Lymphocytes bearing Fc γ receptors in rheumatoid arthritis. III. Immunoregulatory function associated with Facb rosette-forming cells

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SUMMARY A subpopulation of mononuclear cells (PBMNC) that express Fc receptors with specificity for the Cy2 region of IgG may be detected by rosette formation with calf erythrocytes coated with the Facb fragment of rabbit IgG. These Facb-R⁺ cells are found in increased numbers in the peripheral blood of patients with rheumatoid arthritis (RA). Studies have been carried out to identify the functional properties of these cells in healthy and rheumatoid subjects. Facb-R⁺ cells were shown to lack both natural killer and antibody-dependent cytotoxic activity. Depletion of Facb-R⁺ cells from both healthy and rheumatoid PBMNC resulted in a marked suppression of pokeweed mitogen (PWM) stimulated IgG synthesis but had no effect on T cell proliferation induced by phytohaemagglutinin, concanavalin A, or PWM. The addition of Facb fragments to PBMNC cultures also caused inhibition of PWM-driven IgG production. In this assay rheumatoid PBMNC were significantly less sensitive to Facb-mediated suppression than healthy control cells. Our results suggest that Facb-R⁺ cells are involved in the antibody-mediated feedback regulation of immunoglobulin synthesis and that this mechanism is impaired in patients with RA.

Key words: rheumatoid arthritis, antibody synthesis, feedback inhibition, Fc receptor.

The accompanying paper¹ describes the surface marker expression of a subpopulation of Fc γ receptor (FcR)-bearing mononuclear cells which may be identified by their ability to form rosettes with Facb-coated calf red blood cells (CRBC). These Facb-R⁺ cells have been found in increased numbers in the peripheral blood of patients with rheumatoid arthritis.² Facb-R⁺ cells lack conventional markers of T and B lymphocytes but express certain antigens, including OKM1 and HLA-DR, which may also be detected on monocytes.¹ The pattern of surface phenotype observed for Facb-R⁺ cells is therefore similar to that described for L cells,^{3–5} although

Facb-R⁺ cells are clearly associated with a monocytic lineage.

A number of functions have been ascribed to this class of non-T, non-B, FcR⁺ cells. These include both natural killing and antibody-dependent cellular cytotoxicity^{4–8} and regulatory effects on lymphocyte activity. The latter include the stimulation of lymphocyte proliferation^{5,7,9,10} and both enhancing and suppressive effects on antibody production.⁵ In this report the properties of Facb-R⁺ cells from both healthy individuals and rheumatoid patients have been investigated in a variety of assays of lymphocyte function.

Materials and methods

Patient selection and mononuclear cell preparation Mononuclear cells (PBMNC) were prepared from heparinised peripheral blood samples taken, with informed consent, from healthy volunteers and from patients with classical or definite rheumatoid arthritis.

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ritis, as described in the accompanying paper.¹ PBMNC were separated by density flotation over Ficoll-Paque (Pharmacia).¹¹

Preparation and use of Facb fragment. Facb fragments were prepared by plasmin digestion of a purified IgG fraction of rabbit anti-CRBC, and PBMNC expressing surface receptors for the Facb portion of IgG were detected by a rosette assay as previously described.² In some experiments PBMNC were depleted of Facb-R⁺ cells as follows: PBMNC were incubated at a final concentration of 1×10^6 /ml with an equal volume of 1% v/v Facb-sensitised CRBC on ice for 1 hour. Rosetted cells were separated from unrosetted cells by density flotation over Ficoll-Paque. Unrosetted cells were considered Facb-depleted only after testing by re-rosetting with Facb-sensitised CRBC. As a control total PBMNC were sham-depleted with non-sensitised CRBC.

Lymphocyte proliferation. The effects of addition of Facb fragment and of depletion of Facb-R⁺ cells on proliferative responses of PBMNC to certain mitogens were examined. Cells were cultured in 200 μl aliquots of RPMI 1640 (Gibco) at a concentration of 5×10^5 /ml. The culture medium contained 10% heat-inactivated fetal calf serum (Gibco), 4 mM glutamine, and 100 U/ml penicillin-streptomycin (Gibco). Quadruplicate cultures of PBMNC were aliquoted into round-bottomed microtitre plates (Nunc). In order to achieve optimal mitogenic stimulation three separate dilutions of concanavalin A (con A, Sigma), phytohaemagglutinin (PHA, Gibco), and pokeweed mitogen (PWM, Gibco) were used. Cell cultures were incubated for 68 hours at 37°C in humidified air containing 5% CO₂. Subsequently each culture was supplemented with 0.5 μCi ³H-thymidine (specific activity 5 Ci/mmol, Amersham). After a further 4 hours at 37°C the cells were harvested and the uptake of radioisotope measured by liquid scintillation spectrometry.

PWM-driven immunoglobulin synthesis. Duplicate cultures of PBMNC (1×10^6 /ml in RPMI 1640 supplemented as above) were set up in sterile culture plates (Nunc). Cultures (volume 1 ml) contained either total PBMNC or PBMNC depleted of Facb-R⁺ cells by rosetting. Cells received either no mitogen or a 1:200 dilution of PWM (Gibco) in calcium- and magnesium-free salt solution (CMFSS). Cells also received varying amounts of rabbit Facb fragment (0–15 μg/ml). After incubation for seven days at 37°C the supernatants were harvested and stored at –70°C until analysed for IgG content.

Enzyme-linked immunosorbent assay (ELISA). Polystyrene Gilford system cuvettes were coated with sheep polyvalent antihuman immunoglobulins (Sigma). After washing, doubling dilutions of either test supernatants or standard IgG preparations were

added. After one hour at 37°C and a further washing step an optimum dilution of rabbit antihuman IgG conjugated to alkaline phosphatase (Sigma) was added. A further incubation and final wash cycle were followed by addition of developing substrate (disodium *p*-nitrophenol phosphate). Absorbance readings were taken from each well at 405 nm (Gilford EIA Manual Reader) and concentrations of IgG in the supernatants estimated from the standard curve.

Cytotoxicity assays. Natural killer (NK) assays were performed on total PBMNC cultures and those depleted of Facb-R⁺ cells. The method used was a variation of that used by Pape *et al.*¹² Cells from the erythroleukaemic line K562 were maintained in RPMI 1640 medium supplemented as above. Washed cells (3×10^6 /ml) were labelled with ⁵¹Cr by incubation with 100 μCi Na₂⁵¹CrO₄ (Amersham) for one hour at 37°C and washed three times before use as targets. The assays were performed in plastic LP3 tubes (Luckham). Target cells were adjusted to a concentration of 2×10^4 /ml and 250 μl added to an equal volume of PBMNC at a final concentration of 1.2×10^6 or 6×10^5 cells/ml giving a lymphocyte:target ratio of either 60:1 or 30:1. After incubation for 16 hours at 37°C the cells were sedimented by centrifugation at 200 *g* for 10 minutes. Radioactivity present in the pellet and supernatant was measured in a gamma-counter (LKB Wallac) and the amount of specific cytotoxicity expressed as a percentage of ⁵¹Cr released from the target cells corrected for spontaneous release from K562 cells cultured in medium alone.

Antibody-dependent cytotoxicity (K cell activity) of PBMNC and Facb-R⁺ depleted cells was investigated using the method of Panayi and Corrigan.¹³ IgG-coated Chang cells labelled with ⁵¹Cr as above were used as targets.

Results

Immunoglobulin synthesis. When increasing amounts of Facb fragment were added to PWM-stimulated PBMNC cultures, a dose-dependent suppression of IgG production resulted (Fig. 1). The data demonstrate that this suppression was significantly less marked in cultures from patients with RA than in cultures from age and sex-matched controls ($p < 0.01$, Mann-Whitney U test). Baseline levels of IgG production, i.e., responses to PWM without addition of Facb fragment, were lower in the rheumatoid cell cultures (933 ± 1109 ng/ml) than in healthy control cultures (1236 ± 1230 ng/ml), but this difference was not statistically significant in these experiments. Responses to Facb fragment were very similar in all control cultures tested, but cells from

Fig. 1 The effect of rabbit Facb fragment on PWM-stimulated IgG synthesis. Results are expressed in percentage terms (mean $\pm$ SEM relative to IgG production in PWM-stimulated cultures without addition of Facb (=100%). Rheumatoid PBMNC are significantly less sensitive than healthy control cells to inhibition by Facb at 1.5 and 15 μ g/ml ($p < 0.05$ Mann-Whitney U test).

two of the 10 rheumatoid patients failed to suppress antibody production irrespective of the dose of Facb fragment added.

The possible mechanisms underlying the Facb-mediated suppression of antibody production were studied by investigating the effect of Facb-R⁺ cell depletion on PWM-stimulated IgG synthesis. As illustrated in Table 1, this procedure led to a marked inhibition of IgG production by both healthy and rheumatoid PBMNC. The addition of Facb fragment to cultures of Facb-R⁺ depleted PBMNC had no further effect. The low levels of IgG synthesis by these cells were not caused by a loss of monocytes, since significant numbers of these cells remained in the preparation as identified by non-specific esterase staining.

Lymphocyte proliferation. The depletion of Facb-R⁺ cells from PBMNC cultures had no signifi-

cant effect on lymphocyte proliferation stimulated by either con A, PHA, or PWM as measured by ³H-thymidine incorporation into DNA (Table 2). The proliferative response of PBMNC to the three mitogens was also unaffected by the addition of Facb fragments to the cultures (data not shown).

Cellular cytotoxicity. When PBMNC were depleted of Facb-R⁺ cells, there was no reduction of NK activity measured at two separate lymphocyte:target cell ratios (Table 3). The mean spontaneous release of ⁵¹Cr was $23.3 \pm 1.3\%$. K cell activity against Chang cells was also not significantly altered on depletion of Facb-R⁺ cells (Fig. 2) but was abrogated by the removal of all FcR⁺ (EA rosette forming) cells.

Table 1 The effects of Facb-R⁺ cell depletion and the addition of Facb fragments on PWM-driven IgG production by PBMNC

Source of PBMNC	% PWM- driven IgG production	
	- Facb	+ Facb
Healthy controls (n=6)		
Non-depleted	100	32 $\pm$ 9
Facb-R ⁺ depleted	46 $\pm$ 19	52 $\pm$ 18
RA patients (n=5)		
Non-depleted	100	64 $\pm$ 9
Facb-R ⁺ depleted	31 $\pm$ 16	28 $\pm$ 14

Results are presented as the mean $\pm$ SEM IgG production expressed as a percentage of the response in non-depleted cultures (=100%). Rabbit Facb fragment was added at a final concentration of 1.5 μ g/ml.

Table 2 Effect of Facb-R⁺ cell depletion on mitogen-induced lymphocyte proliferation

Mitogen		³ H-thymidine incorporation (cpm) by PBMNC	
		Non-depleted	Facb-R ⁺ depleted
Con A	10 μ g/ml	3207 $\pm$ 1047	3084 $\pm$ 1551
	50 μ g/ml	3915 $\pm$ 1357	4159 $\pm$ 963
	100 μ g/ml	2966 $\pm$ 1391	3451 $\pm$ 1315
PHA	0.2 μ g/ml	2488 $\pm$ 453	2371 $\pm$ 598
	1 μ g/ml	3673 $\pm$ 946	4453 $\pm$ 1546
	5 μ g/ml	2451 $\pm$ 554	3054 $\pm$ 1280
PWM	1:2000	2645 $\pm$ 1458	807 $\pm$ 688
	1:400	4142 $\pm$ 2110	2880 $\pm$ 1334
	1:40	2936 $\pm$ 1376	2196 $\pm$ 662

Results are expressed as ³H-thymidine incorporation (cpm/10⁶ PBMNC) and are the mean $\pm$ SEM of six experiments using rheumatoid cells. Similar data (not shown) were obtained in eight experiments using healthy control PBMNC.

Table 3 The effect of Facb-R⁺ cell depletion on NK cytotoxicity of PBMNC

PBMNC	% Specific cytotoxicity (⁵¹ Cr release) at different lymphocyte:target cell ratios	
	60:1	30:1
Non-depleted	20.8±7.2	16.4±6.1
Facb-R ⁺ depleted	20.9±11.4	18.1±9.0

Results are presented as the mean±standard deviation of five experiments. The spontaneous ⁵¹Cr release from the target cells was 23.3±1.3%.

Fig. 2 Antibody-dependent cellular cytotoxicity of PBMNC fractions at various effector : target cell ratios. Results are presented as % specific ⁵¹Cr release (corrected for release in the absence of antibody). Data points represent the mean ± standard deviation of seven experiments. ●—● PBMNC; ○—○; Facb-R⁺ depleted PBMNC; △—△ FcR⁺ depleted PBMNC.

Discussion

Mononuclear cells expressing FcR have been shown to mediate a number of functions including natural and antibody-dependent cytotoxicity⁶⁻⁸ and both enhancing and suppressive effects on lymphocyte function.^{5,7,9,10} Although forming part of the FcR⁺ population, Facb-R⁺ cells do not exhibit cytotoxic activity. This supports the conclusion drawn from the surface phenotype analysis, namely, a lack of identity

between Facb-R⁺ cells and the L cell population described by Horwitz and coworkers.^{3,4,7} The lack of cytotoxic activity by Facb-R⁺ cells is also consistent with the observations of elevated levels of these cells,² but normal NK function¹⁴ and ADCC¹³ in rheumatoid peripheral blood.

Our results indicate a role for Facb-R⁺ cells in certain lymphocyte responses involved in antibody synthesis but not others such as T cell proliferation. The ability of Facb fragments to inhibit PWM-driven IgG synthesis might be due to the activation of a suppressor mechanism or to the blocking of a positive, accessory function of Facb-R⁺ cells. These possibilities were investigated by depleting PBMNC of Facb-R⁺ cells before culture. Removal of a suppressor population should result in enhanced IgG production, whereas loss of accessory cells would diminish antibody synthesis. Our results clearly indicate that Facb-R⁺ cells are necessary for PWM-stimulated IgG production and that this function may be blocked by Facb fragments. The addition of Facb fragments to Facb-R⁺ depleted cell cultures had no effect, supporting the assumption that their effect is mediated via Facb-R⁺ cells. Depletion of Facb-R⁺ cells did not result in a significant loss of monocytes as judged by staining for non-specific esterase. The 'accessory' function of Facb-R⁺ cells is consistent with the demonstration of class II MHC antigens (HLA-DR) on these cells¹ a property shared by all antigen-presenting cell populations.¹⁵ Further experiments are in progress to investigate antigen-presenting functions of Facb-R⁺ cells using specific antigens. Our data on the functions of Facb-R⁺ cells are similar but not identical to those described for L cells by Lobo.⁵ These include inhibition of immunoglobulin synthesis following treatment with immune complexes and enhancing effects on lymphocyte proliferation. Further studies are required to determine whether, under certain circumstances, human Facb-R⁺ cells may be stimulated to exert a direct suppressive action, although some evidence for such an effect has recently been obtained in mice.¹⁶

Results from the present study do indeed show similarities to those obtained in mice where Facb-R⁺ cells were unaffected by delayed hypersensitivity reactions to oxazolone or to sheep erythrocytes but were elevated during the secondary antibody response to the latter antigen.¹⁶ This report also demonstrated that antigen-specific Facb fragments could dramatically suppress specific antibody production in vivo and that Facb-R⁺ cells might therefore mediate IgG-dependent feedback inhibition of further antibody synthesis. Such feedback inhibition has been recognised for many years¹⁷ and has been considered to operate either via a direct blockade of B lymphocytes¹⁸ or by interfering with T-B cell

interactions.¹⁹ The present study clearly shows that, although PWM-dependent IgG synthesis is markedly inhibited by Facb-R⁺ cell depletion, mitogen-induced T cell proliferation is virtually unaffected. This suggests that Facb-R⁺ cells do not interact significantly with T lymphocytes, and, since Facb-R⁺ cells do not have any characteristics of B lymphocytes,¹ our studies might be taken to support Hoffman's concept of feedback inhibition of antibody production, namely, an effect on T-B cell co-operation.¹⁹

Although the precise mechanism whereby Facb-R⁺ cells are involved in feedback inhibition remains to be elucidated, our results strongly suggest that rheumatoid PBMNC are significantly less sensitive to such regulation than healthy cells. This might be due to the increased numbers of Facb-R⁺ cells in the rheumatoid samples. However, this increase is small (up to 5-fold) compared with the decreased sensitivity of RA cells to inhibition by Facb fragments (100-fold, Fig. 1). There is evidence that rheumatoid Facb-R⁺ cells are in an activated state when isolated ex vivo (in preparation) and thus could be less susceptible to the effects of ligands added in vitro. Alternatively, some rheumatoid B cells might be inherently resistant to feedback suppression by IgG antibody. Such a selective immunoregulatory defect affecting an autoimmune response would lead to excessive antibody production with the likelihood of serious consequences, including immune complex deposition and subsequent inflammatory tissue damage.

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