Serum Epstein-Barr virus DNA, detected by droplet digital PCR, correlates with disease activity in patients with rheumatoid arthritis

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Abstract

Objective

To study the prevalence of asymptomatic activation of Epstein-Barr virus (EBV) in patients with rheumatoid arthritis (RA) and to analyse the correlation of serum EBV DNA with the disease activity.

Methods

The level of EBV DNA was determined by droplet digital PCR assay from the serum of 46 DMARD naive early RA (ERA) and 22 chronic RA (CRA)-patients at study onset. Follow-up samples from 31 ERA and 16 CRA patients were obtained after starting or modifying the anti-rheumatic treatment. EBV DNA was also measured from 33 healthy controls and 9 patients with adult onset Still's disease (AOSD). Disease activity was assessed by the disease activity score (DAS28).

Results

At baseline, EBV DNA was detected in the serum of 7 of the 46 ERA patients all of whom had moderate or high disease activity. In the follow-up samples, 11 of 31 patients were EBV DNA positive. At baseline EBV positive patients had significantly higher disease activity ($p=0.036$) and the concentration of EBV DNA correlated significantly with DAS28 ($r_s=0.333$, $p=0.024$). EBV DNA was detected in 3 of 22 CRA patients at study onset and in 8 of 16 in the follow-up samples. At follow-up EBV positive patients had significantly higher DAS28 ($p=0.027$) and the concentration of EBV DNA correlated significantly with DAS28 ($r_s=0.724$, $p=0.002$). Only one of the healthy controls and none of the AOSD patients were positive for EBV DNA.

Conclusion

Active RA is associated with a lytic EBV infection which may have a role in the pathogenesis of RA.

Key words
Epstein-Barr virus, rheumatoid arthritis, ddPCR, DAS28

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Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disease which, in spite of improved treatment, can result in severe joint destruction and a reduced life span. RA typically presents with frequent flares with unknown cause and the pathogenesis of RA has remained elusive (1). Central to the pathogenesis is the formation of autoantibodies against citrullinated proteins. Viruses, in particular Epstein-Barr virus (EBV), have been implicated in the pathogenesis of RA.

Epstein-Barr virus is a widespread virus in the humans and it infects over 90% of the human population worldwide. Primary infection of EBV typically occurs in early childhood. After the primary infection, EBV persists mostly in B-lymphocytes in a latent form and expresses only a limited amount of its genes essential for viral persistence. Occasionally, latent EBV reactivates and enters into the lytic cycle of infection, during which new virus particles are produced and released from cells (2). Factors triggering the reactivation *in vivo* are poorly known, but the immune response of host is likely to play a significant role. The lytic gene expression and virus replication is known to be induced by the differentiation of EBV-infected B-lymphocytes into antibody-producing plasma cells (3).

Several studies have suggested that EBV contributes to the pathogenesis of RA. Increased levels of serum EBV-specific antibodies against Epstein-Barr virus nuclear antigen (EBNA), viral capsid antigen (VCA) and early antigen (EA) proteins, have been detected in patients with RA as compared to healthy individuals (4). Further, the number of circulating EBV-infected B-lymphocytes is increased in RA patients. Also the EBV DNA levels in the peripheral blood mononuclear cells, saliva, and synovium are higher compared to healthy controls (5-7). High B-lymphocyte immunoglobulin production in response to EBV stimulation in early RA has been associated with increased joint erosiveness and exacerbation of the disease (8). The association of lytic virus infection and activity of RA has not been clearly established. Presence of EBV DNA in cell-free blood samples indicates an on-going lytic infection. There is little evidence to show that lytic virus and its gene products contribute to EBV-associated diseases.

Anti-rheumatic medication can also influence the EBV activation. Methotrexate treatment enhances the expression of BMRF1, the early viral gene of EBV lytic infection (9). In contrast, azathioprine, cyclosporine, cyclophosphamide, mycophenolic acid, or prednisone did not markedly activate the EBV lytic gene expression (9). EBV-positive lymphoma is associated with methotrexate therapy (10). The effect of biological drugs on the reactivation of EBV is poorly known. However, the presence of detectable EBV DNA in the bone marrow and whole blood samples of RA patients, has been demonstrated to predict better clinical response to rituximab treatment, as compared to patients negative for EBV DNA (11, 12).

We investigated the association between serum EBV DNA, the indicator of the lytic phase of EBV, and the clinical disease activity of RA. We also assessed the effect of conventional synthetic disease-modifying anti-rheumatic drugs (sDMARD) and biological disease-modifying anti-rheumatic drugs (bDMARD) on the EBV DNA load in the serum. The results suggest that asymptomatic reactivation of EBV could contribute to high disease activity in RA and thus play a role in the pathogenesis of RA.

Materials and methods

Study design

EBV DNA-levels were measured from serum samples of patients with RA collected before and after the start of synthetic or biological disease-modifying anti-rheumatic drug (DMARD) treatment. Samples were collected at two time points with a baseline between September 2005 and September 2014 and follow-up 20.7 (SD=13.6) months after the baseline visit. Patients were recruited from the rheumatology clinic of Helsinki University Central Hospital after informed consent. The study was approved by the independent review board of the Helsinki and Uusimaa Hospital District (no 240/2004, date 16.6.2004) and included the guidelines

Table I. Disease characteristics of early (ERA) and chronic (CRA) rheumatoid arthritis patients.

	Baseline			Follow-up		
	ERA (n= 46)	CRA (n=22)	Total (n=68)	ERA (n= 31)	CRA (n= 16)	Total (n= 47)
Age, years	51 (15)	52 (11)	51 (14)	55 (13)	53 (13)	54 (13)
Male, n (%)	7 (15)	4 (18)	11 (16)	5 (16)	2 (13)	7 (15)
Female, n (%)	39 (85)	18 (82)	57 (84)	26 (84)	14 (88)	40 (85)
Disease duration, months	10 (18)	186 (117)	67 (106)	33 (24)	216 (117)	95 (112)
DAS28 remission (<2.6), n (%)	4 (9)	4 (9)	8 (12)	18 (58)	7 (44)	25 (53)
DAS28 LDA (2.6-3.2), n (%)	7 (15)	3 (14)	10 (15)	10 (32)	1 (6)	11 (23)
DAS28 MDA (>3.2-5.1), n (%)	26 (57)	10 (46)	36 (53)	3 (10)	6 (38)	9 (19)
DAS28 HDA (>5.1), n (%)	9 (20)	5 (23)	14 (21)	0 (0)	2 (13)	2 (4)
DAS28	4.1 (1.4)	4.1 (1.5)	4.1 (1.4)	2.3 (0.9)	3.0 (1.8)	2.5 (1.3)

All values are mean (SD) unless otherwise stated
DAS28: disease activity score in 28 joints; LDA: low disease activity; MDA: moderate disease activity; HAD: high disease activity.

Table II. Medications used by the patients.

	Baseline			Follow-up		
	ERA n=46	CRA n=22	Total n=68	ERA n=31	CRA n=16	Total n=47
sDMARDs:						
Methotrexate	0 (0)	15 (68)	15 (22)	25 (81)	11 (69)	36 (77)
Leflunomide	0 (0)	8 (36)	8 (12)	1 (3)	3 (19)	4 (9)
Cyclosporine	0 (0)	1 (5)	1 (2)	0 (0)	1 (6)	1 (2)
Podophyllotoxin	0 (0)	3 (14)	3 (4)	1 (3)	2 (13)	3 (6)
Sulphasalazine	0 (0)	8 (36)	8 (12)	14 (45)	4 (25)	18 (38)
Hydroxychloroquine	0 (0)	13 (59)	13 (19)	18 (58)	9 (56)	27 (57)
Aurothiomalate	0 (0)	2 (9)	2 (3)	0 (0)	0 (0)	0 (0)
bDMARDs:						
Adalimumab	0 (0)	0 (0)	0 (0)	0 (0)	2 (13)	2 (4)
Etanercept	0 (0)	0 (0)	0 (0)	0 (0)	10 (63)	10 (21)
Anakinra	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)	1 (2)
Certolizumab	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)	1 (2)
Rituximab	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)	1 (2)
Other medications:						
Corticosteroids	0 (0)	18 (82)	18 (27)	3 (10)	9 (56)	12 (26)
NSAID	9 (20)	5 (23)	14 (21)	6 (19)	5 (31)	11 (23)
Analgesic	9 (20)	7 (32)	16 (24)	9 (29)	5 (31)	14 (30)
Folic acid	1 (2)	15 (68)	16 (24)	26 (84)	11 (69)	37 (79)

All values are n (%).
sDMARDs: synthetic disease-modifying anti-rheumatic drugs; bDMARDs: biological disease-modifying anti-rheumatic drugs.

of the Declaration of Helsinki. Patients with RA were diagnosed according to the American Rheumatism Association classification criteria (13). Disease activity score (DAS28) was calculated from the number of tender and swollen joints, patient's global assessment, and erythrocyte sedimentation rate (ESR) (14). Patients were divided in to four groups based on the disease activity of RA with DAS28<2.6 equalling remission, 2.6–3.2 low disease activity, 3.2–5.1 moderate disease activity and DAS28>5.1 high disease activity (15).

Study population

Serum samples used in this study were collected from a total of 68 patients with RA, comprising 46 DMARD naive early RA (ERA) patients and 22 patients with chronic RA (CRA) treated with synthetic DMARDs and being candidates for treatment with biological DMARDs. Serum EBV DNA was also analysed from the serum of 21 healthy age-matched controls, 12 additional healthy controls, and from 9 patients with adult onset Still's disease (AOSD). The characteristics of RA patients are depicted in Table I. There was a statistically significant positive correlation between age of patients and DAS28 (r_s=0.264, p=0.030). The follow-up group consisted of 47 patients with 31 ERA and 16 CRA patients. The study population was ethnically homogenous all being Caucasian.

Anti-rheumatic treatment

After the baseline visit, treatment with synthetic DMARDs was initiated in ERA patients. Medications either as monotherapy or in different combinations consisted of methotrexate (MTX), leflunomide (LEF), cyclosporine (CSP), podophyllotoxin (PPT), sulphasalazine (SASP), hydroxychloroquine (HCQ) and aurothiomalate. CRA patients were on synthetic DMARD treatment already at study onset. Except for one patient, biological DMARD treatment was started in all CRA patients after the first visit. Biological drugs consisted of adalimumab, etanercept, anakinra, certolizumab or rituximab. The biological DMARD treatment was added on to the synthetic DMARDs the patients were using at baseline. In addition, all patients were also treated with non-steroidal anti-inflammatory drugs (NSAIDs), analgesics and corticosteroids, depending on disease activity. Medications used are depicted in Table II.

Serum samples and manual extraction protocol

Blood samples drawn at the time of clinical examination were centrifuged to obtain the serum fractions. Samples were divided into aliquots and kept at -70°C prior to testing. Manual extraction of DNA from frozen serum samples was performed using the QIAamp® cador® Pathogen Mini Kit (Qiagen, Hilden, Germany). DNA extraction protocol was used to extract DNA from 200 µl of serum, resulting in a final elution volume of 50 µl.

Quantification of serum EBV DNA expression levels with ddPCR and qPCR

Droplet digital PCR (ddPCR) was used to quantify the amount of EBV DNA in serum samples. The ddPCR is a highly sensitive technology that has a capabil-

ity for absolute nucleic acid quantification without establishment of a standard curve (16). Technology is based on Poisson correlation enabling the precise calculation of DNA concentrations at low template copy numbers (17, 18). It is proven to have a low detection limit and to be sensitive enough to detect only few molecules. Droplet digital hydrolysis probe (ddPCR SuperMix for probes, Bio-Rad Laboratories CA, USA) assays were performed using QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories). The final volume of 20 µl reaction mix contained 8 µl of extracted DNA sample, 10 µl supermix, 1 µl primers and 1 µl hydrolysis probe. Primers were diluted in a final concentration of 900 nM and hydrolysis probe in final concentration of 250 nM. The annealing temperature was 59°C.

EBV DNA quantification was also conducted on a LightCycler 96 Real Time PCR system (Roche) for comparison of sensitivity and specificity of the PCR methods. FastStart Essential DNA Probes Master was used to detect EBV DNA and the final volume of 20 µl reaction mix contained 8 µl of extracted DNA sample, 1 µl primers and 1 µl hydrolysis probe. Primers were diluted in a final concentration of 500 nM and hydrolysis probe in a final concentration of 100 nM. The annealing temperature was 60°C.

The serum EBV DNA was detected with PCR primers for the non-coding region of EBV genome originally used and described elsewhere (19). The forward and reverse primer sequences were 5´-TTTGGACCCGAAATCTGACACT-3´ and 5´-GCCAACCATAGACCCGCTTC-3. A dual fluorescence-labelled oligomer FAM-5´-CCATTTTGTCCCCACGCGCG-3´-BHQ was used as a probe. The amplicon size was 152 bp. Primers and probes were ordered from Metabion International AG (Munich, Germany).

DdPCR assay detects lower EBV DNA concentrations than qPCR assay

For comparison, the presence of EBV DNA in the serum samples was measured with ddPCR and qPCR assays. In this study the qPCR technology was not sensitive enough to detect low DNA concentrations in several samples, whereas ddPCR assay was still able to detect as low as 44 copies/ml of DNA in samples (Supplementary Table I). Also the Cq values of qPCR assay increased above 34 and the variability at this level was high requiring more replicates and thus absolute quantification with ddPCR is more suitable for EBV DNA detection in serum samples.

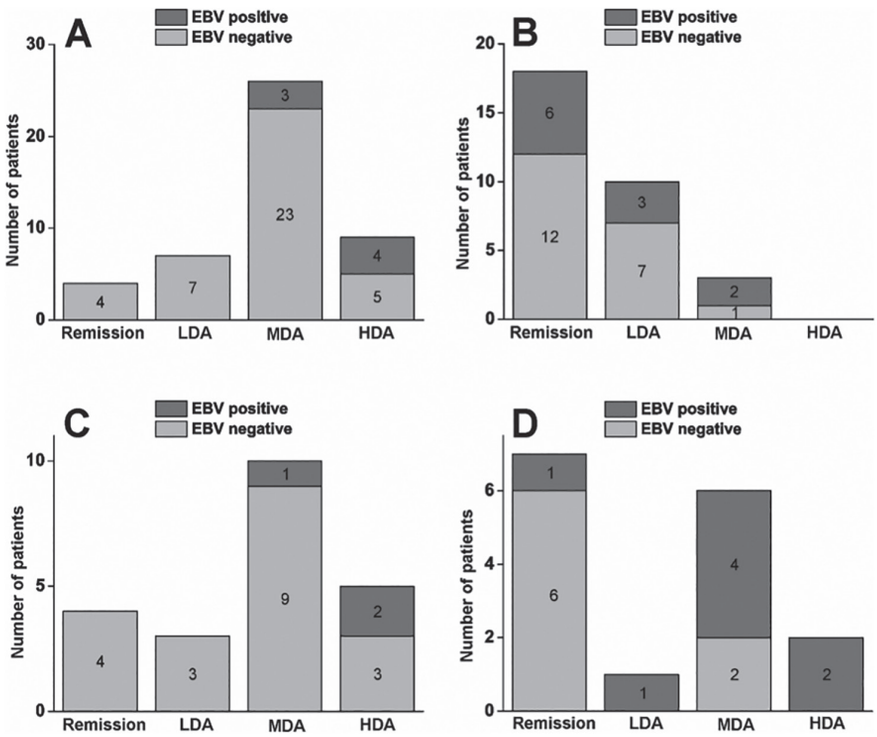

Fig. 1. The proportion of patients with positive EBV DNA in serum in the different disease activity groups (DAS28). **A**: early RA (ERA) patients at baseline and **B**: ERA patients at follow-up visit. **C**: chronic (CRA) patients at baseline and **D**: CRA patients at follow-up visit. Patients were divided into groups based on disease activity of RA: DAS28 <2.6 remission, DAS28: 2.6–3.2 low disease activity (LDA), DAS28: 3.2–5.1 moderate disease activity (MDA) and DAS28: >5.1 high disease activity (HDA).

Statistical analysis

Data analysis was conducted with SPSS v. 24 (SPSS GmbH, Munich, Germany). The patient's demographics and disease activity were described using median and interquartile range. The differences in age and DAS28 between EBV positive and negative groups were compared using Mann-Whitney U-test. The differences in all other parameters between EBV positive and negative groups were compared using Fisher's exact test. Spearman's correlation was applied to determine the association between EBV concentration and DAS28. A p-value of <0.05 was considered significant. Wilcoxon signed-rank test was used to determine the effect of RA medications on EBV DNA load.

Results

Disease activity of early and chronic RA patients is higher in patients with EBV DNA in serum

In patients with early RA at baseline EBV DNA was detected in serum of 15.2% (7/46) patients (Fig. 1A). EBV DNA was observed only in patients with moderate or high disease activity (Fig. 1A) and EBV positive patients showed higher disease activity (p=0.036) (Fig. 2A). There was also a significant positive correlation between the concentration of EBV DNA in serum and the disease activity (r_s=0.333, p=0.024) (Fig. 3A).

Anti-rheumatic treatment with s-DMARDs was started in all ERA patients. 15 patients were lost to follow-up. Of the 31 patients with follow-up information 25 started MTX, 14 started SASP, and 18 started HCQ alone or in different combinations. In the follow-up visit, the median disease activity (DAS28) had decreased, but the fre-

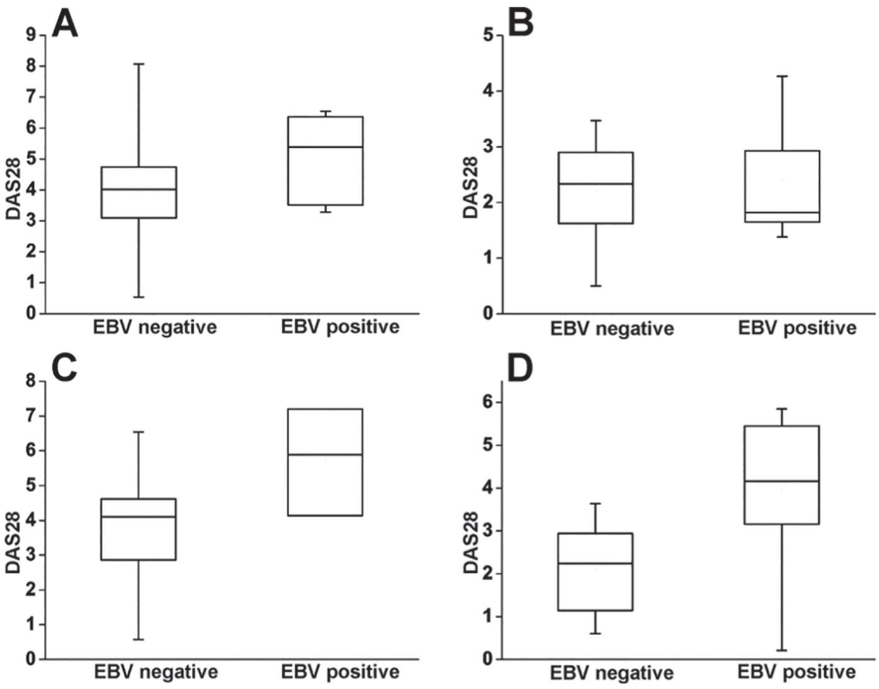

Fig. 2. Disease activity score (DAS28) of EBV negative and positive RA patients. Box plot shows median, interquartile range, minimum and maximum.
A: early RA (ERA) patients at baseline and **B**: ERA patients at follow-up visit.
C: chronic (CRA) patients at onset of the study and **D**: CRA patients at follow-up visit.
A: p=0.036; **B**: p=0.836; **C**: p= 0.077; **D**: p=0.027.

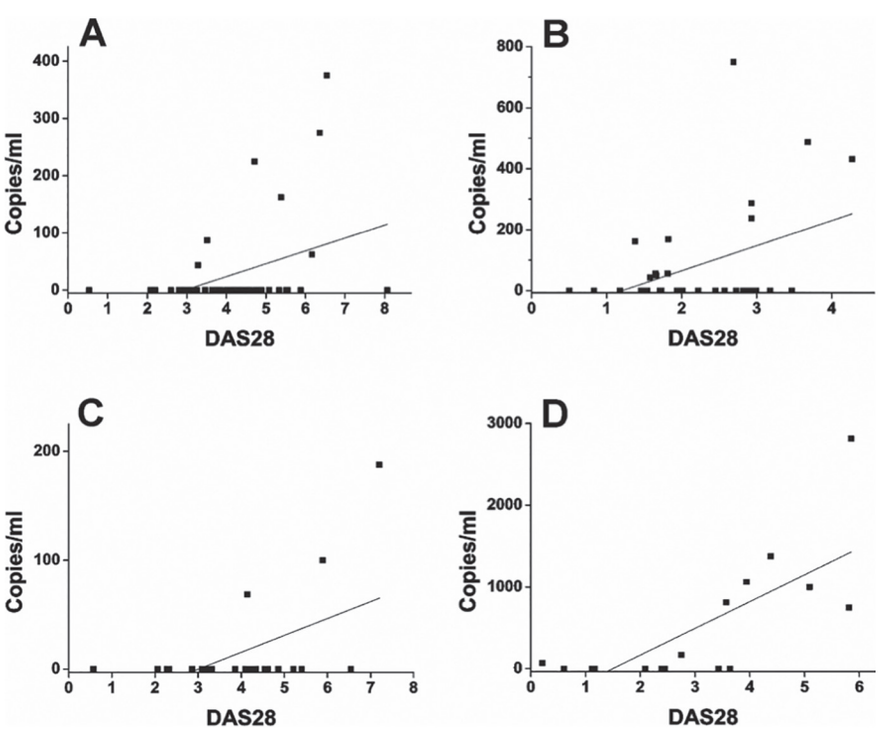

Fig. 3. Correlation between EBV DNA concentration and disease activity score (DAS28) in RA patients. **A**: early RA (ERA) patients at baseline and **B**: ERA patients at follow-up visit. **C**: chronic (CRA) patients at onset of the study and **D**: CRA patients at follow-up visit.
Trend lines represent a positive correlation between EBV DNA concentrations and DAS28. EBV concentration correlated with disease activity DAS28 in ERA baseline and CRA follow-up visit: **A**: r_s=0.333, p=0.024; **B**: r_s=0.157, p=0.400; **C**: r_s=0.406, p=0.061; **D**: r_s=0.724, p=0.002.

quency of EBV DNA positive patients had increased from 15.2% to 35.5% and EBV DNA could be observed also in patients with mild disease (Fig. 1B). Six patients negative for EBV at baseline were positive in the follow-up visit and five patients were positive both in baseline and in the follow-up visit with increased concentrations of EBV DNA in the follow-up samples. In the follow-up samples there was no statistically significant difference in disease activity between EBV positive and negative groups (Fig. 2B) and no significant correlation existed between EBV concentration and disease activity (Fig. 3B). However, higher EBV DNA concentration was observed in patients with higher disease activity (Fig. 3B).

At baseline, EBV DNA was detected in serum of 13.6% (3/22) of CRA patients (Fig. 1C). Similarly to ERA patients, EBV DNA was detected only in patients with moderate or high disease activity and concentrations of EBV DNA tended to be higher in patients with more active disease (Fig. 3C).

After the first visit, biological therapy was started to 93.8% (15/16) of CRA patients. In the follow-up visit, 50% (8/16) of the CRA patients were found positive for EBV DNA (Fig. 1D). EBV positive patients had significantly higher disease activity (p=0.027) (Fig. 2D) and EBV DNA concentration showed a significant positive correlation with the disease activity (r_s=0.724, p=0.002) (Fig. 3D). 6 patients were lost in the follow-up data of which none were positive for EBV at the baseline visit. 5 patients negative for EBV in baseline were EBV positive at follow-up visit and 3 patients were positive for EBV both at baseline and at follow-up visit with increased concentrations of EBV in the follow-up samples.

Taken together, significant association between serum EBV DNA and disease activity was observed both in ERA and CRA patients. Rheumatoid factor was positive in 75% (51/68) of patients. No significant association was found between EBV DNA positivity and rheumatoid factor.

In addition to differences in DAS28 between EBV positive and negative patients, the EBV negative group was

significantly younger than EBV positive among CRA patients at follow-up visit (p=0.035) (Supplementary Table II). Otherwise there were no statistically significant differences between the EBV positive and negative groups.

EBV DNA in healthy controls and in patients with adult onset Still's disease

To study the prevalence of the lytic EBV-infection in healthy individuals, EBV DNA was measured from serum of 21 age-matched healthy controls, from 12 non-matched healthy controls, and from 9 patients with adult onset Stills disease (AOSD). Two of the AOSD patients had active disease with high ferritin and CRP levels. No EBV DNA was detected in the serum of the AOSD patients. Of the 33 healthy controls, all except one were found negative for EBV DNA, with 118.7 copies/ml of EBV DNA detected in the single positive sample (data not shown).

Effect of anti-rheumatic treatment on serum EBV DNA load

None of the ERA patients used anti-rheumatic treatment at baseline, but all of them used sDMARDs at follow-up visit (n=31). At onset of the study, 95.5% (21/22) of CRA patients used sDMARDs but none of them used bDMARDs. At the follow-up, all CRA patients used sDMARDs and 93.8% (15/16) of patients also used bDMARDs. To assess the effect of individual drugs on the EBV DNA load we performed Wilcoxon signed-rank test on those patients who had started sDMARD or bDMARD treatment.

In the ERA patients, the serum EBV DNA concentration increased significantly (p=0.003) after start of sDMARD treatment. Methotrexate alone or in combination with other sDMARDS was started for 25 of 31 patients and in these patients serum EBV DNA concentration at follow-up was significantly increased (p=0.008). Nine of the ERA patients started MTX treatment with no other changes in the medication and in these patients, the concentration of serum EBV DNA increased significantly (p=0.043). For six of the ERA patients MTX treatment was not started. Of these 4/6 started a combination of SASP and HCQ with no other changes in medication. In this group EBV DNA concentration increased in 2 patients, however due to low sample number the overall increase was not statistically significant. In CRA patients, bDMARD was started in 93.8% (15/16) patients. After start of bDMARD treatment, the concentration of EBV DNA increased significantly (p=0.012). TNF inhibitors were started for 14/16 patients and in these patients serum EBV DNA increased significantly (p=0.018).

Discussion

The present study shows that by using the novel ddPCR method, DNA of EBV can be found in the serum of a significant number of RA patients. DdPCR was chosen for the method of analysis as it allows the detection of lower concentrations of viral DNA in the samples than conventional qPCR and thus makes possible the comparison of small differences in DNA concentrations. The amount of EBV DNA in the serum samples was in many cases low, but never the less the EBV positivity correlated significantly with disease activity. The presence of cell-free DNA in serum suggests an activation of EBV and an on-going lytic phase of the EBV infection. In the lytic phase, virus particles are produced and released from cells into the plasma. In addition to lytic infection, serum EBV DNA could also originate from dying apoptotic B-lymphocytes. However, EBV is a mitogen, driving the proliferation of infected B-lymphocytes (2) and, furthermore, EBV infection inhibits the apoptosis of B-cells (20). Thus, apoptotic B-cells can be considered an unlikely source of serum EBV DNA.

Increased EBV DNA load has been observed in peripheral blood mononuclear cells of RA patients. In addition, studies have reported an association between EBV-specific antibodies and RA. The present findings demonstrate that a subset of RA patients have an asymptomatic EBV viraemia, which also correlates with disease activity. Patients with higher disease activity also had a higher amount of EBV DNA in serum. The significance of this asymptomatic activation of EBV is not clear. It might be a consequence of immune activation caused by active RA. EBV virus has been shown to activate the NFκB signalling in macrophages and so potentially increasing RA activity (21). Thus, even if the EBV lytic phase would be a consequence of active RA, EBV viraemia might lead to further activation of the immune system and in an increased RA activity.

Several patients with positive EBV DNA at baseline were positive for EBV DNA also at the follow-up visit approximately 20 months later. Persistent EBV viraemia is relatively common in patients with immunosuppression because of organ transplants (22). Rheumatoid factor has been demonstrated to trigger the lytic phase of EBV in latently infected B-cells (23). RA patients using anti-rheumatic medication are immunocompromised and may thus have impaired ability to fully control the EBV infection. Several primary immunodeficiencies are known that specifically result in reduced ability to control EBV infection and into persistent EBV viraemia (24). Theoretically, a polymorphism in these signalling routes could also lead into reduced ability to control latent EBV infection in RA patients.

Contrasting results have been obtained regarding the effect of anti-rheumatic medication on the activation of EBV. Methotrexate treatment has been shown to activate the release of infectious lytic EBV from latently infected cells (9). However, in some studies, neither conventional anti-rheumatic medication (9) nor biologic treatment (25) had an effect on the prevalence of EBV activation. In the present study, both synthetic and biological anti-rheumatic treatments increased the serum concentrations of EBV DNA. Methotrexate and TNF inhibitors as a group significantly increased the EBV DNA in serum, but with respect to other synthetic DMARDs or biological DMARDs, the patient numbers were too limited to draw definite conclusions.

In conclusion, subset of RA patients have an asymptomatic EBV viraemia which correlates with disease activity. An asymptomatic EBV can stimulate the immune system and thus might increase the activity of RA inflammation and/or decrease the response to the anti-rheumatic medication.

References

1. ANGELOTTI F, PARMA A, CAFARO G, CAPECCHI R, ALUNNO A, PUXEDDU I: One year in review 2017: pathogenesis of rheumatoid arthritis. *Clin Exp Rheumatol* 2017; 35: 368-78.
2. THORLEY-LAWSON DA: Epstein-Barr virus: exploiting the immune system. *Nat Rev Immunol* 2001; 1: 75-82.
3. LAICHALK LL, THORLEY-LAWSON DA: Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus *in vivo*. *J Virol* 2005; 79: 1296-307.
4. TOUSSIROT E, ROUDIER J: Pathophysiological links between rheumatoid arthritis and the Epstein-Barr virus: an update. *Joint Bone Spine* 2007; 74: 418-26.
5. BALANDRAUD N, MEYNARD JB, AUGER I *et al*.: Epstein-Barr virus load in the peripheral blood of patients with rheumatoid arthritis: accurate quantification using real-time polymerase chain reaction. *Arthritis Rheum* 2003; 48: 1223-8.
6. NEWKIRK MM, WATANABE DUFFY KN, LECLERC J, LAMBERT N, SHIROKY JB: Detection of cytomegalovirus, Epstein-Barr virus and herpes virus-6 in patients with rheumatoid arthritis with or without Sjögren's syndrome. *Br J Rheumatol* 1994; 33: 317-22.
7. TAKEDA T, MIZUGAKI Y, MATSUBARA L, IMAI S, KOIKE T, TAKADA K: Lytic Epstein-Barr virus infection in the synovial tissue of patients with rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 1218-25.
8. JOKINEN EI, MOTTONEN TT, HANNONEN PJ, MAKELA M, ARVILOMMI HS: Prediction of severe rheumatoid arthritis using Epstein-Barr virus. *Br J Rheumatol* 1994; 33: 917-22.
9. FENG WH, COHEN JI, FISCHER S *et al*.: Reactivation of latent Epstein-Barr virus by methotrexate: a potential contributor to methotrexate-associated lymphomas. *J Natl Cancer Inst* 2004; 96: 1691-702.
10. BACHMAN TR, SAWITZKE AD, PERKINS SL, WARD JH, CANNON GW: Methotrexate-associated lymphoma in patients with rheumatoid arthritis: report of two cases. *Arthritis Rheum* 1996; 39: 325-9.
11. MAGNUSSON M, BRISSLERT M, ZENDJANCHI K, LINDH M, BOKAREWA MI: Epstein-Barr virus in bone marrow of rheumatoid arthritis patients predicts response to rituximab treatment. *Rheumatology* (Oxford) 2010; 49: 1911-9.
12. VALLEALA H, KAUPPI MJ, KOURI VP, KORPELA M: Epstein-Barr virus in peripheral blood is associated with response to rituximab therapy in rheumatoid arthritis patients. *Clin Rheumatol* 2015; 34: 1485-8.
13. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
14. PREVOO ML, VAN 'T HOF MA, KUPER HH, VAN LEEUWEN MA, VAN DE PUTTE LB, VAN RIEL PL: Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995; 38: 44-8.
15. FRANSEN J, VAN RIEL PL: The Disease Activity Score and the EULAR response criteria. *Rheum Dis Clin North Am* 2009; 35: 745-57, vii-viii.
16. PINHEIRO LB, COLEMAN VA, HINDSON CM *et al*.: Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal Chem* 2012; 84: 1003-11.
17. STRAIN MC, LADA SM, LUONG T *et al*.: Highly precise measurement of HIV DNA by droplet digital PCR. *PLoS One* 2013; 8: e55943.
18. YE W, TANG X, LIU C, WEN C, LI W, LYU J: Accurate quantitation of circulating cell-free mitochondrial DNA in plasma by droplet digital PCR. *Anal Bioanal Chem* 2017; 409: 2727-35.
19. BEUSELINCK K, VAN RANST M, VAN ELDERE J: Automated extraction of viral-pathogen RNA and DNA for high-throughput quantitative real-time PCR. *J Clin Microbiol* 2005; 43: 5541-6.
20. GREGORY CD, DIVE C, HENDERSON S *et al*.: Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* 1991; 349: 612-4.
21. KUNG CP, RAAB-TRAUB N: Epstein-Barr virus latent membrane protein 1 modulates distinctive NF- kappaB pathways through C-terminus-activating region 1 to regulate epidermal growth factor receptor expression. *J Virol* 2010; 84: 6605-14.
22. MORTON M, COUPES B, ROBERTS SA *et al*.: Epstein-Barr virus infection in adult renal transplant recipients. *Am J Transplant* 2014; 14: 1619-29.
23. YANG L, HAKODA M, IWABUCHI K *et al*.: Rheumatoid factors induce signaling from B cells, leading to Epstein-Barr virus and B-cell activation. *J Virol* 2004; 78: 9918-23.
24. COHEN JI: Primary immunodeficiencies associated with EBV disease. *Curr Top Microbiol Immunol* 2015; 390: 241-65.
25. TORRE-CISNEROS J, DEL CASTILLO M, CASTON JJ, CASTRO MC, PEREZ V, COLLANTES E: Infliximab does not activate replication of lymphotropic herpesviruses in patients with refractory rheumatoid arthritis. *Rheumatology* (Oxford) 2005; 44: 1132-5.