The Integrity of Mammary Alveolar Cells in Two Consecutive Lactations[1] (36250)

HOWARD S. PITKOW, RALPH P. REECE, AND GEORGE L. WASZILYCSAK

Departments of Animal Sciences and Environmental Sciences, Rutgers University, New Brunswick, New Jersey 08903

Reece and Warbritton (1), using the colchicine technique, reported that mammary glands of rats pregnant during the first lactation exhibited very little mitotic activity between the fifth day of lactation and the second parturition. This indirect evidence indicated that some of the secretory cells present in the first lactation were probably present in the second lactation in rats pregnant during the first lactation. Hence, it was desirable to obtain direct evidence on this problem.

The use of tritiated thymidine to label alveolar cells has the advantage that labeled cells could be traced through successive lactations through autoradiography. Although cell numbers can be estimated by DNA content, the DNA procedure can not be used to determine whether or not cells are involuting in instances where hyperplasia is also occurring. However, by using tritiated thymidine to label cells and the DNA procedure to estimate cell numbers, the percentage of "carry-over" of alveolar cells from the first lactation to the second lactation can be determined in animals pregnant during the first lactation.

Materials and Methods. Virgin female rats of the hooded Norway strain, weighing 160 to 170 g, were mated. They were maintained at a temperature of 72 ± 2°F and under 10 hr of artificial light daily. The animals received Purina Laboratory Chow and water *ad libitum*. Prior to parturition, female rats were either isolated or cohabitated with a sexually mature male rat in a lactating cage. In cohabitated female rats, vaginal smears were examined each morning following parturition for the presence of sperm (day 1 of pregnancy) until day 3 of lactation at which time the male rat was removed and the litter was standardized at 6 pups. To aid implantation, litters were removed from mother rats at 4:00 p.m. on day 5 of pregnancy and returned at 8:00 a.m. on day 6 (2).

Rats that received tritiated thymidine were injected subcutaneously on the third day of lactation with 0.3 μCi/g of body weight (sp act = 6.7 Ci/mmole; New England Nuclear, Boston, MA), since La Ganga (3) found that mammary proliferation was greatest at this time in lactating hooded Norway rats. The effect of tritiated thymidine on mammary function (litter growth index and nucleic acid content) was determined in rats sacrificed on day 30 of the first lactation and in rats pregnant during the first lactation and sacrificed on day 4 of the second lactation. The percentage of alveolar cells present in the first lactation that was present in the second lactation (carry-over percentage) of rats pregnant during the first lactation was based on two criteria: the mean labeled daughter cell index and the mean total mammary gland DNA content found after injecting tritiated thymidine on day 3 of the first lactation. Rats were sacrificed on day 3 of the first lactation (1 hr after injection), on day 4 of the second lactation (with and without a nonlactating period prior to the second parturition), and on day 41 of the second lactation (replacement litter on day 21 of lactation). The nucleic acid content of the mammery glands was determined by the method of Tucker and Reece (4) on either the 3 right, the 3 left, or the 6 abdominal-inguinal mammary glands. When the nucleic acid content was determined on 3 glands, the value obtained was doubled to

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indicate total nucleic acid content.

The dip-coating technique of autoradiography involved a histological procedure (5) and an autoradiographic technique (6), both of which were modified. After each rat was sacrificed, 0.25 in. of mammary tissue from either the right or left side was dissected at the level of the femoral vein. The tissue was fixed in Bouin's fluid, embedded in paraffin, and sectioned at 6 μ parallel to the abdominal surface of the gland. Sections were mounted, deparaffinized, hydrated, and Kodak NTB-3 liquid emulsion was applied essentially as described by Baserga (6); the preparations were stored for exposure at 4° for a period of 15 days. The exposed radioautographs were developed at 18° for 4 min in D-19 Kodak developer, washed in water, and fixed for 7 min in Kodak general fixer. The autoradiograms were stained with hematoxylin and eosin and mounted in Permount, as described by Humason (5).

A sample size of 1000 cells/mammary section was used to quantitate alveolar cell proliferation and carry-over since Traurig (7) observed an insignificant decrease in the value of the 95% confidence limits of mammary gland cells from sample sizes greater than 1000 cells. A cell was considered to be labeled if 5 or more reduced silver grains were observed in the emulsion overlying the nucleus. Parameters for each group of rats were individually measured and analyzed for significant differences by Student's t test.

Results. A subcutaneous injection of 0.3 μCi/g of body wt of tritiated thymidine on day 3 of the first lactation did not significantly inhibit mammary gland growth or secretion in rats lactating 30 days or in rats on day 4 of the second lactation and pregnant during the first lactation, when compared to noninjected rats (Table I). Following injection of tritiated thymidine, blood samples were collected on days 3 (8 hr after injection), 12, 21, and 30 of lactation. These blood samples, lyophilized and nonlyophilized, were counted through liquid scintillation, and the highest total blood activity of lyophilized blood samples was found on day 3 (12.8×10^{-2} μCi). Through histological autoradiographic grain counts, it was determined that an injection of 12.8×10^{-2} μCi of tritiated thymidine was insufficient to label mammary alveolar cells. Therefore, alveolar cell carry-over could be determined since the amount of tritiated thymidine available 8 hr after injection was insufficient to label mammary alveolar cells.

On day 3 of lactation, 1 hr after injection, 8 rats were sacrificed; and mean values for labeled daughter cells, total DNA, total RNA, and RNA/DNA ratio were 10.9%, 23.01 mg, 39.74 mg, and 1.73, respectively.

When rats were: (i) injected with tritiated thymidine on day 3 of the first lactation; (ii) lactating continuously; and (iii) sacrificed on day 4 of the second lactation the mean values for labeled daughter cells, total DNA, total RNA, RNA/DNA ratio, and percentage carry-over from day 3 of the first lactation were 6.2%, 29.40 mg, 43.56 mg, 1.46, and 72.9%, respectively. Corresponding values for rats with a 7- or 8-day nonlactating period prior to the second parturition were 4.7%, 29.91 mg, 47.28 mg, 1.56, and 56.0%, respectively (Table II, Fig. 1). Except for the labeled daughter cell index and percentage carry-over, the differences between the means of the two groups of rats were not significant. The labeled daughter cell index of rats without a nonlactating period (6.2%) was significantly greater ($p < .01$) than that of rats with a nonlactating period (4.7%). The percentage of alveolar cell carry-over in those rats without a nonlactating period (72.9%) was significantly greater ($p < .05$) than that in rats with a nonlactating period (56.0%). Therefore, even though the mean DNA values between the 2 groups of rats were not significantly different on day 4 of the second lactation, there was a significant difference in the percentage of alveolar cell carry-over.

When the second lactation was extended from day 4 to day 41, in order to determine whether or not cells formed on day 3 of the first lactation maintained their integrity as long as cells formed thereafter, the mean values for labeled daughter cells, total DNA, total RNA, RNA/DNA ratio, and percentage carry-over were 5.8%, 26.63 mg, 55.92 mg, 2.09, and 62.1%, respectively. The RNA

TABLE I. Effect of Tritiated Thymidine on Mammary Growth and Function of Rats on Day 30 of Lactation and of Rats Pregnant During the First Lactation and Sacrificed on Day 4 of Second Lactation.

Treatment[a] and day of sacrifice	No. of rats[b]	Body wt (g)	Litter growth index (g)[c]	DNA (mg)	DNA (mg/100 g of body wt)	Total RNA (mg)	RNA/DNA ratio
Noninjected, day 30 of lactation	10	242 ± 3.9[d]	9.9 ± 0.37	19.08 ± 0.89	7.88 ± 0.30	29.73 ± 2.74	1.53 ± 0.08
Injected, day 30 of lactation	10	220 ± 4.5	9.8 ± 0.37[e]	18.25 ± 0.41[e]	8.31 ± 0.16[e]	24.73 ± 1.65[e]	1.34 ± 0.07[e]
Noninjected, day 4 of 2nd lact.	8	238 ± 4.4	—	28.87 ± 1.25	12.12 ± 0.52	44.18 ± 2.18	1.63 ± 0.05
Injected day 4 of 2nd lact.	8	245 ± 2.6	—	29.40 ± 1.36[f]	11.99 ± 0.53[f]	43.56 ± 3.67[f]	1.46 ± 0.07[f]

[a] Injected rats received 0.3 μCi/g of body wt of tritiated thymidine on day 3 of first lactation.
[b] Litters standardized at 6 pups on day 3 of lactation.
[c] Mean daily litter weight gain between days 6 and 11 of lactation.
[d] Mean ± SE.
[e, f] Not significantly different from corresponding values of control group.

TABLE II. Nucleic Acid Content, Labeled Cells, and Percentage "Carry-Over" of Mammary Alveolar Cells from Day 3 of First Lactation to Days 4 and 41 of Second Lactation in Rats Pregnant During the First Lactation.

Group	No. of rats	Body wt (g)	Total[a] DEEET (mg)	DNA (mg)	DNA (mg/100 g of body wt)	Total RNA (mg)	RNA/DNA ratio	Labeled[b] cells (%)	Carryover[c] (%)
Day 3, first[d,e] lactation	8	214 ± 4.2[h]	671.9 ± 30.7	23.01 ± 0.98	10.72 ± 0.30	39.74 ± 1.99	1.73 ± 0.06	10.9 ± 0.4	—
Day 4, second lactation[d] without nonlactating period	8	245 ± 2.6	883.5 ± 62.9	29.40 ± 1.36[i]	11.99 ± 0.53	43.56 ± 3.67[j]	1.46 ± 0.07	6.2 ± 0.3[k]	72.9 ± 4.5[l]
Day 4, second lactation[d,f] with nonlactating period	8	252 ± 5.0	945.2 ± 66.0	29.91 ± 1.56[i]	11.87 ± 0.64	47.28 ± 3.80[j]	1.56 ± 0.06	4.7 ± 0.4	56.0 ± 5.3
Day 41, second lactation[d,g]	8	281 ± 6.7	985.4 ± 64.9	26.63 ± 0.59[i]	9.51 ± 0.30	55.92 ± 4.86[j]	2.09 ± 0.16	5.8 ± 0.3	62.1 ± 4.6[m]

[a] Dried, ethanol–ether extracted tissue; doubled wt of one side of abdominal-inguinal mammary glands to obtain total wt.

[b] Based on labeled daughter cells.

[c] Determined by comparing mean mitotic index and DNA values on day 3 of first lactation with corresponding mean values of either day 4 or day 41 of second lactation.

[d] Tritiated thymidine inj. (0.3 μCi/g of body wt) on day 3 of first lactation.

[e] Sacrificed 1 hr after inj.

[f] Litters removed on day 18 of lactation.

[g] Pups removed on day 21 of second lactation and replaced with 3- or 4-day-old pups.

[h] Mean ± SE.

[i, j] Not significantly different.

[k] Significantly greater ($p < .01$) than 4.7; [l] Significantly greater ($p < .05$) than 56.0.

[m] Not significantly different than 56.0 and 72.9.

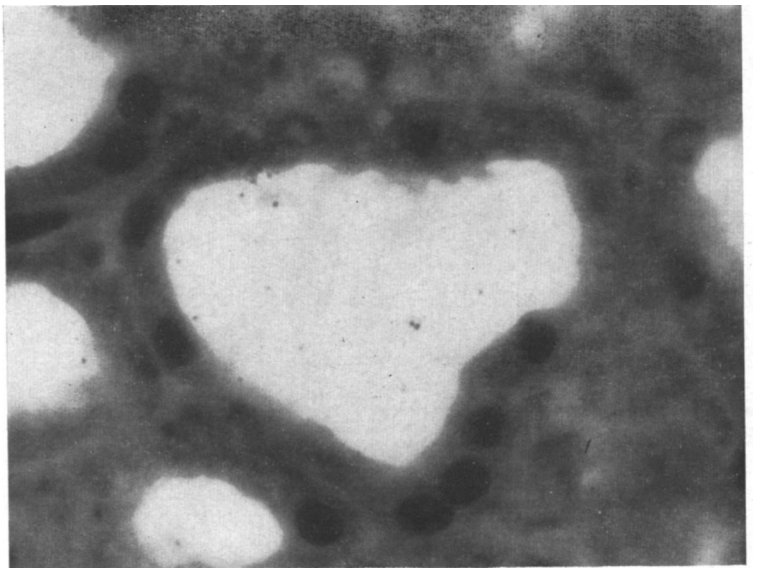

FIG. 1. Photomicrograph of an autoradiographic section of a mammary gland of a rat which had been injected with 0.3 μCi/g of body weight of tritiated thymidine on day 3 of first lactation, which had a 7-day nonlactating period prior to the second parturition, and which was sacrified on day 4 of second lactation (1000×).

and DNA values were not significantly different from the corresponding values found on day 4 of the second lactation, either with or without a nonlactating period. Although the percentage carry-over on day 41 of the second lactation was lower than that found on day 4 of the second lactation, without a nonlactating period, the difference was not significant (Table II).

The rate of cell proliferation on day 3 of the second lactation was determined in rats with, and in rats without, a nonlactating period prior to the second parturition, since there was a significant difference in the percentage of alveolar cell carry-over; whereas total DNA was not significantly different. Labeled daughter cell index, total DNA, total RNA, and RNA/DNA ratio in rats without a nonlactating period were 6.5%, 26.79 mg, 41.12 mg, and 1.54, respectively; and in rats with a nonlactating period the corresponding values were 8.8%, 26.12 mg, 41.70 mg, and 1.60, respectively. The labeled daughter cell index of rats with a nonlactating period was significantly greater ($p < .01$) than that of rats without a nonlactating period; however, other mean comparisons were not significantly different one from another. Therefore, in lactating pregnant rats, a nonlactating period prior to the second parturition resulted in both a decreased alveolar cell carry-over from the first lactation and a greater proliferative activity after the second parturition.

Discussion. Tucker and Reece (8) found that the DNA content of mammary glands of lactating-pregnant (LP) rats decreased significantly between days 21 and 24 of lactation. This decrease in the DNA content of LP rats by day 24 of lactation is in agreement with the present observation that about one-quarter of the alveolar cells present on day 3 of the first lactation involute by day 4 of the second lactation.

When a 7- or 8-day nonlactating period was initiated by removing the litters on day 18 of lactation, a 56.0% alveolar cell carry-over was obtained on day 4 of the second lactation. Paape and Tucker (9) reported that the mammary gland DNA content of pregnant rats, whose litters were removed on day 16 of lactation, declined 28% between days 1 and 4 of the nonlactating period and 4% between day 4 and the end (day 8) of the nonlactating period. However, when the lit-

ters were removed on day 20 of lactation, there was a 33% decline during the 4-day nonlactating period. The 33% decrease in DNA during the nonlactating period is in good agreement with the decreased alveolar cell carry-over observed in this study.

It appears that the combined levels of placental lactogen and pituitary lactogen were sufficient to maintain the integrity of about 75% of the mammary gland parenchyma inasmuch as 73% of alveolar cells were carried-over from day 3 of the first lactation to day 4 of the second lactation. However, when the rats were given a 7- or 8-day nonlactating period, there was no suckling stimulus to initiate the release of pituitary lactogen to aid in maintaining mammary gland integrity. During the nonlactating period there was a greater involution of lactational cells, including labeled cells; this was evidenced by the 56% alveolar cell carry-over. It would appear that placental lactogen alone was not sufficient to maintain the integrity of alveolar cells at the 73% carry-over level observed in rats without a nonlactating period prior to the second parturition.

There was no significant difference in the RNA and DNA values on days 4 and 41 of the second lactation. This seeming lack of alveolar cell involution, when lactation was extended by use of replacement litters, is in agreement with the findings of Bruce (10), Nicoll and Meites (11), and Tucker and Reece (12).

Although the 62.1% carry-over of alveolar cells from day 3 of the first lactation to day 41 of the second lactation was lower than the 73% obtained on day 4 of the second lactation, without a nonlactating period, the two values were not significantly different. This indicated that alveolar cells formed during the first lactation did not involute faster during the second lactation compared with alveolar cells formed during the second lactation. Therefore, many alveolar cells appear to be functional in two consecutive lactations when rats are lactating and pregnant during the first lactation.

The greater loss of alveolar cells in LP rats with a nonlactating period prior to the second parturition than in similar rats without a nonlactating period is compensated for by a greater proliferation of alveolar cells following the second parturition.

Summary. Direct evidence is provided of alveolar cell carry-over from day 3 of the first lactation to days 4 and 41 of second lactation in rats which were pregnant during the first lactation. Rats with a nonlactating period prior to the second parturition had fewer alveolar cells carried-over to the second lactation but had a greater proliferation of alveolar cells following the second parturition than did rats without a nonlactating period.

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