Clin. exp. Immunol. (1978) 32, 504–509.

Rosette formation with goat erythrocytes

A MARKER FOR HUMAN T LYMPHOCYTES

F. PANDOLFI,* J. T. KURNICK, K. NILSSON, K. FORSBECK & H. WIGZELL *Department of Immunology and Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden*

(*Received* 6 *February* 1978)

SUMMARY

We demonstrate the use of goat erythrocytes in a rosette procedure for the classification of human lymphocytes. The population is almost perfectly overlapping with the lymphocytes which form rosettes with sheep red blood cells. 70·2 ± 7·5% of peripheral lymphocytes form rosettes with goat erythrocytes and less than 1% of these cells have surface immunoglobulins. Enrichment of goat rosette-forming cells results in a population with an increased percentage of both goat and sheep rosettes. This population retains activity to the T-cell mitogens Con A and PHA, while the cells depleted of goat rosettes have greatly diminished responses to these same mitogens. Tonsil and spleen lymphocytes form 50·2 ± 6·8% and 24% of goat rosettes respectively, while peripheral blood lymphocytes from patients with CLL rarely form goat rosettes. Cell lines maintained *in vitro* rosetted with goat cells in a parallel fashion to sheep cells. Thus T-cell lines, such as Molt-3, which form rosettes with SRBC also rosette with GRBC, while sheep rosette-negative lines, i.e. Molt-4, are negative for both erythrocytes. B-lymphoid cell lines were negative, as were several lymphoma cell lines. There was a slight variation in the binding of goat cells, depending on the source of the goat. Thus, as in sheep rosettes, some animals were better sources than others, although all the animals tested formed rosettes.

Human lymphocytes are capable of binding goat red cells. The cells which bind to the erythrocytes seem identical to those binding sheep red blood cells, and should be considered as a T-cell population. Preliminary inhibition data suggests that the receptor on T cells is the very same structure for both erythrocytes.

INTRODUCTION

The study and classification of human lymphocytes has long been frustrated by the paucity of markers for different functional subclasses. Although in mice, among the experimental animals, we have surface markers, such as Thy-1 (Raff, 1971), and more importantly, subclass sera such as the Ly series (Cantor & Boyse, 1975), similar discriminatory markers have not been generally available in man.

For B cells we have the classical markers, such as surface Ig (Pernis, Forni & Amante, 1970) and the receptor for EBV (Jondal & Klein, 1973), and mouse red blood cells have been recently utilized for the identification of a subclass of human B lymphocytes (Stathopoulos & Elliot, 1974). The introduction of the sheep red blood cell as a marker for the human T cell (Jondal, Holm & Wigzell, 1972) remains the most useful tool for the identification of this population of cells. Of course, the last few years have

* Present address: III Clinica Medica, Università di Roma, 00161 Roma, Italy.

Correspondence: Dr J. Kurnick, Department of Immunology, Uppsala University, Biomedical Center, Box 582, 75123 Uppsala, Sweden.

0099-9104/78/0600-0504$02.00

given us more sophisticated T-cell markers as well, including anti-T cell sera (Aiuti & Wigzell, 1973; Brouet & Toben, 1976). The possibility of a functional subclassification of human T cells has recently been suggested, using the ability of these cells to bind either IgM or IgG in a rosette assay (Moretta *et al.*, 1975). With this background, it is surprising that the animal kingdom has not been more widely investigated as a possible and helpful source of markers.

Monkey and swine erythrocytes have been utilized (Pellegrino, Ferrone & Theophilopoulos, 1975, Lay *et al.*, 1971), but there are no reports on the use of goat red blood cells (GRBC), except for a report by Minowada, Ohnuma & Moore (1972) showing the ability of a T-cell line to bind erythrocytes of different species, including goat. As the goat is so frequently used in immunology, as a source of antisera, we felt it might be fruitful to study goat red cells for binding to human lymphocytes. If possible, this may serve as a substitute for other markers and may allow the simultaneous use of goat antisera, without the necessity of undertaking troublesome absorptions of natural antibodies.

MATERIALS AND METHODS

Sheep rosette assay. Lymphocytes isolated from peripheral blood (PBL) by centrifugation on a Ficoll–Isopaque gradient were washed twice in phosphate-buffered saline (PBS). The sheep rosettes were prepared as follows: 50 μl of lymphocytes (5×10^6 cells per ml in PBS) were added to 50 μl of sheep red blood cells (SRBC), 1% in PBS and incubated for 10 min at 37°C. After centrifugation at 200 ***g*** for 5 min, the samples were incubated for 1 hr at 4°C, and the pellet was then gently resuspended. Lymphocytes binding three or more SRBC were considered to be rosette-forming cells (SRFC).

Goat rosette assay. GRBC were collected from three different goats. The blood was stored in Alsever's solution for less than 10 days. The GRBC used for the test were washed three times in PBS and resuspended at 1% concentration before use. 50 μl of lymphocytes (5×10^6 cells per ml in PBS) were added to 50 μl of GRBC. 25 μl of FCS adsorbed with GRBC were also added. The tubes were centrifuged for 5 min at 200 ***g*** and subsequently incubated at 4°C for 1 hr. After incubation, the pellet was gently resuspended and 25 μl of trypan blue (0·35% in PBS) were added. Live lymphocytes binding more than three GRBC were considered to be goat rosette-forming cells (GRFC) and 100 or 200 cells were counted for each sample.

Separation of lymphocyte subpopulations. This was achieved by depletion of SRFC and GRFC. The rosettes were stabilized by performing the assay in the presence of dextran (Dextran T–70, Pharmacia, Uppsala, Sweden) at a concentration of 5% in PBS (Brown, Halpern & Wortis, 1975) with 10% of FCS. After centrifugation on a Ficoll–Hypaque gradient for 30 min at 1000 ***g***, subpopulations of rosetting and non-rosetting cells were isolated.

T-cell enrichment was also achieved, using a column of glass beads coated with Ig anti-Ig antibodies (Wigzell, Sundquist & Yoshida, 1972).

Double binding experiments. These were done by pre-incubating the cells with a polyvalent FITC–conjugated antiserum to human Ig (SBL, Stockholm, Sweden) for 30 min at 4°C. After washing, the goat rosette assay was performed.

Stimulation with mitogens. PBL were placed in culture (10^6 cells per ml of RPMI 1640 with 5×10^{-5} M 2–mercaptoethanol, 5% autologous plasma and antibodies). Concanavalin A (Con A, Industrie Biologique Française) was added at a concentration of 5·0 μg/ml and purified phytohaemagglutinin (PHA, Wellcome Research Labs, Beckenham, Kent) at a concentration of 1·0 μg/ml. After 3 days incubation at 37°C in a CO_2 milieu, DNA synthesis was studied by adding 1·0 μCi of tritiated methylthymidine (Sp. act. 5·0 mCi/μg) 6 hr prior to termination. The cells were harvested with a Skatron microharvester and the samples were counted in a liquid scintillation counter.

Con A and PHA blasts were separated after 3 days of culture on a 15–30% FCS gradient. The gradient was generated using a two-chamber gradient maker with the 15% chamber constantly stirred. The separation was checked by morphological criteria and by study of the tritiated methylthymidine incorporation.

Cell lines. Nine lymphoma lines, two myeloma lines, nine leukaemia lines and four lymphoblastoid cell lines (LCL) were studied. Selected references for these lines are found in Nilsson (1978).

Cell lines were maintained in 50 ml Erlenmeyer flasks containing approximately 20 ml of Ham's F-10 tissue culture medium (Gibco, New York) supplemented with 10% foetal calf serum and antibiotics.

For rosette assay, the cells were harvested from logarithmically growing stock cultures and washed three times in PBS. Cells were adjusted to a concentration of 5×10^6 cells per ml.

Sources of lymphoid cells. PBL were obtained from laboratory personnel. Cord blood was collected in heparinized tubes by milking from the placentae of five healthy infants at the time of delivery. Tonsils came from patients undergoing surgical operation for chronic tonsillitis. The spleen was obtained at autopsy from a young transplant donor within 30 min after death. Mononuclear cells from tonsils and spleen were prepared by passage through a fine wire screen and subsequent separation on a Ficoll–Hypaque gradient.

Peripheral blood of patients with chronic lymphocytic leukaemia (CLL) was generously supplied by Dr Simonsson, Uppsala, Sweden.

RESULTS

GRBC from three different animals were all able to bind from 45 to 60% of the tonsil lymphocytes. One of the animals studied gave about 10% higher results and was chosen for subsequent tests.

We routinely incubated the pelleted lymphocytes and the goat red blood cells at 4°C for 1 hr. Incubation at 37°C before centrifugation did not seem to alter the results. Prolonged incubation in the cold did not significantly increase the percentage of rosettes observed. On the contrary, after incubation at 37°C for 1 hr, few or no rosettes were seen on normal lymphocytes.

As shown in Table 1, the percentages of GRFC seem virtually to overlap with the percentages of SRFC in lymphocytes from different sources. The results obtained in PBL of three patients with CLL are also shown in Table 1.

TABLE 1. Percentage of GRFC and SRFC in lymphocytes from PB of normal donors, tonsils, spleen, cord bloods and in PBL of three patients with CLL

Cells	Percentage GRFC	Percentage SRFC
(1) PBL	69	78
(2) PBL	67	74
(3) PBL	69	73
(4) PBL	70	65
(5) PBL	75	72
(6) PBL	74	76
(7) PBL	58	61
(8) PBL	60	56
(9) PBL	78	71
(10) PBL	82	76
mean ± s.d.	70·2 ± 7·48	70·2 ± 7·21
(1) Tonsil	42	48
(2) Tonsil	45	50
(3) Tonsil	56	58
(4) Tonsil	50	62
(5) Tonsil	58	50
mean ± s.d.	50·2 ± 6·8	53·6 ± 6·0
(1) Spleen	24	37
(1) Cord blood	19	23
(2) Cord blood	11	13
(3) Cord blood	23	24
(4) Cord blood	25	22
(5) Cord blood	35	30
mean ± s.d.	22·6 ± 8·76	22·4 ± 6·11
(1) CLL	6	2
(2) CLL	15	3
(3) CLL	1	2
mean ± s.d.	7·33 ± 7·09	2·33 ± 0·58

The results obtained with the separation procedures are shown in Table 2. Goat rosettes as well as sheep rosettes can be used to isolate subpopulations of cells.

Lymphocytes enriched for GRBC binding were able to respond to the T-cell mitogens Con A and PHA, while the depleted subpopulation had a 91% decreased activity to both these mitogens, as shown in Table 3. PBL from two normal donors were also used for a double-binding assay, where we found that almost all the GRFC were negative for surface Ig. Less than 1% of the cells were positive for both the markers.

TABLE 2. Percentage of GRFC (G) and SRFC (S) on subpopulations of lymphocytes from normal donors enriched (+) or depleted (−) for goat and sheep rosettes and on lymphocytes passed through a column of glass beads coated with Ig anti-Ig antibodies (Ig −)

Source of lymphocytes	Subpopulation examinated	Percentage GRFC	Percentage SRFC
PBL	S (−)	4	7
	G (−)	4	3
	S (+)	n.d.	86
	G (+)	80	n.d.
	Ig (−)	84	83
Tonsil	S (−)	1	2
	G (−)	6	4
	S (+)	82	82
	G (+)	81	81

n.d. = Not done.

TABLE 3. [^{3}H]thymidine uptake measured as ct/min in PBL cultured for 3 days

Population	Control	PHA	Con A
Whole population	808 ± 329	77715 ± 7074	32623 ± 1606
GRFC-depleted	1007 ± 139	7849 ± 322 (−91)*	3969 ± 365 (−91)
GRFC-enriched	5456 ± 3560	76246 ± 3152 (−8)	50729 ± 4971 (+42)

* Numbers in parentheses indicate percentage change in the response of the whole population.

In Table 4 we report the results on haemopoetic cell lines. It is clear that the goat and sheep markers coincide nicely in all of the lines tested. Finally, in separated PHA and Con A blasts the percentages of SRFC and GRFC were virtually identical, and showed greater than 80% cells among the PHA blasts and over 70% among Con A blasts in all the experiments.

DISCUSSION

The data presented clearly demonstrate that goat erythrocytes can be used in a rosette assay for the identification of a subpopulation of human lymphocytes. All of the studies thus far indicate that the rosetting lymphocytes are the same T cells which form rosettes with SRBC. Furthermore, there is independent data to suggest that GRFC are T cells. When T lymphocytes were enriched on Ig anti-Ig columns the percentage of GRFC increased. Additional evidence indicating that goat rosettes are formed by human T lymphocytes is provided by the response to mitogens. When GRFC were enriched on Ficoll–Hypaque gradients, these cells were stimulated by the T-cell mitogens PHA and Con A, while the GRFC-depleted cells were virtually devoid of such reactivity. Finally, double-binding experiments showed that almost all GRFC were negative for surface Ig, a B-cell marker.

Our data on human cell lines show that every T-cell line which forms rosettes with SRBC (such as Molt-3, Molt-4F and JM), also binds goat erythrocytes. As these cell lines can be considered a monoclonal proliferation of malignant cells, our data strongly suggest that the goat and sheep red cells

TABLE 4. Percentages of GRFC and SRFC in human haematopoietic cell lines

Cell lines*	Percentage GRFC	Percentage SRFC
Lymphoma lines†	0	0
Myeloma lines‡	0	0
Leukaemia lines (null type)§	0	0
Leukaemia lines (B type)¶	0	0
Leukaemia lines (T type)		
CCRF-CEM	5	4
JM	16	20
MOLT-3	71	75
MOLT-4	0	0
MOLT-4F	15	11
HSB-2	0	0
LCL**	0	0

* According to the classification proposed by Nilsson & Pontén (1975).

† Raji, Daudi, U-698, U-715, DHL-4, DG-75, U-1285, U-937, DHL-2.

‡ U-266, RPMI 8226.

§ KM-3, K 562.

¶ BALL.

** U-1171, GUG 43909, U-255, FL-1525-B.

identify a receptor expressed on the same cell, or it even might be the same receptor. Indeed, the Molt-3 line, which retains its ability to form rosettes with SRBC at 37°C, also rosettes with GRBC under the same conditions. The formation of rosettes with sheep erythrocytes at 37°C has been reported as a characteristic of thymocytes and blasts from patients with acute lymphoblastic leukaemia (T-cell type; Borella & Sen, 1975).

A further attempt to demonstrate the link between sheep and goat erythrocyte receptors also indicates that they could be the same. When sheep rosettes are formed and the red cells subsequently lysed, the ability of the lymphocytes to form sheep rosettes is about 90% diminished. At the same time, the ability to form goat rosettes is similarly impaired. Inhibition of the rosettes is presumably due to covering of the receptor by remnants from the sheep erythrocyte membrane. Though such a negative result can not definitely confirm the impression that one receptor recognizes both sheep and goat red cells, such conclusions may have to await purification of this(these) receptor(s). At the present time, reports on only a few attempts at isolating the sheep receptor are available in the literature (Pyke, Rawlings & Gelfand, 1975; Chisari, Gealy & Edgington, 1977).

Thus we conclude that goat erythrocytes are as good as sheep erythrocytes as a practical marker for human T cells. Whether or not a dichotomy exists between GRFC and SRFC on cells at certain stages of differentation will only become apparent during further studies. In this respect, it may be fruitful to utilize patients with primary immuno-deficiencies, as they selectively express or lack subpopulations of lymphocytes. Such individuals may demonstrate any differences which might exist between the expression of a sheep or goat receptor on human T lymphocytes.

This work was supported by the Swedish Cancer Society and by NIH contract No. NC1-CB-74129-31. Dr J. Kurnick is supported by NIH National Research Service Award No. 1 F 32-AI05479-01.

Spleen cells were kindly provided for by Dr B. Lindblom at the Transplantation Unit for the Uppsala University Hospital.

We appreciate the assistance of Dr O. Sjöberg, at the Blood Center, and Dr L. Olding, from the Department of Pathology at the Uppsala University Hospital, in co-ordinating the supply of clinical specimens.

The technical assistance of Ms Agneta Olofsson is gratefully acknowledged.

REFERENCES

AIUTI, F. & WIGZELL, H. (1973) Function and distribution pattern of human T lymphocytes. I. Production of anti-T lymphocyte-specific sera as estimated by cytotoxicity and elimination of function of lymphocytes. *Clin. exp. Immunol.* **13**, 171.

BORELLA, L. & SEN, L. (1975) E receptors on blasts from untreated acute lymphocytic leukemia (ALL). Comparison of temperature dependence of E rosettes formed by normal and leukemic lymphoid cells. *J. Immunol.* **114**, 187.

BROUET, J.C. & TOBEN, H. (1976) Characterization of a subpopulation of human T lymphocytes reactive with an heteroantiserum to human brain. *J. Immunol.* **116**, 1041.

BROWN, C.S., HALPERN, H. & WORTIS, H. H. (1975) Enhanced rosetting of sheep erythrocytes by human peripheral blood T cells in the presence of dextran. *Clin. exp. Immunol.* **20**, 505.

CANTOR, H. & BOYSE, E. A. (1975) Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses in a differentiative process independent of antigans. *J. exp. Med.* **141**, 1376.

CHISARI, F.V., GEALY, W.J. & EDGINGTON, T.S. (1977) Recovery of soluble sheep erythrocyte receptor from the T lymphocyte surface by proteolytic cleavage. *J. Immunol.* **118**, 1138.

JONDAL, M., HOLM, G. & WIGZELL, H. (1972) Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming non-immune rosettes with sheep red blood cells. *J. exp. Med.* **136**, 207.

JONDAL, M. & KLEIN, G. (1973) Surface markers on human T and B lymphocytes. II. Presence of Epstein–Barr virus receptors on B lymphocytes. *J. exp. Med.* **138**, 1365.

LAY, W. H., MENDES, N. F., BIANCO, C. & NUSSENZWEIG, V. (1971) Binding of sheep red blood cells to a large population of human lymphocytes. *Nature (Lond.)*, **230**, 531.

MINOWADA, J., OHNUMA, T. & MOORE, G. E. (1972) Rosette forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. *J. nat. Cancer Inst.* **49**, 891.

MORETTA, L., FERRARINI, M., DURANTE, M. L. & MINGARI, M.C. (1975) Expression of a receptor for IgM by human T cells *in vitro*. *Europ. J. Immunol.* **5**, 569.

NILSSON, K. (1978) The nature of lymphoid cell lines and their relationship to EB virus. *The Epstein–Barr virus* (eds M. A. Epstein and B. G. Achong). Springer-Verlag. New York. (in press).

NILSSON, K. & PONTÉN, J. (1975) Classification and biological nature of established human haematopoietic cell lines. *Int. J. Cancer*, **15**, 321.

PELLEGRINO, M.A., FERRONE, S. & THEOPHILOPOULOS, A.N. (1975), Rosette formation of human lymphoid cells with monkey red blood cells. *J. Immunol.* **115**, 1065.

PERNIS, B., FORNI, L. & AMANTE, L. (1970) Immunoglobulin spots on the surface of rabbit lymphocytes. *J. exp. Med.* **132**, 1001.

PIKE, K.W., RAWLINGS, G.A. & GELFAND, E.W. (1975) Isolation and characterization of the sheep erythrocyte receptor in man. *J. Immunol.* **115**, 211.

RAFF, M. C. (1971) Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplant Rev.* **6**, 52.

STATHOPOULUS, G. & ELLIOTT, E.V. (1974) Formation of mouse or sheep red-blood cell rosettes by lymphocytes from normal and leukemic individuals. *Lancet*, **i**, 600.

WIGZELL, H., SUNDQUIST, K.G. & YOSHIDA, T.O. (1972) Separation of cells according to surface antigens by the use of antibody-coated columns. Fractionation of cells carrying immunoglobulins and blood group antigens. *Scand. J. Immunol.* **1**, 75.