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LYMPHOCYTE SUBPOPULATIONS

HUMAN RED BLOOD CELL ROSETTES

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SUMMARY

Human red blood cells (HRBC) even without prior neuraminidase treatment, could form rosettes with human peripheral blood lymphocytes *in vitro*. The optimum conditions for forming these rosettes were a pH of 7·0 and a medium with 5% bovine serum albumin (BSA). Rosette proportions became much less at a different pH or using lower concentrations of BSA, or replacing BSA with foetal calf sera (FCS) or human sera. Rosette formation was also promoted by prior treatment of HRBC or lymphocytes with neuraminidase. Mixed rosettes of HRBC and sheep red blood cells (SRBC) showed that HRBC receptors were detectable only on lymphocytes that possessed SRBC receptors, suggesting that HRBC rosette-forming cells were probably thymus-derived (T) cells.

Next, the properties of human red blood cell (HRBC) and sheep red blood cell (SRBC) rosette-forming cells were investigated by comparing the ability of human peripheral blood lymphocytes to form these two types of rosettes after treatment with various inhibitory reagents. HRBC rosettes were relatively more resistant to inhibition with: (1) proteolytic agents, such as trypsin, α-chymotrypsin and pronase; (2) anti-thymocyte serum (ATS); (3) metabolic inhibitors, such as sodium azide and 2,4-dinitrophenol (DNP); (4) cytochalasin B. On further incubation after trypsinization, the lymphocytes recovered some ability to form SRBC rosettes, but continued to lose more of their capability to form HRBC rosettes. All these results were regarded as circumstantial evidence that the HRBC rosettes might represent a subpopulation of human T lymphocytes.

INTRODUCTION

One of the commonly accepted markers for the thymus-derived lymphocytes (T) in humans is the spontaneous SRBC rosette (Jondal, Holm & Wigzell, 1972; Bentwich & Kunkel, 1973). The ability of human lymphocytes to form rosettes with erythrocytes of other species has apparently received less attention. It was initially reported that they did not form rosettes

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with HRBC (Bach, 1973), with the exception of patients with chronic cold agglutinin disease. Rosettes in these patients were probably antigen-recognition cells instead of T- and B-cell markers (Feizi *et al.*, 1973). More recently, a proportion of human lymphocytes was found to have the ability to form rosettes with HRBC, if the HRBC were first treated with neuraminidase (nHRBC). This was thought to be another T-cell marker. These authors were also unable to detect significant HRBC rosettes in peripheral blood using untreated HRBC (Baxley *et al.*, 1973). In this paper a method to induce HRBC rosette formation with untreated HRBC is reported. The method is essentially similar to that of SRBC rosette formation (Yu *et al.*, 1974). However, the requirements for media supplementation and pH were much more stringent than those of SRBC rosettes. Minor variations from these led to a significant decrease in the number of HRBC rosettes. The effect of treatment of HRBC or lymphocytes with neuraminidase on rosette formation was also investigated and compared to rosettes made with untreated cells. The nature of those rosette-forming lymphocytes were examined by forming mixed rosettes with SRBC. Next, lymphocytes were treated with various inhibitory agents and their abilities to form SRBC and HRBC rosettes were compared. If such responses were different, they might constitute evidence that HRBC rosettes formed a subpopulation of human T lymphocytes.

MATERIALS AND METHODS

To obtain HRBC, samples of peripheral blood were drawn from normal volunteers and immediately mixed with equal aliquots of Alsever's solution. These were kept in sterile vials at 4°C for a maximum period of 1 month. To assay rosettes, the red cells were washed three times with isotonic saline and resuspended in 0·5% concentration (6×10^7/ml) in Hanks's balanced salt solution (HBSS) (Grand Island Biological, New York) supplemented with BSA (Path-o-cyte 4, code 81-010, Miles Laboratory, Illinois). Most of the assays described here were carried out with the red blood cells from a single individual (F.B.) of blood group O, Rh-negative. The effect of blood groups on rosette formation will be described in a subsequent paper.

SRBC (Davis Laboratory, Berkeley, California) were also stored in Alsever's solution at 4°C. They were washed three times in isotonic saline and resuspended in 0·5% concentration in HBSS with 10% heat-inactivated FCS previously absorbed with SRBC.

Lymphocytes were separated from peripheral blood by Ficoll–Hypaque gradient (Perper & Zee, 1968) and washed three times with HBSS. They were then resuspended in HBSS in concentrations of 5×10^6/ml.

The method of assaying SRBC rosettes was similar to that reported previously (Yu *et al.*, 1974). For assays of HRBC rosettes, 0·4 ml of HRBC suspension and 0·4 ml of lymphocytes were mixed in plastic tubes (Falcon 2058). They were then incubated at 37°C for 15 min, centrifuged at 200 *g* for 10 min, and kept at 4°C overnight. Prior to assay of rosettes, the pellets were resuspended by a vertical rotating mixer for 2·5 min. One drop of the cell suspension was put into a haemocytometer chamber. One hundred lymphocytes were counted. All tests were done in triplicates, and the proportions of rosettes were expressed as the percentages of the total ± standard errors (s.e.). The s.e. of all results reported here were less than 3·0. A SRBC rosette was defined as a lymphocyte surrounded by three or more SRBC. Because HRBC were larger than SRBC, a HRBC rosette was arbitrarily defined as a lymphocyte surrounded by two or more HRBC. Monocytes were distinguished

by their ability to ingest latex beads, as described previously (Yu *et al.*, 1974).

For treatment with neuraminidase, HRBC and lymphocytes were suspended in concentrations of 2% and 10×10^6/ml respectively in HBSS with 50 units/ml neuraminidase (from *Vibrio cholerae*, Calbiochem, California) at pH 7·4. These were incubated for 60 and 30 min respectively and washed twice with HBSS.

All results reported here represented that of at least two separate experiments.

RESULTS

Effect of pH of media on rosette formation

SRBC rosettes were prepared in media with pH ranging from 6·5 to 8·0. Similarly HRBC rosettes were prepared in 5% BSA in HBSS with pH ranging from 6·0 to 8·0. Whereas the proportions of SRBC rosettes varied only slightly within the tested range of pH, the HRBC rosettes were very much pH-dependent. The optimal pH for HRBC rosette formation was 7·0. At pH 6·5 and 7·4 the proportions of HRBC rosettes were only about half of the maximum (Fig. 1).

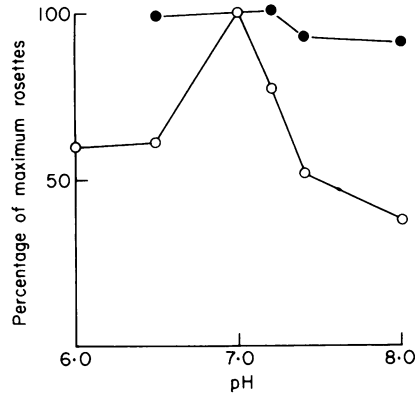

FIG. 1. Effect of pH on rosette formation. (●) SRBC. (○) HRBC.

Effect of BSA concentrations on rosette formation

SRBC and HRBC rosettes were prepared in HBSS with BSA concentrations ranging from 0 to 5%, pH 7·0. There was a slight increase in SRBC rosette proportions when using 5% BSA compared to HBSS alone. On the other hand, there was a striking increase in the HRBC rosette proportions with increasing concentrations of BSA, reaching a maximum at 5% (Fig. 2). When the concentration of BSA exceeded 5%, there was considerable clumping of the red cells and the lymphocytes so that rosette assay became inaccurate.

Effect of different batches of BSA, foetal calf sera and human sera

Three different batches of BSA were tested. These were Path-o-cyte 4 code 81–010 (Miles, USA), crystallized BSA code BV-0102 (Pentex, USA), bovine albumin fraction V A-4503 (Sigma, USA). In addition, since FCS or human AB sera were the usual supplements for promoting SRBC rosettes, we also tested the effect of these on HRBC rosette formation. Five different batches of foetal calf sera were tested (Grand Island Biological,

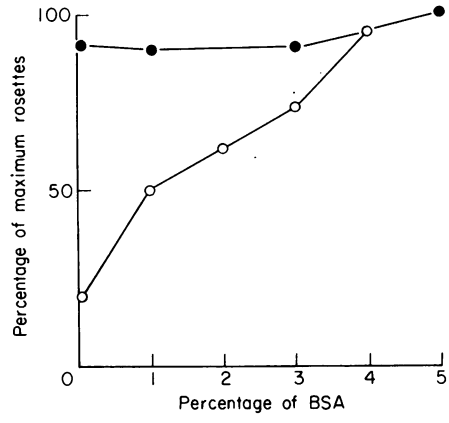

FIG. 2. Effect of BSA concentration on rosette formation. (●) SRBC. (○) HRBC.

New York). These were heat-inactivated at 56°C for 30 min and then absorbed with washed HRBC. Cells were suspended in HBSS with 5% sera at pH 7·0. One batch of AB serum was treated similarly. All three batches of BSA promoted rosette formation to the same extent. Only one batch of FCS (lot number A 334403) was able to promote HRBC rosette formation to the same extent as the 5% BSA. The single batch of human AB sera did not promote rosette formation. Using autologous sera or raising the concentration of human sera to 20% did not alter the results (results not shown).

Effect of concentrations of RBC

HRBC suspensions of different concentrations were used ranging from one to fifteen times the concentration of the lymphocytes (5×10^6/ml). The optimal proportions of the rosettes were obtained with a HRBC:lymphocyte ratio of 12:1. When a concentration exceeding 15:1 was used, the cells became too crowded for accurate assay.

Effect of temperatures and periods of incubation

After centrifugation to pack the HRBC and lymphocytes, one set of tubes was kept at 4°C and another at 37°C. Samples were retrieved and assayed at intervals over a period of 48 hr. The proportions of rosettes formed initially were small. At 37°C these dissociated rapidly so that none could be detected after 4 hr. At 4°C, the proportion of rosettes increased gradually, reaching a maximum at 24 hr. It then declined so that at 48 hr it was about 40% of the maximum.

The time course of HRBC rosette formation was thus similar to that of SRBC formation described by the author previously (Yu *et al.*, 1974).

Effect of neuraminidase treatment

The first report of successful experiments on HRBC rosette formation using neuraminidase-treated HRBC (nHRBC) in 0·1% BSA as the media was made by (Baxley *et al.*, 1973). We repeated these experiments and also compared the results of rosette formation using neuraminidase-treated lymphocytes (nlymphocytes) and also rosette formation using 5% BSA as media. The proportion of rosettes made with nHRBC+lymphocytes in 0·1% BSA

was very similar to that made with HRBC+lymphocytes in 5% BSA. Treatment of lymphocytes with neuraminidase also promoted HRBC rosette formation (Table 1).

TABLE 1. Effect of neuraminidase on HRBC formation

	Experiment 1		Experiment 2	
	BSA (0·1%)	BSA (5%)	BSA (0·1%)	BSA (5%)
HRBC+lymphocytes	19·0±2·5*	45·7±1·3	13·0±0·5	39·7±0·3
nHRBC+lymphocytes	48·5±1·5	56·3±1·9	38·7±0·3	51·7±2·2
HRBC+nlymphocytes	70·3±0·3	80·7±0·7	57·0±0·6	56·0±1·2
nHRBC+nlymphocytes	51·3±0·7	74·7±0·3	53·7±1·2	59·3±0·3

* Percentage rosettes ± s.e.

Mixed rosettes

To test the specificity of the HRBC rosettes, mixed rosettes were formed with 0·5% SRBC and 0·5% HRBC suspensions. Mixed rosettes were also formed with SRBC using either nHRBC or nlymphocytes or both. A mixed rosette is defined as a lymphocyte surrounded by at least two RBC with both SRBC and HRBC present in the rosette. As shown in Table 2, in all combinations, the rosettes seen were either mixed rosettes or pure SRBC rosettes but no pure HRBC rosettes.

TABLE 2. Mixed rosettes

	Mixed rosettes (%)	SRBC rosettes (%)	HRBC rosettes (%)	No RBC (%)
SRBC+HRBC+lymphocytes	23	45	0	32
SRBC+HRBC+lymphocytes	40	32	0	29
SRBC+HRBC+n lymphocytes	28	65	0	7
SRBC+nHRBC = n lymphocytes	26	64	0	10

SRBC and HRBC rosettes in normal individuals

The SRBC and HRBC rosettes were tested simultaneously in the peripheral blood lymphocytes of ten normal volunteers. These were chosen at random from the staff of the laboratories. The average proportion of HRBC rosettes was 28·5±1·4% (range 23·3±1·8%–35±0·5%) and SRBC rosettes was 73·3±1·4% (range 65·3±0·7%–79±0·6%).

Effect of proteolytic agents

Lymphocytes were treated with trypsin (Worthington, New Jersey) at concentrations ranging from 1 to 1000 μg/ml in presence of 10 μg/ml of deoxyribonuclease (Miles, Illinois) for a period of 30 min at 37°C. They were subsequently washed twice and resuspended in concentrations of 5×10^6/ml. HRBC and SRBC rosettes were assayed as described. The proportions of rosettes were then expressed as percentages of those samples not treated by

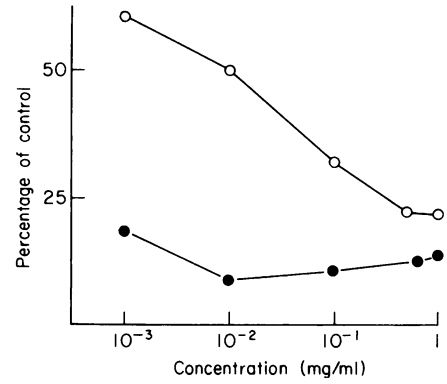

FIG. 3. Effect of trypsin on HRBC (○) and SRBC (●) rosettes.

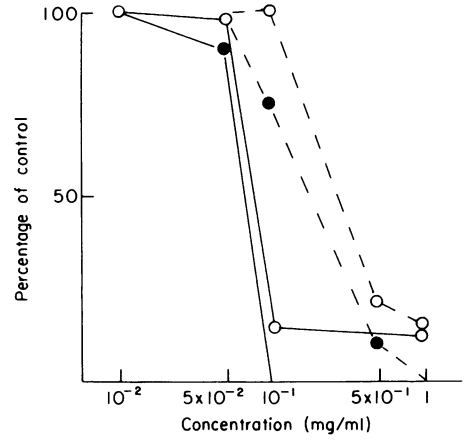

FIG. 4. Effect of pronase (—) and α-chymotrypsin (– – –) on HRBC (○) and SRBC (●) rosettes.

trypsin (control). At high concentrations, the lymphocytes failed to form the two types of rosettes. At relatively low concentrations of trypsin (1 and 10 μg/ml), the lymphocytes retained more of the ability to form HRBC rosettes than SRBC rosettes (Fig. 3).

Lymphocytes were similarly treated with α-chymotrypsin (Worthington, New Jersey) and pronase (Calbiochem, California) at concentrations ranging from 10 to 1000 μg/ml in presence of deoxyribonuclease. Again the HRBC rosettes were more resistant to these two proteolytic agents (Fig. 4).

Effect of anti-thymocyte serum

Lymphocytes were incubated with antithymocyte serum (commercial preparation of horse antihuman thymocyte gamma-globulin, Lot 16, 134–14, UpJohns, Michigan (ATS) in concentrations ranging from 1:2500 to 1:10,000 at 37°C for 60 min. HRBC and SRBC rosettes were then assayed. The proportions of rosettes were again expressed as percentages of that with no antithymocyte sera (control). The HRBC rosette-forming ability was more resistant to inhibition by this antiserum (Fig. 5).

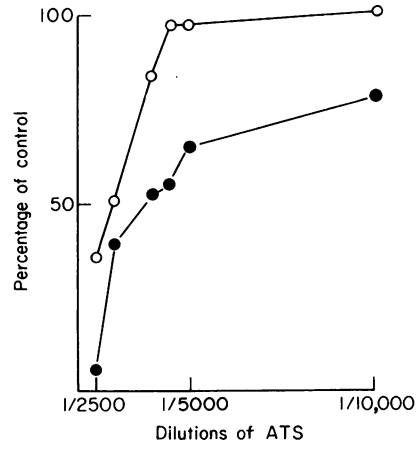

FIG. 5. Effect of anti-thymocyte serum on HRBC (○) and SRBC (●) rosettes.

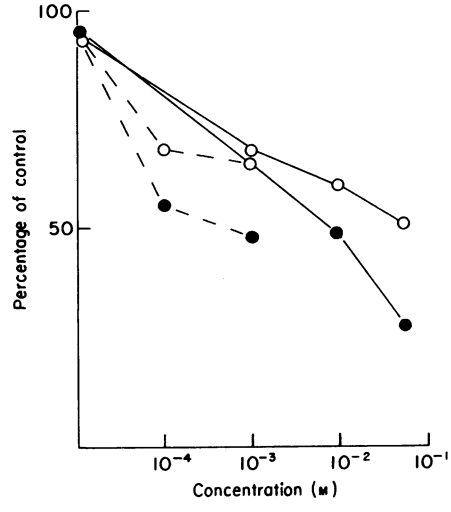

FIG. 6. Effect of sodium azide (—) and 2,4-dinitrophenol (– – –) on HRBC (○) and SRBC (●). rosettes.

Effect of metabolic inhibitors

Lymphocytes were incubated with sodium azide (Matheson, New York) and 2,4-dinitrophenol (DNP) (Eastman, New York) over a range of concentrations from 0·01 to 100 mM and 0·01 mM to 1 mM respectively at 37°C for 60 min. All cells remained viable at the end of incubation. HRBC and SRBC rosettes were assayed and expressed as percentages of control. The HRBC rosette-forming ability was more resistant to inhibition by these two inhibitors than that of SRBC rosettes (Fig. 6).

Effect of cytochalasin B

Lymphocytes were incubated with cytochalasin B (Calbiochem, LaJolla, California) at 37°C for 3 hr. HRBC and SRBC rosettes were assayed. As with other inhibiting agents HRBC rosette-forming ability was significantly more resistant to inhibition than that of SRBC rosettes (results not shown).

Recovery of rosette-forming ability after trypsinization

Lymphocytes lost some of their rosette-forming ability after trypsinization (Fig. 1). They were then incubated at 37°C in RPMI 1640 (Grand Island Biological, New York) for 6 hr. HRBC and SRBC rosettes were assayed before trypsinization, immediately after trypsinization and after 6 hr incubation. The proportion of recovery was calculated by the formula:

$$\frac{\text{rosettes at 6 hr} - \text{rosettes immediately after trypsinization}}{\text{rosettes of untrypsinized lymphocytes at 6 hr}} \times 100.$$

If the proportion of rosettes at 6 hr was less than that immediately after trypsinization, the percentage of recovery became negative in value. Hence, a negative value indicated continual loss of rosette-forming ability after trypsinization.

Results are shown in Fig. 7. There was about 30% recovery of SRBC rosettes at trypsin concentrations 1–100 μg/ml. On the other hand, at all concentrations of trypsin, the HRBC rosettes diminished after further incubation rather than recovery.

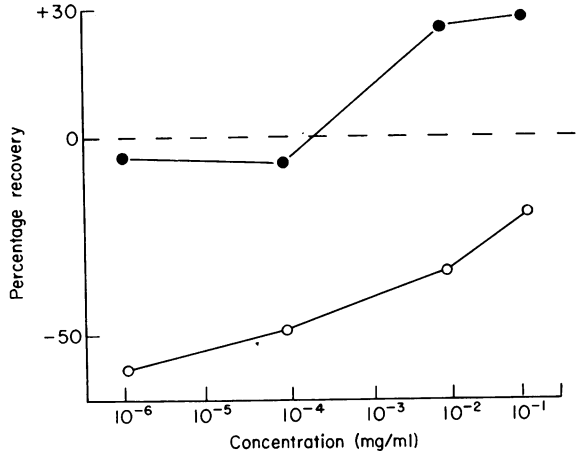

FIG. 7. HRBC (○) and SRBC (●) rosette dissociation.

DISCUSSION

Surface markers for T and B lymphocytes have been a valuable tool for analysis of immunological events (Raff, 1973). The commonly accepted markers for human T lymphocytes have been the cytotoxicity test by anti T-cell antisera (Wortis, Cooper & Brown, 1973) and the SRBC rosettes (Jondal *et al.*, 1972). Both markers, when used with optimal conditions, probably identify nearly all the peripheral blood T lymphocytes in normal individuals.

T lymphocytes, however, are composed of various subpopulations with different properties and functions. In mice, this could be differentiated by the surface density of θ antigen (Cantor, 1973). Similar surface markers are not yet available in human lymphocytes. It was reported recently that a proportion of the T cells in the peripheral blood lymphocytes could form rosettes with nHRBC (Baxley *et al.*, 1973). Although the functional significance of this was not known, it might conceivably represent a subpopulation of the T lymphocytes. The present paper deals with the technique of detecting HRBC rosettes.

Several investigators have mentioned previously that they could not detect human lymphocytes forming rosettes with HRBC. The conditions used were apparently similar to those used for forming SRBC rosettes (Jondal *et al.*, 1972). We found that, under suitable conditions, HRBC rosettes could be formed. The conditions required were more stringent than those for SRBC rosettes, so careful observance of the requirements must be made.

The medium used in these experiments for HRBC rosettes was 5% BSA. The proportions of HRBC rosettes formed increased remarkably with increasing concentrations of BSA, reaching an optimum at 5% BSA. The ability of BSA to promote cell adherence has precedence in red cell typing, where it was found to allow detection of incomplete antibodies on red cells by promoting agglutination. This property was attributed to the relatively high dielectric constant of this media and so lowering the zeta potential of the cells (Pollack, 1965). Since the surfaces of both the HRBC and lymphocytes are negatively charged, they have a natural repulsion to one another (Weiss, 1970). The BSA by reducing the zeta potential helps to overcome the repulsion.

HRBC rosette formation was also pH-dependent. The optimal pH is relatively low compared to physiological pH of 7·4. The pH of the media is another potential factor influencing the surface charge of the cells, as can be demonstrated by electrophoretic mobility. A low pH decreases the electrophoretic mobility. In this case, it may have again favoured cell adherence by lowering the natural repulsion of the cells (Seaman & Heard, 1960; Thomson & Mehrishi, 1969).

The temperature and time of incubation of HRBC rosettes were very similar to the SRBC rosettes. The formation and disssociation of SRBC rosettes had been studied by the author. It was found that at 4°C SRBC rosette dissociation and capping were partially inhibited (Yu, 1974). A similar event probably occurred in HRBC rosettes so that at 4°C, the rate of HRBC formation exceeded the rate of dissociation. This temperature dependency of HRBC rosette formation is in agreement with previous reports using nHRBC (Baxley, 1973).

Different batches of BSA and foetal calf sera were tested for their ability to promote rosette formation. Whereas there was little variation using the three different batches of BSA, all but one of the five FCS gave a lower proportion of rosettes. The contrasting effect of different batches of FCS in promoting lymphocyte reactions has also been noticed in the *in vitro* cultures for antibody production (Watson & Epstein, 1973).

Treatment of HRBC by neuraminidase promoted HRBC rosette formation even with 0·1% of BSA as media. Further, treatment of the lymphocytes by neuraminidase promoted the rosette formation even to a greater degree (Table 1). This is very similar to the effect of neuraminidase on SRBC rosette formation (Bentwich *et al.*, 1973; Gilbertsen & Metzgar, 1973). Neuraminidase removes the *N*-acetyl-neuraminic acid group from the cell surfaces. This may promote rosette formation either by the lowering of the surface charge (Shu & Kung-Ming, 1973) or by exposing more reactive groups. The specificities of these HRBC

rosettes were tested by mixed rosettes with SRBC. It was found that in all instances, the rosettes detected in the mixtures were either mixed HRBC+SRBC rosettes or pure SRBC rosettes, but seldom HRBC rosettes alone. Thus the HRBC receptors were present on lymphocytes with SRBC receptors. Since SRBC rosettes in untreated lymphocytes are T cells, HRBC rosettes in untreated lymphocytes are also T cells.

The proportions of HRBC and SRBC rosettes were assayed simultaneously in ten normal individuals. The proportion of SRBC was $73{\cdot}3 \pm 1{\cdot}4\%$ and probably accounted for most of the T lymphocytes. The proportion of HRBC rosette was similar to that reported using nHRBC instead of untreated HRBC (Baxley *et al.*, 1973). Hence, the two methods probably identified a very similar population.

In conclusion, although both were T-cell markers, the conditions for forming HRBC rosettes and SRBC rosettes were not necessarily identical. SRBC rosettes could be formed over a relatively wide range of pH and BSA concentrations while HRBC rosettes could be formed best at pH 7·0 and 5% BSA media. Further, the proportion of HRBC rosettes in peripheral blood was only about half of the total T lymphocytes detectable by SRBC rosettes. Hence, two possibilities exist. The HRBC rosettes may either be a distinct subpopulation of T lymphocytes or they were formed at random with only some of the T lymphocytes, regardless of their physiological or anatomical differences. One method to resolve this problem is to investigate the nature of the RBC receptors and their interactions with the RBC. If the HRBC rosettes represent a distinct subpopulation of the T cells, most probably the nature of the HRBC and SRBC receptors would be different from each other and so would their interaction with the RBC.

This was investigated by examining: (1) the effects of various inhibitory agents; (2) the turnover of the receptors after trypsinization.

The RBC receptors were treated with three proteolytic agents, trypsin, α-chymotrypsin and pronase. Trypsin and α-chymotrypsin are endopeptidases with different selectivity, while pronase is rather non-specific (Long, 1961). All three agents at sufficient concentrations could completely inhibit both HRBC and SRBC rosette formation. These showed that the peptide linkages affected by trypsin and α-chymotrypsin were present in both SRBC and HRBC receptors. The effect of trypsin on HRBC and SRBC rosettes has already been reported by other investigators. (Baxley *et al.*, 1973; Jondal *et al.*, 1972). Here, we also examined a dose–effect relationship. This showed that the HRBC rosettes were more resistant to trypsinization than SRBC rosettes. Similar results were obtained with α-chymotrypsin and pronase (Figs 3 and 4).

Similarly, the HRBC rosettes were more resistant to inhibition by ATS, sodium azide, 2,4-DNP and cytochalasin B. These inhibitors affect lymphocytes in different ways. ATS coats the cell surfaces and inhibits rosettes either by concealing the receptors directly or by preventing receptor–RBC interaction by steric hindrance (Bach, 1973; Brain & Gordon, 1971; Baxley *et al.*, 1973). Sodium azide and 2,4-DNP are metabolic inhibitors. Their mode of rosette inhibition were not known. They may perhaps interfere with receptor movement or turnover in the cell membrane. Cytochalasin B has also been shown to inhibit SRBC rosettes (Kersey, Hom & Buttrick, 1974). Its mode of action is also unknown, but from its effect on other cell activities, it was suggested that it could in some way modulate the membrane activities of the cells (Henney & Bubbers, 1973). Our results have shown that all these reagents inhibited both HRBC and SRBC rosette formation. However, the SRBC rosettes were adversely affected to a greater extent than the HRBC rosettes.

Receptor 'turnover' was studied by trypsinization. After trypsinization, the rosette receptors were either destroyed or rearranged on the cell surfaces so that rosette formation no longer occurred (Jondal *et al.*, 1972). The extent of this depended on the dose of trypsin. As the trypsinized cells were cultured *in vitro*, they repaired their SRBC rosette-forming ability either by regeneration of the receptors or by their re-arrangement. However, instead of recovering their ability to form HRBC rosettes, they continued to lose more of it. This is perhaps some of the strongest evidence we have found that the receptors for HRBC and SRBC behaved differently.

In conclusion, the various tests we have applied to the HRBC and SRBC rosettes demonstrated at least certain quantitative differences in the behaviour of the two types of rosettes. This can be explained by the following hypotheses: (1) the two types of rosette receptors are entirely different; (2) the receptors are identical but the cells forming the HRBC rosettes were a subgroup of T lymphocytes, perhaps because of their peculiarity in metabolic activity or the density of the receptors on the cell surfaces. Precedence of the latter has been found in murine T lymphocytes where it was observed that although the T_1 and T_2 subpopulations both carry theta antigens on their surfaces, they were more dense on the T_1 than the T_2 (Cantor, 1973).

Either hypothesis strongly suggests that HRBC rosettes are a potential marker for human T-lymphocyte subpopulation. These studies do not indicate whether there is any immunological significance in the HRBC rosette-forming cells. Evaluation of this will have to await tests of their immunological functions and analysis of their proportions in patients with various immunological diseases.

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