A New Concept of Tissue and Tumor Cell Proliferation

Seymour Gelfant

Departments of Dermatology and Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902

Summary

We present a model for cell and tissue proliferation based upon the idea that cycling cells can arrest at three points in the cell cycle: in early G1 (blocked by a G0 barrier); in late G1 (by a G1 block); and in late G2 (by a G2 block). There are four major categories of cells: cycling cells; noncycling G1-blocked cells; noncycling G2-blocked cells; and noncycling G0 blocked cells. These represent the potential proliferating pool in cells of the same type in culture and in tissues and tumors in vivo. The model also includes the possibility of additional subpopulations. The ideas are supported by examples and evidence taken from a wide variety of animal, plant, and tumor tissues in vivo and in vitro. Within this context, we critically review most of the current concepts and schemes of cycling and noncycling cells. We also present a scheme describing the origin and recruitment of all four categories of cycling and noncycling cells, which, with the support of ideas from the literature, leads to new insights regarding cell transformation and tumor growth and to a new tumor kinetic response model.

On a speculative note, we introduce the idea of tissues and tumors as proliferate ecosystems, where the various categories of cycling and noncycling cells (including pre-diversified subpopulations) increase survival value and also serve as a complex adaptive system to fulfill the particular proliferative needs of the tissue or tumor.

Introduction

The concept and the terminology of cycling cells actively moving through G1 → S → G2 → M (where G1 and G2 are gaps, S is the period of nuclear DNA synthesis, and M is the period of mitosis) is clear. For up-to-date and original descriptions of the properties of the G1, S, G2, and M periods and for insights regarding the biochemical and the molecular events that underlie progression through the cell cycle, see 2 outstanding recent publications: a review by Prescott (95) entitled, "The Cell Cycle and the Control of Cellular Reproduction," and a monograph by Baserga (8), "Multiplication and Division in Mammalian Cells."

In contrast, concepts and the terminology of noncycling cells and their relationship to cycling cells are confusing and incomplete. The idea that cells can move out of the cycle or become arrested for extended periods of time but still retain the capability of reactivation was introduced primarily by Lajtha (69) and also by myself (49) in 1963. On the basis of observations on the time pattern of DNA synthesis and mitosis in (rat) liver after partial hepatectomy, Lajtha introduced the concept of "G0," where cells move out of the cycle from the G0 period; where they can remain indefinitely in a resting state (as far as cell division is concerned); and where, upon being recalled by an appropriate stimulus, they exhibit a characteristic delay (in this case about 15 hr) before entering the S period. On the basis of my studies on mouse epidermis, I introduced the term "G1 period cells" to describe a small proportion of epidermal cells that held up or arrested in the G1 period [later shown for an indefinite length of time (89)] and that could enter mitosis immediately, in response to an appropriate stimulus. Also, I developed the concept of "G1 and G2 cell cycle blocks" to depict the point of arrest and to explain the behavior of G2 population cells. In 1969, Epifanova and Terskikh (43) introduced the idea of resting periods out of the cell cycle and used the terminology "R1" and "R2" to distinguish between G1 and G2 population cells. Since then (but not because of that theory), the idea of G1- arrested or G2-blocked cells has been mainly ignored and attention has been focused on G0 models (20). This is understandable because a much greater proportion of cells move out of and back into the cell cycle from the G1 period also; it is more intriguing to look for and discover "some master initiator" (109) that controls and regulates genome replication and cell proliferation from the G1 or the G0 period. (However, there is also the possibility that the cycling behavior of a cell in G1 has already been decided or preprogrammed during the previous S or G2 period of the parent cell. Perhaps this is where we may eventually locate the control mechanisms for cell division.)

Recently, and, for the most part, because of the behavior of normal and transformed cells in culture, G1 and G0 periods have been subdivided and analyzed by a variety of schemes. Temin (116) has subdivided G1 into G1a,b,c. "Gb" is comparable to out-of-cycle G0; it is located in mid-G1 so that there is a delay in entering S upon reentry from G1b; "Gia" is located in late G1, and it represents a stage at which cells are committed to, but have not yet started, DNA synthesis; "Gia" is a phase in early G1 from which cells can move into G1b or G1c, depending upon the degree of stimulation and upon whether cells are normal or transformed. Smith and Martin (108) have subdivided the cell cycle into an "A state" and a "B phase." The B phase includes the conventional S, G2, and M periods and also part of G1; the A state, in one respect, is similar to G0 because it is located in G1 and in this state a cell is not progressing towards division. However, the A state differs from the G0 concept because it implies that cells have a constant probability ("transition probability") of leaving the...
A state and entering the B phase. Using an earlier model of Burns and Tannock (21), DeMaertelaer and Galand (41) have subdivided the cell cycle into a "G phase" and "C phase." These phases are essentially the same as the A state and B phase of Smith and Martin. Pardee (86) has introduced the concept of a unique fixed restriction point "R" located in mid-G1, a critical switching point at which cells shift back and forth from cycling to noncycling states. Pardee implies that malignant cells have lost their R-point control. Martin and Stein (81) propose that the restriction point "R" is not located in mid-G1, but rather at the G1-S boundary, immediately preceding the initiation of DNA synthesis. Radley et al. (97) have introduced the designation "G0 time" to refer to the gap in time between initiation and entry into S in stimulated G0 cells. Baserga (8) does much more than this in his "prereplicative phase" concept. In a purposeful analysis, Baserga and coworkers have mapped the sequence of biochemical events occurring from the point of initiation to the onset of DNA synthesis in a variety of stimulated G0 cells, both in vivo and in vitro. Augenlicht and Baserga (2) introduce the idea of "deep-deeper G0 states," in which the longer cells remain quiescent, the deeper they go into G0, and the longer the prereplicative phase lasts (i.e., the delay before entering S after stimulation). Finally, Prescott (95) advocates the idea of "prolonged arrest" within the cell cycle in the G1 and in the G2 periods, a point of view that hardly acknowledges the concept of G0.

With, as a basis, our earlier concept of G1 and G2 cell cycle blocks and introducing the idea of a "G0 barrier" to account for the behavior of G0 cells, this report presents a new framework to view cycling and noncycling cells. Our model describes 4 major categories of cells: cycling cells, noncycling G1-blocked cells; noncycling G2-blocked cells; and noncycling G0 blocked cells. These represent the potential proliferating pool in cells in culture and in tissues and tumors in vivo. The model also includes the possibility of additional subpopulations. Within this context we review most of the concepts and schemes of cycling and noncycling cells mentioned. We also present a scheme describing the origin and recruitment of noncycling cells which, with the support of ideas from the literature, leads to new insights regarding cell transformation and tumor growth and to a new tumor kinetic response model.

### Concept of Cycling and Noncycling Cells

The idea of G1 and G2 blocks (Chart 1) was introduced by Gelfant (49) in 1963 to explain the existence of a unique population of mouse epidermal cells that were arrested or blocked in the G2 period and that could be stimulated to enter mitosis by wounding or trauma (referred to then as "G2 population cells"). The original model visualized and provided evidence for 2 inherent blocks in the cell cycle: a G1 block between the G1 and the S periods; and a G2 block between G2 and the period of mitosis. That cells arrest in G1 and in G2 at positions located at the G1—S and G2—M transition points rather than in S or in M (73, 120). Further evidence for the locations of the G1 and G2 cell cycle blocks (and the G0 barrier) can be drawn from...
Cycling and Noncycling Cells

What is being proposed in Chart 1 is that the potential proliferating pool in cells in culture and in tissues in vivo is composed of 4 major categories of cells, each having different patterns of cell division as seen in relation to the $G_1$ and the $G_2$ blocks and also in relation to the newly described $G_0$ barrier depicted in Chart 1. Cycling cells are sequentially, asynchronously moving through the cell cycle. The $G_1$ and the $G_2$ blocks in these cells are partially open, implying various physiological states of expansion or retraction. Diurnal variations in labeling indexes and in mitotic indexes (reflecting the number of cells moving from $G_1$ into $S$ and from $G_2$ into $M$) would be an example of different states of expansion of the $G_1$ and the $G_2$ cell cycle blocks in normal, unstimulated tissues in vivo.

Cycling cells can be identified in a number of ways. (a) DNA content profiles obtained by Feulgen cytophotometry or by flow cytofluorometry reveal 3 classes: cells in $G_1$ with nuclear DNA contents of 2C (open circles); cells in $G_2$ with 4C DNA contents (filled circles); and, most importantly, cells with intermediate DNA contents between 2C and 4C representing cells in the process of DNA synthesis (i.e., moving through the S period as shown in the diagram). (b) Cycling cells can be identified in $S$, where they incorporate $[^3H]thymidine$ and can be observed as labeled nuclei in autoradiographs or detected by scintillation counting. (c) Cycling cells can be seen in mitosis (depicted as rounded cells in anaphase in Chart 1).

There are 3 categories of noncycling cells arrested at different points in relation to the $G_1$ and the $G_2$ cell cycle blocks and the $G_0$ barrier. $G_1$-blocked cells arrest late in the $G_1$ period and are located at the $G_1$ block (nuclear DNA contents, 2C). $G_2$-blocked cells are arrested late in the $G_2$ period and are located at the $G_2$ cell cycle block (nuclear DNA contents, 4C). The 3rd category, noncycling $G_0$-blocked cells, is arrested early in $G_1$ by a $G_0$ barrier (Chart 1, dashed lines). These cells have 2C nuclear DNA contents, and they are located at a distance in time from the $G_1$ cell cycle block. For conceptual uniformity, the $G_1$ block is depicted as being closed for $G_0$-blocked cells.

Chart 1 also provides an estimate of the relative proportions and fluctuations of the 4 categories of cells. Most tissues and cell systems are predominantly composed of cycling cells and noncycling $G_0$-blocked cells with relative proportions to one another that depend upon the particular proliferative state of the tissue. Most tissues and cell systems also contain small proportions of both $G_1$- and $G_2$-blocked noncycling cells (although the estimates shown range from 2 to 10%, they may reach as high as 30% during certain stages of tumor growth (92)). The concept in Chart 1 implies that there may be additional subpopulations of cells within the major categories of noncycling cells; these are depicted as triangles, circles, and hexagonal cells. Additional information regarding the dynamic aspects of cycling and noncycling cells and evidence for subpopulations will be provided in other sections of this review.

Procedures for Demonstrating and Distinguishing Cycling and Noncycling Cells

In general, noncycling cells can be demonstrated and distinguished from one another only by experimentally opening the $G_0$ barrier and the $G_1$ and the $G_2$ cell cycle blocks in the same tissue by some form of physical, chemical, or biological stimulus. As depicted in Chart 2, if one stimulates a quiescent or experimentally suppressed tissue and monitors cells entering $M$ and $S$ in autoradiographs at hourly intervals, one observes a prompt and transient increase in the number of mitoses, representing release of $G_2$-blocked cells into $M$; there is also a prompt and transient increase in the number of labeled nuclei in the first few hr, representing release of $G_1$-blocked cells into $S$ (for experimental substantiation of these results, see Chart 3).
S. Gelfant

Then after a delay of about 10 to 20 hr, one observes a 2nd, much larger increase in the number of labeled nuclei, representing Gs-blocked cells entering S after a delay; this is followed by a comparable and subsequent increase in the number of mitoses as depicted in Chart 2.

The following is an outline of another general procedure for demonstrating and distinguishing all 4 categories of cycling and noncycling cells within the same tissue in vivo or in vitro (after DeCosse and Gelfant (40)):

A. To demonstrate cycling and noncycling cells:
   1. Labeled nuclei: evidence for cycling cells.
   2. Unlabeled nuclei: evidence for noncycling cells.

B. To distinguish noncycling cells blocked in G1 or in G2:
   1. Unlabeled 4C nuclei: evidence for G2-blocked cells.
   2. Unlabeled 2C nuclei:
      a. Evidence for G1-blocked cells
      b. Evidence for G0-blocked cells

C. To determine whether unlabeled noncycling cells are G0, G1, or G2 blocked:

Stimulate other samples; keep in presence of [3H]-thymidine
1. Experimentally open G2 block: G2-blocked cells promptly enter M; appear as unlabeled mitoses (and the number of unlabeled 4C nuclei decreases).
2. Experimentally open G0 barrier and G2 block:
   a. Unlabeled G2-blocked cells promptly enter S; appear as labeled nuclei (and the number of unlabeled 2C nuclei decreases slightly).
   b. Unlabeled G0-blocked cells enter S after a delay; also appear as labeled nuclei (the number of unlabeled nuclei is reduced to zero).

For additional procedures to study and to detect cycling and noncycling cells, see the report of Gelfant (50) (Chart 3).

Examples, Evidence, and Analysis of G0-blocked, G1-blocked, and G2-blocked Cells

Noncycling G0-blocked cells. In addition to providing evidence for G0-blocked cells (by recording their characteristic delay in moving into DNA synthesis after stimulation), the examples in Table 1 were chosen and organized to deal with the following issues.

A. Are G0 delay times fixed or variable for cells rendered quiescent by different factors or released by different stimuli? The results with monolayer cultures of Syrian hamster (BHK) cells shown in Table 1A represent 1 line of data used by Pardee (86) to formulate the restriction point hypothesis (described under “Introduction”). Exponentially growing cells were rendered quiescent by 3 different media manipulations; isoleucine or glutamine was deleted from the culture medium or the serum concentration was drastically reduced. After 50 to 60 hr in depleted media, isoleucine, glutamine, or serum was restored (by changing to complete medium). In all 3 cases, the time interval from restoration of complete medium to the onset of DNA synthesis was 10 hr. The mouse embryo fibroblast cell results, also shown in Table 1A, provide another indication of a fixed G0 delay, in response to different stimuli. In these experiments, density-inhibited cell cultures were released by fresh medium containing RNase or hyaluronidase; release by either enzyme resulted in a 10-hr delay.

All other examples in Table 1 do not support the idea of a fixed G0 delay. The experiments in Table 1B illustrate that different stimuli in vivo or different media manipulations in vitro produce very different G0 delay responses in the same cells. The in vivo results on adult rat liver are noteworthy because they show that the delay can vary from 8 to 36 hr, depending upon the experimental stimulus. The experiments on isoleucine-, glutamine-, or serum-deprived Syrian hamster ts cells and on Chinese hamster lung cells are essentially identical with those reported by Pardee, but the results are not in agreement; the G0 delays are quite variable, and they are definitely related to the specific media alteration that moved cycling cells to the quiescent and then back to the cycling state. Also the mouse embryo fibroblast results taken from the experiment quoted in Table 1A show different delay responses to medium change.

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Chart 3. Figures and legends from a report by Choie and Richter (34).
The experimental data in these figures support the idealized results in Chart 2 showing the existence of and the prompt, transient, and independent microscopic fields (—5000 hepatocytes). Each point represents mean ± S.D. of 3 mice. , lead-treated group; ○, controls. Legends have been modified from the original.
### Table 1
Examples of G<sub>0</sub>-blocked cells

<table>
<thead>
<tr>
<th>Tissue*</th>
<th>Stimulus (opener G&lt;sub&gt;0&lt;/sub&gt; barrier)</th>
<th>Enter S after* delay (hr)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Fixed delay: different stimuli, same cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syrian hamster cells</td>
<td>Ileu, Gin, serum</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>Mouse embryo fibroblasts</td>
<td>RNase, hyaluronidase</td>
<td>10</td>
<td>122</td>
</tr>
<tr>
<td><strong>B. Variable delay: different stimuli, same cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermis (m)</td>
<td>Pluck</td>
<td>14</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Carcinogen</td>
<td>18</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Wound</td>
<td>32</td>
<td>49</td>
</tr>
<tr>
<td>Kidney epithelium (r)</td>
<td>Folic acid</td>
<td>18</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Ischemia</td>
<td>20</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Partial nephrectomy</td>
<td>36</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>HgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>48</td>
<td>37</td>
</tr>
<tr>
<td>Liver hepatocytes (r)</td>
<td>Nutritional shift</td>
<td>8</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Partial hepatectomy</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Partial hepatectomy + α-galactosamine</td>
<td>36</td>
<td>72</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syrian hamster ts cells</td>
<td>Ileu</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>Chinese hamster lung</td>
<td>Gin</td>
<td>12</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Ileu</td>
<td>18</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>20</td>
<td>81</td>
</tr>
<tr>
<td>Mouse embryo fibroblasts</td>
<td>Medium change</td>
<td>14</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Digitonin</td>
<td>18</td>
<td>122</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Medium change</td>
<td>14</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Rous sarcoma virus</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>SV40</td>
<td>36</td>
<td>105</td>
</tr>
<tr>
<td>Chick fibroblasts</td>
<td>Insulin</td>
<td>3</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>5</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt; and Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>16</td>
<td>103</td>
</tr>
<tr>
<td>Human lymphocytes</td>
<td>Phytohemagglutinin</td>
<td>20</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Concanavalin A</td>
<td>48</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>96</td>
<td>9</td>
</tr>
<tr>
<td>Mouse macrophages</td>
<td>Melanocyte fusion</td>
<td>3</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>SV40</td>
<td>36</td>
<td>71</td>
</tr>
<tr>
<td><strong>C. Variable delay: same stimulus, different cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue (r)</td>
<td>Wound</td>
<td>72</td>
<td>14</td>
</tr>
<tr>
<td>Epidermis (r)</td>
<td>&quot;</td>
<td>96</td>
<td>14</td>
</tr>
<tr>
<td>Salivary gland (m)</td>
<td>Isoproterenol</td>
<td>22</td>
<td>79</td>
</tr>
<tr>
<td>Kidney (m)</td>
<td>&quot;</td>
<td>30</td>
<td>79</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse macrophages</td>
<td>Melanocyte fusion</td>
<td>3</td>
<td>56</td>
</tr>
<tr>
<td>Chick RBC</td>
<td>&quot;</td>
<td>10</td>
<td>56</td>
</tr>
<tr>
<td>Chinese hamster lung</td>
<td>SV40</td>
<td>10</td>
<td>62</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>&quot;</td>
<td>36</td>
<td>105</td>
</tr>
<tr>
<td>Normal lymphocytes</td>
<td>Phytohemagglutinin</td>
<td>24</td>
<td>63</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>&quot;</td>
<td>6 days</td>
<td>63</td>
</tr>
</tbody>
</table>
Table 1—Continued
Examples of G₀-blocked cells

<table>
<thead>
<tr>
<th>Tissue*</th>
<th>Stimulus (opener G₀ barrier)</th>
<th>Enter S after delay (hr)</th>
<th>Ref.</th>
</tr>
</thead>
</table>

**D. Variable delay: chronological age or time spent in G₀**

**In vivo**
- Liver hepatocytes (r)
  - Weanling: Partial hepatectomy 15 17
  - Adult: " 18 17
  - Old: " 23 17
- Kidney epithelium, connective tissue (r)
  - Weanling: Partial nephrectomy 28 91
  - Adult: " 36 91
- Salivary gland (r)
  - Adult: Isoproterenol 21 96
  - Old: " 48 1

**In vitro**
- Human fibroblasts
  - 5 days stationary: Medium change 8 2
  - 9 days stationary: " 14 2
  - 18 days stationary: " 20 2
- Chinese hamster lung, media depleted
  - 24 hr: Ileu: Complete medium 4 81
  - 24 hr: Gln: " 8 81
  - 24 hr: serum: " 12 81
  - 48 hr: Ileu: " 12 81
  - 48 hr: Gln: " 12 81
  - 48 hr: serum: " 20 81
- Root meristem cells, carbohydrate starved
  - 48 hr: Sucrose 8 121
  - 72 hr: " 10 121
  - 96 hr: " 14 121

**E. Variable delay: single or multiple exposure to stimulus**

**In vivo**
- Salivary gland (r)
  - 3 injections: Isoproterenol 21 96
  - " 12 65
  - " 15 96
- Salivary gland (m)
  - 5 injections: Isoproterenol 30 85
  - " 14 85
  - Kidney epithelium (r)
    - after 2nd and 3rd injections: Lead 24 33
  - " 6 33

**In vitro**
- Human lymphocytes
  - Restimulation Phytohemagglutinin 20 126
  - " 12 126

**F. Variable delay: miscellaneous examples**

**In vivo**
- Liver (r)
  - Partial hepatectomy 18 17
- Liver (m)
  - " 29 4
- Mammary gland (r)
  - Lactation 24 112
- Mammary gland (m)
  - " 48 119
- Liver hepatocytes (r)
  - 10 a.m.: Partial hepatectomy 16 6
  - 4 p.m.: " 21 6
  - 12 a.m.: " 18 6
or to an exposure to digitonin.

The other in vitro results (Table 1B) and those in Table 1C show that the delay response can vary from 3 to 36 hr, depending upon the stimulus, and that, when the same stimulus is applied to different cell types, the G0 delay response (SV40 stimulus) can vary by as much as 26 hr or even 5 days (phytohemagglutinin stimulus) if we consider normal and chronic lymphocytic leukemia lymphocytes as different cell types.

B. The idea that chronological age of the organism or the time spent in the noncycling state is reflected in or related to the G0 delay time appears to be valid. The in vivo and in vitro examples in both animal and plant cells shown in Table 1D establish this generalization and support the concept of "deeper G0 states" proposed by Augenlicht and Baserga (2); they also support our concept of cellular aging, where aging is described as a progressive conversion of cycling to noncycling cells (54) and where there is impaired release to the cycling state with chronological age (53) (also see Chart 4).

C. Table 1E points out the possibility of a "priming effect" (74, 126) produced by repeated exposure to the same stimulus. The implication here is that cells (daughter cells) having recently been exposed to a stimulus respond more quickly to restimulation and enter S after a shorter delay. Such results could be due to less time spent in the noncycling state, as discussed above. For example, decreasing intervals of 76, 52, and 28 hr between a 2nd and 3rd injection of isoproterenol result in corresponding decreasing G0 delays of 26, 21, and 16 hr in rat salivary gland acinar cells (97). In the example of repeated lead injections, the apparent reduction of G0 delay from 24 to 6 hr may have been due to stimulation of cycling kidney cells, because the residual levels of labeling activity 48 hr after the 1st and 2nd injections were still above that in the control, unstimulated kidneys.

The miscellaneous examples of variable G0 delays indicate that there may be species differences and diurnal variations in G0 delays in vivo. The in vitro results on cultured rat liver hepatocytes and on Chinese hamster ovary cells probably reflect time differences between G1-period traverse in cycling cells (because hepatocytes from partially hepatectomized liver are in the cycling state; so are Chinese hamster ovary cells that have been selectively harvested in mitosis) and noncycling G0 delay after stimulation (because adult rat liver hepatocytes are noncycling and so are media-deprived Chinese hamster ovary cells). The last example under Table 1F does not deal with variable G0 delay. It is included to indicate that perhaps the earliest event in the release of noncycling G0 blocked cells may involve cell surface changes (in this case, due to Pronase digestion or alteration of a protein in the cell membrane).

D. The extreme variations in G0 delays listed in Table 1 question the prereplicative concept (8) of a complete and necessary series of biochemical events that occurs from the point of stimulation to the onset of DNA synthesis and that is supposedly responsible for the time lag in entering S after stimulation. The sequence of biochemical events reportedly starts with increases in non-histone protein synthesis and chromatin template activity (early prereplicative phase) and ends with the synthesis of thymidine kinase and thymidylate synthetase (late prereplicative phase). The fact that this can be accomplished in some cells within several hr and in other cells in several days indicates: Explanation 1, that the events are not necessarily continuous and that cells may arrest at different biochemical steps in G0; Explanation 2, that different cells can move through the total sequence of biochemical events at vastly different rates; or Explanation 3, that there is a variable "preprereplicative" delay period for different cells under different conditions. Explanations 1 and 3 both seem reasonable because, as will be shown in Table 2, some noncycling cells (G0 blocked) arrest very late in G0 and seem to be biochemically poised to enter S without having to go through all of the elaborate prereplicative preparations. Also there are indications of a preprereplicative period because under certain experimental conditions ["deep G0" (2)] or in different experimental systems (Ref. 8, p. 120) there are lags in time after stimulation (ranging from 30 min to 24 hr) before any detectable increases in chromatin template activity or non-histone protein synthesis (i.e., the earliest prereplicative phase events). Indeed, it might be worthwhile to investigate the notion of a preprereplicative phase in G0.

Another factor that may account for the great variations in the miscellaneous examples of variable G0 delays involving unstimulated kidneys.
in G₀ delays, particularly those recorded for monolayer cell culture studies in Table 1, may involve confusion in comparing cycling G₁ periods with noncycling G₀ periods. Also, the behavior of cells in density- or media-depleted cell cultures may be an artifactual function of the length of time spent in the media or the method used to render cells quiescent or to the use of serum as the method of stimulation.

In any event, the examples in Table 1 call for a clarification of the prereplicative phase concept. Also, in general, they provide evidence in support of the picture of noncycling G₀-blocked cells shown in Chart 1.

**Noncycling G₀-blocked Cells.** Table 2 presents examples of and provides evidence for the existence of G₀-blocked noncycling cells (Chart 1). The number of examples is small (and hard to come by) because most workers do not ordinarily monitor DNA synthesis immediately after or shortly after stimulation; also the increase in the number of cells entering S from the G₀-blocked state is much less and relatively transient when compared to the subsequent delayed increase in G₀-blocked cells entering DNA synthesis (see Charts 2 and 3 for diagramatic and actual data depictions).

Ideally, the crucial point of evidence for the existence of 2 separate categories of noncycling G₁-blocked and G₀-blocked cells in quiescent tissues is the biphasic appearance of cells entering DNA synthesis in response to the same stimulus in the same population of cells in the same tissue and in the same experiment; this biphasic response is manifested first by the prompt appearance of cells in S (G₁-blocked cells), followed after a lag in time by a 2nd independent surge of cells moving into DNA synthesis (G₀-blocked cells). Therefore, Table 2 presents 2 sets of data to illustrate the biphasic and separate responses of G₁-blocked and of G₀-blocked cells in each experiment. (The tissues lacking G₀-blocked data are known to contain G₀ cells.) Because the examples in Table 2 were gleaned from studies using widely different experimental designs, they will be taken up individually.

The original observation of G₀-blocked cells came from our studies on mouse ear epidermis (references in Table 2) where it was shown that cutting the ear induces a small proportion of noncycling epidermal cells to move into DNA synthesis within 30 min in vivo and within 1 hr when cut ear fragments were incubated in vitro. G₀-blocked epidermal cells enter S about 32 hr after wounding in vivo.

### Table 2

**Examples of G₀-blocked cells**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stimulus (opener G₁ block)</th>
<th>G₀-blocked cells enter S promptly after delay (hr)¹</th>
<th>G₀-blocked cells enter S after delay (hr)²</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear epidermis (m)</td>
<td>Wound</td>
<td>1</td>
<td>32</td>
<td>48, 49</td>
</tr>
<tr>
<td>Body epidermis (r)</td>
<td>Wound</td>
<td>1</td>
<td>96</td>
<td>14</td>
</tr>
<tr>
<td>Tongue epithelium (r)</td>
<td>Wound</td>
<td>1</td>
<td>72</td>
<td>14</td>
</tr>
<tr>
<td>Kidney epithelium, connective tissue (r)</td>
<td>Partial nephrectomy</td>
<td>4</td>
<td>28</td>
<td>91</td>
</tr>
<tr>
<td>Liver (r)</td>
<td>Partial hepatectomy</td>
<td>30 min</td>
<td>16</td>
<td>57</td>
</tr>
<tr>
<td>Liver (m)</td>
<td>Lead</td>
<td>2</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Mammary gland (rabbit)</td>
<td>Prolactin</td>
<td>6</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Hemopoietic cells (m)</td>
<td>Testosterone</td>
<td>6</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Kidney (m)</td>
<td>Irradiation</td>
<td>10 min</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Ascites tumor cells (m)</td>
<td>Transplantation</td>
<td>10 min</td>
<td>12</td>
<td>124</td>
</tr>
<tr>
<td>s.c. capillary endothelial cells (r)</td>
<td>Injections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor cells</td>
<td></td>
<td>6</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>Formic acid</td>
<td></td>
<td>None</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl solution</td>
<td></td>
<td>None</td>
<td>None¹</td>
<td></td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemopoietic cells (m)</td>
<td>Testosterone</td>
<td>3</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Cyclic 3':5'-AMP and cyclic 3':5'-GMP</td>
<td>3</td>
<td>2</td>
<td>24, 27</td>
<td></td>
</tr>
<tr>
<td>Isoproterenol</td>
<td></td>
<td>3</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Carbamylcholine</td>
<td></td>
<td>3</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

* Slow-renewing or quiescent tissues.

¹ In the same cell population, same tissue, and same experiment as those in which G₀-blocked cells appear.

² m, mouse; r, rat.

³ Earliest time period studied.

⁴ For actual data, see Chart 3.

⁵ Quote from Ref. 32: "By autoradiography, there was no labeling whatsoever of endothelial cells 8 and 50 hrs after saline injection." i.e., there are no cycling cells in this tissue.

⁶ In some experiments, enter S within 1 hr after exposure to testosterone or to cyclic 3':5'-AMP (24).
In the next example in Table 2, rat skin and tongue were studied at various intervals after wounding. Within 1 hr there was a marked increase in the numbers of labeled epidermal and tongue epithelial cells located distant from the wound edge, followed 96 or 72 hr later by the appearance of labeled cells in the areas adjacent to the skin or tongue wounds.

The study on kidney tubule and stromal cells after unilateral nephrectomy is interesting because in 4-week-old rats both G₁- and G₀-blocked cells were released, whereas only G₉-blocked kidney cells appeared (after a 30-hr delay) in 4-month-old rats. The data on the percentage of kidney cells labeled in 4-week-old rats were truly biphasic. There was a prompt appearance of labeled tubule and stromal cells within 4 hr (the earliest time period studied); the number of labeled cells rose and then declined at 12 hr (a transient G₁-blocked cell response); 16 hr later (28 hr postnephrectomy) a 2nd and more pronounced wave of labeled kidney cells appeared in S (the appearance of G₀-blocked cells after a 28-hr delay). As indicated in Table 2, Footnote d, examples of G₁-blocked cells entering S 4 to 6 hr after stimulation, rather than more promptly, are probably due to the fact that these were the earliest time periods studied in vivo.

The partially hepatectomized rat liver studies in Table 2 were performed on isolated perfused liver in vitro and also in vivo. The results were comparable. There was a significant increase in [³H]thymidine incorporation 30 min after partial hepatectomy (dpm rose from 1.3 in intact liver to 3.3), indicating the prompt response of G₁-blocked liver cells. Sixteen hr post-partial hepatectomy there was a 2nd and greater increase in [³H]thymidine incorporation (dpm, 13.0), indicating the appearance of G₀-blocked cells in S.

The data in the lead-stimulated mouse liver study so strongly support the concept and the experimental evidence for both categories of noncycling G₀- and G₁-blocked cells (Charts 1 and 2) that they have been reproduced in Chart 3. Regarding the issue of G₁-blocked cells, a single i.c.² injection of lead induces an increase in hepatocyte labeling index within 2 hr after injection. The transient release of G₁-blocked liver cells reaches a peak at 5 hr and drops abruptly at 6 to 8 hr after stimulation. Also, the release of G₁-blocked cells is unrelated to mitotic index data in Chart 3, which reflect the independent behavior of G₀-blocked liver cells. Although mouse liver contains noncycling G₀-blocked cells (Table 1), they were not released by a single dose of lead as administered in these experiments.

Prolactin stimulation of rabbit mammary gland also elicits a biphasic response of alveolar cells entering S phase. The initial appearance of cells in S occurs within 6 hr after stimulation (the earliest point studied). Twelve hr later (or 18 hr after prolactin injection), there is a further, large increase in the number of cells entering DNA synthesis. These results demonstrate and mark the differences between noncycling G₂- and G₀-blocked mammary gland cells. Prolactin is an unusual hormone because it also releases noncycling G₂-blocked pigeon crop gland cells within 30 min after injection (see Table 3 for reference).

The in vivo and in vitro studies on mouse hemopoietic cells in Table 2 demonstrate the existence of and implicate the triggering of noncycling G₁-blocked hemopoietic stem cells into DNA synthesis via hormonal (testosterone), cyclic nucleotide (cyclic 3':5'-AMP and cyclic 3':5'-GMP), adrenergic (isoproterenol), and cholinergic (carbamylcholine) receptor site mechanisms. The in vivo irradiation study indicates that approximately 25% of noncycling mouse bone marrow cells can be stimulated to enter DNA synthesis within 10 min after a low dose of irradiation. This report states: "After excluding alternative explanations, it must be concluded that cells in G₀ have completed all preparations for going into S-phase, or, in other words, that the localization of those G₀ cells in relation to other phases of the cell cycle must be between G₁ and S-phase"; this conclusion supports our depiction of G₀-blocked cells in Chart 1 and our discussion of the prereplicative phase (Table 1).

The 3 separate studies on mouse ascites tumor cells quoted in Table 2 demonstrate the existence and the release of noncycling G₁-blocked cells by transplantation of tumors into the peritoneal cavities of new mice. The differences in how promptly G₁-blocked cells enter S may be related to the time spent in the plateau phase of growth of the tumor transplant (in a manner similar to the deep G₀ state described in Table 1D). Five-day-, 7-day-, and 10-day-growing tumor cells, used in the 3 different reports, resulted in corresponding appearances of G₁-blocked cells in S at 10 min, at 1 hr, and at 2 hr after transplantation to the new host. The 1st report on ascites tumor cells listed in Table 2 also detected a 2nd and delayed increase in tumor cells entering S, 12 hr after transplantation, representing the appearance of G₀-blocked cells in the same experiment. Although the ascites tumor cell results are included in Table 2 to provide evidence for the existence of noncycling G₀-blocked cells, they should not be taken to indicate that this is the predominant category of noncycling cells in tumors. Indeed, most quiescent tumors are primarily composed of noncycling G₀-blocked cells (36).

The results on capillary endothelial cells summarize and emphasize the main points in Table 2: (a) in terms of cell renewal, adult capillary endothelial cells are in a resting, quiescent state (see Table 2, Footnote f); (b) injection s.c. of live tumor cells activates and releases both G₁-blocked endothelial cells (which enter S promptly) and G₀-blocked endothelial cells (which enter S after a delay of 48 hr); (c) injection of formic acid releases only G₀-blocked endothelial cells and serves as an experimental control showing tumor specificity in releasing G₁-blocked endothelial cells; (d) 0.9% NaCl solution injections have no effect on either G₀- or G₁-blocked endothelial cells, thus validating the results of tumor implant and formic acid injections. It is of interest to speculate that the vasoproliferative tumor vascularization effects induced by tumor cells and by the extract tumor angiogenesis factor (46) may be related to the specific release of noncycling G₀-blocked capillary endothelial cells.

The main impact of Table 2 is that it provides evidence for the existence of 2 categories of noncycling cells (within the same tissue), arrested at significantly different, nonoverlapping points in the G₁ period of the cell cycle and that,
in contrast to G₂-blocked cells, G₁-blocked cells arrest at a point late in G₁, where they remain biochemically and temporally poised to enter S, promptly and without need of any extensive prereplicative preparations.

Clarification of Cells in G₁

According to the ideas and the information presented in the charts and tables, a tissue or a given population of cells in vivo or in vitro may contain 3 categories of cells of the same type that are in different physiological and biochemical phases of the G₁ period of the cell cycle. There are cycling cells traversing through the G₁ period, noncycling G₁-blocked cells in various states of G₀ arrest, and noncycling G₁-blocked cells (possibly in various states of G₁ arrest).

Operationally, we can distinguish between cycling cells in G₁ and noncycling G₀- and G₁-blocked cells by long-term [³H]thymidine-combined autoradiographic-cytophotometric experiments, in which cycling cells in G₁ have labeled nuclei with 2C DNA contents and noncycling G₀- and G₁-blocked cells have unlabeled nuclei, also with 2C DNA contents (see “Procedures for Demonstrating and Distinguishing Cycling and Noncycling Cells”). We can distinguish between noncycling G₀- and G₁-blocked cells by observing their prompt (G₁-blocked) or delayed (G₀-blocked) movement into S after stimulating a quiescent tissue (see Table 2). We can distinguish between various states of deep G₀ arrest by correlating the time spent in G₀ with the delay time in entering S after stimulation (see Table 1D). We can distinguish between cells in early G₀ arrest (before they enter deeper G₀ states), which exhibit a short delay in entering S after stimulation, and noncycling G₁-blocked cells, which also exhibit a short delay (or prompt) behavior in entering S after stimulation by releasing G₁-blocked cells after long periods in the noncycling state (see Table 2), thus eliminating participation of cells in early G₀ arrest. Also we can distinguish between cycling cells, which require time to move through the G₁ period, and stimulated G₀ cells by specifically isolating cells known to be either in the cycling or in the noncycling G₀ state. For example see the results on Chinese hamster ovary cells (Table 1F) which compare the effects of replating cycling cells, isolated by mitotic selection with those of stationary G₀-blocked cells after media replenishment. A similar situation may exist for the hepatocyte results obtained from samples from partially hepatectomized and intact liver (also shown in Table 1).

Are there biochemical characteristics or markers that can be used to distinguish any of the above physiological states in G₁? The most significant ideas and investigations in this respect are those reported by Baserga (8) and particularly the study by Augenlicht and Baserga (2). In reviewing the literature, Baserga finds that there are differences in certain cellular proteins between cycling cells in G₁ and noncycling G₀ cells and that there is increased chromatin template activity in activated G₀ cells compared to cycling cells in G₁ (where template activity remains constant and relatively low). In experiments with human fibroblasts at different times after confluence, Augenlicht and Baserga demonstrate that, upon release, cells that have just become quiescent after 5 days in culture show no increase in chromatin template activity, cells that have been stationary for 9 days show an early increase in template activity 1 hr after stimulation, and cells that have been quiescent for 18 days show a delayed increase in chromatin template activity 5 to 10 hr after release from quiescence. There were also correlating shorter and longer G₀ delay times in entering S after 5, 9, and 18 days in the stationary state (see Table 1).

These results implicate a turning off and an activation of specific cell cycle genes that are associated with moving into and out of the noncycling G₀ state but not with moving into or out of the pre-G₀ or G₁ arrest stage. Chromatin template activity in 9-day quiescent and in 18-day unstimulated cultures was lower than in 5-day quiescent cultures. The delayed increase in template activity in stimulated 18-day confluent cultures was taken as an indication that cells go into deeper G₀ states. The delay can also be interpreted according to our notion of a prereplicative phase in G₀ (see discussion of Table 1). It would be interesting to study template activity in other situations such as after replating cycling Chinese hamster ovary cells arrested in mitosis and in cultured hepatocytes from regenerating and intact liver (Table 1). It would also be interesting to see whether there is increased chromatin template activity involved in the release of noncycling G₁-blocked cells, such as in ascites tumors and in hemopoietic cells in vivo (Table 2).

Although in one sense increased chromatin template activity can be viewed as an operational distinction between noncycling G₁-blocked cells and other categories of cells in G₁, because the increase is observed only after stimulation of G₀-blocked cells, studies on this level may lead to discovering the biochemical and metabolic differences between cycling and noncycling cells in the G₁ period of the cell cycle.

Noncycling G₂-blocked Cells. Table 3 (taken from over 50 different examples) demonstrates that noncycling G₂-blocked cells (as depicted in Chart 1) can be found in a wide variety of animal, plant, and tumor tissues both in vivo and in vitro and in slow- or fast-renewing tissues. The examples were also chosen to illustrate the great variety of factors (some general, some specific) that can release G₂-blocked cells in different tissues. All of the examples in Table 3 are supported by 1 or both of the 2 main lines of evidence for the existence of noncycling G₂-blocked cells: Evidence A, their prompt release into mitosis, only after an appropriate stimulus (As point out above, this can occur within 30 min in the example of the effects of prolactin on pigeon crop epithelial cells. See Charts 2 and 3 for diagrammatic and actual data depictions.); Evidence B, their appearance as unlabeled mitoses after stimulation (in most cases) and prior exposure to [³H]thymidine. To illustrate, we (89) labeled mice continuously with [³H]thymidine for more than 6 months and then stimulated regeneration in ear epidermis and in kidney tubular epithelium. The appearance of unlabeled mitoses in both epidermis and kidney epithelium was evidence for the existence of noncycling G₂-blocked cells and for their remaining dormant in the G₂ period indefinitely. Usually, the continuous administration of [³H]thymidine for a period longer than S and G₂, or preferably longer than the total cell cycle time of the...
Table 3
Examples of G_2-blocked cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stimulus (opener G_2 block)</th>
<th>Evidence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear, body epidermis (m)^b</td>
<td>Wound</td>
<td>A, B</td>
<td>50</td>
</tr>
<tr>
<td>Ear (m), plantar (r) epidermis</td>
<td>High temperature</td>
<td>A, B</td>
<td>51, 115</td>
</tr>
<tr>
<td>Hair follicle (m)</td>
<td>Pluck</td>
<td>B</td>
<td>83</td>
</tr>
<tr>
<td>Hairless epidermis (m)</td>
<td>Adrenalin</td>
<td>A</td>
<td>44</td>
</tr>
<tr>
<td>Kidney epithelium (m, r)</td>
<td>Partial nephrectomy</td>
<td>A, B</td>
<td>89, 91</td>
</tr>
<tr>
<td>Kidney epithelium (r)</td>
<td>Ischemia</td>
<td>A</td>
<td>113</td>
</tr>
<tr>
<td>Duodenum (m)</td>
<td>Nutritional shift</td>
<td>B</td>
<td>89</td>
</tr>
<tr>
<td>Rectal epithelium (r)</td>
<td>Wound</td>
<td>A</td>
<td>99</td>
</tr>
<tr>
<td>Liver (m, r)</td>
<td>Lead, azathioprine</td>
<td>A, B</td>
<td>34, 78</td>
</tr>
<tr>
<td>Salivary gland (r)</td>
<td>Isoproterenol</td>
<td>B</td>
<td>64</td>
</tr>
<tr>
<td>Breast tumor (m)</td>
<td>No stimulus</td>
<td>B</td>
<td>82</td>
</tr>
<tr>
<td>Sarcoma (r)</td>
<td></td>
<td>B</td>
<td>92</td>
</tr>
<tr>
<td>Carcinoma (human)</td>
<td></td>
<td>B</td>
<td>35</td>
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<tr>
<td>Ascites tumor (m)</td>
<td>Antilymphocytic serum, azathioprine</td>
<td>A, B</td>
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<tr>
<td>Amelogenic epithelium (rabbit)</td>
<td>Growth</td>
<td>B</td>
<td>110</td>
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<tr>
<td>Esophagus epithelium</td>
<td>Starve-refeed</td>
<td>A</td>
<td>30</td>
</tr>
<tr>
<td>(chicken)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crop gland (pigeon)</td>
<td>Prolactin</td>
<td>A</td>
<td>68</td>
</tr>
<tr>
<td>Wheat, pea, lettuce seeds</td>
<td>Germination</td>
<td>B</td>
<td>3, 15, 45</td>
</tr>
<tr>
<td>Corn, bean roots</td>
<td>Germination</td>
<td>A, B</td>
<td>111, 38</td>
</tr>
<tr>
<td>Flowering shoot apex</td>
<td>Daylight</td>
<td>A</td>
<td>10</td>
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<tr>
<td>Tetrahymena pyriformis</td>
<td>Starve-refeed</td>
<td>A, B</td>
<td>29</td>
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<tr>
<td>In vitro</td>
<td></td>
<td></td>
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<tr>
<td>Mouse spleen cell lines</td>
<td>Scraping</td>
<td>A</td>
<td>31</td>
</tr>
<tr>
<td>Rat thymus lymphocytes</td>
<td>Calcium</td>
<td>A</td>
<td>123</td>
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<tr>
<td>Hamster cheek pouch</td>
<td>Injury</td>
<td>A</td>
<td>60</td>
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<tr>
<td>Hamster embryo cells</td>
<td>Polyoma virus</td>
<td>A, B</td>
<td>77</td>
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<tr>
<td>Chinese hamster cells</td>
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<td>Human embryo fibroblasts</td>
<td>Subcultivation</td>
<td>A, B</td>
<td>75</td>
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<tr>
<td>Human lymphocytes</td>
<td>Yeast factor</td>
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<td>Tobacco pith tissue</td>
<td>Indole acetic acid and kinetin</td>
<td>B</td>
<td>87</td>
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<tr>
<td>Primary root meristems</td>
<td>Nutritional shift</td>
<td>B</td>
<td>120, 121</td>
</tr>
</tbody>
</table>

^a Evidence: A, promptly enter mitosis after being released by appropriate stimulus. For an example of actual data, see Chart 3. B, after stimulation (or in some tumors without experimental stimulation), appear as unlabeled mitoses, having been exposed to continuous [3H]thymidine for long periods of time.

m, mouse; r, rat.

particular cell type, is adequate prior exposure to conclude that unlabeled mitoses (appearing after stimulation) represent noncycling G_2-blocked cells. Because of their relatively small contribution to the total cell population (2 to 10%), one must use appropriate experimental designs to demonstrate G_2-blocked cells. For additional procedures see the paper of Gelfant (50).

Other points of interest in Table 3 are: (a) in some tumors in vivo, G_2-blocked cells are not static and do not require a specific stimulus to be released into M. Rather, there appears to be a steady flow of tumor cells moving into and out of a prolonged G_2-arrested state, because in the 3 examples shown in Table 3 unlabeled mitoses are observed even after 7 to 9 days of continuous infusion of [3H]thymidine and in the absence of any apparent external stimulus. These results support and extend the idea that transformed tumor cells have lost their capacity to (completely) move into a quiescent state (in this case the noncycling G_2-blocked state), a point that will be further alluded to in relation to Chart 4; (b) immunosuppressants, antilymphocytic serum, azathioprine [and hydrocortisone (39)] also release noncycling G_2-blocked cells (see mouse ascites tumor and rat liver examples). This observation may be related to the increased incidence of cancer in transplant patients being treated with immunosuppressants such as corticosteroids and azathioprine (Imuran) (90), if we accept the speculation that the incipient cancer cells were in the noncycling G_2-blocked state and were released to the cycling state by immunosuppressive therapy; (c) and like G_1-blocked cells, G_2-blocked cells become noncycling at a point late (in this case) in the G_2 period where they remain biochemically poised to enter mitosis upon opening of the G_2 cell cycle block.

The information in Table 3, with supporting evidence, establishes the fact that cells can become arrested or noncycling during G_2, a fact that requires consideration and acceptance by the various G_0 models currently being used to describe noncycling quiescent states (alluded to under "Introduction") and by current cell kinetic cancer chemotherapy regimens [which consider all noncycling cells to be in G_0 (7, 61)]. It would also be worthwhile to look into some biochemical characteristics that might dis-
tistinguish noncycling G₂-blocked cells from cycling cells in G₂ and from noncycling G₁- and G₀-blocked cells (see "Clarification of Cells in G₁").

Speculation of Subpopulations

The concept depicted in Chart 1 implies that there may be additional subpopulations within the major categories of noncycling cells. This idea originated from our in vitro studies on stimulated mouse ear epidermis (49) in which additional G₂-blocked cells could specifically be induced to enter mitosis by modifying the sugar, the sodium, or the potassium concentrations of the medium. These G₂-blocked cells were qualitatively different in the sense that they could be selectively and independently activated or inhibited. We therefore postulated the existence of separate glucose-, sodium-, and potassium-responding physiological subpopulations of noncycling G₂-blocked epidermal cells. Other examples, drawn from Table 3, might involve subpopulations requiring very specific or unusual stimuli such as the release of G₂-blocked cells in mouse duodenum by a nutritional shift from low- to high-protein diets, whereas starve-refeeding does not (89); the example of human lymphocytes in vitro where a factor isolated from yeast identifies and releases G₂-blocked cells, but phytohemagglutinin does not (47); and G₂-blocked cells activated by immunosuppressants [a compensatory reaction or a release from immune inhibition (40)].

Possibilities of subpopulations within the other 2 major categories of noncycling cells involve the specific release of G₁-blocked mouse liver cells by a single injection of lead (34), of hemopoietic cells stimulated to enter DNA synthesis within 10 min after a low dose of irradiation (67), and of G₁-blocked capillary endothelial cells by implantation of tumor cells (32). In the noncycling G₀-blocked category, there are indications of subpopulations of rat salivary gland cells that respond only to multiple injections of isoproterenol (97) and of mouse splenic lymphocytes in which different cells require different induction periods (to concanavalin A) to be released from G₀ (59). Other indications of subpopulations of G₂-blocked cells may be taken from the very different G₀ delay times produced by different stimuli in cells of the same tissue (see Table 1). Finally, one could think of subpopulations of cells within each category of noncycling G₁-, G₂-, and G₀-blocked cells, which respond as, and are, potentially transformable (cancer) cells.

The concept in Chart 1 does not allude to subpopulations under the category of cycling cells. It is assumed that there are many subcategories of cycling cells being kept cycling by a wide variety of internal and external regulatory factors. In this situation there would be no G₀ barrier, and what varies is the extent to which the G₁ and the G₂ cell cycle blocks are opened.

Origin and Recruitment of Noncycling Cells, Transformation, and Tumor Growth

The model presented in Chart 1, with supporting evidence (charts and tables), establishes the fact that the potential proliferative pool in cells in culture and in tissues in vivo can be composed of 4 categories of cells of the same type, each having different patterns of behavior as seen in relation to the G₁ and G₂ cell cycle blocks and also in relation to the G₀ barrier; there are cycling cells and 3 categories of G₁-, G₂-, and G₀-blocked noncycling cells. Also there are indications of additional subpopulations within the major categories of noncycling cells. Within this framework, we critically reviewed a number of "G₀" hypotheses and ideas currently being proposed in the literature.

Chart 4 describes the origin of the 3 major categories of noncycling cells and their recruitment to the cycling state when called upon to service tissue proliferative needs. Chart 4 also applies our ideas of cycling and noncycling
cells to the problems of malignant cell transformation and tumor growth.

**Origin of Noncycling Cells (Cellular Aging)**

Chart 4 shows noncycling cells originating from cycling cells in a manner that we have termed "cellular aging" (54). Cellular aging is viewed as a progressive conversion of cycling to noncycling cells in tissues capable of proliferation. In Chart 4, noncycling cells become either G1, G0, or G2 blocked (the major proportion become blocked in G0). In some tissues these noncycling transitions take place and are completed during embryogenesis (pancreas, lens, tongue muscle) or while the animal is still growing (e.g., liver, kidney, bone); these tissues have undergone "immature aging," in contrast to "mature aging" which takes place in other tissues gradually throughout animal senescence (epidermis, epithelium of the gastrointestinal tract). Although the general range of noncycling cells in "aged" tissues is shown to vary from 60 to 90%, in some rapidly renewing tissues such as epithelium of the small intestine less than 5% of the population would be in a noncycling state; in some very slowly renewing tissues such as adult liver, kidney, salivary glands, and capillary endothelium, over 98% of these cells may be in the noncycling state.

**Recruitment of Noncycling Cells (Release from Aging)**

Noncycling cells remain in the G1-, G0-, or G2-blocked states indefinitely until death or until they are specifically recruited to proliferate in response to tissue injury or other proliferative stimuli. This is depicted in Chart 4 as release from aging or opening of the G1 and the G2 cell cycle blocks and the G0 barrier. G1 and G2 blocked cells enter S or M promptly; G0-blocked cells enter S after a delay. (Release from the G0 barrier implies concomitant opening of the G1 block.) The degree, the rate, and the point of release of all 3 categories of noncycling cells may depend upon the stimulus, on whether cells are immature, adult, or chronologically aged and upon the particular tissue (for examples, see subheadings Table 1, B to F, and Tables 2 and 3). The model also indicates that normal cells will in time (after having fulfilled their proliferative functions) revert to their specific noncycling G1-, G0-, and G2-blocked states. For additional references and additional examples that support the above description of the origin and recruitment of noncycling cells (cellular aging transitions), see the reports of Gelfant and Martin (54) and Gelfant and Grove (53).

**The Transition Probability Model (108)**

It is appropriate at this point to comment on the "transition probability" model proposed by Smith and Martin (alluded to as one of the G0 models under "Introduction") and which is gaining increasing acceptance in the literature (81, 86, 102). The following quotations describe their concept:

"Some time after mitosis all cells enter a state (A) in which their activity is not directed towards replication. A cell may remain in the A-state for any length of time throughout which its probability of leaving A-state remains constant. On leaving the A-state, cells enter B-phase in which their activities are deterministic, and directed towards replication. [The B-phase includes the conventional S, G2, and M phases and probably part of G1.] Initiation of cell replication process is thus random... . Cell population growth rates are determined by the probability with which cells leave the A-state... . [i.e., by the transition probability].

To reconcile the ideas quoted above with our concept of noncycling cells, we would have to assume that noncycling G1- and G0-blocked cells have a transition probability value of zero, an assumption that is mildly compatible with the idea of an indeterminate A state. With regard to noncycling G2-blocked cells, we would have to assume that the B phase is not always determinate and directed towards replication, an assumption that is not included in the transition probability model described by Smith and Martin.

There are 2 lines of direct in vivo evidence to support our contention that G0- and G2-blocked cells can remain in the noncycling state indefinitely: (a) noncycling adult (mouse) liver, kidney, pancreas, and salivary gland cells alluded to in Tables 1 and 2 (also adrenal, medulla, transitional epithelial, stomach chief cells, and osteocytes) remain unlabeled even after months of continuous exposure to [3H]thymidine in vivo (28); (b) as pointed out in Table 2, Footnote f, "By autoradiography, there was no labeling whatsoever of endothelial cells 8 and 50 hr after saline injection. i.e., There are no cycling cells in this tissue." The same is true with regard to in vitro cell systems; examples (Table 1) such as human lymphocytes and density-inhibited monolayer cell cultures do not move into DNA synthesis unless an appropriate mitogen is added to lymphocyte cultures or stationary cultures are replated or replenished.

We have also presented direct in vivo evidence (by labeling mice continuously with [3H]thymidine for more than 6 months) that G2-blocked epidermal and kidney cells can remain in the noncycling state indefinitely (89). Thus, although the ideas of an indeterminate A state and a determinate B phase are useful in analyzing the growth characteristics of fast-growing populations of cells (particularly in cell culture), they are not as applicable to the concept of noncycling cells developed in the present report.

**Transformation**

The idea that transformed cells arise from noncycling cells and under certain conditions lose their ability to return to a resting state comes primarily from the reports by Pardee (86), Temin (116), and Baltimore (5). The following quotations support and indicate the derivation of some of the ideas depicted in Chart 4. From Pardee’s paper: "Most animal cells in vivo exist in a nonproliferating state in which they remain viable and metabolically active. They arose from proliferating cells whose metabolic patterns were switched to quiescence at some time during differentiation. In contrast to this pattern of normal differentiation, cancer cells appear to arise from quiescent cells that have been switched back to active proliferation"; and from a review by Baltimore: "...the critical difference between transformed and untransformed cells is that untransformed..."
cells go into a resting state when conditions are not optimal for their growth. Transformed cells continue to grow in such conditions. Transformation is thus an abrogation of the resting state. The point in the cell cycle at which cells rest is somewhere in G1, but not at the border of the S phase.” (Both Pardee’s restriction point “R” and Temin’s “G1b” phase are located in mid-G1.)

Thus, in context of our scheme of cellular aging, Chart 4 depicts transformation (under certain conditions) as a continuous release to the cycling state, with only optional capability of returning to the noncycling state (dashed arrows). More specifically, we imply that transformation can occur (in subpopulations?) in all 3 categories of noncycling cells; accordingly, Chart 4 shows the release of transformed G1-, G0-, and G2-blocked noncycling cells. The in vivo tumor examples alluded to in Table 3 support these ideas to a certain extent by showing that in some solid tumors there is a steady flow of tumor cells moving into and out of a prolonged G1 arrest in the absence of a releasing stimulus. It may be that transformed cells move into a modified cycling state, which reflects the noncycling pattern of behavior from which they originated (in this case, a modified G1-blocked behavior).

Tumor Growth

In a most sensible discussion of tumor growth, Baserga (8) points out:

“It does not take a profound observer to realize that cancer is a disturbance of growth, although apparently it does take an experienced observer to realize that not all disturbances of growth are cancer. This has caused a considerable amount of confusion in the past 20 years since the behavior of certain cells in culture has often been interpreted as a neoplastic transformation by investigators who chose to overlook the characteristics of cancer growth. Thus, the fact that some cells can reach a higher saturation density when plated as a monolayer in cell cultures does not often been interpreted as a neoplastic transformation by investigators who chose to overlook the characteristics of cancer growth. Actually, the diagrams in Chart 4 depict 4 separate systems of cell proliferation that may exist within the same cell type, within the same tissue, and within the same tumor.

The most prominent mechanism is recruiting G0 cells into the proliferating pool [But succinctly, the reason a tumor grows is that the number of new cells produced per unit time [cell birth] always exceeds the number of cells lost per unit time [cell loss].]

The 1st part of the quotation lends encouragement to some of our earlier, admonishing remarks about G0 models derived from the behavior of cells in culture (including certain criticisms of Baserga’s prereplicative concept). More to the point, the overall picture in Chart 4 includes and expands Baserga’s description of tissue and tumor growth. Actually, the diagrams in Chart 4 depict 4 separate systems of cell proliferation that may exist within the same cell type, within the same tissue, and within the same tumor.

The first is the system of residual cycling cells (Chart 4, lower left) that have not undergone the process of cellular aging or conversion to the noncycling state. The proportions of such continuously cycling cells depend upon the tissue and the particular stage of cellular aging. The function of this system is to deal with “normal wear and tear” of a tissue. In this system, cell birth (moving through the cell cycle without obstruction to produce 2 daughter cells) is balanced by cell loss (death due to differentiative sloughing off, or to accidents occurring during mitosis, i.e., during the process of chromosome movement or cytoplasmic cleavage). Speeding up of this system by shortening the cell cycle (even with no concomitant increase in the rate of cell loss) would result in a relatively minor increase in overall cell birth and tissue growth (unless this system represented the only supply of proliferating cells, i.e., before cellular aging occurred).

The 2nd and the major potential proliferative system involves the 3 categories of noncycling G1-, G0-, and G2-blocked cells (plus the system of physiological subpopulations within each category, as described in Chart 1). Over 90% of the cells in any tissue or tumor may be in these noncycling states (most would be in the G0-blocked state). As indicated in the diagram, noncycling cells remain in these blocked states until death (an avenue of cell loss) or until they are released or recruited to the cycling state. Recruitment of noncycling cells may involve either G1-, G0-, G2-blocked or all 3 categories of noncycling cells (this is best illustrated in Chart 1).

Cycling cells, released from the noncycling state (usually in response to cell loss caused by wounding and trauma), represent the 3rd proliferative system. The extent to which they continue cycling determines the number of new cells produced and the overall increase in cell birth. Normally, these released cycling cells revert to the noncycling state after restoration of homeostasis.

The behavior of transformed cycling cells, which have lost their ability to return to the noncycling state, would represent the 4th proliferative system and would result in another method of increasing the rate of cell birth. Thus, as depicted in Chart 4, tumor growth could involve a specific release of transformed G1-, G0-, or G2-blocked noncycling cells, which, because they are unable to return to their prior resting states, continue to cycle. Release of an unregulated transformed system would increase the ratio of cell birth to cell loss and would result in unabated tumor growth.

A final point, not depicted in Chart 4, is that tumors may also contain a relatively large fraction of terminally differentiated, transformed noncycling cells that have lost their ability to be released to the cycling state, but that remain long-lived and therefore do not contribute significantly to tumor cell loss. In terms of the scheme in Chart 4, these cells would represent a fifth system to contend with, especially in trying to design and evaluate therapeutic strategies to eradicate tumors.

Thoughts and Speculations

Customized Cancer Therapy. In an editorial several years ago, Patt (88), having alluded to cancers with high and low growth fractions and to the changing proportions of cycling and quiescent tumor cells during the course of treatment, indicated that the ultimate goal in the management of human cancers would be “customized therapy” that would
take into account and adjust for the cell kinetic and population structure of the tumor. One of the practical implications of the present report is that it provides a more precise and accurate tumor kinetic response model than is currently available in the literature. In view of the recent advances in rapid cell cycle analysis by flow cytometry (58, 66) and by high-speed scintillation autoradiography (42) it would be feasible (using appropriate in vivo and in vitro experimental design) to ascertain and monitor the 4 major categories of cells: cycling cells, noncycling G1-blocked cells, noncycling G2-blocked cells, and noncycling G0-blocked tumor cells before and during the course of treatment. Although precise therapeutic tools to manipulate all categories of cycling and noncycling cells are not available (We are in the process of compiling and analyzing a list of natural and experimental factors that open and close the G0, G1, and G2 blocks), correlation between changing status of cycling and noncycling tumor cells and the clinical condition of the patient would provide the empirical advantage of being able to modify therapeutic regimens during the course of treatment. Also, once we have developed the tools to manipulate the various cycling and noncycling cell transitions, we may be in a position to achieve the ultimate goal of customized cancer therapy.

Tissues and Tumors as Proliferative Ecosystems. The scheme presented in this report of cycling and noncycling G1-, G2-, and G0-blocked cells (shown in Charts 1 and 4), drawn from our own studies and from the literature, indicates that tissues maintain an adaptive system of cell proliferation with the use of the various categories of cycling and noncycling cells to service the actual and the potential proliferative needs of the tissue (and the organism). The fact that noncycling cells can arrest at different temporal and biochemical points in the G1 and G2 gap periods of the cell cycle and that most cells reside in the noncycling state offers the tactical advantage of quiescence (at different points in interphase) over the turmoil involved in the continuous synthesis of the genetic and the mitotic machinery necessary for chromosome replication, chromosome movement, and cytoplasmic cleavage (i.e., the cycling state). The idea of tactical advantage of proliferative quiescence can be supported by quoting the title of an important review by Clarkson (36), “The Survival Value of the Dormant State in Neoplastic and Normal Cell Populations.”

Endogenous and exogenous factors that open the G1 and the G2 cell cycle blocks and the G0 barrier (a) increase the flow of cycling cells (shorten the cell cycle time of cycling cells), (b) release 2 fast-acting renewal systems of G1- and G2-blocked noncycling cells (which enter S and M promptly), and (c) recruit from the major force of noncycling G0-blocked cells [which, released from the G1 barrier, move into the cycling state (S period) after a characteristic delay in time]. Having fulfilled the particular proliferative needs of the tissue by orchestrating the various categories of cycling and noncycling cells, the cells are returned to quiescence by dampening the G1 and the G2 (restriction points?) of cells that remain cycling and by closing the G0 barrier and the G1 and G2 blocks, setting up the 3 major categories of noncycling cells, which return to and come to rest at the G0, G1, and G2 noncycling points of the cell cycle.

Physiological subpopulations within the major categories of noncycling cells (shown in Chart 1) would provide an additional adaptive dimension to the proliferative ecosystem of the tissue. Such cells, capable of being released to the cycling state only by very specific or unusual stimuli, would serve as another restrictive system to secure proliferative quiescence; they would also provide a system of specialized proliferative responses to cope with unusual and unforeseen endogenous and exogenous experiences (including the response of transformation). What is being proposed here is (in a sense) similar to antigen-selected proliferation of preadapted immunocompetent cells (19).

In the same vein of speculation, tumors can be viewed as discrete proliferative ecosystems existing within normal tissues in a parasitic relationship. Tumors are also composed of cycling and noncycling G1-, G2-, and G0-blocked cells; for the same reason as in normal tissues (to increase the probability of survival), tumor cells mainly reside in the quiescent noncycling states (36). Unabated or malignant tumor growth occurs at certain stages or under certain conditions that allow unregulated transformed tumor cells to increase the balance of cell birth over cell loss. Metastasis to new tissue locations would represent an additional tumor-adaptive (parasitic) behavior pattern. The relative proportions of cycling and noncycling tumor cells at metastatic sites may differ from those at the primary tumor site (107), indicating the possibility of separate, independent tumor ecosystems.

An additional complication in trying to understand tissue and tumor proliferative behavior is the possibility that all cells in the cycling state or all cells in the noncycling state may not respond in the same manner to inhibitory or stimulatory control factors. We have already alluded to the idea (Table 1) that chronological age of the organism or the time spent in the noncycling state alters the type of response to the same stimulus. Also, we provided evidence of impaired release to the cycling state with chronological age. The idea that all cells in the cycling state may not be alike is drawn from recent studies by Marks et al. (11, 12, 80) who show that cycling epidermal cells in adult mouse skin, released to the cycling state by gentle skin massage, respond to the inhibitory effect of epidermal G1 chalone (i.e., are prevented from moving from G1 into S). In contrast, cycling epidermal cells in skin of the newborn mouse and cycling epidermal cells in adult hyperplastic epidermis are refractory to the inhibitory effects of epidermal G1 chalone. Thus, in relation to the diagrams in Chart 4, adult epidermal cycling cells released by gentle skin massage would represent normal cells released to the cycling state, cycling epidermal cells in the newborn mouse would represent cells that have not undergone cellular aging transitions, and hyperplastic epidermal cells would be comparable to transformed cycling cells. These results would indicate that endogenous control mechanisms for cycling cells may vary in effectiveness throughout the stages of cellular aging and release from aging as depicted in Chart 4. If, as alluded to above, chalones cannot inhibit hyperplastic epidermis, which from a proliferative point of view is consid-
ered to be in a transformed state (11), one may question the use of chalones in the treatment of tumors (18). Also, unless the growth-inhibitory effects of chalones are unrelated to their transient closing of the G₁ (G₁ chalone) and the G₂ (G₂ chalone) cell cycle blocks, they would be unlikely candidates for cancer therapy, because even if effective on cancer tissues their actions are limited to cycling cells.

A Final Thought: On Transformation. According to the scheme depicted in Chart 4, noncycling cells remain in their noncycling states until death or until they are released to the cycling state (rebirth). In most tissues capable of proliferation in vivo, periodic release to the cycling state is controlled by homeostatic factors such as hormones, other endogenous regulatory molecules, pH and metabolic changes within the cell, or regenerative responses to wounding or to tissue architectural disturbances. We speculate that, if noncycling cells (destined to die) become refractory to normal physiological controls because of chronological age or other reasons, they or other subpopulations of noncycling cells would transform to the proliferative state. Transformation here would be viewed as a compensatory proliferative reaction, to compensate for impaired release of normal noncycling cells, which according to the scheme in Chart 4 would shift the balance of tissue existence in favor of cell loss and tissue death.

Significance

From an investigative point of view, this report puts into perspective most of the concepts and terminologies of cycling and noncycling cells alluded to under “Introduction.” Our concept of cycling and noncycling cells described in the charts and supported by examples and evidence listed in the tables provides a new framework to view a variety of basic and clinical research problems dealing with cell reproduction and tissue proliferation. Of immediate advantage is the awareness that most experimental proliferative systems in vivo or in vitro in normal or in tumor tissues are composed of the 4 major categories of cycling and noncycling cells depicted in Chart 1 (possibly including the prediversified system of noncycling subpopulations). Also of immediate advantage is the scheme in Chart 4, which provides a more precise and accurate tumor kinetic response model than is currently available in the literature.

What is needed now, on a descriptive level, is further independent verification of the model of cycling and noncycling cells presented in the present report, particularly verification of the idea of subpopulations and accumulation of additional examples of noncycling G₁-blocked cells. Also needed now are empirical experimental and therapeutic tools capable of modulating specific cycling or noncycling cell transitions. (We are currently developing a list of natural and experimental factors which can be used to influence these transitions.) What is needed in the not too distant future is to discover the molecular rules (95) that regulate arrest and release of cycling and noncycling cells at the G₀ barrier and at the G₁ and the G₂ cell cycle blocks. It may also be worthwhile to look into the idea that the potential behavior of cycling and noncycling cells is controlled and genetically preprogrammed during the S or the G₂ periods of parent cells.

As an afterthought, I would like to suggest that the cancerous properties of tumor dormancy, metastasis, and repopulation reside in a minority system of tumor cells compatible with our descriptions of noncycling G₁- and G₂-blocked cells and their subpopulations; and that the properties of tumor growth reside (for the most part) in the major system of cycling (≠ noncycling G₂-blocked cell transitions, as presented in this report.

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A New Concept of Tissue and Tumor Cell Proliferation

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