Abnormal responses of rheumatoid arthritis lymphocytes to Epstein–Barr virus infection in vitro: evidence for multiple defects

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SUMMARY Blood lymphocytes from 53 patients with rheumatoid arthritis (RA) and 44 controls were cultured with the polyclonal B cell activator Epstein–Barr virus (EBV). Culture supernatants were removed at weekly intervals and the amount of IgM secreted by the lymphocytes measured by an enzyme-linked immunosorbent assay (ELISA). Three major differences in the pattern of EBV-induced IgM synthesis by RA versus control lymphocytes were observed. Lymphocytes from RA patients, in general, produced less IgM after one week in culture than controls. In contrast, they increased their IgM secretion significantly by the end of the second week, whereas control lymphocyte cultures showed little change in IgM secretion at this time. Control lymphocytes from EBV seropositive individuals produced undetectable amounts of IgM after five weeks in culture. However, lymphocytes from 40% of the RA patients, even though they were EBV seropositive, secreted >2000 ng/ml (μg/l) IgM after five weeks. The data are discussed in terms of defective B and T cell responses to EBV in lymphocytes from patients with RA.

Key words: B lymphocytes, suppressor/cytotoxic cells.

Rheumatoid arthritis (RA) is a disease characterised by the presence of disorders in immunoregulation.^{1,2} The most striking example of this in vivo is the detection of circulating antiglobulins (rheumatoid factors, RF) in the serum of 80% of RA patients, while in vitro a wide variety of experimental techniques have been used to show immunoregulatory defects in cells derived from RA patients.^{1,3}

Epstein–Barr virus (EBV) is a DNA-containing virus of the herpes group. The virus specifically infects human B lymphocytes, and infection is followed by a polyclonal activation of these cells.⁴ These properties make EBV a useful laboratory tool for dissecting various aspects of in-vitro immunoregulation.

The presence of an abnormal immune response to EBV in RA patients was first suggested by the observations of higher titres of antibodies to various

EBV-derived antigens in the serum of RA patients compared with controls.^{5,6} Subsequently many investigators have shown defects in the in-vitro cellular control of EBV-induced B cell activation in RA cells.^{7–12} However, the in-vitro control of EBV-induced B cell activation is undoubtedly complex, involving such factors as susceptibility of B cells to viral infection, the action of natural killer cells, the production of soluble regulatory factors and the role of functionally different T cell subsets. Each experimental protocol tends to highlight one particular aspect of this multifactorial regulatory system, occurring at particular times after EBV infection. We describe here an assay system designed specifically to monitor regulation of EBV-induced B cell activation at several time intervals after viral infection. Extension of this technique to RA cells has shown several differences in the patterns of response given by cells from RA patients compared with normal cells and has suggested ways of further elucidating the defects in EBV regulation evident in RA cells.

Materials and methods

PATIENTS AND CONTROLS

Heparinised peripheral blood was drawn from 53 patients (16 male, 37 female, mean age 58 years, range 24–79) fulfilling the American Rheumatism Association criteria for diagnosis of RA. Treatment regimens included gold injections alone (eight patients), gold injections plus non-steroidal anti-inflammatory drugs (NSAIDs) (17), gold injections plus low dose prednisone (two), NSAIDs alone (13), D-penicillamine plus NSAIDs (five), azathioprine plus NSAIDs (two), chloroquine (one), prednisone plus NSAIDs (one), and unknown (two). Two patients were not receiving drugs. Laboratory personnel and patients in hospital for non-urgent surgery (hernia repair, varicose vein stripping), provided control blood; there were 22 males and 22 females with a mean age of 37 years and a range of 19–71.

EBV SEROLOGY

A standard indirect immunofluorescence method was used to detect IgG antibodies to the EBV viral capsid antigen (VCA) in the serum of all donors, with the P3HR-1 cell line as a source of VCA-positive cells.¹³ Sera were tested at a 1/8 dilution and a positive test taken as evidence of prior exposure to EBV (EBV seropositivity).

PREPARATION OF EBV

Infectious virus was obtained from the supernatant of the marmoset cell line, B95-8¹⁴ (kindly provided by Professor A Rickinson, Birmingham University), and stored in aliquots at -70°C .

LYMPHOCYTE SEPARATION

Blood was diluted 1:1 with Hanks's balanced salt solution, and the mononuclear cells obtained by density gradient centrifugation according to Boyum,¹⁵ using Lymphopaque (Nyegaard). The cells were washed twice with RPMI 1640 containing 5% heat-inactivated fetal calf serum (HI-FCS), counted, and resuspended at 10^9 cells/l in RPMI 1640 supplemented with 10% HI-FCS, 2 mM glutamine, penicillin (10^5 units/l), and streptomycin (100 mg/l).

CELL CULTURE

1 ml aliquots (10^6 cells) were dispensed into 5 ml tubes (Falcon 2003). 100 μl of EBV-containing B95-8 supernatant or 100 μl of supplemented RPMI 1640 were then added to each tube. At least four replicate tubes of virally infected cells were set up for each donor. Cells were cultured at 37°C in a 5%

CO_2 humidified atmosphere. At weekly intervals the tubes were centrifuged at 1500 rpm for seven minutes at room temperature, 900 μl of supernatant was carefully removed, and the cells resuspended in 900 μl of fresh supplemented RPMI 1640.

IGM ASSAY

The amount of IgM secreted into the culture supernatants was measured by an ELISA. Micro-ELISA plates (Dynatech) were coated with 200 μl /well of a 1/1000 dilution of goat antihuman μ chain (Tissue Culture Services) in carbonate/bicarbonate coating buffer. The plates were incubated at 4°C overnight, then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/Tween). 200 μl of a range of IgM standards or of test supernatant was added to each well. The latter were diluted in supplemented RPMI if necessary, in order that the final optical densities obtained fell on the linear part of the standard curve. The plates were incubated at 37°C for two hours, then washed three times as above. Finally 200 μl of a 1/1000 dilution of peroxidase conjugated rabbit antihuman μ chain (Dako) in washing buffer was added to each well. The plates were incubated for two hours at 37°C , then washed three times as above. 200 μl of a chromogenic substrate (2,2'-azino-di-(3-ethylbenzthiazoline) sulphonic acid) dissolved in citrate phosphate buffer to which hydrogen peroxide had been added was added as substrate to each well. After 10 minutes 50 μl of a 3.2 g/l solution of sodium fluoride in distilled water was added to each well to stop the reaction. The optical density of each well was read on a Dynatech ELISA reader at 630 nm. Comparison of the optical densities with the standard curve enabled quantification of the IgM in each test supernatant. Each supernatant was tested in duplicate wells and the mean reading taken.

PROLIFERATION ASSAY

200 μl of peripheral blood mononuclear cells (PBMC) in supplemented RPMI (10^9 cells/l) was dispensed into microplates, and 20 μl of B95-8 supernatant or 20 μl of supplemented RPMI added. The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere. Eighteen hours before harvesting 50 μl of ^{125}I -UdR (uridine deoxyribose) (specific activity 10 mCi/l, Amersham) was added to each well. Plates were harvested using a Titertek multichannel harvester, and the cellular radioactivity was incorporated onto glass fibre discs. The discs were counted and the results expressed as a stimulation index (SI), where $\text{SI} = \text{cpm cells cultured with virus} / \text{cpm cells cultured alone}$.

STATISTICS

Statistical analysis was performed by the Student's *t* test, χ^2 analysis, and the Kolmogorov–Smirnov two-sample test.

Results

AT ONE WEEK OF CULTURE

The amount of EBV-induced IgM secretion in the supernatants of lymphocytes from controls and RA patients after one week in culture was compared (Fig. 1). Overall, larger amounts of IgM were secreted by control relative to RA lymphocytes. Thirty-one out of 53 RA patients had IgM values of 0–200 ng/ml ($\mu\text{g/l}$) compared with 7/44 controls ($p<0.0001$). Only 1/53 RA patients had ≥ 1000 ng/ml IgM compared with 14/44 controls ($p<0.0001$). There was a significant difference between the RA patients and controls ($p<0.002$) as determined by the two-sample Kolmogorov–Smirnov test.

Since gold treatment results in the suppression of lymphocyte responses to the T cell dependent mitogen pokeweed mitogen (PWM),¹⁶ it seemed possible that the reduced IgM secretion by RA lymphocytes after one week of culture with EBV could also be due to this treatment. However, as seen in Table 1, when the one-week EBV-induced IgM responses of lymphocytes from 27 patients treated with gold were compared with those of 24 other patients on a variety of other treatments (including NSAIDs (13 patients), *D*-penicillamine plus NSAIDs (5), azathioprine plus NSAIDs (2), chloroquine (1), prednisone plus NSAIDs (2), and no drug therapy (2)), no differences were seen.

Table 1 Reduced seven-day EBV-induced IgM secretion by RA lymphocytes is not the result of gold treatment

Treatment	IgM (ng/ml)*			
	<200	200–500	500–1000	≥ 1000
Gold (27)†	16‡	9	2	0
Other (24)	13	8	2	1

*ng/ml= $\mu\text{g/l}$.
†Numbers of patients receiving gold or other treatments (NSAID (13), *D*-penicillamine plus NSAIDs (5), azathioprine plus NSAIDs (2), chloroquine (1), prednisone plus NSAIDs (2), and no drug therapy (2)).
‡Number of patients whose lymphocytes cultured with EBV secreted IgM in the ranges shown.

chloroquine (1), prednisone plus NSAIDs (1), and no drug therapy (2)), no differences were seen.

AT THE SECOND WEEK OF CULTURE

The ratio of the amount of EBV-induced IgM secreted by lymphocytes at the end of the second week of culture relative to the first week is shown in Fig. 2. Whereas control lymphocytes tended to give low ratios indicating little change in IgM secretion by the second week of culture, the values obtained for the RA lymphocytes were significantly skewed towards higher ratios. Thus 15/32 control cultures showed little change in EBV-induced IgM secretion in the second week of culture compared with the first (ratios of ≤ 2), whereas only 4/49 RA cultures behaved similarly ($p<0.0001$). However, in 21/49 cultures from RA patients IgM values increased

Fig. 1 EBV-induced IgM secretion by blood lymphocytes from RA patients and controls. IgM was measured in the seven-day culture supernatants by an ELISA. The histograms show the percentage of RA and control donors whose lymphocytes secreted IgM in the ranges indicated; (ng/ml= $\mu\text{g/l}$). Figures in parentheses are the number of individuals studied.

Fig. 2 Increase in EBV-induced IgM secretion by RA and control lymphocytes from the first to the second week of culture. IgM was measured in the seven-day culture supernatant by an ELISA. The supernatants were removed, fresh medium added, and the IgM measured one week later. The histograms show the percentages of RA and control donors with ratios of IgM secreted in the second compared with the first week indicated in the ranges shown; (ng/ml= μ g/l).

more than 10-fold during the second week compared with only 2/32 controls ($p<0.0005$). Population analysis comparing all the RA patients with all the controls by the Kolmogorov-Smirnov test showed a significant difference ($p<0.002$).

AT THE FIFTH WEEK OF CULTURE
EBV-infected lymphocyte cultures from 13/16 EBV seropositive control donors were secreting <200 ng/ml IgM by the fifth week of culture (Fig. 3). Lymphocytes from all these donors had secreted >200 ng/ml IgM in the first week of culture (data not shown), and had therefore 'switched off' their IgM secretion by the fifth week. Three out of six replicate lymphocyte cultures from a single donor (starred) were secreting >2000 ng/ml, while the other three were secreting <200 ng/ml. IgM secretion by two other control donors was very low (<400 ng/ml) and decreasing when compared with the first- and second-week data from the same individuals (data not shown).
In contrast, lymphocyte cultures from 14/38 (37%) EBV seropositive RA patients were still secreting >2000 ng/ml IgM by the end of week 5 and continued to develop into cell lines when transferred to Costar plates, i.e., they were behaving like the cultures from EBV seronegative control and RA donors. Nineteen out of 38 (50%) of the RA patients had 'switched off' their IgM secretion by week 5 (<200 ng/ml), while cultures from one donor were secreting measurable but low values. Cultures from four other donors showed some

Fig. 3 Failure of T cells from some EBV seropositive RA patients to terminate EBV-induced IgM secretion after five weeks of culture. Culture supernatants were replaced at weekly intervals, and IgM measured in the fifth-week supernatant. Each symbol represents an individual EBV seropositive or seronegative RA patient or control. Fourteen out of 38 EBV seropositive patients had IgM values of >2000 ng/ml. Cultures from 4/38 patients showed some tubes with high and some with low secreted IgM ($\dagger$). Three out of six cultures from one control donor (*) secreted >2000 ng/ml IgM, while the remaining three cultures from this donor secreted <200 ng/ml IgM. Cultures from all EBV seronegative donors produced >2000 ng/ml IgM. (ng/ml= μ g/l).

culture tubes with high and some with low IgM levels.

RELATIONSHIP BETWEEN IgM SECRETION AND 125 I-UdR INCORPORATION AT ONE WEEK OF CULTURE

Lymphocyte cultures from 14 controls and 14 RA patients were compared in terms of their EBV-induced IgM synthesis and EBV-induced proliferation as measured by 125 I-UdR incorporation. As shown in Table 2 there was no significant difference in 125 I-UdR incorporation by the RA lymphocytes as measured by the stimulation index compared with the controls ($p>0.6$). However, the RA cultures secreted significantly lower amounts of IgM than the controls, with 6/14 secreting <200 ng/ml IgM compared with 0/14 controls ($0.01 < p<0.025$).

Discussion

The patterns of EBV-induced IgM secretion in vitro by lymphocytes from RA patients and controls differ in three major ways. Firstly, RA peripheral blood mononuclear cells (PBMC) secrete significantly lower amounts of IgM in the first week of culture than do normal cells. Secondly, the synthesis of IgM by RA cells is markedly increased during the second week of culture, while that of control cells is only moderately increased if at all. Finally, cells from a significant proportion of EBV seropositive RA donors continue to secrete large amounts of IgM after five weeks of culture, whereas cultures from EBV seropositive control donors almost invariably fail to produce detectable amounts of IgM at this stage.

The differences in IgM secretion reported in this study are in contrast to the data of Slaughter *et al.*,¹⁷ who found no changes in IgM production by RA compared with normal lymphocytes up to three weeks in culture. The reasons for this discrepancy are unclear, but the latter authors studied many

fewer RA patients and used culture conditions different from those described in this study.

The defects in EBV handling by RA lymphocytes could be a consequence of the disease activity or drug treatments, or both. Although we did not assess the disease activity in all patients, there appeared to be no relationship between disease activity in 12 patients and the pattern of response to EBV. It has previously been shown that gold treatment in vivo inhibits PWM induced differentiation of lymphocytes in vitro.¹⁶ Since many of the patients in this study were treated with gold, it seemed possible that this could contribute to the abnormal response to EBV, especially at week 1. However, comparison of gold versus non-gold patients showed no differences in the pattern of IgM synthesis during the first week of culture with EBV.

The reduced synthesis of IgM in the first week of culture by RA PBMC is unlikely to be due to fewer B cells in their lymphocyte populations, since we and others have shown that both the total number and percentage of circulating B cells in RA patients are not significantly different from those of controls.^{18, 19} There is evidence for heterogeneity in responsiveness to EBV within the normal B cell population.²⁰⁻²³ Our observation that EBV-induced proliferation in RA lymphocytes is similar to control lymphocytes at seven days is consistent with the premise that EBV-induced proliferation and EBV-induced differentiation are two independent processes and leads us to suggest that RA cells may be deficient in that population of B cells which responds to EBV by IgM secretion. This, in turn, could be a consequence of the increased numbers of activated B cells in the circulation of RA patients,^{19, 25} as cells already activated respond poorly to EBV.²⁴ Even though RA lymphocytes may contain fewer EBV-inducible IgM precursor B cells, they must be relatively rich in IgM RF precursors, since there is a high spontaneous and EBV-driven production of IgM RF by RA lymphocytes in vitro.¹⁷

Table 2 Comparison of EBV-induced IgM secretion and 125 I-UdR incorporation by RA and control lymphocytes after one week of culture

Donors	No.	Stimulation index†	IgM (ng/ml)*			
			<200	200-500	500-1000	≥ 100
Controls	14	2.51 ± 0.97	0‡	8	4	2
RA	14	3.53 ± 1.49	6	7	1	0

*ng/ml= μ g/l.

†Quadruplicate cultures were pulsed with 125 I-UdR and harvested 18 hours later (day 7). The stimulation index is the ratio of cpm in the EBV cultures compared with the lymphocytes alone. Values are given as mean $\pm$ SD.

‡Number of donors whose lymphocytes cultured with EBV secreted IgM in the ranges shown.

PBMC from normal donors secrete roughly similar or moderately increased amounts of IgM during the second week of culture. Cultures of control lymphocytes depleted of T cells markedly increase their IgM secretion during the second week of culture (data not shown), suggesting that T cells regulate this response. It is therefore likely that the dramatic increase in IgM secretion between weeks 1 and 2 of culture of RA cells reflects a defect in T suppressor (T_s) mechanism(s). This would be consistent with the work of Tosato *et al.*,¹⁰ who used a similar assay. The suppression of EBV-induced IgM secretion may be mediated by the interferons,^{26 27} and in this regard, γ -interferon production by RA cells in an autologous mixed lymphocyte reaction has been reported to be decreased compared with normal cells.²⁸

The third abnormality in the pattern of response of RA cells to EBV, that of failure to switch off IgM synthesis after five weeks in culture, is the most specific defect, since it was seen in only one of the control donors and then not in all replicate cultures. EBV-specific cytotoxic T cells (T_c cells) develop in in-vitro PBMC cultures from normal EBV seropositive individuals, and these cells mediate regression of proliferating EBV-infected autologous B cells.²⁸ Thus it is likely that these are absent or fail to expand adequately in cultures of lymphocytes from some RA patients. Indeed, defective EBV-specific T_c function in RA, as measured by regression of long-term cultures, has also been reported by other investigators.^{11 29} The process of generation of specific T_c is both interleukin-1 (IL-1) and -2 (IL-2) dependent.³⁰ Impairment of this process in cells from some RA patients may thus be due to an inability to generate or respond to IL-1 or IL-2. Interestingly other workers by different experimental protocols have reported defective production of IL-2 by PBMC from RA patients.³¹

The possible relationship between the putative T_s and T_c cell defects in RA cells described above is unclear. It is worth noting that whereas the majority of RA patients showed the putative T_s defect at two weeks, only lymphocytes from 39% of them failed to switch off IgM secretion after five weeks, i.e., showed evidence of a putative T_c defect. However, this does not formally prove that T_s and T_c functions are mediated by independent T cell subpopulations, since the first defect may reflect low numbers of EBV-specific T_{sc} precursors, which nevertheless can expand sufficiently to mediate regression by five weeks in culture in some RA patients but not in others. In a small number of patients the degree of expansion may be critical, thus explaining why regression can occur in some replicate cultures but not in others.

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