Bovine Placental Lactogen Stimulates DNA Synthesis of Bovine Mammary Tissue Maintained in Athymic Nude Mice (42967)

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Abstract. **Mammary tissue from five midpregnant heifers was transplanted subcutaneously into ovariectomized athymic mice (eight pieces/mouse). After a recovery period of 19 days, mice were injected daily for 5 days with buffer (50 m*M* NH_4HCO_3, pH 7.8) as control, 17β-estradiol (1 μg) plus progesterone (1 mg). Concurrently with the buffer or steroid hormone injections, mice were injected with bovine placental lactogen (0, 5, or 25 μg), bovine prolactin (0, 3.4, or 17.2 μg), or bovine growth hormone (0, 3.4, or 17.2 μg). All mice were injected with 2-bromo-α-ergocryptine (0.1 mg/day). Transplanted bovine mammary tissue was incubated for 4 hr in minimum essential medium containing 1 μCi/ml [^{3}H]TdR. Two pieces were processed for autoradiography and the others were used for DNA assay and total [^{3}H]TdR uptake. Bovine placental lactogen, prolactin, and growth hormone each increased [^{3}H]TdR incorporation into DNA in a linear, dose-response manner. Addition of ovarian steroids to bPL resulted in a significant increase over protein hormones alone. Autoradiographic analysis indicated that the observed differences in DNA synthesis were due to hormonal effects on epithelial, rather than stromal, DNA synthesis. These results provide the first evidence of a mammogenic role of bovine placental lactogen.** [P.S.E.B.M. 1989, Vol 192]

During pregnancy, there is a substantial increase in the number of epithelial cells in the mammary gland. Increase in cell number continues into early lactation in most species, although the extent of lactational mammary growth varies from less than 10% in ruminants (1) to as much as 50% in rats (2). In general, little is known about the hormonal control of the mammary growth during pregnancy. The close association between increases in placental lactogen at about midpregnancy and rapid lobulo-alveolar mammary development in ewes and the occurrence of mammary growth in hypophysectomized pregnant goats provide presumptive evidence that a prolactin-like placental hormone (placental lactogen) influences mammary development in sheep and goat (3). Bromocriptine-treated pregnant ewes had a clear depression of ovine prolactin (PRL) concentrations to values below 0.5 ng/ml without a significant effect on ovine placental lactogen (PL) levels (>200 ng/ml). In pregnant heifers, similar treatments resulted in bovine prolactin (bPRL) levels below 6.5 ng/ml. Unfortunately bovine placental lactogen (bPL) was not measured (4). In both species, PRL depression did not affect mammary gland development. Hence, a mammogenic factor(s) must be present, either by the action of low PRL concentrations or by PL (4).

Placental lactogens have been isolated from several species, including humans, rats, and sheep, but isolation of bPL proved difficult, due at least in part to low tissue concentrations of bPL. Isolation of a 32-kDa molecule capable of displacing radiolabeled prolactin in a rabbit microsomal membrane assay was reported by Arima and Bremel (5). Byatt *et al.* (6) reported an improved method of isolating bPL. Maternal circulating levels of bPL are below 1 ng/ml during entire pregnancy (7, 8). Biologic assays of prolactin-like activity indicate that bPL induces milk constituent synthesis in rabbit mammary tissue and is even more potent than PRL in inducing PRL-induced mRNA accumulation in pigeon crop sac assays (9). However, no substantial biologic response of bovine mammary tissue to bPL has been demonstrated to date. Therefore, the objective of this study was to determine the ability of bPL, alone or in

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combinations with 17β-estradiol plus progesterone, to induce DNA synthesis in bovine mammary tissue xenografted into athymic nude mice.

Materials and Methods

Female outbred athymic nude mice (Harlan-Sprague Dawley Division, Harlan Industries, Madison, WI), 4- to 5-week old, were housed in aseptic conditions in a small animal isolator (CCI equipment, Marietta, OH). Food, water, cages, and bedding were sterilized prior to use. Mammary tissue from five 5- to 6-month first pregnancy Holstein heifers (University of Wisconsin herd) was cut into slices 0.5- mm thick using a Stadie-Riggs tissue slicer. Slices were cut into 1 × 5 × 5 mm and placed subcutaneously (eight pieces per mouse) in ovariectomized athymic mice via a dorsal incision. During surgical procedures, animals were anesthetized with pentobarbital (0.5–0.7 mg/mouse in 0.1 ml of sterile saline). Incisions were closed with autoclips and mice were allowed 19-day recovery periods for tissue to become vascularized before treatment began.

All mice were injected daily for 5 days with 2-bromo-α-ergocryptine (0.1 mg/day, CB-154; Sigma Chemical Co., St. Louis, MO). Simultaneously, mice were treated with either saline (0.9% NaCl containing 1 mg/ml gum arabic) or 17β-estradiol (1 μg/ml) plus progesterone (1 mg/ml) (suspended in saline containing gum arabic). Factored over saline and estradiol plus progesterone treatment was treatment with 0, 5, or 25 μg/day bovine placental lactogen (purified as previously described (6)); 0, 3.4, or 17.2 μg/day bovine prolactin (NIH-bPRL-6); or 0, 3.4, or 17.2 μg/day bovine growth hormone (NIH-GH-b18) (each suspended in 50 m*M* ammonium bicarbonate, pH 7.8). Each treatment was administered to 1 mouse/heifer, for a total of 5 mice-heifers/treatment or 18 mice/heifer.

The day after the last injection mice were killed by cervical dislocation and the transplanted bovine mammary tissue was incubated for 4 hr in Eagle's minimum essential medium (Gibco, Grand Island, NY) containing 1 μCi/ml *methyl*-[^{3}H]thymidine (60 Ci/mmol) (DuPont, Boston, MA). Two pieces were fixed in 10% buffered formalin and processed for [^{3}H]TdR autoradiography as previously described (10). Briefly, the pieces were cut into 5-μm-thick sections (entire piece sectioned) and every sixth section was mounted on glass slides, dipped in Ilford K5 emulsion (Polysciences Inc., Warrington, PA), and stored in light-tight boxes for 2 weeks at 5°C. After 2 weeks of exposure, slides were developed, fixed, and stained with hematoxylin. Cells with five or more silver grains over the nucleus were counted as labeled. Epithelial area was determined with an ocular grid and labeling index defined as labeled cells per mm^2 epithelial area (11). Remaining pieces were frozen in dry ice and kept for DNA assay and total [^{3}H]TdR incorporation into DNA. Frozen grafts were homogenized in saline (0.9% NaCl). Nucleic acids and proteins were precipitated with 10% (w/v) trichloroacetic acid, and washed twice with 10% (w/v) trichloroacetic acid. Lipids were extracted with methanol:chloroform (2:1), followed by ethanol and ether extraction. The extract was further digested with 0.3 *M* KOH, to remove RNA, neutralized with 6 *M* HCl, and further digested with 5% perchloric acid at 70°C for 45 min. An aliquot was neutralized with 1 *M* NaOH and counted for [^{3}H]TdR incorporation. DNA content was determined by the diphenylamine colorimetric method (12).

Data were analyzed as a randomized complete block design (heifer = block). Treatment means were compared using planned orthogonal comparisons to determine main and interaction effects of treatments. Regression analysis was used to describe the response to bPL, bPRL, and bovine growth hormone (bGH). Unless otherwise indicated, tests were two sided with significance at $P < 0.05$ (13).

Results

Bovine mammary tissue was readily accepted by athymic nude mice. Viable epithelia, organized into normal ducts and alveoli, was present in all tissue slices examined by autoradiography.

DNA synthesis (measured by dpm [^{3}H]thymidine/μg DNA) was significantly increased by estradiol and progesterone (indicated by main effect of estradiol and progesterone in statistical analysis) ($P < 0.05$). The effects of bPL, bPRL, and bGH were all statistically significant ($P < 0.05$) (Fig. 1). Regression analysis indicated that the responses were linear over the dose range tested, with no significant quadratic effect ($P > 0.10$ in all cases). There was no significant interaction between bPL and steroid hormone injection ($P > 0.10$). The slope of the response curve was 0.96 in the absence of steroids and 1.23 in the presence of steroids. For bPRL, the slopes of the response curve were 1.94 in the absence of steroid injections and 3.76 in the presence of steroids. These slopes were different at $P < 0.10$, but not at $P < 0.05$. Slopes of the response of thymidine incorporation into DNA to bGH were 0.37 in the absence of steroid injections and 1.5 in the presence of steroid injections. These were not statistically different ($P = 0.12$).

Autoradiographic analysis indicated that most of the differences in DNA synthesis was localized to epithelial cells. Although stromal cells were labeled, their labeling showed no consistent pattern explicable by hormone injections. However, epithelial labeling was increased by steroid hormone injection ($P < 0.05$) and by bPL, bPRL, or bGH ($P < 0.05$ in each case) (Fig. 2). The slope of the response of labeling index to bPL was 10.72 in the absence of steroid injections and 20.14 in the presence of steroid injections. These slopes were different at $P < 0.10$, but not at $P < 0.05$ (exact type I error, $P = 0.06$). Slope of the response of labeling index

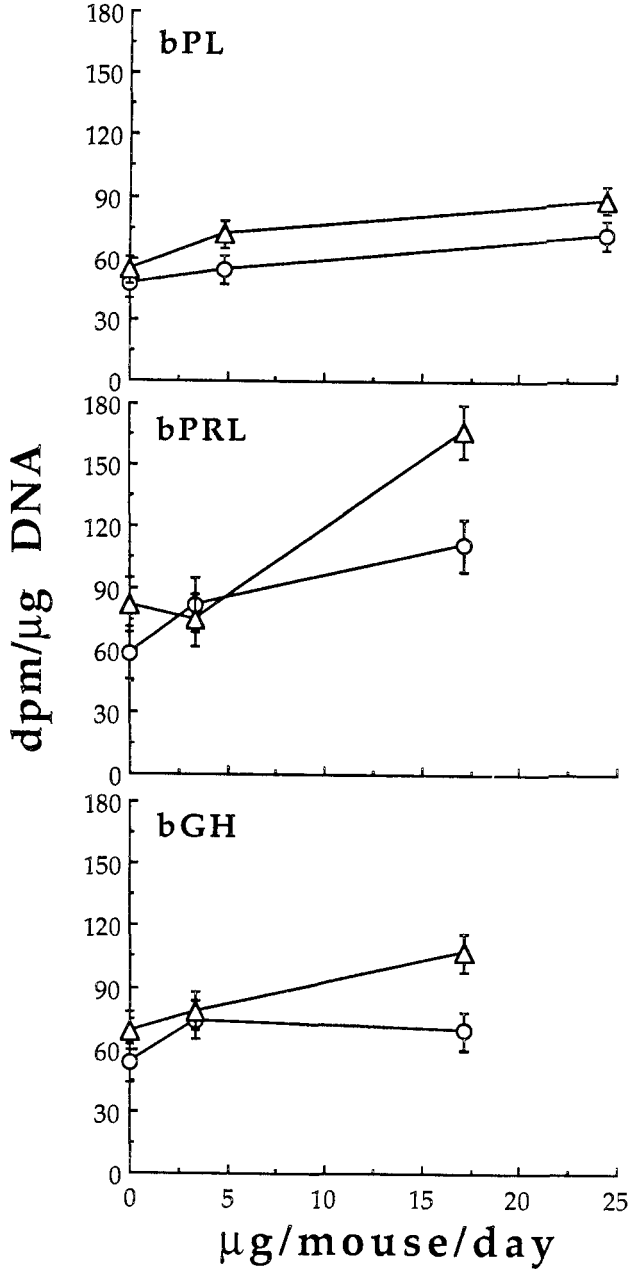

Figure 1. Total [^{3}H]TdR incorporation into DNA of bovine mammary tissue implanted in athymic mice under bPL, bPRL, and bGH stimulation. The athymic mice (n = 90) were injected daily for 5 days with CB-154 plus either buffer (50 mM NH_4HCO_3, pH 7.8), as control (○), or 17β-estradiol + progesterone (△) simultaneously with three levels of bPL, bPRL, and bGH. Each datum point represents the mean ± SE of five heifers. There was a significant linear response ($P < 0.01$) due to bPL and bPRL main effects (averaged over the absence and presence of steroids) and a significant steroid effect ($P < 0.05$), with a nonsignificant interaction ($P > 0.10$). Although the independent effect of bGH was not significant ($P > 0.10$), bGH plus steroids had a significant linear effect ($P < 0.05$), with a nonsignificant interaction ($P > 0.10$). The slopes of the response for bPL and bPL plus steroids were 0.96 and 1.23, respectively. For bPRL and bPRL plus steroids the slopes were 1.93 and 3.76, respectively. The slopes for bGH and bGH plus steroids were 0.37 and 1.15, respectively.

to bPRL was 17.09 in the absence of steroid injections and 24.58 in the presence of steroid injections. These were not different at $P < 0.10$ (exact $P = 0.12$). Slope of the response of labeling index to bGH was 5.94 in the absence of steroid injections and 8.16 in the presence of steroid injections. These slopes were not different ($P < 0.10$).

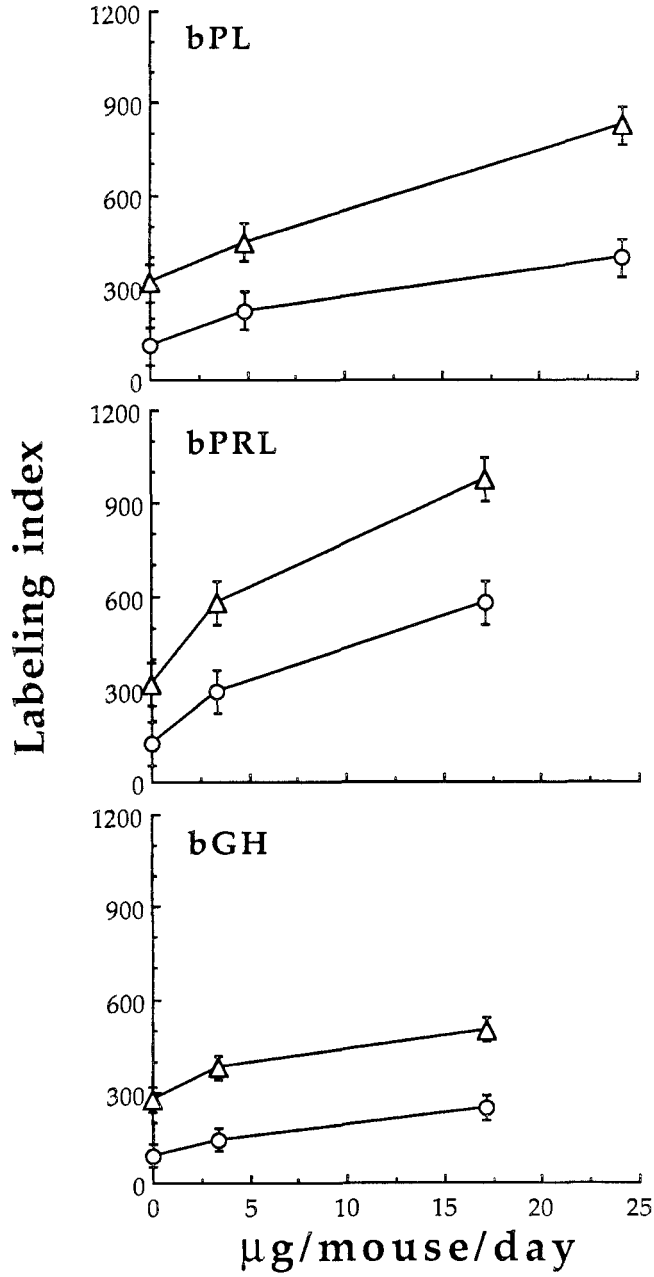

Figure 2. Labeling index of bovine mammary tissue implanted in athymic mice under bPL, bPRL, and bGH stimulation. The athymic mice (n = 90) were injected daily for 5 days with CB-154 plus either buffer (50 mM NH_4HCO_3, pH 7.8), as control (○), or 17β-estradiol + progesterone (△) simultaneously with three levels of bPL, bPRL, and bGH. Each datum point represents the mean ± SE of five heifers. There was a significant linear response ($P < 0.01$) due to bPL, bPRL, and bGH main effects (averaged over the absence and presence of steroids), with a nonsignificant interaction ($P > 0.10$). The slopes of the response for bPL and bPL plus steroids were 10.7 and 20.1, respectively. For bPRL and bPRL plus steroids the slopes were 17.1 and 24.6, respectively. The slopes of the response for bGH and bGH plus steroids were 5.9 and 8.1, respectively.

Discussion

The mitogenic effect of bPL was evident in ovariectomized CB-154-treated mice, even in the absence of exogenous estradiol and progesterone. The response of the tissue to bPL was similar to that obtained with bPRL. However, responsiveness to bGH was lower. This may have been due to the presence of endogenous GH in mice. All mice in these studies were injected with CB-154, which has been shown to decrease PRL release and mammary development in several species, including mice (14). Since CB-154 is not effective in decreasing GH release, the low response to bGH (relative to the response to bPRL or bPL) may have been due to high levels of GH in the mice (relative to circulating PRL levels).

Previous studies indicated that mammogenic agents increase DNA synthesis of bovine mammary tissue maintained in athymic nude mice (11, 15–17). Although independent effects have been examined for some hormones (e.g., estradiol, progesterone, epidermal growth factor), this report represents the first time bPL, bPRL, and bGH have been administered independently. Previous studies indicated that bGH and bPRL together increased bovine mammary tissue DNA synthesis (11, 15), but the two hormones were not examined separately.

McManus and Welsch (18) and McManus *et al.* (19) examined the effects of human placental lactogen (hPL) on DNA synthesis of human breast tissue maintained in athymic nude mice. They observed no effect of hPL alone, but significant synergism between estradiol and hPL on DNA synthesis of xenografted human breast tissue. Since the mice in their studies were not ovariectomized, hPL effects might have been due to the luteotropic effects of hPL. However, McManus and Welsch (18) observed that progesterone injections did not affect DNA synthesis of human breast ductal epithelia maintained in athymic nude mice, whether given alone or in combination with estradiol. As far as we can determine, this represents the first demonstration of a mammogenic effect of purified bPL on bovine mammary tissue.

The effects of each of the lactogens was additive with the effect of steroids on DNA synthesis by xenografted bovine mammary tissue. There is some suggestion from these studies that a synergism exists between steroid and protein hormones in their ability to regulate bovine mammary tissue DNA synthesis. Such a synergism would be consistent with current theories of mammogenesis (1). However, the interactions between steroids and proteins in this study were of marginal statistical significance (type I errors of about 0.1) and are, therefore, inconclusive.

Although results of these studies suggest a mammogenic role for bPL, they do not indicate whether the effect of bPL is direct on the transplanted mammary tissue or mediated via indirect mechanisms. Although there have been no studies with bPL, hPL is known to increase insulin-like growth factor I and insulin-like growth factor II concentrations in rats (20). Since insulin-like growth factor I has been shown to increase DNA synthesis of bovine mammary tissue *in vitro* (21, 22), bPL may not directly affect mammary development. Similar considerations apply to bGH, which has been shown to be mammogenic and galactopoietic *in vivo* (17, 23), but which is generally without effect *in vitro* (24). Similarly, prolactin has been proposed to act on liver to increase production of synlactin (25, 26), which may mediate at least some of the actions of prolactin.

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