i.e., outside the proliferative cycle [6], addition of irradiated bone marrow cells will have a marked stimulating effect on proliferation of the subcultured bone marrow fibroblasts. The impression is created that the growth-stimulating action of bone marrow cells in the presence of 20% serum in the culture medium depends on the stimulating effect on cells in the G_0 phase of the cell cycle. This stimulating effect on cells in the G_0 phase of the cell cycle. This stimulating effect may perhaps be provided by the presence of megakaryocytes among the bone marrow cells [5]; these cells contain platelet growth factors and may secrete them into the cultures. Dependence of proliferation of CFU-F and of their descendants in culture on additional growth-stimulating influences besides those provided by the presence of high concentrations of embryonic serum (20%) in the culture medium, is essential for growth of other types of fibroblasts in the cultures. In fact, for diploid strains of fibroblasts of different origin in culture, a sufficient quantity of growth-stimulating factors for maximal proliferation is present in medium with a high concentration of embryonic serum (20%). Accordingly, to demonstrate the growth-stimulating action of the various additional factors, besides those present in serum, it is necessary to use a cultural system which contains far lower concentrations of serum (usually 1-5%). The growth-stimulating action of the bone marrow cells on CFU-F is thus evidence that bone marrow CFU-F may have an unusual requirement for growth-stimulating factors compared with other fibroblasts. We know that CFU-F of bone marrow in situ are in the G_0 phase [1]. Evidently both CFU-F isolated from the body and bone marrow fibroblasts undergoing passage and withdrawn from the cell cycle ("cultural G_0") are necessary for entry into the G_1 period in higher concentrations of growth factors compared with stimulated fibroblasts in the course of passage, and located in the proliferative pool.

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PROLIFERATIVE POTENTIAL OF HEMATOPOIETIC STEM CELLS (CFU-S) DURING SERIAL PASSAGE IN IRRADIATED MICE IN FOCI OF ECTOPIC HEMATOPOIESIS

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According to the established view, hematopoiesis is maintained by hematopoietic stem cells (HSC), which are capable of self-maintenance [2, 3], i.e., by cells the number of divisions of which is not restricted to any finite value. Nevertheless, it is impossible to prove this by serial passages of hematopoietic cells: After 3-5 transfers the donor's hematopoietic cells are exhausted and the recipient mice either die or revert to the recipient type of hematopoiesis [4]. However, such results do not necessarily prove that the proliferative potential of

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TABLE 1. Proliferative Potential of CFU-S from Foci of Ectopic Hematopoiesis and from Foci of Recipients' Bone Marrow after Serial Passage

No. of experiment	Number of mice	No. of passage	Time between passages, months	Number of surviving mice	Number of daughter CFU-S in 11-day splenic colonies produced by CFU-S*	
					From bone marrow	From transplant
0	50	1	1,5	35	22	68
		2	2,0	16	16	10
		3	2,5	11	42	27
2	32	1	1,5	30	15 (100/100)	28 (100/100)
		2	5,0	23	30 (96/200)	26 (100/70)
		3	2,5	20	9 (78/200)	9 (86/200)
		4	2,0	2	31 (14/100)	5 (49/100)

Legend. *) For bone marrow of intact CBF_1 mice this value is 66.5 ± 4.0 (M ± m; four experiments). In parentheses: numerator — percentage of donor metaphases, denominator — number of metaphases studied.

HSC is limited. It is quite possible that these cells do not withstand the traumatic conversion of bone marrow tissue into a unicellular suspension or repeated mechanical dissociation from their own microenvironment. Furthermore, since their concentration is low, and since with each transfer only a small proportion of the proliferating descendants of HSC is taken, the chances of loss of true HSC with the greatest proliferative potential increase with each successive passage.

To avoid at least some of these technical limitations, we carried out serial passage of hematopoietic tissue in a fragment, without conversion into a unicellular suspension. In this case the traumatizing influence of the procedure of preparing the bone marrow cell suspension and their enforced separation from the stromal microenvironment are absent. During transfer of a whole implant, all HSC contained in it at the time of transfer will undergo passage.

Data on the proliferative potential of HSC, which here is taken to mean their ability to produce daughter CFU-S in splenic colonies after 11 days, were obtained during serial passages of foci of ectopic hematopoiesis in lethally irradiated mice and compared with the proliferative potential of HSC migrating from foci into the recipients' bone marrow.

EXPERIMENTAL METHOD

Experiments were carried out on mice of both sexes $(CBA \times C57BL/6)F_1$ (CBF_1) and $(C57BL \times CBA/T6T6)F_1$ (CBF_1T6) aged 10-16 weeks. The recipient mice were irradiated with ^{137}Cs γ-rays on an IPK apparatus in a dose of 10-12 Gy, and dose rate of 0.25 Gy/min. In the course of 2 h after irradiation syngeneic CBF_1 T6 (experiment No. 2) femoral bone marrow was implanted beneath the renal capsule of recipients (always CBF_1), anesthetized with hexobarbital intraperitoneally (1% solution, 0.25-0.35 ml per mouse). The focus of ectopic hematopoiesis formed 1.5-5 months after transplantation was transferred entirely from the bone beneath the renal capsule of the next recipient. Altogether three or four passages were carried out by this method. To determine the proliferative potential of the CFU-S, hematopoietic cells of ectopic foci and of bone marrow of the recipient mice were injected in a dose of $3 \cdot 10^4$-$4 \cdot 10^4$ cells into mice irradiated in a dose of 12 Gy. After 11 days the number of daughter 8-day CFU-S in the splenic colonies was counted by injecting cells of the colonies in a dose equivalent to 0.2-1 colony into secondary irradiated recipients. The origin of the hematopoietic cells in the foci of ectopic hematopoiesis and in the bone marrow of the chimeras was determined karyologically on the basis of the presence or absence of the T6-chromosome [1].

EXPERIMENTAL RESULTS

The results are given in Table 1. By the time of transfer the number of hematopoietic cells in the transplant was $1 \cdot 10^7$-$3 \cdot 10^7$. The number of hematopoietic precursors migrating from it was sufficient to protect the lethally irradiated mice. As a result of each of the

first three passages 50-80% of recipients survived. Thus the method of transfer of hematopoietic tissue in fragment form proved to be suitable for restoring hematopoiesis in irradiated recipient mice.

The proliferative potential of the bone marrow of the intact mice used was about 70 daughter CFU-S per 11-day splenic colony. After the first transfer, however, this value fell, and the effect for CFU-S from bone marrow was 2-3 times greater than for CFU-S from a focus of ectopic hematopoiesis. During passage there was a further decrease in the proliferative potential both in the foci and in the recipients' bone marrow. In experiment No. 1, after the third passage the proliferative potential of CFU-S in bone marrow and in the implant was higher than after the second passage. It was suggested that this may have been due to reversion and to restoration of hematopoiesis by recipient's cells which had survived irradiation. This hypothesis was tested in experiment No. 2, in which the origin of the hematopoietic cells in the focus and bone marrow was tested karyologically during each passage. It will be clear from Table 1 that partial reversion was observed in the focus and bone marrow as early as after the third passage. After the fourth transfer the protective potential of the donor's hematopoietic cells was almost completely exhausted: Only two of 20 recipients survived. Virtually total reversion had taken place in the bone marrow of the surviving mice: The fraction of donor's cells in it belonged to the recipient. The proliferative potential of CFU-S in the bone marrow under these circumstances was much higher than in the focus, higher even than in the previous passage. The proliferative potential of CFU-S in this transfer was in good agreement with that in cells after the third passage in experiment No. 1. The hypothesis of reversion of hematopoiesis to the recipient's type as the cause of the increased potential of CFU-S after repeated passages was thus confirmed. HSC surviving after irradiation evidently have a proliferative potential which corresponds approximately to the potential of unirradiated cells which have undergone one passage.

Exclusion of traumatic factors associated with the procedure of obtaining a single-cell suspension and with repeated mechanical separation of HSC from their microenvironment, which can be achieved by transfer of hematopoietic cells in the form of unseparated hematopoietic tissue, did not abolish the phenomenon of lowering of the proliferative potential of HSC during their passages. The results are in fact in good agreement with those obtained by serial passages of a suspension of bone marrow cells injected intravenously, when reversion was observed after three or four transfers [3].

The most probable cause of reduction of the proliferative potential of HSC is accidental loss of cells with high proliferative potential during passage. With intravenous passages a relatively small number of hematopoietic cells is distributed each time throughout the hematopoietic system of the irradiated recipient. During transfer of hematopoietic tissue in the form of a fragment, all the cells preserved in the focus at the time of transfer commence passage each time. Loss of cells with high proliferative potential from the focus is evidently less than loss due to distribution of the donor's cells throughout the recipient's hematopoietic system. This may be the explantation of the higher proliferative potential of CFU-S from the focus compared with bone marrow cells after the first passage. If cells with high proliferative potential were able to maintain themselves, their concentration in the focus by the time of the next transfer ought to have been restored to its initial level, and the results of each subsequent passage would not differ from those of the previous passage, i.e., the proliferative potential of CFU-S from the foci ought to be higher always than that in bone marrow. Since this did not happen, it must be accepted that the concentration of donor's cells with high proliferative potential invariably decreases. Consequently, the results are yet another important, although indirect, argument in support of the view that cells responsible for recovery and maintenance of hematopoiesis in irradiated mice are unable to maintain themselves.

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