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Increase in autologous erythrocyte binding by T cells with ageing in man

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SUMMARY

Studies made of 115 normal subjects of both sexes aged 20–94 years showed that autologous rosette-forming cell levels in human peripheral blood lymphocytes were related to age and sex. However, no such relationship was found in response to phytohaemagglutinin performed in the same subjects. The nature of these autorosettes in human peripheral blood lymphocytes was studied. A significant decrease in autorosette levels was observed after passage through nylon wool at high RBC/WC ratios, and at any ratio after E-rosette depletion. The results suggesting a T-cell origin of autorosettes were confirmed by the existence of mixed autologous erythrocyte and sheep red blood cell rosettes.

INTRODUCTION

The cellular basis of autoimmunity and its relationship to ageing has long been a matter of extensive investigation. The demonstration of autoantigen-binding cells among normal lymphoid cells in the mouse and in man has represented an important advance in that respect. For the last 2 years we have been engaged in the study of autologous erythrocyte-binding cells and have shown that in mouse spleen or thymus some lymphocytes can bind autologous erythrocytes to form rosettes (Charreire & Bach, 1975b). A relationship between autorosette levels and thymic function has been clearly demonstrated in the mouse since the level of autorosette-forming cells (A-RFC) increases with ageing and after adult thymectomy (ATx), and can be brought back to normal values with *in vivo* treatment by thymic extracts (Charreire & Bach, 1975a). In man, the relationship between thymic function and A-RFC found in peripheral blood lymphocytes (PBL) appeared to be less clear, although an increase in autorosettes with age was noted in a preliminary study (Charreire & Bach, 1974). Several investigators have failed to observe such relationships with age and sex, probably because of differences in the techniques used (Sandilands *et al.*, 1975a; Gluckman & Montambault, 1975a).

In this paper, we have examined the role of age and sex on autorosette formation in human PBL in larger series of subjects, and the correlation between autorosette level and immunological function (assessed by PHA-induced transformation). In addition, we have studied the main A-RFC characteristics which indicate that they belong to a T-cell subset.

MATERIALS AND METHODS

Subjects. Healthy donors of both sexes (fifty-eight females and fifty-seven males), aged 20–97 years, were tested. Forty-nine were less than 50 years of age, thirty-six were between 50 and 80, and thirty over 80 years of age.

Cell isolation. Human blood was collected on heparin, diluted 1/3 in Hanks' medium and layered on a Ficoll–Angiocontrix mixture (24 vol. 9% Ficoll, Pharmacia, and 10 vol. 30% sodium iotalamate, Guerbet). After centrifugation at 400 *g* for 15 min, cells at the interface were removed and washed twice in Hanks' medium.

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Nylon filtration. The method used was based on that described by Greaves & Brown (1974). In brief, a 5 ml plastic syringe was filled with 300 mg nylon fibers (Fenwall Leukopak) previously washed in 0·2 N HCl, rinsed with distilled water and dried. The syringe-barrel column was washed with pre-warmed (37°C) Hanks' medium supplemented with 10% heat-inactivated foetal calf serum (FCS), and 5×10^7 cells in the same medium were then incubated in the column for 30 min at 37°C and eluted slowly with 15 ml of 10% FCS–Hanks at 37°C.

Autorosette test. The method used was based on that described previously in mice (Charreire & Bach, 1975b): neither lymphocytes nor erythrocytes were treated by enzymes and no serum was added. Various ratios of autologous red blood cells to white cells (A-RBC/WC) ranging from 4/1 to 128/1 were tested. Contact between erythrocytes and lymphocytes was favoured by a 5 min centrifugation at 200 *g*. After overnight incubation, rosettes were scored in a haemocytometer and their number expressed per 1000 nucleated cells. Rosettes were defined by the agglutination of at least three erythrocytes around a lymphoid cell.

E-rosette test. This test has been previously described (Bach, 1973). In brief, 3×10^7 fresh sheep erythrocytes were mixed with 10^6 mononucleated cells in Hanks' medium supplemented with 25% foetal calf serum previously adsorbed with sheep and human erythrocytes. These conditions have been shown to be optimal. The cell mixtures were incubated for 30 min at 37°C, centrifuged for 5 min at 200 *g* and stored overnight at 4°C before being resuspended and read in a haemocytometer.

Mixed rosette test. 3×10^7 sheep red blood cells and $2{\cdot}4 \times 10^7$ human autologous erythrocytes were mixed simultaneously with $1{\cdot}5 \times 10^6$ lymphocytes in Hanks' medium without serum. The rosettes were then performed as usual and scored after overnight incubation at 4°C.

Monocyte evaluation. Peroxidase staining was used (Preud'homme & Flandrin, 1974). Rosette smears were fixed with 0·25% benzidine and 0·5% sodium nitroprussiate alcoholic solution. After washing, the slides were covered for 5 min with a mixture of 1 vol. of the alcoholic solution and 1 vol. of a solution of hydrogen peroxide. The slides were stained with Giemsa after two additional washings. Peroxidase-positive cells were scored.

Surface-immunoglobulin determination. Mononucleated cell suspensions were incubated at 4°C for 30 min with a fluorescent polyvalent anti-Ig serum (Behring) and surface Ig-bearing cells were scored.

E-rosette depletion. E rosettes were set up as described, except that all volumes were multiplied by four and the incubation at 4°C did not exceed 2 hr. Rosette suspensions were layered carefully on Ficoll–Angiocontrix mixture and tubes were centrifuged at room temperature for 30 min at 400 *g*. The interface layers were collected, pooled, and washed twice in Hanks' medium.

PHA response. Stimulation with phytohaemagglutinin M (PHA M Difco) was studied in flat-bottomed microplates (Falcon No. 3040); 4×10^5 lymphocytes in Eagle's minimum essential medium (Gibco) supplemented with 2·5% antibiotics (penicillin and streptomycin) and 20% inactivated human AB serum were incubated with PHA in a final volume of 0·2 ml. Six concentrations of PHA ranging from 10 μg/ml to 1000 μg/ml were tested. Each determination was performed in quadruplicate. After a 3-day incubation at 37°C in a humidified 5% CO_2 atmosphere, the cultures were pulsed with 1 μCi of [^{3}H]thymidine for 18 hr at 37°C, and harvested on a filter with the MASH apparatus (Microbiological Associates). The amount of [^{3}H]thymidine incorporated in the cells was determined by liquid-scintillation counting. Results were expressed by the average values in counts per minute (ct/min) given by each sample.

RESULTS

Relation of A-RFC with age and sex

A-RFC numbers were evaluated in fifty-eight females (F) and fifty-seven males (M) classified in three age groups: group I, aged 20–49 (twenty-five F and twenty-four M); group II, aged 50–79 (thirteen F and twenty-three M); and group III, over 80 years of age (twenty F and ten M). Each test was done with six different A-RBC/WC ratios. Autorosette numbers were found to increase along with this ratio, particularly in older subjects. Results presented in Fig. 1 represent A-RFC levels at a RBC/WC ratio of 64/1 in the three age groups in men and women.

In each group, the A-RFC percentage was higher in women than in men. This phenomenon was observed at all A-RBC/WC ratios but was significantly only at ratios higher than 32/1 and in groups II and III. A-RFC values were respectively 9·4 per 1000 and 3·5 per 1000 in women and in men ($P<0{\cdot}01$) for group II; and 14·1 per 1000 and 6·8 per 1000 ($P<0{\cdot}05$) for group III at an A-RBC/WC ratio of 64/1 (Table 1).

An increase with age was observed for both male and female subjects, but to different extents (Table 1). However, in women, a significant increase in A-RFC levels was found when comparing group I and II but not group II and III, at an A-RBC/WC ratio of 64/1. In men, a significant augmentation of A-RFC levels was observed only after 80 years of age (Table 1).

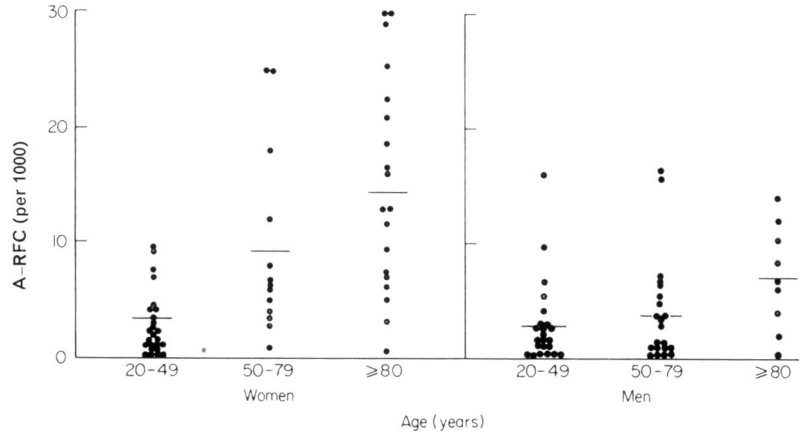

FIG. 1. Autorosette levels in three age groups of normal subjects (A-RBC/WC = 64/1).

PHA response with ageing

PHA response was studied in 124 normal subjects of various ages classified in the same age groups described above. No difference in PHA response was noted in relation to sex or age, whatever the PHA dose utilized.

Nature of human PBL A-RFC

Monocyte participation in autorosette formation. Cell suspensions isolated on a Ficoll–Angiocontrix gradient contain a mixture of mononucleated cells, including lymphocytes and monocytes, in various proportions. In order to investigate the presence of monocytes among autologous RFC, the peroxidase-staining technique was performed on rosette smears. In all suspensions containing an average of 18·1± 2·9% monocytes, no more than 3·6± 1·2% formed rosettes with autologous erythrocytes (Table 2). This small amount represents 12·0± 1·5% of total autorosettes scored. These results, observed at any

TABLE 1. Relationship of A-RFC level (per 1000 lymphocytes) with age and sex

Age (years)	Women		Men
20–49	2·60± 0·5 (25)	←n.s.→	2·5± 0·7 (24)
	↑		↑
	s.*		n.s.
	↓		↓
50–79	9·4± 2·3 (13)	←s.†→	3·5± 0·9 (23)
	↑		↑
	n.s.		s.‡
	↓		↓
80	14·1± 2·1 (20)	←s.‡→	6·8± 1·5 (10)

Ratio of A-RBC/WC = 64/1; all results mean± s.e. (by Student's *t*-test). Numbers in parentheses = number of patients for each result. n.s. = Not significant; s. = significant; arrows show which results are being compared for a significant difference.

* $P < 0{\cdot}001$; † $P < 0{\cdot}01$; ‡ $P < 0{\cdot}05$.

TABLE 2. Percentage of monocytes forming autorosettes evaluated after peroxidase staining

Experiment	Sex	Age (years)	No. cells counted	Lymphocytes (%)	Monocytes (%)	Monocytes forming rosettes (%)	
						Related to total monocytes	Related to total autorosettes
1	M	74	942	77·1	22·9	1·4	10·3
2	M	72	975	72·1	27·9	2·2	10·7
3	F	86	1023	81·7	18·3	1·4	7·9
4	F	80	1200	80·7	19·3	1·0	11·8
5	F	86	830	88·9	11·1	9·0	19·0
6	F	88	800	91·3	8·7	6·9	12·2
Average ± s.e.	—	—	—	81·9 ± 2·9	18·1 ± 2·9	3·6 ± 1·2	12·0 ± 1·5

RBC/WC ratio (ranging from 4/1 to 128/1), show a weak participation of monocytes in autorosette formation. The percentage of monocytes among A-RFC was not different in men and women.

Effect of nylon filtration. Filtration through a nylon-wool column was used to remove monocytes and B lymphocytes from cell suspensions, resulting in an enrichment of mature T cells (Greaves & Brown, 1974). In all reported experiments T lymphocytes were identified by E rosetting and B lymphocytes by fluorescent anti-Ig staining.

The results of nylon-column purification of human PBL are given in Fig. 2. In these experiments the mean incidence of surface Ig-positive cells was 26·5 ± 3·1% before filtration and 2·8 ± 1·4% after filtration. After passage through nylon wool, A-RFC levels decreased, but to a different extent according to the A-RBC/WC ratio used. At a low ratio (4/1) no change in A-RFC values was noted before or after filtration. Conversely, significant differences ($P<0·05$) were already found at a medium ratio (16/1) and became highly significant ($P<0·01$) at a high ratio (64/1). Indeed 41% of A-RFC were lost by nylon filtration at medium ratio, reaching 77% removal at a high ratio. Thus, a disparity appears in autorosettes found in human PBL according to the number of erythrocytes used for rosette formation.

Effect of E-rosette depletion. In order to assess further the T- or non-T-cell nature of autorosettes, E-rosette depletion experiments were undertaken. Most E-RFC were removed by centrifugation of the

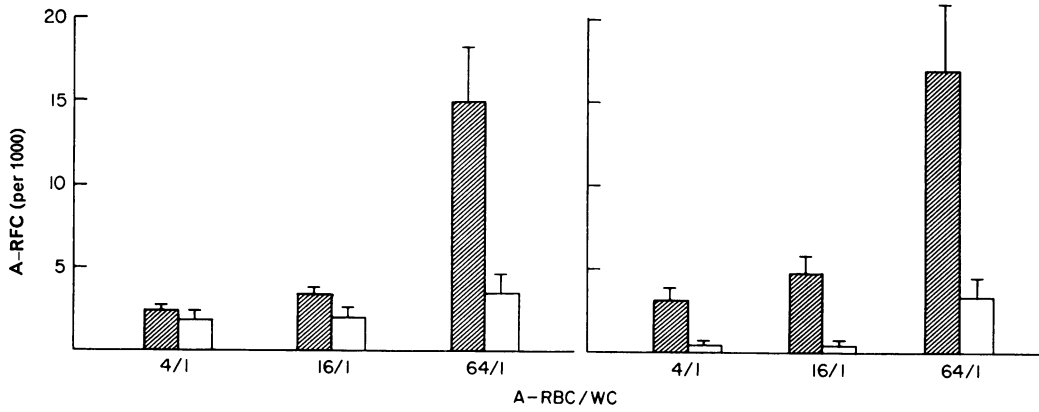

FIG. 2.

FIG. 3.

FIG. 2. Effect of nylon filtration on percentages of autorosettes (mean ± s.e. of seven experiments). (▨) Before filtration; (□) after filtration.

FIG. 3. Effect of E-rosette depletion on percentages of autorosettes (mean ± s.e. of six experiments). (▨) Before depletion; (□) after depletion.

TABLE 3. Percentage of double rosettes formed with sheep erythrocytes and human autologous erythrocytes

Rosette formation in the presence of	Cells in rosettes		
	Sheep RBC (%)	Autologous RBC (per 1000)	Sheep and autologous RBC (per 1000)
Sheep RBC	63·8±2·3	—	—
Autologous RBC	—	1·9±0·4	—
Sheep and autologous RBC	48·2±2·9	0·3±0·1	0·9±0·1

Mean ±s.e. of nineteen subjects.

E-rosette suspension on a Ficoll–Angiocontrix gradient. The preparation of control cells centrifuged on the same gradient without previous rosette formation showed no changes in autorosette levels or in percentages of T and B lymphocytes, although 50% of the initial cell number was lost.

In the experiments presented in Fig. 3, the average number of remaining T lymphocytes in the harvested cell population was 17·8±4·5%. Under these conditions, at least 80% of autorosettes disappeared. A similar loss in A-RFC numbers was observed whatever the A-RBC/WC ratio. The results show a large T-lymphocyte participation in autorosette formation.

Direct relationship with T-cells. To confirm the T-cell nature of A-RFC, mixed rosette experiments were performed with sheep erythrocytes and human autologous red blood cells. Both types of red cells were added simultaneously. Mixed rosettes were defined as at least two erythrocytes of each type bound to a lymphocyte.

Results presented in Table 3 show the existence of mixed rosettes: about 75% of A-RFC bear the double specificity demonstrating a T-cell origin of these autologous rosettes. Further evidence of the T-cell nature of A-RFC has been provided by the high incidence of autorosettes found in a thymoma. In this case, 96·5% of E rosettes were formed by these thymoma thymocytes, and autologous rosettes reached 320 per 1000 thymocytes at the high RBC/WC ratio of 128/1.

DISCUSSION

Discrepancies between our results and those of others (Baxley *et al.*, 1973; Kaplan, 1975; Gluckman & Montambault, 1975b; Sandilands *et al.*, 1975b) in the evaluation of A-RFC in PBL from healthy donors is probably explained by differences in technical parameters. In our hands, A-RFC values, where no enzyme pre-treatment is performed, are very low (from 0 to 30 per 1000 lymphocytes at higher A-RFC/WC ratios), as was also found by Baxley *et al.* (1973) who found high rosette values only after erythrocyte treatment with neuraminidase, from less than 1%–30%. In addition, it should be noted that significantly high autorosette values were only scored in our study in subjects over 49 years old, and that in each age group important fluctuations are observed.

The parallel increase in A-RFC levels and A-RFC/WC ratios suggests that autorosette-forming lymphocytes show heterogeneous avidity for autologous erythrocytes: A-RFC found at low A-RBC/WC ratios are probably more avid than those revealed at high ratios, whether or not this observation relates to differences in the number of autologous erythrocyte receptors expressed on the PBL surface.

The results reported here confirm, in man, our previous reports in mice showing an increase of autorosette levels with ageing (Charreire & Bach, 1975b). The relationship between thymus function and A-RFC demonstrated in adult thymectomized mice, however, is still difficult to establish clearly in man. Our data on cell fractionation indicate that human A-RFC are T cells, as also found by other workers (Baxley *et al.*, 1973; Kaplan, 1975; Gluckman & Montambault, 1975b; Sandilands *et al.*, 1974). More precisely, A-RFC studied by our technique might belong to a T-cell subset as indicated by the low

figures found (0·5–2%) by partial nylon retention, already described in the mouse to be a property of immature T cells (Charreire & Bach, 1975b). This latter interpretation of A-RFC as immature T cells would fit with the low A-RFC level found in PBL in contrast to the high level found in the thymus gland (Sandilands *et al.*, 1974) and on thymoma (Baxley *et al.*, 1973).

PHA responsiveness was studied simultaneously with A-RFC tests in order to assess any correlation between these two T-cell properties in aged subjects.

The similar PHA response obtained with lymphocytes from young or old donors after *in vitro* simulation is not in agreement with results obtained by Weksler & Hütteroth (1974), showing that lymphocytes from old donors present a strongly decreased response to plant mitogens and to allogeneic lymphocytes, compared with lymphocytes from young donors. However, our data are in agreement with those of Teague *et al.* (1970), who reported normal PHA response in ageing mice, or those of Hallgren *et al.* (1973) showing no more than 10% low PHA responses in subjects over 70 years of age.

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