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# Increased EA-rosette formation by lymphocytes from patients with rheumatoid arthritis

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SUMMARY

A modified method for estimating erythrocyte-antibody (EA) rosette formation of human peripheral blood lymphocytes (PBL) reveals consistent differences between rheumatoid arthritis (RA) patients tested and healthy control subjects. Using this method we find an average of $27 \pm 0{\cdot}8\%$ (standard error of mean) of PBL from 120 RA patients forming EA rosettes in contrast to only $6 \pm 0{\cdot}6\%$ of PBL from ninety-five healthy controls, and $7 \pm 0{\cdot}9\%$ from eighteen patients with systemic lupus erythematosus (SLE).

This difference is not due to monocytes forming EA rosettes or to T-cell sheep red blood cell (SRBC) binding. The concentration of antibody used in our assay appears to highlight the RA–control differences—suggesting a possible qualitative difference in EA-binding capacity. We find no correlation between EA binding and disease duration or rheumatoid factor titre.

The assay is susceptible to technical variation, and the effects of antibody concentration, lymphocyte to SRBC ratio, method of blood collection and lymphocyte-separation procedure have all been evaluated.

## INTRODUCTION

There is considerable evidence that lymphocytes play a direct active role in the pathogenesis of RA. Lymphocyte infiltration of the synovia of affected joints is a well recognized concomitant in RA. Drainage of thoracic duct lymphocytes can alleviate acute arthritic episodes which recur when the cells are returned to the patient intravenously (Pearson, Paulus & Machleder, 1975). These observations, together with the increased understanding of the nature of lymphocytes and their various subpopulations, have led many workers to examine lymphocyte populations and their activities in RA patients. Much of this work is conflicting, possibly for methodological reasons. Amongst the most interesting is the observation of Bach, Delrieu & Delbarre (1970) that in patients with RA the proportion of PBL which shows *in vitro* binding of antibody-sensitized red cells (EA) to form rosettes is increased. They found that in normal subjects $0{\cdot}6\%$ or less of PBLs formed EA rosettes, but higher values occurred in $70\%$ of patients with RA. This work was confirmed by some investigators (Sany *et al.*, 1975; Waltzing & Bloch-Michel, 1971) but not by others (Durance, Micheli & Fallet, 1974; Lea & Ward, 1972).

The present paper is a reappraisal of the work of Bach *et al.* (1970), using greatly modified methods. We have shown that in almost all RA patients studied the population of lymphocytes in the peripheral blood which form EA rosettes *in vitro* is significantly increased. In contrast this population is very low in healthy controls and in patients with SLE. EA rosettes formed by monocytes and SRBC rosettes with T lymphocytes do not make a significant contribution to this phenomenon. The method described provides an assay which is consistently abnormal in both sero-positive and sero-negative RA patients and which is not influenced by the severity or duration of disease.

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## MATERIALS AND METHODS

120 patients with the diagnosis of definite or classical RA on American Rheumatism Association (ARA) criteria (1958) were studied. Eighty of these patients had a significant level of IgM rheumatoid factor in their serum (as assessed by Rose Waaler assay, after the method of Whillans & Fischman, 1958). Eighteen patients with SLE (on ARA criteria) and ninety-five healthy control subjects (comprising hospital personnel and out-patients with non-arthritic disease) were also tested.

*Isolation of lymphocytes.* 10 ml of venous blood was defibrinated in a flask containing 15 × 3 mm glass beads. Lymphocytes were then isolated by the method of Harris & Ukaejiofo (1970) with minor modifications. Blood diluted 1:4 with 0·9% saline was layered onto 10 ml of a Ficoll–Hypaque gradient [86 ml of 7·83% Ficoll (Pharmacia) mixed with 20 ml of 50% Hypaque (Winthrop)] and then centrifuged at 7700 *g* for 8 min at 18°C. Lymphocytes which collected just below the serum gradient interface were harvested with a Pasteur pipette, washed twice with 0·9% saline, centrifuged for 8 sec at 7700 *g* before being resuspended in saline to a final concentration of $8 \times 10^6$/ml. Viability was assessed by trypan blue exclusion. Usually > 90% of the cells excluded the dye. To exclude the possibility of a significant selectivity of lymphocyte subpopulations by the Ficoll–Hypaque separation procedure, seven experiments were carried out isolating lymphocytes on Ficoll–Hypaque and, in addition, by a gelatin-sedimentation polystyrene-bead column method (Thomson, Bull & Robinson, 1966).

To examine the effect of collection methods, blood was collected on occasions into EDTA and heparin tubes, and lymphocytes were isolated through Ficoll–Hypaque gradients.

*Preparation of antibody-sensitized SRBC.* SRBC were collected in Alsever's solution (Campbell *et al.*, 1970), stored for at least 1 week before use and, when required, were washed three to six times in calcium- and magnesium-free veronal-buffered saline (VBS) at pH 7·2, prepared 24 hr in advance. A 1% suspension of SRBC was made in a 1:2000 dilution of rabbit anti-SRBC antibody (Rabbit haemolytic serum, Burroughs Wellcome VD 15) in VBS.

This mixture was incubated for 30 min at 37°C. The cells were then washed three times in VBS, centrifuging at 600 *g* for 10 min. Control SRBC were prepared in the same manner, but no haemolytic serum was used.

The effect of varying concentrations of antibody was investigated by sensitizing the SRBC with a range of rabbit haemolytic serum concentrations from 1:100 to 1:20,000.

*Formation of EA rosettes.* In the standard assay, rosettes were prepared by mixing 0·1 ml of lymphocyte suspension ($8 \times 10^5$ lymphocytes) with 0·1 ml of SRBC suspension ($2 \times 10^7$ SRBC) in 0·1 ml of 0·9% saline using round bottomed siliconized glass tubes (internal diameter 13 × 100 mm). These were then centrifuged at 200 *g* for 8 min at room temperature. Immediately before counting, the pellet was resuspended by gently shaking the tube and carefully syringing the suspension three times with a Pasteur pipette. One drop of this suspension was mixed on a slide with one drop of 0·001% acridine orange in VBS. A cover slip was added and the slide was examined for rosette formation using a combination of incident u.v. light with transmitted tungsten light under a Zeiss standard 14 microscope. A lymphocyte with three or more adherent SRBC was counted as a rosette. Monocytes were excluded from the count on the basis of their size and morphology. Acridine orange binds to DNA and RNA and fluoresces under UV light. This highlights nuclear morphology, and aids in the distinction between lymphocytes and monocytes, and makes non-rosetting lymphocytes more clearly visible (Brøstoff, 1974). In some experiments monocytes were distinguished by smearing the rosette preparation on a glass slide with foetal calf serum (FCS), air drying and then staining for myeloperoxidase activity (Undritz, 1973).

In each subject both EA rosettes and non-sensitized SRBC rosettes (E rosettes) were assayed. 200 lymphocytes were counted in each sample. The net EA-rosette count was determined by subtracting the E rosette count from the total number of lymphocytes forming EA rosettes. Samples from a healthy subject and from a patient with proven RA were used as controls with each batch of tests.

The effect of lymphocyte to SRBC ratio on rosette formation was examined over the range 1:5–1:100 by varying the lymphocyte suspension.

## RESULTS

### *EA-rosette incidence with disease*

The proportion of PBLs forming EA rosettes is shown in Fig. 1. Results are grouped according to diagnosis. Each point represents the assay on one subject and repeat samples are not shown. A mean of $6 \pm 0{\cdot}6\%$ of PBL from healthy donors formed EA rosettes. In contrast EA rosette formation by PBL was significantly raised in RA—a mean of $27 \pm 0{\cdot}8\%$. There is very little overlap between these two groups.

Some results have been expressed as absolute numbers of EA rosette-forming cells/ml of whole blood as well as percentage of PBL-forming rosettes. Fig. 2 demonstrates that this modification has not affected the distinction between RA and control data ($P < 0{\cdot}0005$ for absolute EA counts). The trend in the early results led us to define a negative result as 14% or less and a positive result as ⩾ 18%. Any tests giving figures in between were retested. About one-third of the assays were read blind.

Many people have been tested repeatedly. 7% of subjects showed a change of category in EA-rosette formation on different occasions. This 7% comprised six healthy control subjects having EA-rosette results above 14% (three in the RA range) and 10 RA patients showing EA-rosette formation below 18% (six in the control range) on first testing. On each occasion retesting in conjunction with known negative and positive controls showed EA-rosette results within the expected range.

A mean of 3% of PBL from RA patients and 5% of PBL from control subjects formed rosettes with unsensitized SRBC under the conditions used for this assay.

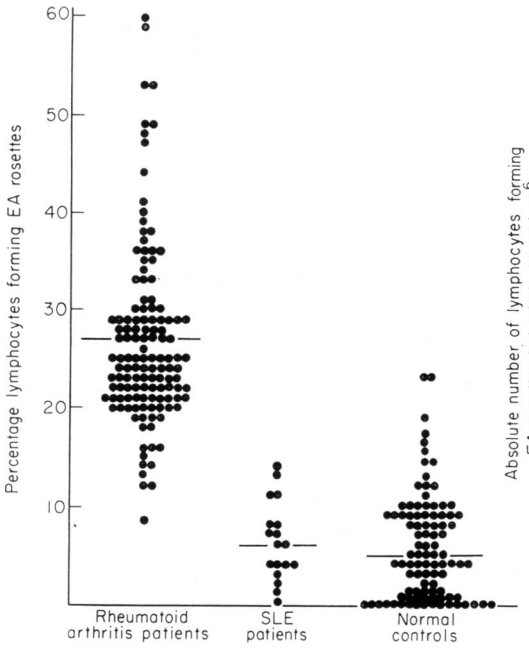


FIG. 1.

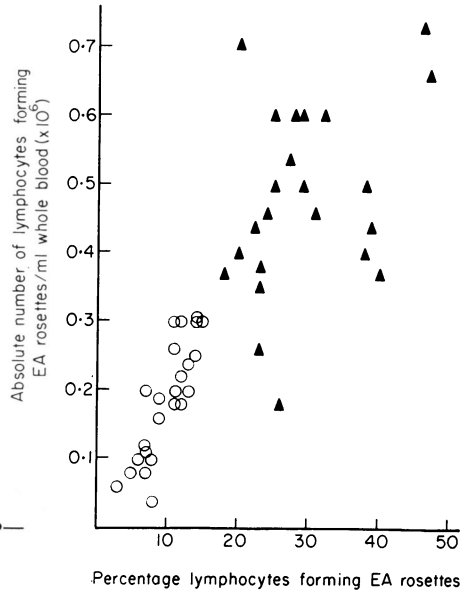


FIG. 2.

FIG. 1. Percentage of lymphocytes forming EA rosettes. Each point represents the net EA-rosette count for a single individual and results are grouped according to diagnosis.

FIG. 2. Comparison of the percentage of EA-binding lymphocytes with the absolute number of EA-binding lymphocytes/ml of whole blood in RA patients (▲) and in normal controls (○).

PBL from SLE patients showed EA-rosette figures within the normal range—a mean of 7±0·9%. Preliminary results with small groups of patients with Reiter's disease, psoriatic arthritis and Heberden's osteoarthropathy indicate that their PBL EA-rosette incidence follows the raised pattern seen with RA patients. In contrast, PBL from patients with monoarticular osteoarthritis, septic arthritis, ankylosing spondylitis and tuberculosis showed normal EA-rosette numbers. SRBC treated with normal rabbit serum did not show significant rosette formation.

*Influence of rheumatoid factor in vivo*

The presence of rheumatoid factor *in vivo* does not appear to influence rosette formation *in vitro*. Both sero-positive and sero-negative RA patients showed similar rosette numbers. There was no correlation between titre of rheumatoid factor (Rose Waaler) and proportion of PBL-forming EA rosettes (Fig. 3).

*Duration of disease*

No relationship between duration of disease and incidence of EA rosettes was apparent (Fig. 4).

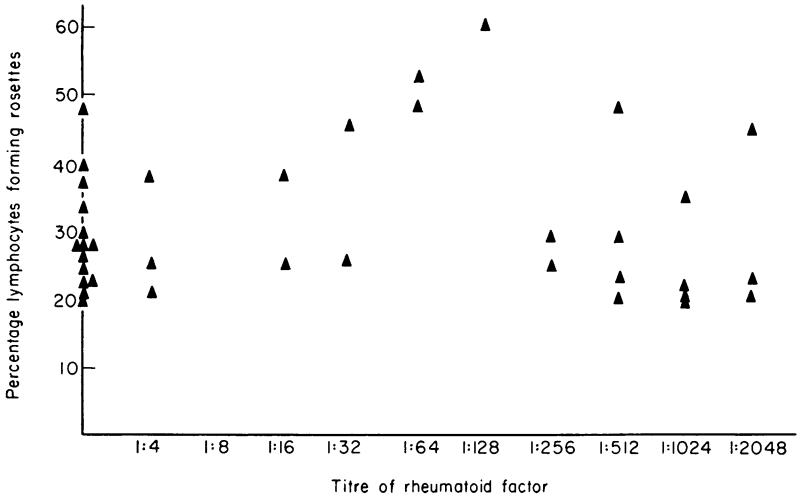


FIG. 3. Relationship between the titre of rheumatoid factor and the percentage of EA-binding lymphocytes from RA patients.

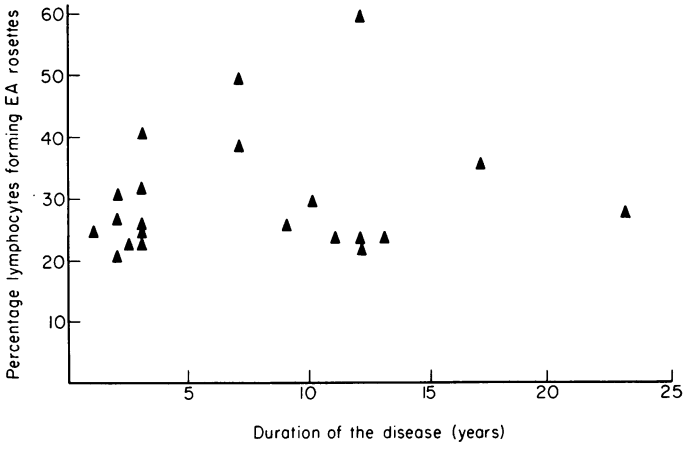


FIG. 4. The duration of disease related to EA-rosette formation of RA patients.

TABLE 1. Effects of different methods of blood collection

| | Percentage of lymphocytes forming rosettes | | | | | |
|---|---|---|---|---|---|---|
| | Controls | | | RA patients | | |
| | E | EA | EA–E | E | EA | EA–E |
| Method of collection | | | | | | |
| Defibrination | 5±2 | 16±3 | 11±4 | 3±2 | 29±2 | 26±3 |
| EDTA | 2±1 | 12±1 | 10±1 | 0±0·5 | 20±2 | 20±2 |
| Heparin | 4±2 | 10±4 | 6±3 | 2±1 | 26±4 | 24±1 |

All results are the arithmetic mean of four experiments ± the standard error. Significance was assessed by the Student's *t*-test. There was no statistically significant difference in EA binding by lymphocytes collected by the three methods used.

*Collection of samples*

Table 1 shows a comparison between EA-rosette numbers obtained from defibrinated blood and from matching blood samples collected into EDTA or heparin. No significant difference in numbers of PBL-forming EA rosettes was seen. The binding between SRBC and lymphocytes from some RA patients was weaker in the presence of platelets.

*Influence of lymphocyte-separation technique*

Yields of lymphocytes from the standard direct-spin method employed were low in many cases, averaging 41% for normal subjects and 40% for RA patients. To exclude possible selective loss of lymphocytes during purification, in seven experiments lymphocytes were separated from duplicate samples by the standard technique and by the gelatine-sedimentation polystyrene-bead column method, where yields of lymphocytes average 50–60% and were almost monocyte-free. No significant difference in EA-rosette numbers was seen between PBL separated by the two techniques—EA rosettes averaging 23% and 26% for RA PBL separated by the single spin and polystyrene-column methods respectively, and 7% and 9% for control subjects.

*Concentration of sensitizing antibody*

The effect of varying the concentration of antibody used to sensitize the SRBC has been examined and results are shown in Fig. 5. Differences between EA-rosette-forming PBL from RA patients and

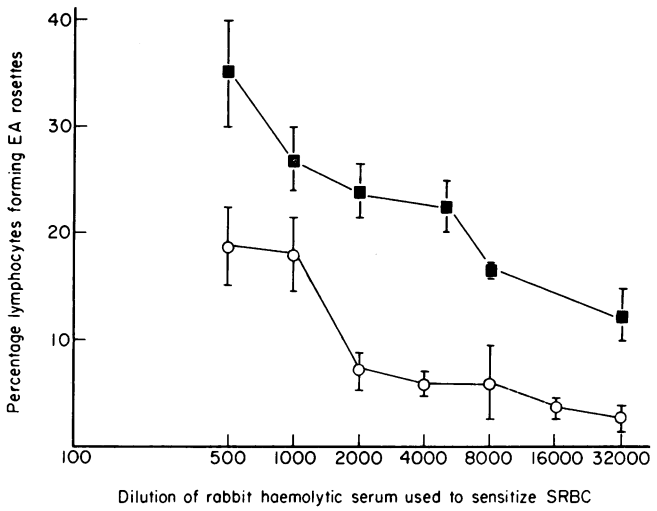


FIG. 5. Effect of concentration of sensitizing antibody on lymphocyte EA binding. Each point represents the mean ± standard error of the mean of nine experiments. RA patients (■); controls (○).

controls exist throughout the sensitizing antibody concentration range from 1:500 down to 1:20,000. The proportion of lymphocytes forming EA rosettes increases with concentrations above 1:2000, and with control lymphocytes rises from a mean of 6% to 17% at 1:500.

*Lymphocyte to SRBC ratio*

EA-rosette formation is not significantly affected by varying the lymphocyte to SRBC ratio unless the ratio falls to less than 1:10 when rosette numbers decline.

*Monocyte contamination*

Myelo-peroxidase stains have shown the monocyte contamination of our lymphocyte preparations to be low, i.e. mean 5% in control preparations and mean 10% in those from RA patients. At 1:2000

dilution of sensitizing antibody, the dilution used in the standard assay, only 11% of monocytes in the control preparation and 14% of those from RA patients were able to form rosettes.

## DISCUSSION

Peripheral blood lymphocytes from patients with RA consistently formed more EA rosettes *in vitro* than PBL from healthy control subjects or patients with SLE. Little overlap was observed between healthy controls and patients with RA. Subjects in the overlap range were usually retested, with rosette formation returning to the expected range, suggesting a transient deviation of rosetting activity. This distinction between EA-rosette formation in RA and control patients existed whether the results were expressed as percentage of PBL-forming rosettes or as absolute numbers of PBL-forming rosettes per ml of whole blood. There was no correlation between EA-rosette numbers and duration of RA or the presence of IgM rheumatoid factor.

The procedure described identifies a subpopulation of PBLs which seems very likely to be implicated in the pathogenesis of RA. In addition, this relatively simple assay offers clinical information of particular value in the diagnosis of sero-negative RA and in the distinction between SLE and RA. The significance of results in other forms of arthritis remain unclear and larger numbers are being studied.

In proceeding to characterize the altered EA-binding capacity found in RA lymphocytes we first examined three major potential sources of error—monocyte contamination, direct T-lymphocyte–SRBC binding and selectivity of our lymphocyte collection procedure.

*Monocyte contamination.* The purification method used for most experiments provides lymphocytes which are contaminated with low numbers of monocytes. Monocytes also carry Fc receptors and so are capable of forming EA rosettes (Berken & Benacerraf, 1966). This introduces a potential source of error as our results are expressed as EA rosettes formed by PBL. Within the limits of the technique monocytes are excluded from the counts on the basis of their size and morphology as demonstrated with acridine orange. However, it is likely that some monocyte EA rosettes might be included in the counts when their morphology was obscured by many adherent red blood cells.

It is clear on two grounds that monocyte rosettes have contributed very little to our recorded EA-rosette numbers. Myelo-peroxidase stains of smears of the rosette population revealed that, with the low concentration of sensitizing antibody employed in our routine assay, only a small percentage of monocytes are able to form rosettes (11% in normal controls and 14% in RA patients). Thus the maximum error, assuming that all monocyte rosettes were counted as lymphocyte rosettes, would be 3% in RA patients and 1% in controls. Secondly, when lymphocytes were purified by the polystyrene-adherence column method, less than 1% of the effluent cells were monocytes. Yet as many or more of the lymphocytes prepared in this manner formed EA rosettes compared with lymphocytes purified by the standard gradient-centrifugation technique.

*T-lymphocyte SRBC rosettes.* Problems in interpretation may also arise because of binding of SRBC by T lymphocytes. Under appropriate technical circumstances almost all T cells will form rosettes with unsensitized SRBC, and thus might largely obscure the EA-rosette numbers. We have therefore set the technical circumstances of our assay to exclude most E-rosettes. This phenomenon is influenced by (a) particular batches of SRBC used (personal observation); (b) temperature changes during assay where 37°C incubation then cooling to 4°C gives strongest union (Jondal, Holm & Wigzell, 1972; Lay *et al.*, 1971; Pang, Baguley & Wilson, 1974); (c) ion constituents of the supporting medium, where divalent cations greatly improve the strength of E-rosette binding (Jondal *et al.*, 1972; Pang, Wiggins & Wilson, 1975; Wybran *et al.*, 1975); (d) duration of incubation during the assay procedure.

By selecting isotonic saline and $Ca^{2+}$ and $Mg^{2+}$ free VBS as the supporting medium, preparing rosette at 18°C and resuspending the cells from the pellet immediately after centrifugation, the proportion of PBL forming E rosettes is reduced to a very low level (5% for controls, 3% for RA patients).

Further evidence showing that the RA–control differences in rosette formation are almost entirely a result of EA binding by PBL is presented in a subsequent paper where we show trypsin treatment of

PBL before rosette formation (a procedure which abolishes the E-rosette phenomenon; Jondal *et al.*, 1972) did not alter EA-rosette numbers.

Finally, pilot studies, recently performed using the method of Hallberg, Gurner & Coombs (1973) employing ox red cells rather than SRBC as the indicator cell for EA-rosette formation, indicate that the RA–control difference for lymphocytes binding EA is independent of the red cell marker system used. These results will be presented in detail in a later communication.

*Comparison with results from other groups.* A number of investigations, following the original observation by Bach *et al.* (1970), have resulted in conflicting reports (Sany *et al.*, 1975; Waltzing & Bloch-Michel, 1971; Durance *et al.* 1974; Lea & Ward, 1972).

Our results demonstrate much higher numbers of PBL-forming EA rosettes than found by Bach *et al.* (1970) (e.g. 12–50% compared with 0·6–2·5% in RA). EA-rosette formation is one of a number of techniques for identifying cells with receptors for the Fc portion of IgG (Froland, Wisløff & Michaelsen, 1974; Hallberg, 1974; Dickler, 1974; Basten, Warner & Mandel, 1972). By different methods and techniques, up to 59% of PBL from healthy donors have been shown to carry Fc receptors (Hallberg *et al.*, 1973). At the 1:2000 concentration of sensitizing antibody used in this assay we find an average of 6% of normal PBL formed EA rosettes, suggesting the method detects only a proportion of cells with Fc receptors. By increasing the concentration of sensitizing antibody the proportion of normal lymphocytes forming rosettes increases to $17 \pm 4\%$, indicating that more Fc receptors could be detected (Fig. 3). Thus any comparison of results from different investigators must take cognisance of the antibody concentration on the indicator cells. Bach's group (1970) used a low concentration of antibody for sensitization, and as we have shown rosette numbers are sharply reduced as antibody concentration is decreased.

Examination of the lymphocyte : SRBC ratios used for EA-rosette formation showed that optimal rosette numbers occurred when SRBC outnumbered lymphocytes by at least 10:1. The method of Bach *et al.* (1970) employed a suboptimal ratio of 5:1. The WHO Technical Report (1974) on detection of human lymphocyte receptors advocated L:RBC ratios over 1:20. Our routine method uses 1:25.

The choice of the indicator cell used in the assay is important. Bach *et al.* (1970) used human red cells coated with anti-Rh antibody. Some investigators have found this system unsatisfactory while others have detected a mean of 39·2% human lymphocytes forming EA rosettes (Froland & Natvig, 1973), emphasizing that major differences may occur with modifications in technique.

*Possible selectivity of lymphocyte separation methods.* The difference in EA-rosette formation between PBL from RA and control or SLE subjects might arise because of selection out of subpopulations of lymphocytes by the purification method used. Although such selection would still be of considerable interest, its interpretation might depend on the characteristics of the missing cells rather than those remaining for the assay. WHO technical memoranda on identification of populations of human lymphocytes advise against defibrination as a preliminary to cell separation methods (WHO/IARC Sponsored Workshop on Human B and T Cells, 1974). There appeared little difference in our results whether blood was defibrinated or collected into heparin or EDTA. Platelets in non-defibrinated blood interfered with the strength of rosette formation by apparently competing with EA for the EA-binding sites on lymphocytes but the numbers of binding lymphocytes were not significantly altered. When a purification method not employing gradient centrifugation (polystyrene-bead column) was used there was no significant difference in EA-rosette formation. Thus the observed differences in EA-rosette formation seem very unlikely to be due to the use of a selective purification method.

Having excluded the important areas of technical difficulty we are left with a highly significant increase in the EA-rosetting activity of PBL from RA patients. Fig. 3 shows clearly that the selection of a 1:2000 concentration of antibody to sensitize the SRBC results in a low proportion of PBL from control subjects forming EA rosettes and exaggerates the differences between the RA and control lymphocytes. This suggests that there is also a qualitative difference in the EA binding.

The nature of the lymphocyte EA receptor, and the observed qualitative difference are examined in the succeeding paper.

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