ASSAY OF ANTILYMPHOCYTIC PREPARATIONS BY INHIBITION OF ERYTHROCYTE ROSETTE FORMATION

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Lymphocytes from the human tonsils can form rosettes with sheep's erythrocytes. Antilymphocytic antibodies inhibit rosette formation, and the immunoglobin isolated from antilymphocytic serum is more active in this respect than the original serum.

No satisfactory methods of assaying the specific activity of antilymphocytic preparations yet exist. Methods such as the lymphoagglutination test or determination of cytotoxic and blast-transforming activity do not show sufficiently high correlation with the specific activity of the preparation. Inhibition of erythrocyte rosette formation could be a suitable test for this purpose. Investigations have shown that some lymphoid cells from an animal of one species, if immune to erythrocytes of an animal of another species, can fix these erythrocytes on their surface in vitro, thus forming "rosettes" [9, 10]. As a rule some human circulating lymphocytes are capable of forming rosettes with sheep's erythrocytes without special preliminary immunization. This phenomenon has been used for the titration of antilymphocytic preparations, which inhibit rosette formation [2, 3].

The object of the investigation described below was to assay the activity of antilymphocytic sera (ALS) and of globulin isolated from them on the basis of their ability to inhibit rosette formation.

EXPERIMENTAL METHOD

Horse serum and horse immunoglobulin against human lymphocytes, prepared at the Moscow Research Institute of Epidemiology and Microbiology by subcutaneous immunization of horses with a cell suspension from human tonsils were used in the investigation. The ALS were inactivated at 56°C and absorbed until total disappearance of the hemagglutinins. Antilymphocytic globulin was isolated by fractionation of the serum with ammonium sulfate. The activity of the preparations used varied in the lymphoagglutination test from 1:2500 to 1:4000, and in the cytotoxic test from 1:1280 to 1:10240. Lymphocytes for the inhibition of rosette formation test, in Biozzi's modification [4], were obtained from freshly removed human tonsils. The tonsils were washed in ice-cold medium No. 199, cut up finely with scissors in a cold Petri dish, and then crushed in a glass homogenizer with the addition of a small volume of medium. The resulting suspension was filtered through three layers of Kapron and washed in medium No. 199 by centrifugation at 1000 rpm. The suspension was then filtered once again through

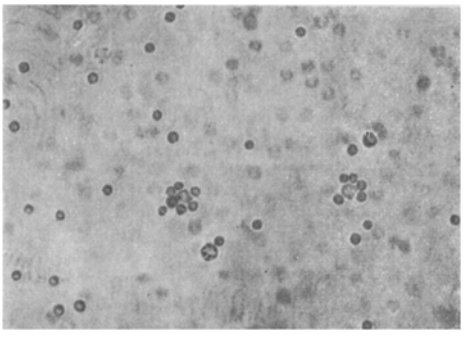

Fig. 1. Formation of rosettes by lymphoid cells from human tonsils and sheep's erythrocytes, 150×.

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TABLE 1. Rosette Formation by Tonsillar Lymphoid Cells

Material	Tot. no. of specimens	No. of rosettes in specimens (in ‰)								Mean per thous. cells
		1	2	3	4	5	6	7	8	
Cells from tonsils of 1 donor	6	17,6	21	0	41	4,3	22,3	—	—	21,3
Cells from tonsils of 4 donors	8	10	13,4	12,5	20	13,4	7,5	5,8	21	13,0

TABLE 2. Inhibition of Rosette Formation by the Action of ALS

Index	ALS concentration (in μg protein/ml)						
	40000	4000	400	40	4	0,4	0,04
No. of rosettes per thous. lymphocytes	0	0	0	2,8	6,3	10,0	13,1
Inhibition of formation (in percent of control)	100	100	100	80	52	24	0

TABLE 3. Inhibition of Rosette Formation by the Action of Antilymphocytic Globulin of Different Batches

Batch of globulin	Titer in lympho-agglutination test*	Titer in cytotoxic test*	Percentage inhibition in concn. shown below (in μg protein/ml)						ED_{50} (in μg protein/ml)	
			0,04	0,4	4	40	400	4000	ED_{50}	95% confidence interval
1	1:2 500	1:1 280	19	40	44	92	100	100	1,01	0,96—1,10
2	1:2 000	1:5 120	38	48	56	98	100	100	0,52	0,50—0,55
3	1:2 500	1:10 240	27	29	37	94	100	100	1,30	0,87—2,00
4	1:4 000	1:10 240	8	42	67	94	100	100	1,00	0,69—1,50

*scaled for 5% protein solution.

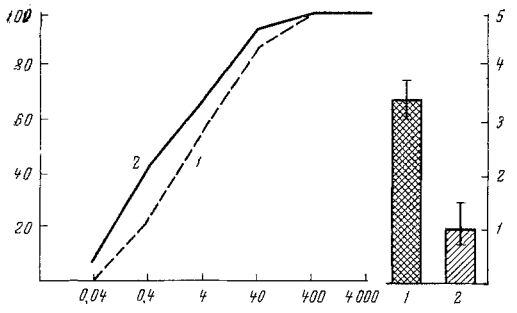

Fig. 2. Comparative activity of ALS (1) and of antilymphocytic globulin isolated from it (2) in inhibition of rosette formation test. Abscissa, dose of preparation (in μg protein/ml); ordinate, on left: percentage inhibition of rosette formation, on right: ED_{50} (in μg protein/ml).

Kapron and three times, drop by drop, through a tuberculin needle, and then made up to a concentration of $6 \cdot 10^6$ cells/ml. All manipulations with the suspension were carried out in tubes immersed in ice. Next, 0.5 ml of the suspension was placed in test tubes with a conical base and 0.25 ml complement (guinea pig serum) in a dilution of 1:10, and 0.5 ml of human antilymphocytic serum or globulin in the same dilution were added. The tubes were closed with rubber stoppers and kept at 37°C for 1.5 h. After this time, 0.5 ml of a suspension of sheep's erythrocytes, in a concentration of $24 \cdot 10^6$ cells/ml, was then added to the tubes and they were kept in a refrigerator at 4°C for 18-20 h. At the end of this time the tubes were carefully agitated by turning them in a vertical plane, care being taken to ensure that the contents of the tubes washed the stopper. The frequency of agitation was about 20 oscillations per minute for 10 min. The suspension was then quickly transferred to a Bürcker's chamber and the number of rosettes (containing at least 4 erythrocytes) per thousand lymphocytes in the first sample of suspension was counted under a magnification of 450 times. The titration was carried out with standard concentrations of the preparations. Normal absorbed horse serum was used as the control. Statistical analysis of the results was carried out by Pogozhev's modification [1] of the probit method, and the mean active dose (ED_{50}), i.e., the dose reducing the number of rosettes by half compared with the control, was calculated for each batch of the preparation.

EXPERIMENTAL RESULTS

Human tonsils were found to contain antibody-producing cells capable of forming rosettes spontaneously with sheep's erythrocytes, like the lymphocytes of the blood (Fig. 1). The number of rosettes formed by the tonsillar lymphoid cells of different persons varied from 0 to 41 per thousand cells. To allow standardization of the method, a mixture of cells from the tonsils of several donors was used; the number of rosettes in it varied from 5.8 to 21 per thousand cells (Table 1).

Treating the cell suspension with ALS inhibited rosette formation and a clear relationship was found between the effect of inhibition of rosette formation and the concentration of antilymphocytic antibodies (Table 2). For example, ALS in a concentration of 400 μg protein/ml or above completely inhibited, and in a concentration of 0.4-40 μg/ml, partially inhibited rosette formation. The value of ED_{50} for the batch of ALS investigated was 3.47 (3.16-3.80) μg protein/ml.

Four batches of horse immunoglobulin against human lymphocytes were investigated in the same way, and were compared in one experiment with the reference serum. The results of 3 such experiments are summarized in Table 3.

All the batches of antilymphocytic globulin studied possessed high activity, significantly higher than that of the ALS (Fig. 2). In all doses the globulin gave a higher percentage of inhibition than the serum from which it was isolated.

It can thus be concluded that human tonsils contain antibody-producing cells capable of forming rosettes spontaneously with the sheep's erythrocytes. Rosette formation can be inhibited by means of antilymphocytic preparations, and a direct relationship exists between the percentage inhibition of rosette formation and the concentration of antilymphocytic antibodies. The activity of the preparation can therefore be characterized quantitatively by determining the value of ED_{50}. The nature of the phenomenon of rosette formation has not yet been finally settled. It is undoubtedly an immunologic phenomenon by virtue both of its specificity and of the relationship existing between the number of rosettes and the level of antierythrocytic antibodies in the blood serum [4, 7, 8]. As a rule, the rosette-forming cells are also antibody-producing [8, 11], although it has been shown that cells which are mediators of the delayed hypersensitivity reaction are also capable of forming rosettes [5]. The mechanism of inhibition of the rosette phenomenon by ALS likewise has not yet been explained. Antilymphocytic antibodies covering the surface of the lumphocyte possibly do not allow attachment of the sheep's erythrocytes. The possibility likewise cannot be ruled out that other, more profound changes have taken place in the lymphocytes. The degree of correlation between the ability of the preparation to inhibit rosette formation and its effectiveness as an immuno-depressant likewise requires precise analysis. Such a correlation has been established for ALS against dogs' lymphocytes [6].

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