Demonstration of Simultaneous Rosette and Plaque Formation by Splenic Lymphocytes in the Rabbit[1] (37020)

NICOLA DONNELLY AND DIETER H. SUSSDORF

Department of Microbiology, Cornell University Medical College, New York, N. Y. 10021

Methods currently available for the study of cellular immune activity include the rosette assay as described by Zaalberg (1) and by Nota *et al.* (2), which detects antigen-binding cells, and the plaque assay of Jerne, Nordin, and Henry (3) for the enumeration of single antibody-secreting cells. Although both techniques have been used extensively, the relationship of antigen-binding (rosette-forming) cells to antibody-forming (plaque-forming) cells is not clear. It was thought earlier that rosettes and plaques were both produced by antibody-secreting cells (1, 2, 4–6); however, recent studies indicate that this is not so (7, 8). Wilson has shown that at the peak of a primary response to sheep erythrocytes (SRBC), less than 10% of cells which have formed rosettes are subsequently capable of forming plaques. Conversely, no plaque-forming cells (PFC) could be induced subsequently to form rosettes (8). He has also shown that as the immune response progresses, the proportion of cells capable of forming both rosettes and plaques decreases. Wilson's method, which involves transfer of cells from one assay system to the other by micromanipulation, is complex and time-consuming and allows examination of only limited cell numbers.

This report presents a direct assay which permits screening of large cell populations for the identification and enumeration of rosette-forming (RFC), plaque-forming (PFC), and rosette-plus-plaque-forming cells (RPFC). Formalinized sheep erythrocytes (f-SRBC), which retain normal surface antigenic properties but are resistant to lysis by antibody and complement, were used to link the suspension-centrifugation rosette test of McConnell *et al.* (9) with the very sensitive monolayer plaque assay of Kennedy and Axelrad (10).

[1] This work was supported by NIH grant 5T01AI00281 and NSF grant GB-23957.

Materials and Methods. Preparation of formalinized SRBC. Formalinization of SRBC was based on the method of Csizmas (11). Thirty milliliters of SRBC in Alsever's solution, drawn less than 10 days before use, were washed (500*g*, 4°, 15 min) 5 times in saline (0.85% NaCl). After the final wash, 5 ml of packed cells were resuspended in cold saline to a volume of 40 ml and placed into a 125-ml flask. Ten milliliters of formaldehyde solution (37%, Certified A.C.S., Fisher Scientific Co.), removed from a previously unopened bottle, were adjusted to pH 5.5–6.0 with 1 *M* NaOH and then introduced into dialysis tubing of 2.5-cm width (Union Carbide Corp.). The tubing was submerged in the SRBC suspension and the flask gently agitated at room temperature on a mechanical shaker (100 cycles per min). After 3 hr, the contents of the dialysis tubing were emptied into the flask and the formaldehyde-SRBC mixture was allowed to shake for 12 additional hours. The suspension was then strained through cotton and washed 6 times in cold saline. The formalinized SRBC were stored as a 30% saline suspension at 4°.

Testing of formalinized SRBC. One milliliter of the stock f-SRBC suspension was washed 2 times and the sedimented cells were resuspended in cold phosphate-buffered saline (PBS; 7.2 g NaCl, 1.48 g Na_2HPO, 0.43 g NaH_2PO_4/liter; pH 7.2). Their concentration was adjusted to 2×10^8 cells/ml by hemacytometer count (approximately equivalent to and subsequently referred to as a 1% suspension). Nonspecific clumping was never observed, but occasional preparations con-

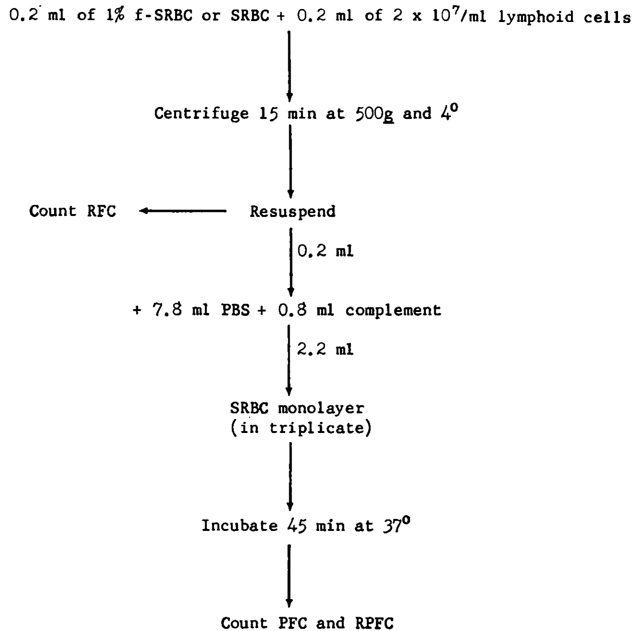

FIG. 1. Outline of combined assay for RFC, PFC, and RPFC (see text for abbreviations).

tained distorted or swollen cells and were discarded. Such deformities can be caused by formaldehyde solutions that had undergone prolonged exposure to air or may be due to formalinization of old or damaged SRBC's.

The f-SRBC in the 1% suspension were reexamined microscopically, and, if found morphologically normal, tested for surface antigenic properties in a suspension-centrifugation rosette assay (9). CBA mice were immunized 4 days previously by intraperitoneal injection with 0.2 ml of a 20% saline suspension of SRBC. A suspension of spleen cells was prepared and tested for rosette-forming activity as described below (see *Combined assay*), using both f-SRBC and untreated SRBC.

Rosette-forming ability of f-SRBC was essentially an all-or-none phenomenon. Sixty percent of the f-SRBC preparations exhibited reactivities nearly identical to those of untreated SRBC preparations. Only f-SRBC preparations with rosette-forming activities paralleling those of untreated SRBC's were used for subsequent assays. Stock suspensions of such preparations retained their initial reactivities for 1 to 2 weeks.

Combined assay. Suspensions of lymphoid cells were assayed for rosette-forming, plaque-forming, and rosette-plus-plaque-forming activities as outlined in Fig. 1. The cells were tested with f-SRBC and untreated SRBC in parallel runs. Untreated SRBC were used to recheck the rosette-forming capacity of the f-SRBC preparation.

The first part of the combined technique was a modification of the suspension-centrifugation rosette assay of McConnell *et al.* (9). If splenic lymphoid cells were to be assayed, the spleen was removed and carefully teased in a small petri dish containing cold PBS. The tissue fragments were then pressed through a 200-mesh wire screen. The resulting cell suspension was washed 3 times in cold PBS (200*g*, 4°, 10 min) and adjusted to a concentration of 2×10^7 nucleated cells/ml by hemacytometer count.

Two-tenths-milliliter aliquots of the lymphoid cell suspension were mixed with 0.2 ml of 1% f-SRBC or 0.2 ml of 1% untreated SRBC [standardized photometrically as described by Campbell *et al.* (12).] The two preparations were centrifuged at 500*g*, 4°, for 15 min, and then gently resuspended with a Pasteur pipette. A portion of the suspension was used to make a rosette count in a Levy–

TABLE I. Rosette-Forming (RFC), Plaque-Forming (PFC), and Rosette-Plus-Plaque-Forming Cells (RPFC) in Spleens of Normal Rabbits and Rabbits Injected Intravenously with 3×10^8 SRBC/kg 7 Days before Assay.[a]

Treatment	Rabbit no.	Rosette assay performed with				
		f-SRBC			SRBC	
		RFC[b]	PFC[c]	RPFC	RFC	PFC
Injected with SRBC	1	8200	477	53.0	7500	402
	2	5400	376	58.7	5500	374
	3	6700	405	18.5	5800	396
		6770 ± 806[d]	419 ± 35	43.4 ± 12.5	6270 ± 609	391 ± 9
Uninjected	4	850	3.0	0	460	3.5
	5	320	2.7	0	250	0.8
	6	710	0.8	0	140	1.3
	7	380	5.1	0	190	4.0
		565 ± 125	2.9 ± .9	0	260 ± 70	2.4 ± .8

[a] Spleen cells were first rosetted with formalinized SRBC (f-SRBC) or untreated SRBC and then incorporated into a monolayer plaque system.
[b] All activities expressed per 10^6 nucleated cells.
[c] Plaques produced by both rosette- and non-rosette-forming cells.
[d] Mean ± standard error.

Hausser hemacytometer. A lymphoid cell surrounded by 5 or more f-SRBC or SRBC was identified as a RFC. Generally, 18,000–36,000 lymphoid cells were scanned.

The second part of the procedure was based on the monolayer plaque assay of Kennedy and Axelrad (10). Two-tenths-milliliter of each rosette mixture was diluted in 7.8 ml cold PBS to give a concentration of 2.5×10^5 nucleated cells per ml. Eight-tenths-milliliter of guinea pig complement (Baltimore Biological Laboratories), previously adsorbed once with SRBC (1 volume cells, 9 volumes complement) and diluted 1:4 in cold PBS, was added to the diluted rosette suspension. Two- and two-tenths-milliliter aliquots of this mixture were plated into each of 3 petri plates containing a SRBC monolayer prepared as described by Kennedy and Axelrad (10).

The petri plates were incubated at 37° for 45 min, then examined microscopically with a 2.5× objective to enumerate the total number of plaques formed. PFC's were visible as central lymphoid cells surrounded by a clear zone in the SRBC monolayer (Fig. 2A). Approximately one-third of each plate containing f-SRBC was also examined with 10× and 40× objectives to identify RPFC and to determine the ratio of PFC to RPFC. If a plaque had been formed by a cell which also bound antigen, a rosette of unlysed f-SRBC was seen fixed in the center of the clear zone (Fig. 2B and C).

Sample assay. Three New Zealand White female rabbits (Camm Research Institute, Wayne, New Jersey), with an average weight of 3.5 kg, were injected with 3×10^8 washed SRBC per kg into the marginal ear vein. Their spleens and those of 4 unimmunized control rabbits were assayed for RFC, PFC, and RPFC seven days later, at a time when both rosette- and plaque-forming activities were near their peaks (Donnelly, unpublished data).

Results and Discussion. By making use of Csizmas' observation that formalinized SRBC's are resistant to lysis by antibody and complement (11), we developed an assay in which lymphoid cells capable of both binding antigen and secreting antibody can be visualized (Fig. 2B and C). Such cells are first rosetted with f-SRBC and then incorporated into a monolayer plaque assay system.

Table I presents data obtained from assays for splenic RFC, PFC, and RPFC in SRBC-immunized and unimmunized rabbits. The animals were sacrificed on day 7 after im-

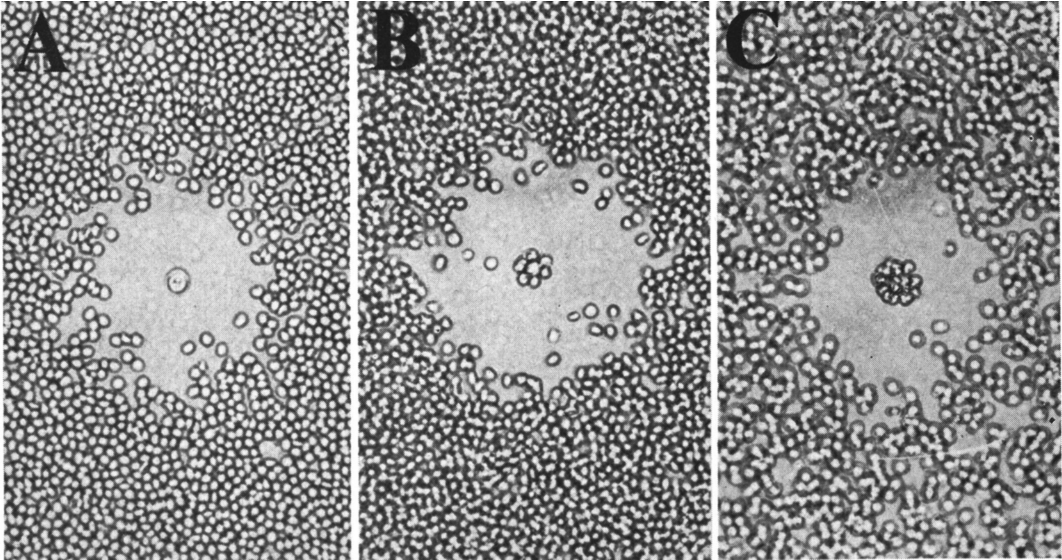

FIG. 2A. Anti-SRBC plaque formed by a splenic lymphoid cell which secretes antibody but does not bind antigen. B and C: Plaques formed by cells which both secrete antibody and bind antigen; 360×.

munization, at which time mainly IgM antibodies are formed (13). Comparison of RFC counts when spleen cells from immunized animals were rosetted with either f-SRBC or untreated SRBC provided a further check on the reactivities of the f-SRBC suspensions. The close correlation between counts of RFC formed with f-SRBC and those formed with untreated SRBC (group means of 6770 and 6270 RFC/10^6 nucleated cells, respectively) demonstrated the unimpairment of rosette-forming ability by formalinization. The difference between the mean number of f-SRBC rosettes and SRBC rosettes formed by cells from unimmunized rabbits (group means of 565 and 260 RFC/10^6 nucleated cells, respectively) was not significant as determined by the t test ($p > 0.5$). The difference was due to the variability inherent in counting small absolute numbers of RFC, which ranged between 2 and 8 per hemacytometer grid.

Wilson (8), and Shearer and Cudkowicz (6) have shown that the rosette procedure itself does not influence the subsequent ability of lymphoid cells to form plaques. Our data demonstrate that this observation also held true when f-SRBC were used for rosetting. As can be seen in Table I, there was no reduction in the mean number of PFC/10^6 after rosetting with f-SRBC as compared with SRBC-rosetting. Differences between these means were statistically not significant.

The group means for RFC, PFC, and RPFC per 10^6 spleen cells from immunized rabbits gave a RFC/PFC ratio of 16.2. The RPFC/RFC and RPFC/PFC ratios were 0.006 and 0.111, respectively. Thus, 7 days after immunization, less than 1% of splenic RFC's also formed plaques, and approximately 10% of plaques were produced by RFC's. These calculations indicate that near the peak of a primary anti-SRBC response in rabbits, RFC's and PFC's are largely two independent populations. This conclusion is in agreement with Wilson's findings for mice (8).

Summary. Formalinized sheep erythrocytes, which retain their surface antigens but are resistant to lysis by antibody and complement, were used to link a suspension-centrifugation rosette assay with the monolayer plaque assay. This technique permitted screening of large cell populations for the identification and enumeration of antigen-binding cells, antibody-secreting cells, and cells which both bind antigen and secrete antibody.

1. Zaalberg, O. B., Nature (London) **202**, 1231 (1964).
2. Nota, N. R., Liacopoulos-Briot, M., Stiffel, C., and Biozzi, G., Comp. Rend. Acad. Sci. **259**, 1277 (1964).
3. Jerne, N. K., Nordin, A., and Henry, C., *in* "Cell Bound Antibodies" (B. Amos and H. Koprowski, eds.), p. 109. Wistar Inst. Press, Philadelphia (1963).
4. Zaalberg, O. B., Van der Muel, V. A., and Twisk, M. J., J. Immunol. **100**, 451 (1968).
5. Moav, N., and Harris, T. N., J. Immunol. **105**, 1501 (1970).
6. Shearer, G. M., and Cudkowicz, G., J. Immunol. **101**, 1264 (1968).
7. Greaves, M. F., Moller, E., and Moller, G., Cell. Immunol. **1**, 386 (1970).
8. Wilson, J. D., Immunol. **21**, 233 (1971).
9. McConnell, I., Munro, A., Gurner, B. W., and Coombs, R. R. A., Int. Arch. Allergy **35**, 209 (1969).
10. Kennedy, J. C., and Axelrad, M. A., Immunol. **20**, 253 (1971).
11. Csizmas, L., Proc. Soc. Exp. Biol. Med. **103**, 157 (1960).
12. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H., "Methods in Immunology," 2nd ed., p. 121. Benjamin, New York (1970).
13. Sussdorf, D. H., J. Infect. Dis. **121**, 545 (1970).

Received Sept. 1, 1972. P.S.E.B.M., 1973, Vol. 142.