Rosette formation in rheumatoid and non-rheumatoid states

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The principle of immunocyto-adherence (Zaalberg, 1964; Biozzi, Stiffel, Mouton, Bouthiller, and Decreusefond, 1967) has been used by Bach, Delrieu, and Delbarre (1970a, b) to demonstrate, on the surface of human lymphocytes, receptors for human red blood cells (HRBCs) coated with rabbit gamma-globulin, the red cells being bound to the lymphocytes to form rosettes. These workers found increased numbers of rosettes in patients with rheumatoid arthritis and certain other diseases, including gout, systemic lupus erythematosus, and juvenile chronic polyarthritis. This type of rosette is referred to as the rheumatoid rosette.

Other investigators (Serre, Simon, Mandin, Clot, and Sany, 1971; Waltzing and Bloch-Michel, 1971) have reached roughly similar conclusions, but Lea and Ward (1972) were unable to confirm their findings.

Lymphocyte preparations may be made by a number of different techniques, some involving filtration, others not. The process of filtration through fibre has been said to eliminate bone-marrow derived (B) lymphocytes in mice (Greaves and Hogg, 1971; Bianco, Patrick, and Nussenzweig, 1970), but it has not yet been established whether rheumatoid rosettes are due to B lymphocytes, or to thymus-dependent (T) lymphocytes.

The aims of this study were:

- (1) To apply the rheumatoid rosette test to a wide population, including subjects with rheumatic diseases.
- (2) To compare rheumatoid rosette counts using lymphocytes prepared with and without filtration.
- (3) To identify the type of cell forming rheumatoid rosettes.

Material and methods

RHEUMATOID ROSETTES

Patients

A total of 236 tests was carried out, using lymphocytes from 190 subjects made up as follows:

Healthy controls	68
Classical or definite rheumatoid arthritis	34
Probable or possible rheumatoid arthritis	16
Osteoarthritis	32
Gout	10
Miscellaneous	30

Lymphocyte separation

(a) By filtration

10 ml. heparinized blood was incubated at 37°C. for 15 min. in a 10 ml. syringe containing well-teased nylon fibre (Ref: 2B-035-B, Laboratoire Roger Bellon, 92 Neuilly, France). The blood was then allowed to run out, being washed through by 30 ml. of Hanks's balanced salt solution (HBSS). Two volumes of this cell suspension were layered on to one volume of a mixture of Ficoll and Triosil (specific gravity 1077), and centrifuged at 1,000 g. for 15 min. The interface containing a lymphocyte-rich population was aspirated, and the cells were washed three times and suspended in HBSS at a concentration of 2,000–4,000/mm³.

(b) Without filtration

To 10 ml. heparinized blood were added 30 ml. of HBSS, the mixture layered on to Ficoll-triosil, centrifuged, and the cells collected and suspended in the manner described above.

Sensitization of HRBCs

Fresh blood from a healthy donor of Group 0, D-negative, taken in acid citrate dextrose, was centrifuged and the cells were washed three times in physiological saline. The cells were suspended at a concentration of 2 per cent. in HBSS, and mixed rapidly with an equal volume of rabbit anti-human haemagglutinin (HA) (Institut Pasteur, Paris) of three different concentrations: that used for the Waaler Rose test in the detection of rheumatoid factor (HA1), and a half (HA½) and a quarter (HA¼) of that concentration (In earlier tests, HA½ and HA¼ were used; in later tests HA1 and HA½). The mixtures were incubated for 60 min. at 37°C., and the sensitized cells were washed three times and re-suspended in HBSS at 2 per cent. Sensitization was confirmed by passive haemagglutination.

Rheumatoid rosette formation

Haemolysis tubes were prepared, containing:

- 0.2 ml. lymphocyte suspension (filtered or unfiltered)
- 0.1 ml. sensitized HRBC suspension
- 0.1 ml. HBSS.

The tubes were centrifuged at 200 g. for 5 min. and the cells re-suspended by turning the tubes slowly (10 r.p.m.) for 10 min., on a Multipurpose Rotator (Scientific Industries, Springfield, Mass.)

The rosettes were then counted in a haemocytometer, and the results expressed as the number of rosettes per 1,000 lymphocytes. A rheumatoid rosette was identified as a lymphocyte with four or more HRBCs bound to its surface.

Slide preparations were also made in some cases by the use of a cytocentrifuge (Shandon) and the smears stained and examined to show the morphology of the cells.

It was repeatedly confirmed that no rosettes were formed if the HRBCs were not sensitized. This was so both with filtered and unfiltered lymphocytes.

Reproducibility

Three categories of rosette count were defined: 0 to 6, 6 to 10, and more than 10 rosettes per 1,000 lymphocytes.

Using filtered lymphocytes, the rheumatoid rosette test was repeated in each of thirteen subjects, each pair of tests being done with HRBCs sensitized with two dilutions of HA, so that 26 pairs of tests could be compared. The reproducibility was quite good. Of the 26 paired tests, there was no change in category in nineteen.

Using unfiltered lymphocytes, the rosette test was repeated four to eight times in only three subjects, and so the results were less meaningful. Of the twelve paired tests, there was no change in category in seven.

SPONTANEOUS ROSETTES

Uncoated sheep red blood cells (SRBCs) were used, the method of Lay, Mendes, Bianco, and Nussenzweig (1971) being followed. Only those lymphocytes binding five or more SRBCs were counted as rosettes.

COMBINED ROSETTES

To lymphocytes prepared without filtration were added both sensitized HRBCs and unsensitized SRBCs. The two sets of red blood cells were suspended in HBSS in concentrations of $4 \times 10^4/\text{mm}^3$, and equal volumes of each added to the lymphocyte suspension to give a ratio of 40 to 60 erythrocytes to each lymphocyte. The tubes were then treated as for spontaneous rosettes; they were centrifuged at 200 g. for 5 min., kept in an ice bath for 60 min., and then agitated gently to resuspend the cells. Rosettes were counted and typed both in a haemo-

cytometer, and in dried preparations on slides. SRBCs were readily distinguished from HRBCs by their smaller size.

Results

RHEUMATOID ROSETTES

In the rheumatoid rosette test, with filtered lymphocytes (Table I), no clear difference could be found between normal and abnormal subjects, nor between the clinical groups.

A separate comparison was made of classical or definite rheumatoid arthritis with probable or possible rheumatoid arthritis, using filtered lymphocytes. The results are shown in Table II. The difference was not statistically significant ($P > 0.4$ for HA₁, $P > 0.8$ for HA₂).

Table II Comparison of rheumatoid rosettes in classical or definite rheumatoid arthritis and probable or possible rheumatoid arthritis

Diagnosis	No. of tests	Mean rosette count/ 10^3 lymphocytes	
		HA ₁	HA ₂
Classical or definite rheumatoid arthritis	16	16.7	7.6
Probable or possible rheumatoid arthritis	8	12.4	9.1

Two series were carried out comparing filtered and unfiltered lymphocytes. In the first, the two groups were made up of different individuals; in the second, the individuals were the same in each group. In the first series, 181 tests were done. There were consistently fewer rosettes using filtered lymphocytes, the mean count being markedly reduced (Table III). In the second series, filtered and unfiltered lymphocytes from the same individuals were compared for rosette formation with red cells sensitized at one or two concentrations of HA. In all, 29 such comparisons

Table I Mean rheumatoid rosette counts and the significance of the difference between the means of patient groups and controls

Diagnosis	No. of tests	Mean rosette count/ 10^3 lymphs HA ₁	S.D. of difference between means of patient groups and controls	Mean rosette count/ 10^3 lymphs HA ₂	S.D. of difference between means of patient groups and controls
Rheumatoid arthritis	16	16.7	5.8 $P > 0.7$	7.6	15.8 $P > 0.7$
Osteoarthritis	26	24.9	26.8 $P > 0.7$	13.3	5.3 $P > 0.9$
Gout	4	33.2	16.3 $P > 0.2$	23.8	9.1 $P > 0.2$
Controls	21	15.2	—	12.4	—

were made. Again the mean rosette count was considerably less with filtered lymphocytes (Fig. 1).

COMBINED ROSETTES

These were made with lymphocytes from two subjects; one healthy, and one with ankylosing spondylitis.

In the fresh preparations examined in the haemocytometer, there was no instance, in either case, of a lymphocyte binding both human and sheep erythrocytes (Table IV, opposite).

It was possible to make cytocentrifuge preparations only from subject II and, because of the higher cell density, a greater number of rosettes could be examined than in the fresh preparations. Of a total of 206 rosettes, 135 were spontaneous (sheep) rosettes, 49 were rheumatoid (human) rosettes, and 22 were mixed rosettes (defined as a lymphocyte bearing four or more of one type of red cell and any number of the other type).

The make-up of these 22 mixed rosettes can be seen in Fig. 2 (opposite).

Discussion

Comparing the rheumatoid rosette counts, using lymphocytes prepared with and without filtration, it is clear that filtration removes large numbers of rosette-forming cells. It is known that more monocytes may contaminate lymphocytes prepared without filtration, and indeed in cytocentrifuge preparations we have observed some rosettes formed by cells with

FIG. 1 Rheumatoid rosette counts using filtered and unfiltered lymphocytes in 29 comparisons. The mean count fell from 14.3 (unfiltered) to 6.5 (filtered)

Table III Comparison of rheumatoid rosette counts using filtered and unfiltered lymphocytes (First series)

Diagnosis	Lymphocytes	No. of tests		Mean rosette count/ 10^3 lymphocytes			
		HA $\frac{1}{2}$	HA $\frac{1}{4}$	Filtered		Unfiltered	
				HA $\frac{1}{2}$	HA $\frac{1}{4}$	HA $\frac{1}{2}$	HA $\frac{1}{4}$
Rheumatoid arthritis	Filtered	20	20	3.7	0.7	21.0	7.7
	Unfiltered	5	5				
Osteoarthritis	Filtered	5	5	4.0	0.5	7.5	3.4
	Unfiltered	3	3				
Gout	Filtered	8	8	4.2	0.1	22.8	8.3
	Unfiltered	3	3				
Controls	Filtered	26	17	8.6	1.6	22.1	14.3
	Unfiltered	30	20				
Total		100	81	20.5	2.9	73.4	33.7
Mean				5.1	0.7	18.3	8.4

Table IV *Fresh preparations of rheumatoid, spontaneous, and combined rosettes in two subjects*

Subject	Rheumatoid rosettes/ 10^3 lymphocytes		Spontaneous rosettes/ 10^3 lymphs	Combined rosettes			
				HA1/sheep RBCs		HA $\frac{1}{2}$ /sheep RBCs	
	HA1	HA $\frac{1}{2}$		Lymphs/ 10^3 binding human RBCs	Lymphs/ 10^3 binding sheep RBCs	Lymphs/ 10^3 binding human RBCs	Lymphs/ 10^3 binding sheep RBCs
I (Healthy)	14.2	9.3	290	3.8	260	2.6	290
II (Ankylosing spondylitis)	6.2	4.1	370	6.7	113	13.0	110

FIG. 2 *Numbers of human and sheep RBCs in mixed rosettes*

the morphology of monocytes. But these would account for only very few of the increased numbers of rosettes seen with unfiltered cells, by far the majority being due to lymphocytes. It has been shown that, in mice, B lymphocytes are eliminated by filtration (Greaves and Hogg, 1971; Bianco and others, 1970), and it may be that, in man, B lymphocytes are responsible for rheumatoid rosettes. If this is so, it would seem inappropriate to perform the rheumatoid rosette test as originally proposed (Bach and others, 1970a), using lymphocytes specifically depleted of the type most likely to produce this phenomenon.

The results of the combined rosette test support the conclusion that rheumatoid rosettes are largely due to B lymphocytes. Spontaneous rosette formation with SRBCs has been shown to be a specific property of T lymphocytes (Lay and others, 1971; Jondal, Holm, and Wigzell, 1972; Wybran, Carr, and Fudenberg, 1972). The fact that very few lymphocytes bound both SRBCs and HRBCs shows that the great majority of rheumatoid rosettes do not have the rosetting properties of T cells.

In the cytocentrifuge preparations, only 22 mixed rosettes were seen. Of these, only five bore four or more HRBCs on their surfaces, thereby being classed

as rheumatoid rosettes, and of these five, only two bound more than four SRBCs. Therefore, of the 54 lymphocytes binding HRBCs, 52 (96 per cent.) were B lymphocytes, and only two were T lymphocytes.

Rheumatoid rosette tests were done chiefly on lymphocytes from patients with rheumatoid arthritis, osteoarthritis, and gout, and from healthy controls, and no distinguishing features emerged from this study. Most of these tests were done using filtered lymphocytes which, it now seems, consisted largely of T lymphocytes. But despite the indications that rheumatoid rosettes are due almost entirely to B lymphocytes, we were unable to show increased rheumatoid rosette counts in rheumatoid arthritis even with unfiltered lymphocytes.

Since rheumatoid rosettes have been found in all groups, it must be assumed that this is a general phenomenon, and that the rosettes are not rheumatoid at all. This might be explained by the existence of receptors for IgG, such as have been demonstrated on lymphocytes from mice and chickens by Basten, Miller, Sprent, and Pye (1972) and Basten, Warner, and Mandel (1972) and more precisely identified as receptors for Fc by Paraskevas, Lee, Orr, and Israels (1972).

Summary

Rheumatoid rosette formation was investigated in 236 tests, using lymphocytes from 190 subjects. Filtration of the blood to produce the lymphocyte suspension eliminated a high proportion of rosette-forming cells. That the eliminated cells were B lymphocytes is supported by the demonstration that, using unfiltered lymphocytes, only two of 54 rheumatoid rosettes were due to lymphocytes identified as T cells by spontaneous rosette formation.

The rheumatoid rosette count was not increased in patients with rheumatoid arthritis, nor in osteoarthritis, nor gout.

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