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The Number of Functioning Hemopoietic Clones in Transplantation-Restored Bone Marrow of Mice Depends on the Dose of Transplanted Cells

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The clone composition of mouse bone marrow restored by transplantations of different doses of hemopoietic cells containing the unique genetic markers at the expense of integration of human adenosine deaminase gene is analyzed. Low dose of donor hemopoietic cells accelerates reversion of hemopoiesis to the recipient type and decreases the number of functioning clones. This finding confirms that a stem hemopoietic cell cannot maintain itself and its proliferative potential is limited. For long and stable repopulation of the recipient subjected to gene therapy, the maximum possible number of transduced stem hemopoietic cells should be transplanted.

Key Words: *primitive stem hemopoietic cell; retrovirus gene transfer; splenic colony-forming units; clonal succession*

Hemopoiesis is maintained by stem hemopoietic cells (SHC) capable of differentiating into all hemopoietic cell lines and self-maintaining [3]. However, recent findings argue against this postulate. In hemopoietic cells of irradiated mice restored by transplantation, hemopoiesis is maintained throughout the life span by tens of simultaneously functioning small short-lived clones — SHC derivatives successively replacing each other [5]. Short life of the clones indicates that SHC are incapable of self-maintenance and their proliferative potential is limited. If so, the number of functioning hemopoietic clones depends on the dose of transplanted hemopoietic cells and, eventually, on the number of injected SHC. This study was undertaken to test this hypothesis.

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MATERIALS AND METHODS

Experiments were carried out on 12-16-week-old (C57Bl/6×CBA) CBF_1 mice; males were the bone marrow donors and females were the recipients. The recipients were irradiated: total dose 12 Gy, 2 sessions at a 3-h interval, ^{137}Cs. Persistent murine bone marrow culture was maintained as described elsewhere [4]. The donors were injected with 5-fluorouracil (Sigma, 150 mg/kg intravenously) or hydroxyurea (Serva, 1 g/kg intraperitoneally) 6 times at 6-h intervals 2 days before the experiment. Human adenosine deaminase (ADA) gene was transduced in mouse SHC as described previously [7]: hemopoietic cells were prestimulated to divide in a sublayer of 3-4-week-old bone marrow culture without exogenous cytokines, and irradiated (total dose of 15 Gy); after 2 days the sublayer with the cells transferred into a retrovirus-producing fibroblast culture (hADA GP+

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E86) pretreated with mitomycin C. After 48-h incubation in the producer culture, bone marrow cells were washed and injected in two doses to lethally irradiated recipients: 0.8 (low dose, 20 mice per group) and 8 (high dose, 10 mice per group)×10^6 cells. After 3, 6, 8, and 10 months bone marrow samples were obtained by puncture of the femoral bone under light ether narcosis. Bone marrow cells of restored mice were injected to repeatedly irradiated recipients, which were sacrificed after 11 days, and individual colonies were isolated from the spleen for analysis of foreign sequences in the DNA. The donor origin of a colony was confirmed by polymerase chain reaction (PCR) using primers specific for the Y-domain of males (5'CTCCTGATGGACA AACTTTACG3' — sense and 5'TGAGTGCTGAT GGGTGACGG3' — antisense codons), and incorporated human ADA sequence was also detected by PCR using human ADA specific primers (5'GACAA GCCCAAAGTAGAACTGC3' — sense and 5'TGA CCCCGAAGTCTCGCTCC3' — antisense codons). The DNA of ADA-positive colonies was then analyzed by Southern blot hybridization in order to detect the unique sites of ADA integration [8]. A total of 970 individual splenic colonies were examined. The results were processed using Student's *t* test. The number of clones was calculated from the polynomial distribution formulas [5].

RESULTS

A foreign gene can be incorporated only by proliferating cells [9]. Therefore, 2-5 days before sacrifice bone marrow donors are usually treated with 5-fluorouracil to improve the efficacy of transduction, presumably both at the expense of induction of SHC proliferation and increase in the relative concentration of SHC due to death of the main population of proliferating cells [6]. The mechanism of 5-fluorouracil effect is, however, unknown. More than 99% of splenic colony-forming units (CFUs) die 2 days after its injection, the majority of them without entering the cell cycle. Therefore, we investigated for the first time the possibility of increasing

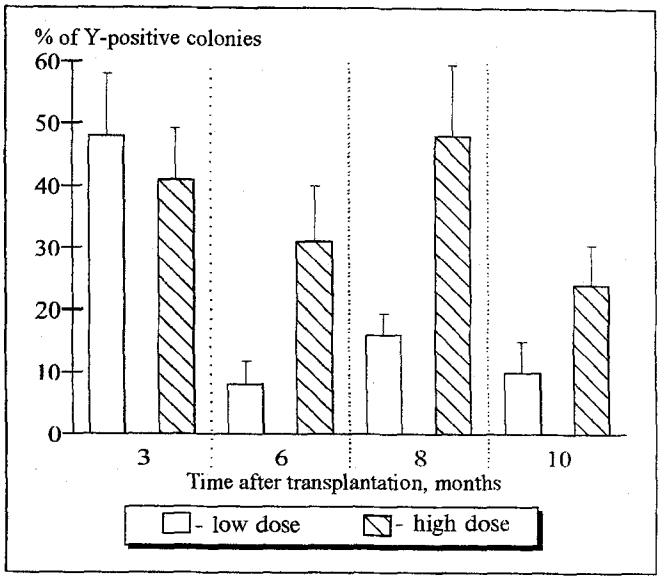

Fig. 1. Percentage of donor CFUs in the bone marrow of irradiated mice restored by transplantations of low and high doses of retrovirus-labeled hemopoietic cells.

the efficacy of a foreign gene transduction in the SHC after 6 injections of hydroxyurea which affects only the cells in the S period of cell cycle and kills the same proportion of CFUs at the expense of their successive mobilization in the cell cycle after each injection [2]. Both methods of cytostatic treatment provided high efficacy of the ADA gene incorporation in CFUs (92% for 5-fluorouracil and 100% for hydroxyurea, 26 and 29 CFUs, respectively). These data indicate that the effect of 5-fluorouracil is due to its capacity to eliminate the cells in the cycle.

The concentration of CFUs in the bone marrow of restored mice was markedly decreased (10-30 times in comparison with healthy mice or mice transplanted normal bone marrow) at all terms of the experiment (Table 1), which confirms the repopulation effect of SHC cultured with exogenous cytokines [11] or subjected to gene transfer [5]. There were no differences between the recipients restored with bone marrow from donors treated with 5-fluorouracil or hydroxyurea during all periods of the study; therefore, the data from both groups were united. More-

TABLE 1. Concentration of CFUs per 10^6 Cells in the Bone Marrow of Restored Mice

Group	Time after transplantation, months			
	3	6	8	10
5-fluorouracil, low dose	12.3±2.7	—	3.0±1.5	—
Hydroxyurea, low dose	10.9±1.6	13.3±3.5	7.5±1.1	12.3±3.8
5-fluorouracil, high dose	11.0±2.6	11.7±3.9	7.2±1.6	8.7±1.8
Hydroxyurea, high dose	14.6±5.8	8.1±3.3	3.0±1.2	13.6±3.5

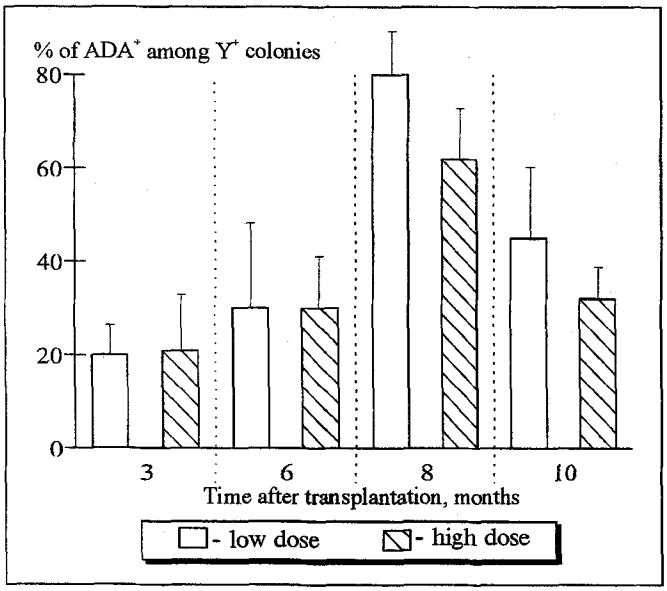

Fig. 2. Percentage of ADA gene-labeled donor CFUs in the bone marrow of irradiated mice restored by transplantation of low and high doses of retrovirus-labeled hemopoietic cells.

over, the content of CFUs in mice restored by low and high doses of hemopoietic cells was the same. Therefore, the production of CFUs per transplanted SHC increases considerably under conditions of hemopoietic stress [10].

At the same time, the duration of donor hemopoiesis depends on the dose of transplanted cells. The proportion of donor CFUs was 30-50% throughout the entire experiment in mice restored with a high cell dose, whereas in animals restored with a low dose reversion to recipient hemopoiesis was observed; 6-10 months after irradiation, the donor genotype was identified only in 10-15% of CFUs (Fig. 1). The relationship between reversion and dose of transplanted bone marrow but not incorporation of foreign sequence in the SHC is confirmed by the fact that the percentage of transduced CFUs during all periods was the same in all recipients (Fig. 2). Together with previous findings [5], this confirms that the repopulation potential of normal SHC is not higher than that of transduced cells.

Every transduced SHC contains a unique marker permitting the identification of individual clones of hemopoietic cells, which allowed us to investigate the dynamics of clones in restored mice. Polyclonal hemopoiesis with a simultaneous function of many small clones was observed in all groups. The size of a clone was usually so small that it could be detected only in individual CFUs of a derivative colony but not in the total DNA from the same bone marrow sample. Life span of the clones was short, no longer than the intervals between tests (3 months), because each clone was detected only once during the experiment, which confirms our previous results [5]. The number of clones depended on the cell dose and was 13.5±2.9 for small dose and 27.7±7.0 for high dose, which was in good agreement with the half lower content of donor cells in animals restored with low dose. Interestingly, the content of donor precursors differed 2-3 times in animals restored with 10 times differing bone marrow doses, as shown by estimation of the primitive SHC by the limiting dilution method [1]. Probably, at higher doses of hemopoietic cells not all transplanted SHC repopulate for a long time.

Our results indicate that the number of functioning hemopoietic clones directly depends on the number of transplanted SHC, which points to their limited proliferative potential and inability of self-maintenance. Besides the theoretical significance of these data, they are important for gene therapy: the maximum available number of transduced cells should be used in order to attain a stable effect.

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