IMPROVED METHOD OF IDENTIFICATION OF CELLS CARRYING ANTIGEN-BINDING RECEPTORS FOR LYMPHOCYTES IN THE ROSETTE FORMATION TEST

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One method of identifying cells carrying antigen-binding receptors is the rosette formation test [2, 6, 7], based on the phenomenon of adhesion of a corpuscular antigen to the surface of a lymphoid cell expressing antigen-binding receptors of corresponding specificity [1, 5]. Existing modifications of the rosette formation test can be conveniently used only at the height of the immune response, when the population of rosette-forming cells (RFC) is sufficiently numerous. If RFC are found in the early stages of the immune response, or if the immune response is weak, the results of the test usually have to be read simultaneously by several workers [4]. It is just as difficult to determine the level of RFC among a population of nonimmune lymphocytes, although investigations of this type give basically important results. Work with unstained preparations is fatiguing and is possible only under low power of the microscope. Under these circumstances it is not always possible to distinguish reliably between rosettes and small groups of agglutinated erythrocytes.

The aim of the present investigation was to devise a more accurate and less laborious method which would allow the RFC population to be determined easily even when only a few are present in the cell population. The method consists in principle of applying test erythrocytes to a monolayer of lymphocytes, after which the unbound erythrocytes are removed by washing.

To form the monolayer coverslips were used, and after thorough defatting each coverslip was divided into four parts. To create a stable positive electric charge on the prepared coverslips, a solution of poly-L-lysine (from Sigma, USA) with a concentration of 50 μg in 1 ml distilled water, was applied, after which the excess of polylysine was removed by washing with distilled water, and the coverslips were dried in air. A drop of the test suspension of lymphocytes in a concentration of 3×10^7 cells in 1 ml Hanks' solution was applied to the coverslip with its polylysine film, and kept for 15 min at 4°C. The coverslip was rinsed in the same solution and next treated with a 5% solution of human serum albumin to block the free sites on the polylysine film. The coverslips were placed in flat-bottomed flasks (to flatten the bottom of a penicillin flask paraffin wax or epoxide resin was used). The suspension of test erythrocytes in a concentration of 15×10^7 cells in 1 ml of Hanks' solution was added to the flasks containing the coverslips. The erythrocytes were sedimented by centrifugation at 200g for 5 min on a centrifuge equipped with bucket rotor. The coverslip was then placed for 10 min with the monolayer underneath in the well of a hemagglutination plate, filled with Hanks' solution, to remove most of the nonadherent erythrocytes. The remaining free erythrocytes were removed by successively rinsing the coverslip in three jars with Hanks' solution. The preparation was fixed for 5 min with 0.5% glutaraldehyde solution and dried in a 20% solution of human serum albumin. The dried preparations were postfixed with methyl alcohol and stained with azure–eosin by Romanovsky's method. The results of the test were read under the microscope with an immersion system in two stages. The first stage consisted of counting rosettes in not less than 50 fields of vision. In the second stage the number of lymphocytes was counted in five fields of vision in different parts of the preparation. The results were expressed as the number of RFC per 10,000 lymphocytes.

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TABLE 1. Detection of RFC in Lymphocyte Suspension and among Lymphocytes in Monolayer

Day after immunization	Method					
	monolayer (50 fields of vision)			suspension (one chamber)		
	lymphocytes	RFC	RFC/10^4 cells	lymphocytes	RFC	RFC/10^4 cells
Before immunization	15 700	7	4,5	1 680	0	0
1st	13 800	13	9,4	1 690	0	0
2nd	15 700	6	3,8	1 990	0	0
3rd	13 500	20	14,8	1 600	3	18,7
4th	12 700	51	40,2	1 650	11	66,7
8th	15 700	44	28,0	1 490	13	87,2
14th	13 700	35	25,6	2 250	8	35,6
23rd	12 700	36	28,4	1 570	3	19,1

Legend. To determine the RFC population a mixture of cells from the spleens of five mice was used at each time.

It must be emphasized that when this test is carried out medium 199 must not be used either to prepare the monolayer or to prepare the erythrocyte suspension. Experience shows that when lymphocytes are sedimented in medium 199 the cell density in the monolayer on a polylysine substrate is very low, and erythrocytes resuspended in the same medium adhere nonspecifically to the spaces between the lymphocytes in the monolayer. This phenomenon is evidently attributable to a soluble factor which appears in the supernatant within 1-3 min after addition of lymphocytes to medium 199. The action of this factor cannot be blocked by serum proteins.

To assess the effectiveness of the proposed method the kinetics of accumulation of RFC in the spleen of (CBA × C57BL/6)F_1 mice immunized intravenously with sheep's red blood cells (SRBC) in a dose of 5×10^8 SRBC per mouse was investigated. The RFC population was counted by the method described above in a monolayer and by the usual method in a suspension [3]. The results are given in Table 1.

It will be clear from Table 1 that the RFC population in unimmunized animals and during the first 2 days after immunization was virtually impossible to determine by the usual method. Meanwhile the proposed method enabled RFC to be identified easily in the same cell population. The somewhat larger number of RFC found by the suspension method than by the monolayer method can be explained by the discovery of a certain number of antibody-forming cells, forming rosettes of the "morula" type together with antigen-binding lymphocytes. Figures of this sort were not formed in the monolayer at all, and all rosettes were of the "corona" type.

Similar results can be obtained on a lymphocyte monolayer formed on a hydrophobic plastic surface treated with phytohemagglutinin (PHA) on account of ligand binding of the lectin with the carbohydrate component of the glycoprotein receptors of the lymphocytes. For this purpose, a solution of PHA (from Reanal, Hungary) in a concentration of 5 μg/ml in distilled water was applied to polystyrene plates or to coverslips coated with polystyrene, dissolved in dichloroethane. When this method of monolayer formation was used, free sites on PHA were blocked by means of normal mouse serum.

The use of this method of performing the rosette formation test thus gives many advantages compared with methods described previously. The proposed method greatly facilitates the identification and counting of RFC in the early stages of the immune response and in nonimmune animals. Furthermore, the possibility of examining large numbers of cells with maximal magnifications of the light microscope, and also the absence of an erythrocyte background in the preparation, enhance the accuracy of the investigation and make it less laborious.

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