

SHEEP RED CELL BINDING TO HUMAN LYMPHOCYTES
TREATED WITH NEURAMINIDASE; ENHANCEMENT
OF T CELL BINDING AND IDENTIFICATION OF
A SUBPOPULATION OF B CELLS*

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Sheep red blood cell rosette formation has been demonstrated in a number of laboratories to be a specific marker for human T cells (1-3). A variety of different methods have been utilized which have shown some differences in the percentages of T cells that form these rosettes. The procedure of Jondal and Wigzell (2) gives values close to 100% for peripheral blood T cells and for thymocytes. Recently double labeling experiments utilizing markers for B cells along with the rosette system have indicated that independent cells are involved although rare double labeled cells may be observed (3). The simplicity and specificity of the procedure have led to wide clinical use of this method and valuable information has been obtained (3, 4).

In the course of investigations on the T cell receptor for the sheep red blood cells, it was noted that neuraminidase treatment of peripheral blood lymphocytes considerably enhances their capacity for rosette formation. A study of this system indicated that a population of presumed B cells also formed rosettes after such treatment.

Materials and Methods

Lymphocyte Separation.—Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood of normal adults and of patients with chronic lymphocytic leukemia (CLL), by Ficoll-Hypaque (F/H) gradient centrifugation followed by passage over nylon columns in some experiments. For sheep red blood cell rosette (SRBC-R) formation the cells were suspended in Hanks' balanced salt solution (HBSS) at a concentration of 5×10^6 cells/ml. For immunoglobulin staining and aggregate binding the cells were suspended at a concentration of $15-20 \times 10^6$ cells/ml in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) pH 7.2 containing 0.02% Na azide (BSA-PBS). Monocytes in these preparations were quantitated by latex bead phagocytosis.

Preparation of SRBC-R.—Rosettes were prepared according to the method described previously (3). 0.1 ml of 0.5% SRBC in HBSS, 0.1 ml lymphocyte suspension (5×10^6 cells/ml), and 0.02 ml of heat-inactivated pooled human AB serum (Grand Island Biological, Co., Grand

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Island, N. Y.) which had been previously absorbed with an equal volume of packed SRBC, or of HBSS, were mixed thoroughly and incubated at 37°C for 5 min. The mixture was then centrifuged at 50 g for 5 min at room temperature and incubated at 4°C for 60 min. The cell pellet was resuspended by very gently rocking the tubes. A minima of 200 lymphocytes were counted in a hemacytometer and lymphocytes were counted as rosette positive if any SRBC were adherent to them. (Small rosettes, if 1-2 SRBC adhered to a lymphocyte and large rosettes, if 3 or more SRBC adhered to one cell.)

Separation of Rosettes from Nonrosette Lymphocytes.—1.5 ml of SRBC-R containing suspension was layered on 1.5 ml of the F/H mixture, used for the lymphocyte isolation, in a 12 × 75 mm polypropylene tube (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), and centrifuged for 20 min at 400 g. The nonrosetting lymphocytes remained at the interface between the cell suspension and the F/H mixture. The lymphocytes at the interface were pipetted off carefully and the percentage of rosettes in both this layer and the rosette-containing bottom layer counted.

Immunoglobulin Reagents and Fluorescence.—A polyvalent antihuman immunoglobulin antiserum, raised in rabbits and conjugated with tetramethyl rhodamine isothiocyanate, was utilized as described previously (5). The antiserum was specific for Ig and was broadly reactive with mu, gamma, kappa, and lambda determinants. It was routinely centrifuged to remove aggregates immediately before use. Aggregated human immunoglobulin was prepared and conjugated to tetramethyl rhodamine isothiocyanate according to the method previously described (5).

Immunofluorescence staining of lymphocyte surfaces for Ig and for aggregate binding was performed as described previously (5). In some experiments lymphocytes were first stained with fluorochrome conjugates and then incubated with SRBC as described for preparing SRBC-R. In these experiments the SRBC-R and the fluorescence of the lymphocytes were studied and counted in the same preparation.

Neuraminidase.—Two preparations of neuraminidase from *Vibrio cholerae* (VCN) were utilized in these studies. The first was obtained from Calbiochem., San Diego, Calif. The second preparation was a highly purified enzyme preparation free of aldolase, protease, and lecitinase C activity, kindly provided by Dr. R. Doljansky. Various concentrations of enzyme or an equivalent volume of HBSS were added to cell suspensions and incubated at 37°C for 30 min. After incubation, both control- and VCN-treated cells were washed in HBSS, re-counted, and distributed for either fluorescence staining or rosette formation, as previously described.

Electron Microscopy.—Cell pellets were fixed in 1.5% redistilled glutaraldehyde in 0.1 M sodium cacodylate and postfixated in 1.0% osmium tetroxide (6). After brief glutaraldehyde fixation pellets were incubated in colloidal iron particles according to the method of Gasic et al. (7). The preparations were en bloc stained with 1.0% aqueous uranyl acetate, dehydrated, and embedded in Epon. These sections were stained with uranyl acetate and lead citrate and examined in a Siemens 101 electron microscope (Siemens Corp., Medical Industrial Div., Iselin, N. J.).

RESULTS

Effect of Neuraminidase on SRBC-R Formation by Normal PBL.—Treatment of normal human PBL by neuraminidase was found to increase the percentage of PBL which formed SRBC rosettes. This effect was dependent on the concentration of the enzyme; reaching its maximum at 25-50 U to 10⁷ cells/ml. Under these conditions, up to 94% of PBL formed rosettes (Table I). The average increase in percentage of rosette-forming cells was 13%. However, in one normal donor (S.F.) known for a relatively low number of rosette-forming cells and high B cell counts, a greater increase in the percentage of rosette-

forming cells, from 61% to 86%, was observed. No further increase of rosette formation was observed when higher enzyme concentration or longer incubation periods were used. Both preparations of neuraminidase gave similar results.

The enhancement of rosette formation by VCN was also apparent in the size and strength of the rosettes formed. Most of them were composed of more than six SRBC attached to the central lymphocyte and at the same time no small rosettes containing only two or three red cells were observed as in the untreated preparations. In addition the rosettes were much more stable and

TABLE I
Effect of Neuraminidase on SRBC-R Formation by Normal Human Lymphocytes

Subject	Untreated rosettes	Neuraminidase-treated* rosettes
	%	%
Z. B.	80	93
S. P.	81	94.5
R. B.	82	94
D. M.	73	91
P. W.	83	92.6
C. D.	84	93
P. J.	79	93.5
S. F.	61	86
Mean	78.9	92.2
Range	61-84	86-94.5

* Concentration of 50 U/ml.

less prone to disruption by manipulation. Under these conditions, the lymphoid cells were alive as judged by trypan blue exclusion and appeared morphologically intact and unagglutinated when viewed through a light microscope. Large cells and small cells which picked up latex particles were excluded from the counts. Neuraminidase-treated SRBC whether reacted with normal or with VCN-treated lymphocytes did not affect the rosette formation.

Characterization of the Lymphocytes Affected by Neuraminidase.—Studies were directed toward finding the explanation for the increased percent of SRBC rosettes after neuraminidase treatment. Ig staining and uptake of aggregated γ -globulin were unaltered and the same percentage of PBL stained with the two B cell reagents before and after neuraminidase treatment. A direct assessment of the populations bearing both markers was next performed. Normal PBL either before or after incubation with VCN, 50 U to 10^7 cells/ml, was first incubated with rhodamine-conjugated anti-Ig or aggregated γ -globulin, thoroughly washed, and then rosette-forming capacity determined. Thus, it was possible to directly determine how many labeled cells, i.e. B cells, also formed rosettes. The results of representative experiments are shown in Table II. It can be seen from these experiments that after neuramini-

dase treatment a significant number of presumed B cells with surface Ig formed rosettes.

In order to further characterize this subpopulation the effect of neuraminidase on an enriched normal B cell population was studied. Such enriched populations were obtained by separation of rosetting from nonrosetting cells over F/H. One portion of the nonrosetting cells from the interface was incubated with VCN after which a determination of their surface Ig and rosetting capacity

TABLE II
Increase in the Percentage of Lymphocytes Showing Combined Ig Staining and SRBC Rosettes after Neuraminidase Treatment

	Rosettes	Ig staining	Rosettes [‡] + Ig staining
	%	%	%
Exp. I normal PBL	80	20	0
VCN-PBL	93	19	10
Exp. II normal PBL	82	16	1
VCN-PBL	93	17	11
Enriched B cells*	14	86	2
VCN-enriched B cells	63	80	42

* Prepared by one step centrifugation after initial rosetting.

‡ Mixed Ig-staining rosettes were determined in separate experiments.

was performed; another portion was analyzed separately for Ig staining within the rosettes. The results of a representative experiment are shown in Table II. It can be seen that a marked enrichment of B cells (86%) and a parallel decrease in the number of rosette-forming cells (14%) were obtained. However, after VCN treatment 63% of the cells in this population formed rosettes. No significant decrease in the number of Ig-staining cells was observed, although there was some evidence of a decrease in the intensity of the surface staining of some cells. The double labeling experiments on the VCN-treated enriched B population revealed a marked increase in cells containing both markers together (i.e. Ig-bearing lymphocytes-forming rosettes). This effect of the enzyme on B cells was apparent whether the rosetting was performed in the presence or absence of absorbed AB serum. These effects were also unrelated to monocytes in some of the preparations; passage over nylon columns did not affect the double labeled cells.

Rosette Formation After VCN Treatment of CLL Lymphocytes.—The majority of the lymphocytes from patients with CLL do not form SRBC-R, but have B lymphocyte markers. Five such cases where the leukemic cells showed Ig staining, uptake of aggregated γ -globulin and very few rosettes were studied. After neuraminidase treatment there was no significant increase in rosette formation, in striking contrast to the findings on normal PBL.

Additional Experiments Including Electron Microscopy.—A number of experiments were carried out to study the alterations on the lymphocyte surface through the action of neuraminidase. It would be expected that the cell surface charge would be altered and clear evidence for such alteration was obtained. Charged colloidal iron particles are known to bind to red cell membranes and can be visualized by electron microscopy (7). This binding is lost after neuraminidase treatment. Similar findings were made on lymphocytes in the present study. T cells which formed SRBC rosettes showed a coating of the iron particles over their surface; this completely disappeared after neuraminidase.

Previous studies (3) have shown that the SRBC rosettes on T cells have an unusual characteristic in the electron microscope. In contrast to other types of rosettes the red cells in this situation show point attachment or in narrow zones to the lymphocyte surface. After neuraminidase treatment a striking change was observed and broad zones of attachment and intimate association of the red cell and lymphocyte plasma membranes were observed. These observations will be published in detail elsewhere.

DISCUSSION

The present observations suggest that after neuraminidase treatment of T cells additional sites are exposed on the lymphocyte surface resulting in broad zones of attachment of the SRBC and an increase in the number of cells bound to each lymphocyte. Such a process would also explain the more stable rosettes that were observed.

The finding that the enzyme effect also caused a population of presumed B cells to acquire the SRBC rosette-forming property was unexpected. This was clearly evident from double labeling experiments where lymphocytes which showed Ig on their surface with fluorescent antisera also formed rosettes. These same cells also had the Fc receptor as determined by the uptake of aggregated γ -globulin, another specific characteristic of B cells. The experiments indicated that approximately half of the presumed B cells acquired the rosette-forming property; the other half showed no such effect irrespective of the amount of neuraminidase and duration of treatment. It would appear that the leukemic lymphocytes in the five cases of CLL studied related to this neuraminidase resistant population since they too failed to form rosettes. The interpretation of these findings is not entirely clear. The most likely explanation is perhaps that there are two distinct B cell populations that can be identified by the neuraminidase effect. Alternatively it might be that the affected Ig-bearing cells form a subpopulation of T cells that have an Fc receptor and perhaps adsorb Ig from the serum. Some evidence for such T cells has been obtained in the mouse (8). It is known that the percentage of peripheral blood lymphocytes that have the complement receptor is consistently lower than the percentage bearing Ig (5). This difference has been ascribed to the very different techniques utilized but perhaps this relates to the present findings. Experi-

ments are underway to elucidate this question. The fact that neuraminidase treatment had marked effects on the rosette system is not surprising in view of previous observations on the increased absorptive capacity and sensitivity of lymphocytes to a variety of antibodies (9, 10).

SUMMARY

Neuraminidase treatment of normal human lymphocytes enhances their capacity to form SRBC rosettes; more red cells are bound and the rosettes are more stable. Under these conditions approximately 90 % of peripheral blood lymphocytes form rosettes. In addition to the effects on T cells, another population of lymphocytes which possess surface immunoglobulin and have the Fc receptor acquire the rosette-forming property after neuraminidase. This subpopulation of 'B' cells represents approximately half of the lymphocytes with surface immunoglobulin but is not found among the leukemic lymphocytes of patients with chronic lymphocytic leukemia. Electron microscope observations indicate close approximation and intimate association of the red cell and lymphocyte membranes after neuraminidase.

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