EFFECT OF COLONY-STIMULATING ACTIVITY ON HEMATOPOIESIS IN ORGAN CULTURES OF EMBRYONIC MOUSE LIVER

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KEY WORDS: embryonic liver culture; precursor cells of granulocytes and macrophages; CFU-S; colony-stimulating activity.

The presence of colony-stimulating activity (CSA) in a jelly-like medium is essential for the formation of colonies of granulocytes and macrophages *in vitro* from hematopoietic precursor cells. However, there are as yet no clear ideas on the mechanism of regulation of granulo-monocytopoiesis *in vivo* and on the physiological role of CSA. Hence the interest in the study of the effect of CSA on hematopoietic cells in cultures in which the structures of the hematopoietic tissue are preserved and hematopoietic precursor cells can make contact with other cells, including stromal cells, so that the conditions closely resemble those obtaining *in vivo*. Two systems of cultures of this kind are known: embryonic liver in organ culture [3, 4] and adult bone marrow on a substrate of marrow stroma [7].

In this investigation the effect of CSA on hematopoiesis was studied in long-term cultures of mouse embryonic liver, where CFU-S, colony-forming units of granulocytes and macrophages (CFU-C), and differentiation of mature cells (neutrophils and macrophages) were maintained for several weeks [1].

EXPERIMENTAL METHOD

Liver fragments from 17-18-day (CBA × C57BL)F_1 mouse embryos were explanted in organ cultures. The methods of culture, of removing the cells from the cultures, and of counting them were described previously [5]. The composition of the medium was: serumless medium (from Gibco, USA), 20% calf serum, 2% mouse serum (CBA), 1% L-glutamine (200 mM), 5 mg% gentamicin. About half the volume of the medium was replaced by fresh twice a week. CFU-S was determined by the method in [11]. CFU-C was determined by the agar culture method [10] in Kondratenko's modification [2]. The nutrient medium (based on Dulbecco MEM medium) contained 25% embryonic calf serum, 3% sodium bicarbonate (5% solution), 0.4% of a solution of nonessential amino acids (MEM 100 ×), 0.4% L-glutamine (200 mM), 0.4% sodium pyruvate (100 mM), 0.6% solution of gentamicin (10 mg/ml), 2% HEPES buffer, 0.3% agar (from Difco, USA), and 10% CSA. Medium conditioned by mouse spleen cells (MSC), from a 7-day culture with pokeweed mitogen [9] was used as CSA. Medium from the cultures was centrifuged (3000g, 15 min), concentrated 4-6 times by means of a PM 10 filter (from Amicon, Holland), and kept at –20°C. Activity of the resulting MSC was tested in agar culture which showed that the efficiency of colony formation with cells of 18-day embryonic liver was 69 ± 11 CFU-C/10^5 cells, and with adult bone marrow cells it varied from 58 to 112 in different experiments. When the action of CSA was tested in embryonic liver organ culture, 10% MSC was added to the nutrient medium initially and with every change of medium. At each testing time cells were taken from at least two culture dishes. To determine the significance of the difference in the number of CFU-S and CFU-C between experimental and control cultures, the Student–Fisher t test was used.

EXPERIMENTAL RESULTS

The results of one of the two experiments are given in Table 1 and Fig. 1. Immediately after explantation the cultures contained 11 × 10^6–13 × 10^6 nucleated cells, 900-1000 CFU-S, and about 4000 CFU-C. After an initial period of reorganization, when the number of precursors and, in particular, the total number of hematopoietic cells, among which cells of the neutrophilic and macrophagal series were becoming the predominant populations, decreased, sta-

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TABLE 1. Effect of MSC on Number of Hematopoietic Cells and Their Precursors in Embryonic Liver Organ Culture (M ± m)

Time of culture, days	Experimental conditions	Number of cells in culture, $\times 10^5$ (M ± σ)	CFU-S		CFU-C	
			per 10^5 cells	per culture	per 10^5 cells	per culture
9	Control	9,7±0,98	41,7±2,1	404,5±20,6	230,4±7,0	2234,9±68,0
	MSC	19,4±1,39	20,9±2,4	405,5±47,0	115,5±19,8	2240,7±382,6
20	Control	16,25±0,9	41,0±4,4	666;1±72,2	106,7±2,2	1733,9±36,4
	MSC	24,6±1,29	4,3±1,0	105,8±25,2**	23,5±3,7	578,1±85,6**
21	Control	14,6±0,85	39,9±2,7	582,5±40,0	148,6±7,0	2169,6±102,2
	MSC	17,0±1,3	5,6±0,7	95,2±12,7**	20,2±5,4	343,4±91,8**
30	Control	12,0±1,1	25,0±2,6	300,0±31,2	98,3±11,2	1179,6±134,4
	MSC	18,0±1,34	4,5±0,9	81,0±16,6**	32,2±4,5	579,6±81,0*
31	Control	7,45±0,6	41,9±2,7	312,2±20,0	160,5±7,3	1195,7±54,4
	MSC	10,2±0,7	11,1±1,3	113,2±13,0**	16,2±4,3	165,0±43,8**

Legend. *P < 0.02, **P < 0.001.

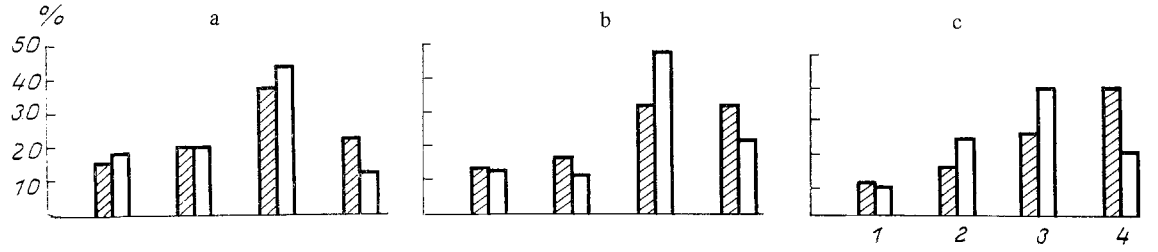

Fig. 1. Histogram of cellular composition of embryonic liver culture: effect of MSC. Unshaded columns — MSC; shaded columns — control. a) 20th day, b) 30th day, c) 31st day of culture. 1) Blast and lymphoid cells, 2) proliferating neutrophilic granulocytes, 3) nonproliferating neutrophilic granulocytes, 4) monocytes and macrophages. Eosinophilic and basophilic neutrophils and erythroid cells accounted for not more than 3%.

ble hematopoiesis was established within at least 4.5 weeks. In the control cultures (without addition of MSC) differentiation of CFU-C and mature cells took place, with the production mainly of neutrophilic granulocytes. In the presence of MSC, by the 9th day the relative number, and later the absolute number also of both types of precursors fell in the cultures. However, until 4.5 weeks no further decrease was observed in either CFU-S or CFU-C, and production of differentiated cells actually increased in this experiment, more especially on account of granulocytes (Fig. 1). Presumably the addition of MSC shifted equilibrium toward the more intensive production of differentiated cells with a lower level of maintenance of precursors. This effect differs from that observed by other workers in a system of bone marrow cell culture in association with stromal cells, when the addition of unpurified conditioning medium from a culture of L cells caused a progressive decline in the level of precursors, especially CFU-S, and cessation of granulocytopoiesis, with survival predominantly of macrophages. This noxious effect was not specific, for purified preparations of CSA did not affect bone marrow cultures, and the addition of antibodies against colony-stimulating factor together with unpurified conditioning medium preserved the toxic effect of the latter [8]. In the opinion of these workers, in long-term bone marrow cultures optimal conditions for granulo-monocytopoiesis already exist, and further stimulation of differentiation of CFU-S and CFU-C by CSA is prevented either by factors liberated from the stromal layer or by close intercellular interactions. The conditions in embryonic liver organ culture are perhaps not so optimal for granulo-monocytopoiesis as in liquid bone marrow culture on a stromal substrate. Whatever the case, the concentration of CFU-C (but not CFU-S) and the ratio of granulocytes to macrophages in embryonic liver cultures under our conditions was always lower than in bone marrow cultures. That may be why the possibility of further stimulation of more intensive production of mature cells of the neutrophilic and monocytic series still remains present in a system of embryonic liver organ culture.

The specificity of action of CSA from MSC in embryonic liver culture, however, remains questionable. The possibility likewise cannot be ruled out that MSC contains another factor (not colony-stimulating) which is responsible for the fall of CFU-S (and CFU-C?) level, and which may act indirectly through the stroma of the cultured liver. For example, cultures with MSC were found to differ appreciably in their external appearance from the controls, in their smaller zone of growth and a reduction in the total mass of the culture.

Examination of a series of increasingly complex hematopoietic systems from the agar culture to the living organism shows a decrease in the effect of the exogenous source of CSA: in a system of disconnected cells in a jelly-like medium dependence of CFU-C on CSA is complete, in embryonic liver organ culture CSA causes only a shift of differentiation toward the formation of mature cells, and the presence of CSA in bone marrow cultures on stroma no longer has any appreciable effect; finally, there is no firm evidence that granulopoietin is a colony-stimulating factor *in vivo* [6].

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EFFECT OF EXPERIMENTAL MODIFICATION OF THE GLUCOCORTICOID RHYTHM ON CIRCADIAN FLUCTUATIONS OF GLUCOSE TOLERANCE IN RATS

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When the insular function of the pancreas is normal it is rare to find significant circadian fluctuations in the blood sugar level [13] despite the discontinuous pattern of food intake and stress, and alternation of work and rest. This is largely due to the well-marked circadian rhythm of response of the β-cells to stimulating factors [10], which has also been demonstrated in experiments *in vitro* [8]. If the function or reliability of the β-cells is reduced, the role of the contrainsular hormones is enhanced, especially that of the glucorticoids, with their precise circadian rhythm, in the formation of daily fluctuations in the blood sugar. Stimulating glucose processing in reactions of gluconeogenesis, increasing the sensitivity of β-cells to glucose [12], acting in relation to many factors as insulin antagonists in target tissues, and inhibiting the binding of insulin with receptors [5], glucocor-

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