Rapid Publication

Abnormally Elevated Frequency of Epstein-Barr Virus-infected B Cells in the Blood of Patients with Rheumatoid Arthritis

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Abstract. Patients with rheumatoid arthritis (RA) are known to have in vitro regulatory T cell abnormalities relating to Epstein-Barr virus (EBV). In this report, we asked whether patients with RA have more circulating EBV-infected B cells than normals. To address this question, we determined the frequency of spontaneously transforming B cells in the peripheral blood of 18 normals, 15 patients with RA, and 8 patients with systemic lupus erythematosus (SLE). The mean frequency of spontaneously transforming B cells in RA patients was $10.1/10^6$ B cells, which was significantly greater than that of the normal controls, $2.8/10^6$ B cells ($P < 0.005$). The group of patients with SLE did not differ from the normals ($P > 0.4$). In further studies undertaken to investigate as to whether RA B cells are more easily transformed by EBV than normal B cells, we determined that the frequencies of transforming B cells in the presence of exogenous EBV were similar in RA patients and normals. Lymphocytes obtained from patients with RA demonstrate a profound T cell defect in their EBV-specific suppression, as measured in vitro; there was no direct correlation, however, between this in vitro T cell abnormality and the number of circulating EBV-infected B cells. Thus, patients with RA, as a group, have abnormally elevated numbers of circulating EBV-infected B cells, and this abnormality most likely derives from a complex dysregulation of the defense mechanisms for infection with EBV.

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Introduction

Epstein-Barr virus (EBV)[1] preferentially infects B lymphocytes (1) that become activated by this virus to proliferate (2), differentiate to immunoglobulin production (3), and transform into long-term culture lines (4). Like other herpes viruses, EBV persists for life after primary infection (5), and has the potential for continuously inducing B cell activation. Several immunoregulatory T cell mechanisms have been identified in vitro which act to limit or suppress B cell activation by EBV (6–9). If these in vitro T cell effects are also relevant to the in vivo control of EBV, then patients with defects in these processes might be expected to reflect this abnormality with evidence of an increased number of EBV-infected B cells in vivo.

A number of recent observations have demonstrated that infection of B cells with EBV is abnormally regulated in rheumatoid arthritis (RA). Most patients with this disease have higher than normal serum antibody titers to EBV-associated antigens (10, 11). Lymphocytes from patients with RA become transformed into EBV-infected continuous B cell lines more readily than those from normals (12, 13). This enhanced transformability has been reported to be due to diminished T cell control of EBV-activated B cells (13). Other regulatory T cell defects

1. *Abbreviations used in this paper:* CyA, cyclosporin A; EBV, Epstein-Barr virus; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

relating specifically to EBV have also been identified in patients with RA, including a diminished control of EBV-induced B cell proliferation and immunoglobulin production (13–15).

To test whether patients with RA have an increase in the number of EBV-infected circulating B cells, we have determined the frequency of spontaneously transforming B cell precursors in patients and in normal controls. Our results indicate that patients with RA have, as a group, abnormally elevated numbers of spontaneously transforming EBV-infected B cell precursors in the peripheral blood.

Methods

Study populations. The study population consisted of 15 patients with classical RA (mean age, 45 yr), 8 patients with systemic lupus erythematosus (SLE) (mean age, 38 yr), 2 normal newborns, and 18 normal controls (mean age, 35 yr). 14 of the patients with RA were positive for rheumatoid factor at the time of the study. 8 of the patients with RA were treated with nonsteroidal anti-inflammatory drugs only; the remaining 7 patients with RA and all patients with SLE were treated with low-dose corticosteroids and/or low-dose immunosuppressive agents. Antibody titers to EBV-specific antigens were determined by immunofluorescence techniques, as previously described (16). All patients and normal adults were EBV-seropositive. Antibody titers to EBV of 10 of the patients with RA, 5 of the patients with SLE, and all the normal adults have been previously reported (15). The remaining patients had anti-EBV antibody titers consistent with these reported cases.

Cells and cultures. Unseparated mononuclear cells and cell subsets enriched for B and T cells were obtained as previously described (9). Precursor frequencies of transforming B cells were determined by limiting dilution analysis of multiple microcultures. Mononuclear cells or B cell enriched populations were cultured at serial twofold reductions in cell densities (1×10^6/well, 0.5×10^6/well, 0.25×10^6, etc.) with a feeder layer of autologous irradiated (3000 rad) T cells (1×10^5/well) in 96 well flat-bottom microtiter plates (Costar, Cambridge, MA). At least 20 replicate cultures were established at each cell density. Cyclosporin A (CyA) was prepared as previously described (17), and was used in culture at the final concentration of 1 μg/ml. In some experiments, EBV (filtered supernatant of the B95-8 cell line, containing $\sim 10^6$ transforming units/ml) was added in 50 μl quantity to the microtiter wells. The microcultures were incubated for 6 wk at 37°C in a humidified atmosphere containing 5% carbon dioxide. The fraction of B cells in mononuclear and non-T cell populations was determined by fluorescence microscopy following treatment using a fluorescein isothiocyanate conjugated goat anti-human immunoglobulin reagent (Cappel Laboratories, Cochranville, PA). T cell suppression of EBV-activated B cells was determined in 12-d cultures of 0.5×10^6 EBV-infected B cells incubated alone or mixed with autologous T cells (2.0×10^6), as previously described (9, 15).

Assay systems and analysis of the data. Outgrowth of transformed lymphocytes was assessed after 6 wk of culture by visual examination using phase-contrast microscopy, according to criteria previously described (6). A well was considered transformed when both large single cells and clumps of large cells were recognized. Outgrowth at 6 wk was also determined in parallel by measuring DNA synthesis, as previously described (6), and expressing the results as counts per minute. Results of microscopic evaluation and proliferation were highly correlated when a counts per minute value > 1,000 was considered to be evidence of outgrowth. A well was considered transformed when it was positive by either microscopic assessment of outgrowth or proliferation. All wells were scored as positive or negative by these criteria.

The precursor frequency of transforming B cells was obtained by Poisson analysis, using the minimum chi square analysis method described by Taswell, as previously reported (18). All experiments had a P value (goodness of fit test) > 0.05, and the frequency determined by minimum chi square analysis was within 15% of that determined by the maximum likelihood method. When there were no transformed wells observed (there were thus no precursors for transformation among the over 30×10^6 mononuclear cells cultured), the precursor frequency was conservatively considered to be <1 in 10×10^6 mononuclear cells. In actuality, the precursor frequency was very likely to be lower than this; with a precursor frequency of 1 in 10×10^6, there is a 95% chance of having at least one transformed well with 30×10^6 cells cultured. The precursor frequencies obtained in this manner were corrected for the percentage of B cells in the mononuclear cell population and expressed as precursor frequency per 10^6 B cells.

The number of immunoglobulin-secreting cells produced in 12-d cultures established to assess EBV-specific T cell suppression was determined by a reverse hemolytic-plaque assay, as previously described (9).

To directly assess the EBV genomic DNA in RA patients and controls, cellular DNA was extracted from the peripheral blood mononuclear cells of 8 normals (6 adults and 2 newborns) and 12 RA patients. Cellular DNA was also obtained from three EBV-negative cell lines derived from American Burkitt's lymphomas and used as control. After denaturation by treatment with 1 M sodium hydroxide and neutralization by addition of 1 M ammonium acetate, 10 μg of each DNA preparation was transferred to nitrocellulose membranes using a slot blot apparatus (Schleicher & Schuell, Keene, NH). ^{32}P-labeled probes of EBV DNA were prepared by nick translation of denatured viral DNA isolated from virions released by the B95-8 cell line. Hybridizations were performed under stringent conditions in 50% formamide at 37°C for 16 h in the presence of 10×10^6 cpm of EBV [^{32}P]DNA with a specific activity of 2.5×10^8 cpm/μg. Following hybridization, the nitrocellulose blots were extensively washed and exposed to XAR-5 x-ray film (Kodak) with intensifying screens at −70°C.

Geometric means, SEM, and correlation coefficients were calculated by conventional formulas; differences between group means were determined by Student's t test. To calculate geometric means and compare group means, precursor frequencies of <1 in 10^6 B cells were considered to be 1 in 10^6 B cells.

Results

Functional inactivation of EBV-immune regulatory T cells by CyA. After primary infection with EBV, regulatory T cells are generated that have been shown to profoundly inhibit the activation of autologous B cells by EBV in vitro (6–8). CyA inhibits the activation of these EBV-specific suppressor T cells (17), and facilitates spontaneous and EBV-induced outgrowth of EBV-infected lymphoblastoid cell lines (19). To determine whether addition of CyA completely removes T cell inhibition of EBV-induced B cell transformation without directly affecting the B cells, we compared the frequencies of B cell outgrowth in cultures of purified non-T cells with the frequencies in cultures of unseparated mononuclear cells supplemented with CyA (1 μg/ml). This comparison was made both for transformation induced

Table I. Comparison of Two Methods for Determining Precursor Frequencies of B Cell Outgrowth

Cell cultures*	Precursors/10^6 cultured cells	% B cells	Precursors/10^6 B cells
Mononuclear cells			
+ CyA + EBV	349	7.8	4,464
Non-T cells + EBV	1,633	33.8	4,830
Mononuclear cells			
+ CyA	2.7	5.8	47.3
Non-T cells	12.0	18.2	45.3

* Multiple replicate cultures of mononuclear cells and non-T cells were established with or without EBV and CyA (1 μg/ml) and scored for the presence or absence of outgrowth after 6 wk.

by exogenous EBV and for spontaneous transformation. As shown in Table I, CyA was found to effectively remove T cell inhibition of B cell outgrowth by EBV, and not to otherwise influence, by enhancing or inhibiting, the process of B cell transformation by EBV.

A representative experiment showing the precursor frequency determination of spontaneously transforming B cells using mononuclear cells with CyA is shown in Fig. 1. The plot of B cell numbers per culture versus the log of the frequency of negative cultures yielded a straight line. This indicated that the technique used was valid for the detection of outgrowth by the progeny of a single EBV-infected precursor B cell, and that only one cell type was limiting in these cultures. In this experiment, 1 in 21,800 B cells transformed (1 in 16,400–1 in 32,800, 95% confidence interval).

Abnormally elevated frequencies of spontaneously transforming B cell precursors in rheumatoid arthritis. Studies were undertaken to determine the precursor frequency of spontaneous B cell outgrowth from the peripheral blood of 18 normal adults, 15 patients with RA, and 8 patients with SLE. Since CyA completely removes the inhibition of B cell outgrowth exhibited by EBV-immune T cells, all experiments were performed using mononuclear cells in the presence of 1 μg/ml CyA. The results, shown in Fig. 2, are expressed as the number of precursors/10^6 B cells. In normals, the frequency of spontaneous B cell outgrowth varied from 10 to <1 precursor/10^6 circulating B cells, with a mean of 2.8/10^6 B cells. By contrast, in patients with RA, the mean precursor frequency of spontaneous B cell outgrowth was 10.1/10^6 cells, with 9 of these patients (60%) having a higher frequency than observed in any of the normals ($P < 0.005$, comparing RA and normals). Two of the patients with SLE (25%) had >10 precursors/10^6 B cells, but as a group SLE patients did not significantly differ from normals ($P > 0.4$). The difference between RA and SLE patients was marginally significant ($P < 0.1$), owing to the small number of SLE patients studied. Thus, patients with RA, but not those with SLE, had significantly more spontaneously transforming EBV-infected B cell precursors in the peripheral blood than found in normals. Each of the two subgroups of patients with RA, those receiving

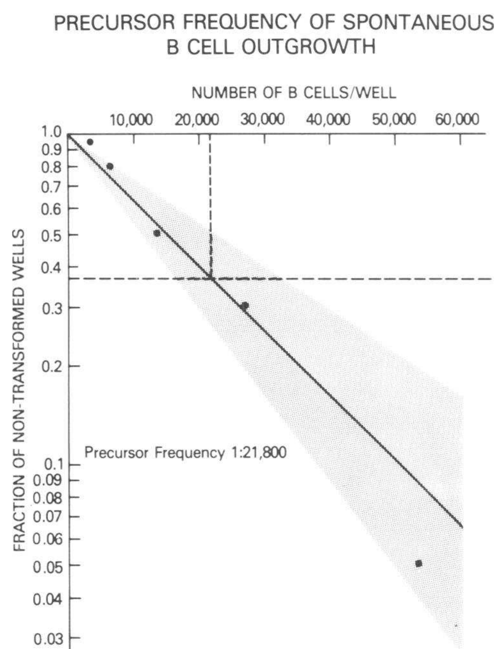

Figure 1. Representative limiting dilution analysis of spontaneous B cell outgrowth in vitro. Multiple replicate cultures of mononuclear cells established in the presence of CyA (1 μg/ml) were scored at 6 wk for the presence or absence of transformation. In this experiment, 1 in 21,800 B cells underwent spontaneous transformation. The shaded area gives the 95% confidence interval for this determination.

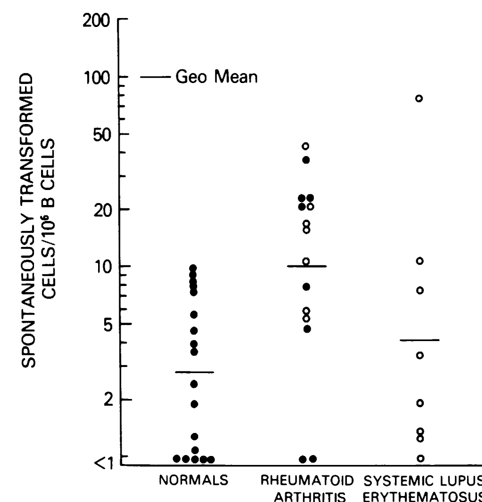

Figure 2. Precursor frequencies of spontaneous B cell outgrowth in normal subjects, patients with RA, and patients with SLE. Closed circles indicate individuals receiving either no treatment or nonsteroidal anti-inflammatory drugs; open circles indicate patients receiving steroids and/or cytotoxic drugs.

nonsteroidal anti-inflammatory drugs (seven patients), and those receiving steroids and/or cytotoxic drugs (eight patients), had higher mean values of spontaneously transforming B cell precursors than normals ($P < 0.05$ in each case).

We asked whether elevated frequencies of spontaneously transforming B cells were associated with an increase in the number of EBV genome copies in the peripheral blood cells of patients with RA as compared with normals and performed blot hybridization of mononuclear cell DNA using a probe of EBV derived from the B95-8 strain. The sensitivity of the assay used was ~1 copy of EBV genome. In spite of the high stringency conditions, faint hybridization bands of roughly similar intensities were observed with DNA obtained from EBV-negative cell lines, from cord bloods, and from the peripheral blood of EBV-seropositive normals and patients with RA (Fig. 3). These results indicate the presence of a significant degree of background hybridization, probably related to the presence of sequences of nucleotide homology between the cellular and viral DNA (20), thus preventing the detection of rare EBV genome copies within a mixed lymphocyte population.

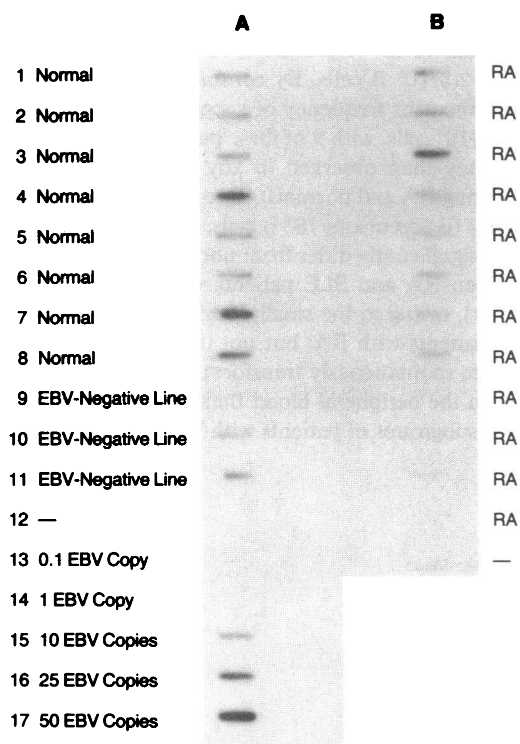

Figure 3. Content of EBV DNA in peripheral blood mononuclear cells of normal individuals and patients with RA. Each filter was probed with 10×10^6 cpm of nick-translated ^{32}P-labelled EBV DNA prepared with EBV of the B95-8 strain. Positions A 13 to A 17 contain known quantities of EBV DNA corresponding to 0.1, 1, 10, 25, and 50 EBV genome equivalents. All the remaining bands represent the hybridization of 10 μg quantities of cellular DNA. Positions A 1 through A 6 contain mononuclear cell DNA from 6 EBV-seropositive normal adults, A 7 and A 8 mononuclear cell DNA from 2 newborns, and B 1 through B 12 mononuclear cell DNA from 12 patients with RA. Positions A 9 and A 11 contain DNA obtained from three EBV-negative cell lines derived from American Burkitt's lymphomas.

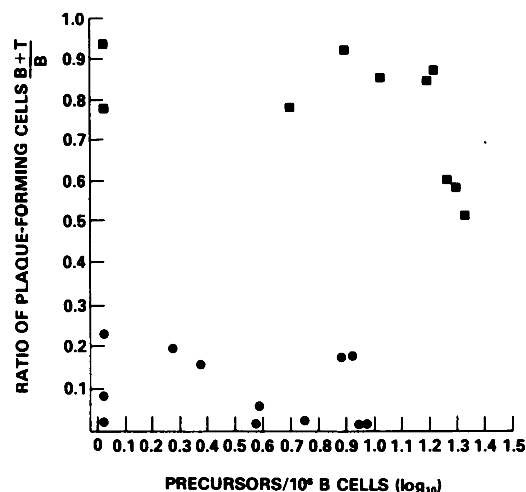

Figure 4. Relationship between the frequency of spontaneously transforming B cells in the circulation and the degree of EBV-specific "late-acting" suppression for 10 patients with RA and 12 normals. Precursor frequencies are expressed as precursors/10^6 B cells ($\log_{10}$). T cell suppression is shown as the ratio of plaque-forming cells generated by 12-d cocultures of 0.5×10^6 EBV-activated B cells with 2.0×10^6 autologous T cells and the plaque-forming cells produced by 12-d cultures of 0.5×10^6 EBV-activated B cells alone (a ratio of 1 denotes no suppression, while a ratio of 0 denotes complete suppression). Results for the normals are shown as closed circles (●), while results for the patients are shown as closed squares (■).

Relationship between frequency of spontaneous B cell outgrowth and EBV-specific T cell suppression. It has been previously reported that T cells from normal individuals who have been previously infected with EBV manifest in vitro a "late-acting" suppression of autologous B cells exogenously infected with EBV (9). Since this EBV-specific suppressor T cell function is markedly defective in most patients with RA (15), we asked whether a correlation could be found between the number of EBV-infected B cells in the circulation and the degree of EBV-specific T cell suppression in vitro. For this purpose, EBV-specific T cell suppression was assessed by measuring the number of plaque-forming cells produced in 12-d cultures of EBV-activated B cells incubated alone or mixed with autologous T cells. As shown in Fig. 4, RA patients formed a group with both decreased T cell suppression (expressed as ratio of plaque-forming cells produced by autologous cocultures of EBV-infected B and T cells as compared with EBV-infected B cells alone) and elevated numbers of spontaneously transforming B cells (ex-

pressed as precursors/10^6 B cells $\log_{10}$) as compared with the normals. Within each group, however, there was no significant correlation between the degree of T cell suppression in vitro, and the number of spontaneously transforming B cells present in vivo ($P > 0.05$ for patients and normals).

Frequencies of EBV-induced immortalization in normals and in patients with rheumatoid arthritis. It has been previously reported that while most human B cells have a surface receptor for EBV, only a proportion of the circulating B cells can be infected and immortalized by exogenous EBV in vitro (21). We asked whether patients with RA differ from normals with respect to the number of B cells that can be immortalized by EBV. To address this question, we determined the precursor frequencies of peripheral blood B cells which could be induced to transform in the presence of exogenous EBV for six normals and six patients with RA. These determinations were performed using purified B cells and a feeder layer of autologous irradiated (3,000 rad) T cells in the presence of nonlimiting amounts of exogenous EBV (Table II). The mean frequency of EBV-induced outgrowth was 8.4/10^3 B cells in normals (range, 1.1–31.4), and 4.6/10^3 B cells in RA patients (range, 2.9–9.7) ($P > 0.05$). Thus, the frequency of peripheral blood B cells which could be immortalized by exogenous EBV was not significantly different in RA patients as compared with normal controls.

Discussion

In this report, we have shown that patients with RA have abnormally elevated numbers of circulating EBV-infected B cells, as evidenced by the occurrence of spontaneous B cell outgrowth at a higher frequency than in normals. Since our patients with RA had normal numbers of B cells, as generally observed in RA, these results indicate that patients with this disease usually have an expanded pool of EBV-infected B cells in the circulation.

Table II. Precursor Frequencies of B Cell Immortalization Induced In Vitro by EBV in Normals and Patients with RA

	Number of precursors/10^3 B cells*	
	Normal individuals	Patients
	1.1	9.7
	31.2	4.9
	18.2	6.1
	4.8	2.9
	9.7	3.5
	14.0	7.9
Geometric mean	8.4	4.7

* Multiple replicate cultures of B cell-enriched mononuclear cells were established in the presence of EBV, and scored for the presence or absence of outgrowth after 6 wk; the results, analysed by Poisson statistics, are expressed as number of transforming precursors/10^3 B cells.

The presence of abnormally elevated numbers of EBV-infected B cell precursors in the peripheral blood is not a consistent feature of all autoimmune diseases, since all but two of our patients with SLE had a normal number of circulating EBV-infected precursors. In addition, the increase seen in RA patients appeared not to be solely related to therapy, since SLE patients receiving similar treatment did not show a significant increase in the number of EBV-infected B cells.

A direct quantitation of the viral burden in the peripheral blood was attempted by the use of a very sensitive blot hybridization technique capable of detecting one copy of EBV DNA. By this technique, patients with RA and normals appeared to have similarly low numbers of EBV genome copies in the peripheral blood. However, a substantial nonspecific background hybridization was apparent, consistent with the presence of nucleotide sequences of specific homology between the cellular and the viral DNA (20). Thus, a precise estimate of viral DNA copies in the peripheral blood of the study group was not possible.

During a primary infection with EBV in acute infectious mononucleosis, B cells infected with EBV are present at a high frequency in the peripheral blood. In this illness, 5–19% of the circulating non-T cells express the EBV-associated nuclear antigen, EBNA (22), and spontaneous outgrowth of EBV-infected cells occurs with a frequency of $\sim 300/10^6$ mononuclear cells (23). After primary infection with EBV, viral nucleic acid sequences can be found to persist within the infected B cells, probably for the lifetime of the host. As a result, B cells obtained from EBV-seropositive, but not from EBV-seronegative normal individuals, can give rise to long term cell lines in vitro without the addition of exogenous virus (5).

The origin of this low-grade B cell infection with EBV is not as yet clear. These circulating EBV-infected B cells may represent the progeny of B cells originally infected with EBV at the time of primary infection, which have maintained the viral information through cell replication. This type of cell-virus interaction is commonly observed in EBV-infected human B cell lines carried in vitro. Alternatively, EBV-infected B cells could derive from a continuous low-grade viral production in the oropharinx and subsequent infection of previously uninfected B cells (24). Indeed, a fraction of EBV-seropositive normals continue to shed transforming EBV particles in the saliva long after primary infection (25). Interestingly, EBV shedding occurs normally in patients with RA (26). We asked whether B cells from patients with RA can be infected and immortalized by EBV more easily than normal and examined the frequency of B cell outgrowth in the presence of exogenously added virus. In normals, while most circulating B cells have a surface receptor for EBV, only a proportion can be infected and activated by the virus at any time (18, 21). Our results indicate that the precursor frequency of B cells that can be immortalized by exogenous EBV is similar in patients with RA and normals,

suggesting that the increased number of circulating spontaneously transforming cells in RA patients is not due to an abnormal susceptibility to transformation of RA B cells.

Cellular immune control mechanisms are believed to play an important role in the defense against infection with EBV, both during acute infectious mononucleosis and after recovery from primary infection (6–9). A number of cellular mechanisms for control of EBV infection have been identified in EBV-seropositive normals, including T cell inhibition of EBV-induced B cell proliferation (7), suppression of EBV-induced immunoglobulin production (9), cytotoxic T cell activity directed against EBV transformed B cells (8), and natural killer activity (27). The relative contribution of these cellular regulatory processes that were studied in vitro to the control of in vivo latent EBV infection is still not clear. Interestingly, it has been reported that T cells from patients with RA have a number of in vitro immunoregulatory defects relating specifically to EBV, including impaired T cell control of proliferation (14), immunoglobulin production (15), and outgrowth of autologous EBV-infected B cells (12, 13). Specific killing of autologous EBV-infected cells, however, has been reported to be normal in patients with RA (28). We attempted to correlate frequencies of EBV-infected B cells in the circulation with degree of T cell suppression of immunoglobulin production by autologous exogenously EBV-infected B cells. No correlation was found. While all patients with RA revealed a marked T cell defect in the control of EBV-induced B cell activation in vitro, only some had abnormally elevated frequencies of EBV-infected B cells in the circulation. This finding indicates that a marked defect in EBV-specific suppression in vitro may be compensated in vivo, perhaps by another immune mechanism of control, and confirms the previously recognized complexities of regulation of EBV infection.

The present study demonstrates that many patients with RA have abnormally elevated numbers of circulating EBV-infected B cells and suggests that in this disease, infection with EBV is abnormally regulated not only in vitro but also in vivo. The existence of abnormally elevated numbers of EBV-infected B cells in patients with RA may contribute to a persistent state of enhanced B cell activation in this disease.

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