for A and ϕ within the segment (1) yields definite values of the group A and ϕ.

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Stem Hematopoietic Cells with Inserted Foreign Gene: Proliferative Activity and Proliferative Potential in the Long Term after Transplantation into Irradiated Mice

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The transfer of the human adenosine deaminase gene to murine stem hematopoietic cells is performed on an irradiated sublayer of a long-term bone marrow culture by the conventional method of retroviral transduction with cytokines and by stimulation of stem cells without cytokines. The efficiency of gene transfer into colony-forming units (CFUs) with the aid of cytokines is 72% and without them it is 50%. In irradiated mice reconstituted with the retrovirus-infected bone marrow cells the donor hematopoietic activity is preserved during a 1-year period. The proliferative activity of CFUs of chimeric cells 6 months after the reconstitution was the same and did not depend on the mode of gene transfer. The spleen repopulation activity is lowered in all the groups of chimeric mice 6-12 months after reconstitution.

Key Words: *stem hematopoietic cell; retrovirus gene transfer; proliferative activity; proliferative potential; adenosine deaminase; CFUs*

Methods have now been developed for transferring foreign genes to the genome of mammalian somatic cells. The most effective among these methods is retroviral transfection, the technique allowing for the insertion of foreign DNA in the genome of stem hematopoietic cells (SHC). It has been demonstrated that SHC containing a neutral gene are capable of repopulating the hematopoietic system of irradiated animals and of maintaining hematopoiesis over significant periods of time [3,7]. However, the main properties of SHC, such as the development potential, proliferative potential, and proliferative activity of the posterity, have not been compared in sufficient detail for cells carrying a cell marker and intact cells. Meanwhile, cells with a cell marker differ from intact cells not only in the presence of foreign DNA in their genome but

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also being stimulated for a long time with pharmacological concentrations of hematopoietic growth factors during gene transfer. This stimulation causes proliferation of resting SHC, which may change the major characteristics of these cells over a prolonged period or forever.

We studied the proliferative potential and proliferative activity of SHC of mice reconstituted with bone marrow cells in which the gene coding for human adenosine deaminase (ADA) had been inserted. In order to investigate the possible influence of cytosine prestimulation on the properties of SHC we have developed a method of gene transfer without cytosine prestimulation.

MATERIALS AND METHODS

Experiments were performed on 12-16-week-old BDF1 mice. Bone marrow from females was grafted into males. The mice were irradiated with a total dose of 12 Gy: two sessions with a 3-h interval in a ^{137}Cs IPK apparatus. A long-term culture of bone marrow cells was maintained by the method of Dexter [2]. The GP+E86/hADA retrovirus producer cell line, containing the coding sequence for human ADA expressed under the promoter of human phosphoryl kinase, was used for the gene transfer. These cells were kindly provided by Dr. D. Williams (Indianapolis). It was demonstrated that these cells never produce a replication-competent virus [5]. The titer of produced retroviral vector was more than 10^6 colony-forming cells (CFC)/ml. The cells were cultured in Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). One day prior to the experiment the producer cells were treated with mitomycin C (5 μg/ml, Serva) for 2.5 h and seeded at a 1:3 ratio in αMEM medium supplemented with 20% FCS. The donor mice were treated with 5-fluorouracil (150 mg/kg, Sigma). The gene transfer was performed in two ways: 1. Bone marrow cells were stimulated by a combination of growth factors (human recombinant interleukin-6, 50-100 U/ml + rat recombinant growth factor for stem hematopoietic cells (50 ng/ml, both from Amgen) for 2 days and transferred to mitomycin C-treated retrovirus producer for 2 days with the addition of the specified cytokines to the culture medium (αMEM with 20% FCS). 2. The donor hematopoietic cells were explanted on an irradiated (15 Gy) bone marrow sublayer (3-4-week culture) in Fisher medium with 20% horse serum without exogenous cytokines. The cells were harvested with a scraper after 2 sec and transferred to the producer line in αMEM medium with 20% FCS without growth factors. In both cases the cell concentration was $1.5\text{-}2\times10^6$/ml; polybrene (4 μg/ml, Sigma) was added to the medium during culturing on the producer. After transduction the hematopoietic cells were injected intravenously into the irradiated mice. Two months or one year following the procedure bone marrow was derived from the femurs of the mice under ether anesthesia and grafted into irradiated mice for the analysis of CFUs. The proliferative activity of CFUs was determined in chimeras by the suicide method with oxyurea (1 mg/ml). The number of spleen colonies, proliferative potential (the number of daughter CFUs/colony), and the presence of the foreign ADA gene were analyzed in the secondary recipients. The cells derived from 1/5 of the colony were injected into the secondary recipients. DNA

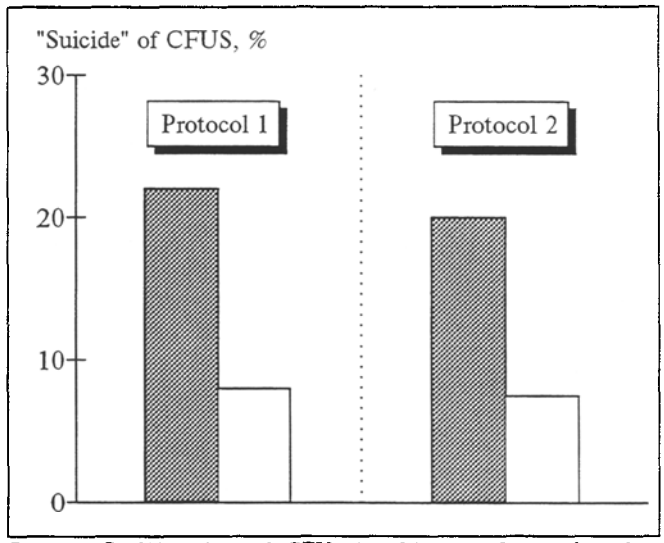

Fig. 1. Proliferation of CFUs in chimeras 6 months after reconstitution. Black bars: low dose of bone-marrow cells; white bars: high dose of bone-marrow cells.

TABLE 1. Characteristics of Lethally Irradiated Mice Reconstituted with Hematopoietic Cells Carrying a Cell Marker

Group/№ of chimera	Means of transduction	Injected cell dose	Number of chimeras	Chimeras surviving at 6 months	Chimeras surviving at 12 months
1/1-4	Protocol 1	1.0×10^5	4	3	2
2/5-8	(with cytokines)	1.0×10^6	4	4	1
3/9-12	Protocol 2	1.5×10^5	4	3	2
4/13-27	(without cytokines)	2.2×10^6	15	15	11

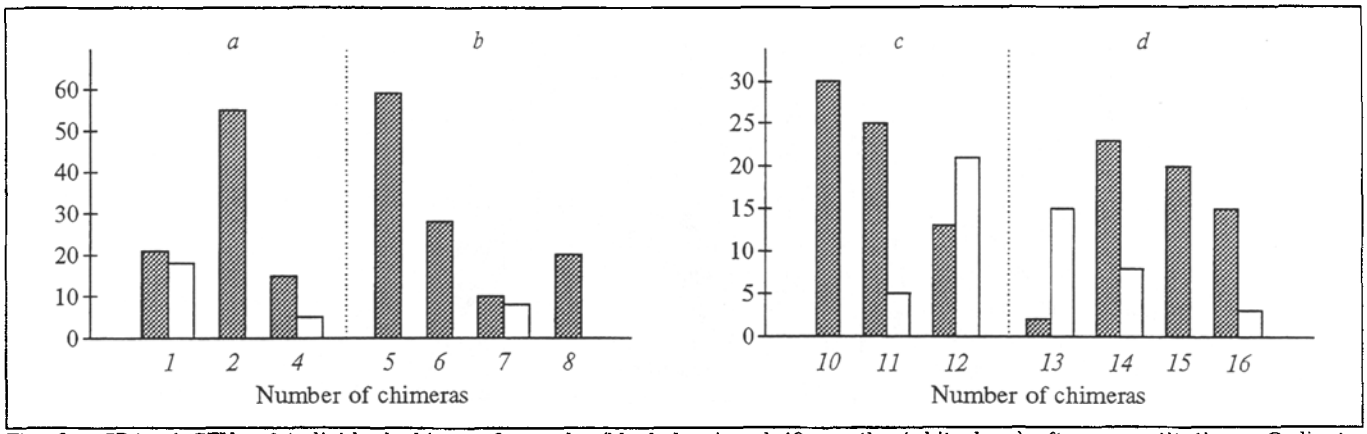

Fig. 2. SRA of CFUs of individual chimers 6 months (black bars) and 12 months (white bars) after reconstitution. Ordinate: number of daughter CFUs per 11-day-old spleen colony. *a*, *b*) protocol 1; *c*, *d*) protocol 2, low and high dose of bone-marrow cells, respectively.

was isolated from the cells forming half of the colony, cleaved with EcoR1 (the vector used in this study has only one restriction site for this enzyme), electrophoresed in a 0.8% agarose gel, and transferred to nylon filters as described elsewhere [6]. The Hind III fragment of ADA cDNA (1.2 Kb from psK-ADA [4]) was used as a probe in blot-hybridization. For the identification of the donor origin of CFUs, i.e, for exclusion of inversion of the chimeras, the Y-probe from pDP1122 plasmid containing 1.5 kilobase pairs (Kb) EcoR1/ Hind III fragment of murine Zfy-2 cDNA [4] was used; the plasmid was a generous gift of Dr. L. Brown (Cambridge).

RESULTS

The efficiency of transfer of the human ADA gene in murine CFUs was very high when pharmacological concentrations of cytokines were used during transduction: 52 CFUs out of the studied 72 CFUs (72%) contained 1-6 copies of the exogenous sequence per genome. Physiologically more favorable stimulation conditions (incubation on an irradiated sublayer of a long-term culture of the bone marrow) also provided successful transfer of the gene, although the efficiency of transfer was lower: only 9 of 18 CFUs (50%) contained the human ADA gene. These findings permit a comparison of the fate of SHC transplanted in an irradiated organism depending on the stimulation with high, not physiological concentrations of hematopoietic growth factors. Table 1 shows the data on lethally irradiated mice reconstituted with varied doses of bone marrow cells carrying the human ADA gene. At 6-12 months after the procedure, all the surviving mice were nonreversed chimeras, i.e., hematopoiesis was maintained by the donor (male) cells. Thus, neither the insertion of a foreign gene by itself nor prestimulation with cytokines reduced the repopulation potential of the primitive SHC that provide for long-term maintenance of hematopoiesis.

The proliferative activity of CFUs in chimeras was studied 6 months after reconstitution (Fig. 1). The results proved to be practically the same when two protocols for gene transfer were used. In the mice reconstituted with a high dose of hematopoietic cells the proportion of CFUs that were in the cell cycle was 10%, i.e., it did not differ from the norm. Fundamentally different results were obtained in the mice reconstituted with a small dose of bone marrow cells: the "suicide" rate was more than 20% and differed significantly from the control. It should be mentioned that the "suicide" rate for the CFUs carrying the human ADA gene was twice as high as in intact cells. However, since the number of colonies containing the marker gene is small (only 9 out of 42), the difference is statistically insignificant. Previously we showed that the number of primitive SHC in mice reconstituted with a low dose of normal bone-marrow cells remains reduced during a long time [1]. Here we demonstrate a second difference of such chimeric mice which had been reconstituted with bone-marrow cells carrying a cell marker: this is a prolonged (several months) maintenance of increased proliferative activity of CFUs, one of the early hematopoietic precursors. This is additional convincing evidence corroborating the concept that SHC, which cannot restore balanced hematopoiesis after their number has dropped below a certain threshold value, lack a genuine capacity for self-renewal.

The self-renewal capacity of CFUs was characterized by their splenic repopulating activity (SRA), i.e., by the number of daughter CFUs in 11-day-old spleen colonies. This index was inves-

tigated in individual chimeras 6 and 12 months after reconstitution. As expected, SRA activity was considerably decreased in all chimeras: 20-60 vs. 150 CFUs per colony in intact mice (Fig. 2). A decrease in SRA has been observed in animals reconstituted with bone-marrow cells carrying no marker [2]. Thus, in this case cells which had undergone transduction did not differ from native cells, and the insertion of the human gene had no significant effect on their proliferative potential. Interestingly enough, in all the chimeras reconstituted with the bone marrow stimulated with cytokines the proliferative potential of CFUs dropped continuously, while in 2 out of 5 animals reconstituted with bone marrow not stimulated by cytokines the proliferative potential at 12 months was higher than at 6 months after the procedure (Fig. 2). This may confirm our data indicating that when cytokines are used there is no clonal succession of hematopoietic cells such as is revealed after reconstitution of hematopoiesis with SHC not stimulated by cytokines [6]. The clones replacing each other naturally have different SRA. Further studies of hematopoiesis in chimeras at later periods will shed more light on this issue.

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