

SURFACE MARKERS ON HUMAN T AND B LYMPHOCYTES

I. A LARGE POPULATION OF LYMPHOCYTES FORMING NONIMMUNE ROSETTES WITH SHEEP RED BLOOD CELLS

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The rosette technique is a method of visualizing receptors on cell surfaces. The principle is the following: The suspected receptor-bearing cells are mixed with signal cells which carry the corresponding receptor-binding substance on their surface. That substance is either naturally occurring on it or artificially coupled to it. The receptor-bearing cells will then bind the signal cells around their surface and form "rosettes."

It has been shown that there exists a small population of spontaneous rosette-forming lymphocytes (RFL)¹ in the unimmunized mouse corresponding to a certain antigen, and that the size of this population will rise after immunization with that antigen (1). These rosettes are called immune rosettes. The RFL consist of antibody-producing cells and of antigen-sensitive cells of bone marrow origin (B lymphocytes) (1). Whether thymus-derived (T) lymphocytes can form rosettes remains uncertain (2); some data support this view. Spontaneous RFL have immunoglobulin receptors, and their rosette-forming ability can be blocked by pretreating the lymphocytes with anti-Ig sera or anti-lymphocyte sera (ALS). There is a positive correlation between the immunosuppressive effect in man of ALS and its blocking capacity of these RFL (3). Such blocking is thus used for testing ALS before use in clinical practice.

In 1970 some workers (4-7) observed that sheep red blood cells (SRBC) formed spontaneous rosettes with human lymphocytes (E rosettes) in a very high percentage, too high to be explained as being caused by binding to immunocompetent cells reactive against SRBC.

In the present work the E-binding lymphocytes have been investigated for Ig determinants on their surface and for erythrocyte-antibody-complement (EAC)-binding capacity. These complement-covered RBC will bind to the lymphocytes by the receptor for activated C'3 (8). Further characterization of E rosette formation is also presented.

Materials and Methods

Lymphocytes.—Purification was carried out on a Ficoll-Isopaque gradient. 9 g of Ficoll (Pharmacia Fine Chemicals, Inc., Stockholm, Sweden) was dissolved in 100 ml of distilled

¹ *Abbreviations used in this paper:* ALS, anti-lymphocyte sera; B lymphocytes, lymphocytes of bone marrow origin; BSS, balanced salt solution; EAC, erythrocyte-antibody-complement; FITC, fluorescein isothiocyanate; HRBC, human red blood cells; HTL, intracellular thymus-specific antigen; PBS, phosphate buffer solution; RFL, rosette-forming lymphocytes; SRBC, sheep red blood cells; T lymphocytes, thymus-derived lymphocytes.

water. 20 ml of Isopaque (Nyegaard & Co., Oslo, Norway) was mixed with 25 ml of distilled water. 96 parts of Ficoll was mixed with 40 parts of Isopaque. 3 ml of the gradient was overlaid with 6–8 ml of whole blood in plastic tubes and spun at 850 g for 30 min at 4°C. The cells were removed from the interface and the concentration was adjusted to 4×10^6 /ml in balanced salt solution (BSS; Militärapoteket, Stockholm, Sweden).

SRBC.—SRBC were stored at 4°C in Alsever's solution 1:1 and used until hemolysis occurred. Before use the cells were washed twice and adjusted to a 0.5% suspension (approximately 80×10^6 /ml) in BSS.

Test Procedure for E Rosettes.—0.25 ml of the lymphocyte suspension was mixed with 0.25 ml of 0.5% SRBC and incubated at 37°C for 5 min. The mixed cell suspension was spun at 200 g for 5 min and then incubated in ice for 1–2 hr. The supernatant was removed and the top layer of the pellet was gently resuspended by shaking. One drop of the cell suspension was mounted on to a glass slide, covered by a cover slip, and sealed. 200 lymphocytes were counted, and all lymphocytes binding more than three SRBC were considered positive.

Labeling of Ig-Bearing Lymphocytes.— 2×10^6 of lymphocytes were incubated with one drop of a polyvalent rabbit anti-human Ig serum (anti-IgG, IgA, IgM, kappa, and lambda; Cappel Laboratories, Downingtown, Pa.) and then washed twice with BSS. A second incubation was performed with one drop of a sheep anti-rabbit Ig serum (State Bacteriological Laboratories, Stockholm, Sweden) and washed twice with BSS. Both antisera were conjugated with fluorescein isothiocyanate (FITC). The pellet was resuspended in one drop of glycerol:PBS (1:1). 200 cells were counted, and all cells displaying more than two to three sharply stained spots were considered positive. The highest serum concentration failing to give nonspecific staining was used.

Test Procedure for EAC Rosettes.—Human red blood cells (HRBC) from heparinized whole blood were washed twice with BSS. A 5% suspension in BSS was reacted with a rabbit anti-HRBC serum diluted 1:1000 (inactivated whole serum prepared by injecting AB + HRBC in a rabbit). 5 ml of the antiserum was mixed with 5 ml of the cell suspension and incubated at 37°C for 1 hr. The cells were washed twice in BSS. 5 ml of human C' (fresh serum) was added in a concentration of 1:10 to the 5 ml cell suspension and incubated at 37°C for 1 hr. The cells were finally washed twice and adjusted to a 0.5% suspension (approximately 80×10^6 /ml). Further procedure was followed as described for E rosette formation with the exception that the rosettes were resuspended by vigorous mixing with a whirl-mixer. 200 cells were counted under a sealed cover slip.

Test Procedure with Mixed E^{Fitc} and EAC.—SRBC (or E) were conjugated with FITC as follows: 0.5 ml of packed SRBC were washed twice in 0.9% NaCl. The cells were mixed with 3.5 ml of 0.9% NaCl, pH 9.0–9.5. 1.4 mg of FITC in 1.0 ml of 0.9% NaCl with the same pH was then added, and the suspension was kept at room temperature for 1 hr with occasional shaking. The cells were washed three to four times.

E and EAC were then mixed to a 0.5% solution. Incubation for rosette formation was performed as for E rosettes. 200 cells were counted under a sealed cover slip.

RESULTS

Lymphocytes Forming E Rosettes.—The percentage of E rosettes from peripheral blood varied from 52 to 81% in eleven cases (Tables I–III). Almost 100% of the thymocytes formed E rosettes. The percentage of E-binding lymphocytes varied slightly according to their location on the glass slide. The figures in the tables are from representative fields with little cell clumping and healthy appearing cells. The binding between lymphocytes and SRBC is very weak so care must be taken when resuspending the rosettes. No movement of the cells on the glass slide should be allowed. A small contamination of other leukocytes

(less than 5% of granulocytes and monocytes) is demonstrated by May-Grünwald Giemsa-stained smears. By spinning down the rosettes in a cytocen-

TABLE I
Lymphocytes from Peripheral Blood

Donor No.	Percentage of E-binding cells	Percentage of IG-bearing cells	Percentage of both IG-bearing and E-binding cells
	%	%	%
1	60	44	0
2	76	20	0
3	52	43	0
4	73	28	0
5	68	35	0
6	75	20	0

Donors 1-5 represent young healthy donors. Donor 6 is a female patient with sarcoidosis (age 21). In this case lymphocytes from both peripheral blood and thymus were studied. Each test was performed separately.

TABLE II
Lymphocytes from Thymus

Donor No.	Percentage of E-binding cells	Percentage of IG-bearing cells
	%	%
6	98	0
7	99	N.D.

Case 6 is the same as No. 6 in Table I. Case 7 is a male patient with sarcoidosis (age 21). Each test was performed separately.

TABLE III
Lymphocytes from Peripheral Blood

Donor No.	Percentage of EFite-binding cells	Percentage of EAC-binding cells	Percentage of IG-bearing cells	Percentage of both IG-bearing and EFite-binding cells	Percentage of both EFite- and EAC-binding cells
	%	%	%	%	%
8	60	27	25	0	0
9	65	30	34	0	0
10	68	33	31	0	0
11	81	25	22	0	0
12	71	27	26	0	0

Donors 8-12 represent young healthy donors. Each test was performed separately.

trifuge (I.V.A., Stockholm, Sweden), it is also shown that rosette formation only occurs around cells with lymphocyte morphology. After 1-2 hr incubation in an ice bath, rosettes were observed with more than one layer of SRBC surrounding the lymphocytes.

Lymphocytes Forming EAC Rosettes.—In five cases studied between 25 and 33% of lymphocytes were found to form EAC rosettes (Table III). In these rosettes the HRBC are very tightly bound to the lymphocytes and the rosettes were shaken vigorously before investigation. These rosettes can also be produced and kept at 37°C, in contrast to the E rosettes.

Lymphocytes Forming E Rosettes and Lymphocytes Forming EAC Rosettes.—No mixed rosettes were observed where the lymphocytes would bind both SRBC and HRBC (Table III). This was possible to determine as the HRBC and the SRBC differ in size. Occasionally the rosettes were in clumps and it was difficult to exclude mixing of the signal cells bound to the surface. This difficulty was overcome by labeling the SRBC with FITC which allowed inspection of the

TABLE IV
Lymphocytes from Peripheral Blood (CLL)

Donor No.	Percentage of E-binding cells	Percentage of Ig-bearing cells
	%	%
1	4	92
2	6	87
3	3	84

Case 1 is a male age 67. Case 2 is a female age 57. Case 3 is a male age 63. All three patients have chronic lymphatic leukemia (CLL). Each test was performed separately.

rosettes in UV light. The EAC rosettes were observed as black spaces between the evenly spread SRBC^{Fite} and SRBC^{Fite} rosettes.

Lymphocytes Bearing Ig Determinants.—20–44% of peripheral lymphocytes were stained but no thymocytes (Tables I–III). All cells that displayed more than two to three brightly stained spots were considered positive.

Lymphocytes Forming E Rosettes and Lymphocytes Bearing Ig Determinants.—No E rosette-forming lymphocytes were found that were also carrying Ig determinants on their surface (Tables I and III). Most of the lymphocyte surface was available for inspection as the SRBC were mostly bound in a ring around the surface and did not cover it completely. Some nonrosette-forming leukocytes that were also negative for anti-Ig labeling were observed. This might be due to the small contamination of granulocytes and monocytes. No inhibition of E rosette formation occurred after anti-Ig treatment of the lymphocytes.

Clinical Tests.—Lymphocytes from three patients with chronic lymphatic leukemia were examined. These lymphocytes were uniformly low in E binding and correspondingly high when tested with anti-Ig sera (Table IV). 11 Burkitt lymphoma lines grown in vitro and three biopsies were examined and showed no binding of E or EAC. Some lines were positive for anti-Ig labeling.

Further Characterization of E Rosette Formation.—Lymphocytes pretreated with 3.2 mM sodium iodoacetate for 30 min at 37°C failed to form rosettes. The lymphocytes were alive according to the trypan blue exclusion test.

Rosette formation was reduced in Ca^{++} - and Mg^{++} -free buffer and was completely impaired in the presence of ethylenediaminetetraacetate (EDTA). Rosette formation was optimal at pH 7.0–8.0 and dropped considerably at higher or lower pH.

Pretreatment of lymphocytes with 0.25% trypsin for 30 min at room temperature abolished their rosette-forming capacity while maintaining good viability (Tables V and VI). These E-binding tests were performed under slightly dif-

TABLE V
Regeneration of Rosette Formation after Trypsin Treatment

Incubation	Percentage of cells forming E rosettes	
	Trypsin treated	Untreated
	%	%
None	1	40
At 4°C	1	29
At 37°C	27	20

Trypsin treatment of lymphocytes.

TABLE VI
Treatment of Trypsinized Lymphocytes with Serum

Treatment	Percentage of cells forming E rosettes	
	Trypsin treated	Untreated
	%	%
Human serum (fresh)	2	47
Human serum (heat inactivated)	1	53
Fetal calf serum (heat inactivated)	1	34
None	1	36

Treatment of trypsinized lymphocytes with human serum or fetal calf serum.

ferent conditions from those described above which explains the low percentages. Trypsinization of SRBC had no effect. When incubated at 37°C in complete medium with fetal calf or human AB serum for 18 hr, rosette-forming capacity was restored (Table V). During incubation at 4°C lymphocytes did not recover rosette formation. Regeneration of rosette formation was not prevented in the presence of cyclohexamide in a concentration which inhibited 90% of protein synthesis (25 $\mu\text{g}/\text{ml}$).

Rosette formation could not be conferred to trypsinized lymphocytes by treating them for 1 hr in 50% fresh human serum or fetal calf serum (Table VI). The same result was obtained when lymphocytes were treated with medium from human lymphocytes cultivated for 4 days at 37°C.

DISCUSSION

Coombs et al. (4) found that SRBC formed rosettes with human lymphocytes in a very high percentage, too high to be explained by binding to immunocompetent cells reactive against SRBC. Only living cells could form this nonimmune type of rosette. No inhibition occurred when the lymphocytes were pretreated with anti-Lg sera.

Brain et al. (5, 6) showed that such rosette formation is uncorrelated with serum anti-SRBC agglutinin levels. Lymphocytes from umbilical cord blood could also form rosettes, as could lymphocytes from baboons and vervet monkeys. Red blood cells from some other species studied (rat, rhinoceros, and warthog) would not bind to human lymphocytes in the same high percentage. When correlating the inhibition, cytotoxic, and leucoagglutinating titers of ALS (raised in horses) obtained at different times after immunization with the survival time of skin allografts on vervet monkeys, it was found that the peak of the inhibition titer appeared to provide a good index of the immunosuppressive activity. These peaks were of short duration in contrast to the cytotoxic and agglutinating titers.

Bianco et al. (7) found that maximal adherence occurred when first contact between the cells was established at 37°C and was followed by incubation at low temperature. With further incubation at 37°C the rosettes disintegrated. In five cases where they studied lymphocytes and thymocytes from the same donors, a significantly higher percentage of E rosettes was found among the thymocytes. RBC from the horse, ox, rabbit, guinea pig, and mouse could not form rosettes.

Recently Yata et al.² studied the presence of an intracellular thymus-specific antigen (HTL) in E- and EAC-binding human lymphocytes. They found that E adhered to the majority of thymocytes and to 10–30% of lymphocytes from peripheral tissues. Almost all E-binding cells were HTL positive. In contrast, cells binding EAC were HTL negative. From these data they conclude that E-binding cells are of thymus origin. Unfortunately SRBC were used for producing EAC which leaves some doubt about the receptor involved. In the thymus for instance they demonstrated approximately 40% of EAC-binding cells.

We have found approximately 52–81% of E-binding lymphocytes in peripheral blood with our test conditions (Tables I–III). This is a higher percentage than has been found by the previous investigators (4–7). Using a double immunofluorescent labeling technique for detecting lymphocytes carrying a high density of immunoglobulin determinants on their surface, it was shown that these latter cells could not form E rosettes. By extension from studies in

² Yata, J., I. Takeda, and T. Tachibana. 1971. Human lymphocyte subpopulations; human thymus-lymphoid tissue (HTL) antigen positive lymphocytes forming rosettes with sheep erythrocytes and HTL antigen negative lymphocytes interacting with antigen-antibody-complement complexes. Submitted to *Clin. Exp. Immunol.*

the mice they are considered B cells (9).³ It was also shown that EAC-binding lymphocytes, which are also considered B cells (10), did not bind SRBC. This was done by a mixed rosette procedure where C' was fixed to HRBC. When lymphocytes were mixed with SRBC and HRBC no mixed rosettes occurred. Since the percentage of cells positive for the B markers and the percentage of cells binding SRBC added together equal approximately 100%, it is suggested that all or at least a major population of all human thymus-derived lymphocytes will bind to SRBC under these conditions. It is also noted that the percentage Ig-carrying cells roughly corresponds with the percentage of EAC-binding cells. This would imply that man in this respect resembles mice and carried the C'3 receptor on all bone marrow-derived lymphocytes. When testing three cases of chronic lymphatic leukemias, their lymphocytes were found to bind E very poorly and to be correspondingly highly positive for anti-Ig. These cases might therefore be called B lymphocyte diseases. This is in line with the finding of Wilson et al. (9). Also three Burkitt biopsies and eleven Burkitt lines grown in vitro were tested. They were uniformly negative for E and EAC binding. Some were positive for anti-Ig.

The E rosettes are not formed by virtue of Ig receptors, and they show much weaker binding characteristics than the immune rosettes. Only living cells are rosette forming and sodium iodoacetate (an inhibitor of glycolysis) blocks the reaction. At 37°C the E rosettes will disintegrate. Rosette formation requires bivalent positive ions and can be abolished by EDTA. Pretreatment of the lymphocytes with trypsin removed their E-binding capacity. Trypsinized lymphocytes put into culture will regenerate the rosette-forming ability which cannot be restored by merely treating the lymphocytes with serum. Possibly the rosettes are formed by virtue of a rapidly released or metabolized substance on the cell surface which behaves as a trypsin-sensitive structure.

As a marker the rosette formation has some disadvantages. The binding is so weak that the technical conditions have to be optimal. Even then, it may be impossible to exclude that some rosettes disintegrate on their way to the microscope.

Possible implications of this human T lymphocyte marker are several: (a) Reclassification of human lymphoid diseases according to the T and B cell concept. This might be of value for better understanding of etiologic and pathogenetic mechanisms. (b) It may also be valuable knowledge from a therapeutic point of view as an additional means of characterizing the immunological status in man during other nonimmunological diseases and after different kinds of treatment (X-ray, cortisone, etc.). (c) Blocking of rosette formation might provide a more sensitive test for ALS than the presently used blocking test with immune rosettes since the latter are only present at the 1% level. Perhaps block-

³ Froland, S. S., and J. B. Natvig. 1971. Surface-bound immunoglobulins on lymphocytes from normal and immunodeficient humans. Submitted to *Scand. J. Immunol.*

ing of E rosette formation also might be more specific since it has not been proven that immune rosettes comprise T cells. These speculations are based on the assumptions that the T cells are the most important cells during these conditions. (d) As a possible way to enrich human B cells, T cells have hitherto not been shown to bind to antigen-coated columns. Perhaps the affinity between T lymphocytes and SRBC under certain conditions can be used for nonspecific retention of T cells. The T cells may stick to SRBC fixed on to some column material and could then be eluted by incubating the column at 37°C.

SUMMARY

By using the two criteria (a) high density of immunoglobulin determinants on the cell surface and (b) presence of receptors for C'3 on the cell surface for defining bone marrow-derived lymphocytes, it is indirectly shown that all or at least a major population of human thymus-derived lymphocytes under certain conditions will form nonimmune rosettes with sheep red blood cells (SRBC). Almost all thymocytes tested from two different donors formed rosettes. The SRBC rosettes are not formed by virtue of immunoglobulin receptors and form only around living cells. Positive bivalent ions are required for rosette formation since EDTA will block rosette formation. Sodium iodoacetate will also block rosette formation demonstrating the dependence on an intact glycolytic pathway. Rosette formation is temperature dependent and will not appear at 37°C. Trypsin treatment of lymphocytes will abolish their SRBC-binding ability which cannot be restored by treating them with fresh donor serum or fetal calf serum, but which will reappear after culturing the lymphocytes. It is suggested that these rosettes are formed by a rapidly released or metabolized receptor substance on the living cell surface which behaves as a trypsin-sensitive structure produced by the cells themselves.

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REFERENCES

1. Bach, J. F., J. Y. Mullner, and M. Dardenne. 1970. In vivo specific antigen recognition by rosette forming cells. *Nature (Lond.)*. **227**:1252.
2. Bach, J. F., F. Reyes, M. Dardenne, C. Fournier, and J. Y. Mullner. 1971. Rosette formation, a model for antigen recognition. Cell interactions and receptor antibodies in immune responses. *In* Proceedings of the Third Sigrid Juselius Symposium. Academic Press Inc., New York. 111.
3. Bach, J. F., M. Dardenne, and C. Fournier. 1969. In vitro evaluation of immunosuppressive drugs. *Nature (Lond.)*. **222**:998.
4. Coombs, R. R. A., B. W. Gurner, Anne B. Wilson, G. Holm, and B. Lindgren. 1970. Rosette formation between human lymphocytes and sheep red blood cells

- not involving immunoglobulin receptors. *Int. Arch. Allergy Appl. Immunol.* **39**:658.
5. Brain, P., J. Gordon, and W. A. Willetts. 1970. Rosette formation by peripheral lymphocytes. *Clin. Exp. Immunol.* **6**:681.
 6. Brain, P., and J. Gordon. 1971. Rosette formation by peripheral lymphocytes. II. Inhibition of the phenomenon. *Clin. Exp. Immunol.* **8**:1.
 7. Bianco, C., V. Nussenzweig, W. H. Lay, and N. F. Mendes. 1971. Binding of sheep red blood cells to a large population of human lymphocytes. *Nature (Lond.)*. **230**:531.
 8. Michlmayr, G., and H. Huber. 1970. Receptor sites for complement on certain human peripheral blood lymphocytes. *J. Immunol.* **105**:670.
 9. Wilson, J. D., and G. J. V. Nossal. 1971. Identification of human T and B lymphocytes in normal peripheral blood and in chronic lymphocytic leukemia. *Lancet*. **2**:788.
 10. Nussenzweig, V., C. Bianco, and P. Dukor. 1971. Membrane receptors for antigen-antibody-complement complexes on lymphocytes. Cell interactions and receptor antibodies in immune responses. *In* Proceedings of the Third Sigrid Juselius Symposium. Academic Press Inc., New York. 75.