in which the frequency of reaction after autohemostimulation was 5-15% above the initial level. These differences can evidently be explained on the grounds that additional scatter or activation of NCILCT which were present in the recipients before hemostimulation, but which were not revealed because of their low activity, takes place in the course of hemostimulation.

These results not only confirm the existing view on the stimulating action of injections of autologous blood, but they can also be used for applied purposes: to raise NCILCT activity in diagnostic sera.

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EFFECT OF RABBIT ANTISERUM TO MOUSE BRAIN ON BONE MARROW CELLS FORMING GRANULOCYTE-MACROPHAGE COLONIES IN AGAR CULTURE *in vitro* AND *in vivo*

A. I. Kolesnikova, A. G. Konoplyannikov, T. N. Semenets, V. P. Kaplan, and A. M. Poverennyi

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KEY WORDS: hematopoietic stem cells; rabbit antiserum to mouse brain; colony-formation in agar cultures in diffusion chambers *in vivo* and in agar cultures *in vitro*; thymus.

It was shown previously [8, 9, 12, 13] that rabbit antiserum against mouse brain (RASMB) possesses activity against mouse pluripotent hematopoietic stem cells (CFU-S), for treatment of bone marrow with this antiserum considerably reduces their ability to form colonies in the spleen of lethally irradiated mice. It has been suggested that activity of RASMB against hematopoietic stem cells is due to the presence of an antigenic marker for CFU-S. However, specificity of RASMB for CFU-S has not been finally proved. The writers have postulated [4, 15] that RASMB inactivates not the pluripotent hematopoietic stem cells, but a different cell population contained in bone marrow and playing a subsidiary role relative to hematopoietic stem cells in splenic colony formation. This hypothesis is based on the following experimental data: Injection of intact syngeneic thymocytes into recipient mice together with RASMB-treated bone marrow considerably reduces the effect of the antiserum [4, 15].

Interaction between thymus-dependent lymphocytes and hematopoietic stem cells has been described [5, 6].

Research Institute of Medical Radiology, Academy of Medical Sciences of the USSR, Obninsk.

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TABLE 1. Effect of RASMB on Colony Formation by Mouse Bone Marrow Cells in Diffusion Chambers *in vivo*

Experiment No.	Conditions	Number of colonies formed in chamber	
		absolute (M ± m)	percent of control
1	Control	44,7±5,5	100
	RASMB	19,2±2,9	43
2	Control	38,7±1,6	100
	RASMB	13,0±2,9	34
3	Control	35,3±2,8	100
	RASMB	6,9±2,1	20
4	Control	35,3±1,2	100
	RASMB	12,6±1,0	36
	$RASMB_t$	13,7±1,4	39
	Normal rabbit serum	36,6±1,9	115
5	Control	31,7±1,5	100
	RASMB	15,3±2,0	48
6	Control	29,0±2,3	100
	RASMB	10,6±1,5	37
7	Control	29,5±1,4	100
	RASMB	8,4±0,9	29

Legend. A constant number of nucleated bone marrow cells ($0.2 \cdot 10^5$) was transplanted into each chamber.

It has been shown that RASMB has no effect on the formation of granulocyte-macrophage colonies in agar cultures (CFU-C) *in vitro* [8] and does not inactivate late erythrocytic precursor cells [7].

This paper describes the results of a study of the effect of RASMB on mouse bone marrow cells forming granulocyte-macrophage colonies in diffusion chambers (CFU-DC) *in vivo* and also on mouse and human bone marrow cells forming CFU-C *in vitro*. In our opinion this is particularly interesting because CFU-DC and CFU-C differ in their degree of committedness: CFU-DC are closest in relation to pluripotent hematopoietic stem cells [11].

EXPERIMENTAL METHOD

Experiments to culture bone marrow cells in diffusion chambers were carried out on male (CBA × C57BL)F_1 mice aged 2-3 months. Rabbit antiserum (RASMB) was obtained by the method in [9]. RASMB exhausted with thymocytes was used in one experiment. Bone marrow cells were obtained by flushing out the medullary cavities of three femora from different mice in 4 ml medium No. 199. The resulting suspension, containing about $3 \cdot 10^7$ cells in 1 ml, was incubated with RASMB for 1 h at 37°C (0.1 ml suspension, 0.1 ml antiserum for normal rabbit serum, and 0.1 ml medium No. 199). After incubation the cells were washed once with medium No. 199 by centrifugation at 1000 rpm for 10 min, the supernatant was drawn off, and the residue was resuspended in 1 ml of fresh medium. To prepare a suspension of thymocytes, the thymus glands removed from four mice were washed with medium No. 199, carefully freed from connective tissue, cut into small pieces, and the cells were washed out with medium No. 199. The resulting suspension, like the bone marrow suspension, was filtered through four layers of Kapron gauze and the number of nucleated cells was counted.

Culture of bone marrow cells with or without the addition of intact thymocytes was carried out in diffusion chambers sterilized with γ-rays in a dose of 40,000 Gy [1, 2, 10]. Explanted cells were added to nutrient medium containing 0.32% agar, and the diffusion chambers were filled with it (0.2 ml per chamber). The rings and bottoms of the chambers were made from Plexiglas, and above the chambers were sealed with a millipore filter with a pore diameter of 0.23 μ. The chambers were filled through the hole in the ring with medium containing the cells, and after solidification of the agar the holes were sealed; the chambers were then sutured in pairs into the peritoneal cavity of mice irradiated in a dose of 5 Gr only, 24 h before implantation of the chambers. The chambers were removed 5-6 days later and the number of colonies formed in them was counted.

Human bone marrow from sterile puncture material was cultured in petri dishes by the method in [14]. As the source of colony-stimulating factor (CSF) peripheral blood leukocytes from a donor were used and were placed in the lower layer of nutrient medium with 0.5% agar in a concentration of 10^6 cells/ml medium. After solidification of the agar bone marrow

TABLE 2. Effect of RASMB on Colony Formation by Mouse and Human Bone Marrow Cells in Agar Cultures *in vitro*

Expt.	Explanted cells	Conditions	Number of explanted cells	Number of colonies formed in chamber	
				absolute (M ± m)	percent of control
1	Human bone marrow cells	Control	$2 \cdot 10^5$	70,7 ± 2,0	100
		RASMB	$2 \cdot 10^5$	67,0 ± 5,9	95
2	CBA mouse bone marrow cells	Control	$1 \cdot 10^5$	22,0 ± 4,2	100
		RASMB	$1 \cdot 10^5$	24,5 ± 5,3	111

cells were placed on the lower layer in a concentration of $2 \cdot 10^5$/ml medium. The agar concentration in the top layer was 0.32%. After culture for 10-12 days at 37°C in an atmosphere of a mixture of air with 10% CO_2 the number of colonies formed was counted.

Bone marrow cells of CBA mice were cultured in Leighton's tubes [3]. Ascites fluid obtained from mice with Ehrlich's adenocarcinoma was used as the source of CSF. The concentration of cells in the nutrient medium containing 0.32% agar was $1 \cdot 10^5$/ml. Colonies were counted after culture for 7 days at 37°C in an atmosphere of a mixture of air with 10% CO_2. Groups containing 50 or more cells were taken to be colonies.

EXPERIMENTAL RESULTS

The results of experiments to study the effect of RASMB on CFU-DC (Table 1) showed that incubation of the bone marrow cells with this antiserum caused a marked decrease in efficiency of colony formation: In all cases (seven experiments) the number of colonies formed in the chambers was 29-48% of the control value. Exhaustion of the RASMB with thymocytes ($RASMB_t$) did not reduce its activity against CFU-DC. Normal rabbit serum had no effect on their number (experiment No. 4).

The results of experiments to study the effect of RASMB on the formation of granulocyte-macrophage colonies by human and mouse bone marrow cells in agar cultures *in vitro* (CFU-C) are given in Table 2. They show that antiserum against mouse brain tissue did not affect the CFU-C contained in human and mouse bone marrow. These results are in full agreement with those obtained previously [8], which was interpreted as evidence of the absence of an antigenic marker on CFU-C by contrast with CFU-S. It was accordingly postulated that CFU-S lose their antigenic marker in the course of differentiation. The information obtained in the experiments to study the action of intact syngeneic thymocytes on splenic colony formation by bone marrow cells treated with RASMB allows another suggestion to be put forward on the nature of the differences observed in the effect of the antiserum. It can be postulated that CFU-C do not require helper cells inactivated by RASMB to form colonies in agar cultures *in vitro*, by contrast with CFU-S and CFU-DC, which do require such helper cells during colony formation in the spleen of lethally irradiated mice or in diffusion chambers.

According to their ability to be inactivated by rabbit antiserum against mouse brain tissue, CFU-DC contained in mouse bone marrow are thus analogous to the pluripotent hematopoietic stem cells that form colonies in the spleen of lethally irradiated mice. The results of the present investigation confirm earlier data [11] that CFU-C and CFU-DC are not identical: In their degree of committedness CFU-DC occupy an intermediate position between CFU-S and CFU-C.

Since the writers showed previously [4, 21] that injection of intact syngeneic thymocytes simultaneously with RASMB-treated bone marrow into recipient mice considerably restores splenic colony formation, it was natural to suggest that the addition of such thymocytes to diffusion chambers may also increase colony formation during culture of cells treated with RASMB. However, experiments showed that thymocytes form colonies by themselves when explanted into diffusion chambers (Table 3). The yield of CFU-DC for all cases averaged 5.4 ± $0.5/10^6$ thymocytes, i.e., there were approximately 300 times more of them in the bone marrow than in the thymus. Colonies formed by thymocytes differed from those formed by bone marrow cells. In bone marrow cultures granulocyte and macrophage colonies accounted for about 20

TABLE 3. Colony Formation by Mouse Thymocytes in Diffusion Chambers *in vivo*

Expt.	Conditions	Number of thymocytes explanted into chamber	Number of colonies formed in chamber	
			absolute (M ± m)	percent of control
1	—	$2 \cdot 10^5$	1,1±0,4	—
		$1 \cdot 10^6$	5,3±0,9	—
2	—	$4 \cdot 10^5$	1,2±0,6	—
3	—	$6 \cdot 10^5$	No growth	—
4	—	$2 \cdot 10^6$	15,4±1,1	—
5	—	$4 \cdot 10^6$	29,0±2,7	—
6	—	$2 \cdot 10^6$	17,8±2,0	—
	Control	$4 \cdot 10^6$	28,7±1,4	100
	RASMB	$4 \cdot 10^6$	14,6±1,4	51
	$RASMB_t$	$4 \cdot 10^6$	20,3±1,6	71
7	Control	$2 \cdot 10^6$	11,8±0,9	100
	RASMB	$2 \cdot 10^6$	9,2±1,3	78
	Control	$4 \cdot 10^6$	30,0±1,6	100
	RASMB	$4 \cdot 10^6$	19,6±2,0	65
8	—	$0,4 \cdot 10^5$	No growth	—
	—	$1 \cdot 10^5$	" "	—
	—	$2 \cdot 10^5$	" "	—
	—	$4 \cdot 10^5$	0,4±0,1	—
	—	$1 \cdot 10^6$	4,5±0,7	—
	—	$2 \cdot 10^6$	7,0±0,6	—
	—	$4 \cdot 10^6$	18,1±1,1	—
9	—	$2 \cdot 10^5$	0,4±0,2	—
	—	$4 \cdot 10^5$	1,4±0,5	—
	—	$1 \cdot 10^6$	6,3±1,2	—
	—	$2 \cdot 10^6$	15,0±2,1	—
	—	$4 \cdot 10^6$	23,5±1,3	—

and 80%, respectively, and colonies formed by fibroblast-like cells were rare (under 1%). Granulocyte colonies in thymocyte cultures, however, were rare (under 1%), and macrophage colonies and colonies formed by fibroblast-like cells accounted for about 90 and 10% respectively. It will be noted that there were practically no pluripotent hematopoietic stem cells in the thymus. The nature of the CFU-DC found in this investigation in the thymus is not yet clear. Like CFU-DC, they are sensitive to the action of RASMB (Table 3). The presence of CFU-DC among thymocytes naturally made it more difficult to assess the role of syngeneic thymocytes in abolishing the inhibitory effect of the antiserum. Preliminary results indicate the absence or weakness of this effect, for when mixtures of bone marrow cells treated with serum and intact thymocytes were explanted into the chamber the yield of colonies was about equal to the sum of the yields of colonies for individual components of the mixture. Meanwhile the possibility cannot be ruled out that conditions necessary for realization of the function of the helper cells (if such exists for CFU-DC) were not present in these experiments. The study of this possible role of thymocytes, in the writers' view, is very urgent and will be the subject of future research.

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CHARACTERISTICS OF THE SECRETORY APPARATUS OF MEMORY T CELLS

S. N. Bykovskaya, T. A. Kupriyanova, and M. O. Raushenbakh

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KEY WORDS: memory T cells; H-2 haplotype; cytolytic activity.

Much research has been devoted to the study of the mechanism of action of cytolytic T lymphocytes (CTL) on allogeneic or syngeneic target cells with modified H-2 haplotype [6, 9, 11].

The writers showed previously that a rough and smooth endoplasmic reticulum, tubular structures and a complex of tubular structures, immature secretory granules, empty vesicles, and a hypertophied Golgi complex (GC), facing the zone of contact with the target cell, are present in the cytoplasm of T lymphoblasts adsorbed on the surface of target cells and possessing high cytolytic activity, evidence of active synthesis and liberation of secreted material [1-3].

Depression of cytolytic activity in a mixed lymphocyte culture (MLC) is accompanied by disappearance of lymphoblasts and large lymphocytes and by the appearance of small and medium-sized lymphocytes, i.e., memory cells [4].

The object of the present investigation was to study the functions and ultrastructure of the secretory apparatus of memory T cells in MLC, which have the property of being specifically adsorbed on the surface of target cells, but do not cause their lysis in a 3-hour cytotoxic test.

EXPERIMENTAL METHOD

Inbred BALB/c ($H\text{-}2^d$) and C3H ($H\text{-}2^k$) mice aged 8-12 days were used. To obtain CTL, an MLC was used by the method in [7]. Reacting spleen cells from BALB/c mice were mixed in the number of $2\cdot10^6$/ml with $1\cdot10^6$ stimulating spleen cells in 1 ml, irradiated with a dose of 1000 R, from C3H mice in medium RPMI-1640 containing $3\cdot10^{-5}$ M 2-mercaptoethanol, 15% embryonic calf serum (ECS), $2\cdot10^{-3}$ M L-glutamine, 5 mM Hepes, and 100 units each of penicillin and streptomycin to 1 ml medium. The cells were cultured in "Sani-Glass" flasks in an atmosphere with 5% CO_2 at 37°C for 4, 5, and 8 days.

On the day of the experiment the number of living cells was counted after staining with eosin and trypan blue. The dimensions of the cells were determined under phase contrast, using an ocular micrometer. The cells were divided into small (5.5-7.0 μ), medium-sized (7-8 μ), and large lymphocytes and lymphoblasts (>8.5 μ); the maximal diameter of cells of this last category did not exceed 13.2 μ. The lymphocytes were fractionated by spontaneous sedimentation at 1 g in ECS by the method in [12]. To determine DNA synthesis the cultures were treated with ^3H-thymidine (spontaneous activity 14 Ci/mmole, from the Radiochemical Center, Amersham, England) for a period of 18 h. Later the cells were treated by the method described previously [13]. Radioactivity was measured with a Mark-2 β-spectometer. The result was expressed as the arithmetic mean of three determinations of the number of counts per minute.

Laboratory of Endogenous Carcinogens, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. M. Zhdanov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 93, No. 6, pp. 85-87, June, 1982. Original article submitted October 22, 1981.

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