SIMULTANEOUS DETERMINATION OF T, B, AND MIXED ROSETTE-FORMING LYMPHOCYTES IN HUMAN PERIPHERAL BLOOD

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Preliminary treatment of erythrocytes in suspension with trypan blue did not affect their ability to take part in the spontaneous rosette formation test, but enabled them to be distinguished when mixed with other erythrocytes after staining with methyl green–pyronine in fixed preparations. The presence of a small subpopulation of lymphocytes binding both types of indicator particles was demonstrated by the use of a mixture of these erythrocytes with erythrocytes sensitized by antibodies and complement.

KEY WORDS: rosette formation; T and B lymphocytes.

The ability of lymphocytes to form rosettes with various indicator particles, namely sheep, mouse, avian, and monkey erythrocytes, erythrocytes sensitized with antibodies and complement, anti-rhesus sera, etc. [1-3, 6, 8, 9, 11], is widely used in modern clinical immunology. It is suggested that each of the particles interacts with a particular type of immunocompetent cell (with T or B lymphocytes). In that case by counting the number of rosettes formed with the various particles, an idea can be obtained of the size of these populations. This application of the method, based on the above-mentioned property of the lymphocytes, obviously demands careful verification of its specificity. The most widely used indicator particles are sheep's erythrocytes and erythrocytes sensitized by antibodies and complement. Data showing that lymphocytes forming rosettes with sheep's erythrocytes belong to the T lymphocyte population, whereas those forming rosettes with erythrocytes sensitized by antibodies and complement belong to the B lymphocyte population have been obtained in experiments to detect the markers to T or B populations on the surface of rosette-forming lymphocytes (RFL) [4, 10], and also by analysis of the distribution of these cells in the lymphoid organs of healthy persons [14] and of patients with immunodeficient states [5, 10, 15].

In the investigation described below an attempt was made to estimate directly the specificity of the rosette-formation test by the simultaneous use of a mixture of indicator particles.

EXPERIMENTAL METHOD

The test object was healthy human peripheral blood. The test was carried out with leukocytes purified from contamination by erythrocytes by brief osmotic shock and the results were read in preparations stained with methyl green–pyronine [3]. To detect T lymphocytes unsensitized sheep's erythrocytes were used. In addition, under the same experimental conditions, sheep's erythrocytes previously stained with trypan blue were used. For this purpose a 4% mother solution of the dye (Chemapol, molecular weight 960,871 batch 095106) in physiological saline was filtered through paper filters. Equal volumes of the filtered dye and a 2.5% suspension of sheep's erythrocytes were carefully mixed and the mixture was incubated at 37°C for 30 min with periodic shaking. After 30 min the erythrocytes so treated were washed four times by centrifugation in physiological saline and resuspended to form a 0.5% suspension.

To detect B lymphocytes human erythrocytes sensitized with rabbit antierythrocytic antibodies and fresh human serum as the source of complement were used [2]. Simultaneous detection of T and B RFL was carried out as follows. First, to form rosettes by T lymphocytes with erythrocytes previously stained with trypan blue

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TABLE 1. Rosette Formation with Sheep's Erythrocytes Stained in Suspension with Trypan Blue, with Human Erythrocytes Sensitized with Antibodies and Complement, and with a Mixture of Particles

Donor	Indicator cells				
	st.SE	sen.HE	st.SE + sen.HE		
	T RFL	B RFL	T RFL	B RFL	mixed RFL
1	72	9	69	6	4
2	35	17	23	9	6
3	39	14	28	8	4
4	60	18	53	8	5
5	61	17	68	4	2
6	70	16	62	8	3
7	63	14	56	7	7
8	48	13	48	9	2
9	60	15	50	11	4
10	58	12	55	7	2
$M \pm m$	56,6±3,8	14,5±0,8	51,2±5,5	8,4±0,9	3,9±0,5

Legend. 1) Here and in Table 2: st.SE – stained sheep's erythrocyte, sen.HE – human erythrocyte sensitized by antibodies and complement. 2) Number of RFL given in %.

TABLE 2. Distribution of Lymphocytes into Subgroups Based on Number of Indicator Particles

Subgroup	Number and type of indicator particles mixed	Mean proportion of total number of lymphocytes (M ± m), %
I	0 st.SE + 0 sen.HE	9,6±2,1
II	1 or 2 st.SE	10,7±0,7
III	1 or 2 sen.HE	6,4±1,7
IV	1 st.SE + 1 sen.HE	3,5±0,5
V	2 st.SE + 1 sen.HE	2,9±0,4
VI	2 sen.HE + 1 st.SE	1,5±0,4
VII	2 st.SE + 2 sen.HE	1,9±0,4
VIII	3 st.SE + 1 or 2 sen.HE	11,0±1,3
IX	3 sen.HE + 1 or 2 st.SE	3,5±0,4
X	3 st.SE + 3 sen.HE	3,9±0,5
XI	≥3 st.SE	40,2±4,2
XII	≥3 sen.HE	4,9±0,5

in suspension, the mixture of cells was incubated at 37°C for 5 min, sedimented by centrifugation (200g, 5 min), and kept for 60 min at 12°C [3]. After 60 min the residue was carefully resuspended, the tubes were heated to room temperature, and to detect the B lymphocytes 0.1 ml of a 0.5% suspension of human erythrocytes sensitized by antibodies and complement was added. The mixture was centrifuged (200g, 5 min) and glutaraldehyde was added in a final concentration of 0.6%. The subsequent procedure of preparation and staining was similar to that described by Petrov et al. [3]. The reaction was read under the light microscope, and RFL binding at least three erythrocytes were counted. Structures formed by lymphocytes fixing at least three indicator particles of different types were classed as mixed rosettes.

EXPERIMENTAL RESULTS

The study of the ability of peripheral blood lymphocytes to form spontaneous rosettes with unstained and stained sheep's erythrocytes showed that the number of rosettes formed with the unstained erythrocytes and with erythrocytes previously treated with trypan blue was practically identical in each concrete donor. The mean values for these two groups likewise did not differ significantly (57.8 ± 3.1 and 56.6 ± 3.8%, respectively). The number of lymphocytes forming rosettes with allogeneic erythrocytes in human blood is small (2-4%). Analogous treatment of human erythrocytes with trypan blue did not change their ability to undergo fixation on the surface of lymphocytes.

The results of determination of RFL by the use of sheep's erythrocytes stained in suspension, human erythrocytes sensitized with antibodies and complement, and a mixture of these particles are given in Table 1. On average 56.6% of peripheral blood lymphocytes formed rosettes with the stained sheep's erythrocytes and 14.5% did so with the sensitized human erythrocytes. When a mixture of particles was used, both T and B RFL were detected, and also a certain number of lymphocytes which bound at least three indicator particles of either type. The number of T and B RFL was less when a mixture of particles was used than by a separate method of

detection. However, the total number of lymphocytes forming rosettes with sheep's erythrocytes (51.2%) and of lymphocytes binding both types of particles (3.9%) did not differ significantly from the mean number of rosettes formed by the use of sheep's erythrocytes only (56.6%). A similar picture also was observed with respect to the population of lymphocytes binding erythrocytes sensitized by antibodies and complement. The mean number of lymphocytes forming mixed rosettes in the group of donors studied varied from 2 to 7%, with a mean value of 3.9%. For a detailed analysis of the reaction subgroups of lymphocytes were distinguished (Table 2) depending on the type and number of bound particles.

While this investigation was in progress a number of publications appeared on the same topic, namely to determine the RFL population during the simultaneous use of a mixture of indicator particles [7, 8, 12, 13]. For this purpose Mendes et al. [12] treated zymosan granules with complement and thus obtained indicator particles for the detection of B lymphocytes. However, it should be pointed out that zymosan particles differ in size from sheep's erythrocytes. Chiao et al. [7] used nucleated pigeon's erythrocytes sensitized with anti-erythrocytic antibodies and complement as B indicators. Shevach et al. [13] used sheep's erythrocytes and larger guinea pig erythrocytes, after fixing complement. However, the size of sheep's erythrocytes and of guinea pig's erythrocytes vary, and for that reason in some cases it is difficult to determine unambiguously to which species a given erythrocyte belongs.

The spontaneous rosette formation reaction also was carried out with erythrocytes previously treated in suspension with trypan blue. After fixation and staining with methyl green–pyronine, such an erythrocyte appears claret-colored, unlike the untreated, pink erythrocytes. As the results show, this procedure did not affect the ability of the erythrocytes to react by rosette formation. Later, erythrocytes treated with trypan blue solution were used as indicator particles for T lymphocytes in the simultaneous determination of T and B RFL in the same preparation. The second component in the reaction consisted of human erythrocytes sensitized by specific antibodies and complement [2]. The number of mixed RFL, according to data in the literature, is about 2% [7, 8, 12, 13]. In the present experiments the mean number of these lymphocytes in healthy persons was 3.9% and it varied in different individuals from 1 to 7%. A definite difficulty is that RFL are always a population of cells with different numbers of bound particles (Table 2). The criterion of a rosette, a lymphocyte with at least three erythrocytes bound to it, is very conventional. A lymphocyte binding at least three indicator particles of one type or another was arbitrarily taken to be a true mixed rosette. Depending on the criterion adopted, it is evident that the number of mixed rosettes must vary. The significance and nature of lymphocytes capable of giving mixed rosettes are uncertain. At present only a few suggestions can be put forward. 1) Human lymphocytes, besides T and B subpopulations, may perhaps consist also of other subpopulations [8]; 2) lymphocytes forming mixed rosettes are precursor cells, which subsequently differentiate into either T or B lymphocytes; 3) such lymphocytes are a subpopulation or a stage of development of either T or B lymphocytes which later lose one of their markers; 4) quantitative differences in the distribution of the surface markers, which vary depending on the stage of development or of the cell cycle of the lymphocyte [7], may perhaps exist between T and B populations. In healthy subjects the number of lymphocytes forming mixed rosettes is small, but in pathological conditions their number may rise sharply [13] or, on the other hand, may be reduced to zero [8]. Consequently, the formation of such rosettes can be regarded as a test for the functional norm of the immunocompetent cells.

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