# CHANGES IN THE NUMBER OF STEM CELLS DURING CULTIVATION OF MOUSE BONE MARROW ON A BED OF FIBROBLAST-LIKE CELLS

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The number of colonies was counted in the spleen of irradiated mice after injection of a culture of mouse bone marrow cells. The colony-forming units were found to persist in the culture only for a short time. The use of a previously grown bed of fibroblasts of bone-marrow origin did not affect the preservation of the colony-forming units or the dynamics of the change in their number.

KEY WORDS: bone marrow; hematopoietic stem cell; tissue culture.

The creation of conditions for cultivation of hematopoietic tissue whereby the powers of the hematopoietic stem cell are preserved is of great importance for the solution of problems connected with the origin, proliferation, and differentiation of cells and the possibility of controlling differentiation experimentally. However, the problem of self-maintenance of polypotent stem cells under tissue culture conditions *in vitro* still remains far from solved.

During the few attempts that have been made to preserve hematopoietic stem cells *in vitro* a sharp decrease in their number was observed during the first few hours in culture [1]. As cultivation continued *in vitro* the number of colony-forming units (CFUs) continued to remain low. A more detailed study of the life span of hematopoietic stem cells of bone marrow in liquid culture has shown that the number of CFUs falls sharply during the first few days and rises on the seventh to ninth days, after which it again falls to the control level [2]. Processes of hematopoiesis (self-maintenance of hematopoietic stem cells, production of committed cell clones, etc.) are exposed to the influence of many factors which determine the proliferation and differentiation of hematopoietic stem cells. One such factor is the state of the stroma of the hematopoietic organs. It is accordingly interesting to study the effect of a bed of fibroblast-like cells of mouse bone marrow origin as stromal elements on the preservation of CFUs in liquid culture.

The object of this investigation was to study the dynamics of the number of hematopoietic stem cells during cultivation of mouse bone marrow *in vitro*, using a bed of fibroblast-like cells of bone-marrow origin.

## EXPERIMENTAL METHOD

Male (CBA × C57BL/6)$F_1$ hybrid mice weighing 20-22 g were used. Bone marrow cells were obtained by flushing out the femora with culture medium, passed through needles of successively smaller diameter, and filtered through Capron. The cells were cultivated at 37°C in penicillin flasks in a medium consisting of equal parts of medium No. 199 and Eagle's medium with the addition of 20% inactivated bovine serum, and in an atmosphere of air with 6% $CO_2$; the total volume of the medium was 2 ml, with $10^7$ cells to 1 ml. The medium was changed on the fifth day of growth of the culture, when 1 ml of the top layer was removed and replaced by the same volume of fresh medium. The monolayer of fibroblast-like cells was grown in advance under the same conditions for 1 week. The cell concentration during growth of the monolayer was $10^7$ cells/ml.

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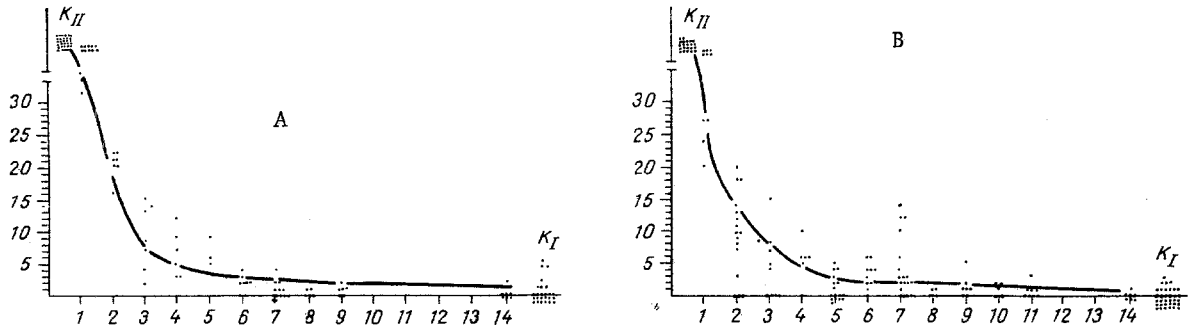


Fig. 1. Dynamics of number of CFUs during cultivation of mouse bone marrow cells: A) cultivation without, B) with bed of fibroblast-like cells. Each point corresponds to number of colonies in spleen of one animal. $K_I$) Group of animals not receiving transplanted bone marrow; $K_{II}$) group of animals into which intact bone marrow was transplanted. Abscissa, times of cultivation (in days); ordinate, number of colonies in spleen.

For the morphological analysis of the cells in culture cover slips were placed in penicillin flasks with the suspension of bone marrow cells. Cells growing on the glass, and also films of the cell suspension, were fixed with methyl alcohol and stained with azure–eosin.

The number of hematopoietic stem cells in the culture grown with or without a bed of fibroblasts was determined in the course of 14 days of cultivation by the method of Till and McCulloch [3].

The mice were irradiated in a dose of 750 R on the RUM-17 apparatus (175 kV, 15 mA, filters 0.5 mm Cu + 1 mm Al, dose rate 53 R/min. The irradiated animals were divided into three groups: Group 1 received an injection of an intact suspension of bone marrow cells in a dose of $2 \cdot 10^5$ cells per animal, group 2 received a suspension of bone marrow cells at different stages of cultivation, in a dose of $10^7$ cells per animal (the increase in the dose of cells injected was due to a decrease in the CFU content during cultivation), and group 3, into which no bone marrow was transplanted, served as the control for endogenous colony formation. Colonies were counted in the spleen on the ninth day after irradiation. The material was fixed with a mixture of formalin, alcohol, and acetic acid (9:3:1).

## EXPERIMENTAL RESULTS

To exclude the role of the fibroblast bed as the possible source of stem cells, a suspension of cells forming the monolayer (seventh day of growth) was injected into irradiated recipients. The results of these tests showed that cells forming the monolayer were unable to form colonies in the spleen. Further confirmation was given by morphological analysis: On the seventh to eighth day of growth the monolayer consisted chiefly of fibroblast-like and polynuclear cells with characteristic degenerative changes.

No significant differences were found in the number of CFUs in the suspensions of bone marrow cells cultivated with or without a bed of fibroblast-like cells (Fig. 1). After injection of intact bone marrow cells into irradiated mice ($2 \cdot 10^5$ cells per animal) confluent growth of colonies was observed in the spleen. During cultivation the number of CFUs fell exponentially. In the first 2 days the number of CFUs was considerable, but starting from the fourth to fifth days and continuing until the 14th day practically no exogenous colonies were detected. Morphological analysis of the injected cell suspension showed that at these times of cultivation the commonest cells were of the fibroblast-like type, but a few mature granulocytes, cell forms incapable of forming colonies in the spleen, also were preserved. The significant increase in the number of CFUs found on the seventh to ninth day by Meints and Goldwasser [2], using similar methods of cultivation, was not observed in this case. The results agree with those of Delmonte and Mumford [4], who found a tenfold decrease in the number of CFUs as early as on the third day of cultivation. Similar rates of decline of colony formation were observed by Dexter et al. [1].

The results thus indicate that under the conditions of cultivation used the hematopoietic stem cells retained their powers *in vitro* for not more than 2-3 days. Cultivation of the bone marrow cells on a previously grown bed of fibroblast-like cells of bone marrow origin had no significant effect on the preservation of the hematopoietic stem cells or the dynamics of the change in their number.

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# IMMUNITY TO TUBERCULOSIS IN THYMECTOMIZED ADULT MICE

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Thymectomy performed on adult animals 6 months or more before infection with *Mycobacterium tuberculosis* considerably reduces resistance to tuberculous infection; the longer the time after the operation, the greater the decrease in resistance. Disturbances of immunity connected mainly with damage to thymus-dependent cells (depopulation of the thymus-dependent zones, a decrease in the tuberculin sensitivity of the skin and the cytotoxic action of lymphocytes on antigen-containing target cells).

KEY WORDS: thymectomy; tuberculosis; immune response.

The work of Miller [8] and Metcalf [7], and subsequently many other authors has shown that the thymus in the neonatal period is the central organ of immunity. Investigations carried out mainly in the last decade [3-6, 9, 10] have demonstrated that the thymus plays an important role in immunogenesis in the adult state also. These investigations are based primarily on the study of the action of thymectomy or irradiation followed by protection of the bone marrow on the individual phenomena of immunity. The effect of thymectomy in adult animals on infectious immunity, including immunity to tuberculosis, has received much less study.

In the investigation described below the state of immunity to tuberculosis was studied after thymectomy in adult mice.

## EXPERIMENTAL METHOD

Thymectomy was performed on CBA mice at the age of 14 weeks (the technique of the operation was fully described earlier [2]). The mice were divided into groups, with 30 in each group, 2, 6, and 12 months after the operation and they were infected intravenously, simultaneously with mice of the same age undergoing mock operations, with a virulent strain $H_{37}Rv$ in a dose of 0.05 mg.

Five mice from each group were killed after infection purely for histological study of the immunocompetent organs, and 10 mice from each group were killed 3 weeks after infection. In this latter group a morphological investigation was made of the immunocompetent organs and lungs and hypersensitivity of delayed type was studied (the cytotoxic effect by the method of Averbakh et al. [1], tuberculin skin tests). The tuberculin skin tests were read after injection of Koch's old tuberculin (0.05 ml) into the footpad of the mouse's hind limb; thickening

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