Influence of Differentiation and Cell Kinetics on the Susceptibility of the Rat Mammary Gland to Carcinogenesis

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ABSTRACT

The susceptibility of the rat mammary gland to carcinogenesis decreases with aging and is nullified by pregnancy and lactation. This work was carried out to determine whether these differences in susceptibility to carcinogenesis are the result of variations in cell kinetics induced by both aging and parity. Cell cycle (Tc) and growth fraction (GF) were studied in the mammary gland epithelium of young virgin, old virgin, and parous Sprague-Dawley rats. For the study of Tc, five young virgin, five old virgin, and five parous rats received an i.p. injection of 1.7 μCi [3H]thymidine per g body weight. Mammary gland biopsies were taken at 2-hr intervals for the first 24 hr and every 4 hr thereafter up to 72 hr. GF was determined in two young virgin, two old virgin, and two parous rats in which an osmotic minipump releasing 1 μCi [3H]thymidine per hr was implanted i.p. After 5 days, the animals were killed, and the mammary glands were removed. Mammary gland biopsies and whole glands were fixed in Bouin’s fluid and embedded in paraffin. Deparaffinized sections were processed for autoradiography. The labeled mitosis wave, labeling index, and GF were determined. The results indicated that, in young virgin rats, mammary gland Tc was 9.9 hr in the most undifferentiated structures (terminal end buds), lengthening to 17.3 hr in terminal ducts (TD) and to 28.2 hr in alveolar buds (AB). In the mammary gland of old virgin rats, Tc was 18.75 hr in terminal ducts in regression, 20.57 hr in TD, and 30.75 hr in AB. In parous rats, mammary gland Tc was 23.92 hr in TD and 49.63 hr in AB. Lengthening of Tc in all of these structures was due to a lengthening of G1, while the duration of the other phases of the cell cycle remained unchanged. GF was 0.55 in terminal end buds of young virgin rats, decreasing to 0.39 and 0.13 in TD and AB, respectively. In terminal ducts in regression of old virgin rats, GF was 0.19, decreasing to 0.054 and 0.030 in TD and AB, respectively. In TD and AB of parous rat, mammary gland GF was 0.009 and 0.004, respectively. These results suggest that the high susceptibility to carcinogenesis that has been demonstrated in young virgin rats is due to the presence of a large proliferative compartment, mainly in terminal end buds and TD, while the low susceptibility of parous animals is due to the formation of a large compartment of nonproliferating cells, and that those cells still proliferating have a longer G1 than do those of young and old virgin rats.

INTRODUCTION

Breast cancer risk in women is associated with nulliparity or a late first full-term pregnancy (17, 19, 42, 43, 91), while early pregnancy seems to reduce the risk to a woman of developing breast carcinoma (17, 34, 42, 43, 88). The mechanism(s) by which an early pregnancy in humans prevents the development of breast cancer late in life has not yet been elucidated (11, 19, 34, 42, 43, 49, 88, 91). The use of adequate experimental models that mimic the human situation, such as the induction of rat mammary carcinomas by DMBA administration, has shown to be a useful tool in elucidating this problem (20, 29, 58, 77–79). The study of the pathogenesis of these carcinomas revealed that they arise from undifferentiated structures, TEB and TD, which are present in the mammary gland of virgin rats; on the other hand, benign lesions such as adenomas, fibroadenomas, hyperplastic alveolar nodules, and cysts originate from more differentiated structures, such as AB and alveoli (76, 77, 79). When the undifferentiated structures TEB and TD disappear from the mammary gland, as occurs after pregnancy and lactation, DMBA administration fails to induce carcinomas, and only a few benign lesions develop in carcinogen-administered parous animals (77). The protection induced by pregnancy extends after the physiological process is over, since parous rats in which the gland has completely regressed from its lactational or pregnant state are still refractory to develop carcinomas. The disappearance of TEB and the decreased number of TD in parous animals is accompanied by a significant reduction in the mitotic and labeling indices in the structures present in the gland (77, 78).

The role of the degree of differentiation of the mammary gland in the susceptibility to carcinogenesis is supported by both experimental and epidemiological evidence. For example, the highest incidence of DMBA-induced mammary carcinomas occurs when animals are inoculated at young ages, a time at which the number of TEB and TD and the level of DNA synthesis are at their highest (32, 82). It has been shown that in humans breast tissue seems to be more sensitive to ionizing radiations when exposure occurs between the ages of 15 and 19 years (10, 51, 84). This indicates that events occurring during the early years of a woman’s life have a significant effect on the undifferentiated or not completely differentiated breast tissue. It is also known that certain variables such as rate of DNA synthesis (2, 7, 15, 39, 57, 60), rate of cell proliferation (9, 35, 50, 59), length of the G1 and S phases of the cell cycle, and cellular competence in DNA repair (9, 54) influence cell and tissue susceptibility to carcinogenesis.

It is the objective of this paper to determine whether changes in cell kinetics in the various structures of the mammary gland are responsible for both increased susceptibility to carcinogen-

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esis, as found in virgin animals, and protection or refractoriness to carcinogenesis, as occurs in parasitized animals. In addition, we want to establish whether differences in cell kinetics among the various structures of the gland might account for the difference in site of origin of benign and malignant lesions of the mammary gland.

MATERIALS AND METHODS

Animals. Noninbred male and female Sprague-Dawley rats were purchased from Spartan Research Animals, Inc., Haslett, Mich. They were housed 4 animals/cage and received water and food ad libitum. The environment was controlled for temperature at 24 ± 1°C (S.D.) and for 12 hr of light and 12 hr of darkness. The rats were separated into 3 groups: young virgins, which consisted of 45- to 50-day-old virgin female rats; old virgins, which consisted of 180-day-old virgin female rats; and parous, which consisted of 180-day-old rats that underwent 2 pregnancies and lactations, as described previously (76, 77). Vaginal smears from each animal of the 3 groups were obtained daily for at least 1 week prior to the experiment in order to ensure regularity of the estrous cycle. The vaginal smears were stained by the method of Papanicolaou, and the phases of the estrous cycle were determined by conventional cytological criteria.

LMW. Five animals from each of the 3 groups were inoculated while in estrus with a single i.p. injection of 1.7 μCi [3H]dThd (specific activity, 25 mCi/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) per g body weight. Mammary gland biopsies were taken at 2-hr intervals during the first 24 hr and every 4 hr thereafter up to 72 hr postinjection.

Double Labeling. Two animals/group received an i.p. injection of 1.7 μCi [3H]dThd per g body weight when they were in estrus, followed 2 hr later by an i.p. injection of 2.5 μCi [14C]dThd (specific activity, 54 mCi/mmol; Amersham/Searle) per g body weight. The animals were killed 60 min after the last injection, and the mammary glands were removed, attached to the skin pelt, and fixed for light microscopy as described in Ref. 77.

GF. GF was determined in 2 rats/group in which an Alzet Model 1701 osmotic minipump that released 1 μCi [3H]dThd per hr was implanted i.p. when the animals were in estrus. Mammary gland biopsies were taken at 24, 64, and 96 hr, and the animals were killed at 120 hr postimplantation of the minipump (GFs).

Sample Collection. The mammary glands of those animals that were double-inoculated were rapidly excised and fixed in Bouin’s fluid for 10 hr, dehydrated, and embedded in paraffin. One whole gland was embedded in each paraffin block, and each gland was oriented flat so that the longitudinal sections of the mammary gland tree could correspond with the 2-dimensional development observed in whole-mount preparations. The blocks were semiserially sectioned at a thickness of 5 μm. Biopsies taken from the other groups were similarly fixed and processed for light microscopy.

Autoradiography. Deparaffinized 5-μm sections were covered with liquid photographic emulsion (Kodak NTB-2), which was applied to slides by the dipping method. The slides were stored at room temperature for 4 hr and stored for 2 to 4 weeks at 4°C in light-proof boxes containing dessicant. The slides were developed with Microdol X (Kodak) and processed in Kodak developer. Autoradiographs were stained with hematoxylin and eosin and dehydrated, and coverslips were mounted with Permount (Fisher Scientific Co., Pittsburgh, Pa.).

In double-label studies, double-emulsion autoradiography was used to distinguish cells labeled with either [3H]dThd, [14C]dThd, or both. The first emulsion was applied, exposed for 2 weeks, and then processed as described above. Prior to the application of the second emulsion, the slides were dipped in 5% nitrocellulose in amylacetate (Ernest Fullam Inc., Schenectady, N.Y.) in order to protect the staining during subsequent steps and to prevent the weaker β particles from penetrating into the second emulsion. The second emulsion was exposed for 3 weeks.

Microscopic Analysis. The [3H]dThd LI was determined by a count of the number of labeled nuclei and by the total number of cells that composed the epithelium of those TEB, TD, TDa, ducts, AB, and alveoli that could be clearly identified in either longitudinal or cross-sections. The histological description of these structures has been reported previously (76, 77, 82) (see also Figs. 3, 5, 6, 7, and 9 in Ref. 77). An average of 6 slides/gland/animal were counted. The [3H]dThd LI was calculated according to the following equation:

\[
LI = \frac{\text{Labeled cells}}{\text{Total cells}} \times 100
\]

The percentage of labeled mitosis was determined by counting the number of labeled and nonlabeled mitoses found in the epithelium of TEB, TD, TDa, ducts, AB, and alveoli. An average of 200 mitoses from each given structure per gland per animal was counted. Mitotic figures from late prophase until late anaphase were counted. In the count of labeled mitoses, those cells with a threshold of 5 grains/mitosis were included. The percentage of labeled mitosis curves was constructed by hand, and each point represents the mean ± S.D. of 5 animals. The results were evaluated by Student’s t test for assessing the significance of a difference.

GF was determined by a count of the number of labeled nuclei, the number of labeled and of nonlabeled mitoses, and the total number of cells (53) that composed the epithelium of the structures mentioned above. An average of 3000 cells/gland/animal were counted. GF was expressed by means of the following equation (52):

\[
GF = \frac{\text{Labeled cells/cells}}{\text{Labeled mitoses/mitoses}}
\]

or as GFs, that is, the total number of labeled nuclei per 100 cells after 5 days of continuous infusion of [3H]dThd. The mean and S.D. were calculated for each point, and the results were evaluated by Student’s t test for assessing the significance of a difference.

\[
T_s = \frac{1}{T_5}
\]
where $^{14}$C is the number of cells labeled with $[^{14}$C]dThd (second emulsion), $^{3}$H is the number of cells labeled only with $[^{3}$H]dThd (bottom emulsion), and $T_{5}$ is the interval in hr between the administration of $[^{3}$H]dThd and $[^{14}$C]dThd.

$T_{5}$ was also calculated from the curve of LMW by measuring the width of the first peak from the 50% level on the ascending portion of the wave to the corresponding point on the descending limb of the curve (Charts 1 to 3). $T_{5}$ was also calculated from the curve of LMW by measuring the interval between the time of injection of $[^{3}$H]dThd and the appearance of the first labeled mitosis. $T_{M}$ was obtained by measuring the time that it took for the percentage of labeled mitoses to go from 0 to its highest point in the first peak of the wave.

$T_{C}$ was calculated by measuring the distance between the center of the first and second peak of the LMW. When the second peak was not obtained because of dampening of the curve, $T_{C}$ was calculated using the following equation (4, 33):

$$T_{C} = \frac{T_{5} \times GF_{S}}{LI}$$  \hspace{1cm} (A)

$T_{A}$ was estimated by subtraction:

$$T_{A} = T_{C} - (T_{5} + T_{M} + T_{S})$$  \hspace{1cm} (B)

The population-doubling time ($T_{p}$) or uncorrected $T_{C}$ was calculated using the equation (4, 33, 87):

$$T_{p} = \frac{T_{5}}{LI}$$  \hspace{1cm} (C)

Equation C is valid only when all the cells are proliferating and uniformly distributed along the cycle, so that the frequency of cells in any one phase will be proportional to the time taken to pass through such a phase. Under these conditions, $T_{p}$ also expresses the turnover time, i.e., the time required for the production of a number of cells equal to that already present.

**RESULTS**

The structure of the mammary gland of young virgin, old virgin, and parous rats has been described in previous publications (76, 77, 79, 82).

In young virgin rats, the mammary gland was composed of numerous ducts ending in dilated, club-shaped TEB, AB, or TD (Chart 1) (77). In TEB, the percentage of mitoses that were labeled at various times after $[^{3}$H]dThd injection allowed us to draw by hand 4 well-defined peaks before a dampening of the curve occurred after 36 hr of the pulse label (Chart 2). The first wave reached a maximum of approximately 80%, and the second wave reached 40% of labeled mitoses. The second, third, and fourth waves of labeled mitosis in the TEB had the same height, and the area under these waves was smaller than that under the first wave. $T_{p}$ lasted 9.9 hr in the first cycle and was slightly shortened in the second and third cycles before lengthening again in the fourth and fifth cycles. This phenomenon has been described in other tissues as well (22, 40, 67). The smoothness of the curves obtained from the TEB indicated a certain degree of synchronism that was similar to that reported in the intestinal epithelium of irradiated mice (40). In TEB, the trailing edge of the first and of all subsequent waves dropped to less than 10% of labeled mitoses, which was in contrast with the progressive rise of the troughs commonly observed in most in vivo systems (22). This observation suggested either that these structures were composed of extremely synchronous cells or that each point, representing the mean ± S.D. of 5 samples/point with very small interanimal variations, gave more accurate values (Chart 2).

In TD, a first peak with 90% of labeled mitoses and a second peak with 65% of labeled mitoses were observed with a dampening of the curve 30 hr post-pulse label. $T_{C}$ obtained from the curve for the percentage of labeled mitoses, was 17.3 hr (Chart 2; Table 1). In AB, only one peak with 70% of the labeled mitoses was observed, and a second peak did not appear, even when samples were obtained up to 72 hr after pulse labeling. It was necessary, therefore, to calculate $T_{C}$ using Equation A; $T_{C}$ thus estimated was 28.18 hr (Charts 1 and 2; Table 1). Calculations of $T_{C}$ in TEB and TD using the technique of the percentage of labeled mitoses resulted in values which were very similar to those obtained from Equation A (Table 2). When Equation A was used, it was assumed that the cell population was in a steady state, in which cell loss occurred randomly with respect to the phases of the cell cycle. In order to be able to use Equation A, it was required to know the growth fraction or size of the proliferative pool. The similarity of results obtained by both methods (Tables 1 and 2)
Length of $T_e$ in the various structures present in the mammary gland of young virgin, old virgin, and parous rats corrected and uncorrected for $G_0$.

<table>
<thead>
<tr>
<th>Rats</th>
<th>Structure</th>
<th>$T_{G1}^a$</th>
<th>$GF_b$</th>
<th>$T_e^c$</th>
<th>DNA Li$^d$</th>
<th>$T_e^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young virgin</td>
<td>TEB</td>
<td>7.20</td>
<td>0.55</td>
<td>11.65</td>
<td>0.34</td>
<td>21.18</td>
</tr>
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<td></td>
<td>TD</td>
<td>7.47</td>
<td>0.39</td>
<td>20.81</td>
<td>0.14</td>
<td>53.36</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>8.67</td>
<td>0.13</td>
<td>28.18</td>
<td>0.042</td>
<td>16.75</td>
</tr>
<tr>
<td>Old virgin</td>
<td>TDR</td>
<td>7.60</td>
<td>0.19</td>
<td>18.75</td>
<td>0.077</td>
<td>98.70</td>
</tr>
<tr>
<td></td>
<td>TD and ducts</td>
<td>7.60</td>
<td>0.054</td>
<td>20.57</td>
<td>0.020</td>
<td>383.00</td>
</tr>
<tr>
<td>Parous</td>
<td>TD and ducts</td>
<td>7.40</td>
<td>0.0097</td>
<td>23.92</td>
<td>0.003</td>
<td>2,466.66</td>
</tr>
<tr>
<td></td>
<td>AB and alveoli</td>
<td>8.20</td>
<td>0.030</td>
<td>30.75</td>
<td>0.0081</td>
<td>1,025.00</td>
</tr>
</tbody>
</table>

Constituent parts of the cell cycle in the terminal structures present in the mammary gland of young virgin, old virgin, and parous rats.

<table>
<thead>
<tr>
<th>Phase length (hr)</th>
<th>Rats</th>
<th>Structure</th>
<th>$T_{G1}^a$</th>
<th>$T_e^c$</th>
<th>$T_m^b$</th>
<th>$T_a^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young virgin</td>
<td>TEB</td>
<td>1.08 ± 0.11$^d$</td>
<td>7.20 ± 0.80</td>
<td>1.20 ± 0.20</td>
<td>0.72 ± 0.42</td>
<td>9.93 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>TD</td>
<td>1.00 ± 0.01</td>
<td>7.47 ± 0.83</td>
<td>1.40 ± 0.40</td>
<td>7.47 ± 0.12</td>
<td>17.33 ± 0.42</td>
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<tr>
<td></td>
<td>AB</td>
<td>1.00 ± 0.01</td>
<td>8.67 ± 0.61</td>
<td>1.20 ± 0.20</td>
<td>17.31 ± 1.14</td>
<td>28.18 ± 1.98$^e$</td>
</tr>
<tr>
<td>Old virgin</td>
<td>TDR</td>
<td>4.40 ± 0.40</td>
<td>7.60 ± 0.40</td>
<td>1.60 ± 0.19</td>
<td>5.15 ± 0.99</td>
<td>18.75 ± 0.99$^f$</td>
</tr>
<tr>
<td></td>
<td>TD and ducts</td>
<td>4.40 ± 0.40</td>
<td>7.60 ± 0.40</td>
<td>1.40 ± 0.20</td>
<td>7.12 ± 0.08</td>
<td>20.57 ± 1.08$^f$</td>
</tr>
<tr>
<td></td>
<td>AB and alveoli</td>
<td>3.60 ± 0.40</td>
<td>8.20 ± 0.72</td>
<td>1.73 ± 0.12</td>
<td>17.25 ± 1.41</td>
<td>30.75 ± 2.70$^f$</td>
</tr>
<tr>
<td>Parous</td>
<td>TD and ducts</td>
<td>1.05 ± 0.05</td>
<td>7.40 ± 0.20</td>
<td>1.40 ± 0.20</td>
<td>14.07 ± 0.19</td>
<td>23.92 ± 0.64$^f$</td>
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<tr>
<td></td>
<td>AB and alveoli</td>
<td>1.05 ± 0.05</td>
<td>10.13 ± 1.40</td>
<td>2.80 ± 0.20</td>
<td>35.65 ± 5.20</td>
<td>49.63 ± 6.86$^d$</td>
</tr>
</tbody>
</table>

$^a$ The constituent parts of the cell cycle have been obtained from the curve of labeled mitoses.
$^b$ Calculated by using the equation: $T_m = T_e - (T_a + T_{G1} + T_s)$.
$^c$ Mean ± S.D.
$^d$ Calculated in these structures using Equation A.
$^e$ Calculated by using Equation A.
$^f$ Calculated by counting the number of labeled cells after 1 hr pulse of $[^3H]^dThd$ and expressed as labeled cells/100 cells.

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The mammary gland of old virgin rats contained a very small number of TEB and, although these structures were slightly dilated, club-shaped terminal structures very similar to those TEB present in the mammary gland of young virgin rats, they were smaller and contained fewer cells per section; therefore, they were considered to be TD in a state of regression from TEB and were called TDR (Chart 3) (see also Figs. 5 and 6 in Ref. 77). In TDR, a first peak with 60% and a second peak with 17% of labeled mitoses were observed. $T_e$ obtained from the curve of labeled mitoses was 21.6 hr (Chart 4); $T_e$ calculated using Equation A was 18.75 ± 0.99 hr (Table 1). In TD, only the first wave of labeled mitoses was observed; therefore, the length of the cell cycle was calculated using Equation A, with a result of approximately 21 hr (Table 1; Charts 3 and 4). In AB and alveoli, only one wave of labeled mitoses was observed. Calculation of the cell cycle using Equation A resulted in a length of 30.75 hr, which was markedly prolonged with respect

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Chart 3. Schematic representation of terminal structures in the mammary gland of old virgin rats. The diagram of the cell cycle shows the relative length of the various phases of the cycle for each structure.

to its length in TDm and TD present in the same gland (Table 1; Charts 3 and 4).

Parous rat mammary glands after 40 to 42 days of postlactational involution resembled those of virgin animals, although some quantitative and qualitative differences were observed. TEB and TDm were completely absent, and TD were markedly reduced in number in parous rats with respect to young and old virgin rats (Chart 5) (77). The number of AB was about the same as that observed in old virgins, and lobules were 3 to 4 times more numerous in parous rats than in young or old virgin rats (77). In TD and AB, a well-defined first wave of labeled mitoses was observed (Chart 6). Calculation of the cell cycle using Equation A resulted in a length of 23.92 hr for TD and 49.63 hr for AB and alveoli (Charts 5 and 6).

Constituent Parts of the Cell Cycle of the Various Structures of the Mammary Gland. From the curves of labeled mitoses, it was possible to determine the length of the different constituents of the cell cycle. As shown in Table 1, while the length of G1 varied from approximately 1 hr (0.72 ± 0.42 hr) in the TEB to 17 hr (17.31 ± 1.14 hr) in the AB of young virgin rat mammary gland, no significant variations in the length of G2, which lasted approximately 1 hr in all the structures, were observed.

In the mammary gland of old virgin rats, the length of G1 in TDm was about 5 hr (5.15 ± 0.99 hr), a value significantly longer than that observed in TEB and shorter than that in TD of young virgin rats (Table 1). In TD, the length of G1 was 7.12 ± 0.08 hr and in AB and alveoli 17.25 ± 1.41 hr, values that were very similar to those of young virgin rats. Although G2 varied in length less than G1, certain differences were nevertheless observed. G2 in TDm, TD, and AB of old virgin rats was 3 to 4 times longer than that of young virgin and parous rats (Table 1).

In parous rat mammary gland, the major differences in the constituents of the cell cycle were observed at the level of G1, which was prolonged to 14.07 hr in TD and to about 36 hr in AB (Table 1). G2 was identical in TD and AB and had values similar to those observed in young virgin rats (Table 1).

The length of the period of DNA synthesis (Ts), calculated by measuring the width of the first peak from the 50% level on the ascending limb of the wave to the corresponding point on the descending curve or by means of the double-labeling technique, demonstrated the constancy of Ts not only for the different structures within a single gland but also in animals of different age or parity. Table 3 shows that Ts values determined by the double-labeling technique were slightly, but not significantly, shorter than Ts values calculated from the LMW (Table 3, Sections A and C). In order to test whether Ts values varied between similar structures present in a single mammary gland, the individual values of Ts for each structure in each group of glands were determined (Table 3, Section B).

GF. GF, which indicates the size of the proliferative compartment, was calculated by labeling the cells with a continuous infusion of [3H]dThd for 5 days (GF5). In most of the structures, labeling reached its maximum on the fourth day after pump

Chart 4. Percentage of labeled mitoses as a function of time after flash labeling with [3H]dThd in TDB, TD and ducts, and AB and alveoli in the mammary gland of old virgin rats. Points, mean of 5 samples. Bars, S.D.
implantation (Chart 7). The size of the proliferative compartment differed significantly among the various structures present in a single gland and between similar structures whether they were in the mammary gland of young virgin, old virgin, or parous rats (Table 4). The largest proliferative compartment was found in TEB of young virgin rats which had a GF of 0.55 against 0.39 in TD and 0.13 in those AB present in the same gland. The differences between these compartments were significant (p < 0.01).

In the involuted postlactational mammary gland of parous rats, a drastic decrease in GF was observed. GF was 0.0097 in TD and 0.0049 in AB; that was a 40- and 27-fold decrease with respect to the GF of TEB and AB of young virgin rats (Table 4). Aging in itself resulted in a reduction of GF, although of a lesser magnitude than that induced by parity, since old virgin rats that were age-matched with parous rats had a GF of 0.19 in TD, 0.05 in TD, and 0.03 in AB, which represented a 3-, 7.2-, and 4.6-fold decrease, respectively, when compared with the value of GF in TEB, TD, and AB of young virgin rats (Table 4). The differences in GF between young virgin and old virgin and between old virgin and parous mammary glands were statistically significant (p < 0.01).

Even though a significant proportion of mitoses were labeled in the TEB, TD, and AB of young virgin and in the TD of old virgin rat mammary glands after 5 days of continuous [3H]dThd infusion (Chart 7, A and B), no labeled mitoses were observed in TD and AB of old virgin and parous rat mammary glands (Chart 7, B and C). These broad differences between the percentage of labeled mitoses and of labeled cells in TD and AB of old virgin and parous rats indicated that a significant fraction of nonproliferating cells existed in those structures. When GF of TEB, TD, and AB of young virgin and of TD, TD, and AB of old virgin rats was calculated using the equation of Mendelsohn (52, 53), the values obtained were similar to those obtained from the percentage of labeled cells for GF (Table 4). The values of GF in TD and AB of old virgin and parous rats thus obtained were used in Equation A for the determination of Tc.

Values increased in inverse proportion to the size of GF (Table 2). The difference between the values of Tc and Tc indicated that the cells were not growing in an exponential fashion.

**DISCUSSION**

The results presented above indicate that in the mammary gland of young virgin rats the various structures that developed as a consequence of progressive differentiation of the gland, namely TEB, TD, and AB, have markedly different GF, which is highest in the least differentiated structure, the TEB, and decreases progressively in the more differentiated structures, TD and AB. Differentiation results also in a progressive lengthening of Tc, which is mainly due to a lengthening of G1. The use of autoradiographic methods for the study of the cell cycle in vivo allowed us to determine the presence of 4 waves of labeled mitoses in TEB. Although there are few reports describing so
Student's t tests were done on all possible comparisons. Ts values that were determined by double labeling did not differ significantly from those obtained by the LMW technique, except in ID and AB of parous rats in which p < 0.05.

### Table 3

<table>
<thead>
<tr>
<th>Rats</th>
<th>Structure</th>
<th>$^{3}$H</th>
<th>$^{14}$C</th>
<th>$^{14}$C/$^{3}$H</th>
<th>$T_s^{a}$ (hr)</th>
<th>$T_s^{c}$</th>
<th>LMW $T_s^{d}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young virgin</td>
<td>TEB</td>
<td>3430</td>
<td>5240</td>
<td>5590</td>
<td>3.16</td>
<td>6.32</td>
<td>5.17 ± 3.60</td>
</tr>
<tr>
<td></td>
<td>TD</td>
<td>640</td>
<td>910</td>
<td>920</td>
<td>2.86</td>
<td>5.72</td>
<td>5.54 ± 1.64</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>690</td>
<td>710</td>
<td>1850</td>
<td>3.71</td>
<td>7.42</td>
<td>5.37 ± 1.28</td>
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<tr>
<td>Old virgin</td>
<td>TEB, TD, and ducts</td>
<td>1200</td>
<td>1580</td>
<td>2780</td>
<td>3.63</td>
<td>7.27</td>
<td>6.23 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>AB and alveoli</td>
<td>1280</td>
<td>1670</td>
<td>2950</td>
<td>3.61</td>
<td>7.22</td>
<td>5.51 ± 3.15</td>
</tr>
<tr>
<td>Parous</td>
<td>TD and ducts</td>
<td>220</td>
<td>330</td>
<td>310</td>
<td>2.91</td>
<td>5.82</td>
<td>4.69 ± 2.83</td>
</tr>
<tr>
<td></td>
<td>AB and alveoli</td>
<td>390</td>
<td>920</td>
<td>550</td>
<td>3.76</td>
<td>7.54</td>
<td>6.77 ± 2.94</td>
</tr>
</tbody>
</table>

- **A** Ratio of the number of cells labeled with $^{14}$C to the number of cells labeled with $^{3}$H.
- **B** Obtained by double-labeling technique, averaging the values in all TEB, TD, ducts, AB, and alveoli present in the mammary gland of 5 animals/group.
- **C** Obtained by double-labeling technique, counting the number of cells labeled with both isotopes in 100 cells each of TEB, TD, ducts, AB, and alveoli present in the mammary gland of 5 animals/group.
- **D** Obtained from the curve of labeled mitoses.

$^{a}$ Mean ± S.D.

With aging, TEB become transformed into TDB, which have a smaller GF than do TEB, but still a larger GF than do more differentiated structures within the same gland. In TD and AB, a lengthening of $T_s$ similar to that observed in young virgins is observed in the old virgin rats.

With pregnancy and lactation, the mammary gland becomes completely differentiated (77). In the postlactational period, the mammary gland regresses to a different morphological level than does that of virgin rats of the same age (77). These morphological differences are accompanied by changes at the level of the GF and $T_s$ that are more markedly pronounced than are those induced by aging. In TD of parous rat mammary gland, GF$_{5}$ is 40-fold smaller than in similar structures in young virgin rat and 6-fold smaller than in old virgin rats. These results indicate that pregnancy is a mechanism more efficient than aging in reducing the proliferating compartment to a minimal level.

These observations suggest that the susceptibility of virgin rats (76, 79, 82) and the refractoriness of parous rats (78) to carcinogenesis are due to topographic differences in cell ki-

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J. Russo and L. Tait, unpublished observations.
nomic parameters and in the size of the proliferative compartment, both of which are controlled by gland differentiation.

Dividing cells have a higher oncogenic response than do nondividing cells (6, 25). The fact that TEB have the largest proliferative compartment, with the shortest $T_c$ and $G_1$, explains the selectivity by which mammary gland carcinomas arise from these structures (76, 79). The interaction of carcinogens with DNA and its significance have been discussed (55, 56, 83). It has been shown that rapidly proliferating liver tissue of newborn or of partially hepatectomized mice and rats is susceptible to OMBA bound to the DNA isolated from either immature or partially intact liver of the adult is resistant (37, 45, 46, 64). OMBA is nondividing cells (6, 25). The fact that TEB have the largest

<table>
<thead>
<tr>
<th>Rats</th>
<th>Structure</th>
<th>Total no. of cells</th>
<th>No. of labeled cells</th>
<th>GF</th>
<th>% of labeled mitoses</th>
<th>GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young virgin</td>
<td>TEB</td>
<td>4,276</td>
<td>2,383</td>
<td>0.55</td>
<td>87.35</td>
<td>0.62</td>
</tr>
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<td></td>
<td>TD</td>
<td>3,798</td>
<td>1,464</td>
<td>0.39</td>
<td>85.00</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>2,352</td>
<td>306</td>
<td>0.13</td>
<td>66.66</td>
<td>0.19</td>
</tr>
<tr>
<td>Old virgin</td>
<td>TDs</td>
<td>797</td>
<td>151</td>
<td>0.190</td>
<td>83.30</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>TD and ducts</td>
<td>8,610</td>
<td>465</td>
<td>0.054</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>AB and alveoli</td>
<td>6,550</td>
<td>198</td>
<td>0.030</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Parous</td>
<td>TD and ducts</td>
<td>3,314</td>
<td>32</td>
<td>0.0097</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>AB and alveoli</td>
<td>10,630</td>
<td>52</td>
<td>0.0049</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Liver*</td>
<td>Intestine*</td>
<td>12,500</td>
<td>361</td>
<td>0.032</td>
<td>52.68</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,422</td>
<td>3,034</td>
<td>0.883</td>
<td>95.00</td>
<td>0.92</td>
</tr>
</tbody>
</table>

a Total number of labeled cells/100 cells after 5 days of continuous infusion of $[3H]$dThd.

b After 5 days of continuous infusion of $[3H]$dThd.

c Determined by a count of the fraction of labeled cells and labeled mitosis after 5 days of continuous $[3H]$dThd infusion and applying the equation of Mendelsohn (52):

$$GF = \frac{Labeled\ cells/cells}{Labeled\ mitoses/mitoses}$$

d ND, not detectable.

e GF$_s$ and GF of liver and small intestine did not differ significantly in the 3 groups of animals studied; therefore, the values obtained for each group were pooled.

Table 4: Comparison of the GF of mammary gland terminal structures with that of liver and intestine

It is known that variations in cell cycle length among different cell types are due mainly to variations in the length of $G_1$, with the durations of the $S$ phase (6 to 8 hr) and the $M$ phase (1 hr) being relatively constant (61). However, it has been reported that steroid hormones, known to be involved in the process of differentiation of the mammary gland (8, 18, 30, 66, 68, 69, 85, 86), when administered to ovariectomized mice, reduce the length of the $S$ phase from 20.1 to 10.7 hr (13, 14), which is in the same range of values reported here for virgin and parous rats. Other authors have measured the length of the $S$ phase in the mouse mammary gland and have found variations to be between 10 and 14 hr, according to the physiological state of the host (3). The discrepancy between the values of $T_s$ that we obtained in virgin rats and those obtained in virgin mice (20 hr) and in rat mammary gland in vitro (17.6 hr) (38) can be explained as methodological. Our results were obtained in vivo from double labeling and LMW by counting cells in well-defined structures in paraffin sections of mammary glands, while other authors have used squash preparations of the mammary gland (13, 14) or they have determined $T_s$ in in vitro conditions (38). In the rat mammary gland, the lengthening of $G_1$ indicates polycyclic hydrocarbons in vitro require that at least one cell division take place for transformation and at least 3 additional cell divisions take place for the expression of the transformed state (35). whereas cells in TEB divide 2.4 times in 24 hr, the cells in TD, which have a lower proliferative compartment and longer $T_c$, have the chance to divide only 1.4 times, and in AB, with a $T_c$ of 28 hr, only two-thirds of the cycle has taken place in the same time period. Therefore, we postulate that TEB could be transformed within the first 24 hr of carcinogen administration, while TD would have a much lower oncogenic potential, and that AB may not undergo malignant transformation, although they are able to develop benign lesions such as adenomas, fibroadenomas, hyperplastic alveolar nodules, and cysts (79).
that certain cells are arrested for a long period of time in this phase of the cycle. On the other hand, the decrease of the growth fraction from 0.55 in the TEB to 0.13 in the AB of young virgin rats indicates that most of the cells have entered in a nonproliferating compartment or G0 during the process of differentiation from TEB to AB (79). Pregnancy and lactation induce the same changes, although more pronounced, on the size of the proliferative compartment and length of Tc. However, it is debatable whether the cells leave the cycle entering G0 or a quiescent state during which they do not divide for long periods or whether they are arrested in a prolonged G1 phase (Chart 8) (5, 21, 24, 47, 61, 65, 73, 74). A broad difference between the percentage of labeled mitoses and the percentage of labeled cells in TD and AB is observed in old virgin and parous rat mammary glands. The lack of labeled mitoses indicates that the interval between the end of DNA synthesis and the beginning of a visible mitosis is lengthened by permanence of the cells in G2; this could be explained in the old virgin rat, in which a lengthening of G2 was observed, since the presence of a quiescent state in G2 has been suggested (26). Another possibility is that the labeled cells traverse G2 and mitosis so rapidly that they are no longer detectable as labeled mitoses and are arrested finally in G1 or enter G0. If the cells are treated at this point in vivo with a carcinogen, they are refractory to carcinoma development (77, 78), and in vitro they are less susceptible to the effect of DMBA (80), which explains the refractoriness of the mammary gland to DMBA-induced carcinogenesis in parous rats (77, 78).

In primary cultures of the mammary gland of parous rats, the cells start growing after a lag phase longer than that of young and old virgin rats. They also have a lower number of doublings before the cultures enter confluence (81). These differences indicate that the cells of parous rats are more limited in the proliferative capacity, which may be due to a more committed function or differentiation. Similar results have been reported for the mammary gland of pregnant mice (89). In tissue culture, various conditions shift the cells into G0 (28, 36, 48, 62, 63, 72). The physiological mechanisms that could trigger the shift of cells into a quiescent state or into G0 in vivo are not known. Both pregnancy and lactation could, as it is demonstrated here, be the first known physiological conditions for inducing in vivo the entrance of mammary gland epithelial cells into a quiescent state (Chart 8). In other organs, such as the liver, most of the cells are in G0 (Table 4). It is known that cells that are in G0 in the liver can be rescued if a partial hepatectomy leading to regeneration of the remnant of the organ is done (61). We do not know if the cells that are in G0 in the parous rat mammary gland can be rescued by a physiological stimulus such as another pregnancy or if the proliferation thus induced occurs at the expense of the cells that are proliferating with a lengthened Tc. It has been postulated that the crucial control event for the regulation of growth seems to reside in G1. Evidence has accumulated for the existence of a restriction or commitment point in mid- to late G1, at which time a cell decides whether to initiate DNA synthesis and undergo division or to cease proliferation (1, 28, 36, 48, 61–63, 72). We postulate that pregnancy modifies the G1 of the proliferating cells in such a way that carcinogenic initiation and promotion cannot break the restriction point for manifesting transformation. Besides, the cells that are cycling with a slow pace in the mammary gland of parous rats, having a G1 longer than that of young and old virgin rats, could have enough time to repair any damage inflicted by the carcinogen before it is fixed in the genome (2, 9, 15, 16, 35, 39, 48, 50, 54, 57, 59, 60). It has been demonstrated in tissue culture that cells in early or mid-G1 phase are capable of repairing prelethal radiation-induced damage (41), whereas the damage becomes fixed in S phase (54). It has also been demonstrated that most of the DNA repair that is going to occur in any given cell takes place within 24 hr (23, 70). A lack of repair of the DNA damaged by the carcinogen occurs when the cells either lack sufficient time to complete the repair, such as in observed in rapidly cycling cells (16), e.g., TEB epithelium, or have low levels of DNA repair enzymes (71). In the case of the mammary gland of virgin rats, we have evidence that repair takes place in vivo, 5 which makes us reject the possibility of a deficit in repair enzymes.

In conclusion, the partial protection that aging and the almost total refractoriness to carcinogenesis that parity induce in the mammary gland are due to differentiation of the gland, with the consequent enlargement of the nonproliferating compartment and lengthening of Tc at the expense of G1. Therefore, it is suggested that the prophylaxis of breast cancer must be oriented toward those mechanisms that physiologically induce a shifting of the proliferating cells to a quiescent state or, conversely, toward reducing potentially carcinogenic exposures to glands which are in their more proliferative phase.

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Differentiation, Cell Kinetics, and Carcinogenesis


Influence of Differentiation and Cell Kinetics on the Susceptibility of the Rat Mammary Gland to Carcinogenesis

Jose Russo and Irma H. Russo


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