Kinetics of Hematopoietic Clones in Reconstituted Mice

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 123, No. 2, pp. 180-183, February, 1997
Original article submitted October 20, 1995

In mice reconstituted with genetically marked bone marrow, hematopoiesis is shown to be effected, over a period of 14 months after transplantation, by numerous locally existing and short-lived cell clones that succeed one another as the potential of the clonogenic precursor cell is depleted. In each reconstituted mouse, several dozens of hematopoietic clones are functional in the bone marrow. Immortal self-maintaining stem cells with unlimited proliferative potential are not detected in this system.

Key Words: *hematopoietic stem cell; retrovirus gene transfer; clonal succession; reconstituted mice*

A major problem in understanding the mechanisms by which cellular self-renewing systems, such as skin, intestine, and hematopoietic organs are maintained, concerns the main properties of stem cells in these systems. Are these cells capable of self-maintenance, i.e., are in fact immortal cells that can divide without differentiation (which is what the currently prevailing doctrine holds) or do they possess only a limited proliferative potential and are expended over the lifetime sequentially, producing cell clones that succeed one another (a process termed clonal succession [4])? No answer to this question has been provided as yet even for the hematopoietic system which has been the subject of more study than any other. Although it has been repeatedly demonstrated that donor hematopoiesis is inevitably and rapidly depleted when bone marrow is passaged *in vivo* [3], this does not necessarily mean that the proliferative potential of the hematopoietic stem cell (HSC) is limited, since depletion may result from damage to HSCs during the transfer procedure, primarily because of their repeated separation from the hematopoietic environment. The recently developed method of HSC marking by foreign DNA using retrovirus gene transfer has enabled investigators to trace the fate of individual clones. Marked HSCs have been shown to produce a few clones of hematopoietic cells which gradually (over several months) repopulate the entire hematopoietic system. Only one clone is usually retained (much less often 2 or 3 clones), which repopulates the recipient's hematopoietic system and is capable of supporting hematopoiesis even after transfer to a second irradiated recipient [1,5,9]. One shortcoming of these studies was that hematopoiesis in each recipient was examined only once; therefore, it was impossible to follow the kinetics of individual clones. Since the sensitivity of the methods used was relatively low, only giant clones representing no less than 10% of all hematopoietic cells in the sample could be identified.

The limitations inherent in the existing models are circumvented by our method whereby bone marrow can be repeatedly obtained from a live mouse, and clones can be studied at the level of one cell (colony-forming unit-spleen, CFU-S). Using this method we were able to show that hematopoiesis in reconstituted mice is maintained by a multitude of short-lived small clones of hematopoietic cells, which confirms that the proliferative potential of HSCs is limited and that clonal succession occurs in the hematopoietic system.

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0007-4888/97/0002-0155$18.00

MATERIALS AND METHODS

In this study 12- to 16-week-old (C57Bl/6×DBA_2) BDF_1 hybrid mice were used as bone marrow donors (males) and recipients (females). Recipients were irradiated in a dose of 12 Gy — two fractions at a 3-h interval from a ^{137}Cs radiator. A long-term bone marrow culture was established by the method of T. M. Dexter *et al.* [2]. Donor mice received an intravenous injection of 5-fluorouracil (Sigma) at 150 mg/kg body weight 2 days before the experiment. The human adenosine deaminase (ADA) gene was transduced as described by B. D. Luskey *et al.* [7]. Briefly, hematopoietic cells were preliminarily stimulated to divide using two protocols. In protocol 1, they were cultured for 2 days with high concentrations of two exogenous cytokines, human recombinant interleukin-6 (100 units/ml; Amgen) and rat recombinant stem cell growth factor (50 ng/ml; Amgen), and then transferred to a mitomycin C-pretreated retrovirus-producing fibroblast line (hADA GP+E86) in the presence of the indicated growth factors. In protocol 2, donor cells without exogenous cytokins were explanted to the 15 Gy-irradiated adherent cell layer of a 3- to 4-week old long-term bone marrow culture, and 2 days later the cell-containing layer was removed with scraper and transferred to the hADA GP+E86 line without growth factors. After a 48-h incubation on the producer cell line (both protocols), bone marrow cells were washed and injected into lethally irradiated recipients. After 1.5-12 months, bone marrow was repeatedly extracted from the reconstituted recipients by thigh puncture under light ether anesthesia. Bone marrow cells from recon-

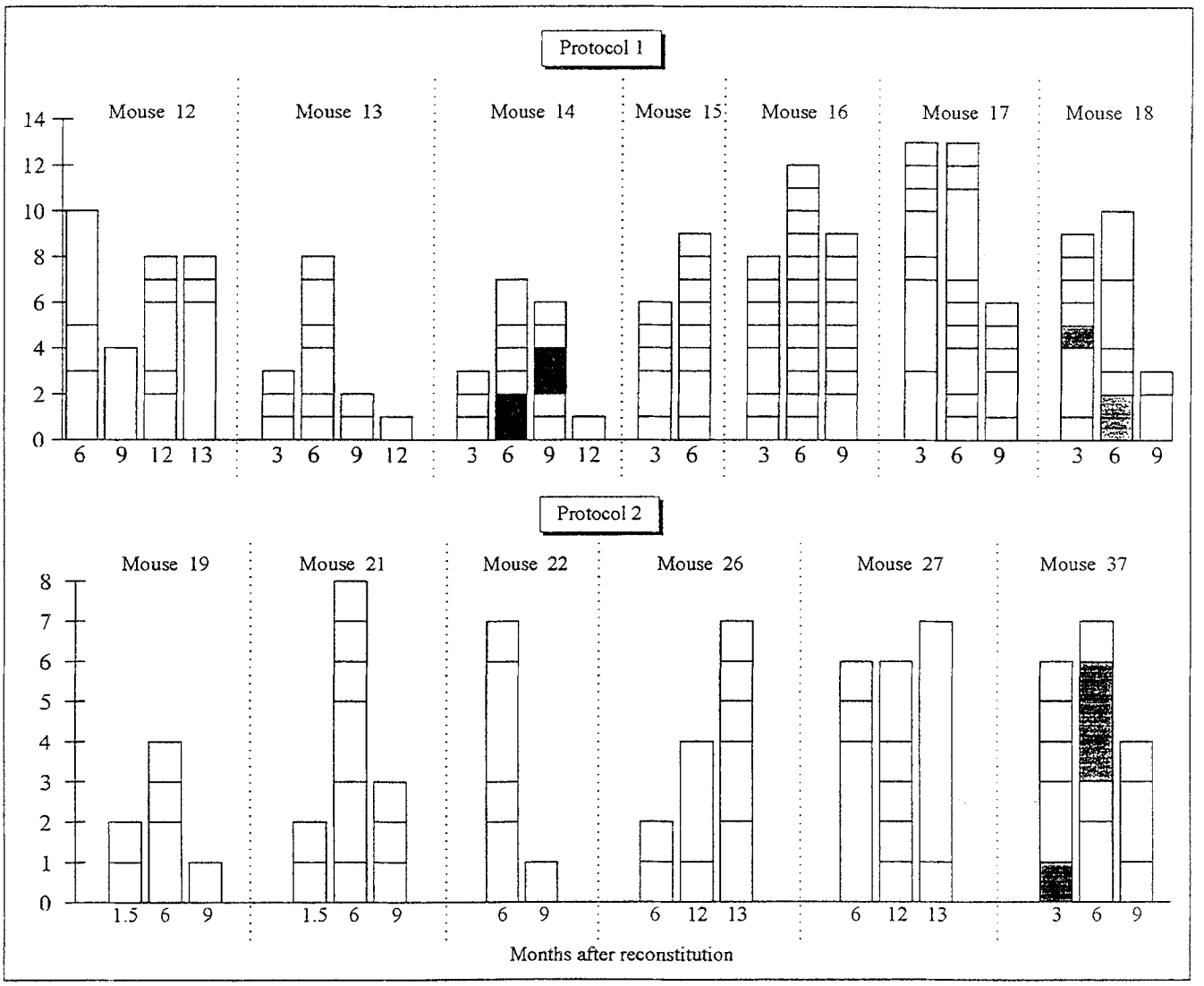

Fig. 1. Variations with time in the number of marked clones in reconstituted mice. Each rectangle represents the number of splenic colonies with a unique integration site, the size of rectangle corresponding to the number of colonies having the same marker. Shaded rectangles denote repeating clones. Ordinates: number of individual clones.

TABLE 1. Make-up of Hematopoietic Clones from Different Areas of Hematopoietic Territories in Mice 13-14 Months After Their Reconstitution[1]

	Markers in hematopoietic organs of reconstituted mice			
mouse	shank	thigh	spleen	thymus
1	—	0	*	+
12	—	0	+	—
18	0	0*	+	0
26	0	*	*	+
27	—	—	0	*
40	—	0	*	*
44	0	0	—	+

Note. [1]Symbols denote unique clones in individual mice (similar symbols are used for different mice); dashes indicate no data.

stituted mice were then injected into second irradiated recipients which were killed 11 days after transfer to isolated individual colonies from the spleen. The donor origin of a colony was checked with the polymerase chain reaction using primers specific for the Y region of males (5'CTCCTGATGGACAAACTTTACG3' (sense primer) and 5'TGAGTGCTGATGGGTGACGG3' (antisense primer); the presence of the incorporated human ADA sequence was also ascertained with the polymerase chain reaction using primers specific for human ADA (5'GACAAGCCCAAAGTAGAACTGC3' (sense primer) and 5'TGACCCCGAAGTCTCGCTCC3' (antisense primer). The ADA DNA of positive clones was then assayed for the number of incorporated ADA copies by Southern blot hybridization [8]. Mice were killed 12-14 months after reconstitution, DNA was isolated from their bone marrow, spleen, and thymus and assayed as above. A total of 247 individual CFU-S clones were followed over time in 44 reconstituted mice.

RESULTS

The efficiency of gene transfer to primary CFU-S proved to be very high: the ADA sequence was incorporated by 58 of the 68 CFU-S studied (85%), and several copies per genome were frequently incorporated. Gene transfer to primitive HSCs that provide prolonged maintenance of hematopoiesis was also effective. Marked CFU-S were regularly detected throughout the observation period of 1.5-14 months. Since CFU-S are a short-lived cell population, the time of their existence in culture and in the body being only about 1 month [6], the human ADA-containing CFU-S were, therefore, not the initially marked cells but rather descendants of the primitive HSC that had incorporated the gene in the process of transfer.

The kinetics of CFU-S clones in mice during 12 months after their reconstitution is shown in Fig. 1: a large diversity of clones (10 or more) were detected in each mouse over the observation period. It is easy to calculate using formulas of polynomial statistics that such a diversity could only result if the total number of existing clones was somewhere between 40 and 50. The polyclonality of hematopoiesis persisted throughout the experimental period with no tendency toward a decrease in the number of clones in the first few months after reconstitution. Another characteristic feature of hematopoiesis in reconstituted mice was the short life-span of the clones. As seen from Fig. 1, most of the clones were detected only once; only 7 clones were detected twice, at an interval of 3 months. Moreover, when some of the mice were killed 1 month after the last examination of CFU-S clones (13 months after the restoration of hematopoiesis), the clonal make-up was found to have changed completely. Consequently, the life-span of clones was, as a rule, less than 3 months. The clones that had disappeared never reappeared. These findings provide good evidence for the existence of clonal succession in the hematopoietic system, which indicates that the proliferative potential of HSCs is limited.

A new characteristic of clones, their small size, is very important. Table 1 shows some clones found in several hematopoietic territories. The vast majority of clones were different in different parts of the hematopoietic system. Thus, clones in the bone marrow were distinct from those in the spleen or thymus and even clones in the thigh were distinct from those found in the shin. This suggests that the migration of cells between hematopoietic organs is slight even in reconstituted mice (i.e., in those which have sustained an extremely severe hematopoietic stress) and that each of such small areas (in comparison with the hematopoietic system as a whole) has its own set

of functioning clones in these animals. In our study several dozens of clones were found to be functioning in the thigh of each reconstituted mouse at each examination time. A more striking proof of the small clone size was the failure to detect the particular clone in the total bone marrow DNA. As a rule, clones could be detected only at the level of individual precursors, CFU-S, in a given bone marrow sample. In the same bone marrow sample, the marker was not detectable by blot hybridization in the total DNA from the sample, and mature cells of the clone therefore constituted much less than 10% of cells in the sample.

This study showed for the first time that hematopoiesis is sustained by a multiplicity of local short-lived small hematopoietic clones that succeed one another as the potential of the clonogenic precursor cell is depleted. Immortal self-maintaining stem cell are not detectable in this system.

This work received support from the Russian Foundation for Basic Research and the International Science Foundation (M5X300 and M5X00, M4M300, and M4M00).

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