can be explained by the fact that the membrane of some lymphocytes shown by staining with 1% trypan blue solution to have died still remained capable of fixing sheep's red cells for some time. This fact was verified by performing the rosette-formation test with completely dead cells obtained after treatment with large doses of ALS and complement. Some of these cells were found to fix 1, 2, or in some cases even 3 red cells to themselves. In experiments with lymphocytes treated with ALS and complement in the presence of HEDPA, the percentages of rosette-forming cells and of cells transformed under the influence of phytohemagglutinin were virtually indistinguishable from the control. Statistical analysis of the data showed that the effect of HEDPA is significant ($P < 0.05$).

It can thus be concluded from the results of these investigations that the potassium salt of HEDPA protects T-lymphocytes against immune injury caused by ALS and complement, possibly as a result of binding of Ca ions. Some injured lymphocytes, considered to be dead on the basis of staining with 1% trypan blue solution, could still form rosettes with sheep's red cells. Finally, the blast-transformation test was shown to be more sensitive than the rosette-formation test for assessment of the viability of T-lymphocytes.

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DEPENDENCE OF THE FUNCTIONAL ACTIVITY OF IMMUNOCOMPETENT MOUSE SPLEEN CELLS ON THEIR HEMATOPOIETIC MICROENVIRONMENT

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Accumulation of erythroid cells and granulocytes, changes in the relative and absolute numbers of T-, B-, and "null" lymphocytes, and also sharp inhibition of the primary immune response were found in the spleen of CBA mice treated with specific antierythrocytic serum (AES). The inductive phase of immunogenesis proved to be sensitive to the action of AES. Injection of syngeneic macrophages and splenocytes did not overcome the immunodepression. The results can be regarded as evidence of the existence of functional dependence between the immunocompetent cells of the spleen and their nonlymphoid hematopoietic microenvironment.

KEY WORDS: immune response; T- and B-cells; microenvironment.

The mouse spleen is an important peripheral organ of immunity. In the plasma cells of its red pulp, most of the humoral antibodies of the organism are synthesized in response to intraperitoneal or intravenous injection of an antigen [1]. Meanwhile marked hematopoiesis takes place in the spleen. Cells of the erythro-, myelo-, and megakaryocytic type coexist with lymphocytes. It may be that a hematopoietic microenvironment is essential for the normal functioning of immunocompetent cells. If this is true, a change in the ratio between hematopoietic nonlymphoid components of the red pulp or injury to them may be reflected in the intensity of the immune response. There are as yet only solitary communications that suggest the existence of a functional

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TABLE 1. Changes in Number of AFC in Spleens of Mice Treated with AES

Index	Control (normal rabbit serum)	Times of immunization of mice with SRBC					
		24 h before injection of AES	simultaneously with first injection of AES	Days after injection of AES			
				1st	4th	8th	15th
AFC per 10^6 karyocytes	641,0	633,6	338,0*	10,04*	17,87*	241,6*	709,4
	(574,7—711,7)	(577,3—694,7)	(280,9—396,7)	(8,25—12,22)	(14,46—22,08)	(206,0—285,7)	(525,4—957,8)
AFC per 10^6 lymphocytes	856,8	857,0	448,0	18,66*	31,98*	563,6*	884,5
	(816,1—918,4)	(806,8—910,4)	(316,4—634,4)	(16,2—21,37)	(27,76—36,86)	(492,5—645,0)	(443,7—1763,0)
AFC per spleen	81 300,0	56 240,0	34100,0*	1 639,0*	2 562,0*	24 800,0*	89 480,0
	(76 510,0—	50 470,0—	(30 120,0—	(1 315,0—	(2 035,0—	(20 590,0—	(64 610,0—
	—95 505,0)	—62 660,0)	—38 560,0)	—2 040,0)	—3 225,0)	—29 880,0)	—123 900,0)

Legend. Mean values of AFC given with their confidence intervals in parentheses. Here and in Tables 2 and 3: *) values differing significantly ($P < 0.05$) from control.

TABLE 2. Changes in Number of Different Types of Nucleated Spleen Cells of Mice Treated with AES (M ± m)

Type of karyocytes	Control (normal rabbit serum)	Days of investigation after treatment of mice with AES			
		1st	4th	8th	15th
Lymphoid:					
%	74,0±3,0	62,0±2,27*	56,0±3,9*	38,0±4,3*	78,0±1,12
10^6	105,7±1,9	127,8±3,6*	142,8±8,8*	47,6±0,2*	91,6±9,3
Granulocytic:					
%	17,0±2,15	17,0±1,9	33,0±3,6*	50,0±3,8*	13,0±0,5
10^6	26,1±3,0	35,0±3,8	84,0±8,4*	63,1±2,2*	15,2±1,2
Erythroid:					
%	4,0±1,29	14,0±1,97*	8,0±1,5	8,0±1,3*	3,5±0,5
10^6	6,0±0,6	31,5±0,05*	16,0±0,1*	9,9±0,5*	4,0±0,5
The rest (plasma-cells, mega-karyocytic, reticular):					
%	4,4±0,86	6,6±0,9	3,6±0,76	4,2±0,86	5,5±0,8
10^6	6,0±2,0	13,5±1,7	9,3±1,1	5,2±0,5	6,2±0,1
Number of karyocytes in spleen, 10^6	142,0±13,5	20,62±1,7*	255,0±2,3*	127,0±15,0	117,0±13,6

connection between the immunocytes of the spleen and the remaining hematopoietic cells which surround them [2].

EXPERIMENTAL METHOD

Male CBA mice weighing 20-24 g were used in the experiments. The animals received an intraperitoneal injection of antiserum against mouse erythrocytes in a dose of 0.2 ml daily for 3 consecutive days. To obtain antierythrocytic serum (AES) chinchilla rabbits were immunized with mouse erythrocytes in a dose of $5 \cdot 10^8$ cells per injection. The first injection of antigen was given intradermally in Freund's complete adjuvant, the next injections (after 4 weeks) intravenously on two consecutive days. The AES obtained on the 7th day after the end of immunization was heated to 56°C for 40 min and then exhausted with mouse blood plasma and a suspension of lymph node and thymus cells and peritoneal macrophages. This serum, in the cytotoxic test with complement, did not damage lymphocytes or macrophages, in the agglutination test it did not agglutinate platelets, and it did not give a cross reaction with the test antigen, sheep's red cells (SRBC). The AES was diluted 1 : 20 and, with an agglutination titer of 1/8, it was used for the experiment. Control animals were injected with normal rabbit serum, treated and diluted in the same way as the AES. The mice were immunized intraperitoneally with SRBC ($3 \cdot 10^8$) 24 h before injection of the AES, simultaneously with the first injections of AES, and 1, 4, 8, and 15 days after treatment of the animal with AES. Antibody-forming cells (AFC) in the spleen were counted 4 days after immunization by Jerne's direct test [3]. In some experiments mice were injected intraperitoneally with $F(ab')_2$-fragments of antierythrocytic antibodies with an agglutination titer of 1/16, obtained by the method of Madsen and Rodkey [4], in a dose of 0.2 ml daily for 3 days, and also with immune complexes (IC) obtained by treatment of mouse erythrocytes with AES. The percentage of T-lymphocytes was determined (in the cytotoxic test using rabbit serum against mouse brain, exhausted with blood and liver [5]) and the percentage of B-lymphocytes carrying receptors for complement on their surface (by the method of rosette-formation with SRBC loaded with antibodies and mouse complement [6]) were determined in a suspension of spleen cells of unimmunized mice on the first, fourth, eighth, and fifteenth days after injection of AES, and the ratio between T- and B- lymphocytes (T/B) was calculated. At all times the total number of nucleated cells was determined in the suspension of splenocytes of the experimental animals and the relative proportions of different types of these cells were counted in films stained with hematoxylin and eosin. Statistical analysis of the results by Student's t-test showed a log-normal distribution of AFC [7].

EXPERIMENTAL RESULTS

As Table 1 shows, in animals treated with AES before or at the same time as injection of antigen (SRBC) there was a small decrease in the number of AFC in the spleen. The decrease in the number of AFC was most marked only when immunization was carried out on the first or fourth days after the injection of AES. Consequently, the inductive phase of immunogenesis was most sensitive to the action of AES. A marked depressive effect of AES was observed for not more than one week, for on immunization of the mice on the 8th and 15th days of the investigation the number of AFC was the same as in the control.

In response to injection of AES into the experimental animals in vivo at least two processes were observed: the formation of IC (erythrocytes–antierythrocytes) capable of blocking the activity of the macro-

TABLE 3. Number of T-, B-, and "Null" Lymphocytes in Spleens of Mice Treated with AES (M ± m)

Type of lymphocyte	Control (normal rabbit serum)	Days of investigation after treatment of mice with AES			
		1st	4th	8th	15th
T-cells:					
%	34,5±1,3	20,0±1,3*	26,0±2,6*	24,6±3,3*	28,0±4,4
10^6	48,4±2,4	41,2±2,3*	63,4±3,4*	30,7±0,5*	32,3±1,4
B-cells:					
%	28,3±1,64	27,0±1,5	17,7±1,5*	17,0±1,0*	20,0±2,2*
10^6	39,5±1,5	55,6±2,6*	43,1±1,3	21,4±1,3*	23,2±0,1*
Null lymphocytes:					
%	12,8±2,5	15,0±3,0*	12,3±2,4	0,0	32,8±9,5
10^6	17,8±2,24	31,0±3,3*	36,3±9,5	0,0	36,6±7,6
T/B	1,2	0,74	1,4	1,48	1,4

phagal system, and stimulation of erythropoiesis, caused by the erythrocytopenia. These factors evidently play an essential role in the depression of the immune response to SRBC. To determine the role of IC in immunodepression caused by AES, a suspension of IC in which the antibody content was approximately twice the dose of antibodies in the AES obtained by the main group of animals was injected into mice. Immunization with SRBC was carried out 24 h after the injection of IC. The number of AFC in the spleen was 1/3-1/4 of that in the control [198.3 (158.7-247.8) per 10^6 karyocytes and 20,120 (15,400-26,290) per spleen], but nevertheless still one order of magnitude higher than the level of AFC observed after injection of antierythrocytic antibodies (AES) not bound with IC (Table 1). Consequently, depression of the immune response in animals treated with AES was due not to the formation of an IC in them, but to other mechanisms. This is shown indirectly also by the fact that intravenous injection of syngeneic peritoneal macrophages in a dose of $5 \cdot 10^6$ into animals previously receiving IC enabled the immune response to be fully restored to the control level, whereas injection of macrophages in the same dose or of normal syngeneic splenocytes ($3 \cdot 10^8$) into mice treated with AES did not lead to an increase in the number of AFC in the spleen. Furthermore, injection of $F(ab')_2$-fragments of antierythrocytic antibodies forming IC incapable of interacting with the Fc-receptors of the macrophages depressed the primary immune response, although to a lesser degree than AES [126.6 (75.7-212.0) AFC per 10^6 splenic karyocytes].

A suspension of spleens of the experimental animals was subjected to cytological analysis on the 1st, 4th, 8th, and 15th days after injection of AES (Table 2). On the 1st and 4th days, i.e., during the period of greatest depression of the immune response, a considerable increase was observed in the absolute number of nucleated spleen cells, including lymphocytes. The relative number of lymphocytes under these circumstances, starting from the first day of the investigation, fell gradually to reach a minimum by the 8th day, mainly on account of an increase in the proportion of granulocytes and erythroid cells.

Among the nucleated spleen cells under normal conditions there were 34.5% of T-cells and 28.3% of B-cells carrying receptors for complement. About 12% of lymphocytes could not be classed by the method used as either T- or B-cells. They were called "null" lymphocytes [8]. After injection of AES into the animals (Table 3) the relative percentage of T-cells fell by 1.3-1.7 times between the 8th and 10th days. The relative number of B-cells was below the control starting from the 4th day of observation. An increase in the absolute number of lymphocytes calculated per spleen from the 1st day was due to an increase in the number of B-lymphocytes and mainly to an increase in the pool of null cells. On the 4th day the increase in the total number of splenic lymphocytes took place on account of T-cells. The number of T- and B-lymphocytes was low characteristically in the spleen on the 8th day and no null cells were present at that time. On the 15th day the number of null lymphocytes did not differ significantly from the control, whereas the number of T- and B-cells was still below normal. The increase in the number of null lymphocytes on the 1st day after injection of AES could be taken as evidence of a disturbance of differentiation of the immunocompetent cells.

An extremely important factor for the obtaining of a marked cooperative immune response is the optimal ratio between T- and B-cells. In the present experiments the T/B ratio in the control was 1.2, falling to 0.74 24 h after injection of AES, and at later stages of the investigation it was over 1 (Table 3). The decrease in the T/B ratio to 0.74 on the first day was not, however, the essential cause of the decrease in the number of AFC at that time, for as A. A. Yarilin and É. F. Polushkina found, significant depression of the primary immune response to SRBC in mice was observed only if the T/B ratio did not exceed 0.5. By the 4th day, when the immune response was depressed to about the same degree as on the 1st day, the T/B ratio was 1.4. In this case other factors probably play an important role in immunodepression.

The more profound depression of the primary immune response to SRBC in mice treated with AES than the immunodepression caused by injection of IC obtained in vitro, the decrease in the number of AFC in the spleen under the influence of $F(ab')_2$-fragments of antierythrocytic antibodies, the failure of the immune response to SRBC to be restored after injection of syngeneic macrophages or splenocytes into animals treated with AES, the change in the cellular composition of the erythromyeloid complement of the spleen in these animals, and disturbance of the normal numbers of T-, B-, and null lymphocytes – all these facts suggest the presence of functional interconnections between the immunocytes of the spleen and their nonlymphoid hematopoietic microenvironment.

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