Kinetic Parameters of Bone Marrow Stem Cells Using In Vivo Suicide by Tritiated Thymidine or by Hydroxyurea

By F. Vassort, M. Winterholer, E. Frindel, and M. Tubiana

The kinetics of colony-forming units (CFU) of the bone marrow in mice were studied using a method involving the suicide of cells in vivo by $^3$H-TdR or hydroxyurea. After selectively killing cells in S phase, the surviving cells traversed S phase in a cyclic fashion. This enabled the duration of the cycle and of the S phase of CFU to be measured—8 hr and 4 hr, respectively. It was shown that quiescent stem cells pass into the proliferative compartment. A computer model helped to explain the experimental results. The feedback mechanism seemed to be initiated by the depletion of the compartment of maturing cells. This hypothesis is supported by the fact that the CFU response to S-phase cell killing was similar in two strains of mice and was independent of the proportion of CFU in S phase, which was approximately 0% in the C57BI and 20% in the C3H mice.

Since the studies of Quastler and Sherman, the duration of the cell cycle has been measured using the labeled mitosis technique for many normal and pathologic tissues. However, it is not possible to apply this technique to the study of marrow stem cells, as they cannot be identified morphologically. Some workers have attempted to approach this problem indirectly.2,3

In an earlier study, we examined the effects of hydroxyurea on stem cells, employing the splenic colony method of Till and McCulloch.5 Hydroxyurea kills cells in the S phase, provoking cyclic variations in the percentage of stem cells in S that enable the duration of their cell cycle to be estimated. This was found to be 10 hr. Nevertheless, these results must be interpreted with caution, as the surviving cells could have been modified by hydroxyurea.

Furthermore, these experiments and earlier results showed that hydroxyurea stimulated the entry of quiescent stem cells into the cycle. This stimulation could be the consequence of a feedback mechanism either within the stem cell compartment or dependent on the depletion of the marrow maturation compartment. To attempt to investigate these two alternative hypotheses, two strains of mice were used: C3H mice, which in our laboratory have

From Institut Gustave-Roussy, Villejuif, France.

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20% ± 5%[4] of their stem cells in DNA synthesis; and C57Bl mice, whose stem cells are virtually quiescent.

In C3H mice, the same strain employed to study the effects of hydroxyurea, the stem cells in S were selectively killed by giving high doses of tritiated thymidine (3H-TdR). The incorporation of the 3H-TdR into the cells in S phase causes them to die, without having any effect on cells in other phases of the cell cycle. The second experiment used C57Bl mice in order to study the mechanism of recruitment of stem cells into cycle uniquely as the result of depletion of the maturation compartment, since practically none of the stem cells in these mice is in DNA synthesis.

MATERIALS AND METHODS

Two strains of mice were used: female C3H, aged 2-3 mo and weighing 20-30 gr; and C57Bl, aged 3 mo.

In the 3H-TdR suicide in vivo experiments, each mouse was injected with 1 mCi 3H-TdR (specific activity 25 Ci/mM) intraperitoneally. For the 3H-TdR suicide in vitro,7 the cells were incubated in Hanks' medium containing 250 μCi 3H-TdR/ml. The hydroxyurea (HU) was prepared immediately before injection, and a dose of 0.5 mg/g body weight was given intraperitoneally.

Principle of Method Used to Measure Duration of Cell Cycle

In the percentage labeled mitosis (PLM) method,1 the cells in the S phase were labeled by a brief exposure to 3H-TdR, and the passage of this cohort of cells through mitosis was identified by radioautography.

In the current experiments, the cells in S were killed selectively by the administration of 1 mCi 3H-TdR to the animals in vivo. After this selective killing, the passage into the S phase of the cohort of cells that were initially in the G1 and G2 phases followed. The percentage of the stem cells in S was measured by the 3H-TdR suicide in vitro technique.7

Experiments

Tritiated thymidine was injected intraperitoneally into three series of 24 C3H mice. Bone marrow was taken at scheduled times following injection during the next 24 hr. All donor marrow was pooled from both hind limbs of three donors. Each experimental point is, therefore, based on three series of three mice each. The number of colony-forming units (CFU) was determined by counting the spleen colonies formed after the injection of the marrow into 12 recipient mice irradiated with a dose of 900 R. The proportion of stem cells in S phase was determined by the in vitro 3H-TdR suicide method described by Becker et al.7 Each determination was based on counting at least 10 spleens in each normal and suicide bone marrow group.

In the study on C57Bl mice, 3H-TdR experiments were as follows: 3H-TdR was injected into two series of 18 C57Bl mice. At each of the various intervals following the injection, two groups of three mice were killed, and their bone marrow was collected and pooled before injection into recipient mice. This was then examined to determine the number of CFU and the percentage in the S phase, as described above.

The procedure and number of mice were identical for hydroxyurea experiments.

Simulation Experiments on a Computer

To interpret the results of these experiments, a method of model simulation, as described by Valleron and Frindel,8 was adapted that enables a cell population to be simulated on a computer. The mathematical model was based on the hypothesis that there are two compartments, proliferative (P) and, quiescent (Q) in G0 phase. The parameters involved
Table 1. CFU Number per Leg in C3H and C57Bl Mice

<table>
<thead>
<tr>
<th>Time After Injection (hr)</th>
<th>C3H Mice</th>
<th>C57Bl Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H-TdR</td>
<td>HU</td>
<td>3H-TdR</td>
</tr>
<tr>
<td>Control</td>
<td>2050 ± 120</td>
<td>2050 ± 120</td>
</tr>
<tr>
<td>1</td>
<td>1600 ± 240</td>
<td>1680 ± 150</td>
</tr>
<tr>
<td>4</td>
<td>1350 ± 305</td>
<td>1400 ± 130</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>1520 ± 250</td>
<td>1200 ± 210</td>
</tr>
<tr>
<td>10</td>
<td>1300 ± 180</td>
<td>1500 ± 260</td>
</tr>
<tr>
<td>12</td>
<td>1170 ± 220</td>
<td>1250 ± 110</td>
</tr>
<tr>
<td>16</td>
<td>1150 ± 260</td>
<td>1300 ± 140</td>
</tr>
<tr>
<td>20</td>
<td>900 ± 130</td>
<td>1150 ± 120</td>
</tr>
<tr>
<td>24</td>
<td>820 ± 110</td>
<td>1380 ± 160</td>
</tr>
</tbody>
</table>

in the simulation were the growth fraction and the parameters of the cell cycle, i.e., the means and variances of the duration of the various phases (T\textsubscript{M}, T\textsubscript{G1}, T\textsubscript{G2}, and T\textsubscript{S}). It is assumed that the duration of the phases in a proliferating population has a log-normal distribution. In generating the quiescent compartment, it is supposed that each cell at its birth has a certain probability of entering the G\textsubscript{0} phase and remaining in this phase for a very long period of time. The death of cells that were in S at the beginning of the experiment was simulated, as well as the time course changes in the percentage of those surviving cells that enter into S.

Computations were carried out under two hypotheses. In the first model, it was assumed that the growth fraction was constant. In the second, there was a recruitment of resting stem cells. A rapid and massive entry of stem cells into cycle over a period of a few hours has been simulated. The parameters introduced were the percentage of cells entering into the P compartment, the duration of the flux, and the moment of their entry into P. On an empirical basis, these last parameters were adjusted by trial and error, so as to obtain a satisfactory agreement with the experimental findings.

RESULTS

The number of CFU in C3H mice decreased as soon as the first hour after injection of 3H-TdR or HU, whereas the CFU count in C57Bl mice decreased only after the fourth hour (Table 1).

The curve of Fig. 1 was obtained from the mean of three independent experiments for each point. In the C3H control mice (Fig. 1), the percentage of CFU in S was 20\%. An hour after injection with 3H-TdR, the percentage of cells in S was virtually nil, by 4 hr it had risen to 22\%. Subsequently, the percentage of cells in S followed a cyclic pattern with the maximums at 12 hr and 20 hr separated by a minimum at 16 hr. The maximum percentage of CFU in S was 35\%. The duration of the cycle (T\textsubscript{C}) and the S phase (T\textsubscript{S}) was estimated graphically by measuring the interval in time between two corresponding points on the ascending and the descending slopes of the curves at the midpoint of the wave of the cells in S. These values were 8 hr for T\textsubscript{C} and 4 hr for T\textsubscript{S}.

In C57Bl control mice, where two individual experiments per point were performed, the percentage of CFU in S phase was between 0\% and 5\% and
Fig. 1. Percentage of CFU in S phase in C3H mice after administration of 1 mCi of tritiated thymidine (black circles) and after 10 mg of hydroxyurea (open circles).

remained at this level for 6 hr after injection of $^3$H-TdR (Fig. 2). Subsequently, the percentage rose rapidly to reach a value of 40% at 12 hr. After the injection of hydroxyurea (Fig. 2), the percentage of CFU in the S phase evolved in a similar fashion.

Figure 3 shows the curves produced by the computer for models of this experiment using C3H mice and based on $^3$H-TdR suicide in vivo. The parameters of the cycle were: $T_{G_1}$, 2.0 hr; $T_s$, 4.0 hr; $T_{G_2}$, 1.5 hr; and $T_m$, 0.5 hr ($\sigma_{G_1} = 0.2$, $\sigma_s = 0.1$, $\sigma_{G_2} = M = 0.0$).

**DISCUSSION**

The experiments reported in this paper provide data on the duration of the cell cycle of stem cells and on the mechanism of homeostasis within the stem cell compartment.

The cyclic variation in the percentage of cells in S enables the duration of the cell cycle and the S phase to be determined as previously reported. The
first peak of cells in S occurred 4 hr after the injection of \(^{3}\text{H-TdR}\), which is compatible with the in vivo suicide effect of \(^{3}\text{H-TdR}\). The incorporation of \(^{3}\text{H-TdR}\) by the cells in S causes their death but does not slow the progression of the surviving cells through the cycle. After a rapid entry into S, the cohort of cells traverse the other phases of the cycle and the S compartment is then partially depleted. The second peak occurs after a time interval equal to the duration of the cell cycle (Tc). In fact, the height of the peak at 12 hr is higher than the first wave at 4 hr. This phenomenon suggests that quiescent cells must be entering the proliferative compartment.

Cells in S phase can also be killed by in vivo administration of hydroxyurea. Variation in the percentage of CFU in S phase after HU administration has been previously reported by us\(^4\) and by Chaffey and Hellman.\(^9\) By comparing the curves obtained after the injection of \(^{3}\text{H-TdR}\) with those obtained after injection of HU, it is apparent that HU not only kills cells in S phase, but also causes a retardation of about 4 hr for the passage of surviving cells into S phase. Furthermore, the cell cycle studied by the method of HU suicide is 2 hr longer than the cell cycle studied by the \(^{3}\text{H-TdR}\) suicide technique.
A duration of $T_c$ of 8 hr and $T_s$ of 4 hr for CFU is similar to that observed in recognizable proliferating marrow cells. Morse et al. have also found a $T_c$ of 8–10 hr in differentiated erythroid cells. On the other hand, the 6-hr cycle time for CFU reported by Lajtha et al. is shorter than our estimate. The reason may be that the conditions of his experiments were different and that the duration of the cycle may have been at its lowest value at the time of transplantation of CFU into irradiated mice, when all the cells were in the proliferative compartment and were hyperstimulated.

The computer simulation confirmed the conclusions about the durations of the cell cycle and the $S$ phase (Fig. 3A). To account for the oscillations observed in the percentage of CFU in $S$, the duration of the $S$ phase would have to be between 3 and 5 hr with $T_c$ of about 8 hr.

The increase of the percentage of the stem cells in $S$ observed after the injection of HU or of $^3$H-TdR in C3H mice is, in part, due to the recruitment of quiescent cells. The results obtained with C3H mice demonstrate the entry of quiescent stem cells into the cycle but cannot help distinguish between the two possibilities of the stimulatory feedback mechanism which could be switched on by the depletion of either the CFU compartment or of the maturation compartment.

To elucidate this problem, we used C57Bl mice in which nearly all stem cells are quiescent, whereas in the C3H mice about 20% of stem cells are in

![Diagram](image)
S phase. However, in both strains approximately 50% of differentiated cells are in DNA synthesis.

Three observations were made using C57Bl mice (Fig. 2): the delay of the appearance of cells in S phase of 6–8 hr indicates that the entry into G1 takes 4–6 hr; the rise in the number of CFU in S phase is rapid and increases from 0% to almost 50% between 8 and 12 hr; and the peak of the proportion of cells in S is between 40% and 47%, demonstrating that 80%–95% of CFU have entered the cycle.

Using a computer model with the above data on the C3H experimental results, it was found that the kinetics of the passage of quiescent cells to cycling cells were similar in both C57Bl and C3H mice. Thus, it can be assumed that the mechanism of feedback is identical in the two strains of mice, despite the differences in the proliferative status of the CFU. Therefore, the feedback seems to be due to depopulation of the differentiated compartment, which is the only one depleted in the case of the C57Bl mice.

Another interesting observation shown by the model is that the duration of the cell cycle of the newly recruited cells is similar to that observed in the surviving cells after 3H-TdR treatment.

Regulation of stem cell proliferative activity has been discussed in the literature.\textsuperscript{11–13} The early increase of the percentage of CFU in S found in our experiments show that quiescent CFU can be stimulated by factors other than the number of circulating blood cells. Moreover, the number of cells in the differentiated and in the precursor compartment is maintained by a feedback mechanism within the bone marrow. The repair of the differentiated compartment is accomplished at least partly by influx from the stem cell compartment, as already suggested by Morse et al.\textsuperscript{11} These authors followed, in hyper-transfused mice, the repair of response to erythropoietin of the committed erythroid compartment after injury by HU, and they observed a significant decrease in the number of CFU between 4 and 14 hr after HU administration.

Our data further demonstrate that this efflux of stem cells is observed also in normal mice after selective injury of the differentiated compartment and is concomitant with the entry into cycle of a large proportion of previously quiescent stem cells. This effect of HU should not be overlooked when it is used in patients for its synchronizing ability.

One could ask whether the depopulation of the differentiated compartment triggers only the differentiation of CFU or if it also triggers the proliferation of CFU. It is difficult to answer this question. Our data suggest that the first event is the efflux of CFU and that entry into cycle of CFU occurs later on, perhaps as a consequence of the depletion of the CFU compartment. Whatever the sequence of events, it can be concluded that the stimulation by a feedback mechanism is due to the depletion of the maturing cell compartment.

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