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Clonal Succession in the Hemopoietic System: Number of Primitive Hemopoietic Stem Cells and Clone Lifespan

N. I. Drize, T. V. Todriya, and I. L. Chertkov

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The fate of individual primitive hemopoietic stem cells is studied by retroviral gene transfer technique. Tens of small hemopoietic clones with a lifespan no longer than 1 month simultaneously function throughout life in lethally irradiated mice reconstituted with bone marrow cells. The disappearing clones are not detected again, which confirms the clonal succession. The number of primitive hemopoietic stem cells in mouse bone marrow has been directly estimated: 1 per 8000 hemopoietic cells, or 30×10^3 per mouse, which is at least ten times higher than expected.

Key Words: *primitive hemopoietic stem cell; retroviral gene transfer; splenic colony-forming unit (sCFU); clonal succession*

Hemopoiesis is a continuous process of generation of enormous quantities of blood cells of at least 8 differentiations. All these cells originate from the same precursor: a primitive hemopoietic stem cell (pHSC), a polypotent cell with a high proliferative potential, although not immortal. These cells are in general not involved in the cell cycle, being in the deep G_0 period. They start proliferation and differentiation in succession, forming clones of hemopoietic cells replacing each other. This process has been theoretically predicted and called clonal succession [9]. It was indirectly demonstrated in studies on the time course of hemopoietic cells with inactivated paternal or maternal X chromosome in heterozygous female cats [2] and in women [6]. Clonal succession was directly proven in irradiated animals reconstituted with stem cells marked by a foreign DNA sequence [3,11,12,14]. The first quantitative characteristics of this process have been recently studied [1,5]. Hemopoiesis is maintained due to functioning of tens of small local (in various compartments of the hemopoietic system) clones of hemopoietic cells with a lifespan, as a rule, no longer than 3 months. The actual lifespan of these clones has not been determined, because analyses were carried out only once in three months.

We investigated the lifespan of clones in mice monthly reconstituted with marked bone marrow (BM), which helped us more precisely determine the lifespan of these clones and their total number. For the first time the number of pHSC, the cells maintaining the hemopoiesis throughout life, was directly determined.

MATERIALS AND METHODS

Hybrid (C57Bl/6×CBA2) CBF_1 mice aged 12-16 weeks were BM donors (males) and recipients (females). The recipients were irradiated in a dose of 12 Gy (two fractions with 3-h intervals) on a ^{137}Cs device made for Institute of Blood Transfusion. Continuous mouse BM culture was prepared as described previously [4]. Donor mice were intravenously injected with 5-fluorouracyl

Hematology Research Center, Russian Academy of Medical Sciences, Moscow

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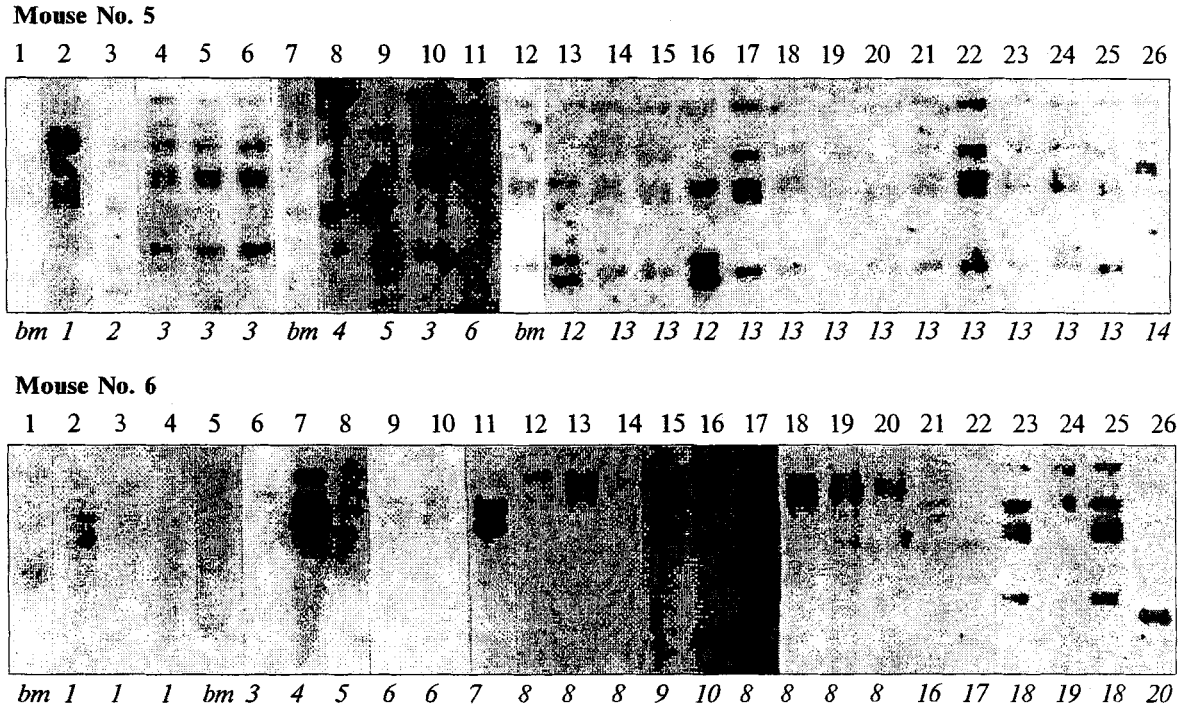

Fig. 1. Molecular markers of representative clones. Figures under blots correspond to clone number in Fig. 2; *bm*: total bone marrow DNA; figures on the tops are blot numbers.

(150 mg/kg, Sigma) 2 days before the experiment. Transduction of human ADA gene in mouse HSC was carried out as described previously [13]. The mitosis of hemopoietic cells was stimulated in irradiated (15 Gy) sublayer of a 4-week BM culture without exogenous cytokines; after 2 days, the sublayer was scraped off and transferred into a retrovirus-producing fibroblast culture (hADA GP+R86) irradiated in a dose of 50 Gy. After 48-h incubation with fibroblasts, BM cells were washed and transplanted to lethally irradiated recipients in a dose of 0.5×10^6 cells. After 4-10 months BM of reconstituted recipients was obtained by puncture of the right and left femur under light ether narcosis. Bone marrow cells of reconstituted mice were injected to repeatedly irradiated recipients, which were sacrificed after 10 days. Individual splenic colonies were isolated and assayed for the presence of a foreign DNA sequence. The donor origin of the colony was identified by PCR with primers specific for the male Y-region: the sense 5'CTCCTGATGGACAAACTTTACG3' and the antisense 5'TGAGTGCTGATGGGTGACGG3'. The presence of incorporated human ADA sequence was

TABLE 1. Number of Donor sCFU (Y^+) and Proportion of ADA-Positive Colonies in Reconstituted Mice

Mouse No.	Time after reconstitution, months											
	4		5		6		7		8		9	
	Y^+, %	ADA^+/Y^+	Y^+, %	ADA^+/Y^+	Y^+, %	ADA^+/Y^+	Y^+, %	ADA^+/Y^+	Y^+, %	ADA^+/Y^+	Y^+, %	ADA^+/Y^+
1	100	8/21	88	0/15	—	0/8						
2	94	5/16	97	8/28	69	0/19	76	1/19				
3	96	13/23	100	3/25	82	0/22	42	0/8				
4	60	5/13	92	0/22	87	0/13						
5	88	6/22	100	6/17	100	4/22	95	9/21	100	1/5	96	18/25
6	80	6/22	100	23/30	96	9/22	95	12/18	71	0/5	100	6/24
7	96	2/26	91	0/10	—	0/20						
8	100	27/37	90	23/27	91	26/31	100	13/15	100	19/25	82	9/14
9	80	10/16	95	16/18	93	4/13	90	10/19	100	22/23	93	4/26
12	63	17/24	73	14/27	59	5/24	50	0/16			80	0/16

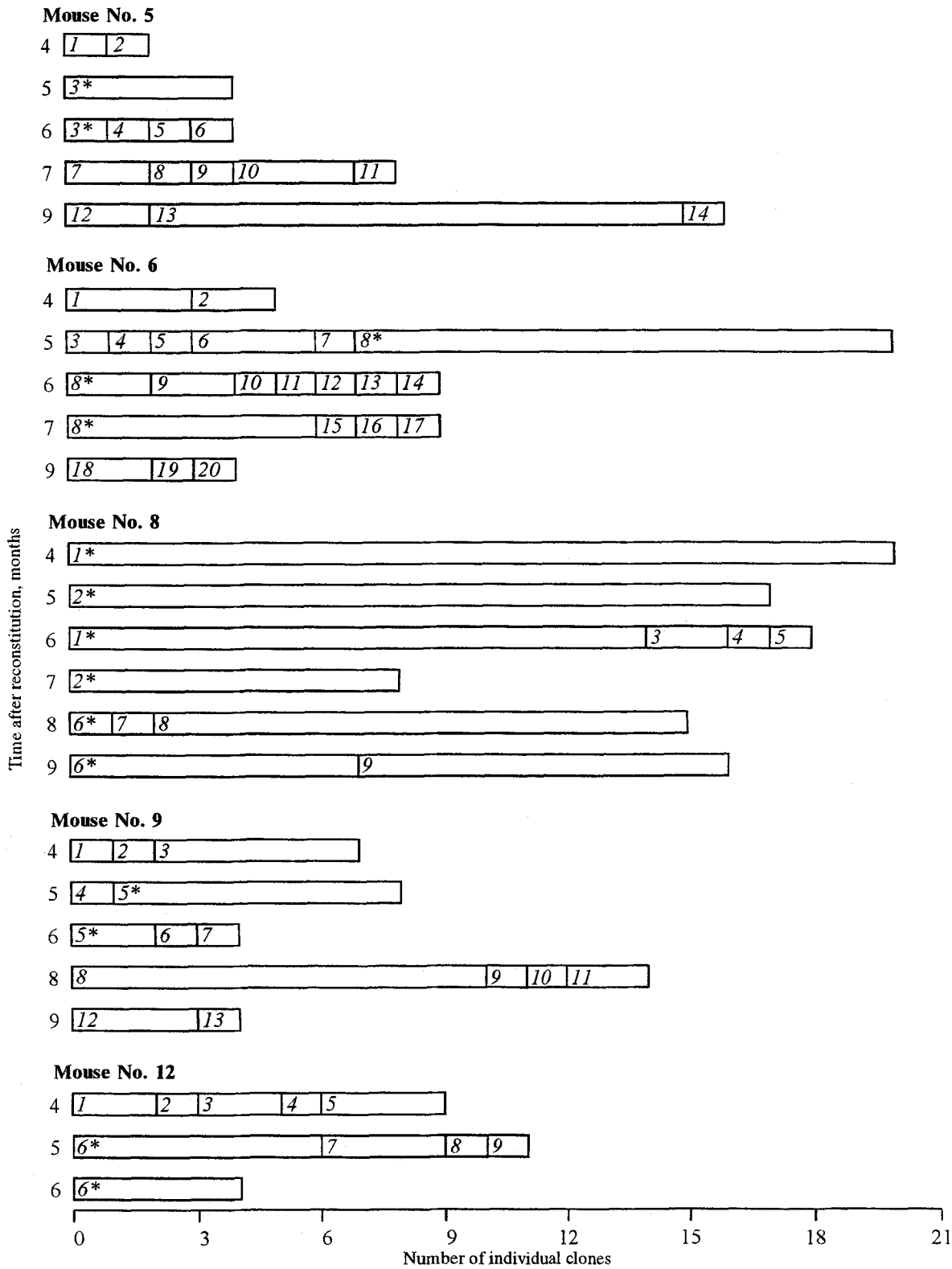

Fig. 2. Time course of clone succession in reconstituted mice. Each rectangle represents splenic colonies with a unique provirus integration site. Figures show clone numbers; *persistent clones.

tested by PCR with primers specific for human ADA: the sense 5'GACAAGCCCAAAGTAGAACTGC3' and the antisense 5'TGACCCCGAAGTCTCGCTCC3'. DNA of ADA-positive colonies was then analyzed by Southern-blot hybridization for detecting unique sites of ADA integration. A total of 1114 individual splenic

colonies were tested. The results were processed using Student's *t* test. The number of clones was estimated using the polynomial distribution formulas [5].

RESULTS

Donor cells accounted for 70-100% cells in surviving mice and maintained hemopoiesis during the entire experiment. Only one mouse (No. 3) reversed to recipient hemopoiesis 7 months after reconstitution (Table 1). Polyclonal hemopoiesis with simultaneous functioning of 1-7 clones was observed in all mice. The clone lifespan was about 1 month. Out of 64 clones studied, only one persisted for 4 months, one for 3 months, and three for 2 months. Other clones (59-92%) were detected in only one analysis, and the disappearing clones never reappeared (Fig. 1). These data confirm clonal succession (consecutive replacement of hemopoietic clones).

The detected variety of clones helped us estimate the total number of clone-forming cells in a mouse (pHSC). The more mature than pHSC precursors labeled by gene transfer persisted for no more than 1-3 months [10,14], and we started analyses 4 months after transplantation of marked BM to lethally irradiated mice, when all labeled donor hemopoietic cells, including the SCFU, were derivatives from initially labeled pHSC. The number of functioning clones was estimated as described elsewhere [5]. About 25 clones were detected during a month. The percentage of labeled sCFU among donor cells was about 50% (Table 1). If we presume that pHSC carrying no marker function at least not worse than the labeled ones, the mean number of hemopoietic clones is 50 per month.

The lifespan of mice used in our experiments is 2-2.5 years. Therefore, about 600 clones function throughout life. It was previously shown that the clones function locally, and very rarely the same clone was detected in different hemopoietic territories. For example, different clones function simultaneously in the femur and tibia, femur and spleen, humerus and tibia [5]. We examined the time course of the clones only in the femurs, which represent about 10% of all BM, and hence, about 6000 clones function at all hemopoietic territories throughout life. Each mouse received cells initially contained in 1.5 femurs of donor mice. During culturing (4 days) pHSC divide no more than twice. Therefore, the femur contain at least 1500 pHSC and hence, their concentration in the hemopoietic system (the femur contains 12×10^6 cells) is 1 per 8000 hemopoietic cells. These estimations yield the minimum content of pHSC, assuming that the size of all clones is similar. But clones can vary in size, which is proven by different numbers of sCFU representing one clone (Fig. 2). This factor can only decrease the number of clones estimated for this model. The presence of individual large clones in an extreme case repopulating the hemopoietic system for a long time [3,8,11] and their physiological significance remain unclear. Usually oligo- or monoclonal hemopoiesis is regarded as the first stage of tumor transformation of hemopoietic cells. In fact, this can reflect normal distribution of clone size and lifespan. We evaluated the incidence of leukemic transformation in reconstituted animals with oligo- and monoclonal hemopoiesis.

We demonstrated that the actual content of pHSC in the mouse hemopoietic system is at least 10-50 times higher than was reported [7,15]. This explains why despite a short lifespan of individual clones the population of pHSC is virtually inexhaustible and even by the end of life their total number decreases negligibly (by 2-3%) and this decrease cannot be detected by comparing the repopulation potential of young and old mice [7]. In general, similarly to many other molecular and cell systems, the structure of the hemopoietic system is characterized by excessiveness. The functions of many genes in the genome can be realized via different pathways; hundreds of oocytes can be maximally utilized throughout life of a mammal, although hundreds of thousands are intended for the purpose; the regeneration capacity of liver cells is much higher than is actually utilized, *etc.*

Coming to such a conclusion with regard to the hemopoietic system, we must specify that we experimented with pHSC containing a foreign gene, and though such cells, at least at the level of sCFU, are characterized by a normal proliferative potential [5], the procedure of gene transfer requires obligatory exit of the cell from the dormant status and entry into the S period of the cell cycle. We do not know whether pHSC which once started proliferating differ from intact dormant cells of this compartment of the hemopoietic system.

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