Proliferative Kinetics of Human Hematopoietic Cells during Different Growth Phases in Vitro

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SUMMARY

The proliferation kinetics of an established human hematopoietic cell line (SK-L7) derived from the peripheral blood cells of a patient with acute myeloblastic leukemia were studied during different phases of cell growth with thymidine-3H and autoradiographic methods. As the population changes from logarithmic to stationary growth, the S, G2, and G1 phases of the cell cycle become longer and more variable; G1 is most affected, and an increased proportion of cells remain in G1. Both nutrient deficiency and the extent of cell confluence affect the cell cycle as the cultures approach saturation density; the changes are reversible if the cells are fed and their population density is reduced. The kinetic behavior of SK-L7 cells in many ways resembles that of leukemic cells in the marrow in acute leukemia, and this system may be a useful in vitro model to study growth-regulatory factors and chemotherapeutic effects.

INTRODUCTION

A number of fundamental problems regarding the cytokinetics of acute leukemia are difficult to resolve by further studies in patients because of limitations in clinical investigation. Most kinetic studies of human leukemia have indicated that the generation times of leukemic cells are usually longer than those of the corresponding normal hematopoietic cells and that a significant fraction of the leukemic population is dormant and therefore relatively resistant to chemotherapy (10, 12, 14, 18, 36, 41, 45). Because of ethical and technical considerations, these studies were usually carried out during advanced disease, and it is possible that the kinetic parameters of the leukemic cells might be more nearly normal during early disease. It has been demonstrated in a number of experimental cancers in vivo that growth slows progressively as the disease advances (4, 23, 24, 31, 32, 40–43, 46, 47, 49, 50). The slower growth rate may be due to a smaller GF, a longer cell cycle, increased cell loss (due to cell death, exfoliation, or metastases) or a combination of these factors; the relative contribution of each may vary in different tumor systems.

Rather than use an animal model, we have investigated the possibility that a long-term culture of primitive human hematopoietic cells (SK-L7) (16) may, under appropriate conditions, mimic the proliferative behavior of leukemic cells in acute leukemia. It appears reasonable to anticipate that a leukemic cell population during advanced disease in vivo may be simulated by an in vitro population of hematopoietic blast cells in (or approaching) the stationary growth phase. This paper reports the changes which occur in the kinetic behavior of SK-L7 cells as their growth changes from logarithmic phase to stationary phase and, after subculturing, back to log phase.

MATERIALS AND METHODS

Origin of Cell Line

The SK-L7 line was derived from the peripheral blood of a child with acute myelomonocytic leukemia (16), and the cells have been growing continuously in suspension culture for over 4 years. Although the patient had a white blood cell count of 425,000/cu mm, and almost all the cells placed in culture were leukemic, it is not definitely established whether the cell line originated from leukemic or from normal lymphoid cells (9, 48, 57).

Culture Conditions

Unless otherwise noted, the cells were grown in Erlenmeyer flasks in McCoy 5a medium (modified) supplemented with 10% FCS at 37°C in an atmosphere of 5% CO2 and 95% air. The cells do not adhere to glass and grow in suspension in small clumps which settle to the bottom of the culture vehicle; the clumps are easily dispersed by shaking, and accurate counts can be obtained with a hemocytometer. The cellular content of different-sized clumps was determined by picking up individual clumps with micropipets and diluting them in appropriate volumes of counting fluid. Viability was estimated by trypan blue dye exclusion. Population-doubling times were determined by fitting a least-squares regression line to the log of cell concentration versus time.

Labeling Procedures and Radioautography

For the cell cycle studies, cells were incubated for 30 min
with thymidine-$^3$H (0.2 $\mu$Ci/ml; specific activity, 6 to 11 Ci/mmole). A 1000-fold excess of unlabeled thymidine was then added, and the cells were washed twice with fresh prewarmed medium (without added thymidine) and resuspended at the desired cell concentration. Samples were processed for radioautography with Kodak AR-10 stripping film (15), and cells having 4 or more grains over the nucleus were classified as labeled; background counts usually averaged less than 1 grain per nucleus. One thousand or more cells were counted to determine the thymidine-$^3$H LI, and 5000 were counted for the MI.

For the continuous infusion studies, 1-liter cell cultures were infused with thymidine-$^3$H in 0.9% NaCl solution at a constant rate of 5 ml/24 hr with a Watkins Chronofuser pump (United States Catheter and Instrument Corporation, Glens Falls, N. Y.). The initial loading dose of isotope was 0.05 to 0.1 $\mu$Ci/ml, and the rate of infusion was 0.01 to 0.1 $\mu$Ci/ml of culture medium per 24 hr.

A 30-min incubation with 1-$\mu$Ci or higher concentrations of thymidine-$^3$H per ml caused inhibition of cell growth, but concentrations of 0.2 $\mu$Ci/ml or less did not have any detectable effect, at least during the 96-hr period of observation. Continuous infusion of thymidine-$^3$H at concentrations of 0.2 $\mu$Ci/ml/24 hr or more caused inhibition of cell growth, but 0.05 $\mu$Ci/ml/24 hr or less had no discernible effect.

**Generation Time and Cell Cycle Phases**

Labeled Mitoses Method. One hundred or more mitoses were counted for each sample. The median generation time ($T_G$) and durations of the S, G2, and G1 phase were determined according to conventional methods (19). The duration of the mitotic phase ($T_M$) was estimated by the formula: $T_M = (M/LI)T_G$ (29, 55). Although this probably underestimates $T_M$ slightly in an exponentially growing population, it is not affected by the possible presence of a dormant cell component which may cause significant error in the more commonly used formulae:

$$T_M = MI \times T_G, \text{ or } T_M = MI \times T_G/0.693.$$

Grain Count-halving Method. A least-squares regression line was fitted to the logarithms of the data points (median grain counts). The average $T_G$ was taken as the halving time of this line, correcting for error due to lightly labeled cells passing below the grain count threshold upon division (22).

**RESULTS**

**General Growth Characteristics**

If cells from a log phase culture are transferred to fresh prewarmed medium at a starting concentration of about $10^5$ cells/ml and left undisturbed, they continue to grow without much lag for 3 to 4 days during which they double about 3 to 4 times. The MI, thymidine-$^3$H LI, and total thymidine-$^3$H incorporation begin to fall appreciably before the decrease in the growth rate is apparent (Chart 1). If no fresh medium is added, growth then ceases rather abruptly and viability (as estimated by dye exclusion) begins to fall; the total cell count remains fairly constant for several more days because the dying cells do not lyse immediately. During this period, the glucose concentration falls from an initial level of 3 mg/ml to about 1 mg/ml (at the end of 1 week). If the starting cell concentration is higher (0.5 to $1 \times 10^6$/ml), the population will undergo fewer doublings but will reach a higher final cell count (2 to $3 \times 10^6$/ml). If the starting concentration is lower ($10^2$ to $10^4$), the cells undergo more doublings (with about the same or slightly faster doubling time) but do not attain as high a final cell count. If cells at their maximum concentration (stationary phase) are transferred (at a lower cell concentration) to fresh medium
before their viability has decreased appreciably, most of them survive and, after a time lag, again begin to grow logarithmically. Even if stationary phase cells are left in the same "spent" medium but their concentration is merely reduced (i.e., to 1 to 2 x 10^5 /ml), the population will undergo 1 or 2 doublings, although they will not reach as high a concentration as in fresh medium. With these cell concentrations, if the medium is supplemented with 30% FCS, the cells will usually not grow to any higher final concentration than in 10% FCS. Moreover, if 10 to 30% fresh FCS is added to a culture which has already reached stationary phase (for 1 day) in 10% FCS (while removing an equal volume of spent medium to maintain the same cell concentration), no significant increase in cell density occurs, although loss of viability occurs less rapidly. Thus serum deficiency is not the sole reason that the cells fail to exceed their characteristic (maximum) stationary phase density.

Effect of Frequent Feeding

If cells are fed daily, they will reach a higher maximum concentration, and viability will remain good for a longer time (Chart 2). Nevertheless, the viability gradually decreases to about 50% and remains at that level for at least several weeks. The mitotic and flash thymidine-^3H LI's decrease appreciably but never reach zero, and cell division is balanced by cell death.

Effect of Agitation

If left undisturbed, the cells settle to the bottom of the culture vehicle where they grow in clumps which increase progressively in size as they approach their maximum population density. During log phase, the grossly visible clumps contain several hundred to about 10,000 cells which can easily be dispersed by shaking. If the culture is not shaken until the cells approach stationary phase, vigorous shaking results in a higher cell concentration, probably by promoting better diffusion of oxygen and other nutrients. The cells in shaken cultures settle out in smaller aggregates which adhere to each other and form a 3-dimensional, chain-like labyrinth on the bottom of the flask. The influence of shaking on the final cell concentration is illustrated by the following experiment.

After subculturing, cells were fed daily (shaking only after changing the medium) until they reached stationary phase at about 2.6 x 10^6 cells/ml. Then 20 ml of the suspension were added to each of 18 commercial 100-ml media bottles. Half of the bottles (Group A) were shaken approximately every 2 hr; the others (Group B) were not disturbed after the start of the experiment. The medium was changed at the start of the experiment and 22 and 34 hr later by gentle pipetting to avoid disturbing the (Group B) cells. For prevention of cell loss, Group A cells were not shaken for 4 or 5 hr prior to the medium change. When thymidine-^3H was added to the Group B cells, the label was added dropwise with very gentle stirring to minimize disturbance of the clumps.

The results are shown in Chart 3. The cell concentration, mitotic and flash thymidine-^3H LI's were greater in the shaken than in the undisturbed bottles.

Effect of Bottom Surface Area

When the volume of fluid is relatively large as compared to the surface area of the bottom of the culture vehicle, the cells reach stationary phase at a relatively low concentration even if fed and shaken daily, and the clumps become almost confluent on the bottom of the flask. For example, if 1 liter of medium...
is added to a 4-liter Erlenmeyer flask (bottom surface area, 250 sq cm; ratio of volume to surface area, 4:1) with a starting cell concentration of 1 to 2 \( \times 10^5 \) cells/ml, the culture will generally not exceed a final concentration of 2 to 2.5 \( \times 10^6 \) cells/ml of medium (equalling 2.5 \( \times 10^9 \) total cells in flask or 10\(^7\) cells/sq cm of bottom surface area). If the volume of culture fluid is relatively less (e.g., 100 ml/500-ml Erlenmeyer flask; bottom surface area approximately 56 sq cm; ratio of volume to surface area, 1.8:1), and the cells are fed and shaken daily, the cells will grow to a higher concentration before reaching stationary phase (about 5.5 \( \times 10^6 \) cells/ml of medium), but the maximum concentration attainable per unit of bottom surface area is still about the same (5.5 \( \times 10^8 \) total cells in flask or approximately 10\(^7\) cells/sq cm). If, on the other hand, cells are grown in only 10 ml of medium in a 250-ml flask (bottom surface area about 35 sq cm; ratio of volume:surface area, 1:3.5) and fed and shaken daily, the final concentration reached will be about 7 \( \times 10^6 \) cells/ml of medium. However, in this case there will only be about 2 \( \times 10^6 \) cells/sq cm of bottom surface area, and the cell clumps do not become confluent.

In the 1st 2 experiments, there was excess nutrient, and growth was limited by the bottom surface area, but in the 3rd case, nutrient deficiency seemed to be the growth-limiting factor.

Medium depth per se is relatively unimportant since cells will grow to equally high concentrations (per ml of medium) in different flasks in which the depth varies from 1 to 10 cm, providing they do not exceed the maximum concentration attainable with respect to the bottom surface area (about 10\(^7\) cells/sq cm).

### Cell Cycle Measurements

Cell cycle (labeled mitoses) studies were performed during each phase of population growth. Charts 4 and 5 show the growth of the cultures in preparation for the different studies.

**Log Phase.** Cells were suspended in fresh prewarmed medium at a concentration of 2 \( \times 10^5 \) cells/ml. After daily feeding for 3 days, the concentration was readjusted to 2 \( \times 10^5 \) cells/ml, and the labeling experiment was begun. In order to ascertain the cytokinetic stability of the population, the labeled mitoses experiment was performed twice, the 2nd time 18 months after the 1st (Chart 4, *Logarithmic Phase II*).

The labeled mitoses curves are shown in Chart 6, and the cell cycle measurements derived from these curves are given in Table 1 together with the population-doubling times.

**Semistationary Phase.** After a lag period of 1 day following subculturing, the cells grew exponentially for about 2 days (Chart 4). They were fed with fresh medium daily, beginning on the 3rd day when the cell concentration was about 8 \( \times 10^5 \) cells/ml; by this time the growth rate had decreased, but the population was still increasing slowly during the cell cycle study which was started on Day 7.

The 1st wave of labeled mitoses is broader and somewhat less sharply defined in the semistationary culture (Chart 6) than in log phase, and the broadened character of the 2nd wave in the former population suggests that the G\(_1\) phase has become highly variable. Because the peak of the 2nd wave failed to reach the 50% level, \( T_G \) was estimated by equating it to the time interval between the maximum labeling percentages of the 2 waves. The resulting estimate of \( T_G \) as 34 hr was confirmed by determining the time interval between the midpoint of the 50% levels of the 1st wave (12 hr) and the midpoint of the 30% levels of the 2nd wave (45.6 hr); this interval is 33.6 hr. The cell cycle measurements are summarized in Table 1.
Stationary Phase. Three experiments were performed, differing in the methods used to reach a steady state. For Experiments I and II, the cells were fed daily (with shaking only after feeding) until their concentration reached a plateau of 2.3 and 2.7 x 10^6/ml, respectively (Chart 5). Under these conditions, there was excess nutrient, and growth was limited by the bottom surface area. The cell concentrations were adjusted to 3.0 and 4.7 x 10^6/ml, respectively, and labeled mitoses experiments were performed on each (Chart 7).

For Experiment III, the cells were fed only twice (Chart 5). Net population growth ceased by Day 6 at a concentration of about 1.5 x 10^6/ml, and the cell cycle study was carried out (with frequent shaking) between Days 7 and 9 (Chart 7). In this case, the cells stopped growing before they had reached their potential maximum concentration with respect to bottom surface area (i.e., at 6 x 10^6 cells instead of 10^7 cells/sq cm), and nutrient deficiency was probably the main growth-limiting factor.

The stationary phase populations exhibited broad, highly variable 1st waves. The average durations of the G2 and S phases varied greatly under the different experimental conditions (Table 1). The prolongation of the S phase was especially marked in Experiment III as cell viability declined, presumably largely because of nutrient deficiency.

Constancy of Rate of DNA Synthesis

In order to obtain some estimate of the average rate of DNA synthesis as a function of position within the S phase, log and semistationary cells were given a 30-min pulse of thymidine-3H, and then grain counts of cells in mitosis were determined at intervals (Chart 8). Whereas there was considerable variability in the labeling intensity of individual cells at any given location in S, the median grain count was fairly constant except at the beginning and end of the S period when it was reduced. While it is possible that a marked deviation in the median grain count during the S phase might be due to factors such as variation in intracellular pool sizes, activity of thymidine kinase, etc., rather than changes in the rate of DNA synthesis, it appears unlikely that a relatively constant median grain count level would fortuitously result from such factors if the average rate were in fact variable.

Our findings are similar to those reported by Alpen and Johnston (1) for nucleated erythroid cells in dogs.

Determination of Generation Time by Grain Count-halving Method

Median grain counts of interphase cells during the labeled mitoses experiments are plotted in Chart 9. The generation time estimates are 24 hr for the log phase and 35 hr for the semistationary phase cells.

Continuous Infusions of Thymidine-3H

Continuous infusions of thymidine-3H were given during each growth phase under conditions similar to those described above (as shown in Charts 4 and 5). The curves of the percentages of labeled cells increased linearly at first (Chart

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>LI (%)</th>
<th>MI (%)</th>
<th>T_G, labeled mitosis (hr)</th>
<th>T_S (hr)</th>
<th>T_G1 (hr)</th>
<th>T_G2 (hr)</th>
<th>T_M (hr)</th>
<th>T_G1-halving time (hr)</th>
<th>T_M-halving time (hr)</th>
<th>Doubling time (hr)</th>
<th>T_S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Phase I</td>
<td>50</td>
<td>2.1</td>
<td>18</td>
<td>12</td>
<td>3.7</td>
<td>1.8</td>
<td>0.50</td>
<td>24</td>
<td>26</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Log Phase II</td>
<td>52</td>
<td>1.9</td>
<td>20</td>
<td>13</td>
<td>4.2</td>
<td>2.3</td>
<td>0.50</td>
<td>35</td>
<td>55</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Semistationary phase</td>
<td>29</td>
<td>1.1</td>
<td>34</td>
<td>16</td>
<td>13.7</td>
<td>3.7</td>
<td>0.60</td>
<td>35</td>
<td>55</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Stationary Phase I</td>
<td>26</td>
<td>0.7</td>
<td>24</td>
<td></td>
<td>4.7</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stationary Phase II</td>
<td>24</td>
<td>0.6</td>
<td>32</td>
<td></td>
<td>6.6</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stationary Phase III</td>
<td>26</td>
<td>0.5</td>
<td>42</td>
<td></td>
<td>3.6</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aT_M = (MI/LI) X T_S.
Transition from Stationary to Logarithmic Growth Phase

Cells were grown to a stationary concentration of $4.5 \times 10^6$ cells/ml with daily feeding. An aliquot of $3.2 \times 10^8$ cells was then diluted in 1000 ml of prewarmed fresh medium, and the experiment was begun (Chart 11). The cell concentration remained almost constant for about 12 hr (lag phase) before beginning to increase exponentially; by the end of 72 hr, the concentration had reached $1.5 \times 10^6$ ml, indicating that slightly more than 2 doublings had occurred. The MI increased abruptly 5 to 6 hr after dilution of the cells but then leveled off at about 1% for about a day before increasing further to around 2%. The flash thymidine-$^3$H LI increased rapidly after

10, although with decreasing slopes for log, semistationary, and stationary phases, respectively.

The rates of change of the slopes of the curves as they approach their final values give some indication of the variability of generation times; a more gradual change suggests a larger variability, particularly of G$_1$. Thus the $T_G$ variability apparently increases from log to semistationary to stationary phase.

Both the semistationary and stationary phase labeling curves appear to have leveled off prior to the completion of the infusions, suggesting that about 8% of the semistationary cells and 17 to 28% of the stationary cells were in a dormant state (i.e., in G$_0$ or in a greatly prolonged G$_1$ phase).
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Viable Cell Count

Chart 11. Transition from stationary to log phase. An aliquot of stationary phase cells was diluted in 1000 ml of fresh prewarmed medium in a 4-liter Erlenmeyer flask at a starting concentration of $3.2 \times 10^7$/ml. The cells were shaken every 2 to 4 hr but not fed for the duration of the experiment. Viability remained over 90% throughout. Small aliquots were taken at intervals for determination of cell count, MI, and flash thymidine-3 H LI.

a 12-hr lag and then remained at about 50% during log phase growth. As the cells again approached stationary phase, both the mitotic and flash LI's again declined.

In another experiment, stationary cells were diluted in fresh prewarmed medium to a starting concentration of $1.8 \times 10^7$/ml, and the culture was then infused continuously with thymidine-3 H for 53 hr (Chart 12). As in the preceding experiment, the cells again lagged for about 12 hr before growing exponentially to $7.6 \times 10^7$/ml at 53 hr (>2 doublings); after about 4 hr, the thymidine-3 H LI began to increase rapidly from the stationary level (about 35%) and reached 95% at 20 hr; after 40 hr, 98 to 99% of the cells were labeled.

The following observations indicate that the great majority of cells in early stationary phase (before viability declines) are capable of resuming log phase growth: (a) very few dead cells were found in either experiment; (b) almost all cells were labeled by the end of the 1st day in the 2nd experiment; and (c) including the lag phase, the doubling time in the 2nd experiment was about 24 hr (the starting cell concentration was too high to permit more than about 2 doublings in the 1st experiment).

GF during Different Growth Phases

The GF was estimated by 3 methods.

Comparison between $T_S$ Ratio and Flash LI. According to this method (29), the GF is equal to the ratio of the observed flash LI to the expected LI of the proliferating cell component. On the assumption that the proliferating cells can be approximated by a simple steady state model, the expected LI is equal to $T_S$. Thus $\text{GF} = \text{LI} + \left(\frac{T_S}{T_G}\right)$.

Utilizing the data from Table 1, we have the following estimates of GF:

\[
\text{Log Phase I, GF} = \frac{50}{0.67} = 0.75
\]

\[
\text{Semistationary phase, GF} = \frac{29}{0.47} = 0.62
\]

\[
\text{GF estimates for stationary phase cells were not obtained, as } T_G \text{ could not be measured. It is evident that this method yields GF values considerably less than the maximum LI reached during continuous infusion of thymidine-3 H (Chart 10).}
\]

Method of Mendelsohn. In this method (37), the GF is equal to the ratio of LI of interphase to mitotic cells, determined after the labeled mitoses curve has decayed to a relatively constant level. The GF estimates are:

\[
\text{Log Phase I, GF} = 0.85
\]

\[
\text{GF estimates for stationary phase cells were not obtained, as } T_G \text{ could not be measured. It is evident that this method yields GF values considerably less than the maximum LI reached during continuous infusion of thymidine-3 H (Chart 10).}
\]

Chart 12. Continuous infusion of thymidine-3 H during transition from stationary to log phase. An aliquot of stationary cells was diluted in 1000 ml of fresh prewarmed medium in a 4-liter Erlenmeyer flask at a stationary concentration of $1.8 \times 10^7$/ml. Thymidine-3 H was infused at a constant rate of 0.01 μCi/ml of medium per hr. The cells were shaken every 2 to 4 hr but not fed during the experiment. Viability remained over 85% throughout.

\[
\text{Continuous Infusion Method. This method utilizes the observed initial rate of increase of interphase LI during continuous thymidine-3 H exposure for estimating the GF. The initial fractional rate of increase of the LI of the proliferating cell component is equal to the observed initial rate of LI increase of the total population, divided by the GF, provided that this initial rate is determined sufficiently early so that no labeled cells will have divided. The former rate is approximately equal to the inverse of the generation time of the proliferating component (neglecting } T_G \text{ variability). Thus } \left(\frac{1}{T_G}\right)^{-1} = \frac{R_0}{\text{GF}} \text{ or } \text{GF} = \frac{R_0}{T_G}, \text{ where } R_0 \text{ is the observed (initial) rate of increase in the LI during the infusion.}
\]

With the values of $R_0$ obtained from the data in Chart 11 and the labeled mitoses $T_G$'s from Table 1, we obtain:

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Method of Mendelsohn. In this method (37), the GF is equal to the ratio of LI of interphase to mitotic cells, determined after the labeled mitoses curve has decayed to a relatively constant level. The GF estimates are:

\[
\text{Log Phase I, GF} = 0.85
\]

\[
\text{Semistationary phase, GF} = 0.76
\]
Log Phase (with average $T_G$, 19, of I and II), GF = 0.052 × 19 = 0.99

Semistationary phase, GF = 0.026 × 34 = 0.89

These results compare quite well with the maximum LI values achieved during continuous infusion (Chart 10). However, the GF estimates are highly sensitive to small changes in $R_0$, and it is possible that the results are in part fortuitous.

The differences between the estimates of GF with the various methods are probably due to the fact that each is based on a different set of simplifying (and perhaps invalid) assumptions. A more realistic mathematical model of this system has been proposed (23) and should reduce uncertainty in the GF estimate.

**DISCUSSION**

The rate of growth of cell populations both in culture and in experimental tumor systems invariably decreases as the population density approaches some maximum value (20, 21, 24, 27, 33, 58–60). The mechanism of growth retardation may vary in different systems. In some tumors, the decreased growth rate is due mainly to a smaller GF (24, 52), whereas in others it is due primarily to a prolongation of the cell cycle (6, 24, 31, 52, 60).

The cytokinetic changes which occur in cultured cells as they become confluent also differ among 3T3 (28, 54) and other monolayer cells (27, 30, 34, 35), as well as among multilayer cells (8) or cells in suspension culture (58). It is therefore of interest to compare the behavior of human hematopoietic cells with that of other mammalian cell lines and to analyze the possible mechanisms of growth control.

The generation time of log phase SK-L7 cells is about 19 hr, and their doubling time is about a day (Table 1); almost all cells are dividing, and the majority of them show little variability in $T_G$ during log phase growth. It was difficult to obtain an accurate estimate of $T_G$ in semistationary phase with the labeled mitosis method, because the 2nd wave was damped, probably because of variability in $G_1$. We therefore compared the applicability of the median grain count-halving time method for estimating $T_G$ with the labeled mitoses method (Charts 6 and 9). The results from the former were only slightly longer than those obtained from the labeled mitoses curves (Table 1); this is in agreement with results which we have obtained with several other cell lines.

The generation time measured from consecutive labeled mitotic waves of log phase SK-LN1 cells [derived from normal lymphocytes (9)] was 17 hr and that of log phase SK-RCS1 cells [derived from reticulum sarcoma cells (17)] was 25 hr. The durations of the S phases were 11 and 15 hr, respectively, in these 2 cell lines (56). Aoki and Moore (2) measured the cell cycle during log phase in 6 established human hematopoietic cell lines. Their estimates of S were similar to our results, but the population-doubling times (generation times were not measured directly) were generally longer than we found. This may be because they started their measurements at higher cell concentrations (3 to $6 \times 10^5$ cells/ml) and growth may have already begun to slow.

As SK-L7 cells approach stationary phase, the cell cycle becomes progressively longer and more variable. While the mean generation time doubled as the cells passed from log to semistationary phase, $T_G$ increased by one-third, $T_G$ doubled, and $T_G$ increased by a factor of almost 4 (Table 1). $G_1$ also appears to have undergone the greatest increase in variability. Although the mitotic time was slightly prolonged during stationary phase, the fact that the MI decreased proportionally more than the LI (Table 1) indicates that the duration of mitosis is altered the least. The GF also decreases, as evidenced by the fall in the flash thymidine-3H LI (despite the prolonged S phase) and by the lower final LI following continuous infusion of thymidine-3H. Regardless of the uncertainty about the precise change in GF that occurs as the cells approach stationary phase, it is nevertheless evident from the continuous thymidine-3H infusion experiments that most of the cells continue to progress slowly through the cell cycle, with the greatest delay occurring in passage from $G_1$ to S. There is therefore no complete halt in any particular phase of the cell cycle, as has been observed in some other systems during density-dependent inhibition of cell growth (26, 34, 35, 53, 54).

If stationary SK-L7 cells are fed and shaken frequently, proliferation slows but does not entirely stop, and the cell concentration remains static as proliferation is balanced by cell death (Chart 2). If cells that have reached stationary phase are rescued by dilution in fresh medium before their viability has declined, almost all will recover and resume exponential growth (Charts 11 and 12).

The following factors have been reported to be important in the regulation of cell division as cells become confluent and the rate of growth slows or stops: (a) inhibitory factor(s), (b) nutrient exhaustion, and (c) cell density-dependent inhibition (size of clumps, cell density per unit surface area, etc.).

**Inhibitory Factor(s).** Inhibition of growth in crowded cultures has been attributed to accumulation of diffusible inhibitory factors (20, 30, 61) or to local changes in the medium in the immediate vicinity of the cells which are not necessarily reflected in the whole medium (39). Numerous attempts to demonstrate that SK-L7 cells produce a diffusible inhibitor were unsuccessful, although our experiments did not exclude the possibility that a locally active or short-acting and labile inhibitor might exist in crowded cultures. It is difficult to devise definitive experiments to demonstrate an inhibitor with the latter properties while excluding other growth-limiting factors. The precise role of specific inhibitors in regulating the growth of different cell types remains uncertain at present, and this subject represents an important challenge for future investigation.

**Nutrient Exhaustion.** Fasting has been shown to slow the cell cycle of tumor cells *in vivo*, and feeding will stimulate the initiation of cell growth or accelerate the phases of the cell cycle (5, 7). Hahn et al. (27), using HA2 Chinese hamster monolayer cells, found that unfed cultures reached a plateau of growth earlier and at a lower cell concentration than did fed cell cultures, although viability remained excellent. A large proportion of plateau cells were in $G_1$ (or a $G_1$-like phase), and prolongation of $G_1$ and striking increases in the length and variability of the S phase were observed. Watanabe and Okada
In this study, growth under conditions of nutrient deficiency (Chart 5, 
Stationary Phase III) was compared to that in a frequently fed culture (Chart 4, 
Semistationary Phase). The cell concentration reached a maximum significantly sooner in the unfed culture, in which the S phase 
(provided cells as well, as indicated by the low thymidine-3H LI) became markedly prolonged and variable (Chart 7). Under the given conditions, the G2 phase was not significantly prolonged by nutrient deficiency (Table I), in 
contrast to the results reported by others using different cell lines (5, 7, 27, 58, 62). The mitotic time of our cells was also 
very low saturation density, and the inhibited cells all remain 
early release of cells from an extended G2 phase, (e) 
interplay of multiple factors.

The kinetic behavior of SK-L7 cells during their semistationary growth phase appears to be most comparable to 
that of human leukemic cells in vivo during advanced disease (10, 12, 18). The full stationary phase in vitro is an extreme 
situation and is probably not applicable to any leukemic population in vivo except perhaps in rare situations when the disease has been left untreated and the patient is in extremis. The flash thymidine-3H LI's of semistationary phase cells in 
culture are significantly lower than those of log phase cells; similarly, acute leukemic cells in the marrow frequently have 
lower LI's during advanced disease than during early relapse, and their LI's are almost always lower than those of normal 
erythrocyte and granulocyte precursors (10, 12–14, 18, 29, 51). As SK-L7 cells approach stationary phase, the 2nd peak of 
labeled mitoses becomes progressively lower and more ill 
defined. Likewise, labeled mitoses curves of human leukemic 
cells during advanced disease in vivo show a well-defined 1st 
wave, but no 2nd wave is generally discernible (14). The lack of a clear 2nd wave indicates that the labeled cohort of cells 
when some cells were removed by scraping a line in a dense 
culture, the frequency of mitosis again increased.

The SK-L7 cell line (16) was initially diploid but later 
became near-tetraploid (38). Although it is uncertain whether 
the line originated from leukemic cells or from normal lymphocytes (9, 57), it has certain neoplastic properties, such 
as heterotransplantability into immunologically tolerant rats 
(48). The SK-L7 line is a good model system of a highly 
confluent cell population; its saturation density per unit 
(bottom) surface area is about 10^7 cells/sq cm, which is about 
10 times higher than that reached by 1S1 cells (8) and more 
than 100 times higher than that reached by 3T3 cells (54).

When SK-L7 cells are allowed to reach a maximum cell 
density without agitation, the durations of G2 and S and 
probably also G1 are prolonged, even though adequate nutrient is supplied (Charts 5 and 7, Stationary Phase I and II). 
Simply shaking the culture vehicle to disperse the clumps will 
to some extent enhance cell division, but the effect is transient 
and not great (Chart 3). A block in G2 has been observed in 
other cell systems, and several authors (5, 7, 26, 34, 35, 58) 
have emphasized the importance of the G2 phase in the 
regulation of cell proliferation. In this study, it appears that 
while dispersed cells are arrested and die rather selectively in 
G1 or S when they are deprived of sufficient nutrients, 
undisturbed (densely contacted) but adequately fed cells may 
show a relatively greater prolongation of G2 than of S. Under 
the latter conditions, impaired local diffusion of nutrient 
probably is a significant factor, but mere mechanical 
compression causes different cytokinetic effects than simple 
nutrient deficiency in dispersed cultures. It therefore appears 
that the various phases of the cell cycle are affected to 
different extents under different circumstances and that 
growth regulation is highly complex and dependent on the 
interplay of multiple factors.

Following release from a highly confluent condition by 
diluting the cells, the following sequence is observed: (a) an 
early release of cells from an extended G2 phase, (b) 
acceleration of passage of cells in G1 to S, and (c) shortening 
of the S phase and reconstitution of the whole (log phase) cell 
cycle.

The kinetic behavior of SK-L7 cells during their semistationary growth phase appears to be most comparable to 
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fails to divide in synchrony a 2nd time but enters a prolonged (and variable) G₁ phase (10).

The results of continuous thymidine-³H infusion experiments during semistationary phase are also similar to those found in vivo in acute leukemia during advanced disease (10, 12, 18). Because there is a significant fraction of unlabeled "resting" cells in a prolonged G₁ as the population approaches stationary phase (Chart 11), stationary phase cells are killed much less rapidly than log phase cells by S phase-active drugs such as hydroxyurea or arabinosycytosine (3, 25). Similarly, the rate of conversion of leukemic cells from G₁ to S is generally so slow during advanced disease that it usually takes 10 or more days of therapy with cytotoxic doses of such drugs to achieve the 2-log₁₀ cell kill which is generally necessary to cause a remission (11, 18).

Although there are many similarities to acute leukemia, the SK-L7 system is an imperfect model in 2 respects: (a) whereas SK-L7 cells are almost all capable of dividing repeatedly without maturing, only a fraction of acute leukemic cells in man can generally do so; there is good evidence that many undergo partial maturation and die spontaneously (10-12, 18). (b) Whereas almost all SK-L7 cells in early stationary cultures quickly resume logarithmic growth after their population density is lowered, acute leukemic cells in the marrow do not usually behave in this manner; after the latter's population density has been greatly reduced by drugs that kill the actively dividing cells, a significant fraction of surviving cells still persists in G₁ for extended periods and hence remains relatively insensitive to such drugs (11, 18). Nevertheless, despite these imperfections, the SK-L7 system appears to be a useful model in which to study growth-regulatory factors and chemotherapeutic effects, isolated from the enormous complexity of the marrow environment in vivo.

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Akio Todo, Annabel Strife, Jerrold Fried, et al.

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