffer and CO_2 were used to maintain culture pH near 7.4. Phenol red was present in media as a pH indicator unless otherwise indicated. Mac-T cells were plated at a density of 2 X 10^4 cells/ml/well in 24 well dishes (Nunc, Denmark). Mac-T cells were incubated in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) for 24 h to allow cells to attach to plastic. Media containing FCS was then removed and test media added. Test media was often added alone or with basal serum (1% FCS) to facilitate possible growth effects of mitogens. After 72 h exposure to test media all wells were harvested and cells diluted in EDTA-PBS, sonicated and prepared for DNA assay as per Labarca and Paigen (1980) described in chapter 2.

Conditioned Media

Conditioning Phase

Fib-T cells and Mac-T cells were plated in separate dishes at a density of 2.6 X 10^4 cell/ml in 15 ml DMEM + 10% FCS or together at a density of 1.3 X 10^4

Mac-T cells/ml and 1.3 X 10^4 Fib-T cells/ml in a series of 100 mm petri dishes (Falcon, Becton-Dickson, Lincoln Park, NJ). Medium with serum was removed after 24 h and replaced with 0%, 1.5%, and 5% FCS/DMEM with no steroids, with E (1 ng/ml), P (5 ng/ml) or E + P to yield the types of conditioned media in Table 1. After exposure for 96 h, media was harvested from each plate. Cells were harvested and assayed for DNA as previously discussed. Media was filter sterilized through .2 μm filters to ensure no cells were present.

Growth Phase

Conditioned media was mixed 1:1 with fresh DMEM. However, supplemental FCS was added to half the 0% and all the 1.5% conditioned media samples. Table 2 shows serum in conditioned media, serum added to growth media, and total serum present in conditioned media sample used in growth phase of conditioned media experiment.

Radioimmunoassays

Filter sterilized serum from heifers previously described was prepared for radioimmunoassay (RIA).

E and P assays were done in duplicate for each animal using commercial coat-a-count tube method (Diagnostics Product Corp., Los Angeles, CA)

Radioimmunoassay of PRL and GH was performed in duplicate for each animal as per Barnes et al. (1984).

Dr. McFadden, University of Idaho, assayed samples for IGF-I (Herring and McFadden, in press). Serum from all heifers was pooled for each time period, so only 1 serum sample (in quadruplicate) was analyzed for each time.

Statistical analysis

The general linear models procedure (PROC GLM) of SAS (SAS, 1988) was

used to analyze dependent variables. Growth of Mac-T cells in hormones was analyzed using Dunnett's test (Zar, 1984) when treatments were compared to a control value. Tests between treatments were compared with contrasts. F values from non-orthogonal contrasts were replaced by the Bonferroni F test (Zar, 1984). All tests comparing growth of Mac-T cells in various hormones used only treatment in the model. Conditioned media experiments were analyzed separately for each serum concentration (as they were performed). Steroid additives were compared to no steroid addition for each cell type using Dunnett's test. Also, each cell type without steroid additions was compared to fresh media without steroids using Dunnett's test. The conditioned media experiment model contained cell type and steroid and cell type x steroid. Finally, RIA data were evaluated using Dunnett's test by comparing post treatment serum data to the pretreatment value. The model for RIA contained treatment. Data were expressed as least square means and considered significant if the F statistic probability was less than .05, unless otherwise noted.

RESULTS

Hormones on Growth of Mac-T Cells in Culture

Growth of Mac-T cells was not stimulated by E at any dose alone or in the presence of basal serum, (Chapter 2, Fig. 11). Additionally, growth of Mac-T cells in phenol red free media with or without 1% FCS and/or E (10 ng/ml) was similar to growth in phenol red media with the same characteristics. P was unable to stimulate growth at any concentration with or without 1% FCS (Chapter 2, Fig. 12), also. Concomitantly, combinations of E and P were not able to cause increased growth of Mac-T cells in the absence (Chapter 2, Fig. 13) or presence of 1% FCS

(Chapter 2, Fig. 14). Moreover, pretreatment of cells with E for 48 hours, followed by treatment with P did not stimulate growth (Fig. 1).

Serum from Heifers Treated with E on Mac-T Growth

Because samples of serum pooled across heifers at each time point show little capacity to differentially support Mac-T cell proliferation (data not shown), serum from individual heifers were evaluated only for pre treatment and +120 h sample periods. Overall, serum recovered before and 120 h following initiation of E treatment were not different in stimulating Mac-T cell growth (Fig. 2). Individual analysis of serum from the heifers revealed 9 without overall change and 1 heifer with a decreased ($P < .05$) ability to support Mac-T cell growth at 120 h compared with 0 h. Adding E to culture of cells in serum harvested from heifers before treatment (Pre) did not change growth rate of Mac-T cells compared to serum without exogenous E additions (Fig. 3).

Conditioned Media

Conditioned media stimulated growth of Mac-T cells more than fresh media whether conditioned by Mac-T cells, Fib-T cells, or a combination of the two cell types with no FCS additions or .75% total FCS. (Fig. 4) ($P < .01$). When total FCS in media was 2.5% or higher Mac-T cell conditioned media did not stimulate growth of Mac-T cells more than fresh media, but Fib-T cell conditioned media still exhibited higher growth than fresh media ($P < .05$) (Fig. 5). Addition of steroids to cells in the conditioning phase of incubations did not affect growth of Mac-T cells except for Fib-T cell conditioned media when no FCS was supplemented in condition or growth phases. In this case, incubations with E or E+P, but not P produced Fib-T conditioned media which was a more potent stimulator of Mac-T cell proliferation than Fib-T conditioned media cultured in

the absence of steroids ($P < .05$) (Fig. 6). Fibroblast conditioned media was always more stimulatory than Mac-T conditioned media or fresh media, even though cell number present in conditioning phase were consistently less in Fib-T conditioned plates by the end of the conditioning period.

Radioimmunoassays

Table 3 contains results of all RIAs. Estrogen values increased dramatically following E treatment. Serum E levels peaked near 1 ng at 96 and 120 hours following treatment. Progesterone levels remained low at all times ($<$ 100 pg/ml). Serum IGF-I decreased significantly by 96 h ($P < .05$) and decreased further by 120 h. However, GH levels increased by 48 h and peaked at 96 h ($P < .05$). Finally, serum PRL values were low and showed no pattern related to time.

DISCUSSION

Mammary epithelial proliferation in prepubertal heifers is stimulated by E, but not P (Woodward et al., 1991; Chapter 1). However, unlike murine studies (Shyamala and Ferenczy, 1984), E does not cause proliferation of stromal cells prior to epithelial cell proliferation in the bovine. Moreover, E, P or any combination of these two steroids were unable to increase bovine mammary epithelial proliferation *in vivo*. Researchers have previously demonstrated phenol red may exhibit mild E-like effects (Berthois, et al., 1986). However, growth of Mac-T cells was not increased by phenol red or by E in phenol red free media (data not shown). Thus, it appears that E must not be acting directly on the epithelium to elicit epithelial proliferation. Murine experiments have demonstrated P receptors increase in mammary epithelium to be mediated by E; these increases may be responsible for indirectly causing growth of epithelium (Haslam and

Shyamala, 1979; Haslam 1988 a,b,c; Wang et al., 1990). However, we primed Mac-T cells for 48 h with E, removed this media and exposed cells to P spiked media for an additional 48 h, but discovered no apparent differences in growth of Mac-T cells.

Mammary growth by E has also been indicated to be endocrine in action by several researchers (Sirbasku and Benson, 1979; Sirbasku, 1980; and Sirbasku and Leland, 1982). Thus, E induced release of hormones into the general circulation that will reach mammary epithelium and stimulate growth appeared to be a reasonable hypothesis. However, when serum from heifers harvested before and after initiation of E treatment was used to culture Mac-T cells, growth of Mac-T cells was not different. Thus, serum from heifers after E treatment does not contain a complex of mitogens able to stimulate mammary epithelial cell growth different from those in serum from heifers before E treatment.

Hormone and growth factor levels in serum were altered. Serum E levels approximated previously reported levels for cows induced into lactation (Monk et al., 1973). However, PRL levels remained unchanged, unlike similar murine studies (Liberman et al., 1978; Shull and Gorski, 1985; Pan and Chen, 1990). Likewise, serum P concentrations were not affected by E treatment. GH levels, however, increased by 48 h and peaked by 96 h. However, GH has not been shown to increase mammary epithelial growth. GH has been shown to increase IGF-I concentrations in serum, however (Phipps, 1988; Kleinberg et al., 1990). IGF-I is stimulatory to mammary epithelium of several species (Imagawa et al., 1986; Cullen and Lippman, 1989; Constantino et al., 1991), including the bovine (Shamay et al., 1988; Campbell and Baumrucker, 1989). Oddly, IGF-I levels exhibited a significant decline with E treatment. However, similar results with increases in GH

and no increases or decreases in IGF-I have been reported previously (Wiedemann et al., 1976). Although serum concentrations of these hormone were altered significantly by treatment with E, there were no differences in capacity of serum to support mammary epithelial cell proliferation.

Earlier we reported that mammary epithelial cells do not require prior proliferation of adipocytes or fibroblasts to induce their proliferation when heifers were treated with E (Woodward et al., 1991; Chapter 1). However, this does not exclude paracrine mediated growth increases exerted by E. Several researchers have demonstrated fibroblastic or adipocytic mediated mammary epithelial proliferation (McGrath, 1983; Levine and Stockdale, 1984; Haslam, 1988d). However, similar experiments in the bovine are lacking.

We demonstrated conditioned media from Mac-T cells or Fib-T cells or combination of the two cell types increases growth of Mac-T cells more than fresh media alone. Additionally, Fib-T cell conditioned media appears to increase growth more readily than Mac-T cell conditioned media, especially when expressed on a per cell basis. Although all cells were plated at the same density, Mac-T cells grow faster than Fib-T cells under the same stimuli, thus there were approximately 3 times as many Mac-T cells as Fib-T cells by the end of the condition media phase of the experiment. Addition of E, P or both steroids to Mac-T cells or Mac-T cells + Fib-T cells during the conditioning phase did not increase subsequent growth of Mac-T cells. However, when no serum is supplemented, E or E+P caused an increase in the mitogenic action of Fib-T cell conditioned media on Mac-T cell growth. Thus, E mediated proliferation of bovine mammary epithelium may be paracrine in action; though more research is needed to fully ascertain the mechanism of estogen's action.

SUMMARY III

Estrogen does not directly cause proliferation of Mac-T cells, nor does it cause autocrine release of any growth factor directly mitogenic to Mac-T cells. However, E does not appear to be responsible for endocrine release in serum of growth factors directly mitogenic to bovine mammary epithelial cells. However, several endocrine changes are occurring in serum that may be indirectly responsible for Mac-T cell growth. For example, mammary tissue IGF-I may be increased by increasing circulating GH levels without altering circulating IGF-I levels.

Conditioned media experiments revealed Mac-T cells, Fib-T cells and both cells cultured together release growth factors into the media that are capable of increasing Mac-T cell proliferation. Moreover, Fib-T conditioned media is more effective at increasing Mac-T cell growth than Mac-T cell conditioned media. Also, Fib-T cells can be stimulated by E to increase mammary epithelial cell growth. Data is still sketchy as to whether this increase in Fib-T cells by E can result in increases in Mac-T cell proliferation. However, this area holds promise as a method for E mediated bovine mammary epithelial cell proliferation to occur and further research is warranted.

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Table 1. Conditioned media experiment (conditioning phase) design. Media obtained from this phase was used to grow Mac-T cells in the growth phase.

Cell Type	FCS Concentration	Steroid Additions
Mac-T	0	None, E, P, E+P
	1.5	None, E, P, E+P
	5.0	None, E, P, E+P
Fib-T	0	None, E, P, E+P
	1.5	None, E, P, E+P
	5.0	None, E, P, E+P
Mac-T + Fib-T	0	None, E, P, E+P
	1.5	None, E, P, E+P
	5.0	None, E, P, E+P

Table 2. Amount of serum added during conditioning and growth phases and total serum present during growth phase of conditioned media experiment.

Serum in Conditioning Phase (%)	Serum in fresh DMEM added before Growth phase (%)	Total serum present during Growth phase (%)
0	0	0
0	1.5	0.75
1.5	1.5	1.5
5	0	2.5

Table 3. Radioimmunoassays of estrogen, progesterone, insulin like growth factor-I, growth hormone and prolactin in serum from 10 Holstein heifers before and several periods after treatment with estrogen.

Hormone Assayed	Time After Estrogen Treatment					
	PRE	+24	+48	+72	+96	+120
Estrogen (pg/ml)	63	522	603*	580	1293*	790
Progesterone (pg/ml)	<100	<100	<100	<100	<100	<100
IGF-I Pooled (ng/ml)	104*	92*	106*	91*	77*	67*
Growth Hormone (ng/ml	10.7	10.6	14.1	16.1	24.3	16.2
Prolactin (ng/ml)	5.6	0.9	1.0	2.8	5.7	2.7

* Assay pooled sample only

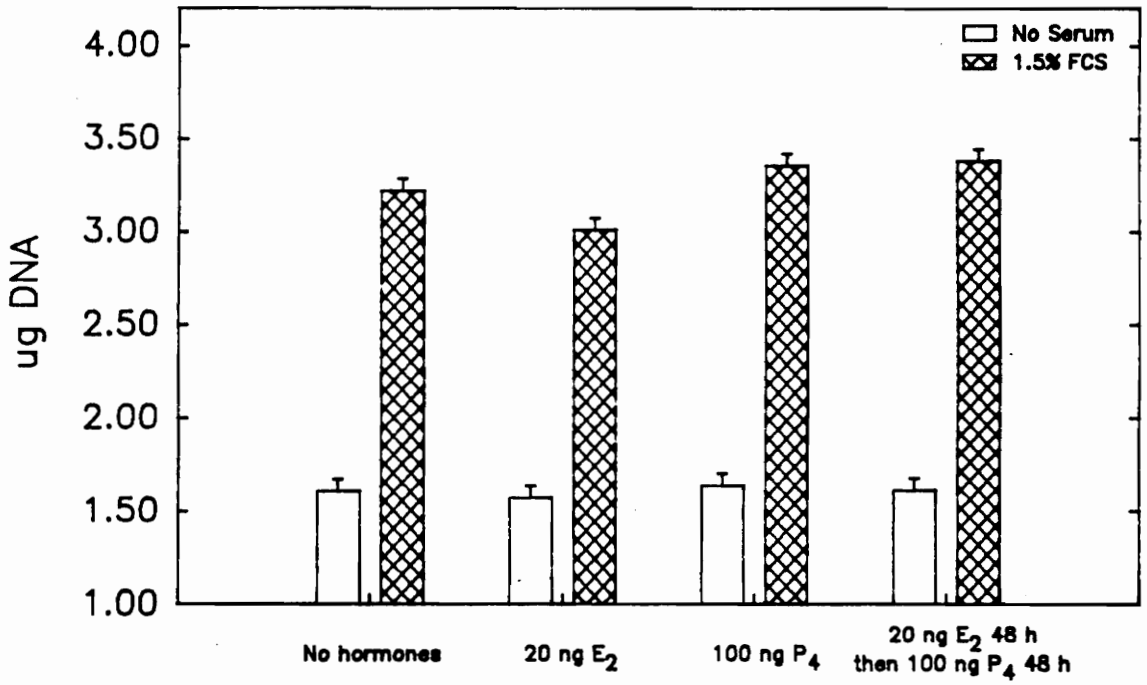

Figure 1. Growth responses of Mac-T cells cultured in estrogen, progesterone, or estrogen + progesterone after priming with estrogen previously for 48 h.

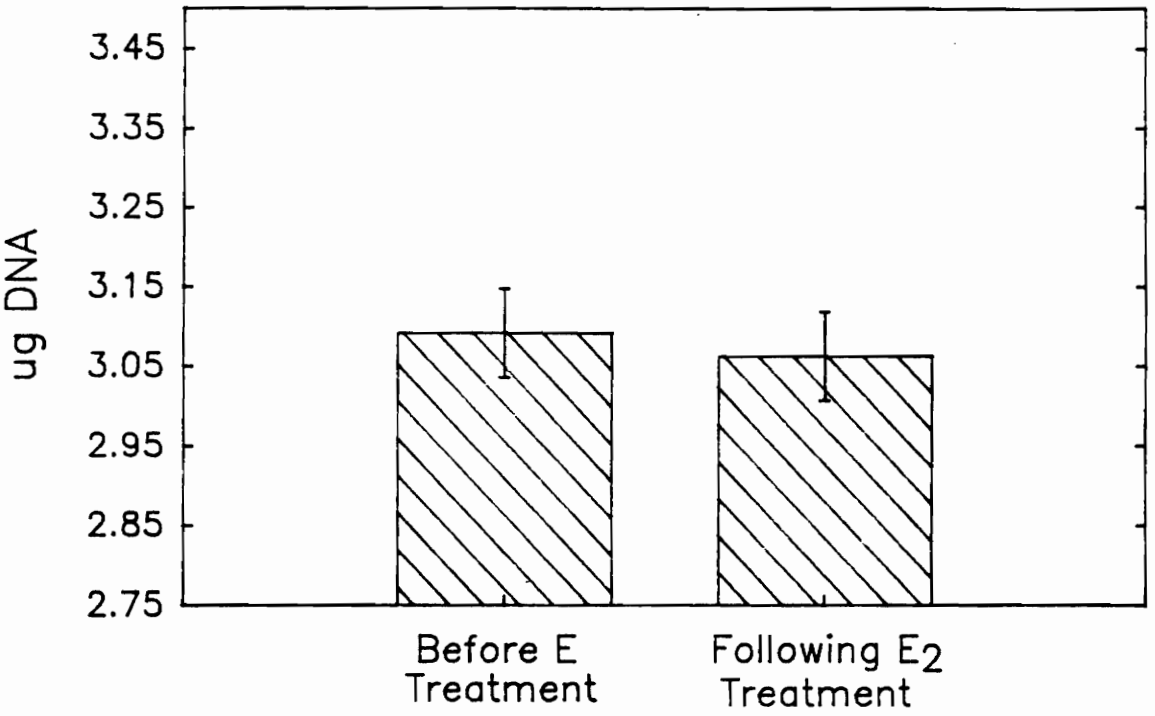

Figure 2. Growth responses of Mac-T cells cultured in 4% serum from prepubertal heifers before and 120 h after treatment with estrogen.

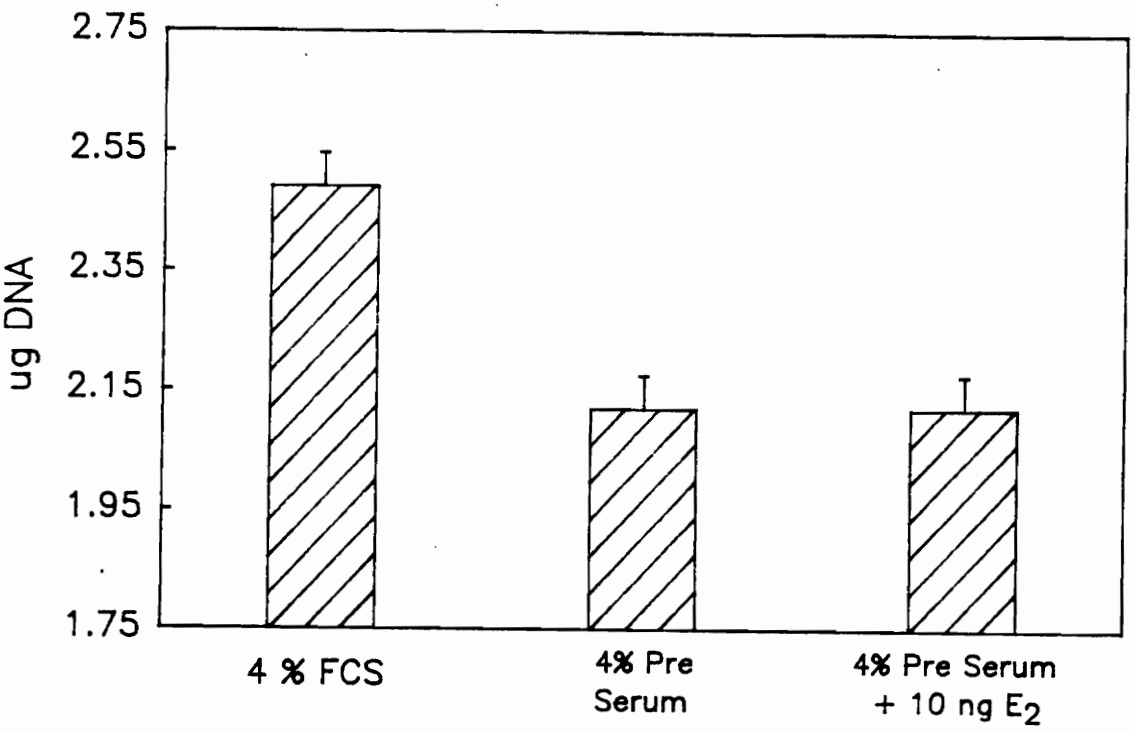

Figure 3. Growth responses of Mac-T cells cultured in 4% fetal calf serum, 4% serum from prepubertal heifers before treatment, or 4% serum from prepubertal heifers before treatment with 10 ng estrogen/ml.

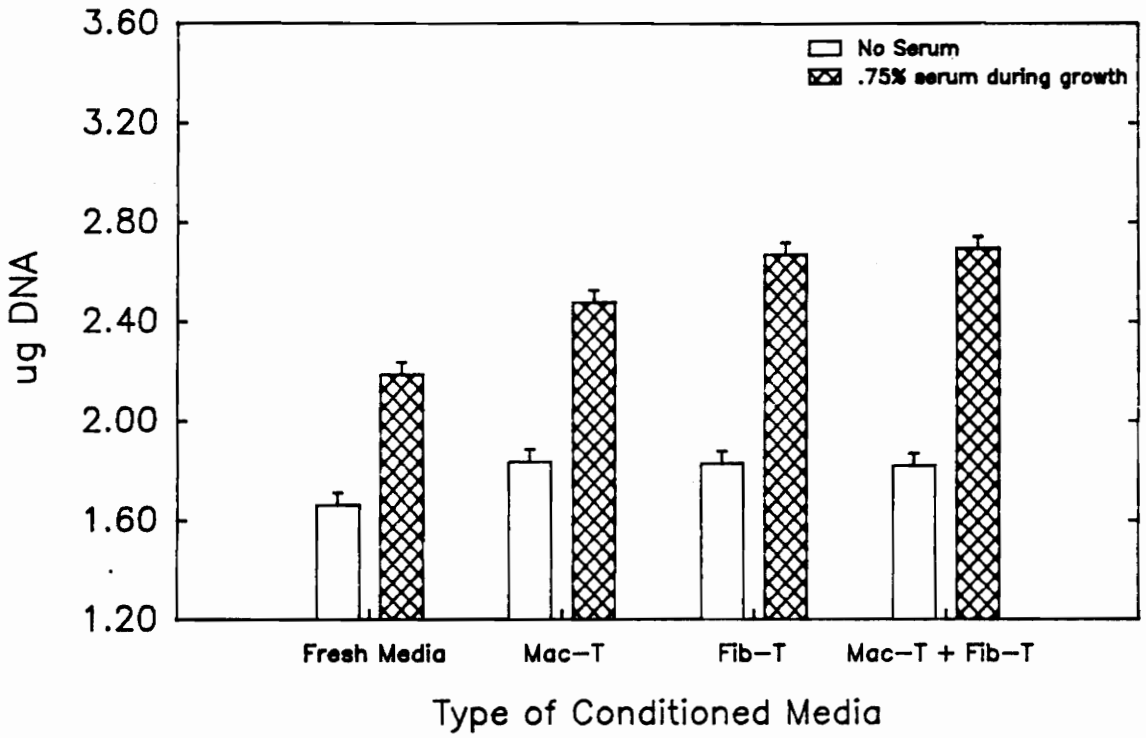

Figure 4. Growth responses of Mac-T cells cultured in media conditioned by Mac-T cells, Fib-T cells, both cells, or fresh media, with no fetal calf serum or .75% total fetal calf serum added.

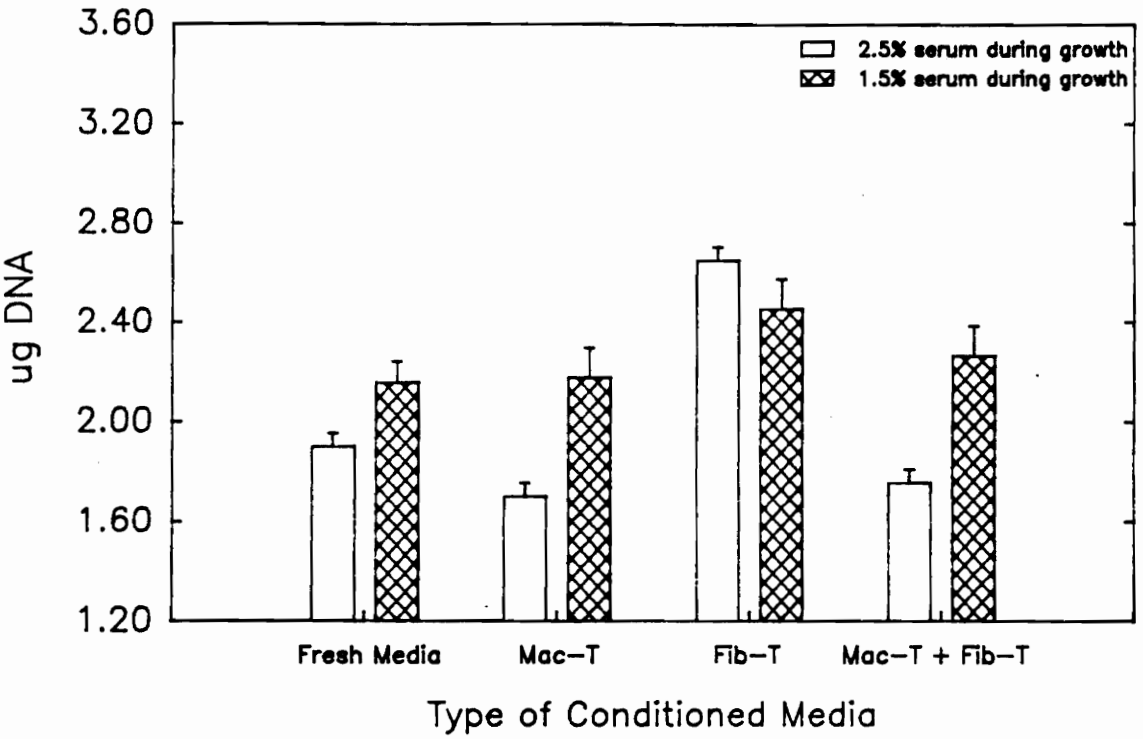

Figure 5. Growth responses of Mac-T cells cultured in media conditioned by Mac-T cells, Fib-T cells, both cells, or fresh media, with 1.5% or 2.5% total fetal calf serum added.

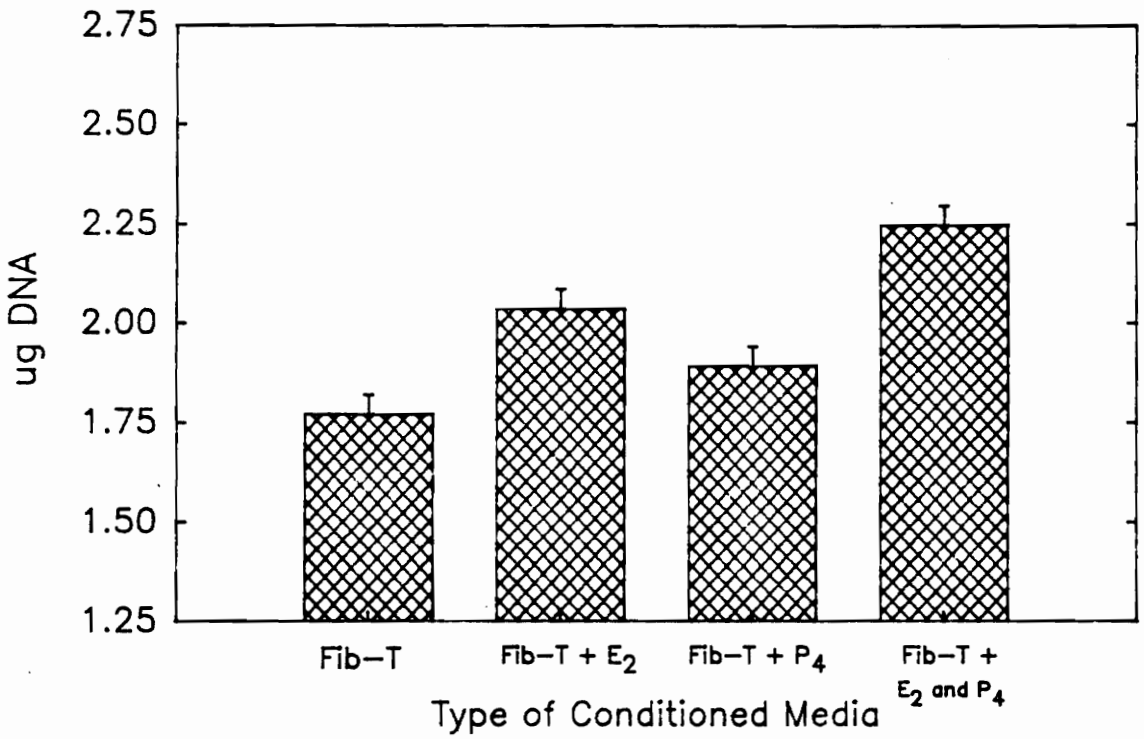

Figure 6. Growth responses of Mac-T cells cultured in media conditioned by Fib-T cells with no steroids, estrogen, progesterone, or estrogen + progesterone.

VITA

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