The Egyptian Rheumatologist 41 (2019) 255–259

Contents lists available at ScienceDirect

The Egyptian Rheumatologist

journal homepage: www.elsevier.com/locate/ejr

Elevated Cytomegalovirus and Epstein-Barr virus burden in rheumatoid arthritis: A true pathogenic role or just a coincidence

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ARTICLE INFO

Article history:
Received 24 February 2019
Accepted 28 March 2019
Available online 2 April 2019

Keywords:
CMV
EBV
Rheumatoid arthritis
Disease activity

ABSTRACT

Background: Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) have created great interest as their immunomodulatory action and latent forms could play a role in the advance of autoimmune diseases.
Aim of the work: To investigate the viral load of CMV and EBV in the serum of rheumatoid arthritis (RA) patients' and study their association with the clinical and laboratory profiles.
Patients and methods: The study included 50 RA patients and 32 healthy controls. Disease activity score (DAS28) was assessed and the medications received reported. Quantitation of CMV and EBV DNA in serum of all subjects was achieved by real-time polymerase chain reaction.
Results: Patients were 38 females and 12 males with mean age of 43.1 ± 12 years, disease duration of 6.5 ± 5.7 years; age of onset 36.6 ± 12.8 years. CMV DNA was detected in serum of 68% (34\50) patients, while EBV DNA was detected in 40% (20\50). Fourteen (28%) patients had both EBV and CMV DNA detected in serum. No viruses were detected in serum of 10/50 (20%) patients or in healthy controls. The mean viral load of CMV and EBV detected were 42005 ± 24805 copies/ml and 18756 ± 24937 copies/ml respectively. A significant increased frequency of anemia ($p < 0.0001$), Raynaud's ($p < 0.0001$), oral ulcers ($p = 0.014$) and arthritis ($p < 0.0001$) was detected in those infected with CMV *versus* those infected with EBV.
Conclusions: A high incidence of CMV and EBV was detected in RA patients with increased viral load than described previously. Frequencies of RA disease manifestations are significantly higher in CMV infected patients compared to those infected with EBV.

1. Introduction:

Rheumatoid arthritis (RA) is perhaps the most common inflammatory arthritis, affecting 0.5–1% of the general population worldwide. It is a disease of joints and the abnormal systemic immune response can lead to a variety of manifestation. Although the precise cause remains uncertain, environmental and genetic influences clearly are factors [1]. Gene polymorphism has been reported in Egyptian RA patients [2,3]. Similar to most inflammatory and autoimmune disorders, aberrant immune responses to environmental challenges contribute substantially. Among these challenges, infectious agents are undisputed leaders [4]. The socioeconomic impact of RA is remarkable due to the long-term articular damage and reduced functional capacity [5]. Interplay of pain, fatigue, activity and disability may lead to a high frequency of sleep disturbances in these patients [6]. Co-morbid depression [7], metabolic syndrome [8], thyroid [9] and urological dysfunction [10] as well as an increased frequency of osteoporotic fractures [11] have been reported in Egyptian RA patients. Within a small window of time, starting treatment early leads to a better disease outcome [12].

Peer review under responsibility of Egyptian Society of Rheumatic Diseases.
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https://doi.org/10.1016/j.ejr.2019.03.003
1110-1164/

Since the 1870s, an impressive list of microorganisms suspected of provoking RA has formed, and the list is still growing [4]. It was assumed that exposure to infection could prompt the development of RA and might contribute to disease flares [13]. Many hypotheses support viral infection role in RA pathogenesis. A wide range of viral infections have been alleged including Parvovirus B19, Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Hepatitis B and C viruses (HBV and HCV) and Herpes simplex virus (HSV) [4,13]. In Egyptian RA patients, there has been a rising attention to the impact of HCV on disease activity and functional status [14]. Moreover, the differentiation between HCV-related polyarthropathy and RA may be intricate especially early in the disease course [15]. Yet, the relation to other viruses has not been sufficiently studied. Firstly, some viral antigens were reported to be major immunogenic epitopes in RA patients ensuing in establishment of a molecular mimicry hypothesis in the pathogenesis [13,16]. Secondly, RA may develop as a result of multiple viral communities. Viruses could act as an adjuvant in the development of autoimmune diseases by non-specific motivation of innate immune responses via complement receptors, Toll-like receptors, mast cells and dendritic cells [17]. Thirdly, viral agents may trigger RA when linked with other factors such as smocking tobacco, psychological stress, ethnic differences, inflammation, or chronic joint tissue micro-trauma. Fourthly, some hypotheses assert that RA development results from immune hypersensitivity to viral infections with loss of tolerance to self-antigens [16].

Both EBV and CMV belong to the human Herpes virus family with a high seroprevalence in adults; In the USA, Europe and Australia, CMV seroprevalence is variable, ranging between 36 and 77%; while in developing countries and in particular sub-Saharan Africa, CMV is highly endemic with a seropositivity rate up to 100% [18]. The worldwide prevalence of EBV is estimated to be more than 90% of the populations [19]. Both viruses are characterized by persistence and latency, with acute replicative reactivation of infections [16]. Compared to a single infection with one virus, a combined infection with two or more of these viruses may increase the risk for development of RA in a rate more than that of a simple additive effect [4]. The present study aimed to investigate the association of viral load of EBV and CMV in the serum of RA patients and with their clinical and laboratory profiles.

2. Patients and methods:

The study included 50 Rheumatoid arthritis (RA) patients recruited from the Internal Medicine and Rheumatology and Rehabilitation Departments, Fayoum University Hospitals, during the period from January to June 2018. Patients were diagnosed according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria [20]. Patients eligible to be enrolled in this study were those with RA with age <60 years old with no other systemic rheumatic diseases. Exclusion criteria were patient age ≥60 years old, pregnant and lactating females, patients with known renal, hepatic or gastrointestinal diseases, previous or concurrent malignant diseases were all excluded, in addition to patients with history of solid or hemopoietic organ transplantation, or those with, sepsis or critically ill patients. Thirty-two age and sex matched healthy controls were included in the study. The study was approved by the Local Research Ethical Committee of Fayoum University and conforms to the provisions of the Declaration of Helsinki in 1995. All patients gave their informed consent prior to their inclusion in the study.

All participants were subjected to clinical assessment in the form of full history and thorough clinical examination. Disease activity for RA patients was assessed using the Disease Activity Score in 28 joints (DAS28) [21]. A DAS28 value >5.1 corresponds to a high disease activity, between 3.2 and 5.1 corresponds to a moderate activity, between 2.6 and 3.2 corresponds to a low activity and a value <2.6 corresponds to remission.

Investigations were performed in the form of complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), hepatic and renal function tests, rheumatoid factor (RF) by latex agglutination (Rose-Waaler test) and antinuclear antibody by indirect immunofluorescence on Hep-2 cells.

Quantitation of CMV and EBV DNA in serum was performed by real-time polymerase chain reaction. Serum was separated from blood drawn by centrifugation. Each sample was divided into two aliquots; stored at −70 °C until being tested. DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Valencia, CA) as described by the manufacture. Quantitation of CMV and EBV DNA was carried out using CMV- and EBV specific primers sequences targeting the conserved 105-bp region of the major immediate-early antigen in the CMV genome and 97 bp region of the conserved Epstein-Barr nuclear antigen 1 (EBNA-1) region in the EBV genome as described by the Artus® CMV TM PCR Kit and the Artus® EBV TM PCR Kit (QIAGEN, Valencia, CA), respectively. Quantitative real-time PCR assay amplification, data acquisition, and data analysis were carried out on ABI 7000 real-time PCR system (Applied Biosystems, Life technologies). The Artus EBV & CMV TM PCR kit primer and probes are proprietary and not made publicly available. Four Quantitation Standards were included in each run to generate a standard curve ranging from 5×10^4 copies/μl, 5×10^3 copies/μl, 5×10^2 copies/μl and, 5×10^1 copies/μl for EBV and 1×10^4 copies/μl, 1×10^3 copies/μl, 1×10^2 copies/μl and 1×10^1 copies/μl for CMV. To adjust this copy number to copies/mL for patient samples, a conversion factor was used. The detection limit of the Artus CMV TM PCR Kit and the Artus EBV TM PCR Kit is 0.20 copies/μl and 5.3 copies/μl respectively. For either reactions, a negative control was added in the form of PCR grade water in addition to the internal control which was used to manage the DNA isolation procedure and to check for possible PCR inhibition.

2.1. Statistical analysis

Data was analyzed using SPSS (Statistical package for the social sciences) version 20. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test. Spearman-rho method was used to test correlation between numerical variables. A p-value <0.05 was considered significant.

3. Results

Fifty RA patients (38 females and 12 males with mean age 43.1 ± 12 years) and 32 age matched controls (22 females and 10 males with mean age 31.1 ± 11.5 years) were studied. The mean disease duration was 6.5 ± 5.7 years and age of onset 36.6 ± 12.8 years. The clinical and laboratory manifestations of RA patients at the time of the study are demonstrated in Table 1.

The CMV DNA was detected in 68% (34\50) of patients, while EBV DNA was detected in 40% (20\50) of patients. Fourteen (28%) patients had both EBV DNA and CMV DNA detected in serum. No viruses were detected in serum of 10/50 (20%) patients. Both CMV DNA and EBV DNA were not detected in healthy controls with a viral load below the detection limit for both kits. The mean viral load of CMV and EBV detected were 42005 ± 24805 copies/ml and 18756 ± 24937 copies/ml respectively (ranging from 10,230 to 74,200 copies/ml for CMV and from 2100 to 80,700 copies/ml for

Table1
Clinical manifestations and laboratory investigations of rheumatoid arthritis patients.

Parameter mean ± SD (range) or n(%)	RA patients (n = 50)	
Arthritis O/E	36	(72)
Oral ulcers	6	(12)
Raynauds	10	(20)
Cutaneous vasculitis	8	(16)
ESR (mm/h)	66.2 ± 36.2	5–150
CRP (mg/L)	1.1 ± 4.8	0.0–24
Hemoglobin (g/dL)	11.9 ± 1.7	8–16.5
WBC count ($\times 10^9$/L)	7.8 ± 2.8	3.4–14.2
Platelets count ($\times 10^3$/ul)	285.3 ± 73.9	158–473
blood urea (mg/dL)	25.9 ± 13.9	0–82
Serum creatinine (mg/dL)	0.74 ± 0.24	0.0–1.2
ALT (IU/L)	21.7 ± 13.2	0–50
AST (IU/L)	21.6 ± 12.04	0–58
RF positive	32	(64)

O/E: on examination, ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; WBC: white blood cell; ALT: alanine transaminase; AST: aspartate transaminase; RF: rheumatoid factor; ANA: antinuclear antibody.

EBV). The CMV and EBV loads were comparable between females and males in patients and control.

When comparing the frequencies of RA disease manifestations in CMV infected patients *versus* those infected with EBV, there was a significant increase in the incidence of anemia, Raynaud's phenomenon, oral ulcers and arthritis in those infected with CMV ($p < 0.05$) (Table 2). According to DAS28, 12 cases were in remission, 12 with low activity, 26 having moderate activity and none had high activity. Distribution of EBV and CMV infection in relation to diseases activity and immunosuppressives received by the patients is demonstrated in Table 3.

In RA patients, the EBV DNA load significantly correlated with age ($r = 0.72$, $p = 0.02$) and disease duration ($r = 0.7$, $p = 0.02$), while no significant correlation was found between CMV load with age ($r = 0.26$, $p = 0.32$) or disease duration ($r = 0.32$, $p = 0.21$). No significant correlation was detected between DAS28 with CMV or EBV loads ($r = 0.33$, $p = 0.1$ and $r = 0.31$, $p = 0.14$ respectively).

To test whether the increased EBV and CMV load was the consequence of an immune suppressive drug treatment, patients on methotrexate (n = 42) showed no significant correlation of the dose with the viral loads ($r = -0.19$, $p = 0.35$ for CMV and $r = 0.34$, $p = 0.1$ for EBV, respectively). Patients receiving leflunomide (n = 12) showed no significant relation with the viral load ($r = -0.02$, $p = 0.93$ for CMV and $r = -0.24$, $p = 0.26$ for EBV). 26 were on prednisolone with no significant correlation with viral load ($r = -0.18$, $p = 0.4$ for CMV and $r = -0.1$, $p = 0.65$ for EBV).

4. Discussion

Infections are considered potent causes of life-threatening complications in rheumatic diseases patients [22]. It is well recognized that dissemination of viruses in blood as CMV and EBV has now been established as a substantial risk factor for development of concomitant clinical abnormalities in these patients [4,13,22]. CMV primarily infects macrophages and monocytes but also infects dendritic cells, fibroblasts, epithelial cells and endothelial cells while EBV primarily infects B cells but can also exists in latent phase within nasopharyngeal epithelial cells [23].

Detection of cell-free DNA in serum samples is generally acknowledged to be a marker of active infection [24]. The present study has investigated active infection with EBV and CMV in 50 RA patients and 32 healthy controls by detecting viral load of both viruses in serum. The current results revealed that CMV DNA was detected in 68% of patients *versus* 40% for EBV**.** The mean viral load was 42005 ± 24805 copies/mL and 18756 ± 24937 copies/mL for CMV and EBV respectively. Neither CMV DNA nor EBV DNA were detected in serum of healthy controls. In agreement, Mourgues et al. [25] found the EBV viral load positive in serum of eight RA patients out of 22 (36%) but the mean viral load value was 1777.2 ± 3518.3 copies/ml. They detected in their study only one patient with a positive CMV viral load (4.5%) with 2337 copies/ml which is much lower than that reported in the present study. On the other hand, Alvarez-Lafuente et al. [24] reported much lower percentage than the present results (2% for CMV and 0% for EBV serum samples). A strengthening explanation for the high incidence of CMV in RA patients in the present study is the high prevalence in the general population and the endemic state of CMV in sub-Saharan Africa with a rate approaching 100% in some areas [18]. Both viruses in the present study showed a relatively high viral load especially CMV, one of the possible explanations is the defects in cellular immunity in RA which may results in increased viral load, another explanation is the reactivation of these viruses as a consequence of immunosuppressive drug treatment [24].

This work indicated a significant correlation of EBV load with patients' age and disease duration. However, there was no supporting evidence from other studies.

Unexpectedly, our results detected some RA patients suffering from Raynaud's phenomenon, oral ulcers and vasculitis. RA was previously reported as a cause of secondary Raynaud's phenomenon with prevalences, ranging from 2.7%1 to 17.2% [26]. Rheumatoid vasculitis is usually associated with longstanding seropositive disease, erosive, and it has been reported to be more frequent among patients with circulating immune complexes [27]. In RA patients leflunomide is prescribed in active rheumatoid and psoriatic arthritis. Oral ulcers were reported in 3–5% of leflunomide medicated RA patients [28].

The present work was attempting to compare the frequencies of RA disease activities in CMV infected patients as opposed to those infected with EBV; to our knowledge this is the first time to target this issue in spite of the implication of both viruses in RA pathogenesis [4,13]. A significant increased frequency of anemia,

Table 2
Frequency and viral load of detected serum cytomegalovirus (CMV) and Epstein-Barr virus deoxyribonucleic acid (EBV DNA) with various clinical and laboratory variables in rheumatoid arthritis (RA) patients.

Parameter (n° of patients)	Viral load in RA patients (n = 50)				
	n(%)/affected parameters			Copies/ml mean ± SD (range)	
	CMV	EBV	*p*	CMV	EBV
Arthritis O/E (36)	32 (88.9)	18 (50)	**<0.0001**	42079 ± 25616 (10230–97000)	20379 ± 25884 (2100–80700)
Raynaud's (10)	10 (100)	2 (20)	**<0.0001**	34384 ± 25209 (10320–74200)	2265 ± 165 (2100–2430)
Vasculitis (8)	6 (75)	4 (50)	0.302	41750 ± 31995 (10230–74200)	10950 ± 12516 (2100–19800)
Oral ulcers (6)	6 (100)	2 (33.3)	**0.014**	37840 ± 25337 (10320–60200)	2265 ± 165 (2100–2430)
Anemia (12)	12 (100)	2 (16.7)	**<0.0001**	43802 ± 20496 (10320–74200)	2100 ± 0 (2100–2100)
Leucopenia (4)	4 (100)	2 (50)	0.103	58600 ± 22062 (43000–74200)	2100 ± 0 (2100–2100)
RF (32)	22 (68.8)	16 (50)	0.126	41703 ± 23101 (10320–74200)	18435 ± 28266 (2100–8070)

RA: rheumatoid arthritis; O/E: on examination; CMV: cytomegalovirus; EBV: Epstein-Barr virus; RF: rheumatoid factor. Bold values are significant at $p < 0.05$.

Table 3
Distribution of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infection in relation to the disease activity score (DAS28) and immunosuppressive treatment in rheumatoid arthritis (RA) patients.

Virus positivity n (%)	DAS28 score			Immunosuppressive		
	Mild (n = 12)	Moderate (n = 26)	Remission (n = 12)	MTX (n = 42)	LFN (n = 12)	Steroids (n = 26)
CMV	10 (83.3)	22 (84.6)	2 (16.6)	26 (61.9)	10 (83.3)	20 (76.9)
EBV	4 (33.3)	14 (53.8)	2 (16.6)	20 (47.6)	2 (16.7)	18 (69.2)

RA: rheumatoid arthritis; CMV: cytomegalovirus; EBV: Epstein-Barr virus; DAS28: disease activity score in 28 joints; MTX: methotrexate; LFN: leflunomide. None of the patients had a high DAS28.

Raynaud's phenomenon, oral ulcers and arthritis has been found in those infected with CMV compared to those infected with EBV. Most of the other parameters also tended to increase in CMV. On studying the profiles of immune response for CMV and EBV *in-vitro* in RA patients, Davis et al. [23] suggested that an immune response to latent CMV but not EBV infection could play a critical role in the progression of inflammation and structural damage of joints in these patients. The increased RA disease activities in the present study may be due to up-regulation of leukocyte immunoglobulin-like receptor1 (LIR-1) by CMV, which was reported to be associated with premature aging, more severe joint disease and increased disease activities in RA patients [29]. Another explanation is the existence of CD4+CD28− T-cell phenotype which was defined only in CMV-infected individuals; among patients with RA only patients who are CMV-seropositive carry CD4+CD28− T cells. The CD4+CD28− T cells are augmented in patients with RA, dermatomyositis and polymyositis. These cells are also detected in lower frequency in healthy CMV-seropositive controls. The frequency of CD8+CD28− T cells is reported to be strongly correlated with disease activity in patients with polymyositis [30].

Although many previous studies had reported the role of immunosuppressive drug in reactivation of latent viral infections [22,29–31] yet no significant correlation was found regarding any of immunosuppressive drug therapy administrated by RA patients included in the present study with CMV or EBV viral load. In harmony, Lossius et al. reported that EBV load was nearly the same in patients receiving or not receiving immunosuppressive drugs [32]. Even after long-term treatment with methotrexate there is no increase in EBV load [22]. Also Valleala and colleagues founds no association of EBV DNA load in peripheral blood in RA patients treated with leflunomide [33]. It can be used as a treatment option for human CMV [24,25]. It might be clinically beneficial in preventing EBV-induced lymphoproliferative disease in patients with high EBV loads [34]. A large-scale study declared the efficacy of using leflunomide in preventing CMV reactivation in post allogeneic stem cell transplant, its role seems limited, however its effect may be considerable when CMV copy number is less than 2000/ml [35]**.** This might explain the absence of association between leflunomide and viral load in the current study as all detected cases of either CMV or EBV had viral loads above 2000 copies/ml.

The reduced clinical input is among the study limitations and further analyses in view of the patients' disease characteristics, activity and medications received are recommended.

In conclusion, RA patients have increased seroprevalence of CMV and EBV signifying more frequent viral reactivation and increased viral loads. Frequencies of RA disease activities are significantly higher in CMV infected patients compared to those infected with EBV. Additional efforts are required to entirely translate how these viruses may modulate the immune system in RA patients. An interdisciplinary approach will be necessary to better understand the pathways by which both viruses might influence the pathogenesis and progress of RA.

Conflict of interest

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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