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**Effect of Bovine Placental Lactogen and other
Placental Proteins on Growth and Differentiation
of Cultured Bovine Mammary Cells**

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Effect of Bovine Placental Lactogen and Other Placental Proteins on Growth and Differentiation of Cultured Bovine Mammary Cells.

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Abstract

Bovine placental lactogen (bPL) interaction with bovine prolactin (bPRL) was studied in mammary tissue obtained from 19-d pregnant rabbits and 5- to 6- month primiparous Holstein heifers. Mammary explants were incubated for 72 h in medium containing insulin, cortisol and various concentrations of bPRL and bPL. Casein phosphorylation was measured in the rabbit and bovine tissue after 4 h pulse with [^{32}P] orthophosphate and quantitative densitometry of autoradiographs. α -Lactalbumin content of homogenates was measured only in bovine mammary explants by RIA. Increasing levels of bPRL or bPL (0.6 to 22.7 nM) progressively increased casein phosphorylation in rabbit mammary gland, with bPL being somewhat more potent at low doses than bPRL. While an additive effect was detected for treatments with 0.6 to 4.5 nM bPRL plus 0.6 and 1.2 nM bPL, an inhibition of bPRL- or bPL-induced response was observed for treatments with 22.7 nM bPRL plus 0.6 to 22.7 nM bPL or vice versa. In bovine mammary tissue, bPRL stimulated casein phosphorylation and α -lactalbumin content in the homogenate in a dose-dependent response, whereas bPL was not capable of stimulating either end point. Bovine PRL-induced response for treatments ≥ 4.5 nM bPRL was partially inhibited by increasing levels of bPL. This inhibition was detected for both casein phosphorylation and α -lactalbumin content. No direct mitogenic effect of bPL was observed in the collagen gel culture of undifferentiated bovine mammary epithelial cells, indicating that its eventual mitogenic activity *in vivo* is mediated through other factors. It had, however, strong lactogenic, PRL-like effects in explants from lactating bovine mammary gland in Nb2 cell bioassays. Other lactogenic hormones such as bPRL, oPRL and hGH behaved similarly. Other growth factors such as IGF-I and EGF exhibited remarkable mitogenic activity on undifferentiated mammary epithelial cells and strongly synergized with each other and with unknown mammary growth factors found in fetal calf serum or in extracts of kidney, uterus, pituitary and mammary gland.

Objectives of the Research

The objectives of this study were to evaluate the different forms of bPL and other proteins secreted by cells of the fetal cotyledon for their ability to stimulate mammary epithelial cell growth and differentiation and activity. Two different in vitro models were used to study effects on mammary growth. The NB2 tumor cell system and collagen gel-embedded normal mammary tissue was used. Lactogenesis was determined by the ability of the bovine tissue to synthesize and/or to secrete alpha-lactalbumin, casein, and milk fat in response to hormonal stimuli.

Report Body

INTRODUCTION

Factors controlling the growth and development of domestic animals are of paramount interest to animal agriculture. Animal growth is controlled by a number of hormones including growth hormone (GH), somatomedins, thyroid hormones, androgens, etc. It is probable that GH is the most important, being required for normal growth and also capable of stimulating additional growth when circulating GH concentrations were increased. Currently, there is considerable interest in the development of biological agents to modify animal growth. In particular, there is an increasing interest in the potential for manipulation of the somatotrophic axis to increase productivity of domestic animals.

Growth factors are typically produced by a variety of cell types and act on their target cells over short distances. Although they usually function as mitogens, they sometimes act to inhibit cellular proliferation, or to stimulate differentiation and to promote anabolic processes in growth arrested cells. The bovine placenta is a potentially rich source of physiological biological response modifiers. A prolactin-like factor (bovine placental lactogen, bPL) was isolated from bovine placenta. In addition, the bovine placenta produces a variety of messenger RNA (mRNA) closely related to prolactin but distinct from bPL. In the process of studying the mammatogenic activity of bPL, we have observed substantial weight gains in mice treated with proteins isolated from secretory granules from binucleate cells of the bovine placenta. The objective of this study was to determine the ability of partially fractionated placental proteins, alone or in combination with 17 β -estradiol plus progesterone, to increase body weight in peripuberal ovariectomized mice.

MATERIALS AND METHODS

Placental Protein Isolation

Reproductive tracts from about midpregnant cows were obtained from a slaughterhouse. Placental proteins were partially fractionated according to the method used for bPL purification (Byatt *et al.*, 1986). Briefly, the secretory granules from the binucleate cells were released from the cells by gentle homogenization and subjected to differential centrifugation. This granular fraction was separated on a discontinuous Percoll (Pharmacia Inc., Piscataway, NJ) gradient with layers of density 1.03, 1.04, 1.06, and 1.08 g/ml. Subsequent to lysis of the granules, insoluble proteins and membranes were removed by high speed centrifugation and the supernatant was applied to a 5 x 70 cm column of Sephadex G-75 Superfine (Pharmacia).

Proteins were eluted with 50 mM ammonium bicarbonate buffer, pH 7.8, at 4°C and absorbance monitored at 280 nm using a Gilson model 260-12 spectrophotometer with a 0.2 ml flow cell (Gilson Medical Electronics Inc., Middleton, WI). The presence and concentration of bPL were measured with a radioimmunoassay (RIA) which recognizes all forms of bPL (Byatt *et al.*, 1987). Three pools were made based on bPL content: A high molecular weight pool (HMW, > 35,000 daltons), which was eluted from void volume to the fraction prior to bPL. An intermediate molecular weight pool (IMW, 14,000-35,000 daltons), which was the bPL-containing fractions and a low molecular weight pool (LMW, < 20,000 daltons), which represented the proteins eluted after the bPL fractions. Sucrose was added (20 mg/100 ml) to every pool prior to lyophilization. Protein concentration was

estimated by the bicinchoninic acid protein assay method (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions and using bovine serum albumin as a standard.

Two-dimensional (2-D) gel electrophoresis

2-D electrophoresis was performed according to the method of O'Farrell (1975) by the Kendrick Laboratory (Madison, WI). The isoelectric focusing was carried out with 1.5% pH 5-7, 1.5% pH 5-8, and 1.0% pH 3.5-10 ampholines (LKB Instruments, Rockville, MD) at 400 V for 12 h. For the last 30 min the potential was increased to 800 V. The pH gradient in the tube gel extended from pH 4.1-8.3, as measured by colored acetylated cytochrome isoelectric point (pI) markers (Calbiochem-Behring, La Jolla, CA) run in an adjacent tube. Purified vitamin D-dependent calcium binding protein, molecular weight 27,000 daltons (pI 4.2) was added to the samples as an internal pI marker. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards to the agarose which sealed the tube gels to the slab gels: myosin (220,000 daltons), phosphorylase A (94,000 daltons), catalase (60,000 daltons), actin (43,000 daltons), and lysozyme (14,000 daltons). These standards appear as fine horizontal lines on the silver stained 13% acrylamide slab gel.

Animals

Female outbred ND4/SWISS mice (Harlan-Sprague Dawley Division, Harlan Industries, Madison, WI) 21-24 g BW were used. Mice were fed commercial laboratory animal diets and given water *ad lib*. Ovariectomy was performed in mice under anesthesia with pentobarbitol (0.5 to 0.7 mg/mouse in 0.1 ml sterile saline). A suture was placed in the muscle and peritoneal layer. Skin was closed with autoclips. Mice were allowed 7-d recovery period before treatment began.

Treatments

Trial 1: Three groups of mice (8 mice/treatment) were injected s.c. daily (8:00 AM) for 10 d with 2-bromo-ergocryptine (0.1 mg/day, CB-154, Sigma Chemical Co.) plus either buffer (50 mM ammonium bicarbonate, pH 7.8, as control), or 17 β -estradiol (1 μ g/d) plus progesterone (1 mg/d, E+P) (Sigma Chemical Co.). Placental protein treatments were HMW (2.3 mg protein/d), IMW (2.3 mg protein/d), and LMW (0.5 mg protein/d). They were injected simultaneously with either control or E+P. Estradiol and progesterone were suspended in 0.1 ml saline (0.9% NaCl containing 1 mg/ml gum arabic). Placental protein fractions were resuspended in 0.1 ml of 50 mM ammonium bicarbonate buffer, pH 7.8. Four h after the last injection, mice were killed by barbiturate overdose and body weights were recorded.

Trial 2: A dose-response curve for HMW fraction was done. One group of mice (3 mice/treatment) were injected s.c. daily for 10 d with CB-154 (0.1 mg/day) plus either buffer (50 mM ammonium bicarbonate, pH 7.8, as control) or HMW (1, 3, 10, 30, 100, 300, and 1,000 μ g protein/d). Four h after the last injection, mice were killed as before and body weights were recorded.

Mammary Gland Development

Fourth (inguinal) mammae were removed from mice in the first trial. One mammary gland from each mouse was spread on a Dacron raft and fixed in acetic acid:ethanol (1:3). Glands were stained with carmin-alum and whole mounts examined for development. Mammary gland development score was assigned on a scale of one to six, with a score of 6 representing maximum development, as in late pregnancy. The contralateral mammary gland from each mouse was frozen on dry ice for subsequent DNA analysis. Glands were homogenized in 150 mM NaCl and proteins and nucleic acids precipitated with an equal volume of 10% TCA. Lipids were extracted with sequential washes with methanol:chloroform (3:1), ethanol and ethyl ether. The resulting dry fat free tissue was weighed and RNA extracted with 0.1M KOH for 3 hours at 37°C. The samples were neutralized with HCl and acidified with 5% PCA. Samples were centrifuged and the supernatant used to estimate RNA by the orcanol reaction (Albam and Umbreit, 1974). The precipitate was hydrolyzed with 5% PCA (70°C, 45 minutes) and DNA content estimated by the diphenylamine reaction.

Lactogenesis in Bovine Tissue

Cutting of explants from either heifer or rabbit mammary gland began within 20 min of removal of tissue. Small strips of tissue containing a minimum of adipose and connective tissue were diced into explants of approximately 2 mm². Four explants were cultured on 12-mm diameter Millicel-HA, 0.45 µm pore size tissue culture plate insert (Millipore, Bedford MA), in 1.2 ml of medium in polystyrene culture dishes (24-well dishes, well 16-mm in diameter; Cell Wells, American Scientific Products, McGaw Park, IL). Medium for all experiments consisted of MEM with Earle's salts and L-glutamine, supplemented with 20 mM HEPES (Sigma), 5 mM sodium acetate (Mallinckrodt Inc., St. Louis, MO), 26 mM sodium bicarbonate (Sigma), 50 µg/ml gentamycin sulfate (Sigma) and, for the bovine mammary explants, 2% steer serum. Medium, antibiotics, buffers and steer serum all together, were adjusted to pH 7.2, then sterilization was carried out by filtration (Nalgene filterware, 0.2µm, Nalge Sybron Corp., Rochester, NY). Insulin and cortisol were added to the medium to a final concentration of 1 µg/ml. Bovine PRL and bPL were used at concentrations of 0, 0.6, 1.2, 2.3, 4.5 and 22.7 nM, either individually or together for a total of thirty six treatments. During the 72 h incubation, the culture dishes were maintained at 37 C in an atmosphere of 95:5 O₂:CO₂.

Determination of casein synthesis

Casein synthesis was estimated from the incorporation of [³²P] orthophosphate into casein. Explants were pulsed with 45 µCi of [³²P] per well (37.5 µCi/ml) for 4 h before termination of the culture. Radiolabeled explants were rinsed several times with MEM then, homogenized in 250 µl of 10 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma), 0.01% (vol/vol) Tween 20 (Sigma). Tissue was homogenized with a ground glass Potter-Elvehjem type homogenizer (American Scientific Products). The homogenate was then centrifuged at 8700 x g for 5 min using a microfuge B (Beckman Instruments Inc., Palo Alto, CA) and the supernatant was frozen overnight. Protein content in the supernatant was estimated by the bicinchoninic acid protein assay method (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions and using BSA as standard. Quantitative densitometry of autoradiography

An autoradiographic image analysis system of the type which has been utilized previously in this laboratory (14) was used to analyze the density of the bands corresponding to casein. Autoradiographs were placed on a light box and intensity data from a 256 x 256 diode array with 14-bit analog-digital resolution was collected by a two-dimensional diode array scanner (Tracor Northern Inc., Middleton, WI), cooled to 5 C with a Peltier-effect cooler. The resulting picture was analyzed for optical density with a desktop computer system. Areas of interest (casein bands) were integrated by selecting both the area and a local background. A linear regression was used to estimate the areas in the standard curves as a function of CPM/mm². The equation used was: square root of area = 6.01 + 19.15 logCPM/mm² (r²= 0.98). Finally, total CPM per casein band was calculated by multiplying the CPM/mm² times the area of the disc containing the isotope used to make the standard curves.

alpha-Lactalbumin assay

a-Lactalbumin was quantified in the homogenate of mammary tissue by radioimmunoassay (RIA) using an antibody raised in rabbits against a-lactalbumin (11). The a-lactalbumin tracer used in the RIA was radioiodinated by the Iodo-gen method (Pierce). Briefly, 10 µg of Iodo-gen was dissolved in 100 µl of methylene chloride then, 2.5 µg was applied onto the surface of a reaction vessel and dried thoroughly under a stream of nitrogen gas at room temperature. Two and one half µg of a-lactalbumin was added to the reaction vessel followed by 0.5 mCi [¹²⁵I] NaI. The reaction was allowed to proceed for 10 min and then stopped by loading the reaction mixture onto a Sephadex G-25 column (coarse; Pharmacia, Piscataway, NJ). The eluant buffer used was a-lactalbumin RIA buffer [40 mM sodium phosphate, 40 mM sodium chloride, 10 mM EDTA, 0.1% (wt/vol) sodium azide, 0.125% (wt/vol) gelatin, pH 7.3, containing 0.01% (vol/vol) Tween 20]. Mean specific activity of the [¹²⁵I] a-lactalbumin was 55 µCi/µg protein. Mammary tissue homogenate supernatant was diluted with a-

lactalbumin RIA buffer. The a-lactalbumin standards were also made in the same buffer. [125 I] a-lactalbumin (20,000 to 25,000 CPM/tube) and a-lactalbumin antisera (R3225/3; 1:20,000) were added and incubated 24 h at 4 C. Bound and free a-lactalbumin were separated by using a second antibody against rabbit immunoglobulins raised in goat. The a-lactalbumin antibody did not crossreact with BSA, b-lactoglobulin, and a-, b- or k-casein (11). Sensitivity of the assay was 0.2 ng/ml, and the usable range of the assay was between 1 to 30 ng/ml. The intraassay coefficient of variation was 9.2 % and the interassay coefficient of variation was 7.2 %.

Statistical analysis

Data from the growth experiments were analyzed as a randomized complete block design (replicate = block). Treatment means from trial 1 were compared to control using Dunnett t test. Non-linear regression analysis was used to describe the response in trial 2 to HMW fraction. Unless otherwise indicated, tests were two sided with significance at $P < 0.05$.

Heterogeneous variances were observed in the raw data of the lactogenesis experiments. Therefore, base 10 logarithm transformation was performed for statistical analysis, in order to distribute the data normally with homogeneous variances. Transformed data were analyzed by mixed model ANOVA, with heifers considered random blocking factors and treatments fixed. Regression analysis was used to relate log (measurement) to log (bPL+1), log (bPRL+1) and log (bPL+1)*log (bPRL+1). All statistical tests were 2-sided with significance set at $P \leq 0.05$ (15). Transformation was only for statistical analysis. Data presented were untransformed, and standard errors of means (SEM) were adjusted to remove block (animal) effect (16).

Tissue preparation

Mammary tissue from healthy Israeli-Friesian 4- to 6-month-old female calves was excised aseptically immediately after slaughter and put into 100 ml M-199 supplemented with antibiotic-antimycotic mixture. The tissue was transferred to the laboratory, cut into small pieces, and placed in a 500-ml Erlenmeyer flask containing M-199 supplemented with collagenase (1 mg/ml), hyaluronidase (1 mg/ml), and bovine insulin (1 mg/ml) in a ratio of 10 ml medium/1 g tissue. The flask was swirled on a gyratory water bath at 100 rpm at 37 C for 3-4 h. During this period tissue fragments were further dissociated by occasional passage through a 10-ml pipette with a large orifice. One hour before termination of the enzymatic digestion, 0.5 ml 0.04% DNase/100 ml digest was added. At the end of the incubation, the suspension was passed through a Nitex filter (200 mm), and the organoids were collected by centrifugation (4 min at 300 X g). The organoids were washed three to five times with M-199 and kept on ice until embedded in collagen. Clumps of epithelial cells in a minimal volume of M-199 were added to the cold collagen solution, yielding a final concentration of $4-8 \times 10^5$ cells/ml. Cell number was determined by counting the nuclei after treatment with 0.2% crystal violet in 0.1 M citric acid. Cell viability was determined with trypan blue. The collagen-cell suspension (0.5 ml) was then overlaid on 0.3 ml pregelled collagen in each well of 24-multiwell plates and allowed to gel at room temperature. As soon as the layer gelled, 1 ml M-199 containing the appropriate additives was added. The cells were cultured at 37 C in an air-CO₂ (95:5) atmosphere, and the medium was changed every 48 h.

Thymidine incorporation

Cells were pulsed with 1 mCi [3 H]thymidine for 24 h. The medium was aspirated from the wells. Gels were lifted carefully with broad-tipped forceps and placed in 1.5-ml Eppendorf tubes. Fifty microliters of 4.3 M acetic acid were added to each tube, and the tubes were incubated for 20 min at 37 C to dissolve the gels. The cells were recovered by centrifugation (5 min at 500 X g) and washed consecutively with 4% perchloric acid (PCA), 80% ethanol (EtOH), 100% EtOH, and 4% PCA. After hydrolysis in 0.5 ml 6% PCA (80 C; 1 h), the suspension was mixed with 10 ml scintillation liquid and counted in a Beckman b-scintillation counter.

Bovine lactating mammary gland explant culture

Preparation of explants from bovine lactating mammary tissue was carried out as previously described (16), and medium was changed every 24 h. Fat synthesis was determined by measuring incorporation of $\text{CH}_3\text{-}^{14}\text{C}\text{OOH}$ into the fatty acid fraction for 3 h after 4 days of culture with a selected hormonal regimen (10). α -Lactalbumin secretion into the medium was determined by RIA (10). The intra- and interassay coefficients of variation were, respectively, 5.8% and 12.5%, and the sensitivity was 0.6 ng/tube. Glucose uptake was determined by measuring the glucose content of the medium before and after incubation, as described previously (10).

Nb2 Rat Lymphoma Culture

The assay for the lactogenic hormonal activity was performed using Nb2-11C rat lymphoma cells as described previously (Gertler et al 1985).

RESULTS

Effects of Placental Proteins on Mouse Body Weight

In the first experiment, 17 β -estradiol plus progesterone (E+P) treatment increased mouse body weight by 2.7% over control after a 10-d injection period. Final weights were not significantly different ($P > 0.10$) with values of 20.9 g and 21.5 g for control and E+P, respectively. Partially fractionated bovine placental protein treatments increased BW 8.5%, 4.4%, and 20.0% over control after a 10-d injection period for LMW, IMW, and HMW, respectively. Addition of E+P did not increase body weight response to placental protein fractions. The main effect of the placental protein fractions (averaged over the absence and presence of steroids) were 3.7%, 3.5%, and 16.5% increase over control (average of buffer and E+P treatments) for LMW, IMW, and HMW, respectively (Table 1). The effect of the HMW fraction was significant ($P < 0.01$) by Dunnett's t test, but the LMW and IMW fraction effects were not ($P > 0.10$).

Table 1. Effect of placental extracts (low, medium (Med.) or high molecular weight) (control = buffer alone) with or without steroid hormone treatment (control = saline containing 1 mg/ml gum arabic; E+P = 1 μg + 1 mg estradiol + progesterone in saline containing 1 mg/ml gum arabic) on body weight, mammary development score, wet weight, dry fat free tissue (DFFT), DNA or RNA.

Placental Fraction	Steroid	Body Wt.	Development Score	Wet Wt. (mg)	DFFT (mg)	DNA (μg)	RNA (μg)
Control	Control	20.9	2.1	120	1.90	71	76
Low	Control	22.7	2.5	123	1.93	79	76
Med.	Control	21.9	2.6	136	2.01	86	78
High	Control	25.1	2.4	133	1.98	82	80
Control	E+P	21.5	3.7	163	2.47	107	95
Low	E+P	21.2	4.8	177	2.74	131	127
Med.	E+P	22.2	5.2	189	3.10	155	110
High	E+P	24.3	5.6	178	3.40	184	127
Pooled SE		0.9	0.2	3.3	0.24	4.7	4.5

Effects of Placental Proteins on Mammary Gland Development

There was a significant synergism between the steroid hormones and the high molecular weight fraction. Mammary gland development, as assessed by either development score, dry fat free tissue, total DNA or total

RNA was significantly increased by the high molecular weight fraction if exogenous estradiol and progesterone were administered ($P < 0.05$), but was ineffective in increasing mammary development when administered alone ($P > 0.05$) (Table 1). The HMW fraction did not substantially increase either wet weight ($P > 0.05$ and < 0.10) or RNA/DNA ($P > 0.10$). The intermediate molecular weight fraction increased mammary development, using the same criteria as before ($P < 0.05$). However, this fraction showed some mammary activity in the absence of exogenous estradiol and progesterone, although it was more active if estradiol and progesterone were also administered. LMW fraction stimulated mammary development in the presence of exogenous estradiol and progesterone, but was less active than either the HMW or IMW fractions.

In the second experiment, HMW fraction significantly increased ($P < 0.05$) BW gain/10-d period as levels of HMW increased from 0 to 1,000 μg protein/d. The data were fit by regression analysis to a standard growth equation of the form:

$$Y = \frac{A}{1 + ((A/A_0) - 1) e^{-aD}}$$

where Y = mouse body weight gain/10-d period,
A = maximal growth,
A₀ = minimal growth,
a = constant, and
D = high molecular weight fraction (μg protein/d).

$$Y = \frac{4.49}{1 + ((4.49/2.15) - 1) e^{-4.49 \cdot 0.016 \cdot D}}$$

where Y = mice BW gain/10-d period; D = HMW fraction (μg protein/d). Pooled SE was 0.99.

HMW treatments increased BW gain after a 10-d period by 2.1, 2.0, 2.4, 3.5, 3.6, 4.3, 4.6, and 4.8 g/mouse for control, and HMW treatments 1, 3, 10, 30, 100, 300, and 1,000 μg protein/d, respectively. One mouse in the highest dose (1,000 μg protein/d) was designated as an outlier and omitted from the statistical analysis. Its body weight gain was 8.3 g/10-d period.

Experiments with Bovine Tissue Lactogenic Activity:

Bovine mammary explants were used in experiments to measure casein phosphorylation and alpha-lactalbumin as end points. Casein phosphorylation and α -lactalbumin results showed that bPL was not a lactogenic hormone when added to the bovine mammary gland explants. Bovine PRL and bPL stimulated casein phosphorylation in rabbit mammary gland explants in a dose-related response (Table 2). Explants incubated in the presence of increasing quantities of bPRL or bPL alone responded with progressively increasing amounts of casein phosphorylation. Rabbit mammary explants were more sensitive to low levels of bPL than bPRL. In fact, bPL response curve was initially steeper than bPRL. While bPL-induced response was 1.4-, 2.7-, 12.7-, and 12.4-fold over control, bPRL-induced response was 1.1-, 1.5-, 1.8-, and 6.5-fold over control for 0.6, 1.2, 2.3, and 4.5 nM levels, respectively. Half maximal response was achieved at about 2.5 nM bPL, whereas bPRL was about 6.0 nM. Explants exposed to bPRL in the presence of bPL resulted in an additive effect when bPRL levels were 0.6 to 4.5 nM and bPL 0.6 and 1.2 nM (Table 2). This additive effect was lost at any other bPL level. In fact, a partial inhibition in casein phosphorylation was observed when 22.7 nM bPRL level was used in the presence of any bPL level. The same effect was observed at 22.7 nM of bPL in the presence of any level bPRL.

Table 2. Casein phosphorylation in rabbit mammary gland explants after a 72 h incubation and 4 h pulse. Values are expressed as CPM/mm²

bPL nM	bovine prolactin nM					
	0.0	0.56	1.14	2.27	4.54	22.72
0.0	3.0±.65 ^a	3.3±.94	4.6±2.7	5.6±2.7	19.9±8.5	78.8±31.4
0.56	4.3±1.5	5.3±1.4	10.9±5.7	15.8±7.0	33.3±14.8	54.7±21.0
1.14	8.1±4.8	11.9±5.9	10.9±4.0	13.4±7.8	22.54±11.2	54.3±20.0
2.27	38.7±15.5	16.5±5.3	25.5±10.2	28.0±13.3	24.2±10.3	35.4±7.7
4.54	37.64±15.6	29.4±14.5	32.9±11.1	36.2±9.7	36.0±9.0	37.0±6.6
22.72	67.1±24.0	53.7±8.9	44.0±9.1	46.5±10.4	48.9±5.2	46.5±7.7

^a Mean± std. deviation.

Table 3. Casein phosphorylation in bovine mammary gland explants after a 72 h incubation and 4 h pulse. Values are expressed as CPM/mm²

bPL nM	bovine prolactin nM					
	0.0	0.56	1.14	2.27	4.54	22.72
0.0	4.0±2.4 ^a	8.1±2.3	10.0±4.3	10.2±2.5	14.6±3.7	18.3±7.1
0.56	6.8±2.8	7.7±3.0	9.3±3.2	12.0±4.8	14.5±5.2	16.6±5.2
1.14	6.4±2.7	8.0±3.3	9.6±4.6	13.1±8.8	15.2±6.9	16.7±3.0
2.27	9.1±3.5	6.7±3.0	9.8±4.2	12.2±4.8	11.7±4.9	11.7±4.9
4.54	7.7±2.1	7.2±1.8	8.6±3.7	10.3±4.1	12.1±4.2	14.5±4.3
22.72	8.8±2.9	8.1±2.6	8.8±3.4	9.4±2.6	12.2±3.8	15.5±6.5

^a Mean± std. deviation.

Bovine PRL, but not bPL, stimulated casein phosphorylation in bovine mammary gland explants (Table 3). Incubation of bovine mammary explants in presence of bPRL showed a dose-response ($P < 0.01$) of the incorporation of [³²P] into casein, whereas bPL did not stimulate phosphorylation of casein significantly ($P > 0.10$). Bovine mammary explants responsiveness to bPRL was low compared to rabbit mammary explants. While bPRL stimulated casein phosphorylation 2.0-, 2.4-, 2.5-, 3.6-, and 4.5-fold over control, bPL responses were 1.7-, 1.6-, 2.2-, 1.9-, and 2.2-fold over control for hormonal treatment of 0.6, 1.2, 2.3, 4.5, and 22.7 nM. Half maximal response for bPRL was about 2.0 to 3.0 nM. Explants cultured with bPL in the presence of increasing concentration of bPRL showed a partial inhibition in bPRL-induced casein phosphorylation (Table 3). Although there was a general tendency for increasing bPL levels to inhibit bPRL-induced casein phosphorylation when bPRL treatments were ≥ 4.5 nM, a slight increase in casein phosphorylation was present, but only in treatment 22.7 nM bPRL plus 22.7 nM bPL.

An unexpected but potentially very interesting finding is shown in Figure 1. When the heifers were grouped accordingly to their sires' PDs, we were able to see a dramatic increase in the concentration of a-

lactalbumin the cultured bovine mammary explants in response to prolactin. These results are very interesting and should be studied in more detail, in order to gain more insight to the possibility of developing a screening assay in young animals for their genetic potential.

Fig.2 Effect of bovine prolactin on the α -lactalbumin concentration in bovine mammary gland explants of heifers according to their sires' PDs.

DNA synthesis in cell culture

Our results indicate that insulin alone stimulates growth of bovine mammary gland undifferentiated cells. However, its effect is apparent only at extremely high nonphysiological concentrations, and even then its maximum activity was only 25-50% of that achieved with 10% FCS-supplemented medium. On the other hand, IGF-I had significantly higher mitogenic activity at a low concentration; half of its maximum effect was achieved at 30 ng/ml. The presence of a low insulin concentration (12 ng/ml) shifted the half-maximum concentration to 5-6 ng/ml, but did not change IGF-I's maximum effect. The mitogenic activity in cells cultured with FCS-supplemented medium was 2.5-4 times higher than the maximum achieved with IGF-I.

To determine whether the responsiveness of the cells to IGF-I changes with time, we measured the effect of 0-100 ng/ml IGF-I in the presence of 10 ng/ml insulin on DNA synthesis in cells cultured for 8 day. After 48 h (not shown) [3 H]thymidine incorporation was low, and no effect of IGF-I or FCS could be seen. However, after 4 days or more in culture, a significant ($P < 0.05$) dose response was obtained, and the maximum effect varied between 30-50% that of FCS. A response significantly different from that of the control without IGF-I was achieved with 3.1 ng/ml (after 6 or 8 days) or with 12.5 ng/ml after 4 days. The proportion of fibroblasts, as judged by two-dimensional microscopical observations, was less than 10% and did not increase through 2-8 days of the experiment.

Culturing the cells in various combinations of insulin and IGF-I indicated that the effects of insulin and IGF-I are almost absolutely dissociated. At high insulin concentrations (10 mg/ml or more) insulin alone most likely substituted for IGF-I. Similar to results shown in Fig 1, the incorporation of [3 H]thymidine achieved with IGF-I (50ng/ml) plus insulin (10 ng/ml) did not significantly differ from that achieved with 50 mg/ml insulin. However, in the presence of 10 or 50 mg/ml insulin, IGF-I addition slightly but significantly ($P < 0.05$) decreased [3 H]thymidine incorporation. Unlike that of IGF-I, addition of 10% FCS increased the effect of insulin. The effect of 10% FCS was significantly potentiated by the addition of 1-50 mg insulin/ml. Addition of 1% FCS also increased the effect of insulin. The effect of 10% FCS was significantly potentiated by the addition of 1-50 mg insulin/ml. Addition of 1% FCS also increased the effect of insulin (except at 1000 ng/ml), but the effect was less pronounced than with 10% FCS.

In the presence of FCS, IGF-I elevated [^3H]thymidine incorporation by 2 - 2.5 fold and the effect was statistically significant ($P>0.05$; Table 4). A similar effect was achieved by adding EGF (100 ng/ml). When IGF-I and EGF were added simultaneously, a dramatic increase in [^3H]thymidine incorporation occurred in medium supplemented with FCS or FCS plus insulin. This synergistic effect was not seen in the absence of FCS, thus indicating that the simultaneous effect of IGF-I and EGF depends on other factors in FCS. EGF was used in concentrations that exert a maximum effect in serum-free medium. Increasing the EGF concentration to 500 ng/ml in the presence or absence of IGF-I did not increase [^3H]thymidine incorporation (data not shown). The maximal effect of EGF in the presence of 10 ng insulin/ml was obtained at 5 ng/ml (data not shown). Addition of oPRL (5 mg/ml), bGH (5 mg/ml), or both to medium containing 10 ng insulin/ml had little or no effect on [^3H]thymidine incorporation (data not shown). In an additional 2 experiments, the interaction between IGF-I, EGF and FCS was further explored. As shown in Table 4, in these experiments (and others not shown), IGF-I and EGF strongly synergized and addition of both to 10% FCS-supplemented medium enhanced 3 - 3.5 fold in the mitogenic effect.

Earlier we reported that in parallel to elevated [^3H]thymidine incorporation a microscopically visible outgrowth occurred. One type of outgrowth had a needle-like appearance, while the other was star-like. Similar results were obtained in the present work. We observed remarkable outgrowth characterized by a 1.5- to 4-fold increase in the diameter of the organoids, which paralleled the incorporation of [^3H]thymidine after 5-6 days of culture. Although a quantitative analysis was difficult to perform, our general impression was that the various treatments did not affect the ratio between these two types of outgrowth.

Table 4 The Interactive Effect of IGF-I, EGF and FCS on DNA Synthesis in Undifferentiated Bovine Mammary Tissue

Factor added ²	$^3\text{[H]}$ -Thymidine Incorporation (dpm $\times 10^{-3}$ /well) ¹			
	Exp. No. 1		Exp. No. 2	
None	12	$\pm 0.5^a$	12.7	$\pm 1.1^a$
IGF-I	52.0	$\pm 11^b$	48.1	$\pm 5.9^b$
EGF	35.9	$\pm 2.6^b$	29.1	$\pm 3.0^c$
FCS	121.0	$\pm 7.1^c$	206.9	$\pm 18.5^d$
IGF-I + FCS	301.0	$\pm 15.6^e$	437.5	$\pm 38.7^e$
EGF + FCS	242.8	$\pm 14.2^d$	487.9	$\pm 63.0^e$
IGF-I + EGF	116.2	$\pm 6.3^c$	172.1	$\pm 22.1^d$
IGF-I + EGF + FCS	379.8	$\pm 74.0^e$	751.0	$\pm 51.3^f$

¹Values are the Mean $\pm$ SE (n = 3) 24 h pulse after 6 days in culture. Numbers marked with different letters within the same column are significantly different ($p < 0.05$).

²The concentrations of IGF-I and EGF were, respectively, 50 and 20 ng/ml.

Several partially purified or pure fractions obtained from bovine placenta were examined for their ability to promote growth and DNA synthesis of bovine undifferentiated mammary epithelial cells. Three materials were assayed: (a) The high MW (HMW) fraction obtained after G-75 columns (tubes 20 - 30, Fig. 4a, Byatt et al. 1986); (b) low molecular weight (LMW) fraction (see above tubes 40 - 50) and pure placental lactogen (see above Fig. 4b, tubes 27 - 29 after HPLC column).

In order to estimate whether these fractions exhibit a mitogenic effect or interact with other factors, they were tested in basal medium or in basal medium supplemented with IGF-I (50 ng/ml), EGF (50 ng/ml) or FCS (10%). As shown in Table 5, all 3 fractions were devoid of mitogenic activity and did not interact with other factors. Interestingly, the fraction prior to HPLC purification (HMW and LMW) were inhibitory when tested at > 10 mg/ml. It should be noted that these fractions were intensively dialyzed prior to experiments, to

remove any interfering low molecular material. These materials were further tested in two other assays. In the Nb2 bioassay, they exhibited the expected bioactivity that matched their bPL content as measured by RIA (results now shown). In explants from bovine lactating tissues (Table 6), the activity of pure bPL was almost equal to that of bPRL while the LMW fraction did not affect fat synthesis but inhibited alpha-lactalbumin secretion. Glucose uptake indicated no toxic effect.

Table 5 The Effect of Different Fractions from Placenta and Purified Placental Lactogen on DNA Synthesis in Undifferentiated Bovine Mammary Tissue

³ [H]-Thymidine Incorporation (dpmx10 ⁻³ /well) ¹											
Material added											
Fraction ² tested	None			IGF-I ²			EGF ²			FCS (10%)	
HMW mg/ml											
0	12	±	0.5	52.0	±	11	35.9	±	2.6	121	± 7.1
1	10.7	±	0.4	47.8	±	3.2	34.2	±	9.0	118	± 2.6
10	9.0	±	0.4	54.2	±	7.0	14.6	±	6.3	60.5	± 11.8
50	9.1	±	0.3	35.5	±	4.2	5.7	±	2.4	24.4	± 3.3
100	4.2	±	0.1	10.4	±	3.7	7.8	±	2.6	11.8	± 4.6
LMW mg/ml											
0	12.7	±	1.1	48.1	±	5.9	29.1	±	3.0	206.9	± 18.5
2	10.1	±	2.1	35.6	±	5.7	26.5	±	4.2	192.2	± 4.2
10	9.4	±	1.1	43.6	±	3.3	13.4	±	1.3	137.4	± 4.2
40	6.6	±	0.5	40.4	±	2.8	12.4	±	0.1	132.9	± 8.9
120	4.6	±	0.6	29.2	±	5.4	N D			102.1	± 6.7
Pure bPL ng/ml											
0	10.4	±	1.2	21.4	±	1.2	21.4	±	0.7	145.8	± 2.5
10	10.5	±	0.4	24.9	±	2.9	20.1	±	1.8	N D	
50	11.3	±	0.7	23.7	±	0.5	16.0	±	1.8	152.5	± 32.4
200	9.5	±	0.5	N D			10.6	±	1.1	111.7	± 19.9
600	13.6	±	3.0	21.1	±	7.9	19.4	±	5.9	165.1	± 24.4

¹Values are Means ± SE, (n = 3). 24 h pulse after 6 days in culture.

²HMW and LMW fractions were obtained after G-75 Sephadex column (see Byatt et al. 1986, Fig 4a, the respective fractions 20 - 30 and 40 - 50) and pure bPL was obtained after HPLC chromatography (see above Fig 4b fractions 27 - 28). Each fraction was tested in tissue from different cows. The concentrations of IGF-I and EGF were 50 ng/ml. The lactogenic effect of various partially purified and pure bPL fractions was further tested using explant culture (see Table 3). Pure bPL exhibited stimulatory, PRL-like effects on both fat synthesis and alpha-lactalbumin secretion. The LMW fraction had no significant effect on fat synthesis and surprisingly inhibited the alpha-lactalbumin secretion.

The lactogenic effect of various partially and pure bPL fractions was further tested using the explant culture (see Table 3). Pure bPL exhibited stimulatory, PRL-like effects on both fat synthesis and alpha-lactalbumin secretion. The LMW had no significant effect on fat synthesis and surprisingly inhibited the alpha-lactalbumin secretion.

Table 6 The Effect of Bovine Placental Lactogen and LMW Fraction on the Fat Synthesis and Alpha-lactalbumin secretion in Explants from Bovine Lactating Mammary Gland

Hormone added: ¹	mg/ml	Fat synthesis dpm/3 h/mg tissue ²		a-lactalbumin secretion ng/24 h/mg tissue ²		
IF	-	621	± 48 ^c	11.1	±	1.0 ^a
IF + PRL	0.2	1323	± 127 ^{ab}	20.7	±	0.8 ^a
IF + PRL	1.0	1490	± 196 ^a	21.5	±	0.6 ^a
IF + LMW	4.0 ³	794	± 26 ^c	1.6	±	0.1 ^c
IF + pure bPL	2.0	1181	± 56 ^c	19.4	±	0.8 ^{ab}

¹All media included insulin (I) 1 mg/ml and cortisol (F) 500 ng/ml.

²Determined respectively by incorporation of acetate and specific RIA.

³Determined by RIA.

All means marked with different letters within the same column are significantly (P < 0.05) different.

An additional portion of the present work was devoted to the exploration of the existence and the extent of mitogenic factors contained in various tissues. To achieve this aim, several organs from a lactating cow, such as kidney, udder, uterus and pituitary were extracted with either 0.1 M acetic acid (acidic extract) or neutral PBS buffer in a 1:3 ratio. The extracts were dialyzed and assayed for mitogenic activity when added to the medium in the absence or presence of IGF-I (50 ng/ml). The protein content of these extracts varied between 8 - 12 mg protein/ml for the acid and 15 - 20 mg/ml for the neutral extract. Results obtained with the kidney, uterus and the udder extracts are presented in Table 7.

Table 7 Effect of Kidney, Udder, and Uterine Extract on DNA Synthesis

³ [H]-Thymidine Incorporation (dpm × 10 ⁻³ /well) ¹					
Exp. no.	Factor added	Extract added (%)	-IGF-I		+IGF-I
1	None		14.2 ±	0.7	206.7 ± 47.7
	FCS	10	806.0 ±	27.3***	NT
	Kidney acidic extract	1	20.8 ±	1.9*	NT
		10	27.4 ±	1.6***	762.6 ± 64.6***
	Kidney neutral extract	1	24.2 ±	4.2*	NT
		10	165.6 ±	15.4***	921.4 ± 21.8***
	Udder acidic extract	1	27.4 ±	2.8**	NT
		10	38.5 ±	5.8**	281.5 ± 16.5
2	Udder neutral extract	1	75.7 ±	4.3***	NT
		10	187.6 ±	20.2***	894.1 ± 6.5***
	None		13.6 ±	0.6	121.7 ± 1.3
	FCS	10	207.6 ±	20.1**	NT
	Uterine neutral extract	10	25.2 ±	2.5**	302.9 ± 16.9***

¹Values are Mean ± SE (n = 3), 24 h pulse after 6 days in culture. The means marked with asterisks are significantly different from the control without added factors: * - P < 0.05; ** P < 0.01; *** P < 0.001.

As shown, the kidney neutral and to a lesser extent, neutral extracts contain mitogenic factors that very strongly synergize with IGF-I. Similar results were also obtained with the neutral extracts of the udder and the uterus but not with the udder's acidic extract. Thus, the mitogenic factor that synergizes with IGF-I is either destroyed by the acidic treatment or not extract. Pituitary extract (PE) contains powerful mitogenic activity that synergizes with FCS or with IGF and EGF.

The nature of all these mitogenic factors is at present unknown. Further experiments to purify and characterize them are required. Once these experiments are accomplished, we shall be able to ascertain whether these powerful growth factors are secreted to the circulation and affect the proliferation of mammary epithelial cells in vivo.

In view of the apparent discrepancy between the results obtained in the US laboratory by injection of the placental fractions into nude mice carrying bovine tissue and the failure of these fractions to stimulate the undifferentiated mammary tissue 3 additional replicates of the US experiment were carried out and the serum samples sent to the Israeli collaborators for evaluation. Unfortunately the between-replicate variation in the undifferentiated tissue model precluded our ability to evaluate the results (Table 8).

Table 8 Effects of the sera of mice injected with different experimental combinations of placental factors on the DNA synthesis of undifferentiated bovine mammary tissue

3H TdR incorporation DPM/well N = 3									
Replicate	A			B			C		
Treatment in mice	% sera v/v	Mean	SE	% sera	Mean	SE	% sera	Mean	SE
Control	1	100.4	6.9	1	91.5	12.1	1	25.1	-
	2.5	180.6	6.4	2.5	184.5	11.8	2.5	30.8	10.4
Low	1	*22.3	0.5	1	77.9	8.7	1	95.5	4.8
	2.5	109.3	18.6	1.1	82.0	5.3	2.5	116.7	8.5
Med.	1	*123.9	4.7	1	16.1	1.5	1	74.1	10.2
	2.5	102.6	31.5	2.5	87.5	3.9	2.5	160.6	20.3
High	1	55.6	0.4	1	20.6	3.6	1	118.3	4.1
	2.5	159.0	8.7	2.5	18.4	2.2	2.5	N D	
Control +EP	1	31.1	0.4	1	57.9	1.0	1	33.6	0.7
	2.5	116.7	36.0	2.5	111.8	18.2	2.5	94.8	5.6
Low +EP	1	143.5	11.8	1	112.9	11.8	1	68.1	20.3
	2.5	116.2	1.0	1.8	162.2	3.9	2.5	120.3	11.0
Med. +EP	1	71.4	13.5	1	16.1	1.5	1	75.3	20.9
	2.5	157.6	12.8	2.5	87.5	3.9	2.5	111.7	11.2
High + EP	1	63.0	0.3	1	104.4	12.6	1	80.4	13.3
	2.5	182.1	4.8	2.5	98.2	11.9	2.5	51.5	7.1
Control basal I	0.05	8.1	1.8						
+ FCS %	1	71.1	5.8						
	2.5	158.8	14.9						
	5	172.0	44.1						
	10	253.4	13.4						

Control = SALINE, EP = ESTRADIOL (1mg/day) + PROGESTERONE (1 mg/day)

B = Buffer, Low = low molecular weight fraction, High = High molecular weight fraction, Med. = G75 fraction containing bovine placental lactogen. (See also Table 1).

DISCUSSION

Our results clearly suggest the stimulatory effect on mouse body weight of proteins from the secretory granules of the bovine fetal cotyledon. The somatotrophic response was not steroid-dependent whereas a synergistic effect of steroids and the high molecular weight factors were seen in the mammary response. The growth promoting activity was part of a high molecular size pool; larger than either pituitary growth hormone or any of the known growth factors. There is a possibility that such factor(s) might be either a low molecular weight component(s) associated with a higher molecular weight binding protein or aggregated low molecular weight molecules which eluted with a large hydrodynamic volume on the gel filtration column. Whereas our 2-D PAGE analysis under reducing conditions revealed a predominant material with high molecular weight, low molecular weight factor(s) cannot be ruled out.

The results of the dose-response curve for HMW fraction further demonstrated the growth-promoting activity in peripuberal mice of this partially purified protein pool. The response was fitted as a non-linear regression equation commonly used to describe growth. A recovery period of seven days after surgery was critical to seeing the effect. A trial in which the animals were not allowed a 7-d recovery period after surgery resulted in lack of response to HMW treatments. The results obtained using HMW fraction cannot be explained by bPL present in our sample. The amounts of bPL detected by radioimmunoassay (Byatt *et al*, 1986) were 0.9 µg/mg protein (HMW), 11.0 µg/mg protein (IMW), and 1.5 µg/mg protein (LMW).

Although bPL was lactogenic in our positive controls (Rabbit mammary explants), we could not detect a lactogenic response of bovine mammary organ culture to bPL. Two different end points were used to assess lactogenic activity in case bPL was not stimulatory for a particular milk-related end product. Therefore, we have demonstrated that bPL is not lactogenic in homologous species. In fact, previous work done in our lab, bPL was not able to stimulate casein phosphorylation, lipid synthesis and α-lactalbumin content in bovine mammary organ culture. Explants cultured in presence of bPRL plus bPL resulted in a bPL inhibition on bPRL-induced casein phosphorylation and α-lactalbumin content (Table 2 and 3).

One possible explanation of this inhibitory activity of bPL on bPRL-induced response could be the binding of bPL to bPRL receptor(s) with perhaps a similar affinity as bPRL itself. As bPL concentration is raised in the organ culture, a reduction in the bPRL-induced casein phosphorylation and α-lactalbumin is present. Although explants from lactating bovine mammary tissue have an absolute need of PRL for maintenance of casein synthesis, the nature of PRL receptor(s) in bovine mammary gland is still unknown.

Placentae are a rich source of growth promoting molecules. Almost all of the placental protein and polypeptide hormones investigated to date are structurally and functionally very similar, or in some cases, identical, to protein and polypeptide hormones produced by the pituitary gland and hypothalamus. Placental expression of multiple prolactin-related genes may be a common feature among many mammals, whereas the growth hormone-like placental hormones found in primates may be relatively unique. Proliferin (PLF) a member of the PRL-GH family, was reported in the trophoblastic giant cells of mouse placenta and in maternal circulation or pregnant animals. The time of maximal secretion of PLF was different from mouse PL I. PLF function was suggested as an autocrine growth factor for cells in culture and a placental hormone that may regulate the growth or differentiation of fetal or maternal tissues during pregnancy.

Our results suggest the existence of a growth promoting factor(s) in the secretory granules of the fetal cotyledon. The biological role of such factor can only be speculated on at this time. It could be important in either fetal development, maternal mammary development, uterine accommodation or repartitioning of nutrients during pregnancy.

Description of Cooperation

Placental samples were prepared in the US were sent to Israel for testing in the *in vitro* systems established in their laboratories. The sending of samples between the laboratories proved to be an extraordinarily difficult task because of airline security regulations. We found that the only reliable way for this to be done was to have cooperators carry the samples in their personal luggage. A graduate student from the Israeli cooperators

laboratory spent a period of time in the US doing experiments on a different type of mammary tissue . During this period individuals from the two laboratories were able to work together for a period of time and share ideas and technical information. Collaborators on the project generally met at scientific meetings to share information.

Main Achievements of the research

- Developed a model for in vitro proliferation of undifferentiated bovine mammary epithelial cells.
- Factors produced by the bovine placenta have a strong growth promoting effect on whole animal growth
- Factors produced by the bovine placenta stimulate bovine mammary growth in a steroid hormone dependent manner
- Bovine placental lactogen is lactogenic in rabbits but not in cow and is a partial prolactin antagonist in both animals.
- Alpha-lactalbumin production of bovine tissue in response to prolactin is dependent on the genetic potential of the cows for milk production.

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