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Mouse red cell rosette formation by subpopulations of human lymphocytes

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SUMMARY

In six separate experiments, 7·2% of purified peripheral blood lymphocytes from normal donors were shown to form rosettes with mouse erythrocytes. Normal T and B lymphocytes were separated according to their membrane properties, by E-rosette formation or fractionation on anti $F(ab')_2$ column. The results obtained in both the separation procedures used were in good agreement: T lymphocytes were never found to form mouse red cell rosettes. On the contrary, enrichment of suspensions in B lymphocytes resulted in an increased percentage of mouse red-cell rosette-forming cells. Under the technical condition used, peroxidase-positive monocytes were not shown to form such rosettes. However, if all the mouse red cell rosette-forming cells were shown to be of B nature, not all the B lymphocytes form rosettes with mouse erythrocytes and it is not unlikely that this marker could be characteristic for a subset of Ig-bearing B lymphocytes.

INTRODUCTION

Human lymphocytes can be classified according to their surface markers. Thymus derived (T) lymphocytes may be identified by spontaneous rosette formation with sheep red cells (Jondal, Holm & Wigzell, 1972); on the other hand, the presence of surface immunoglobulins and of receptors for the third component of complement and the Fc portion of immunoglobulin characterizes bone marrow-derived B lymphocytes (Schevach, Jaffe & Green, 1973; Brown & Greaves, 1974). Recently it has been demonstrated that chronic lymphatic leukaemia (CLL) lymphocytes, as well as a subpopulation of normal peripheral blood lymphocytes, may form rosettes with mouse red cells (MRC) (Stathopoulos & Elliot, 1974; Gupta & Grieco, 1975). The well-established B nature of the leukaemic cells in the majority of cases of chronic lymphatic leukaemia and the presence of surface immunoglobulins on cells forming rosettes with mouse erythrocytes (Dobozy *et al.*, 1976) strongly suggest that rosette formation with mouse red cells is a B-cell marker.

However, what proportion of B cells can form rosettes with mouse erythrocytes, and the relationships between mouse red cell rosetting and other markers have not yet been defined. In the studies reported here, the distinctive ability of human T lymphocytes to form rosettes with sheep red cells (Dean *et al.*, 1975) and of B lymphocytes to bind to an anti $F(ab')_2$ column (Chess *et al.*, 1975) were used as separation procedures and the distribution of lymphocytes forming rosettes with mouse red cells was investigated in the separated subpopulations as well as in non-T lymphocytes subsets obtained by bovine serum albumin gradient fractionation. Each lymphocytes subpopulation was tested for other membrane markers (surface immunoglobulins, C3 receptor, Fc receptor, E and active E-rosette formation with sheep red cells (SRC)). The results presented below clearly indicate that the mouse red cell rosette is a B-cell marker and suggest that it may be restricted to a subset of human B lymphocytes.

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MATERIALS AND METHODS

Lymphocyte suspensions. Heparinized blood (calciparine R, Choay) from normal donors was drawn by vein puncture, or enriched leucocyte suspensions were obtained by leucopheresis using a cell separator (Haemonetics Model 30). Blood or leucocyte suspensions were diluted in Hanks's balanced salt solution (HBSS) and incubated for 30 min at 37°C with 2 mg/ml of carbonyl iron powder (Koch–Light Laboratories) with constant stirring. The suspension was then layered on Ficoll–Hypaque and centrifuged at 400 *g* for 30 min at 18°C; this treatment resulted in removing polymorphonuclear cells and phagocytes having ingested iron particles.

Membrane markers. (1) *E rosettes and active E rosettes (Ea).* E and Ea rosettes were determined according to Wybran & Fudenberg (1973). Briefly, in the E-rosette test, 5×10^5 lymphocytes in 30 μl of HBSS were centrifugated at 400 *g* with 30 μl of SRC-absorbed foetal calf serum (FCS) and 60 μl of washed SRC adjusted to 350×10^6/ml, then incubated overnight at room temperature. After gentle resuspension by hand, the percentage of rosette-forming cells (RFC) was counted in an haemocytometer. Cells binding three or more erythrocytes were considered as rosetting.

For active E rosettes tests, 5×10^5 lymphocytes in 30 μl of HBSS were incubated for 1 hr in 30 μl of SRC-absorbed FCS at 37°C. Then 90 μl of washed SRC adjusted to 40×10^6/ml were added. After 5 min centrifugation at 200 *g*, pellets were gently resuspended and RFC counted as for E rosettes (Bertoglio & Doré, 1976).

(2) *Detection of C3 receptor, using EAC-rosette formation.* (a) *EAC-complex formation.* A 0·5% SRC suspension was incubated 30 min at 37°C in an equal volume of a subagglutinating dilution of rabbit anti-SRC IgM purified by chromatography on a Sephadex G-200 column. After three washings, SRC-IgM complexes were adjusted to 5×10^8/ml and further incubated for 30 min at 37°C with fresh C57B16 serum diluted 1:5 in veronal buffer (0·005 M, pH 7·3). After three more washings, EAC complexes were adjusted to 10×10^8/ml, stored at +4°C and used within the week.

(b) *EAC-rosette formation.* 10^6 lymphocytes in 50 μl of HBSS were incubated with 50 μl of EAC complexes for 30 min at 37°C. After dilution, the appropriate percentage of RFC was counted as for E-rosettes. Each test was done in triplicate and controls were performed using unsensitized SRC and SRC-IgM complexes.

(3) *Detection of Fc receptor, using EA-rosette formation.* (a) *EA complex formation.* A 5% suspension of washed White Leghorn chicken red cells (CRC) was incubated for 30 min at 37°C in an equal volume of a subagglutinating dilution of rabbit anti CRC antiserum. After three washings in HBSS, the cells were adjusted to a 0·5% suspension.

(b) *EA-rosette formation.* 0·1 ml of lymphocyte suspension adjusted to 2×10^6 cells/ml were added to 0·1 ml of EA complexes and the mixture was centrifuged at 200 *g* for 5 min at +4°C. Rosettes were immediately resuspended and counted as for other rosette tests. Each test included controls using unsensitized CRC.

(4) *Immunofluorescence detection of surface immunoglobulins.* 10^6 cells were pelleted in plastic test tubes and resuspended in 50 μl of an appropriate dilution of FITC-labelled globulin fraction from a polyvalent goat anti-human immunoglobulin serum (Meloy Laboratories Incorporated). After 30 min incubation on ice, three washings were done in a large excess of cold HBSS. The cells were then layered on microscope slides, air dried and processed for detection of monocytes by staining for endogenous peroxidase as described by Preud'Homme & Flandrin (1974).

(5) *Formation of mouse red-cell rosettes.* The technique of Stathopoulos for the demonstration of mouse red cell rosettes (MRC) was applied (Stathopoulos & Elliot 1974). Briefly, to 250 μl of a lymphocyte suspension (4×10^6/ml in HBSS) 50 μl of MRC-absorbed FCS, and 250 μl of a 1% suspension in HBSS of freshly drawn C57B16 mouse erythrocytes were added. The mixture was incubated for 10 min at 37°C, centrifuged 5 min at 400 *g*, and further incubated for 1 hr at +4°C. The percentage of cells forming rosettes with mouse erythrocytes (M-RFC) was counted after gentle resuspension.

Separation procedures. (1) *Using E-rosette formation.* Lymphocytes were allowed to form E-rosettes under similar conditions as in the tests but ten times the reactive volumes were used. After 6–8 hr incubation rosettes were gently resuspended by hand, and the suspension thus obtained was layered on Ficoll–Hypaque and centrifuged at 400 *g* for 30 min at 18°C. This produced at the interface a ring of pure mononuclear cells not having formed rosettes. Cells from the pellet and the ring were separately harvested and treated with 0·85% NH_4Cl for 10 min to lyse sheep erythrocytes, then washed three times and tested.

(2) *Using an anti F(ab')$_2$ column.* T and B lymphocytes were separated by means of an immunoabsorbent column according to the technique of Schlossman & Hudson (1973) as slightly modified by Thierry *et al.* (1976).

(a) *Column preparation.* Anti-F(ab')$_2$ was raised in rabbits by immunization with F(ab')$_2$ fragments obtained by pepsin digestion of human gammaglobulin Cohn fraction II (Pentex, Miles Laboratories) and emulsified in Freund's complete adjuvant. The IgG fraction of antiserum was further purified by ammonium sulphate precipitation and affinity chromatography on Sepharose 4B coupled to human gammaglobulin. After proper dialysis and concentration, antiserum prepared in this way was kept at −20°C until use. Antiserum was linked to Sephadex G-200, previously activated with cyanogen bromide. A 10-ml syringe was filled with coupled Sephadex and further equilibrated at 37°C in medium (RPMI 1640+1% HEPES+2% of 0·02% EDTA solution +15% FCS).

(b) *Preparative separation of T and B lymphocytes.* 2×10^8 lymphocytes were layered on the top of the column. The flow rate was adjusted to 1 ml/min for the first 15 ml of medium and afterwards increased. Complete elution of T lymphocytes was obtained when 50 ml medium had passed through the column. Elution of B lymphocytes was obtained by competition between membrane immunoglobulin and 30 ml of human gammaglobulins added to the column at a concentration of 10 mg/ml.

(2) *Using bovine serum albumin (BSA) gradient.* Non-T lymphocytes obtained by E-rosette depletion were further

sub-fractionated on discontinuous gradient of bovine serum albumine (Leif, 1964). The gradient was built up by layering one upon the other 1 ml vol. of BSA dilutions of densities 1·095, 1·072, 1·064 and 1·058 g/cm^3, respectivly. Lymphocyte suspensions were added at the top of the gradient and centrifuged at 20,000 *g* for 30 min at +4°C. Lymphocytes sedimented according to their buoyant density, and fractions were obtained at interfaces.

RESULTS

Distribution of MRC rosettes in E-RFC- enriched and depleted subpopulations

Centrifugation of E rosettes on Ficoll–Hypaque is an efficient separation method as can be seen from the recovery data shown in Table 1, and yields subpopulations of good purity as assessed by membrane characteristics. This technique, as expected, concentrates monocytes in the E-RFC depleted fraction (Table 2). B-cell markers are highly depleted in E-RFC-enriched fractions while they are enriched in E-RF-C depleted fractions. MRC-rosette forming cells are also depleted in T-cell enriched subpopulations and concentrated in the T cells depleted ones (Table 3), which indicates that E-rosette forming lymphocytes do not form MRC rosettes.

Distribution of MRC rosettes in lymphocyte subpopulations separated by anti F(ab')$_2$ column

Separation of lymphocytes on anti-F(ab')$_2$-coupled Sephadex column is also an efficient procedure (Table, 4). As shown in Table 2, monocytes are not recovered in the retrained or unretrained subpopulations, probably because of their adherent properties. A good degree of enrichment is obtained by this method as shown in Table 5. These experiments show that none of the Ig-negative (effluent) populations bind MRC and that only 25% of the eluted, Ig positive lymphocytes form MRC rosettes. Surprisingly, in the three experiments Ig positive cells failed to bind EA complexes. It is not unlikely that the Fc receptors were blocked by the Ig used for elution of the cells. The Ig solution was not ultracentrifuged and may have contained Ig aggregates, although EA receptors have been reported to be inhibited by 7S IgG (Fröland *et al.*, 1974). These results also ruled out the possibility that so called null lymphocytes form MRC rosettes since they might be expected to be recovered in the effluent population in which only 0·2% of cells formed MRC rosettes.

TABLE 1. Per cent of total input peripheral blood lymphocytes recovered by the rosette separation method

Total recovered	In E-RFC-enriched fraction	In E-RFC-depleted fraction
78·1 (71·3–83)	52·3 (36·5–60)	25·8 (18·8–34·8)

Mean per cent lymphocytes recovered represents the total number of cells recovered in each fraction/total number of cells input.

Figures in parentheses are per cent recovery ranges.

TABLE 2. Per cent peroxidase positive monocytes in separated subpopulations

In unseparated populations	In E-RFC-enriched fractions	In E-RFC-depleted fractions	In populations unretained by anti-F(ab')$_2$ column	In population eluted from anti-F(ab')$_2$ column
7·6	0·6	25·3	0·6	1·9

TABLE 3. Characteristics of E-RFC-enriched and depleted human peripheral blood lymphocytes subpopulations

Peripheral blood lymphocyte populations tested	E-RFC (%)	EA-RFC (%)	EA-RFC (%)	EA-CRFC (%)	M-RFC (%)	S-Ig (%)
Unseparated*	62·2 (53·5–81)	21·2 (18·5–27·5)	11·3 (9–16·5)	19·1 (13·8–26·5)	7·2 (2·3–14·5)	9·6 (3–13·8)
E-RFC-enriched†	81·5 (71·7–91·3)	37·2 (37–37·5)	1·6 (0·3–2·6)	8·3 (4·5–14)	0·1 (0–0·3)	0 —
E-RFC-depleted†	0·9 (0–1·5)	1·1 (0–2·3)	22 (8·5–30·5)	39·9 (32–45·3)	14 (6·5–21·6)	74·8 (69–83)

Results are expressed as percentage of the total population, eventually including contaminant monocytes. Ranges are shown in parentheses.

* Means of 6 separate experiments.
† Means of 3 separate experiments.

TABLE 4. Per cent of total input peripheral blood lymphocytes recovered after separation on anti-F(ab′)$_2$ column

Total recovered	Effluent populations (unretained)	Retained populations (eluted by immunoglobulins)
86·3 (83–92)	71·3 (66·6–76·5)	15·1 (13·4–16·5)

Same legend as Table 1.

Distribution of MRC rosettes in non-T lymphocyte subpopulations separated on BSA gradient

Table 6 shows that buoyant density heterogeneity of non-T cells does not correlate with membrane markers and did not yield sub-populations of cells with distinctive properties. MRC rosette-forming lymphocytes were equally distributed in all sub-fractions with respect to lymphocyte content, and it is thus unlikely that monocytes, which concentrate in low density fractions, form MRC rosettes.

TABLE 5. Characteristics of anti-F(ab′)$_2$ column retained and unretained human peripheral blood sub-populations

Peripheral blood lymphocyte populations tested	E-RFC (%)	EA-RFC (%)	EA-RFC (%)	EA-CRFC (%)	M-RFC (%)	S-Ig (%)
Unseparated*	62·2 (53·5–81)	21·2 (18·5–27·5)	11·3 (9–16·5)	19·1 (13·8–26·5)	7·2 (2·3–14·5)	9·6 (3–13·8)
Effluent population†	77·7 (59–96·5)	25·4 (22·3–28·6)	5·2 (2·6–6·5)	3·8 (2–5·6)	0·2 (0–0·5)	0 —
Eluted population†	0·7 (0–3·1)	0·4 (0·3–0·5)	0 —	39·7 (28·7–52·5)	25·7 (12–49)	70·4 (58·8–82)

Same legends as Table 3.

TABLE 6. Characteristics of subpopulations obtained by BSA gradient fractionation of E-RFC-depleted populations

Fractions obtained at buoyant density of:	Monocytes (%)	Lymphocytes (%)	E-RFC (%)	EA-RFC (%)	EAC-RFC (%)	M-RFC (%)
1·058	73·1	26·9	0	7·2	12	0·5
1·064	41	59	0	15·5	26·5	2·7
1·072	23·9	75·1	3·7	29·4	39·5	4·8
1·095	25·7	74·3	3	18·8	36·7	4·8

Results are expressed as percentage of the total cell population—mean from three separate experiments.

DISCUSSION

Stathopoulos & Elliot (1974) have described formation of rosettes with mouse red cells by chronic lymphatic leukaemia lymphocytes and by a subpopulation of normal peripheral blood lymphocytes. The well-established B nature of CLL lymphocytes and of some other pathological cells such as cells from leukaemic reticuloendotheliosis (Catowsky *et al.*, 1974) strongly suggested that this membrane property could be a B-cell marker. Furthermore, Dobozy *et al.* (1976) demonstrated that MRC-rosette forming lymphocytes bear surface immunoglobulins. The percentage of MRC rosette-forming cells usually parallels that of Ig-bearing cells in unseparated lymphocyte suspensions, and the results presented here are in good agreement with those previously reported.

The two different separation procedures used in these studies gave enrichment of T and B populations by both positive and negative surface characteristics as reported by other investigators (Dean *et al.*, 1975; Chess *et al.*, 1975). E-RFC-positive and immunoglobulin-negative lymphocyte populations were considered as enriched in T cells. B cell-enriched populations were made either from immunoglobulin positive or from E-RFC negative lymphocytes and in the latter case were contaminated with about 25% monocytes. Results obtained with cells obtained in these two different ways were in good agreement: MRC-rosette formation by cells from T-enriched populations never exceeded 0·5% in any experiment and averaged 0·2%. In contrast, filtration of lymphocytes on an anti-$F(ab')_2$ column yielded subpopulations of immunoglobulin positive cells, in which most if not all the MRC rosette-forming cells were found.

From the results it is evident that if all MRC rosette-forming cells are immunoglobulin positive, as shown by Dobozy *et al.* (1976), only part of immunoglobulin-positive cells form MRC rosettes. This is further supported by studies of malignant haemopathies which are thought to arise from a single cell. Among haemopathies of B type, as assessed by other membrane markers, only CLL cells bind MRC (Stathopoulos & Davies, 1976). Thus CLL cells may originate from aB subpopulation and the study of MRC rosette formation by cells from haemopathies of B origin might be of clinical interest and help in the diagnosis of some CLL. MRC rosette formation by human B lymphocytes appears to be different from the rosette formation with monkey red-cells described by Pellegrino, Ferrone & Theofilopoulos (1975) since $20 \pm 6{\cdot}4\%$ of unseparated human lymphocytes and $96 \pm 2\%$ of purified B cells form rosettes with monkey erythrocytes, suggesting that all B lymphocytes bear this marker.

It is well established that monocytes may express C3 and Fc receptors and are thus an important source of potential error in enumerating B lymphocytes. It is of interest to note that, under the technical conditions we used, formation of mouse red-cell rosettes appears to be a marker of B lymphocytes not shared by monocytes. When non-T cells are centrifuged on a BSA gradient, the monocytes are mainly harvested in the low density fractions: in these fractions a small percentage of cells form MRC rosettes. Observations on acute monocytic leukaemia cells, from our laboratory (unpublished) and from Gupta & Grieco (1975) are in good agreement with these findings. However, it cannot be excluded that some phagocytic monocytes form MRC rosettes since such cells would have been removed by our method of preparing the cell suspensions. Furthermore, incubation with carbonyl iron may have led to a selective loss of some B lymphocytes (Zucker-Franklin, 1974) and to an artificial decrease in the percentage of mouse red-cell rosettes.

The relationships between MRC-rosette formation and the presence of B markers other than surface immunoglobulins is not absolutely clear from these experiments. However, it can be hypothesized that if MRC positive lymphocytes possess Fc receptors, these are not in direct relation since total inhibition of EA-rosette formation in the anti-$F(ab')_2$ column experiments had no effect on MRC-rosette formation. Furthermore, preliminary studies on chronic lymphatic leukaemia cells seem to indicate that MRC rosettes may be expressed independently of C3 and Fc receptors on these cells. These results do not allow any conclusion regarding the membrane characteristics of the lymphocytes which form mouse red-cell rosettes. Further studies with double-labelling techniques are now in progress to answer this question.

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