Lymphocytes bearing Fc γ receptors in rheumatoid arthritis. II. Phenotypic characterisation of mononuclear cells forming Facb rosettes in RA

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SUMMARY We have previously reported an increased proportion of Facb-rosette forming cells in the peripheral blood of patients with rheumatoid arthritis in comparison with healthy controls. The present study investigates the surface phenotype of these cells by means of monoclonal antibodies and a variety of rosetting and lymphocyte fractionation techniques. Facb-R⁺ cells were found to lack surface markers characteristic of T and B lymphocytes. Studies with monoclonal reagents showed a positive reaction with OKIa1, OKM1, and another monocyte-specific antibody. Facb-R⁺ cells were recognised by anti-HLA-DR reagents but did not bind the monoclonal antibody 17.15 that recognises a determinant on HLA-DR antigens expressed by lymphocytes but not monocytes. These results show that Facb-R⁺ cells share certain surface characteristics with monocytes, though they are not phagocytic. These observations are consistent with an accessory role for Facb-R⁺ cells in the immune response.

Key words: Fc receptor, surface marker, monoclonal antibody.

Receptors which bind the Fc portion of IgG (Fc γ R) have been demonstrated on the surface of many peripheral blood leucocytes including T lymphocytes,¹ B cells,² null cells,³⁻⁶ monocytes,⁷ and neutrophils.⁸ These receptors play significant roles in chronic inflammation, since they are associated with phagocytosis of IgG aggregates⁸ and antibody-dependent cellular cytotoxicity⁹ and have also been implicated in the regulation of humoral immunity mediated by specific antibody.¹⁰⁻¹² These observations have led to the investigation of Fc γ R expression on mononuclear cells from patients with a variety of autoimmune diseases.

Several authors have reported an increased proportion of peripheral blood mononuclear cells

(PBMNC) exhibiting Fc γ R in patients with rheumatoid arthritis (RA) compared with healthy controls.¹³⁻¹⁶ These authors did not identify the subpopulation bearing the Fc γ R, although Sharpin and coworkers¹⁷ have recently suggested that this increased expression is due to an increase in T_H cells. Other authors have failed to demonstrate any increase in Fc γ R expression on rheumatoid mononuclear cells¹⁸ or rheumatoid 3rd population (L) lymphocytes.^{19, 20} In our laboratory mononuclear cells bearing a receptor capable of binding the Cy2 region of IgG have been detected in peripheral blood by a novel rosette assay employing calf red blood cells (CRBC) sensitised with the Facb fragment of rabbit IgG anti-CRBC.²¹ It has been shown that Facb-R⁺ cells form an increased proportion of PBMNC from patients with RA compared with healthy control subjects and patients with osteoarthritis (OA) or ankylosing spondylitis (AS). In this report a number of techniques have been utilised to investigate the surface characteristics of the Facb rosette forming cells in rheumatoid patients.

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Materials and methods

Heparinised venous blood was obtained from patients with classical or definite active RA receiving only non-steroidal anti-inflammatory therapy and from a group of healthy hospital personnel, age and sex matched where possible.

CELL SEPARATION

Peripheral blood mononuclear cells were obtained by density flotation on Ficoll-Paque (Pharmacia) as described by Bøyum.²² Enriched cell populations were prepared by a variety of rosette techniques and by affinity chromatography.

ROSETTE TECHNIQUES

For all rosette assays approximately 200 PBMNC were counted, with cells surrounded by three or more erythrocytes being scored as positive. Rosette-positive or negative (depleted) populations were separated on Ficoll-Paque and the rosette-positive cells were treated with 0.83% w/v ammonium chloride to lyse the red cells. Effective fractionation of the cell subpopulations was confirmed by rerosetting.

EA and Facb rosettes. FcγR⁺ cells were prepared as previously described.²¹ Briefly, PBMNC were incubated for 60 min at 4°C with complexes composed of a 1% v/v suspension of CRBC sensitised with intact rabbit IgG anti-CRBC or its Facb fragment at optimal concentrations.

E rosettes. For E rosette formation sheep erythrocytes were treated with 2-aminoethylisothiuronium bromide (AET) as described by Kaplan and Clark.²³

C3 rosettes. Human group 0 rhesus negative erythrocytes coated with the third component of complement were prepared by incubation of heparinised venous blood at 37°C in a low ionic strength buffer.^{24 25} These erythrocytes were then washed extensively, adjusted to a 1% v/v suspension, and used to form C3-rosettes by similar methodology to that outlined above for FcγR⁺ cells.

Anti-light chain rosettes. Calf erythrocytes, conjugated either to normal sheep IgG or to sheep IgG anti-human light chain by means of chromic chloride,²⁶ were kindly provided by Dr A. Jehanli (Department of Biochemistry, University of Bath). A 1% v/v suspension of these erythrocytes was used to form rosettes with PBMNC as outlined above. The percentage anti-light chain rosette-forming cells was corrected for any rosettes formed with the normal sheep IgG-coated erythrocytes.

AFFINITY CHROMATOGRAPHY ON HELIX POMATIA LECTIN-SEPHAROSE 6MB

Human T cells have been shown to possess a

receptor which is exposed after neuraminidase treatment and which binds a lectin isolated from *Helix pomatia*—the vineyard snail.²⁷ PBMNC were incubated with neuraminidase, resuspended in phosphate buffered saline (PBS) containing 0.2% w/v human serum albumin (HSA) and 0.02% w/v sodium azide, and were fractionated by passage down a column of *Helix pomatia* lectin-Sepharose 6MB (Pharmacia).²⁸

Adherent cells were eluted from the column in two stages with 0.1 mg.ml⁻¹ and 1.0 mg.ml⁻¹ N-acetyl-D-galactosamine in PBS-HSA-azide. The initial PBMNC, the neuraminidase-treated cells, and the three fractions obtained from the column were assayed for percentage E⁺ and Facb-R⁺ cells.

FLOW CYTOMETRY ANALYSIS

Facb-R⁺ cells were obtained by positive selection from PBMNC preparations using rosette formation and centrifugation through Ficoll-Paque. Rosetted cells were washed three times with PBS, incubated at 4°C for five minutes with 0.83% w/v ammonium chloride, and washed a further three times with PBS. The final wash was performed with ice-cold PBS containing 0.2% w/v sodium azide and 0.5% w/v bovine serum albumin (PBSA). The cells were then reacted for 30 minutes on ice with an optimal dilution of either OKT3, OKM1, OKIa1 (Ortho Diagnostics),^{29 30} or mouse antihuman immunoglobulin (Becton Dickinson), washed and incubated for a similar period with fluorescein isothiocyanate-conjugated rabbit antimouse immunoglobulin (FITC-RaMIg). The cells were finally washed five times with PBSA before analysis on a fluorescence-activated cell sorter (Ortho Model 2103).

Statistical analysis. Paired data were compared by Student's *t* test.

Results

ROSETTE ASSAYS

Mononuclear cells were depleted of different subpopulations (Facb-R⁺, E⁺, sIg⁺) by rosette formation and centrifugation through Ficoll-Paque, and the cells remaining above the density medium were analysed. The results are summarised in Table 1. E rosette depletion resulted in the concentration of Facb-R⁺ cells in the E⁻ fraction. Similarly, depletion of surface Ig⁺ cells by rosette formation with anti-light chain-coated erythrocytes did not remove the Facb-R⁺ cells. Removal of the Facb-R⁺ cells themselves resulted in an increased proportion of C3-R⁺ in the depleted population. Depletion of C3-R⁺ cells by rosetting was found to be impossible owing to the fragility of the rosettes formed.

Table 1 Surface marker analysis of rheumatoid Facb-R⁺ cells by means of rosette assays

Experiment	n	% Rosettes (mean $\pm$ SD)			
		Facb	E	C3	Anti-light chain
PBMNC	4	8 $\pm$ 5		6 $\pm$ 3	
Facb-R depleted	4	3 $\pm$ 1		13 $\pm$ 6	
PBMNC	6	5 $\pm$ 4	62 $\pm$ 9		
E depleted	6	17 $\pm$ 9	6 $\pm$ 2		
PBMNC	4	6 $\pm$ 4			9 $\pm$ 6
Anti-light chain depleted	4	7 $\pm$ 6			1 $\pm$ 1

Peripheral blood mononuclear cell preparations were depleted of either Facb-R⁺, E⁺, or slg (light chain)⁺ cells by centrifugation of the relevant rosettes through Ficoll-Paque. The resulting interface populations were analysed for the presence of Facb-R⁺, E⁺, C3-R⁺, and slg (light chain)⁺ cells.

FRACTIONATION OF PBMNC ON HELIX POMATIA LECTIN-SEPHAROSE 6MB

Neuraminidase-treated PBMNC from both rheumatoid and control subjects were fractionated on an affinity column of *Helix pomatia* lectin-Sepharose 6MB beads with elution of the bound cells with two concentrations of N-acetyl-D-galactosamine (NAcGal). The results given in Fig. 1 are pooled

from both RA and control PBMNC, as there was no apparent difference in the fractionation of the two groups. E⁺ cells were significantly decreased in the unbound fraction compared with untreated PBMNC ($p<0.001$), whereas Facb-R⁺ cells were enriched ($p<0.001$). The cells eluted with the higher concentration of NAcGal were enriched for E⁺ lymphocytes ($p<0.05$) but significantly depleted of Facb-R⁺ cells ($p<0.01$).

BINDING OF MONOCLONAL ANTIBODIES

Fluorescence activated cell sorter (FACS) analysis of Facb-R⁺ cells was carried out following incubation with a variety of monoclonal reagents. Results from these experiments, given in Table 2, show a lack of reaction between Facb-R⁺ cells and OKT3 or anti-Ig, but considerable binding of OKM1 and, to a lesser extent, OKIa1. Rosette assays for Facb-R⁺ cells, E⁺ cells, and slg⁺ cells were carried out in parallel (Table 2). Only 52% of the isolated Facb-R⁺ cells were able to re-form Facb rosettes, perhaps because of modulation of their Fc receptors. This was similar to the proportion of cells binding OKM1. In contrast, only small numbers of cells formed E rosettes or expressed surface immunoglobulins. Owing to the lack of a dual laser FACS system it was impossible in these experiments

Fig. 1 Fractionation of peripheral blood mononuclear cells on *Helix pomatia* lectin-Sepharose 6MB. PBMNC were treated with neuraminidase, resuspended in PBS-HSA-azide, and applied to a column of *Helix pomatia* lectin-Sepharose 6MB. Non-adherent cells were washed through in PBS-HSA-azide and bound cells were eluted in two stages with N-acetyl-D-galactosamine (NAcGal) at 0.1 mg.ml⁻¹ and 1.0 mg.ml⁻¹ in PBS-HSA-azide. Cell fractions were washed in PBS-HSA-azide and assessed for E⁺, FcγR⁺, and Facb-R⁺ cells by rosette formation.

Results are expressed as mean percentage rosette formation $\pm$ 1 SD ($n=6$). Columns A-E represent the following: (A) unfractionated PBMNC; (B) neuraminidase-treated PBMNC; (C) cells not bound to column; (D) cells eluted with 0.1 mg.ml⁻¹ NAcGal in PBS-HSA-azide; (E) cells eluted with 1.0 mg.ml⁻¹ NAcGal in PBS-HSA-azide.

Table 2 Surface marker analysis of rheumatoid Facb-R⁺ cells by means of FACS

OKT3	% positive cells OKM1	OKIa1	slg	% rosettes Facb	E	% positive cells slg ⁺
4±2	48±9	21±8	6±2	52±6	4±1	7±3

*Detected by direct immunofluorescence.

Aliquots of cells were treated with the recommended dilutions of the monoclonal reagents OKT3, OKM1, and OKIa1, and mouse antihuman Ig and were then labelled by the addition of FITC-conjugated rabbit antimouse Ig. The cells were subsequently analysed using a FACS (Ortho). Further aliquots of the same Facb-R⁺ populations were analysed for percentage Facb-R⁺, E⁺, and slg⁺ cells. Results are expressed as the mean ± 1 SD of data from five rheumatoid patients.

Table 3 Interaction of Facb-R⁺ cells with monoclonal antibodies

	% positive Facb-R ⁺ cells
Antimonocyte (BRL)	85± 7
Antibody 7·2 ³¹ (DR framework)	84±11
Antibody 17·15 ³¹ (DR lymphocyte specific)	21± 8

PBMNC were allowed to form Facb rosettes, then incubated with optimal dilutions of the above reagents followed by FITC-conjugated antimouse Ig. Cells were then analysed with a Leitz SM-Lux microscope with epifluorescence. Results are expressed as the mean ± 1 SD of data from five rheumatoid patients (all HLA-DR4⁺).

to determine whether or not the same cells formed Facb rosettes and bound OKM1 and OKIa1. Evidence for this was obtained in further studies carried out by incubating Facb rosettes with an optimal dilution of monoclonal reagents antimonocyte (Bethesda Research Laboratories, Cambridge), antibody 7·2, and antibody 17·15.³¹ Antibody 7·2 binds an HLA-DR framework determinant present on both lymphocytes and monocytes, but antibody 17·15 reacts with a determinant expressed only on lymphocytes bearing certain HLA-DR haplotypes, including DR4.³¹ PBMNC from five rheumatoid patients known to be HLA-DR4⁺ were incubated with these reagents and subsequently with fluoresceinated antimouse Ig (Table 3). The results indicate that Facb-R⁺ cells are recognised by the anti monocyte reagent and by the anti-HLA-DR framework antibody 7·2, but not by the lymphocyte DR-specific reagent 17·15.

Discussion

A considerable body of literature has accumulated in recent years describing altered lymphocyte subpopulations in rheumatoid blood and synovial specimens compared with samples from healthy subjects.³² Various authors have demonstrated in-

creased expression of FcγR on rheumatoid PBMNC,¹³⁻¹⁶ a finding that has been attributed by Sharpin and coworkers to the Tγ subset.¹⁷ The subpopulation of FcγR⁺ cells that express receptors for Facb is also found in greater numbers in rheumatoid patients than in control groups.²¹ However, the data in this report show clearly that these cells are not T lymphocytes. Thus Facb-R⁺ cells do not form E rosettes with AET-treated sheep erythrocytes, do not bind OKT3, and do not express receptors for *Helix pomatia* lectin. Other experiments have shown that this cell population lacks surface membrane immunoglobulin (sIg) and receptors for C3. The lack of slg was demonstrated not only by direct immunofluorescence but also by FACS analysis and by anti-light chain rosette formation, a technique very similar to the sensitive DARR method used by Haegert³³ to identify low levels of slg on some 'null cells'. These negative data suggest that Facb receptors are expressed on a subpopulation of non-T, non-B mononuclear cells.³⁻⁵ The results presented above were obtained with rheumatoid PBMNC, since the increased number of Facb-R⁺ cells in these patients²¹ allow more clear-cut analysis of the data obtained. However, the experiments have also been carried out with healthy control Facb-R⁺ cells, and all the results indicate that these cells have the same surface marker characteristics in health and rheumatoid disease.

In contrast to the negative findings with T and B cell markers, positive staining of Facb-R⁺ cells has been observed with monoclonal antibodies directed against HLA-DR and monocyte surface antigens. Facb-R⁺ cells are not mature monocytes, since previous experiments have shown that they neither phagocytose carbonyl iron nor stain for non-specific esterase.²¹ However, clear-cut evidence for the monocyte-associated lineage of Facb-R⁺ cells has been obtained with the monoclonal reagents 7·2 and 17·15.³¹ Facb-R⁺ cells bind antibody 7·2, thus confirming expression of HLA-DR antigens, but fail to react significantly with the lymphocyte-

specific antibody 17-15. The overall pattern of surface phenotype observed for Facb-R⁺ cells is similar to that described for L cells, that is, non-T, non-B cells⁴ that react with monocyte-related markers and express variable amounts of HLA-DR antigens.^{12 34-36} In contrast to Facb-R⁺ cells, however, L cells are considered to be lymphocytes,⁴ do not react with the BRL antimonocyte reagent,¹² and appear to express HLA-DR antigens or monocyte markers but rarely both.³⁴ This lack of complete identity between Facb-R⁺ cells and the L cell population may be responsible for the different observations of these cells in rheumatoid peripheral blood, where Facb-R⁺ cells are increased²¹ but L cells are not.²⁰

The expression of class II MHC (HLA-DR) antigens on Facb-R⁺ cells and the monocyte-associated lineage of these cells both suggest that they might function as accessory cells in an immune response.³⁷ This possibility is also supported by the morphology of Facb-R⁺ cells revealed by transmission electron microscopy. This shows similarities to known accessory cells such as dendritic cells and Langerhans cells isolated from various tissues (paper in preparation). Experiments in mice suggest that Facb-R⁺ cells play a role in the antibody-mediated suppression of immunoglobulin synthesis.³⁸ Further results obtained with human cells³⁷ agree with the above observations of both positive and negative effects on antibody production and support the hypothesis that Facb-R⁺ cells may be involved in immune regulation in vivo.

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