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Studies on the nature of EA binding by lymphocytes from rheumatoid arthritis patients

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SUMMARY

Investigation of the nature of the increased erythrocyte-antibody (EA) binding activity of peripheral blood lymphocytes (PBL) from rheumatoid arthritis (RA) patients reported in the preceding paper has revealed that IgG is the active class of antibody in this rosette formation. Some IgM binding also occurs. SRBC sensitized with $F(ab)_2$ preparations of IgG do not give rosette formation even at high concentrations. EA binding is inhibited by prior incubation of lymphocytes with heat-aggregated human IgG but antigen–antibody complexes did not give significant inhibition.

The majority of these rosettes were found to be stable at 4°C and room temperature but labile at 37°C.

Enzyme studies with pronase, trypsin, neuraminidase and treatment with sodium azide gave results strongly supporting the conclusion that the increased binding observed is increased Fc-receptor activity. This activity appears not to be a result of Fc binding by cell-bound rheumatoid factor.

A range of titres of antibody and of IgG was used to sensitize erythrocytes to form EA and the enhanced EA-rosette formation by PBL from RA patients occurred throughout the range of concentrations of sensitizing antibody. Significantly more EA were bound by individual lymphocytes from RA patients than control subjects. This data suggest that the Fc receptors on RA lymphocytes are more avid for EA than receptors on lymphocytes from healthy people.

INTRODUCTION

In the preceding paper it has been shown that PBL from patients with RA form significantly more EA rosettes than do those from healthy subjects, or from patients with systemic lupus erythematosus (SLE). The technique of EA-rosette formation is a well recognized method of identifying cells with Fc receptors suggesting that the phenomenon is due to an increase in the number of lymphocytes carrying active receptors of this type. However, the magnitude of the enhanced EA binding is such that it is necessary to exclude other factors, such as an exaggerated reactivity of other receptors on the cell surface or a result of rheumatoid factor bound to the rosetting lymphocytes.

This paper describes the results of studies designed to characterize the nature of the increased EA binding. We have shown that the antibody-sensitized indicator cells are binding to Fc receptors on the lymphocyte surface, and that the density or avidity of these receptors is substantially increased.

MATERIALS AND METHODS

Patients. Two groups of subjects were used—healthy controls and RA patients having definite or classical disease as defined by the American Rheumatism Association (ARA) (1958).

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Rosette preparation. The techniques used were described in the preceding paper. In selected experiments lymphocytes were separated from duplicate blood samples purified by the usual one-step gradient technique and by the gelatin-sedimentation polystyrene-bead column method of Thomson, Bull & Robinson (1966).

Sensitization of SRBC. This was carried out as described in the preceding paper.

The preparation of pure fractions of anti-SRBC serum. Anti-SRBC IgG was prepared from rabbit anti-sheep red cell antiserum (Wellcome rabbit haemolytic serum [RHS]) on a DEAE–cellulose column (Michaelsen & Natvig, 1971). The IgM peak from a Sephadex G-200 column (Flodin & Killander, 1962) was further purified by immunoabsorption (Sapin *et al.*, 1975). The purity of these fractions was confirmed by rocket immunoelectrophoresis (Laurell, 1966). Pepsin digestion of the purified IgG fraction followed by carboxy-methyl-cellulose chromatography yielded a purified $F(ab)_2$ fraction (Nisonoff, Markus & Wissler, 1961). Individual immunoglobulin fractions were used to sensitize SRBC through a range of concentrations. $F(ab)_2$ was used at a dilution equivalent to a 1:100 dilution of haemolytic serum.

Characterization of EA binding. The effect of rheumatoid factor in vitro on the EA-binding capacity of lymphocytes. This was investigated by incubating lymphocytes from patients with RA and from healthy controls in the presence of rheumatoid factor, prior to EA-rosette formation. 4×10^6 purified lymphocytes in 0·5 ml of sera containing IgM rheumatoid factor at titres of 1:256–1:8192 were incubated for 30 min at either 37°C or 4°C. The cells were then washed thoroughly—either three times with isotonic saline or, in some experiments, through discontinuous 50–100% foetal calf serum (FCS) gradients followed by two saline washes. The cells were then tested for EA rosette-forming capacity. Each experiment included samples incubated with autologous sera and sero-negative sera.

Enzyme treatment of lymphocytes. Leucocytes from RA patients and healthy controls in 4×10^6 aliquots were incubated for 30 min at 37°C in solutions of selected enzymes in isotonic saline—0·1% typsin (Sigma Chemical Co., St Louis, Missouri, U.S.A.), 0·1% pronase (Calbiochem, San Diego, California 92112), and 0·05 u/ml of neuraminidase (*V. cholera*, Sigma Chemical Co.). The cells were washed twice in isotonic saline before rosettes were prepared. Additional cell samples were treated with 0·1 M sodium azide (in isotonic saline) for 30 min at 37°C and rosettes were prepared in the presence of the azide. Treated lymphocytes were assayed for viability with trypan blue.

The effect of aggregated IgG and antigen–antibody complexes. Aggregated human IgG was prepared by heating a solution containing 15 mg IgG/ml of phosphate-buffered saline (PBS) at 63°C for 10 min. The aggregates were isolated, redissolved in PBS and separated on a Sephadex G-200 column (Normansell, 1971). Aggregated IgG was collected from the excluded volume of the column. Soluble antigen–antibody complexes were prepared in antigen excess from mouse IgG and rabbit anti-mouse IgG.

4×10^6 lymphocytes were incubated with either 1–2 mg/ml of aggregated IgG or in concentrations of antigen–antibody complexes ranging up to 7 mg/ml for 30 min at 37°C and then assayed for EA-rosette formation.

Heat inactivation of rabbit haemolytic serum. Further heat inactivation of the sensitizing sera was carried out at 56°C for 30 min.

RESULTS

Class and titre of sensitizing antibody

IgG fractions of RHS were almost as effective as whole sera in sensitizing SRBC for EA-rosette formation. Differences in EA binding between lymphocytes from RA patients and control subjects were demonstrable throughout a range of IgG sensitizing concentrations from 1 mg/ml to 0·25 μg/ml (Fig. 1). At the

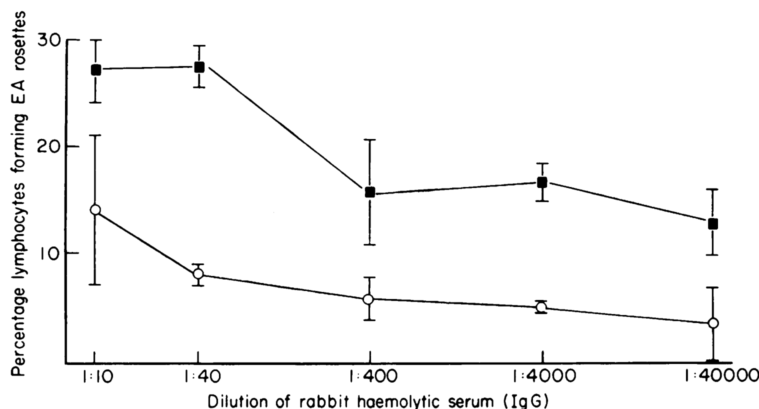

FIG. 1. Effect of concentration of IgG on lymphocyte–EA binding for RA patients (■) and control (○). The 1:10 dilution contains 1 mg of IgG/ml. Each point represents the mean ± standard error of the mean of five experiments.

lowest concentration of IgG (0·25 μg/ml), 3% of control lymphocytes and 13% of RA lymphocytes formed EA rosettes.

Results with IgM fractions gave variable numbers of EA (IgM) rosettes with both control and RA lymphocytes.

Characterization of EA binding

Effect of rheumatoid factor. Incubation with rheumatoid factor led to no significant increase in EA rosette-forming capacity of normal lymphocytes indicating that the phenomenon is not an adoptive one mediated by lymphocytes carrying surface-bound rheumatoid factor. EA binding of lymphocytes from either sero-negative or sero-positive RA patients was similarly unaffected by incubation with the sero-positive sera (Table 1).

Enzyme studies. Table 2 summarizes the EA- and E-rosette formation by lymphocytes pretreated with various enzymes.

EA rosettes. EA-rosette formation by both RA and control lymphocytes was abolished by pronase treatment but was unaffected by 0·1–0·5% trypsin or neuraminidase. Preparation of rosettes in the

TABLE 1. Exclusion of adsorption to lymphocytes of rheumatoid serum factors as an explanation of increased EA rosetting

Treatment	Temperature (°C)	No. of experiments	Percentage of lymphocytes forming rosettes: Controls E	EA	EA−E	RA patients E	EA	EA−E
None	37	12	8	14	6±2	5	32	27±3
Seropositive sera	37	12	14	22	8±4*	8	35	27±6*
None	4	2	6	16	10	6	44	38
Seropositive sera	4	2	10	13	3*	5	40	35*

The results are expressed as the arithmetic mean of all experiments ± the standard error.

* Difference not statistically significant.

TABLE 2. Effect of enzyme treatment on EA-rosette formation

Treatment	Percentage of lymphocytes forming rosettes: Controls, Untreated E	EA	EA−E	Controls, Treated E	EA	EA−E	RA patients, Untreated E	EA	EA−E	RA patients, Treated E	EA	EA−E
0·1% Trypsin	3±2	12±2	9±2	0±0·2	7±3	7±3*	3±1	34±3	31±2	0	28±5	28±5*
0·1 M Sodium azide	5±2	11±3	6±2	0	8±3	8±3*	3±2	28±1	25±2	0	9±5	9±5†
0·1% Pronase	4±2	8±2	4±3	0	0	0*	5±1	29±2	24±2	0	0	0‡
0·05 u neuraminidase	2±1·5	10±0·5	8±1	30±6‡	31±1	1±4*	6±2	30±2	24±4	29±6§	48±15	19±9*

These results are the arithmetic mean of three to eight experiments ± the standard error.

* Difference not statistically significant.

† $P<0.025$. ‡ $P<0.0025$. § $P<0.01$.

presence of sodium azide caused a decrease in EA-rosette formation by lymphocytes from RA patients ($P<0.025$). Lymphocytes from healthy people were unaffected.

E rosettes. In contrast E rosettes were abolished by pronase or trypsin treatment of lymphocytes and in the presence of sodium azide. Neuraminidase augmented E-rosette formation.

The effect of IgG aggregates and immune complexes. When aggregated IgG was present in the supporting medium EA rosetting by RA lymphocytes was significantly reduced ($P<0.0025$).

Antigen–antibody complexes inhibited RA EA-rosette formation by only 25% ($0.1>P>0.05$) and in two of the three experiments increased EA rosetting by control lymphocytes.

Rosette formation with $F(ab)_2$-sensitized EA. When rosettes were prepared using SRBC sensitized with $F(ab)_2$ fractions of IgG antibody, EA-rosette numbers were low, 7±2% for controls and 8±3% for RA patients. E-rosette formation at the same time averaged 6·5±3% indicating that no significant EA rosetting occurred under these conditions. This clearly implicates the Fc portion of the antibody molecule in mediating EA binding.

Heat-inactivated sensitizing serum. Further heat inactivation of RHS before preparing EA had no affect on EA-rosette formation, thus helping to exclude binding of EA to C3 receptors.

Temperature stability of rosettes

Table 3 shows that EA rosettes formed by lymphocytes from RA patients or healthy donors were stable at 4°C and at room temperature, but many, though not all, rosettes of RA patients' lymphocytes disrupted at 37°C during the 120 min incubation. E rosettes were abolished at 37°C.

Numbers of EA bound in rosettes by control or RA lymphocytes

At all concentrations of sensitizing antibody tested, more EA are bound by RA patients' lymphocytes than by those from control subjects. Results show that 69±6% of EA rosettes from RA patients bind ten or more EA compared with 42±5% of control EA rosettes when EA are sensitized with a 1:500 dilution of antibody. Similarly 34% of RA lymphocytes bind fifteen or more EA while only 14% of control lymphocytes were able to do so. If the EA are sensitized with a 1:2000 dilution of the antibody there is a marked 52% decrease in the population of control lymphocytes now able to bind ten or more red cells, but only a 14% decrease in that population from the RA patients.

DISCUSSION

The studies described above appear to verify that the increased EA-rosette formation seen in RA

TABLE 3. Temperature sensitivity of EA rosettes. Percentage of lymphocytes forming rosettes

Temperature	Time (min)	Controls			RA patients		
		E	EA	EA−E	E	EA	EA−E
4°C	0	7±2	10±3	3±3	6±4	32±4	26±3
	30	n.d.	n.d.		n.d.	n.d.	
	120	14±6	18±5	4±2*	8±3	32±8	24±8*
Room temperature	0	5±3	9±3	4±3	6±5	30±4	24±3
	30	n.d.	n.d.		4	23	19
	120	20±6	28±2	8±5*	12±2	40±12	28±14*
37°C	0	6±2	10±2	4±2	5±4	30±3	25±2
	30	0	6	6	0	13	13
	120	0	7±4	7±4*	0	12±5	12±5†

All results are the arithmetic mean of three experiments± the standard error. n.d. = Not done.

* Difference not statistically significant.

† $0.05>P>0.025$.

patients is due to binding to Fc receptors. A number of alternative explanations have been eliminated.

Binding of SRBC to T-lymphocyte SRBC receptors. This question was discussed in detail in the preceding paper. Briefly, any contribution from this reaction was excluded on the grounds that our routine control using unsensitized SRBC resulted in very low numbers of E rosettes being formed. Furthermore, pretreatment of lymphocytes with trypsin prior to rosette formation (a procedure which eliminated E-rosette formation) (Jondal, Holm & Wigzell, 1972) did not affect the levels of EA binding. Finally, ox red cells, which are unable to bind directly to human lymphocytes also demonstrated the altered EA-binding capacity of lymphocytes from RA patients.

Monocyte interference. The question of a contribution to the EA-rosette formation by contaminating monocytes is also discussed in the previous article. Using myeloperoxidase stains it was shown that an insignificant number of monocytes were capable of forming EA rosettes at the concentrations of sensitizing antibody used.

Cell-bound rheumatoid factor. It has been suggested that increased EA binding by RA lymphocytes may be a result of cell-bound rheumatoid factor. It is possible that RA lymphocytes may absorb rheumatoid factor to their surface, or in the case of IgG rheumatoid factor to their Fc receptors *in vivo*, resulting in EA-rosette formation through the Fc-binding activity of rheumatoid factor. This suggestion seems unlikely for the following reasons: (a) EA-rosette formation occurs with lymphocytes from healthy people, although to a much lower extent than RA lymphocytes. An increase in the concentration of antibody on the SRBC surface raises the proportion of normal lymphocytes forming EA rosettes. This indicates that the difference between RA and control lymphocytes in binding EA is not an all-or-none difference but rather one of degree of binding. (b) It is not possible to mimic the increased EA binding of RA lymphocytes by incubating lymphocytes from healthy people with IgM rheumatoid factor-positive sera *in vitro* at either 37°C or 4°C. The sera also failed to increase the EA binding by lymphocytes from sero-negative RA patients. Sera with a range of titres of rheumatoid factor were tried and although IgG reumatoid factor was not estimated it is most likely that at least one of the ten sera tested in these experiments would contain IgG rheumatoid factor. In twelve out of fourteen studies there was a reduction in EA-rosette formation following incubation with rheumatoid sera suggesting some binding of IgG to lymphocyte Fc receptors without a concomitant increase in EA binding capacity. (c) As surface Ig is removed when lymphocytes are incubated with 0·2% trypsin (Dickler, 1974), it seems likely that rheumatoid factor, itself an immunoglobulin, would be similarly removed. However, trypsin treatment of lymphocytes did not inhibit EA binding by RA lymphocytes.

Involvement of other receptors. Interactions between the sensitized SRBC and either surface immunoglobulin or C3 receptors on the lymphocytes has largely been excluded by studies of the effect of enzyme treatment and heat inactivation. Most strikingly, trypsin pretreatment of RA lymphocytes failed to abolish EA-rosette formation even with concentrations as high as 0·5%, a level which had removed surface Ig and abolished C3-receptor activity. Furthermore, the sensitivity of EA-rosette formation to sodium azide is distinctive as both surface Ig and C3 receptors are unaffected by this treatment (Dickler, 1974; Parish & Hayward, 1974; Lay & Nussenweig, 1968).

Role of Fc receptor. With most of the alternative possibilities excluded we now turn to the evidence in favour of the phenomenon being due to Fc-receptor activity. Fc receptors on human and murine lymphocytes bind the Fc portion of IgG (Dickler, 1974; Froland & Natvig, 1973; Basten, Warner & Mandel, 1972). We have shown that the IgG fraction of our RHS does indeed possess most of the reactivity for EA binding to occur. This reactivity is retained even when the sensitizing IgG is titred to 0·25 μg/ml. The Fc portion of the IgG antibody is essential for rosette formation. Even high concentrations of $F(ab)_2$ fractions used to sensitize SRBC resulted in no significant EA-rosette formation.

When EA were prepared using IgM fractions of the RHS some lymphocytes formed rosettes. Whether IgM binds to Fc receptors is a matter of some conflict in the literature. A number of groups have reported that IgM will not mediate EA-rosette formation but Basten *et al.* (1972) have shown that mouse lymphocytes could bind antigen–antibody complexes where antibody was purified IgM. Similarly Moretta *et al.* (1975) showed that human lymphocytes develop the capacity to bind EA (IgM) after 24 hr in culture. We are examining the question of IgM binding further.

Effect of aggregates and immune complexes. Dickler (1974) has shown that human lymphocyte Fc receptors will bind both aggregated IgG and antigen–antibody complexes (where the antibody is an IgG molecule). The competitive inhibition of EA-rosette formation by aggregated IgG in our experiments then fully supports our contention that the enhanced EA-rosette formation by RA lymphocytes is mediated through those cells' Fc receptors. Our inability to demonstrate significant inhibition of EA-rosette formation with antigen–antibody complexes is the only evidence we have found in conflict with the above conclusion. However, the type and size of complexes and aggregates and the subclass of IgG involved can materially affect their ability to bind to Fc receptors (Dickler, 1974; Basten *et al.* 1972; Horwitz & Lobo, 1975; Dickler & Kunkel, 1972). It is possible that the complexes we used were not optimal either in form or concentration for inhibition of EA rosetting. Also at 1:2000 concentration of sensitizing antibody, we are detecting only a proportion of Fc -receptor-bearing lymphocytes. If, therefore, the immune complexes do not saturate all Fc sites the remaining receptors may be sufficient to allow EA rosetting to occur.

Enzyme studies. A number of groups have described the effects of various enzymes and metabolic inhibitors on human lymphocyte receptors. Our studies reported in Table 3 show a pattern of sensitivity of EA binding to various enzymes consistent with the reported effects of these enzymes on Fc receptors. This is most clearly exemplified with trypsin which did not inhibit EA-rosette formation by RA lymphocytes even with trypsin concentrations up to 0·5%. This concentration would ablate SRBC binding to T lymphocytes, remove surface Ig (Dickler, 1974) and abolish C3 receptor activity (Parish & Hayward, 1974; Lay & Nussenweig, 1968). Similarly, the sensitivity of EA-rosette formation by RA lymphocytes to pronase treatment accords with published accounts of pronase deletion of Fc receptors (Dickler, 1974; Horwitz & Lobo, 1975). Neuraminidase treatment of lymphocytes increases the proportion of E rosettes as expected (Froland & Natvig, 1973; Bentwich *et al.*, 1973) while net EA-rosette formation was unaffected. Union of antigen with surface Ig or of appropriate markers with C3 is unaffected in the presence of sodium azide (Parish & Hayward, 1974; Lay & Nussenweig, 1968), but there are conflicting accounts of effects on Fc-receptor activity. Froland, Wisløff & Michaelsen (1974), using 0·015 M sodium azide, were unable to inhibit EA binding to normal human lymphocytes, while Hallberg (1975) showed clear inhibition of EA rosetting. In our hands some reduction of EA-rosette formation by RA lymphocytes occurred with sodium azide but there was little change with lymphocytes from healthy donors. Many of the experiments have clearly indicated we are detecting a heterogeneous population of lymphocytes forming EA rosettes. The populations of cells being assayed appear to have distinctive enzyme and temperature sensitivity and the representation of the various subpopulations is apparently different for RA patients and healthy controls. In fact we are detecting only a small proportion of Fc receptor-bearing cells in controls.

Temperature stability of rosettes. Many EA rosettes were unstable at 37°C, their numbers declining during 120 min incubation, but reaching a plateau supporting a heterogeneous population of lymphocytes forming EA rosettes. This observation is in agreement with the work of Pang (1976, personal communication). Dickler (1974) showed aggregated IgG binding to human lymphocytes decreased by 33% during 4 hr incubation at 37°C.

The data presented above confirms our belief that the greatly increased EA-rosette formation by lymphocytes from RA patients results from EA binding to the Fc receptor of populations of lymphocytes.

Avidity of binding. The experimental conditions we have chosen to demonstrate EA binding are clearly suboptimal for identifying all lymphocytes with Fc receptors. By diluting out the antibody used in sensitizing the red cells to 1:2000 the difference in the EA-rosette numbers formed by RA or control lymphocytes is exaggerated. This suggests that, though Fc receptors are present on many control lymphocytes, their density or their avidity for binding EA is less than that of RA lymphocytes. This conclusion receives further support from consideration of the numbers of EA red cells bound to each lymphocyte. With decreasing concentrations of sensitizing antibody, the numbers of EA binding to control lymphocytes decrease more rapidly than do those binding to RA lymphocytes. This is particularly marked over the range of concentrations down to 1:2000.

CONCLUSION

The increased EA-rosetting capacity of lymphocytes from patients with RA has been clearly established. Extensive studies have revealed no evidence for involvement of any mechanism other than Fc-receptor binding of the EA. We conclude therefore that RA lymphocytes carry Fc receptors that are substantially more dense and/or avid for EA than similar receptors on lymphocytes from healthy people. Double-labelling studies recently carried out marking both surface immunoglobulin (using fluorescent-labelled anti-immunoglobulin) and EA-rosette formation indicate that the majority (85–95%) of lymphocytes forming these rosettes in the RA patients are K lymphocytes. These results will be reported in detail elsewhere.

The observations described in the present and preceding paper appear to have fundamental importance in the understanding of rheumatoid arthritis. Although lymphocytes with enhanced Fc-receptor activity may not be the initiating factor in RA they may well be an essential link in the chain leading to active joint destruction.

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