PROLIFERATION OF HEMATOPOIETIC STEM CELLS IN MOUSE EMBRYONIC LIVER CULTURES

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The use of a vinblastin method has shown that proliferation of hematopoietic stem cells, i.e., cells capable of forming colonies of hematopoietic cells in the spleen of lethally irradiated mice, continues for more than 2 weeks in organ cultures of mouse embryonic liver.

A previous communication [1] described how hematopoiesis continues in organ cultures of embryonic liver for a long time (up to 3 weeks) and hematopoietic stem cells capable of forming colonies in the spleen of irradiated mice can be found. The relative number of colony-forming units (CFU) in the cultures may actually increase during the first week by comparison with the number in embryonic liver cells. However, because of the difficulty of counting the absolute number of cells in organ cultures exactly, it was not clear whether hematopoietic stem cells multiply in the culture or whether they simply survive selectively in vitro by comparison with more mature cells. This is a problem of fundamental importance in connection with assessment of the possibilities afforded by the method of organ cultivation of hematopoietic tissue. None of the existing methods of cultivation can ensure maintenance of the kinetics of the stem cells [7].

The use of the "thymidine suicide" method has shown that the number of CFU in the bone marrow of adult animals is not reduced after a single injection of large doses of thymidine-H^3 in vivo or after exposure to thymidine-H^3 for short periods (20-30 min) in vitro [3]. If large doses of thymidine-H^3 are administered in vivo, about 30% of the CFU still remains at the end of 24 h in the bone marrow [4]. Considering that thymidine-H^3 injures cells only after incorporation into DNA, whereas unincorporated thymidine-H^3 does not cause death of the cells [3], it is evident that a large proportion of the hematopoietic stem cells in the bone marrow of the adult animal are either not participating in the mitotic cycle (in the G_0 period) or are proliferating slowly with a prolonged mitotic cycle. At the same time, during exposure of hematopoietic embryonic liver cells to thymidine-H^3 in vitro, the proportion of dying CFU may reach 40% [3].

Consequently, embryonic CFUs proliferate much more rapidly (or more of them are in a state of proliferation) than CFUs from the bone marrow of adult animals. It was interesting to investigate how these parameters change as the age of the culture increases.

To study the proportion of actively proliferating CFUs, vinblastin was used. This substance has been shown to inhibit the entry of cells into mitosis [6]. Although in some systems higher concentrations of vinblastin can evidently damage cells in other phases of the cycle, in the late G_1-period and S-period [8], investigation of the action of vinblastin on CFUs in vitro has, on the whole, confirmed its suitability for use in determining the proliferative activity of hematopoietic stem cells. In particular, during its action on hematopoietic tissue of adult animals in a stable state, in the course of 24 h vinblastin lowers the number of CFUs to 20-30% of its initial value. Hence, during 24 h up to 70-80% of the hematopoietic stem cells begin mitosis, a figure in agreement with data for the proliferative activity of CFUs obtained by the

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TABLE 1. Action of Vinblastin on CFUs in Embryonic Liver Cultures

Duration of cultivation (in days)	Group of animals	No. of mice	No. of cells injected	No. of colonies*	No. of CFUs per 100,000 cells injected	Proportion of cells dying during treatment with vinblastin (in %)
1	Control	9	35,000	1.9±0.8	5.4	70
	Experimental	10	102,000	1.6±0.5	1.6	
7	Control	8	51,000	23.4±1.7	45.5	87
	Experimental	5	70,000	4.2±0.5	6.0	
12	Control	7	32,000	14.4±1.0	45.0	56
	Experimental	8	126,000	25.0±3.3	20.0	
17	Control	6	43,000	9.7±1.6	22.6	89
	Experimental	7	78,000	2.0±0.8	2.6	

*Two colonies were found in the spleens of 24 irradiated control mice, not receiving hematopoietic cells.

"thymidine suicide" method [4]. If the stable state of hematopoiesis is disturbed, such as in the bone marrow regenerating after irradiation [10] or in the rapidly proliferating bone marrow after transplantation into irradiated animals [12], the proportion of CFUs dying after vinblastin treatment rises substantially. The object of the present investigation was to compare the sensitivity of the colony-forming cells of mouse embryonic liver to vinblastin after their cultivation in vitro for 1-17 days.

EXPERIMENTAL METHOD

Mice of line CBA were used in the experiments. The embryonic liver of 17-18 day embryos was grown in organ cultures [2]. Pieces of liver, 1-2 mm in diameter, were placed on a Millipore filter with a pore diameter of 0.45-0.9 μ, located on the boundary between the liquid (70% medium No. 199, 20% bovine or human serum, 10% chick embryonic extract) and gaseous (95% air + 5% CO_2) phases. The medium was changed every 1-2 days. Vinblastin sulphate was added to some of the cultures 24 h before the cells were used, in a concentration of 1 μg/ml. The explants were washed from the filters with medium No. 199, and the cells were suspended by passing them through a syringe with needles of decreasing diameter, flushed through with medium No. 199. Cells of the control and vinblastin-treated cultures were injected intravenously into syngeneic male CBA mice irradiated 1-2 h previously in a dose of 816-850 R. At each time of the experiment 30, 50, or 100 explants were used. The mice were sacrificed 8 days layer, the spleens were fixed in Bouin's solution, and the number of colonies was counted under a binocular loupe.

EXPERIMENTAL RESULTS

After cultivation in the presence of vinblastin for 24 h, only about one-third of the CFUs still remained. Consequently during this time the hematopoietic stem cells in the culture were actively proliferating. In the course of cultivation the proportion of rapidly proliferating CFUs actually increased, for after cultivation in the presence of vinblastin for 6-7 days the number of remaining CFUs was reduced to one-eighth of their number in the control culture. This shows that the conditions in organ cultures of embryonic liver are suitable for maintaining proliferation of the stem cells and for ensuring a relative increase in the number of CFUs in the culture (calculated per 10^5 cells). With an increase in the duration of cultivation, the rate of CFU proliferation evidently diminishes. Whatever the case, in an 11-12 day culture the proportion of CFUs affected by vinblastin was only about half of the total number of CFUs. It is interesting to note that this decrease took place before the relative number of CFUs in the culture began to fall. In fact, the number of CFUs was virtually the same in the 7-12 day cultures (it was rather less than 1 per 2000 cells) and it was significantly increased only in cultures aged 3 weeks [1]. In the cultures to begin with there is evidently a decrease in the rate of proliferation of the stem cells or of their entry into the G_0-period of the cell cycle, and death or "suicidal differentiation" takes place later.

However, proliferation of the hematopoietic cells again began to take place more rapidly toward the end of the life of the cultures; by the 17th day the proportion of CFUs proliferating in the course of the 24 h period reached 90%. It must be remembered that the proportion of proliferating CFUs was underestimated because the method of organ cultivation does not allow the absolute number of hematopoietic

cells in the culture or its decrease by vinblastin treatment to be determined exactly. There undoubtedly was such a decrease, because to obtain the same number of cells it was necessary to take twice or three times as many explants of vinblastin-treated cultures than of the controls.

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