Search for Correlation of CD8 T Cell Response to Epstein-Barr Virus with Clinical Status in Rheumatoid Arthritis: A 15 Month Followup Pilot Study

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ABSTRACT. ***Objective.*** To assess in a longitudinal 15 month followup study the CD8 T cell response to immunodominant Epstein-Barr virus (EBV) antigens of 17 patients with rheumatoid arthritis (RA); and to seek an association between these responses and both clinical activity/severity of RA and a qualitative PCR for EBV in peripheral blood.

Methods. At each patient's visit every 3 months: (1) RA activity was assessed for Disease Activity Score (DAS-28); (2) a qualitative PCR for EBV was performed; (3) CD8 T cell response to EBV epitopes was screened in peripheral blood, using an autopresentation assay of 13 EBV peptides previously identified as immunodominant targets in RA synovia. Activation of anti-EBV CD8 T cells was evaluated by measuring the release of tumor necrosis factor-α.

Results. The semiquantitative CD8 T cell response to EBV roughly paralleled RA clinical activity in only 4/17 patients. No clear association could be found between positive PCR for EBV (performed at least once in 10/17 patients) and RA activity/severity or fatigue. Reactivity was not qualitatively broader in samples where PCR for EBV proved positive, and most often focused on one or 2 EBV antigens. However, these antigens differed between patients, as did the magnitude of CD8 T cell response to immunodominant antigens at different timepoints for the same patient.

Conclusion. The CD8 T cell response to EBV paralleled clinical activity in only 4/17 patients. Our pilot study does not support the hypothesis that this CD8 response contributes to RA activity/flares, although the quantitative variations in the pattern of this reactivity over time confirmed that control of EBV manifestations was difficult in most patients with RA. (J Rheumatol 2003;30:1673–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS CD8 T CELLS EPSTEIN-BARR VIRUS LYMPHOCYTES

Epstein-Barr virus (EBV) has long been suspected to be involved in the pathogenesis of rheumatoid arthritis (RA), at least as a cofactor[1]. This suspicion was raised by observation of an increased frequency and concentrations of antibodies against specific epitopes on EBV encoded EBNA1 (BKRF1) and EBNA3 (BERF1) antigens, and by the decreased ability of lymphocytes from patients with RA to limit outgrowth of autologous EBV infected lymphocytes[1]. It was later recognized that RA was associated with HLA-DR alleles sharing a QK/RRAA motif also found within the EBV gp110 protein (BALF4)[2], a phenomenon suspected to contribute to the poor control of autologous EBV infected lymphocytes. Recent reports indeed confirm that patients with RA have a decreased T cell response to the EBV gp110 (BALF4)[3], a glycoprotein usually undetectable in virions but essential for EBV replication in human cells[4-6]. Although T cell recognition of other EBV proteins (like BZLF1) has been considered as more crucial to control EBV[7], some investigators have put forward the hypothesis that this decreased response to BALF4 could account for the reactivation of EBV[3] in B cells from RA bone marrow[8] and/or synovium. This possibility would be in accord with recent reports of the presence of EBV genome in RA synovia, especially for patients with the QK/RRAA motif[9], including fibroblast-like synoviocytes[10] or lining cells at the top of villus lesions[11]. Indeed, although humoral and CD8 T cell responses are probably involved in control of EBV infection[12], EBV reactivation seems to be mostly controlled by CD8 T cell responses[13] (mainly lytic epitope-specific CD8 T cells[14]), in a quantitatively and qualitatively changing homeostasis between the virus and the immune system[15]. Extensive screening of the CD8 T cell responses in the synovial fluid of patients with RA showed that T cells specific for EBV epitopes were much enriched in their synovial fluid[16], and were highly focused to a restricted set of immunodominant epitopes, primarily generated during

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the early lytic cycle of EBV[7], while the response to gp110 was by contrast often undetectable[17-19]. Transient reactivation of EBV (favored by a qualitative defect of CD4 and/or CD8 T cell responses toward some EBV critical epitopes) followed by delayed and excessive CD8 T cell responses (toward the same or other EBV critical epitopes) could contribute to RA flares and/or global level of RA activity, in RA and in other disorders with B cell autoimmunity. To investigate this hypothesis, we prospectively gathered clinical and biological data every 3 months from 17 patients with RA over a 15 month period to seek for any correlation between RA activity and (1) CD8 T cell responses to immunodominant EBV peptides in peripheral blood, and (2) the presence of EBV confirmed by polymerase chain reaction (PCR) in peripheral blood. As well we investigated the relationship between EBV resurgence in blood and the qualitative profile of CD8 T cell responses of those 17 patients.

MATERIALS AND METHODS

Patients. The 17 patients were prospectively, randomly, and consecutively selected from RA outpatients followed in our institution. All gave informed consent for that study, which had been approved by the local ethics committee. These 17 patients had typical RA[20] with no overlap with other connective tissue disorders or conditions associated with immunosuppression (except one 70-year-old woman with idiopathic thrombocytemia treated with low dose hydroxyurea). Patients' mean age was 57 ± 16 years (range 26–80). Duration of RA for the group was 11.6 ± 8.2 years (range 2–28). All had positive serology for EBV infection, but none had been diagnosed with infectious mononucleosis and/or had previously had severe sore throat and/or recurrent pharyngitis. All were currently treated with disease modifying antirheumatic drugs, including etanercept in one case (Patient 17), parenteral gold salts in 2 cases, sulfasalazine in 2, and low dose methotrexate (7.5–15 mg weekly) in 12 cases; these prescriptions remained unchanged during the whole study period; 10/17 patients were taking oral steroids. The clinical activity of these patients was very variable at different timepoints, and their overall severity was also quite different (Figure 1, Table 1): the median Disease Activity Score (DAS28) values for the 6 visits ranged from 2.61 ± 0.50 (Patient 12) to 5.97 ± 1.24 (Patient 6). HLA class I genotyping (Centre Régional de Transfusion Sanguine, Nantes, France) was as described[21]. Results of this genotyping for each patient are given in Table 1.

Assessment of disease activity. At each visit, the following data were collected for each patient: DAS28 score (a composite index of both clinical and biological activity[22]), morning stiffness, pain level on an analog scale of 0 (no pain) to 10 (maximal pain), fatigue level on an analog scale of 0 (no fatigue) to 10 (maximal fatigue), and C-reactive protein (CRP) value (mg/l). A DAS28 value > 3.2 indicates active RA.

PCR for the detection of EBV. DNA samples. Peripheral blood samples (7 ml) were collected into EDTA. Peripheral blood mononuclear cells were isolated by Ficoll-hypaque centrifugation and a final suspension of 10^7 cells/ml in phosphate buffered saline was performed. Two aliquots were stored at –20°C until the amplification procedure.

PCR analysis. One aliquot was used as the DNA template for qualitative detection of EBV DNA by PCR. Before amplification, cells were boiled and centrifuged 10 min at 8000 g. A 121 base pair (bp) fragment located in the unique BamH1C region was amplified using the 2 primers EBV1 (5' GACAACTCGGCCGTGATGGA 3') (position 4010–4029) and EBV2 (5' TGAAGTTGGAGGCGGACGAG 3') (position 4130–4111). Amplification reactions were performed in a final volume of 25 µl with deoxyribonucleotides at 200 µM each, 1.5 mM $MgCl_2$, 1 µM of each primer, 2.5 units Taq DNA polymerase (Pharmacia, Guyancourt, France), and 3 µl of DNA sample. The amplification mixture was overlaid with mineral oil, and amplifications were carried out on a PCR processor (Thermal Reactor, Hybaid, Middlesex, UK). After the first denaturation at 95°C for 7 min, PCR was carried out for a total of 35 cycles (denaturation 30 s at 94°C, annealing 30 s at 60°C, extension 1 min at 72°C) with a final extension 5 min at 72°C. The PCR products were hybridized with a biotinylated

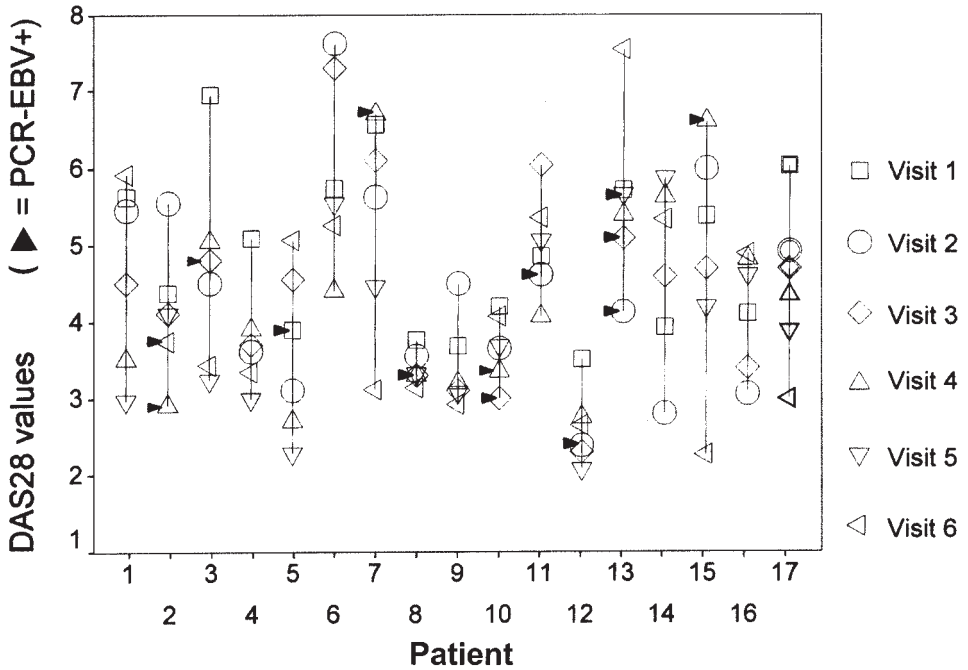


Figure 1. Vertical lines represent fluctuations of DAS28 scores of 17 patients during the 15 months' followup (one visit every 3 months) (upper values: visits during which RA clinical activity was highest; lower values: lowest clinical activity). Arrowheads indicate a positive PCR for EBV.

Table 1. Patients' CD8 T cell responses to EBV peptides and PCR for EBV at each visit. The amount of TNF released in the responding T cell lines was estimated by the WEHI 164 cytotoxic assay[24]: release of TNF < 1 µg/ml was considered negative, 1–10 µg/ml (+), 10–20 µg/ml (++), 20–50 µg/ml (+++), 50–200 µg/ml (++++), > 200 µg/ml (+++++). In rare cases ("?") background noise prevented evaluation of TNF release. Only positive PCR results for EBV are given: remaining results were negative.

Patient/Phenotype	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
1 A11,B8,B27	ENBA3B/A11 + DAS28 = 5.63	EBNA3B/A11 + DAS28 =5.46	– DAS28 =4.51	BRLF1/A2 + DAS28 =3.50	– DAS28 =2.97	– DAS28 =5.92
2 A1,B8,B55	– DAS28 =4.37	BZLF1/B8 +++ DAS28 =5.55	BZLF1/B8 ++ DAS28 =4.10	BZLF1/B8 + **(PCR EBV+)** DAS28 =2.90	BZLF1/B8 ++ DAS28 =4.11	BZLF1/B8 ++ **(PCR EBV +)** DAS28 =3.74
3 A2,A3,B7,B15	– DAS28 =6.95	– DAS28 =4.50	– **(PCR EBV+)** DAS28 =4.82	– DAS28 =5.05	– DAS28 =3.23	– DAS28 =3.43
4 A2,A11,B15,B55	EBNA3B/A11 ++++ DAS28 =5.09	– DAS28 =3.61	– DAS28 =3.70	– DAS28 =3.90	– DAS28 =2.99	EBNA3B/A11 ++ BMLF1/A2 + DAS28 =3.34
5 A2,B44,B49	BMLF1/A2 ++++ **(PCR EBV+)** DAS28 =3.89	BMLF1/A2 ++++ DAS28 =3.10	BMLF1/A2 +++ BMRF1A2 +++++ LMP2/A2 +++++ BZLF1/B8 +++++ EBNA3A/A2 ++++ EBNA3C/A2 ++ BRLF1/A2 ++ DAS28 =4.55	– DAS28 =2.71	BMLF1/A2 +++ LMP2/A2 + BRLF1/A2 + DAS28 =2.28	BMLF1/A2 + DAS28 =5.07
6 A1,A2,B37,B44	BMLF1/A2 + DAS28 =5.74	BMLF1/A2 +++++ BRLF1/A2 +++++ LMP2/A2 ++++ EBNA3A/A2 ++++ EBNA3C/A2 +++ DAS28 =7.62	BRLF1/A2 +++ DAS28 =7.30	LMP2/A2 +/– DAS28 =4.40	– DAS28 =5.54	BMLF1/A2 ++ DAS28 =5.26
7 A2,A29,B44,B50	? DAS28 =6.56	BZLF1/B18 +++ DAS28 =5.63	? DAS28 =6.10	– **(PCR EBV +)** DAS28 =6.72	– DAS28 =4.46	– DAS28 =3.11
8 A2,B51,B55	– DAS28 =3.76	– DAS28 =3.55	– **(PCR EBV+)** DAS28 =3.30	– DAS28 =3.30	– DAS28 =3.32	– DAS28 =3.14
9 A1,A3,B7,B8	– DAS28 =3.68	– DAS28 =4.50	– DAS28 =3.11	– DAS28 =3.20	– DAS28 =3.07	– DAS28 =2.92
10 A2,A31,B35,B40	BZLF1/B35 + DAS28 =4.20	BZLF1/B35 +++++ DAS28 =3.65	BZLF1/B35 + BMLF1/A2 + **(PCR EBV+)** DAS28 =2.99	– **(PCR EBV+)** DAS28 =3.35	BZLF1/B35 + DAS28 =3.68	BZLF1/B35 +/– DAS28 =4.07
11 A1,A3,B8,B44	– DAS28 =4.86	BZLF1/B8 +++ **(PCR EBV +)** DAS28 =4.62	BZLF1/B8 + DAS28 =6.05	BZLF1/B8 + DAS28 =4.07	BZLF1/B8 + DAS28 =5.07	BZLF1/B8 + DAS28 =5.37
12 A2,A24,B39,B40	– DAS28 =3.51	BMLF1/A2 ++ BZLF1/B8 ++++ **(PCR EBV +)** DAS28 =2.38	? DAS28 =2.30	LMP2/B40 + DAS28 =2.75	BMLF1/A2 + DAS28 =2.07	BMLF1/A2 + BZLF1/B8 + BZLF1/B40 ++++ DAS28 =2.65
13 A2,A11,B35,B53	BRLF1/A2 + DAS28 =5.72	BRLF1/A2 + **(PCR EBV +)** DAS28 =4.13	– **(PCR EBV +)** DAS28 =5.12	– DAS28 =5,4	– **(PCR EBV +)** DAS28 =5,67	– DAS28 =7.55
14 A2,B15,B49	– DAS28 =3.93	– DAS28 =2.80	BRLF1/A2 + DAS28 =4.61	BMLF1/A2 + DAS28 =5.64	BMLF1/A2 + DAS28 =5.88	– DAS28 =5.35
15 A1,A2,B7,B18	– DAS28 =5.39	– DAS28 =6.02	– DAS28 =4.70	– **(PCR EBV +)** DAS28 =6.61	– DAS28 =4.20	– DAS28 =2.27
16 A2,A24,B35,B40	– DAS28 =4.11	– DAS28 =3.06	BMLF1/A2 +/– BRLF1/A2 + DAS28 =3.40	LMP2/B40 + DAS28 =4.82	LMP2/B40+ DAS28 =4.62	LMP2/B40+++ LMP2/A2 + DAS28 =4.90
17 A2,A32,B35,B44	BZLF1/B35 ++++ BMLF1/A2 + LMP2/A2 + DAS28 =6.04	BZLF1/B35 ++ BMLF1/A2 + DAS28 =4.93	BZLF1/B35 + LMP2/A2 +/– DAS28 =4.70	BZLF1/B35 + DAS28 =4.35	BZLF1/B35 +++ BMLF1/A2 + DAS28=3.89	? DAS28=3.02

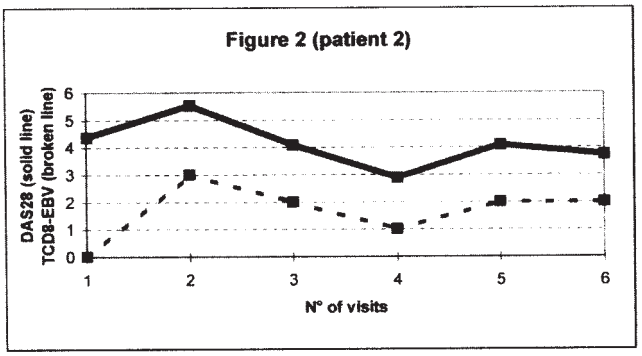
internal specific oligonucleotide probe (5' TGGCCTGGGCGTGAAGCTGACCTTTGGCTCGGCCTCCT 3') (position 4060–4097) using the microtiter plate hybridization assay (ETI-K DEIA, Sorin Biomedica, Saluggia, Italy). To evaluate the sensitivity of our PCR, serial fold-dilutions of the Burkitt's lymphoma cell line Namalwa (2 EBV copies per cell) were prepared and amplified by PCR. It was possible to score the hybridization signal from 2 EBV copies.

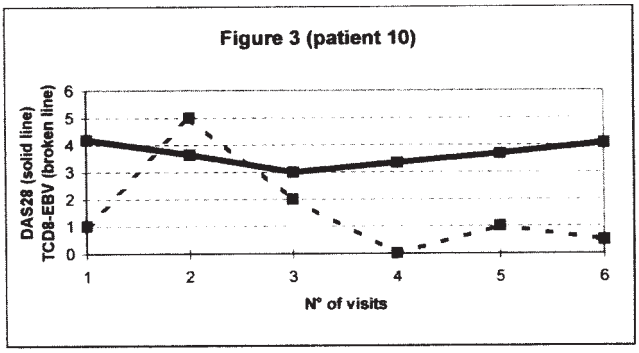
T cell lines. Samples of peripheral blood were taken from the 17 RA patients. Mononuclear cells were isolated by Ficoll/Hypaque density gradient centrifugation. Peripheral blood lymphocytes were maintained in RPMI-1640 supplemented with 10% pooled human serum, 1 mM L-glutamine, and recombinant interleukin 2 (IL-2; 150 IU/ml), hereafter referred to as IL-2 supplemented culture medium (IL-2/CM). Lymphocytes were expanded *in vitro* in IL-2/CM supplemented with purified phytohemagglutinin (leukoagglutinin, 0.5 µg/ml) and irradiated (30 Gy) allogeneic feeder cells (peripheral blood and EBV transformed B lymphoblastoid cells at a 10/1 ratio) as described[18,19]. Cells were maintained for 3 to 4 weeks without restimulation prior to functional analysis.

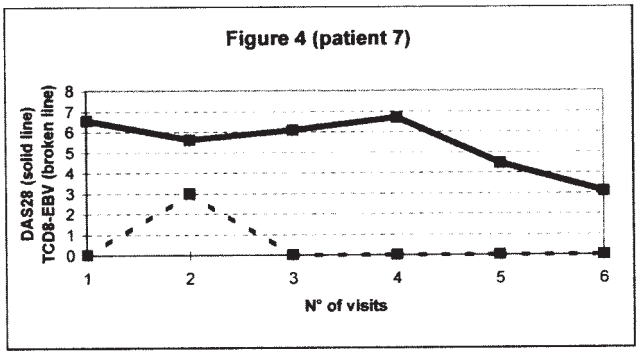
Peptides. Peptides were all synthesized commercially by Genosys (The Woodlands, TX, USA) or Chiron (Chiron Mimotopes Corp., Victoria, Australia). Peptide stock solutions (20 ng/ml in DMSO) were diluted first to 2 ng/ml in acetic acid (1%) and second to the final concentration in RPMI-1640 culture medium. Characterization of EBV epitopes recognized by polyclonal T cells was achieved in an autopresentation assay by assaying the ability of a large set of peptides, previously characterized as dominant peptides for the CD8 T cells' response to EBV[23], to trigger tumor necrosis factor (TNF-α) release by responding T cell lines. The following CD8 T cell epitopes were screened systematically, whatever the HLA alleles of the donor: BMLF1$^{259-267}$/A2, BMRF1$^{208-216}$/A2, BRLF1$^{100-122}$/A2, EBNA3A$^{596-604}$/A2, EBNA3C$^{284-293}$/A2, LMP2$^{329-337}$/A2, LMP2$^{426-434}$/A2, EBNA3B$^{399-408}$/A11, LMP2$^{200-208}$/B4001, BZLF1$^{190-197}$/B8, BZLF1$^{172-183}$/B18, and BZLF1$^{54-64}$/B35.

TNF assays. Production of TNF-α by activated lymphocytes was estimated in an autopresentation assay. To trigger TNF-α release by responding T cells, 3×10^4 polyclonal T cells were incubated for 5 h with individual peptide at 10 µm concentration, and the amount of TNF released in the supernatant was estimated by the WEHI 164 cytotoxic assay[24].

Expression of results. Results for CD8 T cell response to EBV are expressed qualitatively (Table 1) and by a semiquantitative method (Figures 2 to 4): release of TNF < 1 µg/ml was considered negative, between 1 and 10 µg/ml was denoted positive (+), with 10–20 µg/ml (++), 20–50 µg/ml (+++), 50–200 µg/ml (++++), and > 200 µg/ml (+++++). In rare cases (designated "?" in Table 1) background noise prevented a correct evaluation of TNF release by cytotoxic T lymphocytes (CTL). For semiquantitative evaluation of the magnitude of TNF release on a 0 to 6 scale (Figures 2 to 4), the value of the stronger response was considered (i.e., a score of 4 for a sample with CTL release of magnitude ++++), except when 2 or more specificities gave similar levels of response: in that event the superior level was recorded (i.e., 2 scores of 3 for different peptides led to a final score of 4).







Figures 2 to 4. Three patients' DAS28 values (solid lines) and CD8 T cell responses to EBV peptides (semiquantitative scale from 0 to 6; broken lines). Data are shown for one of the 4 patients with a parallel between CD8 T cell response and clinical activity (Figure 2, Patient 2). In contrast, one patient had a burst of CD8 T cell response despite stable RA activity (Figure 3, Patient 10); and one patient had a poor CD8 T cell response with high RA activity (Figure 4, Patient 7).

RESULTS

Clinical and biological activity over the 15 month period. DAS28 scores for each of the 17 patients during the 15 month followup appear in Figure 1. It had previously been defined that a 0.6 variation of the score was clinically significant. A variation of DAS28 ≥ 0.6 was noted between extreme values for 15/17 patients during the followup period.

PCR for EBV in peripheral blood. PCR was positive on one sample or more in 10/17 patients, Patients 2, 3, 5, 7, 8, 10, 11, 12, 13, and 15, although positive result was noted on only one occasion in 7 of these 10 cases. There were no differences in DAS28 (Figure 1, Table 2), semiquantitative evaluation of CD8 T cell response to EBV (Tables 1 and 3), fatigue (Table 4), CRP (Table 5), and broadness of CD8 T cell responses to EBV epitopes (Table 1) between patients with negative PCR and patients with positive PCR.

CD8 T cell responses to EBV peptides: qualitative results. CD8 T cell responses to EBV assayed by TNF-α production were consistently directed toward an immunodominant antigen in 11/17 cases (Table 1): BZLF1 (Patients 2, 10, 11, 17), BMLF1 (Patients 5, 6, 12, 14), BRLF1 (Patient 13), LMP2 (Patient 16), and EBNA3B (Patient 4). However, for

Table 2. DAS28 results (mean ± SD) at each visit, comparing patients with positive/negative PCR result for EBV during the 15 month followup.

	Visit1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
EBV+	4.9 ± 1.2	4.3 ± 1.2	4.4 ± 1.2	4.3 ± 1.6	3.8 ± 1.1	3.7 ± 1.0
EBV–	4.9 ± 1.0	4.6 ± 1.7	4.5 ± 1.4	4.3 ± 0.8	4.1 ± 1.2	4.6 ± 1.3

Table 3. Semiquantitative evaluation of CD8 T cell response (mean ± SD) to EBV immunodominant peptides screened at each visit, comparing patients with positive/negative PCR result for EBV during the 15 month followup.

	Visit1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
EBV+	0.7 ± 1.3	2.3 ± 1.9	1.8 ± 2.2	0.3 ± 0.5	0.8 ± 1.0	0.7 ± 0.8
EBV–	1.4 ± 1.8	1.3 ± 2.2	0.9 ± 1.0	0.7 ± 0.5	0.7 ± 1.1	1.2 ± 1.3

Table 4. Fatigue scores (mean ± SD) [analog scale from 0 (no fatigue) to 100 (extreme fatigue)] at each visit, comparing patients with positive/negative PCR result for EBV during the 15 month followup.

	Visit1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
EBV+	45.0 ± 25.3	29.2 ± 20.1	35.4 ± 26.6	43.9 ± 28.9	29.8 ± 24.8	31.8 ± 28.9
EBV–	45.8 ± 17.1	37.1 ± 22.8	40.3 ± 19.6	35.5 ± 23.9	29.1 ± 30.0	20.2 ± 16.6

Table 5. CRP (mg/l, mean ± SD) at each visit, comparing patients with positive/negative PCR result for EBV during the 15 month followup.

	Visit1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
EBV+	20.9 ± 16.9	12.8 ± 9.8	14.9 ± 18.1	23.4 ± 25.0	12.0 ± 6.6	5.6 ± 2.9
EBV–	29.9 ± 30.7	26.6 ± 29.3	29.6 ± 35.2	19.6 ± 16.5	14.6 ± 14.4	30.8 ± 37.5

most patients, other antigens could elicit a transient and even stronger CD8 T cell response. The broadness of the CD8 T cell response seemed to be no more associated with clinical activity of RA than its magnitude. No association could be found between the broadness response and a positive PCR for EBV on the same sample (Table 1): for instance, PCR remained negative for the 2 samples showing the broader response (visit 3/Patient 5 and visit 2/Patient 6) and was conversely positive in other samples with a seemingly narrow and/or poor CD8 T cell response (Patients 3, 7, 8, 13, 15).

CD8 T cell response to EBV peptides. Semiquantitative results. A striking parallel between clinical activity (assessed by DAS28 scores) and semiquantitative assessment of the CD8 T cell responses (assessed on the magnitude of TNF-α release) was noticed in Patients 2, 6, 14, and 17 (Figure 2, Patient 2). In other patients, either no relationship was determined (Patients 1, 3, 4, 5, 8, 10, 11, 12, 16), including some patients with a burst of CD8 T cell response not associated with clinical flare (Patients 10 and 12) (Figure 3, Patient 10), or the lack of identifiable response to EBV antigens precluded definitive conclusions (Patients 7, 9, 13, 15) (Figure 4, Patient 7). Similar observations were made for CRP and fatigue and the CD8 T cell response to EBV (data not shown).

DISCUSSION

The first aim of this longitudinal pilot study was to look for a correlation between the clinical activity/severity of 17 patients with RA regularly assessed over a 15 month period and both the CD8 T cell response to EBV immunodominant epitopes in patients' peripheral blood and the resurgence of EBV at the same time. We did observe a striking parallel between clinical activity and CD8 T cell response in some patients, suggesting that CD8 T cell response to EBV could sustain the inflammatory process and/or RA itself. However, this was true for a minor subset of patients (4/17). This would be in accord with the hypothesis that RA is probably more a syndrome than a disease, i.e., an abnormal response to EBV (poor initial control, followed by excessive response) perhaps driving the disease process in only a minority of cases. This assumption would also be in line with early results of research into the EBV genome in RA synovia, which proved inconsistently positive; however, this research became much more successful in recent investigations using more sensitive methods, with results of 6%

(2/31)[25], 19% (7/37)[26], 20% (1/5)[27], 24% (8/34)[11], 30%[28], 35% (29/84)[9], 47% (15/32)[29], and 91% (10/11)[30]. Similarly, mRNA EBV transcripts have been more rarely detected by *in situ* hybridization [8% (EBER 1 and 2)[28], 16% (5/32) (EBER1)[29], and 24% (EBER1)[11]] than by more sensitive RT-PCR for EBER1[30].

The lack of relationship between CD8 T cell response and clinical activity of RA in other patients could be ascribed either to the fact that CD8 T cell response to EBV plays no role in the pathogenesis of RA, or to technical limitations. In this respect, and for ethical reasons, only peripheral blood samples were taken (every 3 months) over the 15 month study period: a closer relationship between CD8 T cell response and RA activity might have been observed using synovial biopsies instead of peripheral blood.

The lack of relationship between the PCR results and CD8 T cell responses (Table 1) might be ascribed to the PCR technique we used, which analyzed roughly 30,000 peripheral blood mononuclear cells per reaction, thus offering a higher cutoff than in other studies using more sensitive techniques[11,28]. However, it could also be partly explained by the lag time between EBV reactivation and in the occurrence of maximal CD8 T cell responses. Similarly, a lag between EBV resurgence and RA flares might also account for the negative PCR results during most RA flares, even in those patients for whom CD8 T cell responses were roughly parallel to RA clinical activity (Table 1). However, our overall results support a limited role for EBV in the pathogenesis of RA in most patients studied.

The second aim of the study was to look for a relationship between EBV resurgence in blood and the qualitative profile of CD8 T cell responses to EBV for the 17 patients. Recent longitudinal data on dynamics of antigen-specific CD8 cytotoxic T lymphocytes following primary EBV infection led to the observation that the protracted illness in one patient was associated with a narrowly focused response, while in contrast a broad T cell reactivity to multiple epitopes was noted in healthy virus carriers or for a patient who rapidly recovered from an acute infectious mononucleosis[31]. As the overall strength of cytolytic activity was greater in the patient who could not clear the EBV infection, this led to the hypothesis that the broadness of CD8 T cell responses is of greater importance than its magnitude to achieve good control of EBV, at least for primary infection. This could still be true later. Indeed, several reports have described that long after the primary infection, although CD8 T cell responses to EBV were still highly focused to a restricted set of early lytic proteins (and mainly BZLF1, BRLF1, and BMLF1; 42/342 tested EBV/HLA combinations in a recent report[23]), most individuals had CTL responses to more than one EBV antigen[7,19], including weaker responses to some latent EBV proteins, namely EBNA3A, 3B, and 3C and LMP2 in 10 to 20% of patients. In those studies, the reactivity patterns were similar for CD8 T cells from inflammatory sites and CD8 T cells from peripheral blood of longterm virus carriers free of autoimmune disorders[7]. We made similar observations, as most CD8 T cells from our patients responded to several EBV epitopes, including a dominant antigen (Table 1). However, for individual patients, we noted striking modifications of the pattern of response from the same patient at different timepoints, both qualitatively (Table 1) and semi-quantitatively (Figure 3). As no controls from healthy subjects or other chronic inflammatory processes were used, this could lead to the hypothesis that fluctuations of CD8 T cell response patterns are related to the poorer control of EBV previously described in RA. Our results strengthen observations of a permanently changing homeostasis between virus and immune system, at both quantitative and qualitative levels[14,15], which deserves further study.

Investigations would be even more instructive if CD4 T cell and NK responses were screened simultaneously with CD8 T cell responses. Indeed, assistance from CD4 T cells might be important for maintaining CD8 T cell memory[32] and maturing CD8 T cell function[33], and might directly inhibit the proliferation of EBV infected B cells[34]. Although CD4 T cell responses to EBV have been poorly analyzed to date, hampered by the small size of the CD4 compartment, it would be pertinent to determine the level of T cell response to EBV epitopes according to HLA-DR antigens and/or to the expression of the shared epitope.

In the absence of proof of a relationship between CD4 T cell responses to EBV and RA activity, our longitudinal screening of CD8 T cell responses to immunodominant EBV epitopes does not provide evidence that an excessive and delayed CD8 T cell response to EBV plays a significant part in most RA flares. However, our findings suggest a poor control of EBV infection (similar to that observed in systemic lupus erythematosus[35]), as recently ascribed by Takei, *et al*[36] to low expression of signaling lymphocytic-activation molecule-associated protein (SAP) in peripheral leukocytes or T cells of patients with RA. Indeed, SAP functions as a regulator of a signal transduction pathway initiated by the molecule 2B4 (expressed on NK cells and a subset of CD8 T cells), which plays a leading role in the ability of NK cells to lyse EBV infected cells[36]. Thus, as also described in X-linked lymphoproliferative syndromes (where mutations of the SAP gene have been identified), dysfunction of 2B4/SAP induced signal transduction pathways may be responsible for the ineffective T cell or NK cell response in sustaining the elimination of EBV infected cells in many patients with RA[36].

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