Thymidine Incorporation by Lactating Mammary Epithelium During Compensatory Mammary Growth in Beef Cattle

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ABSTRACT

Ten Hereford cows, 100 d into first lactation, were assigned to treatment or control groups to study compensatory growth of mammary glands. The right udder half of treatment cows was covered to prevent suckling by the calf, whereas control cows were suckled on all quarters. Milk production was estimated the day treatment began and 4 d later by machine milking following removal of calves for 12 h and i.v. injection of oxytocin. Five to 7 d after beginning treatment, cows were killed and mammary tissue was obtained from three regions within left and right glands for in vitro incubation with \(^{3}\mathrm{H}\) thymidine. Deoxyribonucleic acid of lactating udder halves did not increase in response to treatment although RNA: DNA ratio and milk production tended to increase. Incorporation of \(^{3}\mathrm{H}\) thymidine was greater in lactating quarters of treated cows than control cows (35,000 vs. 19,000 cpm/mg of DNA) with greatest incorporation in the basal regions of each gland. Furthermore, greatest incorporation of \(^{3}\mathrm{H}\) thymidine occurred in nonsuckled glands. Autoradiographic analyses confirmed incorporation data and indicated that 81% of proliferating cells were epithelial. Data suggest that proliferation of mammary epithelial cells, within both the lactating and nonlactating glands, occurred in response to milk stasis.

(Key words: lactation, involution, proliferation)

Received August 30, 1989.
Accepted April 2, 1990.

1990 J Dairy Sci 73:3094-3103

INTRODUCTION

Mammary growth continues during lactation in most rodent species (3, 21, 22), although in the hamster, mammogenesis is complete at parturition (18). In ruminants, the situation is less clear. Mammary growth appears to cease with the onset of lactation in sheep (2) but continues into lactation in goats (14). Although it has generally been assumed that little or no mammary growth occurs in cattle during lactation, lactating bovine mammary epithelial cells may be able to divide (9). Whether cell proliferation during lactation in cattle can result in significant mammary growth remains to be determined.

In goats, unilateral inhibition of milk secretion results in a compensatory increase in milk production by the other gland (11). Although this compensatory response apparently is not related to availability of rate limiting substrates (12), the relative contributions of hypertrophy and hyperplasia are unknown. Similarly, preliminary evidence in cows suggests that increased milk production by uninfected quarters of a mastitic udder may compensate for decreased milk secretion by infected quarters (23).

The objectives of the study were 1) to determine whether compensatory hypertrophy and hyperplasia occur in bovine mammary glands when milk is not removed from the contralateral glands, and 2) to determine, if cell proliferation occurs, the cell types and regions within the gland that proliferate.

MATERIALS AND METHODS

Animals

Ten lactating Hereford cows, 100 d into first lactation, were randomly assigned to treatment
Each cow was suckling a single calf. Control cows were permitted to suckle calves on all quarters, and cows assigned to compensatory treatment were permitted to suckle calves only on the left teats. The right udder half of treatment cows was covered with canvas, attached to the udder with tag cement (Nasco, Fort Atkinson, WI), to prevent suckling by the calf.

Cows were machine milked the morning treatment began and 4 d later. Calves and cows were separated for 12 h prior to milking. Cows were then restrained in a squeeze chute, injected in the coccygeal vein with 20 USP units of oxytocin, and milked using a portable quarter milker (4). Five to 7 d after initiation of treatment, cows were killed by a gunshot to the head and exsanguination at the USDA abattoir, Beltsville, MD. Equal numbers of control and compensatory treatment cows were randomly selected for slaughter on a given day. At slaughter, the udder was removed and trimmed of extraparenchymal tissues. Parenchyma of udder halves was weighed and mammary parenchyma was obtained from three regions within the left front, left rear, and right rear quarters. Parenchyma was sampled at the base of the gland adjacent to the gland cistern (zone 1), midway between the gland cistern and dorsal boundary of the mammary parenchyma (zone 2), and near the parenchymal border above the teat (zone 3). Tissue was transported to the laboratory in ice-cold Tris-sucrose buffer (25 mM Tris, .1 mM EDTA, pH 7.4). The remaining tissue from each udder half was ground using a commercial meat grinder (Hobart Corporation, Troy, OH) and aliquots stored at -20°C until assayed for nucleic acid content.

Tissue Incubations

Mammary tissue was sliced with a Stadie-Riggs hand microtome. Triplicate tissue slices (approximately 150 mg) from each zone (three zones, three quarters, 10 cows) were incubated for 2 h in Medium 199 (GIBCO, Grand Island, NY) containing 1 μCi [3H]thymidine/ml (25 Ci/mmol, Amersham, Arlington Heights, IL), under an atmosphere of 5% CO₂ and 95% O₂. Following incubation, tissues were rinsed in .9% saline. One slice per zone was fixed and processed for autoradiography and two slices stored at -20°C for later determination of [3H]thymidine ([3H]Tdr) incorporation.

[3H]Thymidine Incorporation

In order to determine the quantity of [3H]Tdr incorporated, tissue slices were first homogenized in 5 ml of homogenization buffer (.05 M sodium phosphate, 2 M NaCl, 2 mM Na₂EDTA, pH 7.4). Duplicate .5 ml aliquots of each homogenate were then precipitated with .5 ml of ice-cold 10% TCA. The pellet was washed three times with 2 ml of ice-cold 5% TCA and the final pellet solubilized and counted in ACS II scintillation fluid (Amersham). The quantity of DNA in the homogenate was determined by the method of Labarca and Paigen (15), using calf thymus DNA (Sigma Chemicals, St. Louis, MO) as standard.

Udder Half Deoxyribonucleic Acid and Ribonucleic Acid Determination

Ground mammary tissue was homogenized (1:4 wt/vol) in distilled water with a Polytron homogenizer. One milliliter of homogenates was treated with 1 ml of .5N ice-cold perchloric acid (PCA) for 30 min and centrifuged at 3000 × g for 30 min. The pellet was washed three times with 2 ml of ice-cold .2N PCA and then hydrolyzed three times in 2 ml of .5N PCA (70°C, 20 min), cooled on ice, and centrifuged between subsequent hydrolysates. Hydrolysates were pooled, and DNA and RNA contents were determined by the Burton (6) and Ceriotti (7) methods, respectively. Recovery of DNA and RNA standards added to homogenates was 102 ± 2 and 70 ± 5%, respectively.

Autoradiography

Tissues were fixed in modified Karnovsky’s fixative and processed for autoradiography as described in Smith et al. (20). For each zone per quarter per animal, labeled epithelial cells were enumerated within the boundaries of six random microscopic fields defined by the boundary of an ocular grid (100 × 100 μm). The number of epithelial cells within each field averaged 30 ± 1 (SE). Additionally, labeled cells were identified according to cell type. A cell was considered labeled when its nucleus was overlain with 12 or more silver grains. Background labeling was less than one grain per nucleus.

Statistical Analyses

Effect of covering the right udder half (compensatory treatment) on the difference between
pretreatment and posttreatment milk production by the left udder half was analyzed by paired t test. Other statistical analyses utilized the general linear models procedure of SAS (17). Mammary gland composition data were analyzed in accordance with a split-plot design in which effects of compensatory treatment and treatment by udder half interaction were tested. Incorporation of $[^3H]Tdr$ (cpm/μg of DNA) and the percentage of cells autoradiographically labeled were analyzed in accordance with a split-split-plot design, with three zones in each quarter serving as the final split. Effect of treatment was assessed using the animal variation. Udder half effects and interactions were tested using the within animal variation. Zone effects and zone interaction effects were tested using the within quarter variation. Data for incorporation of $[^3H]Tdr$ (cpm/μg of DNA) were log-transformed and the percentage of cells autoradiographically labeled were arcsin-transformed prior to statistical analyses. For ease of interpretation, these data are summarized as arithmetic means of nontransformed data in Figure 1.

RESULTS

Cessation of milk removal from an udder half tended ($P = .104$) to increase milk production by the contralateral glands 4 d later (Table 1). Consistent with milk production data, the ratio of RNA:DNA in lactating quarters of compensatory treatment cows tended ($P = .2)$ to be greater than that in control cows (Table 2). However, DNA content of lactating quarters did not differ ($P > .1$) between compensatory and control treatments (5.2 for treated vs. 5.4 and 5.3 for control. The DNA content of nonsuckled quarters was less ($P = .08$) than that of lactating quarters (4.2 vs 5.4, 5.2, and 5.3), and the RNA:DNA ratio of involuting glands was less ($P < .005$) than that of lactating quarters. Expression of parenchymal composition on a body weight basis did not alter interpretation of data. Mean live body weight of treated heifers (502 kg) did not differ ($P > 1$) from that of control heifers (487 kg).

Incorporation of $[^3H]Tdr$ by mammary parenchymal tissue obtained from lactating quarters of compensatory treatment heifers was greater ($P < .05$) than incorporation in quarters of control cows (Figure 1). Incorporation of $[^3H]Tdr$ in lactating and involuting quarters was greatest ($P < .0001$) in the region of mammary gland nearest the gland cistern and decreased toward the periphery (mammary fat pad). Overall, greatest incorporation of $[^3H]Tdr$ occurred in involuting quarters (all zones, $P < .05$). The same pattern of incorporation was observed when the percentage of mammary epithelial cells labeled with $[^3H]Tdr$ was quantitated autoradiographically (Figure 1). The percentage of labeled epithelial cells was greater in lactating quarters of compensatory treatment cows ($P < .05$) than in control cows, was greatest in involuting glands ($P < .01$), and was greatest,
within a gland, in the region of the gland cistern, decreasing toward the periphery \((P<.01)\). Regardless of treatment, the majority of cells labeled with \(^{3}H\)Tdr were secretory epithelial cells (Table 3, Figures 2, 3, 4). Overall, 81% of labeled cells were epithelial and 19% were stromal. Labeled stromal cells were primarily fibroblasts (95%), but occasionally, labeled endothelial cells or leucocytes were identified.

Morphologically, lactating tissue from control and compensatory treatment cows was indistinguishable, and tissue did not appear consistently different between zones within lactating quarters. However, two distinct characteristics of lactating tissues could be described. Visually, about 40% of the parenchyma appeared to consist of areas containing closely packed alveoli with minimal stromal matrix, and the epithelium appeared less differentiated. In contrast with well differentiated tissue, the cells frequently contained large irregularly positioned lipid droplets, and polarization and clustered apical secretory vesicles were less evident. Labeled epithelial cells were observed among cells in both morphologically defined regions, although labeled epithelial cells appeared to occur more frequently within areas containing cells less well differentiated (Figure 2D). Labeled cells in lactating tissue from each of the three zones (from teat region to periphery) of a compensatory treatment cow are illustrated in Figure 3A, B, and C.

The appearance of mammary parenchyma in involuting glands was markedly heterogeneous with a distinct gradation in histological appearance between zones. Mammary involution appeared greatest near the gland cistern (zone 1) and decreased toward the periphery (zone 3). Generally, alveolar structure and organization were apparent throughout the mammary gland and accumulated secretions were evident. However, cells in zone 1 (near the gland cistern) (Figure 4A and B) frequently appeared swollen, engorged with lipid, and disorganized. Alveoli with areas denuded of cells or with cells containing pyknotic nuclei were also observed.

**TABLE 2. Mammary gland composition in suckled and unsuckled udder halves of treatment and control cows.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parenchyma</th>
<th>Fat</th>
<th>DNA</th>
<th>RNA</th>
<th>RNA:DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left udder half</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.44</td>
<td>1.34</td>
<td>5.4</td>
<td>11.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Treated</td>
<td>2.74</td>
<td>1.33</td>
<td>5.2</td>
<td>12.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Right udder half</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.52</td>
<td>1.50</td>
<td>5.3</td>
<td>11.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Treated</td>
<td>2.29</td>
<td>1.91</td>
<td>4.2a</td>
<td>4.8b</td>
<td>1.2b</td>
</tr>
<tr>
<td>SE</td>
<td>.14</td>
<td>.14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\)Diffs from other means within column \((P<.005)\).

\(b\)Diffs from other means within column \((P<.005)\).

\(c\)Each value represents mean for five heifers slaughtered 5 to 7 d after initiation of treatment. The right udder half of treated cows was covered to prevent suckling of those quarters by the calf while permitting suckling of left quarters. All quarters were suckled in control cows.

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**TABLE 1. Effect of milk stasis in the right udder half on milk production by the left udder half.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Milk from the left udder half</th>
<th>Pretrt.</th>
<th>Posttrt.</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.84</td>
<td>3.04</td>
<td>.25</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>2.58</td>
<td>3.27</td>
<td>.69*</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>.26</td>
<td>.28</td>
<td>.22</td>
<td></td>
</tr>
</tbody>
</table>

\(a\)Diffs from control \((P = .104)\).

\(b\)Each value represents mean for five heifers. The right udder half of treated cows was covered to prevent suckling of those quarters by the calf. All quarters were suckled in control cows. Quantity of milk was determined just prior to the time treatment was initiated (pretrt.) and 4 d later (posttrt.).
TABLE 3. Classification of cells labeled with $[^3\text{H}]$thymidine.$^1$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell type</th>
<th>Number of cells evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epithelium</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>Left udder half</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>82.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Treated</td>
<td>88.2</td>
<td>11.3</td>
</tr>
<tr>
<td>Right udder half</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>69.4</td>
<td>24.2</td>
</tr>
<tr>
<td>Treated</td>
<td>77.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Overall$^2$</td>
<td>80.7</td>
<td>18.5</td>
</tr>
</tbody>
</table>

$^1$Cells labeled with $[^3\text{H}]$thymidine were classified according to cell type as epithelial, fibroblastic, or other (leucocytes and endothelial cells). Values are percentage of total cells evaluated. The right udder half of treated cows was covered to prevent suckling of those quarters by the calf while permitting suckling of left quarters. All quarters were suckled in control cows.

$^2$Mean for all quarters and treatments.

Despite relative lack of organization and differentiated structure, labeled epithelial cells were observed. In areas with alveoli that appeared especially involuted, labeled nuclei occasionally appeared in a position suggesting the labeling of myoepithelial cells rather than secretory epithelial cells. However, as illustrated, most labeling was clearly of alveolar epithelium. Figure 4C and D illustrates the histological appearance of parenchyma in zones 2 and 3, respectively. There was a marked accumulation of secretions, but alveolar structure was intact. At higher magnification (Figure 4E and F) the secretory cells were clearly intact and much less disorganized than cells in zone 1. In fact, cells in zone 3 appeared morphologically similar to those described in mammary glands of nonlactating, pregnant, periparturient cows (1).

**DISCUSSION**

Although secretion of milk did not increase significantly in compensatory treatment quarters, trends in the data suggest that a compensatory response was occurring. Difficulty demonstrating a significant compensatory increase in milk production may have been due to errors inherent in machine milking beef heifers, use of a limited number of milkings to assess milk production, or use of heifers rather than multiparous cows. Although errors associated with machine milking untrained beef heifers most likely were large, the number of milkings was limited in order to maximize normal milk removal by the calf and to minimize the potential disruptive effects of machine milking. Data of Woolford (23) suggest that our failure to detect a compensatory increase in milk production may be due to use of first lactation heifers. These investigators also failed to demonstrate a compensatory increase in milk production by uninfected quarters of mastitic dairy heifers (primiparous), yet a significant increase occurred in the case of multiparous dairy cows. Finally, the lack of a compensatory increase in milk production may have been due to insufficient length of treatment. However, data in goats (11) suggest that such a compensatory response should be observed within 24 h.

Relative rates of $[^3\text{H}]$Tdr incorporation into mammary DNA suggested that compensatory proliferation in mammary parenchyma occurred in response to cessation of milk removal from the opposite udder half. Autoradiographs indicated that increased incorporation of $[^3\text{H}]$Tdr was due to an increase in the percent of mammary epithelial cells incorporating the tracer, which most likely indicated a proliferative response to milk stasis in the opposite udder half. Within the responding quarters, greatest incorporation and labeling of epithelial cells occurred in the basal regions of glands; however, labeled cells were not distinguishable from other mammary epithelial cells. Thus, data do not suggest the presence of a morphologically distinct stem cell population in bovine mammary gland, as reported for mouse mammary gland (19). Indeed, epithelial cells which were
Figure 2. Tissue from lactating mammary glands. Panels A (x340) and B (x680) illustrate the histological and cytological appearance of areas containing fully differentiated epithelial cells and minimal stroma. Panel C (x340) and D (x650) illustrate areas of less well-differentiated cells and more stromal area. Panel D illustrates the presence of several labeled epithelial cell nuclei. In this and all histological figures, the following abbreviations and symbols are used: S = stroma, I = lumen, C with arrow = corpora amylacea, and arrows indicate cell nuclei labeled with $^{3}$H]thymidine.
Figure 3. Tissue from lactating (left) mammary glands of treated cows. Panels A, B, and C (×680) illustrate labeled cells in zones 1, 2, and 3, respectively. Three corpora amylaceae are visible in panel A.
Figure 4. Tissue from unsuckled mammary glands. Panel A and B (x640) show epithelial cells from zone 1. Lumina are filled with material, and epithelial cells exhibit moderate (upper alveoli) to rather extreme degrees of engorgement with lipid and vacuoles (lower alveoli). Labeled nuclei of epithelial cells are also indicated. Micrographs in panels C (x340) and D (x136) were taken from zones 2 and 3, respectively. Outlines of alveoli with intact epithelial cells and secretion-filled lumina are apparent. Panels E and F (x640) show alveoli in zone 3. Although many of the cells have large lipid droplets, cytoplasmic areas are densely stained and cells do not appear swollen. Several labeled epithelial cell nuclei (panels E and F) and two corpora amylacea in the luminal area of alveoli are shown (panel F).

routinely labeled with $[^3]$H]Tdr appeared to be secreting actively. Despite increased incorporation of $[^3]$H]Tdr, mammary parenchymal weights and nucleic acid content did not differ between the lactating (compensatory) glands of control and treated cows, which might be the case if a proliferative response were not initiated until the final days
of treatment, or if the magnitude of response were insufficient to result in a significant increase in cell number. In either case, a rapid secretory response would thus be due to increased activity of existing cells. Although not statistically significant, the tendency toward an increase in ratio of RNA:DNA is consistent with this supposition and consistent with the hypertrophic response observed in the goat (11, 13).

Interestingly, greatest incorporation of [3H]Tdr occurred within mammary glands that were involuting, despite an apparent decrease in cell number (DNA) in those glands. Morphologically, tissue obtained from the region near the gland cistern (zone 1) of these quarters exhibited moderate but variable degrees of involution within the 5 to 7 d-period; e.g., some alveoli showed cells with large lipid droplets and apically displaced nuclei, whereas others contained cells that appeared intact but non-secretory. In extreme cases, alveolar outlines were apparent, but individual cells were denuded from the basement membrane, and former alveolar lumina were filled with secretory material, free cells, and cell debris. The degree of involution appeared greatest in zone 1, and there was a gradation toward the most “normal” appearing epithelium in zone 3. Greatest incorporation of [3H]Tdr apparently occurred in those zones with greatest involution. Local factors, both stimulatory and inhibitory (8, 10, 11), appear to regulate the growth and secretory rate of mammary tissue. If factors that regulate a compensatory growth response originate in involuting glands, then these areas should contain highest and lowest concentrations, respectively, of stimulatory and inhibitory growth factors. Thus, increased incorporation of [3H]Tdr during early mammary involution may not be unexpected. A significant reduction in cell number occurs because cell mortality exceeds cell replication. Although we cannot rule out the possibility that at least a portion of the [3H]Tdr was incorporated for processes of DNA repair, the density of autoradiographic labeling suggests that incorporation of [3H]Tdr was primarily for DNA replication (5).

Our data also indicate that the rates of cell proliferation and involution are not uniform throughout the mammary gland. Greatest rates of DNA synthesis were observed at the basal (gland cisternal) region of all glands (control and compensatory lactating quarters as well as involuting quarters) and decreased toward the periphery. In involuting quarters, histological examination suggested that regression of mammary parenchyma proceeded most rapidly in the basal region of the gland.

In conclusion, proliferation of mammary epithelial cells occurs as a response to milk stasis. A greater number of cells within lactating and involuting glands of treated cows incorporated [3H]Tdr than did cells within lactating glands of control cows. Those cells incorporating [3H]Tdr were primarily (81%) secretory epithelial cells and were morphologically indistinguishable from nonproliferating epithelial cells. Increased cell proliferation in nonlactating quarters suggests that extensive cell turnover occurs during mammary involution whereas increased proliferation in lactating quarters of treatment cows is consistent with compensatory mammary growth in response to involution of contralateral glands. Data suggest that the bovine mammary gland has capacity for continued growth during lactation. Consequently, strategies to increase or to maintain the number of mammary epithelial cells during lactation appear feasible.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of P. Grier and P. Boyle. Our gratitude is also extended to J. E. Keys and J. J. Smith, who assisted with animal handling.

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COMPENSATORY MAMMARY GROWTH


Journal of Dairy Science Vol. 73, No. 11, 1990