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Detection of incorporated iododeoxyuridine in colonies by immunoperoxidase staining: A novel method to measure the proportion of cycling colony-forming cells

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Abstract

In vitro suicide by tritiated thymidine (3H-TdR), hydroxyurea (HU), or cytosine arabinoside (Ara-C) is assumed to reflect the proportion of colony-forming cells in S-phase at the time of exposure. However, these techniques are not always accurate. Nonradioactive iododeoxyuridine (IdUrd) is incorporated into DNA during S-phase and can be detected by monoclonal antibodies. In the present study, a new IdUrd application was developed to investigate the kinetics of hematopoietic progenitor cells. After incubation with IdUrd, colony-forming cells were cultured in semisolid assay. An immunoperoxidase staining protocol was developed to detect IdUrd in cells of colonies in agar. Colony-forming cells in S-phase during the IdUrd exposure were postulated to give rise to IdUrd+ colonies, whereas non-S-phase cells would generate IdUrd− colonies. Toxicity, sensitivity, and IdUrd inactivation studies indicated that progenitor cells could safely be pulse-labeled for 2 hours with 40 μM IdUrd, whereas prolonged labeling with 1 μM IdUrd was at least feasible for 5 days.

Molt-4 cells and normal bone marrow cells were used to compare IdUrd pulse-labeling with 3H-TdR suicide. Part of the Molt-4 cells were enriched for G1- and S-phase cells by counterflow centrifugation. The bone marrow cells were either unstimulated or stimulated with growth factors. As a result, the accuracy of both techniques could be tested in populations with different quantities of S-phase cells. Wide confidence intervals of the suicide technique contrasted with the small confidence intervals obtained with IdUrd pulse-labelling. For instance, the fraction of Molt-4 cells with 27.8% S-phase cells contained 17.7% (confidence interval −8.2 to 43.6%) clonogenic cells in S-phase when determined with 3H-TdR suicide. Of this fraction, the percentage of clonogenic cells in S-phase was 36.2% with a confidence interval of 25.5 to 43.6% when determined with IdUrd pulse-labelling. In our hands, the IdUrd pulse-labelling was more accurate than the 3H-TdR suicide technique.

Thus far, kinetic studies of progenitors have been limited to the determination of the fraction of S-phase cells by suicide techniques. By prolonged IdUrd labeling, it is now possible to determine the proliferating fraction of progenitor cells. Insight into the proliferative behavior of progenitors will help to understand the biology of the hematopoietic system under physiologic and pathological conditions. Furthermore, therapeutic interventions, such as exposure to cytostatic drugs or growth factors, can be evaluated. The proliferative status of progenitors can be studied in vitro by suicide techniques [2,3]. After short-term exposure to 3H-TdR, HU, or Ara-C, the loss of progenitor cells is determined by colony-forming assay. The fractional reduction in cloning efficiency is considered to reflect the proportion of colony-forming cells in S-phase at the time of exposure. The estimation of progenitors in S-phase by suicide techniques may not always be accurate [2,4,5]. It has been reported that non-S-phase cells, probably late G1 cells, can take up 3H-TdR and eventually be killed [4,5]. Nevertheless, the commonly applied 3H-TdR suicide seems to be the most reliable of the available techniques [4].

Previously, IdUrd has become available for kinetic studies. This nonradioactive thymidine analog is incorporated into DNA during S-phase and can be detected by anti-IdUrd antibodies [6,7]. Using flow cytometry (FCM) or immunocytochemistry, S-phase fractions of both normal and malignant cells have been determined [8-12].

To our knowledge, proliferation of hematopoietic progenitors has not been studied with IdUrd. The present study was designed to develop a new IdUrd technique to investigate the kinetic behavior of colony-forming cells. During IdUrd exposure, only progenitor cells in S-phase will incorporate IdUrd into their DNA. Subsequently, when a colony-forming assay is performed, a single progenitor cell will form one colony. Cell division is expected to result in the distribution of parental DNA over the daughter cells. Therefore, detection of incorporated IdUrd in the cells of a particular colony will indicate that it originated from a colony-forming cell that has been in S-phase during the labeling period. Colony-forming cells not in S-phase during exposure will give rise to IdUrd− colonies. The fraction of progenitor cells in S-phase can be determined by pulse-labelling. The total proportion of cycling progenitors can be assessed after a prolonged labeling period.

Labeling of cells with 10 μM IdUrd for 30 minutes proved to be optimal for kinetic analysis by FCM [8,9,13]. With FCM, cells are analyzed immediately after labeling. As a result, dilution of the IdUrd signal due to cell division occurs infrequently. In contrast, IdUrd+ colonies derive from labeled clonogenic cells that have divided several times to form a colony. To prevent that, the IdUrd signal will become unde-
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The concentration used to pulse-label progenitors should be sufficiently high, and the labeling period should be as long as theoretically justified. A 52% reduction in myeloid colonies was observed in a study in which normal bone marrow was incubated with 5 μM IdUrd for 24 hours [14]. IdUrd can be catabolized to 5-Idouracil (IUra) and further degraded [14]. Therefore, toxicity and IdUrd inactivation were considered to be major limitations for prolonged labeling.

In the present study, sensitivity, toxicity, and IdUrd inactivation were evaluated to determine the optimal concentration for pulse and prolonged labeling. A previously published protocol developed for immunologic detection of IdUrd by FCM was improved and adjusted for application on agar cultures [13]. Comparative studies with [3H]-TdR suicide were performed to establish the accuracy of IdUrd pulse-labeling.

It appeared that the fraction of colony-forming cells in S-phase can be determined more accurately with IdUrd pulse-labeling than with [3H]-TdR suicide. Moreover, prolonged IdUrd labeling may create the ability to assess the fraction of proliferating progenitors.

Materials and methods

Bone marrow collection, cryopreservation, and thawing
Normal bone marrow cells were obtained from healthy bone marrow donors. Informed consent was given in all cases. The cells were collected in sterile buffered acid-citrate dextrose (pH 7.0) and enriched for clonogenic cells by flotation centrifugation [15]. In short, cells were mixed with Percoll (Pharmacia, Uppsala, Sweden) to obtain a density of 1.085 g/mL. From this suspension, 15 mL was layered under 30 mL of a 1.067 g/mL Percoll solution with 5 mL Hanks’ balanced salt solution (HBSS) (Flow Laboratories, Irvine, Scotland) on top of it. After centrifugation, the cells with a density of <1.067 g/mL were collected and frozen in liquid nitrogen using a temperature-controlled freezer (Kryo 10; Planer Biomed, Sunbury, Middlesex, UK). Each vial contained 5×10⁶ cells in Iscove’s modified Dulbecco’s medium (IMDM) (Flow Laboratories), supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT), 50 IU/mL penicillin, 50 μg/mL streptomycin (both Flow Laboratories), and 10% DMSO. Just prior to the experiments, cells were thawed in a 37°C waterbath, resulting in a recovery of >90%. Freezing and thawing procedures have been described in detail elsewhere [16].

Recombinant human growth factors
Recombinant human interleukin-3 (rhIL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were a kind gift of Sandoz BV (Uden, The Netherlands). rhG-CSF was kindly donated by Boehring (Marburg, Germany). Final concentrations of 40 ng/mL, 20 ng/mL, and 5 ng/mL of each, respectively, were used and resulted in plateau stimulation.

Pulse and prolonged IdUrd labeling
Normal bone marrow cells (10⁴ cells/mL) were suspended in IMDM supplemented with 20% FCS, 50 IU/mL penicillin, and 50 μg/mL streptomycin. The cultures were either unstimulated or stimulated with the combination of IL-3, GM-CSF, and G-CSF. IdUrd (Sigma, St. Louis, MO) dilutions were prepared from freshly thawed stock solution. Based on the experience with FCM, pulse-labeling was performed for 2 hours at final concentrations of 0.5, 1, 2, 3, 4, 5, and 10 μM IdUrd. In prolonged labeling, IdUrd at final concentrations of 0.5, 1, 2, 3, 4, 5, and 10 μM was added to the suspensions. Cells were labeled for a period of 1, 2, 2, 4, and 5 days. Labeling was performed at 37°C in a fully humidified atmosphere containing 5% CO₂ in air. Subsequently, cells were washed three times with glucose-phosphate-buffered saline (G-PBS). The cell pellet was resuspended in FCS and used for clonogenic assay and cyt centrifugation. In each experiment, one sample not labeled with IdUrd was identically processed and served as negative control. The supernatants of the cell suspensions, incubated for 1 to 5 days with 1 μM IdUrd, were collected before washing and preserved at -20°C to determine the remaining IdUrd content by bioassay.

Clonogenic assay (CFU-GM)
Cells were cultured in IMDM supplemented with 20% FCS, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 0.3% (wt/vol) baclo-acar (Difco, Detroit, MI). Cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF. Duplicate cultures were in 35×10-mm culture dishes (Costar, Cambridge, MA) at 37°C in a fully humidified atmosphere containing 5% CO₂ in air. To obtain 200 to 400 aggregates per dish, 2 to 4×10⁵ cells in a total volume of 0.85 mL were plated. The cultures ended after 5 to 7 days when aggregates consisted of 20 to 60 cells. After counting the clusters (5 to 40 cells) and colonies (>40 cells), the agar cultures were dried by cyt centrifugation and used for immunoperoxidase staining.

Cytocentrifugation of cells
Cells in suspension were sedimented onto a microscope slide by cyt centrifugation (Shandon, Pittsburgh, PA). Filter cards were moistened with 100 μL 5% human serum albumin (HSA) in PBS. Next, 50 μL of a cell suspension with a concentration of 5×10⁵/mL was added in the sample chamber. The samples were centrifuged at 500 rpm during 10 minutes. The sedimented cells were assessed for IdUrd incorporation by immunoperoxidase staining.

Cytocentrifugation of agar cultures
The method used for cyt centrifugation of agar cultures is a modification of the method described by Baines [17]. Cultures were cut into halves and shaken into G-PBS. Each half was mounted on a 76×26-mm alcohol-cleaned glass slide and covered with a cellulose acetate strip (Sepaphore; Gelman Sciences, Ann Arbor, MI). Next, a piece of filter paper (Schleicher & Schuell, Dassel, Germany) was put on top, followed by a filter card. The cultures were partially dried during 30 to 60 minutes at room temperature. Subsequently, the slides were put into cytoclips together with the plastic sample chambers. They were cyt centrifuged in a cytosin centrifuge at 1500 rpm for 10 minutes. After removal of the filters, a thin agar layer, in which the cells were embedded, remained on the glass slide.

Detection of incorporated IdUrd by indirect immunoperoxidase staining
The method used to detect incorporated IdUrd was based on a previously published protocol developed for flow cytometry [13]. A line was drawn around the sedimented cells or dried agar cultures with a Dako Pen (Dakopatts, Glostrup, Denmark) to keep the solutions on the slides. After an initial wash step in PBS, a 1 mg/mL pepsine (Serva, Heidelberg, Germany) 2M HCl solution was applied to make the DNA accessible for the antibodies and to neutralize endogenous peroxidase activity in the cytoplasm. After 30 minutes, 0.1M Na₂B₄O₇ was added for neutralization. Next, pooled human serum (PHS) (20% in PBS) was applied to block aspecific binding sites. Subsequently, a specific monoclonal anti-IdUrd antibody produced in our institute (HN-IU; dilution 1:10) was added [12]. Binding of the anti-IdUrd antibody was visualized by adding the following antibodies: peroxidase-conjugated rabbit antimouse immunoglobulins (dilution 1:100,
Dakopatts), peroxidase-conjugated goat antirabbit immunoglobulins (dilution 1:250; ICN Biomedicals, Costa Mesa, CA), and peroxidase-conjugated rabbit antigoat immunoglobulins (dilution 1:250; ICN Biomedicals). Slides were washed in PBS in between each step. The incorporated, labeled IdUrd was visualized with 0.5 mg/mL 3,3-diaminobenzidin tetrahydrochloride (DAB) (Sigma) and 0.015% H2O2 at pH 7.8 in PBS. After 10 to 12 minutes incubation at 37°C, the slides were washed by running them under tap water for 10 minutes, counterstained with hematoxylin solution (Merck, Darmstadt, Germany), and fixed with alcohol (70, 80, 90, 96, 100, 100, and 100%) and xylene substitute (Shandon). The slides were covered with Malinol (Schmid, König, Germany) and a 24×50-mm glass slide for microscopic examination. In each experiment, a sample not labeled with IdUrd was identically treated and served as negative control.

Scoring
A cell was considered IdUrd+ when its nucleus contained one or more brown-black spots or when the nucleus was colored totally brown-black. Aggregates were considered positive when >50% of the cells in the aggregate were positive for IdUrd. Per experiment, 100 to 200 aggregates were analyzed. The percentage of positive aggregates was calculated as: [number of IdUrd+ aggregates/(number of IdUrd+ aggregates + number of IdUrd-aggregates)]×100. All experiments were analyzed by one individual.

Thymidine suicide
A modification of the method of Becker et al. [3] was used for thymidine suicide. Cells (10^6/mL) were suspended in IMDM supplemented with 20% FCS, 50 IU/mL penicillin, and 50 μg/mL streptomycin, and half of the specimen was exposed for 30 minutes to 3H-TdR (25 Ci/mM and 1 mCi/mL) at 37°C. The final concentration was 100 μCi/mL. Subsequently, cells were washed three times with cold G-PBS containing 100 μg cold thymidine per mL. The remaining cell pellet was resuspended in FCS and used for clonogenic assay. The other half of the cells was not exposed to 3H-TdR, the cells were incubated, washed, and plated in agar identically to the suicided specimen and served as controls. The percentage of progenitor cells in S-phase was calculated as: [(number of control aggregates - number of aggregates after 3H-TdR exposure)/number of control aggregates]×100.

Molt-4 clonogenic assay
Cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 IU/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamin, and 0.3% (wt/vol) bacto-agar. Per experiment, eight 35×10-mm dishes containing 500 cells in 0.85 mL medium were cultured at 37°C in a fully humidified atmosphere containing 5% CO2. After 4 days, the aggregates (eight to 32 cells) were counted. Agar cultures of IdUrd-labeled cells were dried by cytocentrifugation and used for immunoperoxidase staining.

Counterflow centrifugation of Molt-4 cells
Counterflow centrifugation (CC) was performed with a multichamber rotor (Curaré 3000; Dijkstra Vereenigde, Amsterdam, The Netherlands) to enrich Molt-4 cells in G1 and S-phase [18]. The elutriation profiles proved to be identical for the different chambers [18]. One chamber contained control cells, a second chamber thymidine-labeled cells, and a third IdUrd-labeled cells. Cells collected at a decreasing rotor speed showed a progressively larger cell volume and represented distinct populations of cells in transition from G1 phase, through S-phase to G2/M phase. On the basis of DNA histograms, collected fractions were combined to obtain four different fractions representing an “early” and “late” G1, fraction and an “early” and “mid” S-phase fraction. Of each fraction, cells were used for DNA analysis and clonogenic assay.

Bivariate flow-cytometric IdUrd/DNA analysis in suspension
Bivariate staining for IdUrd incorporation and DNA content was performed using the simultaneous proteolytic enzyme digestion and acid denaturation technique [13,19]. The anti-IdUrd FITC conjugated IgG goat antimouse second-step antibody (FITC-IGG) (American Quaiex International, La Mirada, CA) were used for detection of IdUrd. Propidium iodide (PI) was used for DNA staining. IdUrd staining of the cell suspensions was always performed in duplicate, and all incubation steps were executed in the dark. Non-IdUrd-labeled low-density blood cells served as negative controls. Samples were analyzed on a Coulter Epics Elite (Coulter, Hialeah, FL) flow cytometer equipped with a 40 mW Argon ion laser running at 15 mW. A high-pass filter of 610 nm for red fluorescence (PI), a band-pass filter 525/30 for green fluorescence (FITC), and a dichroic mirror of 550 nm were used. A minimum of 20,000 cells was analyzed in duplicate. The fluorescence signals were recorded on a linear scale in list mode. The labeling index was defined as the percentage of IdUrd-labeled cells.

Bioassay for the detection of residual IdUrd after prolonged labeling
Normal bone marrow cells were suspended in IMDM containing 20% FCS and the combination of IL-3, GM-CSF, and G-CSF. The cells were cultured in an incubator for 3 days to attain a high proportion of proliferating cells. After washing with G-PBS, the pelleted stimulated cells were suspended in thawed supernatants and cultured in an incubator for another 24 hours. The used supernatants were collected from cell suspensions and incubated with 1 μM IdUrd for a period of 1 to 5 days. As control, cells were also exposed to freshly prepared 1 μM IdUrd. After incubation, the cells were washed with G-PBS. The cell pellet was resuspended in FCS and used for cytocentrifugation and clonogenic assay. Subsequently, immunoperoxidase staining was performed to determine whether the supernatants contained sufficient IdUrd to label cells in S-phase.

Statistics
Values were expressed as mean ± 95% confidence interval (CI) of the mean. After IdUrd labeling, the binomial distribution was applied to calculate the 95% CI of the mean percentage of progenitors in S-phase. Significance of IdUrd concentration level was tested with variance analysis (ANOVA with Duncan-Waller test) [20].
Results

Development of the detection of incorporated IdUrd

Immunoperoxidase staining. The first step was the adaptation of the protocol for IdUrd detection by immunofluorescence developed for FCM [13]. In our hands, it was difficult and time-consuming to identify aggregates in agar cultures when fluorescence was used; we preferred an immunoperoxidase staining technique. This allowed evaluation by light microscopy. All aggregates, whether positive for IdUrd or not, could easily be visualized after counterstaining. A sandwich method of three subsequent additions of peroxidase-conjugated immunoglobulins was chosen. Intensification of the signal with these three peroxidase-conjugated immunoglobulins proved to be superior to the signal obtained with only one or two peroxidase-conjugated immunoglobulins (data not shown).

Adaptation of the semisolid (agar) culture and culture time.

Instead of the usual 2.0 mL, a reduced volume of 0.85 mL agar per dish was plated. Incorporated IdUrd was more easily accessible for antibodies when the thickness of the agar culture was reduced. Subsequent mitoses of the clonogenic cell are expected to dilute the IdUrd signal, which may result in false-negative colonies. The percentage of IdUrd* bone marrow colonies after 7 culture days (average colony size=64 cells) was the same as determined after 3 days (average cluster size=16 cells; data not shown). This indicated that the immunoperoxidase staining was at least sensitive enough to detect IdUrd in colonies consisting of maximally 64 cells. All cells present in smaller clusters contained at least three spots, whereas most, but not always all, cells of the larger colonies contained one or a few spots (Fig. 1).

Pulse-labeling

Toxicity. The IdUrd toxicity was tested on three different normal bone marrows. The cells were cultured for 4 days in the presence of the combination of IL-3, GM-CSF, and G-CSF to obtain a high proportion of cycling progenitors. IdUrd was added during the last 2 hours of incubation. The number of CFU-GM was not significantly reduced, even after incubation with 80 μM IdUrd (Fig. 2). However, the size of the aggregates cultured after incubation with 80 μM IdUrd was smaller compared to control cultures. Therefore, 80 μM was con-

Fig. 1. Examples of IdUrd* aggregates. A. Normal CFU-GM cultured after 24-hour labeling with 1 μM IdUrd. B. Molt-4 aggregate cultured after pulse-labeling with 40 μM IdUrd.

Fig. 2. Toxicity of IdUrd pulse-labeling with IL-3-, GM-CSF-, and G-CSF-stimulated CFU-GM. Normal bone marrow cells were labeled with various concentrations of IdUrd for 2 hours. Subsequently, the number of CFU-GM was determined and expressed as a percentage of the control cultures (incubated without IdUrd). The graphic represents the mean percentage (+95% CI) of three normal bone marrows.
considered to be toxic. In addition, peroxidase staining was performed on sedimented cells of the suspensions after labeling with the different IdUrd concentrations. All samples contained IdUrd* cells. Part of the cells labeled with 80 pM were covered with a brown smear. It appeared as if their nuclei were no longer intact. This, again, suggested toxicity.

Signal intensity. Cells were pulse-labeled for a period of 2 hours. Because of an insufficient amount of incorporated IdUrd, a shorter period may underestimate the number of clonogenic cells in S-phase, whereas a longer period may result in overestimation. Pulse-labeling with 5 μM IdUrd resulted in a significantly lower percentage of positive aggregates when compared to the other tested IdUrd concentrations. The mean (±95% CI) positive percentage was 22±20 after pulse-labeling with 5 μM vs. 42±8, 37±12, and 45±12 after labeling with 10, 20, and 40 μM, respectively (Duncan-Waller; n=3; p<0.05) (Fig. 3). The percentages of positive aggregates after labeling with 10, 20, and 40 μM IdUrd were not significantly different. The lower percentage of positive aggregates found after 80 μM labeling suggested toxicity toward colony-forming cells that incorporated IdUrd. After labeling with 5, 10, and 20 μM IdUrd, the brown staining in a small percentage (<10%) of the aggregates was too vague to determine whether they were positive or negative. Taking both toxicity and signal intensity into account, 40 μM was considered to be the optimal IdUrd concentration for pulse-labeling.

Sensitivity. Molt-4 cells were used to check whether all clonogenic cells in S-phase were sufficiently pulse-labeled with IdUrd to be detected by immunoperoxidase staining. If not, an underestimation of the percentage of colony-forming cells in S-phase may occur. In contrast to normal bone marrow cells, Molt-4 cells have a high plating efficiency (20 to 50%). Therefore, cell cycle analysis of the total cell population can be considered to approximate the cycling status of the colony-forming cells. The percentage Molt-4 cells in S-phase was determined by FCM using IdUrd incorporation. After pulse-labeling, 40.9% of the overall Molt-4 cell population was in S-phase, whereas 45.6% (41.1 to 50.3; mean ± 95% CI) of the colonies were IdUrd* (Table 1).

In addition, Molt-4 cells were elutriated to create cell populations with an increasing number of S-phase cells. This allowed evaluation of the accuracy of IdUrd pulse-labeling under different conditions. The percentage of S-phase cells increased from 13.2% in fraction 1 to 69.9% in fraction 4 (Table 1). The percentage of IdUrd* CFU-Molt-4 increased from 14.8% (11.4 to 29.0) in fraction 1 to 70.1% (64.9 to 74.8) in fraction 4. The percentage of IdUrd* colonies corresponded strongly with the percentage of S-phase cells.

Comparison of 3H-TdR suicide and IdUrd pulse-labeling. IdUrd pulse-labeling was compared with 3H-TdR suicide, which is considered to be the most reliable suicide technique [4]. Unfractionated Molt-4 cells and Molt-4 cell fractions containing different quantities of S-phase cells were tested. The fractions were obtained by counterflow centrifugation. Comparison between the suicide index and the percentage of IdUrd* colony-forming cells in S-phase determined by 3H-TdR suicide and IdUrd pulse-labeling in CC fractions containing different percentages of S-phase cells

<table>
<thead>
<tr>
<th>CC fraction</th>
<th>Percent S-phase cells</th>
<th>CFU-Molt*</th>
<th>3H-TdR</th>
<th>Suicide index (%)</th>
<th>IdUrd* CFU-Molt*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40.9</td>
<td>106.6±7.6</td>
<td>59.8±9.0</td>
<td>43.9</td>
<td>45.6</td>
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<tr>
<td>1</td>
<td>13.2</td>
<td>14.6±2.0</td>
<td>15.3±3.2</td>
<td>4.8</td>
<td>28.3-59.5</td>
</tr>
<tr>
<td>2</td>
<td>27.8</td>
<td>31.6±5.6</td>
<td>26.0±2.6</td>
<td>-40.4-30.8</td>
<td>14.8</td>
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<tr>
<td>3</td>
<td>45.8</td>
<td>65.9±4.2</td>
<td>26.0±3.4</td>
<td>-8.2-43.6</td>
<td>30.6</td>
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<tr>
<td>4</td>
<td>69.9</td>
<td>92.5±8.0</td>
<td>11.1±3.2</td>
<td>60.5</td>
<td>46.0-58.4</td>
</tr>
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</table>

*Fractions (1 to 4) obtained with CC at decreasing rotor speed. The total fraction represents Molt-4 cells that were not elutriated.

**Mean number (±95% CI; n=8 cultures) of Molt-4 colonies/500 cells.

***Mean percentage (±95% CI; n=8 cultures) of CFU-Molt-4 in S-phase determined by 3H-TdR suicide.

****Mean percentage (±95% CI; n=8 cultures) of CFU-Molt-4 in S-phase determined by IdUrd pulse-labeling.
Table 2. Comparison of the percentage of CFU-GM in S-phase determined by $^3$H-TdR suicide and IdUrd pulse-labeling in four normal bone marrows

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Culture condition</th>
<th>Control CFU-GM (±95% CI, n=6)</th>
<th>$^3$H-TdR CFU-GM (±95% CI, n=6)</th>
<th>Suicide index (%)</th>
<th>IdUrd CFU-GM (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>Unstimulated</td>
<td>154.3±20.9</td>
<td>189.5±19.4</td>
<td>-22.8</td>
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<td></td>
<td>Stimulated</td>
<td>409.0±34.3</td>
<td>343.7±63.7</td>
<td>-48.8-3.2</td>
<td>1.8-9.5</td>
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<td>16.0</td>
<td>33.3</td>
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<td>-7.9-39.9</td>
<td>27.5-39.8</td>
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<tr>
<td>2</td>
<td>Unstimulated</td>
<td>108.7±18.3</td>
<td>107.3±5.2</td>
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<tr>
<td></td>
<td>Stimulated</td>
<td>328.7±12.7</td>
<td>163.3±19.2</td>
<td>-20.3-22.9</td>
<td>4.4-15.0</td>
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<td>40.7-59.6</td>
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<td>3</td>
<td>Unstimulated</td>
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<td>96.0±2.8</td>
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<td>Stimulated</td>
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<td>446.5±84.9</td>
<td>293.5±31.0</td>
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<td>8.2-60.2</td>
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<td>30.4-42.9</td>
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</table>

*Mean number of CFU-GM/0.3X10⁵ nucleated cells (±95% CI, n=6).

Thawed normal bone marrow cells were tested 4 days after liquid culture in the absence (unstimulated) or presence (stimulated) of the combination of IL-3, GM-CSF, and G-CSF. In case 4, freshly obtained bone marrow cells were also tested.

Percentage (±95% CI, n=6 cultures) of CFU-GM in S-phase determined by $^3$H-TdR suicide.

Percentage (±95% CI, n=200 aggregates) of CFU-GM in S-phase determined by IdUrd pulse-labeling.

CFU-Molt-4 revealed no significant differences for the five tested samples (Table 1). The wide confidence intervals of the suicide technique contrasted with the small confidence intervals obtained with IdUrd pulse-labeling.

In a second set of experiments, normal bone marrow cells were cultured for 4 days in the absence or presence of the combination of IL-3, GM-CSF, and G-CSF to generate colony-forming cells with a low and high proliferative activity, respectively. In unstimulated thawed bone marrow, the percentage of CFU-GM in S-phase varied from -23 to 7% when determined with thymidine suicide and from 4 to 10% when assessed with IdUrd pulse-labeling (Table 2). After stimulation with the combination of growth factors, the percentage of CFU-GM in S-phase ranged from 16 to 70% ($^3$H-TdR suicide) and 33 to 56% (IdUrd pulse-labeling). No significant differences were observed between the percentages obtained with $^3$H-TdR suicide and IdUrd labeling. The confidence intervals of the suicide index were again much wider than those of the IdUrd pulse-labeling. The fresh bone marrow sample showed a higher percentage of clonogenic cells in S-phase with both methods, probably owing to the presence of viable CSF-producing monocytes in this sample (Table 2, sample 4).

Prolonged labeling

Toxicity of various concentrations of IdUrd during 5-day incubation. The toxicity was tested on three normal bone marrows. Cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF to obtain a high proportion of proliferating cells. It appeared that prolonged labeling with IdUrd concentrations of 2 μM and higher was toxic for CFU-GM (Fig. 4). The curves obtained with 2, 3, 4, and 5 μM showed no further decrease in CFU-GM number after 4- and 5-day exposure. This could indicate that IdUrd concentrations had decreased below toxic levels. Therefore, the concentration of IdUrd in time was investigated.

Sensitivity. Theoretically, 1 μM IdUrd could be too low to label all clonogenic cells in S-phase. Therefore, the percentage of positive colonies obtained after 24-hour labeling with 5 μM (the highest nontoxic concentration) as well as 1 μM and 0.5 μM were compared. It appeared that about 75% of
the colonies were IdUrd* at all three concentrations tested (Fig. 5). This demonstrated that 1 μM IdUrd was sufficient to label all cycling cells for up to 24 hours.

IdUrd concentration in time. The toxicity study indicated that 1 μM IdUrd was the highest concentration that could be safely used for prolonged labeling. IdUrd can be inactivated, especially in media containing FCS not inactivated by heat (data not shown). The media used in this study contained heat-inactivated FCS. Since 1 μM or less IdUrd was below the detection limit of high-performance liquid chromatography (HPLC), a bioassay was performed. It appeared that IdUrd sufficient to label colony-forming cells for up to 5 days was present in the supernatants. The percentages of positive colonies were the same as obtained after exposure to a freshly prepared 1 μM solution (Fig. 6). However, the intensity of the signal in the colonies, cultured after incubation with the supernatant from days 4 and 5, was reduced.

Discussion

Suicide techniques are available to study the kinetics of colony-forming cells in vitro. The fractional reduction in cloning efficiency after exposure to 3H-TdR, HU, or Ara-C may not always estimate the percentage of S-phase cells correctly [2,4,5]. Calculation of the suicide index is based on the subtraction and division of colony numbers. This is a major disadvantage of all suicide techniques. The confidence intervals of colony-forming assays are substantial. A 95% CI of 10% is not uncommon [3,21]. Statistically, this has a great impact on the accuracy of the suicide index. Calculation of the 95% CI of the suicide index of some publications revealed a variation up to 30% [3,21].

Nonradioactive IdUrd appears to be an attractive alternative for determining the proportion of colony-forming cells in S-phase. In contrast to the suicide techniques, the percentage of IdUrd* colonies per culture dish is independent of the variation in colony numbers. Instead, the exactness of the estimated percentage S-phase cells depends on the total number of analyzed colonies [20].

After adaptation of a protocol developed for immunological detection of IdUrd by FCM, IdUrd* colonies could easily be detected in agar cultures (Fig. 1). The immunoperoxidase staining technique was sensitive enough to detect IdUrd in colonies consisting of maximally 64 cells.

Toxicity and sensitivity experiments indicated that a 2-hour exposure to 40 μM IdUrd was optimal for pulse-labeling. Underestimation of the percentage S-phase cells due to insufficient incorporated IdUrd was excluded by extensive studies with Molt-4 cells (Table 1). Molt-4 cells and normal bone marrow cells with varying percentages of cells in S-phase were used to compare IdUrd pulse-labeling with 3H-TdR suicide. The mean percentage of S-phase cells determined with IdUrd labeling was not significantly different from the percentage obtained with 3H-TdR suicide (Tables 1 and 2). Due to the inevitable variation in colony numbers, the extent of the 95% CI of the suicide index was substantial. Based on the evaluation of only 200 aggregates, a much smaller confidence interval was calculated in case of IdUrd pulse-labeling. Thus, IdUrd pulse-labeling appeared to be more accurate than 3H-TdR suicide. A negative suicide index, which means higher colony numbers in the 3H-TdR exposed fraction, was observed in two samples. Both contained a low percentage of S-phase cells (4.2% and 14.8%, respectively, when determined with IdUrd pulse-labeling). The relatively small number of 3H-TdR killed cells fell probably within the variation of the colony-forming assays. Other authors have

Fig. 5. Signal intensity after prolonged IdUrd labeling.

![Fig. 5](image)

Normal bone marrow cells from three donors were labeled with various concentrations of IdUrd for 24 hours. Subsequently, clonogenic assays were performed. Cultures were ended after 6 days and the Incorporated IdUrd was detected by immunoperoxidase staining. The diagram shows the mean (±95% CI) percentage of IdUrd* colonies for each concentration of IdUrd.

Fig. 6. IdUrd concentration in time. IL-3-, GM-CSF-, and G-CSF-stimulated normal bone marrow cells were incubated with supernatants collected after incubation of cell suspensions with 1 μM IdUrd for a period of 1 to 5 days. As control, cells were also incubated with a freshly prepared 1-μM IdUrd suspension (=day 0). After 24 hours, clonogenic assays were performed. Cultures were ended after 6 days and IdUrd was detected by immunoperoxidase staining. The mean (±95% CI) percentage of IdUrd* colonies reflects the residual amount of IdUrd which could be incorporated.
made identical observations [3,21]. It again shows a limitation of suicide techniques.

Toxicity and inactivation of IdUrd and sensitivity of the IdUrd detection were evaluated to determine the optimal concentration for prolonged labeling. It appeared feasible to determine the proportion of cycling clonogenic cells for a period of at least 5 days during which a concentration of 1 μM IdUrd was used to label the cells. Thus, prolonged IdUrd labeling provides the unique opportunity to determine the proliferating fraction of progenitor cells. With the traditional techniques, only the fraction of S-phase cells can be determined.

During DNA repair, both 3H-TdR and IdUrd will be incorporated into non-S-phase cells. In theory, this could result in an overestimation of cells in S-phase. However, the number of repaired sites is relatively small compared to the doubling of DNA that occurs during S-phase. This suggests only a minor or even no detectable effect of DNA repair on the estimation of cells in S-phase. In some leukemic samples, we observed that even during 3-day labeling with 1 μM IdUrd, <5% of the leukemic clonogenic cells were IdUrd⁺ (data not shown). This additionally supports the limited influence of DNA repair on the estimation of S-phase cells.

Prolonged and pulse-labeling with IdUrd can be applied to study the proliferative behavior of progenitors under physiological and pathological conditions. For example, the effects of growth factors on the in vitro proliferation of normal and leukemic colony-forming cells can be analyzed. Furthermore, cycling data can be correlated to the cytotoxicity of chemotherapeutic drugs. IdUrd can safely be administered to humans [11,12]. Kinetic analysis of in vivo labeled progenitors seems feasible, and the first clinical data are currently being collected in our institute.

The present study shows that the percentage of colony-forming cells in S-phase can be determined more accurately with IdUrd pulse-labeling than with suicide techniques. In addition, prolonged IdUrd labeling makes it possible to study the proliferating fraction of progenitor cells for at least 5 days.

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References