SIMULTANEOUS DETECTION OF T⁻, B⁻, AND D-ROSETTE-FORMING LYMPHOCYTES AND "NULL" CELLS IN MAN

T. I. Grishina and S. Muller

UDC 612.112.94.017.1-087.23

To detect T-, B-, and D-rosette-forming lymphocytes and "null" cells in human peripheral blood a combined rosette-formation test is recommended, using zymosan-complement, sheep's erythrocytes, fixation, and staining the films with amidotriazoate (Triosil) for identification of the lymphocytes.

KEY WORDS: rosette-forming cells; combined detection.

To estimate the numbers of T and B lymphocytes in man the rosette-formation test is nowadays widely used. T Lymphocytes can bind sheep's erythrocytes directly to form so-called spontaneous rosettes – E or T rosettes [2, 6, 8]. The rosette-formation test can be used to identify B lymphocytes if erythrocytes conjugated with antibodies and complement are used as indicator cells [4, 10].

Hitherto in most cases T and B lymphocytes have been counted separately in different preparations. For the sake of unification, standardization, and simplfication of the technique, simultaneous determination of T and B lymphocytes in the same preparation has substantial advantages. Moreover, it can also be used to determine the subpopulation of lymphocytes binding both T and B indicators simultaneously (D lymphocytes). Only isolated reports have been published on this subject [7, 9, 11]. The method meriting the greatest attention is that suggested by Mendes [9], in which zymosan particles conjugated with complement are used as the B indicator. However, this method is very laborious (the reaction is read in suspension under the phase-contrast microscope), and this prevents its wide application in clinical practice. The writers accordingly suggest a more convenient modification of this method.

Investigations were carried out with heparinized blood from healthy donors. Lymphocytes were isolated from the peripheral blood of 21 donors by Böyum's method [5] and from the blood of 14 donors by Veksler's modification of that method, using a 14.5% solution of Verografin (Spofa, Czechoslovakia; equivalents: amidotrizoate, Triosil), pH 7.35. T-Rosette-forming lymphocytes (T-RFL) were determined by the method of Petrov et al. [2]. B-Rosette-forming lymphocytes (B-RFL) were identified by means of zymosan particles (from Tallin Pharmaceutical Chemical Factory), conjugated with complement [1]. Equal volumes of a 0.1% standard suspension of zymosan and complement (diluted 1:5 with physiological saline) were incubated for 30 min at 37°C. The twice washed particles were then diluted with physiological saline to produce a 0.17% suspension. Zymosan not conjugated with complement did not bind with the lymphocytes, in agreement with data in the literature [9]. For the combined rosette-formation test, a special modification* of Mendes' method [9] was used. Instead of group AB serum, medium No. 199 was added to the lymphocytes–zymosan-complement–sheep's erythrocytes system to prevent inactivation of rosette formation, and to prevent damage to the lymphocytes the centrifugation time was shortened to 5 min. The numbers of T-, B-, and D-RFL were counted in fixed preparations by the the method suggested for determination of T-RFL [2]. Taking these modifications into account, the test was carried out by the following method.

To 0.1 ml of a suspension of lymphocytes ($2 \cdot 10^6$ lymphocytes/ml) in medium No. 199 in a siliconized micro-test-tube 0.1 ml of a 0.5% suspension of sheep's erythrocytes and 0.1 ml of a 0.17% suspension of zymo-

*Efficiency Suggestion No. 197 dated April 25, 1977, awarded to T. G. Grishina by the Research Institute of Work Hygiene and Occupational Diseases, Academy of Medical Sciences of the USSR.

Laboratory of Industrial Immunology and Allergology, Research Institute of Work Hygiene and Occupational Diseases, Academy of Medical Sciences of the USSR. Department of Immunology and Section of Immunology, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR A. M. Chernukh.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 85, No. 4, pp. 503-506, April, 1978. Original article submitted June 30, 1977.

 0007-4888/78/8504-0550$07.50

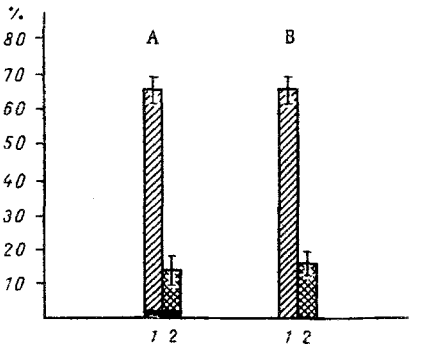

Fig. 1

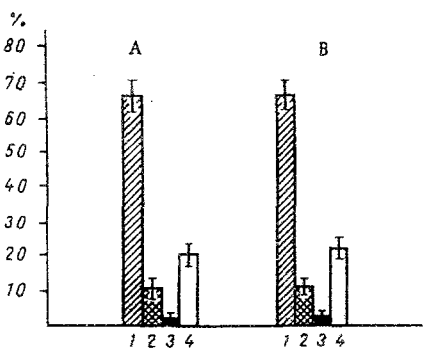

Fig. 2

Fig. 1. Content of T- (1) and B-RFL (2) during combined (A) and separate (B) determination (in %). D-RFL subpopulation indicated in black.

Fig. 2. Relative proportions of T- (1), B- (2), D-RFL (3), and "null" cells (4) during detection of lymphocytes in amidotrizoate (A) and Ficoll (B) gradient in combined rosette-formation test (in %).

TABLE 1. Subpopulations of Peripheral Blood Lymphocytes from Clinically Healthy Donors

Index	RFL						Null cells	
	T		B		D			
	%	thousands/ mm³	%	thousands/ mm³	%	thousands/ mm³	%	thousands/ mm³
M	63,0	1,5	11,3	0,25	1,7	0,04	24,0	0,57
$\pm m$	1,5	0,1	0,8	0,01	0,3	0,005	1,5	0,04
Δ	60,0—66,0	1,3—1,7	8,3—14,4	0,22—0,28	1,2—2,2	0,03—0,05	22,0—26,0	0,48—0,66

Legend. Δ) Confidence interval.

san-complement complex were added. The mixture was incubated for 5 min at 37°C, sedimented for 5 min at 200g, and incubated for 60 min at 12°C. It was then quickly fixed with 0.1 ml of 0.6% glutaraldehyde for 20 min. Distilled water was then added up to the edge of the tube, the suspension was centrifuged for 5 min at 200g, the supernatant was removed with a Pasteur pipet, and films were made on slides from the carefully resuspended residue. The films were dried in the air, fixed for 10 min in methanol, washed for 2 min in distilled water, dried, and stained with methyl green–pyronine by Brachet's method [3]. A coverslip was glued to the film with Canada balsam, and after drying, the film was examined under the microscope with an immersion system. A lymphocyte binding three or more sheep's erythrocytes was taken as a T rosette and a lymphocyte with three or more zymosan particles as a B rosette; a lymphocyte with at least three zymosan particles and with three or more erythrocytes was taken as a mixed rosette.

To compare the combined rosette-formation test with the more usual separate determination of T- and B-RFL parallel experiments were carried out by the two methods. The results are given in Fig. 1 and they show that the number of cells carrying T markers did not differ significantly in the two methods. The number of cells carrying the B-marker when determined separately was a little higher than the number determined by the combined method (15.9 ± 0.7 and 13.0 ± 1.0%, respectively), and this can most probably be attributed to spatial competition between the corpuscular indicators for the lymphocyte receptors in the combined rosette-formation test. However, it must be remembered that the mean number of B-RFL in the combined rosette-formation test did not exceed the limits of variations of the number of B cells obtained by other workers when determining them separately [10]. Not only the number of T- and B-RFL can be determined by this test, moreover, but also the number of D-RFL and of so-called "null" cells, which belong to none of the other three types.

Nevertheless, the use of expensive reagents in short supply remained an obstacle to the widespread use of this method of identifying the cell fractions of lymphocytes in clinical practice. With this in mind, the more accessible method of identification of lymphocytes by means of amidotrizoate in Veksler's modification [1] also was tested. In parallel combined rosette-formation tests with lymphocytes obtained in a density gradient by Böyum's method [5] and in an amidotrizoate gradient by Veksler's method [1], no significant differences were found in the numbers of T-, B-, and D-RFL (Fig. 2).

The results obtained during trials of this modification of the test are given in Table 1. They show that the relative proportions of T and B subpopulations of lymphocytes discovered by this means were in good agreement with values obtained for their numbers in the blood of clinically healthy donors when determined separately [2].

The use of the zymosan-complement complex as B indicator for the quantitative determination of B-RFL in fact revealed certain advantages of this method. First, it is technically simple, and second, the use of a standard zymosan preparation enabled comparable results to be obtained. It is important to note that morphological differences between zymosan particles and sheep's erythrocytes were particularly clearly seen in fixed and stained preparations. Zymosan particles are between one-third and one-quarter the size of sheep's erythrocytes, and when stained they appear bright pink, unlike the ruby erythrocytes; it is therefore much easier to count the RFL than in suspension under the phase-contrast microscope.

The combined rosette-formation test using zymosan-complement, sheep's erythrocytes, fixation, and preparation of stained films for the identification of the test lymphocytes with the aid of amidotrizoate (Verografin, Triosil) can thus be recommended for the detection of T-, B-, and D-RFL and of "null" cells in human peripheral blood.

LITERATURE CITED

1. Kh. M. Veksler, in: Problems in Autoallergy and Practical Medicine [in Russian], Tallin (1975), pp. 210-212.
2. R. V. Petrov, M. A. Stenina, and K. A. Lebedev, Byull. Éksp. Biol. Med., No. 2, 197 (1976).
3. A. G. E. Pearse, Histochemistry, Theoretical and Applied [Russian translation], Moscow (1962), p. 744.
4. C. Bianco, R. Patrick, and V. Nussenzweig, J. Exp. Med., 132, 702 (1970).
5. A. Böyum, Scand. J. Clin. Lab. Invest., 21, Suppl. 67, 1 (1968).
6. P. J. Brain, W. A. Gordon, and P. Willetts, Clin. Exp. Immunol., 6, 681 (1970).
7. J. W. Chiao, V. S. Pantic, and R. A. Good, Clin, Exp. Immunol., 18, 483 (1974).
8. R. R. A. Coombs, B. W. Gurner, A. B. Wilson, et al., Int. Arch. Allergy, 39, 658 (1970).
9. N. F. Mendes, S. S. Miki, and Z. F. Peixinho, J. Immunol., 113, 531 (1974).
10. V. Nussenzweig, C. Bianco, P. Dukor, et al., in: Progress in Immunology (International Congress), New York (1971), p. 73.
11. E. Shevach, R. Edelson, M. Frank, et al., Proc. Nat. Acad. Sci. USA, 71, 863 (1974).