CYTOTOXIC AND SUPPRESSOR ACTIVITY OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES, STIMULATED BY INTERLEUKIN-2 AND PHYTOHEMAGGLUTININ, BEFORE AND AFTER SEPARATION IN A PERCOLL GRADIENT

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The study of the cytotoxic antitumor action of human peripheral blood T lymphocytes (PBL), stimulated by interleukin-2 (IL-2) and lectins, is a promising trend in research into tumor immunotherapy [4, 5, 12]. The formation of antitumor cytotoxic T lymphocytes (CTL) is inhibited by suppressor T cells and monocytes, which prevent IL-2 synthesis by helper T cells [7, 8, 11]. Accordingly, removal of suppressor cells from the human PBL population is an important condition for the effective antitumor action of CTL.

This paper gives the results of fractionation of PBL from healthy blood donors and cancer patients in a Percoll gradient, by means of which human mononuclear blood cells can be separated, depending on their size and density, into fractions enriched with CTL precursors or suppressor cells, and also the results of a study of the PBL composition of fractions of the gradient with the aid of monoclonal antibodies, and their culture in the presence of IL-2 and of phytohemagglutinin (PHA).

EXPERIMENTAL METHOD

Mononuclear cells were isolated [2] from PBL and layered in a number of $(25-50)\cdot10^6$ above a stepwise Percoll gradient (1.077, 1.067, 1.056 g/cm^3). After centrifugation for 10 min at 1300 rpm (450g) cells were collected in interphases on the boundary between Percoll layers with densities of 1.056-1.067 g/cm^3 (fraction 1) and 1.067-1.077 g/cm^3 (fraction 2). Unfractionated PBL, leukocytes of fractions 1 and 2, and also a mixture of lymphocytes of fractions 1 and 2 in the ratio of 1:1, were cultured in plastic flasks in a concentration of $1\cdot10^6$ cells/ml in complete RPMI-1640 medium. The culture medium contained 3-5 U/ml of an active preparation of human IL-2, purified by gel filtration on Sephacryl S-200, and obtained from the Laboratory of Cellular Immunity, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow, or 1 µl/ml of the PHA preparation. After culture for 3-4 days at 37°C in an atmosphere of 5% CO_2 the cytotoxic activity of the lymphocytes was determined on ^{51}Cr-labeled tumor cells, obtained from tumors removed surgically from patients, by trypsinization in a mixture of enzymes [1]. Cytotoxic activity was calculated by the formula: $(a - b)/(c - b)\cdot100$, where a denotes the quantity of ^{51}Cr label released (QLR) into the medium after incubation with lymphocytes, b — QLR in medium without lymphocytes, and c — QLR in medium with tumor cells, destroyed by a 1% solution of sodium dodecylsulfate.

To obtain T cells and monocytes from fraction 1 of the Percoll gradient the lymphocytes of this fraction were incubated on a plastic Petri dish for 45 min at 37°C. T cells were isolated from lymphocytes not adhering to the plastic by adsorption on a column packed with nylon wadding [6] and treated with mitomycin C (MC; 50 µg/ml, 30 min at 37°C). Monocytes were obtained by removing cell populations of fraction 1 of the gradient, adherent to plastic, with versene solution.

The composition of the donors' and patients' PBL before and after fractionation in a Percoll gradient was studied by indirect surface immunofluorescence, 20 µl of a cell suspension containing $(2-4)\cdot10^6$ PBL being placed into the wells of a slide covered with poly-L-lysine (100 µg/ml). The cell monolayer thus formed was treated initially with AKT3, OKT4, OKT8, and OKM1 monoclonal antibodies (Orto-mune) in a dilution of 1:20 for 30 min at 4°C,

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TABLE 1. Cytotoxic Activity (in %) of PBL from Donors and Cancer Patients, Stimulated by IL-2 (A) and PHA (B) before and after Fractionation in a Percoll Density Gradient (M ± m)

Patient No.	Unfractionated lymphocytes	Fraction of Percoll gradient		
		1	2	1+2
Donors				
		A		
1	28,9±1,5	4,5±1,0	51,9±5,7***	35,3±1,1
2	7,3±2,2	0,7±0,4	40,8±0,3	37,0±2,0
3	40,2±4,4	0,3±0,2	65,4±3,4*	22,9±2,8
4	22,3±1,5	3,0±2,7	36,8±2,4	31,5±3,7
5	36,6±11,1	3,7±0,5	56,0±7,1	68,3±5,3
		B		
6	24,8±2,1	4,9±1,3	57,8±5,8	45,6±5,3
7	14,9±4,9	5,0±2,2	46,3±3,0**	20,1±5,7
8	41,0±3,8	6,7±1,6	47,6±3,2	46,5±1,6
9	25,6±2,4	2,6±2,5	34,0±3,2	36,3±2,8
10	33,3±2,8	0,9±0,3	50,5±3,7**	26,9±2,3
Cancer patients				
		A		
1	40,3±3,1	5,8±1,3	78,6±3,7*	25,5±2,5
1a	37,7±6,1	6,3±2,2	75,5±6,4*	26,2±1,1
2	37,9±1,0	6,9±1,8	70,7±2,5*	7,9±2,2
2a	32,7±2,5	1,5±2,4	69,8±8,6*	0,7±2,7
3	13,2±0,2	1,1±0,5	26,4±4,4**	10,1±1,1
4	40,5±5,8	2,2±2,0	53,4±5,8**	24,4±1,3
4a	28,1±2,0	0,1±0,1	39,4±0,1*	3,6±1,0
5	12,7±1,0	2,1±1,0	32,2±2,7*	0,6±1,6
6	7,4±1,4	2,8±0,9	20,0±2,4*	0,1±1,1
6a	11,8±2,0	0,1±0,1	34,0±3,5*	4,3±1,4
		B		
7	0.3±0,3	0,1±0,1	35,8±0,8*	0,1±0,1
7a	0,6±0,7	−0,3±0	12,0±2,1**	−0,2±0,3
8	25,7±5,4	3,6±0,3	49,8±2,9*	6,7±1,9
8a	27,3±2,5	5,9±1,7	58,0±8,3**	9,2±1,6
9	44,0±1,0	3,8±3,0	56,2±3,0*	6,3±2,7
9a	32,5±2,7	0,9±0,7	56,0±2,0*	3,8±1,7
10	10,8±1,1	0,2±0	32,2±0,4*	0,2±0,1
11	10,3±1,3	−2,2±1,2	30,1±5,5**	−0,6±0,3
12	10,9±1,1	2,5±0,1	34,6±3,4*	3,3±0,5
13	6.1±0,2	3,0±0,6	12,4±0,8*	−0,8±0,8

Legend. Cytotoxic activity of cultured donors' lymphocytes was determined in a test against tumor cells from patients with CR (1, 2, 5, 6, 10) and CB (3, 4, 7, 8, 9); of lymphocytes from cancer patients in tests against allogeneic and autologous (a) tumor cells from patients with CR (1, 7, 8, 10, 12), CB (2-6, 9, 11), and melanoma (13). *$p < 0.01$, **$p < 0.05$, ***$p < 0.1$ compared with mixture of lymphocytes of fractions 1 and 2 in the ratio of 1:1.

and then with fluorescein isothiocyanate for 30 min at 4°C. In the direct immunofluorescence method the cell monolayer was treated with B Cell slg Marker antibodies in a dilution of 1:20. The reaction was read by counting the number of fluorescent cells among 300 cells of the monolayer.

EXPERIMENTAL RESULTS

Culture of unfractionated PBL from 13 patients with carcinoma of the rectum (CR), carcinoma of the bladder (CB), and melanoma and from 10 healthy blood donors (Table 1), in the presence of IL-2 and PHA, induced cytotoxic activity against autologous and allogeneic

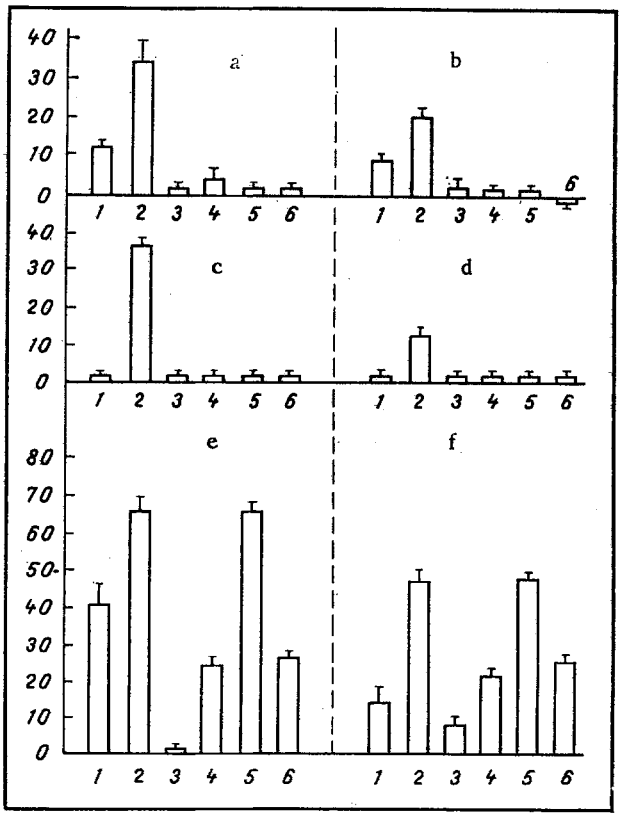

Fig. 1. Cytotoxic activity of lymphocytes from fraction 2 of Percoll gradients from donors and cancer patients after addition to it of T cells and monocytes from fraction 1 of gradients for combined culture. a and b) Cytotoxic activity of lymphocytes from patient No. 6, with CB, stimulated by IL-2 against cells of autologous (a) and allogeneic (b) tumors; c and d) cytotoxic activity of lymphocytes of patient No. 7 with CR, stimulated by PHA against cells of autologous (b) and allogeneic (c) tumor; e and f) cytotoxic activity of lymphocytes of donors Nos. 3 and 7, stimulated by IL-2 and PHA respectively. Horizontally — cytotoxic activity of unfractionated mononuclears (1), and lymphocytes of fractions 1 (2) and 2 (3); of fraction 2 lymphocytes after addition to it of fraction 1 lymphocytes (4) or MC-treated T cells (5) and monocytes (6) from fraction 1 of Percoll gradients, addition at beginning of culture; ordinate, cytotoxic activity (in %).

tumor cells in 12 patients and in all the donors. If the PBL were fractionated in a Percoll gradient and fraction 2 (1.077-1.067 g/cm^3) was used in the tests, cytotoxic activity was increased in every case (on average by 1.7 times for the donors and twofold for the patients). Culture of the fraction 1 lymphocytes did not lead to the formation of cytotoxic lymphocytes. Addition of cells of fraction 1 to the lymphocytes of fraction 2 in the ratio of 1:1 suppressed the induction of cytotoxic activity in all patients but in only 4 of the 10 donors, i.e., cells of fraction 1 exhibited suppressor activity.

In cancer patients the suppressors of CTL were T cells and monocytes isolated from fraction 1 of the Percoll gradient (Fig. 1: a-d). Suppression of formation of antitumor CTL from lymphocytes of fraction 2 in the presence of IL-2 or PHA was observed after the addition of MC-treated T cells or monocytes from fraction 1 of the gradient to it at the beginning of culture. Individual cases of suppression in the blood donors were associated with the action of suppressor monocytes, for suppression of CTL formation from lymphocytes of fraction 2 was observed after the addition of the monocyte fraction to them, and not of MC-treated T cells from fraction 1 of the gradient (Fig. 1: e, f).

Immunofluorescence analysis showed that unfractionated CBL from donors and cancer patients contained only slightly different numbers of T and B cells and of monocytes (Table 2). The patients showed a tendency for the number of T cells and the ratio OKT4/OKT8 to decrease. In fraction 2 of the gradient an increase in the proportion of T cells from 66

TABLE 2. Number (in %) of T and B Cells and Monocytes among PBL from Blood Donors and Cancer Patients, before and after Fractionation in a Percoll Density Gradient (M ± m)

Monoclonal antibodies	Unfractionated lymphocytes	Fraction of Percoll gradient	
		1	2
Donors (n = 10)			
OKT3	66±17	36±14	78±4
OKT4	46±14	30±14	58±29
OKT8	21±10	33±7	13±8
OKT4/OKT8	2,2	0,9	4,4
B Cell sIg Marker	12±10	10±12	10±16
OKM1	27±10	69±25	7±8
Patients with CR (n = 3)			
OKT3	59±6	48±14	92±9
OKT4	38±14	38±19	61±4
OKT8	21±6	28±16	28±6
OKT4/OKT8	1,8	1,4	2,0
B Cell sIg Marker	11±10	14±9	6±5
OKM1	20±11	46±14	10±11
Patients with CB (n = 5)			
OKT3	63±10	41±18	88±10
OKT4	38±19	31±12	63±14
OKT8	21±6	22±12	26±15
OKT4/OKT8	1,8	1,4	2,5
B Cell sIg Marker	16±10	10±3	4±0
OKM1	25±33	43±14	10±10

to 92% and a decrease in the number of monocytes from 27 to 7% were observed. There was also a tendency for the OKT4/OKT8 ratio to increase, and this was particularly marked in the donors. Conversely, in fraction 1 of the gradient there was a decrease in the proportion of T cells to 36% and an increase in the number of monocytes to 69%, together with a tendency for the OKT4/OKT8 ratio to fall. The results of immunofluorescence analysis are in agreement with those of known investigations of the distribution of T and B cells from human blood among fractions of the Percoll gradient and supplement them with information on the number of mononuclear cells with the OKM1 marker [9].

The increase in the cytotoxic effect on tumor target cells after the use of lymphocytes from fraction 2 for culture with IL-2 and PHA can be explained, first, by the enrichment of this fraction of the Percoll gradient by cells of the $OKT4^+$ helper-inducer lymphocyte subpopulation, facilitating more intensive proliferation of CTL from precursor cells of the $OKT8^+$ lymphocyte subpopulation; second, by removal of suppressor T cells and monocytes, concentrated after fractionation in fraction 1 of the Percoll gradient, from fraction 2 of the gradient. The writers showed previously that suppressor T cells, induced by tumor cells in autologous mixed culture of lymphocytes and tumor cells, and detected on the boundary between the Percoll layers with density of 1.067 and 1.056 g/cm^3, belong to the general suppressor-cytotoxic $OKT8^+$ human lymphocyte population. The results are in agreement with data showing that enrichment of the PBL population by T cells leads to increased activity of antitumor CTL formation, but T cells and monocytes are suppressors of killer cells induced by the tumor [7, 8, 11]. The results of fractionation of human PBL in a Percoll gradient, into fractions enriched with precursors of antitumor CTL or suppressor cells, can be used in the cellular adoptive immunotherapy of tumors: 1) to determine suppressor activity of human blood lymphocytes by a method based on fractionation of PBL in a Percoll gradient, along with other known methods of determining suppression activity based on the prostaglandin E_2 level [3] and on the ability of T_8 cells with the Fc-receptor for IgG to suppress proliferation and differentiation of B cells [10]; 2) to remove suppressor cells from the population of human blood lymphocytes in order to enrich it with antitumor CTL followed by culture with IL-2 and lectins.

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EFFECT OF NEUTROPHILOKINES ON THE IMMUNE RESPONSE OF MICE

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An urgent problem in modern immunology is the study of interaction between cells of the immune system. The least studied aspect of this problem is the role of neutrophils in immune reactions and their connection with other immunocompetent cells. The writers showed previously that neutrophils, on incubation in medium 199 without activators or with activators secrete products capable of altering the functional activity of cells of the mononuclear phagocytic system [3, 4]. The nature of these products and their effect on the immune response remain unexplained.

The aim of the present investigation was to study the nature of products of neutrophils and their effect on the immune response of mice.

TABLE 1. Effect of Neutrophilokine Fractions on Induction of AFC-SRBC and AGR Function of (CBA × C57BL)F_1 Mouse Macrophage (M ± m)

Experimental conditions	Immunization with SRBC		Immunization with macrophages	
	number of AFC per spleen (× 10^2)	number of AFC per 10^6 spleen cells	number of AFC per spleen (× 10^2)	number of AFC per 10^6 spleen cells
Control (medium 199)	228,1±24,2	206,7±22,3	70,0±5,67	48,96±3,77
Injection of U_1	138,1±16,8	90,0±9,09	37,5±3,61	30,31±3,38
p	<0,02	<0,01	<0,001	<0,01
A_1	231,3±18,0	238,8±13,6	107,0±11,34	98,09±8,59
p	—	—	<0,02	<0,001
U_2	187,5±17,4	156,9±14,4	53,5±10,3	42,8±10,24
A_2	180,6±13,39	161,9±19,12	77,0±5,15	55,96±6,35
U_5	225,0±25,4	165,8±12,1	71,0±9,79	53,27±11,34
A_5	386,9±19,3	382,0±21,3	116,0±6,19	104,09±10,33
p	<0,001	<0,001	<0,001	<0,001

Legend. Here and in Table 2: p — compared with control.

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