Lymphocytes bearing Fc γ receptors in rheumatoid arthritis. IV. Increased numbers and activation of Facb-R⁺ cells after immunisation of healthy individuals

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SUMMARY Mononuclear cells expressing Fc γ receptors that form Facb rosettes are increased in the peripheral blood of patients with rheumatoid arthritis compared with controls. Healthy individuals with a positive skin response to tuberculin showed a marked increase in numbers of circulating Facb-R⁺ cells three days after challenge, returning to baseline after seven days. No response was observed in subjects showing a negative skin test. A similar increase in Facb-R⁺ cell numbers was measured after intramuscular injection of another specific antigen, tetanus toxoid. In addition to this enhancement of Facb-R⁺ cell numbers, evidence has been obtained that these cells are in an activated state postimmunisation as judged by acquisition of low density and increased expression of class II MHC antigens. Apparently identical changes in Facb-R⁺ cell numbers and activation may be induced in vitro either by culturing sensitised mononuclear cells with specific antigen for three days or by an overnight incubation of normal cells with γ -interferon (γ -IFN). By analogy, therefore, the increased numbers of Facb-R⁺ cells in patients with rheumatoid arthritis are probably induced by γ -interferon generated as part of an antigen driven immune response. In this context it is interesting that patients with Felty's syndrome, in whom neutropenia increases susceptibility to infections leading to the possibility of further stimulation of the immune system by micro-organisms, have particularly high levels of circulating Facb-R⁺ cells.

Key words: class II MHC antigen, γ -interferon, low density.

Several authors have demonstrated an increased expression of Fc γ receptors (FcR) on peripheral blood mononuclear cells (PBMNC) in patients with rheumatoid arthritis.¹⁻³ A subpopulation of PBMNC expressing receptors for the Cy2 region of IgG may be detected by rosette formation with rabbit Facb coated, calf erythrocytes. Cells expressing the Facb receptors are found in increased numbers in the peripheral blood of rheumatoid patients.⁴ Recent studies have shown that Facb-R⁺ cells lack the phenotypic markers of both T and B

lymphocytes but that they express certain monocyte related markers and class II MHC antigens.⁵ Functionally, Facb-R⁺ cells are non-phagocytic⁴ and lack cytotoxic activity but act as regulatory cells in pokeweed mitogen driven IgG synthesis.⁶

The factors which promote the increase in Facb-R⁺ cells in rheumatoid peripheral blood are unknown. Experiments in mice have shown that splenic Facb-R⁺ cells increase in number after secondary but not primary immunisation with a specific antigen, sheep erythrocytes.⁷ In view of this, numbers of circulating Facb-R⁺ cells have been monitored in healthy volunteers undergoing immunisation procedures and in cultures of PBMNC stimulated in vitro with specific antigen. Since such procedures are well recognised to induce cell activa-

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tion, various assays have been performed to determine the state of activation of Facb-R⁺ cells after antigenic challenge in vivo.

Patients and methods

SUBJECTS

Healthy volunteers were recruited from hospital personnel. A group of 25 patients with classical/definite rheumatoid arthritis all with clinically active synovitis and receiving non-steroidal anti-inflammatory drug therapy, and a further 17 patients with Felty's syndrome also consented to enter the study.

IMMUNISATION PROCEDURE

Adsorbed tetanus toxoid vaccine (Wellcome Foundation, London) was given intramuscularly in a 0.5 ml dose of 40 international units. Tuberculin purified protein derivative (PPD) was administered in the form of a Tine test (Lederle Laboratories, Gosport, Hampshire) on the volar surface of the mid-forearm. Both heparinised and clotted venous blood samples were taken at daily intervals before and up to seven days after immunisation, with a final sample taken after 14 days.

MONONUCLEAR CELL PREPARATION

Mononuclear cells were separated from heparinised venous blood by flotation on Ficoll-Paque (Pharmacia) using the method described by Boyum.⁸ PBMNC were further fractionated in some experiments into monocyte and lymphocyte enriched populations by centrifugation through 52% v/v Percoll (density 1.062 g/ml; Pharmacia) at 1000 g for 30 minutes.⁹

ANALYSIS OF MONONUCLEAR CELL SUBPOPULATIONS

The population of PBMNC expressing Facb receptors was enumerated by a rosette assay as previously described.⁴ The expression of HLA-DR antigens on the surface of these cells was examined using a monoclonal anti-DR framework antibody (MAb 7.2), a gift from Professor J Hansen, University of Washington, Seattle.¹⁰ PBMNC at a concentration of 2×10^6 /ml were first rosetted with Facb coated calf erythrocytes and then incubated with doubling dilutions of MAb 7.2 in an ice bath for 30 minutes. After a series of gentle washes in phosphate buffered saline (PBS) the cells were further incubated with a 1:20 dilution of rabbit antimouse IgG-fluorescein isothiocyanate conjugate (Dakopatts, Denmark) on ice for 30 minutes. The cells were gently resuspended and washed in PBS before enumeration of positively stained Facb-R⁺ cells by immunofluorescence microscopy.

MONONUCLEAR CELL CULTURES

PBMNC were washed three times in calcium and magnesium free salt solution and once in tissue culture medium (RPMI 1640, Gibco) before being resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 1 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin.

Fig. 1 Percentage change in Facb-R⁺ cell numbers after immunisation of healthy individuals with tetanus toxoid (upper half) and tuberculin PPD (lower half). Upper histograms and bars represent mean and SD of data from six subjects. Lower graphs represent mean and SD of data from 12 individuals giving a positive skin reaction at 48 hours (▲—▲) and from five subjects with negative skin tests (×—×). Day 3 Tine positive v Tine negative (a): $p < 0.01$, Mann-Whitney U test; Tine positive day 3 v day 1 (b): $p < 0.05$, Wilcoxon signed rank test.

μg/ml streptomycin. Cells from individuals showing a positive Tine test were incubated with increasing doses of tuberculin PPD (Wellcome Foundation, London) in 1 ml aliquots in polystyrene multiwell dishes (Nunc) in 5% CO₂/95% air at 37°C. Well contents were decanted at daily intervals for the enumeration of Facb-R⁺ cells. A similar protocol was followed for healthy PBMNC cultured overnight at 37°C with various concentrations of recombinant γ-interferon (γ-IFN; Biogen) generously provided by Professor G S Panayi (Guy's Hospital, London).

STATISTICAL ANALYSIS

Non-parametric data were analysed with the Mann-Whitney U test or Wilcoxon signed rank test.

Results

IN VIVO RESPONSES TO SOLUBLE ANTIGENS
Six subjects were immunised intramuscularly with tetanus toxoid at a dose of 40 international units. Peripheral blood samples were taken at intervals over 14 days and levels of Facb-R⁺ cells determined. These were expressed relative to day 0 values and are presented in Fig. 1 (upper half). There was a significant increase in numbers of circulating Facb-R⁺ cells from 2.9 (0.5)×10⁴/ml (mean (SD)) on day 0 to 5.9 (2.4)×10⁴/ml on day 3 (p<0.05), returning to preimmunisation values by day 7. Of 17 healthy individuals challenged intradermally with another specific antigen, tuberculin, 12 showed a positive reaction after 48 hours and

Table 1 Percoll density gradient analysis of Facb-R⁺ cells in normal individuals challenged with tuberculin PPD

		Facb-R ⁺ cell numbers (×10 ⁻⁴ /ml)		Total PBMNC numbers (×10 ⁻⁴ /ml)	
		Density <1.062 g/ml	Density >1.062 g/ml	Density <1.062 g/ml	Density >1.062 g/ml
Tine positive (n=4)	Day 0	0.9 (0.2) ^a	2.7 (0.6)	21.9 (5.7)	47.0 (25.0)
	Day 3	17.2 (9.1) ^{a,b}	3.1 (0.9)	19.0 (9.2)	39.3 (13.2)
Tine negative (n=2)	Day 0	2.1 (1.4)	1.9 (0.1)	28.8 (11.0)	60.5 (10.6)
	Day 3	4.6 (0.4) ^b	2.5 (1.1)	19.6 (4.1)	60.3 (16.5)

Results are expressed as mean (SD).

Significant differences are between points (a) p<0.01, Wilcoxon signed rank test; and (b) p<0.05, Mann-Whitney U test.

Fig. 2 Percentage Facb-R⁺ cells positively stained with MAb 7.2 (i.e., expressing HLA-DR antigens) before (×---×) and three days after (▲—▲) immunisation with tuberculin PPD. Graphs represent median data from four subjects. The points indicated by (a) differ significantly (p<0.05, Wilcoxon signed rank test).

five were negative. A marked increase in Facb-R⁺ cell numbers again reaching a peak on day 3 was observed in the Tine test positive group ($p<0.05$), but no change in the distribution of these cells occurred in the negative individuals. Numbers of circulating Facb-R⁺ cells had returned to baseline levels by four days after challenge.

Mononuclear cells further separated by Percoll density centrifugation into populations of density less than and greater than 1.062 g/ml were tested for their ability to form Facb rosettes before and three days after challenge with tuberculin. Whereas the distribution of total PBMNC in the two fractions remained constant in both Tine test positive and negative groups before and after immunisation (Table 1), there was a dramatic increase in the number of Facb-R⁺ cells of density <1.062 g/ml postimmunisation in the Tine positive group ($p<0.01$), but not in the Tine negative group. Before immunisation most Facb-R⁺ cells were found in the fraction of cells with density >1.062 g/ml. Using MAb 7.2, a significant enhancement of class II MHC antigen expression was noted within the Facb-R⁺ cell population three days post-tuberculin challenge as determined by diluting the MAb from 1:50 to 1:400 (Fig. 2). When Percoll fractionated cells were examined from subjects who responded positively to the Tine test enhanced binding of MAb 7.2 was visible in the low density fraction postimmunisation (Table 2).

The rise in Facb-R⁺ cell numbers after immunisation with specific antigens was compared with levels of these cells in groups of patients with active rheumatoid arthritis and Felty's syndrome. The results shown in Fig. 3 demonstrate that patients with Felty's syndrome had considerably higher levels of circulating Facb-R⁺ cells ($12.1(3.0)\times 10^4/\text{ml}$) than the active rheumatoid group ($9.3(5.2)\times 10^4/\text{ml}$, $p<0.01$). Both groups had a higher proportion of Facb-R⁺ cells in their PBMNC than normal

controls ($3.8(1.3)\times 10^4/\text{ml}$, $p<0.01$). Peak levels of Facb-R⁺ cells after immunisation of healthy volunteers with PPD ($9.6(6.0)\times 10^4/\text{ml}$) were comparable with those in the RA group but below the numbers detected in the patients with Felty's syndrome.

IN VITRO RESPONSES TO SOLUBLE ANTIGENS
Mononuclear cells from eight healthy subjects who showed a positive Tine test were cultured in vitro in the presence or absence of PPD (50 $\mu\text{g}/\text{ml}$, the optimal concentration from preliminary experiments). Fig. 4 shows that a significant increase occurred in the proportion of Facb-R⁺ cells after

Fig. 3 Numbers of circulating Facb-R⁺ cells in healthy subjects and patients with active RA and Felty's syndrome. Levels in patients with Felty's syndrome are significantly higher ($p<0.01$, Mann-Whitney U test) than the active RA group, and the levels in both patient groups are higher ($p<0.01$, Mann-Whitney U test) than those in the healthy controls.

Table 2 HLA-DR expression by Facb-R⁺ cells in low and high density fractions postimmunisation of six individuals with tuberculin PPD. Results show the percentage of Facb-R⁺ cells binding MAb 7.2 (median, range)

Dilution of MAb 7.2	Cell density	
	<1.062 g/ml	>1.062 g/ml
1:50	80 (60-100)	60 (33-75)
1:100	71 (67-83)	33 (0-75)
1:400	67 (33-100)	25 (0-40)

Binding of MAb 7.2 (HLA-DR expression) on low density Facb-R⁺ cells is significantly greater than on high density Facb-R⁺ cells at all three dilutions tested ($p<0.05$, Wilcoxon signed rank test).

Fig. 4 Percentage Facb-R⁺ cells in cultures of PBMNC incubated at 37°C either with tuberculin PPD 50 µg/ml (■ — ■) or alone (□ - - - □). a: $p < 0.05$, Wilcoxon signed rank test; b: $p < 0.01$, Mann-Whitney U test.

three days in culture with PPD compared with day 0 values ($p < 0.01$). A gradual fall in Facb-R⁺ cell numbers was observed in control cultures in the absence of antigen. Results similar to those obtained from cell cultures containing PPD were obtained by incubating PBMNC with low concentrations of γ -IFN. This lymphokine at 5 units/ml caused an increase in the proportion of Facb-R⁺ cells from 2.6(1.0)% to 7.2(2.1)% after 18 hours at 37°C ($p < 0.05$). A parallel increase in the expression of class II MHC antigens was also induced on the Facb-R⁺ cells by γ -IFN (Table 3). The enhance-

Table 3 Increased numbers of Facb-R⁺ cells and HLA-DR antigen expression induced by γ -interferon

	Concentration of γ -interferon (U/ml)		
	0	5	10
Percentage of Facb-R ⁺ cells	2.6 (1.0) ^{a b}	7.2 (2.1) ^a	7.1(2.1) ^b
MAB 7.2 binding			
1:100	44 (25-59) ^{c d}	82 (80-86) ^c	75 (70-79) ^d
1:400	44 (33-50) ^c	47 (30-60)	83 (67-100) ^c

Results for percentage of Facb-R⁺ cells are presented as mean (SD); those for % Facb-R⁺ cells binding of MAb 7.2 as median and (range) (n=6).
a, b, c, d, e: $p < 0.05$, Wilcoxon signed rank test.

ment of both the increase in Facb-R⁺ cell numbers and HLA-DR expression was blocked by the addition to the cultures of cycloheximide, an inhibitor of protein synthesis (data not shown).

Discussion

Facb-R⁺ cells have been reported previously to be increased in the circulation of patients with rheumatoid arthritis.⁴ This paper confirms those results and shows that these cells are increased even further in patients with Felty's syndrome. Increased numbers of Facb-R⁺ cells are not unique to patients with rheumatoid arthritis, however, being induced transiently in healthy individuals undergoing a secondary immune response to specific antigens. These observations have been made in different sets of individuals immunised with two different antigens — tuberculin PPD and tetanus toxoid. The kinetics of this response are very similar to data already obtained in mice undergoing secondary challenge with sheep erythrocytes.⁷ Evidence has also been presented that the Facb-R⁺ cells that appear three days after antigenic challenge are in an activated state as judged by the acquisition of low density and the enhanced expression of HLA-DR antigens. It has been suggested that activated cells acquire low density owing to an increase in the cytoplasm:nuc-

leus ratio that accompanies blastogenesis.¹¹ Increased expression of HLA-DR antigens has also been associated with activation of cells, especially T lymphocytes,¹² and has been noted in vivo after immunisation of healthy individuals.¹³ Thus Facb-R⁺ cells appear to undergo biochemical and morphological changes after activation that parallel those observed with other cell types.

Previous data have suggested that the major function of Facb-R⁺ cells is as accessory cells in antibody production.⁶ The increase noted in Facb-R⁺ cell numbers after immunisation may therefore have functional significance. The effective induction of an immune response to specific antigen requires an adequate supply of accessory cells presenting antigens. These cells must express significant quantities of class II MHC antigens with which the antigen is recognised by sensitised T cells.¹⁴ Thus the appearance of a population of Facb-R⁺ cells expressing enhanced levels of HLA-DR antigens would allow the induction of a more effective immune response, with subsequent efficient elimination of the stimulating antigen. These Facb-R⁺ accessory cells would only be required in the early phases of the response and could appear transiently in the circulation after antigenic challenge. Experiments carried out with specific antigen in vitro cultures suggest that cells may be induced by specific antigen to express more Facb receptors rather than or in addition to those appearing from sources such as bone marrow. The disappearance of Facb-R⁺ cells from the circulation five to seven days postimmunisation may reflect migration of these cells into peripheral lymphoid tissue, though no evidence has yet been obtained for this.

It is interesting to note that increased numbers of Facb-R⁺ cells were obtained in vitro after culture of sensitised cells with specific antigen. A more rapid increase in Facb-R⁺ cells was achieved by incubation with γ -interferon. This molecule is secreted by antigen stimulated helper T lymphocytes¹⁵ and is well recognised as an inducer of class II MHC antigens¹⁶ and Fc receptor expression¹⁷ on myelomonocytic cells. Low concentrations of γ -IFN induced a threefold increase in Facb-R⁺ cell numbers and a dramatic enhancement of HLA-DR expression by these cells. These observations provide a likely explanation for the rise in Facb-R⁺ cells three days after antigenic challenge in vivo, the longer time course reflecting the need for initial interaction between antigen and T cells leading to γ -IFN production. The finding of increased numbers of Facb-R⁺ cells in normal individuals undergoing a secondary immune response suggests that a similar process associated with release of γ -IFN might be responsible for the raised numbers of these cells in

patients with rheumatoid arthritis. Increased levels of immune (γ)-IFN have been detected in rheumatoid serum samples.¹⁸ This hypothesis is also supported by the greatly increased numbers of Facb-R⁺ cells seen in patients with Felty's syndrome. The neutropenia suffered by these patients renders them more susceptible to bacterial infections which could provide an additional chronic stimulus to the immune system.

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