10-6-1997

Cell cycle analysis and synchronization of pluripotent hematopoietic progenitor stem cells

G. Prem Veer Reddy
Cheryl Y. Tiarks
Lizhen Pang
University of Massachusetts Medical School

See next page for additional authors

Follow this and additional works at: http://escholarship.umassmed.edu/oapubs

Part of the Cancer Biology Commons, and the Oncology Commons

Repository Citation
Reddy, G. Prem Veer; Tiarks, Cheryl Y.; Pang, Lizhen; Wuu, Joanne; Hsieh, Chung-Cheng; and Quesenberry, Peter J., "Cell cycle analysis and synchronization of pluripotent hematopoietic progenitor stem cells" (1997). Open Access Articles. 285.
http://escholarship.umassmed.edu/oapubs/285

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in Open Access Articles by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
Cell cycle analysis and synchronization of pluripotent hematopoietic progenitor stem cells

Authors
G. Prem Veer Reddy, Cheryl Y. Tiarks, Lizhen Pang, Joanne Wuu, Chung-Cheng Hsieh, and Peter J. Quesenberry
Cell Cycle Analysis and Synchronization of Pluripotent Hematopoietic Progenitor Stem Cells

G. Prem Veer Reddy, Cheryl Y. Tiarks, Lizhen Pang, Joanne Wuu, Chung-Cheng Hsieh and Peter J. Quesenberry
Cell Cycle Analysis and Synchronization of Pluripotent Hematopoietic Progenitor Stem Cells

By G. Prem Veer Reddy, Cheryl Y. Tiarks, Lizhen Pang, Joanne Wuu, Chung-Cheng Hsieh, and Peter J. Quesenberry

Hematopoietic stem cells purified from mouse bone marrow are quiescent with less than 2% of Lin<sup>−</sup> Hoechst<sup>low</sup>/Rho<sup>low</sup>/Sca<sup>−</sup> cells in S phase. These cells enter proliferative cycle and progress through G<sub>0</sub> and into S phase in the presence of cytokines and 5% heat-inactivated fetal calf serum (HI-FCS). Cytokine-stimulated Lin<sup>−</sup> Hoechst<sup>low</sup>/Rho<sup>low</sup> cells took 36 to 40 hours to complete first division and only 12 hours to complete each of 5 subsequent divisions. These cells require 16 to 18 hours to transit through G<sub>0</sub>/G<sub>1</sub> period and 28 to 30 hours to enter into mid-S phase during the first cycle. Up to 56% of Lin<sup>−</sup> Rho<sup>low</sup>/Hoechst<sup>low</sup> cells are high-proliferative potential (7 factor-responsive) colony-forming cells (HPP-CFC). At isolation, HPP-CFC are quiescent, but after 28 to 30 hours of culture, greater than 60% are in S phase. Isoleucine-deprivation of Lin<sup>−</sup> Rho<sup>low</sup>/Hoechst<sup>low</sup> cells in S phase of first cycle reversibly blocked them from entering into second cycle. After the release from isoleucine-block, these cells exhibited a G<sub>1</sub> period of less than 2 hours and entered into mid-S phase by 12 hours. Thus, the duration of G<sub>1</sub> phase of the cells in second cycle is 4 to 5 times shorter than that observed in their first cycle. Similar cell cycle kinetics are observed with Lin<sup>−</sup>/Sca<sup>−</sup> population of bone marrow cells. Stem cell factor (SCF) alone, in the presence of HI-FCS, is as effective as a cocktail of 2 to 7 cytokines in inducing quiescent Lin<sup>−</sup>/Sca<sup>−</sup> cells to enter into proliferative cycle. Aphidicolin treatment reversibly blocked cytokine-stimulated Lin<sup>−</sup>/Sca<sup>−</sup> cells at G<sub>1</sub>/S boundary, allowing their tight synchrony as they progress through first S phase and enter into second G<sub>1</sub>. For these cells also, SCF alone is sufficient for their progression through S phase. These studies indicate a very short G<sub>1</sub> phase for stem cells induced to proliferate and offer experimental approaches to synchronize murine hematopoietic stem cells.

© 1997 by The American Society of Hematology.

MURINE PRIMITIVE long-term repopulating lymphohematopoietic stem cells have been characterized by the absence of differentiated lineage markers (Lin<sup>−</sup>), the presence of cell surface Ly-6 antigen (Sca<sup>+</sup>) and c-Kit, and relatively low levels of staining with the supravital dyes rhodamine-123 and hoechst-33342. Lin<sup>−</sup> cells expressing Sca or c-Kit or showing low staining with rhodamine (Rho<sup>low</sup>) and hoechst (Hoechst<sup>low</sup>) are relatively quiescent and enriched for multifactor-responsive high proliferative potential colony-forming cells (HPP-CFC), although using continuous bromo-deoxyuridine (BrdU) labeling, Bradford et al<sup>1</sup> have shown that most of the Lin<sup>−</sup> Rho<sup>low</sup>/Hoechst<sup>low</sup> cells have entered cell cycle over a 12-week time interval.

The primitive long-term repopulating cells can be induced to proliferate after in vivo exposure to cytotoxic agents, such as 5-fluorouracil (5-FU) or in vitro exposure to cytokines. Particular attention has focused on the possibility of using such proliferative stimuli to expand stem cells or to facilitate retroviral integration in gene therapies.<sup>2-7</sup> These manipulations can result in expansion and cycle activation of primitive surrogate stem cells, HPP-CFCs, but with a concomitant loss of long-term, but not short-term, in vivo engraftment potential,<sup>5,9</sup> and through in vivo hydroxyurea suicide experiments, it was shown that the cells engrafting into nonmyeloablated marrow, spleen and thymus were insensitive to hydroxyurea and, therefore, noncycling.<sup>10</sup> Spleen colony-forming ability of cycling cells was reported to be 50% less than that of normal marrow or velocity sedimented noncycling cells.<sup>11</sup> Studies using purified populations of stem cells showed that 100 G<sub>0</sub>/G<sub>1</sub> cells are capable of protecting 90% of lethally irradiated recipients, whereas the 100 S/G<sub>2</sub>/M phase cells protect only 22% of recipients.<sup>12</sup>

Although these observations show an engraftment defect in mitotically active hematopoietic progenitor/stem cells, they fail to fully establish a cause and effect relationship between the engraftment defect and the cell cycle status of stem cells induced to proliferate. To address this issue more critically, it is important that the same population of purified pluripotent hematopoietic stem cells (PHSCs) in their quiescent state be followed for their ability to engraft at specific phases as they traverse the cell cycle after proliferative stimulation. This would require not only a fuller understanding of the kinetics of cell cycle entry and progression of cytokine-stimulated quiescent PHSCs, but also developing procedures that allow synchronization and enrichment of PHSCs to represent specific phases of cell cycle after proliferative stimulation. In the present studies, we have characterized the cell cycle kinetics of Lin<sup>−</sup> Rho<sup>low</sup>/Hoechst<sup>low</sup> and Lin<sup>−</sup>/Sca<sup>−</sup> stem cells after exposure to a 4-cytokine cocktail consisting of interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor (SCF) or SCF alone and developed experimental conditions that allow their synchronization in early S phase of the first cycle.

MATERIALS AND METHODS

Purification of Lin<sup>−</sup>/Sca<sup>−</sup> and Lin<sup>−</sup> Rho<sup>low</sup>/Hoechst<sup>low</sup> cells from mouse bone marrow. Sorting of the Lin<sup>−</sup>/Sca<sup>−</sup> cells involved recovering marrow from tibiae and femora of 6- to 8-week old Balb/c or C57BLK6 mice under sterile conditions and enriching low-density populations can result in expansion and cycle activation of primitive surrogate stem cells, HPP-CFCs, but with a concomitant loss of long-term, but not short-term, in vivo engraftment potential,<sup>5,9</sup> and through in vivo hydroxyurea suicide experiments, it was shown that the cells engrafting into nonmyeloablated marrow, spleen and thymus were insensitive to hydroxyurea and, therefore, noncycling.<sup>10</sup> Spleen colony-forming ability of cycling cells was reported to be 50% less than that of normal marrow or velocity sedimented noncycling cells.<sup>11</sup> Studies using purified populations of stem cells showed that 100 G<sub>0</sub>/G<sub>1</sub> cells are capable of protecting 90% of lethally irradiated recipients, whereas the 100 S/G<sub>2</sub>/M phase cells protect only 22% of recipients.<sup>12</sup>

Although these observations show an engraftment defect in mitotically active hematopoietic progenitor/stem cells, they fail to fully establish a cause and effect relationship between the engraftment defect and the cell cycle status of stem cells induced to proliferate. To address this issue more critically, it is important that the same population of purified pluripotent hematopoietic stem cells (PHSCs) in their quiescent state be followed for their ability to engraft at specific phases as they traverse the cell cycle after proliferative stimulation. This would require not only a fuller understanding of the kinetics of cell cycle entry and progression of cytokine-stimulated quiescent PHSCs, but also developing procedures that allow synchronization and enrichment of PHSCs to represent specific phases of cell cycle after proliferative stimulation. In the present studies, we have characterized the cell cycle kinetics of Lin<sup>−</sup> Rho<sup>low</sup>/Hoechst<sup>low</sup> and Lin<sup>−</sup>/Sca<sup>−</sup> stem cells after exposure to a 4-cytokine cocktail consisting of interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor (SCF) or SCF alone and developed experimental conditions that allow their synchronization in early S phase of the first cycle.

From the Cancer Center and Department of Cell Biology, University of Massachusetts Medical Center, Worcester, MA.

Submitted September 19, 1996; accepted May 23, 1997.

Supported by National Institutes of Health Grants No. PO1HL-56920 and PO1DK-50222-01.

Address reprint requests to G. Prem Veer Reddy, PhD, Cancer Center, University of Massachusetts Medical Center, 373 Plantation St, Suite 202, Worcester, MA 01605.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1997 by The American Society of Hematology.
MAC-1, and GR-1 were also biotin-conjugated. Lineage-depleted cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti–SCA-1 antibodies (PharMingen, San Diego, CA) and phycoerythrin (PE) streptavidin (Biomedia, Foster City, CA) and sorted on FACS-STAR (Becton Dickinson, San Francisco, CA) to recover FITC-positive and PE-negative cells.

The overall procedure for sorting of Lin⁺, Rho⁺/-/Ho⁺/- cells from murine bone marrow is essentially as described by Wolf et al.¹³ Briefly, this procedure involved recovering lineage-depleted bone marrow cells as described above and labeling them with Rhodamine-123 and Hoechst-33342. The labeled cells were then sorted on FACS-STAR (Becton Dickinson) or on MoFlo (Cytomation, Fort Collins, CO) cell sorter to recover lowest 10% Rhodamine-123 staining and lowest 5% Hoechst-33342 staining cells.

In vitro culture and synchronization of hematopoietic progenitor/stem cells. Immediately after their purification, Lin⁻/Sca⁺ or Lin⁻, Rho⁺/-/Ho⁺/- cells were incubated at a density of 10 to 20 x 10⁶ cells/mL in Dulbecco’s Modified Essential Medium (DMEM; GibCO-BRL, Grand Island, NY) containing 5% heat-inactivated fetal calf serum (HI-FCS; HyClone Laboratories, Logan, UT), glutamine (2.5 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL), and appropriate cytokines at 37°C in a 5% CO₂ containing humidified incubator. Unless otherwise indicated, the concentration and the source of individual cytokines added in different combinations to the media in these studies were recombinant murine SCF (mSCF) at 100 ng/mL (R&D Systems, Minneapolis, MN), mIL-3 at 100 U/mL (Collaborative), mIL-6 at 50 U/mL (R&D), recombinant human IL-11 (hIL-11) at 50 ng/mL (Genetics Institute, Cambridge, MA), IL-1α at 250 U/mL (Hoffmann La Roche, Nutley, NJ), granulocyte-macrophage colony-stimulating factor (GM-CSF) at 2.5 ng/mL (R&D), granulocyte colony-stimulating factor (G-CSF) at 5 ng/mL (Collaborative), basic fibroblast growth factor (bFGF) at 5 ng/mL (R&D), and CSF-1 at 5,000 U/mL (Chiron, Emeryville, CA).

For synchronization of the cells at G₀/S boundary, hematopoietic stem cells exposed to the medium containing a cocktail of 7 cytokines (IL-3, IL-1α, G-CSF, GM-CSF, SCF, CSF-1, and bFGF) described above for 8 hours were treated with 2 µCi/mL of [³H]thymidine (200 µCi/mL) for 20 minutes at 37°C and then analyzed by an autoradiographic suicide assay. This assay was performed essentially as described by Quesenberry and Stanley.¹⁷ The number of cells in S phase were determined in this assay by subjecting an aliquot of cells in culture to a high specific activity [³H]thymidine (200 µCi/mL). Cells were included in 0.5 mL overlayer with a final agar concentration of 0.3%. Culture dishes with the cells were then incubated at 37°C for 14 days in the presence of 5% O₂, 10% CO₂, and 85% N₂ and scored for HPP-CFC using a dissecting microscope.

HPP-CFC typically generated colonies greater than 0.5 mm in diameter, consisting of tightly packed cells. Functionally, these are defined as primitive cells by their relative resistance to 5-FU, their synergistic growth factor requirements, and their copurification with long-term reconstituting cells in vivo.¹¹,¹⁶

H-thymidine suicide assay. This assay was performed essentially as described by Quesenberry and Stanley.¹⁷ The number of cells in S phase were determined in this assay by subjecting an aliquot of cells in culture to a high specific activity [³H]thymidine (200 µCi/mL) for 20 minutes at 37°C and determining their ability to form HPP-CFC in a double-layer agar system described above.

RESULTS

Lin⁻ Rho⁺/-/Ho⁺/- cells in culture with IL-3, IL-6, IL-11, and SCF evidence their first population doubling at between 36 and 40 hours, whereas subsequent population doublings occurred every 12 hours up to 100 hours (Fig 1). Details of the first cycle were determined by pulse labeling with [³H]thymidine. As shown in Fig 2, replicating and nonreplicating cells exhibit a distinct pattern of staining after autoradiography and Giemsa staining of pluripotent hematopoietic stem cells. Lin⁻ Rho⁺/-/Ho⁺/- cells in IL-3, IL-6, IL-11, and SCF (Fig 3A) or in a 7-cytokine cocktail consisting of IL-3, IL-1α, G-CSF, GM-CSF, SCF, CSF-1, and bFGF (Fig 3B) begin to enter S phase at approximately 16 to 18 hours, and the S phase peak is somewhere around 30 hours. In these experiments, about 60% of the cells were in S at 30 hours of culture using a 30-minute [³H]thymidine pulse label, but, when the cells were labeled over 4 hours (from 26 to 30 hours of culture), the nuclei of more than 90% of the cells were labeled with [³H]thymidine (data not shown), indicating that virtually all of the cells transit through the S phase of the first cycle during 30 hours of incubation in cytokines. Transition of all the cells incubated with cytokines through the first cycle is also indicated by a twofold increase in cell numbers (one doubling) by 36 to 40 hours (Fig 1). These data indicate that primitive Lin⁻ Rho⁺/-/Ho⁺/- cells, when stimulated to enter and transit cell cycle, have a G₁ phase of about 18 hours and a total cell cycle length of 36 to 40 hours. Subsequent cycles are very rapid.

Seven-factor responsive HPP-CFC represents a primitive surrogate stem cell assayed in vitro. We determined the
SYNCHRONIZATION OF PHSCs

Fig 1. Growth rate and the doubling time of Lin− Rho−/Ho− cells incubated in the presence of medium containing IL-3, IL-6, IL-11, and SCF. Immediately after their purification, Lin− Rho−/Ho− cells were suspended at 10 × 10^3 cells/mL in medium containing 4 cytokines and incubated at 37°C in a 5% CO2 containing humidified incubator as described in the Materials and Methods. At indicated intervals, duplicate aliquots of cells from growing culture were counted using a hemocytometer. The numbers in the parentheses of the insert indicate the number of experiments from which the values for that particular time point were obtained.

Fig 2. Representative fields of cells showing unlabeled and labeled nuclei at 0 hours (A) and 30 hours (B) after cytokine stimulation of Lin− Rho−/Ho− cells. Experimental procedure for the sorting of the stem cells, their labeling with ^3H-thymidine, autoradiography, and Giemsa staining are as described in the Materials and Methods. Cells with labeled nuclei are indicated by arrows.

Fig 3. Progression of cytokine-stimulated Lin− Rho−/Ho− cells through G1 and into S phase. Lin− Rho−/Ho− were incubated in medium containing a cocktail of either 4 cytokines (IL-3, IL-6, IL-11, and SCF; A) or 7 cytokines (IL-3, IL-1α, GM-CSF, G-CSF, SCF, FGF, and CSF-1; B), and at indicated intervals duplicate aliquots of cells were analyzed for ^3H-thymidine incorporation followed by autoradiography to assess the percentage of cells with labeled nuclei as described in the Materials and Methods. Each set of symbols in (A) represents observations from separate individual experiments.
Table 1. Cell Cycle Status of Lin<sup>−</sup>Rho<sup>low</sup>/Ho<sup>low</sup>-Derived HPP-CFC Cultured in Cytokines

<table>
<thead>
<tr>
<th>Time in Culture</th>
<th>HPP-CFC/100 Cells*</th>
<th>% Killed by 3H-TdR&lt;sup&gt;²&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>28 ± 13</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>28-30 h</td>
<td>31 ± 7</td>
<td>60 ± 10&lt;sup&gt;î&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Seven-factor±responsive HPP-CFC per 100 cells from initially isolated Lin<sup>−</sup>Rho<sup>low</sup>/Ho<sup>low</sup> cells and after 28 to 30 hours in culture with IL-3, IL-11, IL-6, and SCF (see the Materials and Methods).

† Cell cycle status of HPP-CFC as determined by 3H-TdR suicide. These data are from four separate experiments with 4 to 10 culture plates per group in each experiment.

<sup>î</sup> Significantly different from 0 hours value (Wilcoxon rank-sum test; P ≤ .0209).

36 hours with IL-3, IL-6, IL-11, and SCF were subjected to isoleucine-deprivation to arrest them in early-G<sub>1</sub> of subsequent cycle. We observed that isoleucine-deprivation for 26 to 30 hours effectively limited stem cells completing first S phase from progressing through the subsequent G<sub>1</sub> and entering into the second S phase (Fig 4). During this entire period, the cell number doubled once, indicating their arrest immediately after the first cycle, and their viability remained unchanged as determined by their ability to exclude Trypan-Blue. When isoleucine-deprived cells were refed with complete medium, they immediately entered into S phase (Fig 5), exhibiting a short (1 to 2 hours) G<sub>1</sub> for cells that have completed first cycle.

We also evaluated an alternate population of purified hematopoietic stem cells, Lin<sup>−</sup>/Sca<sup>+</sup> cells, stimulated by SCF, SCF plus IL-3, or a 7-cytokine cocktail (Fig 6). Here, approximately 10% of the purified cells were labeled with 3H-thymidine at time 0 in culture, indicating a less quiescent population than that represented by Lin<sup>−</sup>Rho<sup>low</sup>/Ho<sup>low</sup>. The
general profile and the temporal order of their progression through G0/G1 and into S phase is found to be essentially similar to that seen with Lin− Rho−/Ho−/Sca+ cells. Furthermore, the lag period of 16 to 18 hours and the fraction of cells entering into S phase at 28 to 30 hours observed in the presence of SCF alone remained unchanged when SCF was combined with IL-3 (2-cytokine combination) or with IL-3, GM-CSF, IL-1α, bFGF, CSF-1, and G-CSF (7-cytokine combination). Thus, SCF alone in the presence of 5% HI-FCS is as effective as multiple cytokine combinations for inducing the progression of Lin−/Sca+ cells from a quiescent state into proliferative cycle and into S phase. However, supplementing SCF containing media with HI-FCS was found to be essential for cell viability, because, in its absence, a progressive cell death was observed during the first 24-hour incubation period (data not shown). Similarly, HI-FCS alone in the medium, without SCF, was not sufficient for the proliferative stimulation of the cells (data not shown). Thus, these cells require a minimum of serum factors and SCF for their entry into and subsequent progression through the cell cycle.

As can be seen in Fig 6, 50% to 60% of Lin−/Sca+ cells entered synchronously into S phase by 30 hours after cytokine stimulation. To achieve more synchronous entry of cells into first S phase and to establish procedures that allow the maintenance of stem cell synchrony as they complete first S phase and enter into subsequent cycle, we examined the ability of aphidicolin, an inhibitor of DNA polymerase α,18 to reversibly block cytokine stimulated Lin−/Sca+ cells at G1/S boundary. As shown in Fig 7, Lin−/Sca+ cells exposed to a combination of 7 cytokines were limited from entering into S phase after aphidicolin treatment from 8 to 32 hours after cytokine stimulation. This is in contrast to more than 50% of the cells being in S phase in the absence of aphidicolin during the same incubation period (Fig 6). When the cells were released from aphidicolin block, they entered into S phase without any detectable lag period, indicating their arrest at G1/S boundary or in early-S phase in response to aphidicolin. These cells retained a tight synchrony as they completed S phase and traversed through the subsequent phases of cell cycle (Fig 7). Furthermore, as in cytokine-stimulated cells, SCF alone was as effective as the 7-cytokine cocktail in allowing the progression of aphidicolin-synchronized Lin−/Sca+ cells through S phase.

**DISCUSSION**

The present data indicated that highly purified Lin− Rho−/Ho− murine lymphohematopoietic stem cells are quiescent at isolation, but with exposure to a cytokine combination of IL-3, IL-6, IL-11, and SCF, enters and progresses through cell cycle with an initial cycle length of 36 to 40 hours. Subsequent cycles appear to be about 12 hours in length. These data were based on cell numbers and 3H-thymidine labeling and their functional significance was confirmed by induction of quiescent 7-factor HPP-CFC into S phase. Given the length of S phase, these data indicate that essentially all cells traversed S phase. In other studies using whole unseparated marrow exposed to the same cytokine combinations, we have shown that, at 48 hours of culture, the cells show very poor long-term engraftment in either irradiated or normal hosts, but more recent studies indicate that this engraftment defect is reversible over time, although it is virtually impossible to rule out some degree of differentiation within the stem progenitor classes in these experiments. The data cited above indicate that the population retains stem cell-like properties through multiple cell cycle transits. A detailed analysis of the first cell cycle transit indicates a G1 phase of about 18 hours and a total cycle length of 36 to 40 hours. Given the relative fixed minimum of 7 to 8 hours for S, 2 hours for G2, and 1 hour for M, the G1 in the subsequent cycle must be very short, in the range of 1 to 2 hours. The isolate deprivation studies (see below) add further evidence for a very short G1 in cytokine-stimulated Lin− Rho−/Ho− cell cycles after the first cell cycle transit.

Dogma suggests that Lin− Rho−/Ho− cells are dormant and not easily stimulated to divide, but our data suggest that all of these cells are easily induced to enter cell cycle. Studies by Hendriks et al19 on transplantation of highly purified murine marrow stem cells and using PKM-H26 fluorescence labeling indicate that the majority of engrafted cells have divided over a 24-hour period after intravenous infusion. These studies suggest cycle times for primitive stem cells stimulated from dormancy of 24 to 48 hours. This is to be contrasted by recent studies by Bradford et al,1 using prolonged continuous labeling with BrdU, indicating that the average turnover time of Lin− Rho−/Ho− cells in vivo was 4.3 weeks with a time to 50% cycle of 2.75 weeks. These investigators also reported that, over a 12-week labeling period, 89% of Lin− Rho−/Ho− cells incorporated BrdU. This is, of course, a steady-state situation, but these data do not distinguish between a prolonged cell cycle for most cells or...
an intermittent entrance of dormant cells into cell cycle with a shorter cycle length.

Studies of 7-factor–responsive HPP-CFC indicate that this surrogate stem cell was dormant in freshly isolated Lin−Rho−/Ho− cells but was clearly transiting cell cycle by 28 to 30 hours of culture in IL-3, IL-6, IL-11, and SCF. The percentage of HPP-CFC in cycle (Table 1) closely approximated the percentage of Lin−Rho−/Ho− cells that labeled with 3H-TdR (Fig 3).

As shown in Fig 3, cytokine-stimulated cells entered synchronously into S phase, with 55% to 60% of them in S phase at 30 hours. During 90 to 100 hours of incubation with cytokines after the first cycle, about 60% of cells continued to be in S phase as determined by autoradiography analysis of the cells pulse-labeled with 3H-thymidine at 12- to 14-hour intervals (Fig 4, data not shown). This indicates either that a partial synchrony of the cells persisted as they traversed through the subsequent cycles with maximum number of cells in S phase at every 12 to 14 hours examined or that the cells after first cycle grew exponentially, with 60% of the cells in S phase through the remainder of the proliferative period. The latter possibility implies that the S phase of exponentially growing cells constitutes greater than 60% of total cell cycle time during each division after the first cycle. The doubling time of 12 hours with a S period of 7 to 8 hours after the first division observed in the present studies (Fig 1) is consistent with such a possibility.

Alternate populations representing Lin−/Sca+ cells stimulated with SCF alone, SCF plus IL-3, or a cocktail of 7 cytokines also exhibited cell cycle kinetics similar to those observed with Lin−Rho−/Ho− cells. However, unlike Lin−Rho−/Ho− cells, Lin−/Sca+ cells were heterogeneous, with 10% to 15% of them in S phase immediately after their sorting (Fig 6). Their entry into, and progression through, the first cycle essentially remained the same whether they were stimulated with SCF alone or with 7 cytokines. A partial synchrony of these cells was maintained as they traversed through G1 into S phase (Fig 6).

To enrich PHSCs in first S and to acquire the synchrony that can be sustained as the cells transit through the first G2 and M phases, we tested the ability of aphidicolin to reversibly block Lin−/Sca+ cells at their first G1/S boundary. Selective antimitabolites, including aphidicolin (inhibitor of DNA polymerase α), hydroxyurea (inhibitor of ribonucleotide reductase), and methotrexate (inhibitor of dihydropolate reductase), that inhibit DNA replication and DNA precursor synthesis have been used to synchronize a variety of mammalian cells at G1/S boundary.20–21 Our observations show that Lin−/Sca+ cells, like other cell systems, respond to a secondary synchronization at G1/S boundary after aphidicolin treatment (Fig 7). Considering its selective inhibitory effect on DNA polymerase α and its ineffectiveness in altering G1- associated erythroid differentiation of MELC cells in vitro,22 aphidicolin may not show secondary effects that are independent of cell cycle status that it induces.

We tested both the serum-deprivation and the isoleucine-deprivation methods that are commonly used to achieve G0/G1 phase arrest of a variety of cells, including hematopoietic progenitor cells,23–25 for their ability to block PHSCs completing first S phase from entering into second proliferative cycle. Whereas serum (HI-FCS)-deprived PHSCs experienced a progressive death with time (data not shown), isoleucine-deprivation reversibly blocked them in a nonproliferative state after first division (see Figs 4 and 5). As compared with PHSCs in their first cycle, those released from isoleucine-block entered into S phase with a shorter lag (G1) period.

Thus, these studies describe essential cytokine and serum conditions required for the synchronous progression of purified PHSCs from their initial G0/G1 phase into S phase and identify for the first time two specific approaches to synchronize PHSCs at G1/S boundary of their first cycle (aphidicolin-induced synchrony) and at G0/G1 phase of their second cycle (isoleucine-deprivation–induced synchronization of the cells completing first S phase). They also show a relatively long cycle time from dormancy of primitive Lin−Rho−/Ho− hematopoietic stem cells with a subsequent rapid cycle transit and short G1. Given the apparent fluctuation of engraftment with cell cycle status, these results have important implications for clinical marrow transplantation and gene therapy strategies.

ACKNOWLEDGMENT

The authors are thankful to Christina Grimaldi, Judy Reilly, and Dr Ruud Hulspas for their help in sorting bone marrow cells.

REFERENCES

8. Peters SO, Kitter EL, Ramshaw HS, Quesenberry PJ: Murine marrow cells expanded in culture with IL-3, IL-6, IL-11, and SCF acquire an engraftment defect in normal hosts. Exp Hematol 23:461, 1995
12. Flemming W, Alpern EJ, Uchida N, Ikuta K, Spangrude GJ,


