Clin. exp. Immunol. (1975) **22,** 302–310.

Spontaneous autorosette-forming cells in man

A MARKER FOR A SUBSET POPULATION OF T LYMPHOCYTES?

J. C. GLUCKMAN & P. MONTAMBAULT, *Department of Nephrology and U. 27 de l'INSERM, Groupe Hospitalier Pitié-Salpétrière, Paris, France*

(*Received* 29 *April* 1975)

SUMMARY

A subpopulation of human peripheral blood lymphocytes are capable of binding with human (autologous or allogeneic) erythrocytes, forming rosettes. The conditions which lead to autorosette formation are similar to those required for sheep red-cell rosetting. Ageing human erythrocytes are shown to bear less of the determinants involved in the phenomenon than younger ones. Evidence is presented that autorosetting is a T-cell marker. As autorosette-forming cells are very sensitive to the inhibiting effects of ATG they could therefore belong to a T-cell subpopulation.

INTRODUCTION

The formation of spontaneous rosettes between human lymphocytes and sheep red cells under suitable conditions (Lay *et al.*, 1971; Jondal, Holm & Wigzell, 1972) is a constant feature of T cells (Wortis, Cooper & Brown, 1973; Brown & Greaves, 1974). At the present time, this is the most widely accepted marker to identify human T lymphocytes. Recently, following results obtained in mice (Micklem *et al.*, 1970; Micklem, 1971; Charreire & Bach, 1974), rabbits (Siegel & Sherman, 1972) and rats (Gluckman & Legrain, 1975), the capability of a major subpopulation of thymocytes (Baxley *et al.*, 1973) and a minor subpopulation of peripheral blood lymphocytes to bind autologous erythrocytes *in vitro*, forming autorosettes, has been demonstrated in man (Sandilands *et al.*, 1974; Dewar *et al.*, 1974). The relationship between autorosette-forming thymocytes and peripheral lymphocytes is not known, although the thymus dependency of the latter has been demonstrated (Gluckman & Montambault, 1975; Kaplan, 1975).

In the present study, the conditions for autorosette formation by human peripheral blood lymphocytes are determined. Evidence is presented confirming that peripheral blood autorosette-forming cells (ARFC) are T-cells highly sensitive to low doses of anti-thymocyte globulins. The determinants involved on the erythrocyte surface are shown to be less expressed on ageing than on young red cells.

Correspondence: Dr J. C. Gluckman, Department of Nephrology, Groupe Hospitalier Pitié-Salpétrière, 83 Boulevard de l'Hôpital, 75634 Paris, Cedex 13, France.

MATERIALS AND METHODS

Defibrinated blood samples obtained from healthy donors were centrifuged for 15 min at 300 *g*. Thereafter the serum, buffy coat and red cells were collected separately. The lymphocytes were separated by centrifugation on Ficoll–Metrizoate ($d = 1{\cdot}077$ g/cm^3) at 400 *g* for 30 min. The cells were then washed three times and resuspended at $2{\cdot}5 \times 10^6$/ml in Hanks's buffered salt solution (HBSS). The erythrocytes were washed three times in isotonic saline and resuspended at 6×10^7/ml in HBSS. Sheep red cells, stored in Alsever's solution at +4°C, were treated in the same way.

Unless otherwise stated, rosette experiments were conducted in media containing AB serum. These sera were heat inactivated (30 min at +56°C), sheep red-cell absorbed (60 min at +37°C), and kept frozen at −30°C until required.

Rosette assays. Sheep red cell rosettes were formed using the technique of Bach *et al.* (1974) modified according to Pang, Baguley & Wilson (1974). Lymphocytes (5×10^5) were mixed with erythrocytes (15×10^6) in 0·5 ml of HBSS containing 10% AB serum. The cells were incubated at +37°C for 30 min and then centrifuged at 200 *g* for 5 min at room temperature. The pellet was kept overnight at +4°C. The cell mixture was then gently resuspended by end-over-end rotation, and the rosettes fixed by the addition of one drop of cold 2·5% glutaraldehyde. The suspension was mixed and incubated at +4°C for at least 20 min. One drop of 0·2% methylene blue was added to stain lymphocytes, and the percentage of lymphocytes binding three or more sheep red cells (SRFC) was counted in a haemocytometer under a phase contrast microscope. For each tube, approximately 200 cells were examined

Auto- and allorosettes were obtained using a similar technique. Lymphocytes (5×10^5) were incubated at +37°C for 60 min in 0·25 ml HBSS supplemented with 20% stored AB or fresh autologous serum as required. (The fresh sera used were autologous to the lymphocytes in allorosette assays; each time that there was an ABO mismatch between the lymphocyte and the erythrocyte donors, sera were absorbed 90 min at +37°C and 90 min at +4°C, with the red cells.) The assay was otherwise the same as for sheep red-cell rosettes. For each tube approximately 1000–2000 cells were examined.

Throughout the paper, SRFC and ARFC are reported as numbers of rosettes per 100 lymphocytes.

Separation of erythrocytes of different ages on the basis of density difference. Human erythrocytes of different ages were separated on the basis of density differences by centrifugation (Greenwalt, Flory & Steane, 1970; O'Connell, Caruso & Sass, 1965; Prentice & Bishop, 1965). Fresh blood was centrifuged for 15 min at 300 *g* and the buffy coat and most of the serum were removed, so that the haematocrit value of each blood sample was adjusted to approximately 70%. An aliquot of each red cell sample was pipetted off in other tubes to serve as controls. The red cell samples were spun at 1000 *g* for 2 hr at room temperature in an angle head centrifuge. Thereafter, the top and the bottom layers (each representing approximately 5–10% of the total volume) of the packed red cells were removed by gentle aspiration with a 1-ml syringe using an 18 gauge needle. The control samples were spun in an identical manner, but were well mixed before being used. All these samples were collected and then washed three times in 10 ml HBSS for the rosette tests and in saline for the biochemical studies. Compared rosette assays using erythrocytes collected from control tubes, top and bottom layers, were performed.

Age differences of erythrocytes obtained from the top and bottom layers were assessed by comparing their cholinesterase and pyruvate kinase activities (Gluckman, Gattegno & Cornillot, 1975).

Rosette inhibition by equine anti-human lymphocyte globulins. Two batches of globulins were studied: anti-lymphocyte globulins (ALG) obtained after immunization by human chylolymphocytes (Lymphoser Berna, Institut Sérothérapique et Vaccinal Suisse, Berne) and anti-thymocyte globulins (ATG) (Thymoglobuline, Institut Merieux, Lyon). Both were devoid of agglutinins, haemolysins and antiplasma protein antibodies.

The inhibiting effect of these globulins was comparatively assayed on SRFC and ARFC, after serial dilutions in AB or in fresh autologous serum (so that serum concentration in all the tubes remained 10%). Lymphocytes (5×10^5) were added to 50-μl aliquots of dilute globulins, and the mixture was incubated for 60 min at +37°C. Then human or sheep erythrocytes (15×10^6) were added, and the normal rosetting procedure was followed.

Statistical tests. Significance of the differences observed in the experiments were assessed by either unpaired or paired Student's *t*-test as required.

RESULTS

Rosetting conditions

These were assessed by series of paired experiments verifying various parameters of the assay.

Since autorosette formation is related to a weak binding between lymphocytes and erythrocytes (Kaplan, 1975), fixation of rosettes before reading the test was tried. Non-specific sticking of erythrocytes to lymphocytes by glutaraldehyde fixation was not observed. Similar numbers of SRFC ($56{\cdot}3 \pm 4{\cdot}8\%$ versus $57{\cdot}1 \pm 3{\cdot}1$; $n = 7$; $P > 0{\cdot}80$) and of ARFC ($1{\cdot}49 \pm 0{\cdot}17\%$ versus $1{\cdot}28 \pm 0{\cdot}09$; $n = 6$; $P > 0{\cdot}20$) were obtained with or without addition of glutaraldehyde. Glutaraldehyde can thus be used to keep the rosettes stable, avoiding disruption at room temperature and allowing repeated counts to be made.

The effect of varying the erythrocyte/lymphocyte ratio, the number of lymphocytes remaining constant, was next investigated. Fig. 1 shows that the counts obtained reached a plateau beginning at a 15/1 or 30/1 ratio according to the technical conditions of the assay. Unless otherwise stated, a 30/1 ratio was used throughout the study.

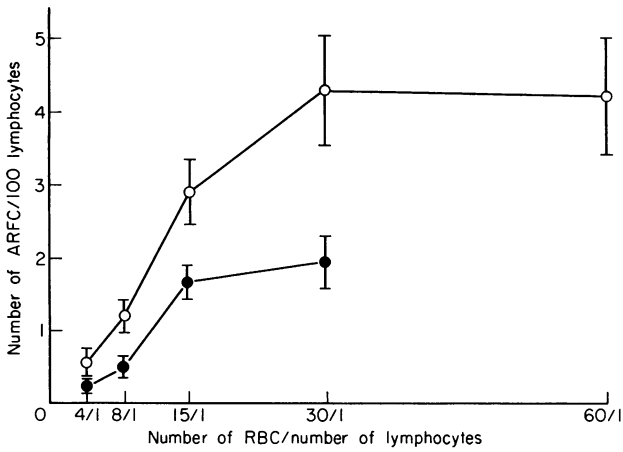

FIG. 1. Autorosette formation in media containing either frozen stored AB or fresh autologous serum. Relationship between erythrocyte/lymphocyte ratio and autorosette counts. (●) Stored serum: eleven experiments. (○) Fresh serum: seven experiments.

The incubation time of the cell mixture at +4°C did not influence rosette counts, provided that incubation lasted at least 60 min. For instance $51{\cdot}6 \pm 2{\cdot}1\%$ SRFC and $1{\cdot}52 \pm 0{\cdot}10\%$ ARFC were observed after 60 min as compared to $53{\cdot}0 \pm 2{\cdot}7$ and $1{\cdot}78 \pm 0{\cdot}22$, respectively, after overnight incubation ($n = 4$; $P > 0{\cdot}70$ and $P > 0{\cdot}20$). In most instances, overnight incubation was used for practical reasons.

The effect of human serum on autorosette formation was studied by experiments in which the cells were mixed in media without serum or additioned with stored or fresh sera (Table 1). SRFC counts did not change whatever type of serum was used. ARFC counts were similar in media without serum or with stored, AB or autologous, sera. Addition of stored AB serum was prefered to the use of non-supplemented media, because of a better lymphocyte viability after overnight incubation. The addition of fresh autologous serum significantly increased the numbers of ARFC observed. Heat inactivation (56° + C, 30 min) of fresh sera did not change ARFC counts, as compared to untreated sera ($3{\cdot}92 \pm 0{\cdot}57\%$ versus $2{\cdot}90 \pm 0{\cdot}58$; $n = 7$; $P > 0{\cdot}05$).

Normal ARFC values

Under the usual rosetting conditions, the mean incidence of ARFC in normal individuals was of 2·02% (s.d. = 1·15; s.e.m. = 0·12; $n = 93$) in media containing stored serum and of 4·20% (s.d. 2·26; s.e.m. = 0·27; $n = 69$) when fresh serum was used. There was no obvious relationship with sex or age. The variability of the values observed could be related to short-lived fluctuations of ARFC, as suggested by serial tests on the same subjects (Table 2). Meanwhile, the average of SRFC was 55·8% (s.d. = 10·7; s.e.m. = 1·5; $n = 51$).

TABLE 1. Effect of human serum on human autorosette forming cells (RFC/100 lymphocytes, mean ± s.e.)

Types of rosettes	Number of paired experiments	Without serum	Stored AB serum	Stored autologous serum	Fresh autologous serum	*P* of the differences observed
SRFC	4		61·1 ± 5·0	57·0 ± 3·5		> 0·40
	7		58·1 ± 3·6		61·3 ± 5·7	> 0·30
ARFC	6	2·39 ± 0·54	2·46 ± 0·56			> 0·90
	5		1·82 ± 0·22	1·82 ± 0·27		> 0·80
	49		1·80 ± 0·18		4·25 ± 0·37	< 0·001

TABLE 2. Serial studies of ARFC in normal donors (ARFC/100 lymphocytes)

Donor	Sex	Time-range* (weeks)	ARFC range in media with: Stored sera	Fresh sera
FRA.	F	24	0·25–1·85 (6)†	1·64–6·64 (5)
PAU.	M	12	0·67–4·60 (6)	3·67–5·14 (3)
GLU.	M	20	2·00–3·11 (3)	3·17–7·45 (3)
JAC.	F	15	0·71–3·83 (3)	1·07–4·46 (2)
DEM.	F	14	0·74–2·43 (3)	5·15–9·27 (2)

* Time range between the first and last determination in the same subject.

† The numbers of determinations performed in each case are in parentheses.

Allo-rosette-forming cells (allo-RFC)

Allo-RFC counts were compared to those of ARFC in the same subjects. In twenty comparisons stored AB serum was used in the medium: 1·84 ± 0·19% ARFC were observed, as compared to 2·45 ± 0·22% allo-RFC ($P > 0·05$). In fifteen comparisons where fresh sera autologous to the lymphocytes were used, 3·66 ± 0·65% ARFC and 3·87 ± 0·43% allo-RFC were obtained ($P > 0·20$). Thus, no statistically significant difference could be observed, although these tests were mainly performed with ABO incompatible cells.

Auto-rosette formation and age of erythrocytes (Table 3)

Separation of erythrocytes of different ages was obtained by centrifugation. The method used is known to separate young and old erythrocytes as demonstrated by enzymatic activity

TABLE 3. Autorosette formation and age of erythrocytes

	Enzymatic activities (i.u./g haemoglobin)		ARFC/100 lymphocytes; mean ± s.e. of 5–8 expt.				
	Cholinesterase (9 expt.)	Pyruvate kinase (5 expt.)	Media with stored serum				Media with fresh serum
			4/1*	8/1	15/1	30/1	30/1
Control erythrocytes			0·29 ± 0·09	0·46 ± 0·12	1·45 ± 0·33	1·92 ± 0·30	4·48 ± 0·92
Top erythrocytes	30·9 ± 2·3	5·0 ± 0·7	0·33 ± 0·09	0·62 ± 0·12	2·22 ± 0·58	2·27 ± 0·41	4·10 ± 0·93
Bottom erythrocytes	23·2 ± 1·9	2·1 ± 0·5	0·27 ± 0·06	0·38 ± 0·06	1·14 ± 0·22	1·02 ± 0·22	2·19 ± 0·42
P of the differences observed							
C versus T			> 0·30	> 0·05	> 0·05	> 0·40	> 0·70
C versus B			> 0·60	> 0·30	> 0·20	< 0·05	= 0·05
T versus B	< 0·001	< 0·01	> 0·50	< 0·05	= 0·05	< 0·01	< 0·02

* Erythrocyte/lymphocyte ratio.

differences, and in the rabbit, by striking half life differences (assessed by ^{51}Cr labelling) between top and bottom layer cells (L. Gattegno, personal communication).

Erythrocytes from the bottom layers had significantly lower cholinesterase and pyruvate-kinase activities than those from the top layers. Hence, cells from the bottom layers displayed characteristics which are generally admitted to be those of ageing cells, while younger cells were obtained in the top layers.

TABLE 4. Rosette inhibition by ALG and ATG (% inhibition: mean ± s.e. of three to six experiments)

μg ALG/10^6 lymphocytes	SFRC	ARFC (stored serum)
25·0	92·5 ± 7·5**	Not tested
12·5	79·5 ± 7·5**	93·9 ± 6·1***
6·25	62·9 ± 1·9***	89·1 ± 6·4***
3·13	33·9 ± 9·2*	79·8 ± 3·7***
1·563	4·4 ± 8·6	63·0 ± 7·0**
0·781	−12·9 ± 6·7	55·6 ± 6·8**
0·391	−4·4 ± 3·0	56·5 ± 8·9**
0·195	Not tested	18·8 ± 10·6
0·098	Not tested	9·0 ± 3·1
0·049	Not tested	−12·1 ± 6·0

ATG (μg/10^6 lymphocytes	SFRC	ARFC	
		Stored serum	Fresh serum
17·8	52·5 ± 13·0*	96·3 ± 2·3***	100**
8·9	41·4 ± 10·6*	88·4 ± 6·0***	90·5 ± 4·7***
4·46	41·3 ± 10·3*	72·6 ± 7·1***	84·5 ± 7·4***
2·23	34·0 ± 7·5*	62·5 ± 4·5***	64·9 ± 9·9**
1·116	5·4 ± 4·0	50·5 ± 9·4**	51·1 ± 6·5***
0·558	−2·0 ± 5·0	39·9 ± 11·3*	32·3 ± 11·0*
0·279	−1·0 ± 2·9	24·9 ± 11·7	8·4 ± 8·5
0·140	Not tested	11·7 ± 12·3	0·5 ± 6·6
0·070	Not tested	−4·9 ± 7·4	Not tested
0·035	Not tested	−6·3 ± 2·7	Not tested

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Probability that the rosette inhibition observed is due only to chance.

To test their ability to form rosettes, either control or top or bottom erythrocytes were mixed with the same lymphocytes at different erythrocyte/lymphocyte ratios. At an 8/1 ratio and over, ARFC counts were significantly lower with bottom than with top erythrocytes. Thus, ageing erythrocytes were less able to bind to autologous lymphocytes.

Rosette inhibition by ALG

It has previously been shown that ARFC appear to be T cells, by using two types of experiments: (1) separation of SRFC by density centrifugation on a Ficoll–Metrizoate gradient has shown that ARFC counts are significantly lower in the SRFC-depleted lympho-

cytes and higher in the SRFC-enriched population, than in the control cells; (2) when rosette formation of lymphocytes with both sheep and human autologous erythrocytes is assayed, most of the rosetting lymphocytes bind either both kind of erythrocytes or sheep red cells alone (Gluckman & Montambault, 1975). Therefore, it was interesting to assess the degree of sensitivity to ALG of SRFC and ARFC, comparatively.

One batch of ALG and one batch of ATG were simultaneously tested on SRFC and ARFC. Both inhibited autorosettes to a greater extent than sheep red-cell rosettes (Table 4). Although more autorosettes were found in media additioned with fresh sera than in media with stored sera, the degree or inhibition was the same in both conditions. At concentrations below those at which complete inhibition of autorosettes occurred, ATG and ALG appeared to cause a redistribution of receptors. This was indicated by the finding that after incubation with the globulins and erythrocytes, most lymphocytes bound erythrocytes at only one end of the cell whereas lymphocytes with 'capped' erythrocytes were seldom observed in control tubes.

DISCUSSION

The results of the experiments reported here confirm previous reports that a population of peripheral blood lymphocytes are capable of binding autologous erythrocytes, *in vitro* (Sandilands *et al.*, 1974; Dewar *et al.*, 1974; Charreire & Bach, 1974). However, several investigators have failed to observe such autorosette formation (Coombs *et al.*, 1970; Lay *et al.*, 1971; Whittingham & Mackay, 1973; Baxley *et al.*, 1973) and this discrepancy is almost certainly due to differences in technique. Actually, Kaplan (1975) has shown that the binding of human erythrocytes by lymphocytes is extremely weak and temperature sensitive. This pitfall seems to be avoided by glutaraldehyde fixation.

ARFC values in normal subjects are low, but the range varies from less than 0·5 to approximately 5% when the assay is performed in media with or without stored sera, and up to 10% when fresh serum is added. Serial tests in the same subjects suggest that short-lived fluctuations may occur. The increase in the counts observed in the presence of fresh serum cannot be related to cell viability variations after overnight incubation. Perhaps, it is possibly due to the fact that only high-density-receptor cells can rosette in the presence of stored serum (or in the absence of serum) whereas, for a yet unknown reason, cells with less receptors are able to rosette in the presence of fresh serum. Experiments are currently in progress to investigate this phenomenon. Allo-RFC counts do not differ significantly from those of ARFC (Dewar *et al.*, 1974; Kaplan, 1975) suggesting that the lymphocyte receptors involved bind to determinants shared by all human erythrocytes.

Separation of erythrocytes of different ages on the basis of density differences was performed to test the hypothesis that autorosette formation might be the *in vitro* reflection of a mechanism involved in removing effete, altered cells, as suggested by us in the rat (Gluckman *et al.*, 1975), and by Baxley *et al.* (1973) in man using neuraminidase-treated human red cells. These authors have demonstrated that the conditions which lead to rosette formation with neuraminidase-treated erythrocytes are similar to those required for sheep red cell rosette formation, and that the binding sites for both kind of red cells may be similar. Indeed, surface sialic acid is known to decrease in old erythrocytes, this phenomenon being related to alterations of the surface glycoproteins due to ageing (Greenwalt & Steane, 1973). On the contrary, our data show that ageing human erythrocytes are less likely to form auto-

rosettes. The difference between these findings could be due to the fact that Baxley used a neuraminidase dosage known to completely remove sialic acid from the erythrocyte surface (Gattegno, Bladier & Cornillot, 1975) and did not mimic the molecular events occurring during erythrocyte ageing. Actually sialic acid differences between young and old erythrocytes do not exceed 10–20% of the young cells content (Gattegno *et al.*, 1975; Gluckman *et al.*, 1975). It may be postulated that neuraminidase, by removing the sialic acid group from the cell surface, can facilitate rosette formation either by unmasking of receptors or by lowering the negative charges on the cell surface (Galili & Schlesinger, 1974). On the other hand, it has recently been shown that ageing erythrocytes bear less carbohydrates on their surface than younger ones (L. Gattegno, personnal communication). Therefore, it can be suggested that autorosette formation with either normal or neuraminidase-treated erythrocytes involves different mechanisms. However, in both instances binding is an active process, inhibited by sodium azide, and the lymphocyte receptors have protein moieties since trypsin treatment prevents binding (Kaplan, 1975).

Human T lymphocytes can be demonstrated by a specific surface antigen, or a surface membrane receptor which binds sheep red blood cells to form non-immune rosettes (Brown & Greaves, 1974; Owen & Fauger, 1974a). Recently, a second receptor has been demonstrated which binds Rhesus monkey red cells to human T lymphocytes (Lohrmann & Novikovs, 1974). In the same way, autorosette formation corresponds to a non-specific binding of human erythrocytes to lymphocytes. There is evidence that ARFC are T cells as demonstrated by SRFC separation experiments and analysis of the frequency of lymphocytes rosetting with both sheep and human erythrocytes (Gluckman & Montambault, 1975), and by the fact that ARFC lack surface immunoglobulins (Kaplan, 1975). Moreover, autorosette formation is completely inhibited by ATG. The observation that at concentrations lower than those required to block completely rosette formation, ATG can induce rosette formation at one pole of the lymphocyte, suggests co-capping of an ATG receptor and the autologous erythrocyte receptor. This suggests that the receptors for human red cells on peripheral lymphocytes are the same as or very closely associated with a T cell-specific antigen. The data do not exclude the possibility of steric hindrance resulting from ATG binding, or the presence of distinct antibody populations directed toward several different T cell-specific receptors, one of which is the receptor for autologous erythrocytes. Similar results on the effects of ATG on SRFC, obtained by Owen & Fauger (1974b), bring evidence that receptors for sheep and human erythrocytes could be the same or very close to one another. So it is possible that autorosettes may be formed at random with only some T lymphocytes. However, the finding that ARFC are more sensitive to ATG than the average SRFC is compatible with the hypothesis that ARFC represent a distinct subpopulation of T lymphocytes, in the same way as Raff & Cantor (1971) have postulated that subpopulations of peripheral T cells can be distinguished by their sensitivities to ATS.

In conclusion, there is some evidence that ARFC could represent a subset population of T lymphocytes. Since a controlling function of thymus-derived lymphocytes in relation to autoimmunity has been ascertained (Allison, Denman & Barnes, 1971) further studies in normal subjects and in pathological circumstances (autoimmune diseases) could provide a clue as to the understanding of this phenomenon. Such experiments are in progress in our laboratory.

The authors wish to acknowledge the excellent technical assistance of Mrs J. Delorme and Ms F. Sanchez, the editorial help of Mrs Debrun, and thank Dr L. Gattegno for the biochemical studies.

REFERENCES

ALLISON, A.C., DENMAN, A.M. & BARNES, R.D. (1971) Cooperating and controlling functions of thymus-derived lymphocytes in relation to autoimmunity. *Lancet*, **ii**, 135.

BACH, J.F., JUDET, C., ARCE, S. & DORMONT, J. (1974) Exploration de la fonction thymique chez l'homme. II. Le phénomène des "rosettes-moutons" marqueur des lymphocytes T chez l'homme. *Nouv. Presse méd.* **3**, 655.

BAXLEY, G., BISHOP, G.B., COOPER, A.G. & WORTIS, H.H. (1973) Rosetting of human red blood cells to thymocytes and thymus-derived cells. *Clin. exp. Immunol.* **15**, 385.

BROWN, G. & GREAVES, M.F. (1974) Cell surface marker for human T and B lymphocytes. *Europ. J. Immunol.* **4**, 302.

CHARREIRE, J. & BACH, J.F. (1974) Self and not-self. *Lancet*, **ii**, 299.

COOMBS, R.R.A., GURNER, B.W., WILSON, A.B., HOLM, G. & LINDGREN, B. (1970) Rosette formation between human lymphocytes and sheep red cells not involving immunoglobulin receptors. *Int. Arch. Allergy*, **39**, 658.

DEWAR, A.E., STUART, A.E., PARKER, A.C. & WILSON, C. (1974) Rosetting cells in autoimmune haemolytic anaemia. *Lancet*, **ii**, 519.

GALILI, U. & SCHLESINGER, M. (1974) The formation of stable E rosettes after neuraminidase treatment of either human peripheral blood lymphocytes or of sheep red blood cells. *J. Immunol.* **112**, 1628.

GATTEGNO, L., BLADIER, D. & CORNILLOT, P. (1975) Ageing *in vivo* and neuraminidase treatment of rabbit erythrocytes: influence on half-life as assessed by ^{51}Cr labelling. *Hoppe-Seyl. Z. phys. Chem.* **356**, 391.

GLUCKMAN, J.C., GATTEGNO, L. & CORNILLOT, P. (1975) Significance of spontaneous autorosettes in rats. *Europ. J. Immunol.* **5**, 301.

GLUCKMAN, J.C. & LEGRAIN, M. (1975) Spontaneous autorosettes in rats. *Transplant. Proc.* **7**, supplement **1**, 407.

GLUCKMAN, J.C. & MONTAMBAULT, P. (1975) Spontaneous autorosettes in man. *Lancet*, **i**, 112.

GREENWALT, T.J., FLORY, L.L. & STEANE, E.A. (1970) Quantitative haemagglutination. III. Studies of separated populations of human red blood cells of different densities. *Brit. J. Haemat.* **19**, 701.

GREENWALT, T.J. & STEANE, E.A. (1973) Quantitative haemagglutination. IV. Effect of neuraminidase treatment on agglutination by blood group antibodies. *Brit. J. Haemat.* **25**, 207.

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

KAPLAN, J. (1975) Human T lymphocytes form rosettes with autologous and allogeneic human red blood cells. *Clin. Immunol. Immunopath.* **3**, 471.

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.

LOHRMANN, H.P. & NOVIKOVS, L. (1974) Rosette formation between human T-lymphocytes and unsensitized Rhesus monkey erythrocytes. *Clin. Immunol. Immunopath.* **3**, 99.

MICKLEM, H.S. (1971) The cellular basis of "self" tolerance. *Immunological Tolerance to Tissue Antigens* (ed. by N. W. Nisbet and M. W. Elves), p. 237. Orthopaedic Hospital, Owestry.

MICKLEM, H.S., ASFI, C., STAINES, N.A. & ANDERSON, N. (1970) Quantitative study of cells reacting to skin allografts. *Nature (Lond.)*, **227**, 947.

O'CONNELL, D.J., CARUSO, C.J. & SASS, M.D. (1965) Separation of erythrocytes of different ages. *Clin. Chem.* **11**, 771.

OWEN, F.L. & FAUGER, M.W. (1974a) Studies on the human T-lymphocyte population. I. The development and characterization of a specific anti-human T-cell antibody. *J. Immunol.* **113**, 1128.

OWEN, F.L. & FAUGER, M.W. (1974b) Studies on the human T-lymphocyte population. II. The use of a T-cell specific antibody in the partial isolation and characterization of the human lymphocyte receptor for sheep red blood cells. *J. Immunol.* **113**, 1138.

PANG, G.T.M., BAGULEY, D.M. & WILSON, J.D. (1974) Spontaneous rosettes as a T-lymphocyte marker: a modified method giving consistent results. SRBC rosettes. *J. immunol. Meth* **4**, 41.

PRENTICE, T.C. & BISHOP, C. (1965) Separation of rabbit red cells by density methods and characteristics of separated layers. *J. cell. comp. Physiol.* **65**, 113.

RAFF, M.C. & CANTOR, H. (1971) Subpopulations of thymus cells and thymus derived lymphocytes. *Progress in Immunology* (ed. by B. Amos), p. 83. Academic Press, New York.

SANDILANDS, G., GRAY, K., COONEY, A., BROWNING, J.D. & ANDERSON, J.R. (1974) Autorosette formation by human thymocytes and lymphocytes. *Lancet*, **i**, 27.

SIEGEL, I. & SHERMAN, W.B. (1972) The interaction of lymphocytes with autologous red cells. *J. Allergy clin. Immunol.* **50**, 65.

WHITTINGHAM, S. & MACKAY, I.R. (1973) Rosette formation by human thymocytes. *Cell. Immunol.* **6**, 362.

WORTIS, H.H., COOPER, A.G. & BROWN, M.C. (1973) Inhibition of human lymphocyte rosetting by anti-T sera. *Nature: New Biology*, **243**, 109.