

AMERICAN UNIVERSITY OF BEIRUT

THE CORRELATION BETWEEN EPSTEIN-BARR
VIRUS DNA LEVELS AND SERUM IL-17 IN
RHEUMATOID ARTHRITIS

by

SARA HABIB JICHE

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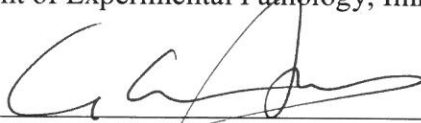
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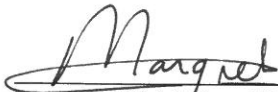
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Title: The Correlation Between Epstein Barr-Virus DNA Levels and Serum Interleukin-17 in Rheumatoid Arthritis

Background: The *Herpesviridae* is a large family of viruses that includes more than a hundred members. Human herpes viruses are large, enveloped, double stranded DNA viruses known for establishing latency and recurrent reactivations. The Epstein-Barr virus (EBV), a herpes virus, infects mostly the B lymphocytes and epithelial cells and establishes latency within memory B cells. The virus then recurrently reactivates and replicates. EBV infection has been previously associated with rheumatoid arthritis (RA), a chronically progressive autoimmune disease. RA is characterized by chronic synovitis and damage of cartilage and bone. IL-17, a proinflammatory cytokine, is produced by Th17 T-lymphocytes as well as other cell types such as monocytes, neutrophils, and natural killer T (NKT) cells. IL-17 is believed to underlie proinflammatory pathways in several autoimmune diseases including RA. In a previous study conducted at the Department of Experimental Pathology, Immunology, and Microbiology, injection of EBV DNA led to enhanced IL-17 production in mice. Therefore, EBV may lead to increased levels of IL-17 in other mammalian systems, such as in humans. Hence, we hypothesize that due to the capability of this virus to cause persistent and repeated infections, its continuous production of DNA would augment autoimmune promoting factors such as IL-17. To assess this possibility we examined whether the EBV DNA load correlates with IL-17 levels in 24 RA patients and 24 non-RA controls.

Methods: Upon obtaining subject consent, blood samples were withdrawn. Peripheral blood mononuclear cells (PBMCs) were then separated from blood samples with Ficoll-isopaque. PBMC DNA was then extracted and quantitative PCR (qPCR) was conducted to assess EBV DNA copy numbers. On the other hand, IL-17 levels were measured in sera samples using an enzyme-linked immunosorbent assay (ELISA).

Results: The average level of EBV DNA copy number per 10^6 PBMCs was 31.73×10^3 in control samples whereas it was 31608.23×10^3 in RA samples indicating significantly higher levels ($p=0.0026$). The average IL-17 levels were also significantly ($p<0.001$) higher in RA subject sera (11.42 pg/ml) compared to controls (7.3 pg/ml). Examining the linearity of the relationship between EBV copy numbers and IL-17 levels denoted a propensity for linearity in RA subjects (Spearman's Rho coefficient= 0.732 , $p<0.001$) but not in controls (Spearman's Rho coefficient= 0.236 , $p=0.267$).

Conclusion: Our data indicates a potential causal relationship between EBV DNA copy numbers and serum IL-17 levels in RA subjects but not in controls. This

implicates EBV DNA in possibly exacerbating the disease. Alternatively, particular genetic factors may underlie both RA and enhanced EBV replication; such genetic factors require further investigation.

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**To my
loving
Family**

CHAPTER I

INTRODUCTION

Rheumatoid arthritis (RA), one of the most common autoimmune diseases, is a chronic progressive disease characterized by chronic synovitis, and damage of cartilage and bone. This disease usually results from a combination of genetic predisposition and environmental triggers. The Epstein-Barr virus (EBV), a member of the *Herpesviridae* family of viruses, has been associated with an increased risk of RA among other autoimmune diseases. Previous studies have emphasized the pathogenic role of EBV in genetically susceptible RA patients. EBV infects B lymphocytes whereby its DNA remains in the host's nucleus in a latent form. It then undergoes persistent and repeated reactivation leading to potential continuous presence of its DNA in the patient's serum. A previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology showed that injecting mice with EBV DNA led to an increase in serum IL-17, a proinflammatory cytokine previously associated with RA among other autoimmune diseases. We hypothesize that EBV DNA would augment autoimmune promoting factors such as IL-17. Therefore, in the current study we aimed at assessing whether EBV DNA is associated with enhanced IL-17 production in humans.

The aims of this study were to:

1. Assess EBV DNA genomic copy numbers in peripheral blood mononuclear cells (PBMCs) as well as IL-17 serum levels in both RA patients and non-RA control subjects.

2. Assess the correlation between EBV DNA levels and IL-17 in RA patients and controls by examining the linearity of the relationship between these two factors in both subject groups.

CHAPTER II

LITERATURE REVIEW

A. Herpesviridae

The Herpesviridae is a family of viruses that includes more than a hundred members; eight are known to infect humans. Human herpes viruses are large (150-200 nm), enveloped, double stranded DNA viruses (1,2). Latency establishment and recurrent infection are typical characteristics of herpes viruses (1). Human herpes viruses are classified into three subfamilies: alpha, beta, and gamma (1). The alpha subfamily includes herpes simplex virus 1 (HSV-1), HSV-2, and varicella zoster virus (VZV). The beta subfamily includes cytomegalovirus (CMV), Human herpes virus 6 (HHV-6) and HHV-7 (1). The gamma subfamily includes Epstein-Barr virus (EBV) and HHV-8 also known as the Kaposi-sarcoma associated herpes virus (1). The subfamilies differ in tissue tropism; the alphaherpesvirinae establish latency in the sensory and cranial nerve ganglia, while the betaherpesvirinae and gammaherpesvirinae establish primary latency in lymphocytes. (1-3).

B. Herpesviridae structure

A *Herpesviridae* virion is composed of four major components. 1. The genome (150 – 250 kbp), double stranded DNA, encodes approximately 80 proteins (4). 2. An icosadeltahedral nucleocapsid (100 nm in diameter) encloses the genome and contains about 162 capsomeres (5,6) 3. A tegument layer or matrix between the nucleocapsid and the envelope surrounds the nucleocapsid. 4. An envelope that is derived from the host

cell membrane; the envelope has about seventeen amorphous proteins (5,6). Mass spectrometry on EBV identified 6 capsid proteins, 12 glycoproteins, and 17 tegument proteins. However, gp350 is the most abundant glycoprotein that the virus uses for attachment to B cells (5).

C. Epstein - Barr virus

In 1964, Epstein and Barr initially recognized the first human tumor virus, EBV, in lymphoblasts from Burkitt's lymphoma (BL) (7). More than ninety percent of the overall populace is asymptotically infected by EBV (8,9). EBV, also known as *Human Herpes Virus 4*, is a member of lymphocryptovirus genus of gammaherpesviruses (10). EBV infects mostly the B lymphocytes and epithelium cells (6). Once the host is infected with EBV, the virus establishes latency within memory B cells throughout the host's lifetime (2). The virus then recurrently reactivates and replicates to allow its spread and latency in B cells (6). Although, it has been documented that EBV can also infect T-lymphocytes *in vitro*, however, EBV shows a major preference for B-cells (6,11).

D. EBV genome and life cycle

The EBV genome is approximately 172 kilo base pairs (kb) (12). The genome is organized in a sequence of unique (U1 to U5), internal repeat (IR1 to IR4), and terminal repeat (TR) domains (4). The direct terminal repeats (TRs) are found on both ends of the DNA with approximately 538 base pairs. TRs have a function in the formation of the episome during latency (13).

The promoter region of EBV nuclear antigens (EBNAs) is found in IR1. In addition, the unique sequence (U_L) is divided into four shorter sequences (U2, U3, U4, and U5) by the other IRs (IR2, IR3, and IR4). Approximately 80 proteins can be translated by open reading frames of the EBV genome (9).

The life cycle of EBV consists of three phases: 1. Maintenance of the virions through lytic replication. 2. *In vivo* latency formation 3. Viral reactivation, replication and synthesis (14).

Lytic replication, which takes place in both epithelial cells and B lymphocytes, results in the production of new virions and expression of most of its proteins. EBV's entry into the epithelial cells is mediated through the binding of its gp350 with CR2 on epithelial cells (15). After this step, CR2 will bind to gp220 allowing further approach of EBV to the membrane of the epithelial cell. Similarly, entry into B cells is mediated by the concerted action of the same proteins; however, in addition, the viral gp42 then interacts with HLA class II on B Cells (15). The immediate early viral proteins BamHI R fragment leftward open reading frame 1 (BRLF1) and BamHI Z leftward open reading Frame 1 (BZLF1) are proteins that are expressed in the lytic phase; they initiate the expression of DNA polymerase, viral capsid antigen (VCA), and the membrane antigen (MA) (16).

Whereas in the latent infection, which happens mostly in B lymphocytes, only a few viral proteins are expressed. In this phase, the viral genome becomes circularized in the host nucleus and exists in an episomal form in the host cells for life (9).

Latency is a characteristic of all herpes viruses; EBV enters a state of latency after lytic replication. Furthermore, the virus can then re-enter the replication phase after latency (14).

During the latent phase twelve genes have been reported to be expressed. Of the 12 genes, 9 encode proteins and the other three lead to the expression of non-coding RNAs. The latently encoded proteins are grouped into two classes, the EBV-encoded nuclear antigens (EBNAs), and the latent membrane proteins (LMPs).

EBNAs include EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP; whereas the latent membrane proteins (LMPs) include LMP-1, LMP-2A, and LMP-2B. In addition, EBV-encoded small RNAs (EBER 1 and 2) and the BamH1-A rightward transcripts (BARTs) are the noncoding RNAs expressed during latency (16). Viral DNA polymerase is needed for the production of new DNA in the new virions during lytic replication. While during latent replication, the host cellular polymerase is accountable for maintaining the viral episome (17).

E. EBV Types and Genomic Variability

Despite the fact that the EBV genome is highly conserved, few genetic variabilities exist. The two EBV types, EBV-1 and EBV-2, differ from one another in their EBNA-2 gene. EBV-1 is prevalent worldwide, whereas EBV-2 is found mostly in Africa (18).

F. EBV pathogenesis

Transmission of the virus occurs mostly through the saliva (19). Other modes of transmission include sexual intercourse, blood transfusion, and organ transplantation,

but such transmission is rare (4,20,21). Some studies show that transmission of EBV through breast milk may occur (22).

Following oral transmission of the virus, EBV enters the oropharyngeal epithelial cells and undergoes lytic replication (19) ; it then spreads into salivary glands, oropharyngeal lymphoid tissues, and tonsils (19) . In the tonsils, EBV encounters B cells where it can undergo lytic replication or latency in memory B cells (23).

Reactivation of the virus may occur after latency leading to new virion production, infection of the oropharyngeal epithelial cells and consequent shedding in the saliva.

Reactivation also reseeds the lymphocytic compartment. Reactivation is thus an essential phase for persistence of EBV infection in the host, and transmission to a naive host (24).

G. EBV diseases and epidemiology

EBV diseases can be categorized into infectious mononucleosis (IM), epithelial diseases, and other associated diseases.

IM is an acute, self-limiting, multisystem disease that in most cases is self-limiting (25,26). Mononucleosis is typically characterized by a clinical triad of fever, sore throat, and adenopathy (4) although it presents with other symptoms such as malaise, myalgia, fatigue and swollen lymph nodes that may last for about 1-2 weeks (25). Moreover, symptoms such as splenomegaly and hepatomegaly may be presented (25). The incubation period for IM is 30 to 45 days (25). EBV infection is mild or asymptomatic in infancy and early childhood whereas in adulthood it may cause IM (2,27,28) . By the age of 25, most adults become seropositive to EBV (4).

EBV is also associated with epithelial diseases such as oral hairy leukoplakia (OHL) and nasopharyngeal carcinoma. OHL is a benign white lesion on the lateral side of the tongue that harbors a copious number of EBV virions (29). OHL mostly occurs in immunocompromised individuals (2,30,31). Other EBV diseases associated with immunodeficiency include post-transplant lymphoproliferative disorders and the X-linked lymphoproliferative syndrome.

In 1997, the World Health Organization (WHO) classified EBV as a 'carcinogenic agent' due to several EBV-associated human cancers (32). Being carcinogenic, EBV plays a role in different types of lymphomas in addition to nasopharyngeal carcinoma (2,6). Due to its major tropism to B cells, the virus has been associated with B-cell lymphomas such as Hodgkin's lymphoma and Burkitt's lymphoma (6,30). EBV leads to Burkitt's lymphoma, which classically presents as a jaw tumor mostly seen in African children. Studies showed that it acts as a cofactor to the c-myc proto-oncogene resulting in overactivation of the latter (33). Whereas, in Hodgkin's lymphoma, EBV inhibits apoptosis of B lymphocytes through its anti-apoptotic protein LMP-2A (34). Moreover, in nasopharyngeal carcinoma, EBV enhances tumor growth through an increase in viral IL-10 that in turn stimulates epithelial cells and CD4 T cells to excessively produce IL-1 α and IL-1 β (35). Another possible mechanism is through the inhibition of apoptosis through an overexpression of bcl-2 (36,37).

In underdeveloped countries, primary infection occurs early in life. By 3 to 6 years of age, around 80% become seropositive (25). In developed countries, primary infection happens between the ages of 10 and 30 (25). More than 90% of adults worldwide are infected with EBV (25). Burkitt's lymphoma is mostly seen in Central

Africa and Papua New Guinea, while nasopharyngeal carcinoma is in Southeast Asia (25).

No effective antiviral therapies exist for treating EBV infections (2,3). Preventative as well as therapeutic vaccines are under investigation. (38-42).

H. Autoimmunity

Autoimmune diseases (AID) result from the failure of self-tolerance resulting in the destruction of host tissues. These diseases usually result from a combination of genetic predisposition and environmental triggers (43).

AIDs are a global, heterogeneous group of diseases with more than 80 types diagnosed in about 100 million people worldwide (44). In the United States, the incidence of AIDs is estimated at approximately 80 per 100,000 person in a year with about 3% percent of the population diagnosed with an AID (45).

In the United States, the leading causes of death among young and middle-aged women is AIDs (45,46). AIDs affect women more than men; 75 % of patients are women (43,45). Although, there are few exceptions such as autoimmune renal disease which affects males more than females (43).

Examples of AIDs include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and type 1 diabetes mellitus. AIDs may target either a specific organ or multiple organ systems (45). For example, in type 1 diabetes mellitus the immune system targets the pancreas only while in SLE several organs including brain, heart, and kidneys are affected (45).

I. Infection and autoimmunity

AIDs are associated with several etiologies that have not been fully understood. Genetic predisposition in addition to environmental factors, such as infections, may lead to the development of an AID (47). Multiple theories explain the cause of autoimmune disorders. Three of these theories include molecular mimicry, epitope spreading, and bystander activation.

1. Molecular mimicry

The cross-activation of auto reactive lymphocytes due to sequence similarities between self and foreign antigens is referred to as molecular mimicry (48).

There are three criteria needed for a foreign antigen to cause an AIDs through molecular mimicry: 1) structural sequence similarity with the human antigen 2) adequate differences between self-epitopes and the foreign epitopes to -allow the trigger of an immune response, and 3) the self-peptide has to be localized on a biologically important tissue where the injury leads to a major consequence. The pathogenesis of AIDs such as MS, Graves' disease and diabetes mellitus has been associated with molecular mimicry (44,49,50) . For example, EBNA-1, a viral component of EBV, shares sequence similarity with the myelin basic protein (MBP), the autoantigen in MS. This may underlie molecular mimicry and hence an autoimmune reaction (45,51,52). Moreover, structural similarities between *Streptococcus Pyogenes* and cardiac proteins may play a role in the pathogenesis of rheumatic fever (53).

2. Epitope spreading

Epitope spreading is defined as the spread of an immune response from an initiating epitope to involve other epitopes resulting from constant damage of self-tissue. Specific Th1 cells are activated in a viral infection. The response then leads to the damage of host tissues causing the release of self-antigens in the circulation. An autoimmune response then leads to the *de novo* production of auto reactive T cells. These self-antigens are then taken up by antigen presenting cells (APCs) and in turn activate auto reactive T cells. Hence, the immunological response against the self-epitope is a result of the foreign antigen that initially provoked the response. On the other hand, epitope spreading may lead to the release of cryptic epitopes. Cryptic epitopes are self-epitopes that are not typically encountered by immune cells. In a virus-induced inflammatory response, there is an increase in antigen processing. This may result in the exposure of these cryptic self-epitopes (54). For example, SLE has been associated with an increase in auto reactive T cells attributed to epitope spreading (55).

3. Bystander Activation

The activation of a non-specific immune response and the proliferation of auto-reactive T cells due to proinflammatory cytokines and chemokines is referred to as bystander activation. (56). In an immune response, consistent inflammatory signals lead to an auto reactive response. Interferon γ (IFN- γ) and tumor necrosis factor- α (TNF- α) lead to the penetration of activated T lymphocytes into the infection site. Exaggerated immune responses in IM have been associated with the pathogenesis of SLE by the activation of auto-reactive lymphocytes (57).

J. Helper T 17 (Th17) Cells and IL-17

T Helper 17 (Th17) cells, defined by their production of IL-17, are a subset of pro-inflammatory T helper cells (58). Th17 cells can produce other ILs such as IL-1 β , IL-22, IL-21, and GM-CSF (59).

The differentiation of Th17 cells necessitates the presence of several transcription factors which include: retinoic acid receptor-related orphan receptor (ROR), IFN-regulatory factor 4 (IRF4), aryl hydrocarbon receptor (AHR), and STAT3 in addition to several cytokines such as IL-1 β , IL-6, and TNF- α (59-62). The function of TGF- β and IL-23 in Th17 differentiation is still questionable (63) and IL-23 may have roles in the differentiation, expansion and stability of Th17 cells (64).

IL-17 was initially recognized as a transcript from a rodent T-cell hybridoma by Rouvier *et al.* in 1993 (65). IL-17, a proinflammatory cytokine, is produced by Th17 T-lymphocytes as well as other cell types such as monocytes, neutrophils, and natural killer T (NKT) cells (65).

IL-17 is a family of 6 members: IL-17A, B, C, D, E, and F; IL-17A and F are the most studied and they seem to play a role in inflammatory responses (66). Although IL-17 has a restricted expression, its receptor is expressed in almost all cells. The IL-17 receptor is a type I trans membrane protein that is unique in its sequence (67). IL-17 binds to its specific receptor found on epithelial cells, endothelial cells, fibroblasts, adipocytes, and osteoblasts. Binding of IL-17 to its receptor activates NF- κ B signaling pathway, which in turn leads to cytokines secretion (68). Subsequently, recruitment of immune cells such as monocytes, neutrophils, and macrophages occurs. Th 17 cells are believed to play a critical role in combating fungal and bacterial infections (69). For

example, a high fungal load was observed in mice with a knocked-out IL-17 receptor (70-72). Oposing its beneficial role in fighting microbial infection, IL-17 is believed to be involved in the pathogenesis of various autoimmune diseases. These include multiple MS, RA, SLE and inflammatory bowel disease (IBD) among many others whereby increased IL-17 levels were detected found in the serum of various types of AID patients. (69,73-78). In addition to its association with autoimmune diseases, IL-17 has a pathogenic role in the rejection of an organ allograft (79).

IL-17 increases T-cell proliferation and enhances the production of antibodies from B-cells and the secretion of IL-1 and tumor necrosis factors (TNF) from macrophages and myeloperoxidase from neutrophils (61,80). Moreover, IL-17 results in the expression of major histocompatibility complex II (MHC II) molecules on dendritic cells (61,79). It also stimulates keratinocytes and epithelial cells to release IL-6 as well as IL-8 (61,80-82). In addition, IL-17 plays a role in the proliferation and maturation of neutrophils (60,80). Furthermore, antimicrobial peptides such as β -defensin are also released by mesenchymal and myeloid cells due to IL-17 (83).

IL-23 seems to be associated with IL-17 in the pathogenesis of several autoimmune diseases such as RA in humans and experimental autoimmune encephalomyelitis (EAE) in mice (48). Mice deficient in IL-23 or in IL-23 receptor are resistant to EAE induction (48,59,62). Moreover, neutralizing the effects of IL-23 or IL-23 receptor in mice inhibits the pathogenesis of EAE (70,71,82).

IL-17 plays a vital role in the pathogenesis of RA as well. After intra-articular administration of IL-17 gene in mice, key features of RA such as bone and cartilage damage were reproduced (84). In addition, studies done on mice have shown that the

inhibition of RA can be achieved by blocking the functions of IL-17 and IL-17 receptor (48,85,86). Furthermore, IL-17 is thought to play a critical role in the chronicity of RA (87). Studies done in mice showed that antibodies used to inhibit IL-17A receptor prevented the progression of arthritis (88).

A study performed at the Department of Experimental Pathology, Immunology and Microbiology at the American University of Beirut (AUB) aimed to examine the effects of EBV DNA on IL-17 production in BALB/c mice. Two groups of mice served as negative controls; one received no injections while the other received sterile water. Three mouse groups were injected with 36×10^3 , 72×10^3 or 144×10^3 copies of EBV DNA. A group injected with staphylococcal DNA was used as a non-viral DNA control. IL-17 levels were then measured in mouse sera using an enzyme-linked immunosorbent assay (ELISA). Results showed that mice injected with EBV DNA had higher levels of IL-17 compared to controls (2).

K. Rheumatoid Arthritis

1. Clinical presentation and epidemiology

Rheumatoid arthritis (RA), one of the most common autoimmune-related diseases, is a chronic progressive disease characterized by chronic synovitis, and damage of cartilage and bone (88). In RA, continuous inflammation leads to painful deformities especially in the fingers, wrist, feet, and ankles (48,89). Other organs that may be affected include: lungs, heart, and kidneys. RA has a prevalence of about 0.5 to 1% of the global population (78), with women being three times more affected than men (90).

Although RA was thought to be mediated by a Th1 response, it is now believed to be mostly driven by a Th17 response (48).

2. Etiology

Like other AIDs, RA pathogenesis is thought to be the result of genetic and environmental interplay (91). Studies indicate that patients with HLA-DRB1, and HLA-B27 are more likely to have RA (92,93). Other loci that have shown correlation with RA include those encoding protein tyrosine phosphatase 22 (PTPNN22) (94), the tumor necrosis factor alpha- induced protein 3 (TNFAIP3) (95), the tumor necrosis factor associated factor-1 and the C5 complement (TRAF1-C5) (95), and the signal transducer and activator of transcription-4 (STAT-4) loci (96) [78]. RA is characterized by the rheumatoid factor, an antibody against the Fc portion of IgG (97). Smoking was reported to be a potential environmental trigger of RA (98). The exaggerated immune response in RA is mediated by several cytokines such as IL-1, IL-2, IL-6, IL-17, TNF- α , TGF- α/β , and IFN- γ (99). These cytokines also lead to the activation of matrix metalloproteinases which in turn lead to joint injury (99). Moreover, TNF α and IL-1 β also contribute to the pathogenesis of RA through the activation of many cell types that include macrophages, osteoclasts that lead to the release of proinflammatory mediators and chemokines such as IL-8 (100). *In vivo* studies revealed that chronic inflammation and cartilage damage can be neutralized by inhibiting the functions of TNF α and IL-1 β (101). However, RA patients were not cured after the administration of anti TNF α and IL-1 β (88).

3. Rheumatoid Arthritis and Infection

Various infectious agents may contribute to RA in genetically susceptible patients (102). These agents include *Borrelia burgdorferi*, mycobacteria, parvovirus, rubella, hepatitis B and C viruses, HIV in addition to *Cryptococcus* (103-106). Studies have shown that EBV is the most common potential infectious trigger for RA (107); various studies have indicated a higher viral load, increased levels of anti-EBV-directed in addition to a compromised cell-mediated control of EBV infected cells in RA patients compared to controls (108). Nevertheless, a recent systematic review and meta-analysis of the literature has shed some doubt on this association; this review indicated that the majority of studies indicating a robust association between EBV and RA suffered from various limitations such as small study population samples (109).

The EBV gp110 protein harbors a sequence of amino acids that resembles one in HLA-DR1 and 4, which are also associated with RA. This has been proposed a possible mechanism for how EBV may trigger RA via molecular mimicry (110,111). EBNA-1 has also been shown to share homology with type II collagen and this has also been implicated in RA (112).

4. Diagnosis

According to the American College of Rheumatology (ACR) (113), a patient is diagnosed with RA upon meeting the following criteria: synovitis in at least one joint, and a score of 6 or more in the following four domains.

1. Number and site of affected joints (Score of 0 to 5)
2. Serological irregularities (Score of 0 to 3) including the rheumatoid factor test and anti-citrullinated protein antibody

3. Increased acute-phase reactants (Score of 0 or 1)

4. Duration of symptoms (Score of 0 or 1).

5. Treatment

Administration of anti-inflammatory agents is at the core of treating RA (114).

Other agents include T cell inhibitors (115) and possibly anti-TNF- α antibodies (116).

CHAPTER III

MATERIALS AND METHODS

A. Blood sample collection

Blood samples were collected from 24 RA subjects (age range 25-72 years, including 3 males and 21 females) and 24 matched non-RA control subjects. Blood was withdrawn and collected in EDTA tubes. All patients were older than 18 years, non-pregnant and fulfilling the American College of Rheumatology (ACR) classification criteria for rheumatoid arthritis (RA). The non-RA subjects were sex and age-matched with patients, non-pregnant and had no immediate family history of autoimmune diseases. Subjects were recruited by Dr. Imad Uthman. Institutional Research Board (IRB) approval was obtained prior to sample collection. Consent forms were read with the subjects and their details were clarified prior to obtaining their signatures. Blood samples were centrifuged for 15 minutes at 1500 rpm to ensure the separation of serum from whole blood. The serum was then stored at -80°C for later use.

B. Separation of Peripheral Blood Mononuclear Cells (PBMCs) by Ficoll-Isopaque

Collected blood was diluted with an equal volume of phosphate buffered saline (PBS). Then, 6 ml of diluted blood were layered on 3 ml of Ficoll-isopaque in a round bottom tube. The tube was then centrifuged for 30 minutes at 1500 rpm. Four layers were observed: 1) plasma 2) white layer or buffy coat (PBMC layer) 3) Ficoll-isopaque and 4) erythrocytes and granulocytes. The buffy coat was collected and washed with an equal volume of PBS. The tube was then centrifuged at 1500 rpm for 15 min.

C. DNA extraction

An equal volume of phenol was added to the buffy coat collected by the Ficoll-isopaque technique; this was then vigorously mixed. Next, the mixture was centrifuged for 15 minutes at 13,000 rpm and the supernatant was collected and transferred to another tube. Sodium acetate (1/10 of supernatant's volume) and 100% cold ethanol (3 times the supernatant volume) were added to the supernatant. The mixture was then stored overnight at -80°C. The tube was then centrifuged for 15 minutes at 13,000 rpm; a pellet was formed and the supernatant was discarded. The DNA pellet was then washed three times using 1 ml of 70% ethanol. Twenty µl of autoclaved distilled water was added to the DNA pellet and then vigorously mixed. DNA concentration was then measured using NanoDrop (Wilmington, DE).

D. Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time PCR (qPCR) was performed in triplicates to quantitate the level of EBV DNA copies in subject DNA samples. Previously published primers (117) detecting a region in the EBER gene and resulting in a product of 107 bp were used. Primers had the following sequence

Forward: 5'-CCCTAGTGGTTTCGGACACA-3'

Reverse: 5'-ACTTGCAAATGCTCTAGGCG-3'.

The Quantifast™ SYBER® green PCR kit (Qiagen, Germany) was used for qPCR reaction mixtures. Manufacturer recommendations were employed. Briefly, each reaction mixture consisted of 25 µl and contained 22 ng subject DNA, 7.5 pmol of each primer and 12.5 µl of the Quantifast SYBR Green PCR master mix.

An EBV DNA standard of 2.4×10^4 whole genomic EBV DNA copies/ μl , as specified by the manufacturer, was obtained from Advanced Biotechnologies (Columbia, MD). This was used to prepare a standard curve. qPCR reaction mixtures for the standard samples were prepared similar to those of subject samples; however, these contained either 2.4, 24, 240, 2400 or 24000 copies of EBV DNA per reaction.

The Bio-Rad CFX96 Real Time System C1000 Thermal Cycler was then employed using the following cycling conditions: 95°C for 5 minutes followed by 39 cycles of 95°C for 10 seconds and 61.4°C for 30 seconds.

Since 22 ng of DNA (employed in each subject qPCR reaction mixture) is the estimated genomic DNA content of 3.34×10^3 cells and the viral load is reported as EBV DNA copies per 10^6 PBMCs, we used the following formula for calculating the DNA load:

$$\text{EBV DNA copies per } 10^6 \text{ PBMCs} = (\text{EBV DNA copy number per qPCR reaction} \times 10^6) / (3.34 \times 10^3).$$

E. Enzyme-Linked Immunosorbent Assay (ELISA) for IL-17

IL-17 levels were measured in patient sera using human cytokine specific ELISA kits (Abcam, Cambridge, UK). Manufacturer recommendations were followed. Samples were performed in duplicates. All samples were diluted 2 times and standards were prepared. The experiment was conducted at room temperature. One hundred μl of Standards and samples were loaded onto the 96 well plate and then incubated for 2 hours. The plate was washed 3 times by adding 300 μl washing buffer per well and aspirating it each time. 50 μl of 1X Biotinylated IL-17 were then added to each well and then incubated for 1 hour. The plate was then washed three times as mentioned above.

One hundred μl streptavidin horse radish peroxidase (HRP) solution was then added to each well. The plate was covered and incubated for 30 min. Wells were washed again and 100 μl of chromogen Tetramethylbenzidine (TMB) One-Step Substrate was added to each well and incubated in the dark for 15 minutes. Finally, 100 μl of Stop Solution were added to each well. Absorbance was measured at a 450 nm wavelength using an ELISA reader (BIO-TEK, Winooski, VT). A standard curve was plotted using Microsoft Excel. Sample concentrations were then calculated using the standard curve formula.

F. Statistical Analysis

PASW Statistics 18 for Windows was used to conduct statistical analysis. Two-sample t-tests were conducted to assess significant differences in means. Spearman's rho coefficient was calculated to assess linear correlations.

CHAPTER IV

RESULTS

A. Average levels of EBV DNA in RA subjects and non-RA controls

The RA patients group had a higher EBV DNA average level when compared to the non-RA controls (Table 1, Figure 1). Average EBV copy numbers were significantly ($p=0.0026$) higher in RA patients (31608.23×10^3) compared to controls (31.73×10^3).

B. Average levels of IL-17 in RA subjects and non-RA controls

The RA patients group had a significantly ($p<0.001$) higher IL-17 average level when compared to non-RA controls (Table 2, Figure 2). While the average level in the RA patients group was 11.42 pg/ml it was 7.3 pg/ml in the control group.

C. Correlation between EBV DNA copy numbers and serum IL-17 in RA patients and non-RA controls

In RA patients, the correlation between EBV DNA copy numbers and serum IL-17 levels indicated a Spearman's Rho Coefficient of 0.732 ($p<0.001$) while the relationship between these two variables in the controls had a Spearman's Rho Coefficient of 0.236 ($p=0.267$). This indicated a statistically significant propensity for a linear relationship between serum IL-17 levels and EBV DNA copy numbers in RA subjects (Table 3, Figure 1) but not in non-RA controls (Table 3, Figure 2).

Table 1: EBV DNA copy numbers in RA subjects and non-RA controls

Status	Mean EBV DNA copy number per 10 ⁶ PBMCs	SD
Controls	31.73 x 10 ³	91.42 x 10 ³
RA patients	31608.23 x 10 ³	48635.29 x 10 ³

Table 2: IL-17 levels in RA subjects and non-RA controls

Status	Mean serum IL-17 (pg/ml)	SD
Controls	7.3012	3.46
RA patients	11.4277	3.44

Table 3: EBV DNA copy numbers per 10⁶ PBMCs and IL-17 serum levels in RA subjects and non-RA controls

RA patient ID	EBV DNA copy number	IL-17 (pg/ml)	Control subject ID	EBV DNA copy number	IL-17 (pg/ml)
RA 30	48825.34	6.18	CTRL 1	812.08	4.89
RA 43	338117.71	7.06	CTRL 50	4651.17	12.11
RA 28	1587846.43	7.06	CTRL 44	26435.08	5.21
RA 44	1232427.60	7.06	CTRL 57	839.53	8.06
RA 47	3787265.87	7.35	CTRL 49	215574.34	12.90
RA 12	3406088.08	8.24	CTRL 2	29101.73	11.48
RA 15	12769343.82	8.24	CTRL 7	243.17	4.49
RA 26	323984.78	9.71	CTRL 41	1728.03	3.22
RA 39	787682.77	10.00	CTRL 56	3957.95	6.24
RA 32	18172443.70	10.29	CTRL 17	104.34	8.30
RA 23	2137270.62	10.29	CTRL 6	1063.88	8.38
RA 1	757568.24	11.47	CTRL 8	463.89	6.48
RA 20	14211958.77	11.47	CTRL 20	8671.32	1.63
RA 11	2175369.45	11.76	CTRL 46	99.72	8.30
RA 24	7608825.72	13.24	CTRL 52	31.97	3.62
RA 17	156879.14	13.53	CTRL 53	58.67	7.51
RA 37	3549287.54	13.82	CTRL 21	844.38	1.63
RA 14	22384496.79	14.12	CTRL 45	408350.46	8.54
RA 13	128286701.73	14.26	CTRL 11	8683.80	6.76
RA 16	135825446.99	14.41	CTRL 3	633.07	9.49
RA 31	70471276.49	14.41	CTRL 48	35601.43	6.40
RA 40	82768949.40	14.71	CTRL 42	393.66	15.37
RA 45	114529161.08	16.18	CTRL 9	13047.04	9.65
RA 18	131280369.36	19.41	CTRL 47	165.35	4.56

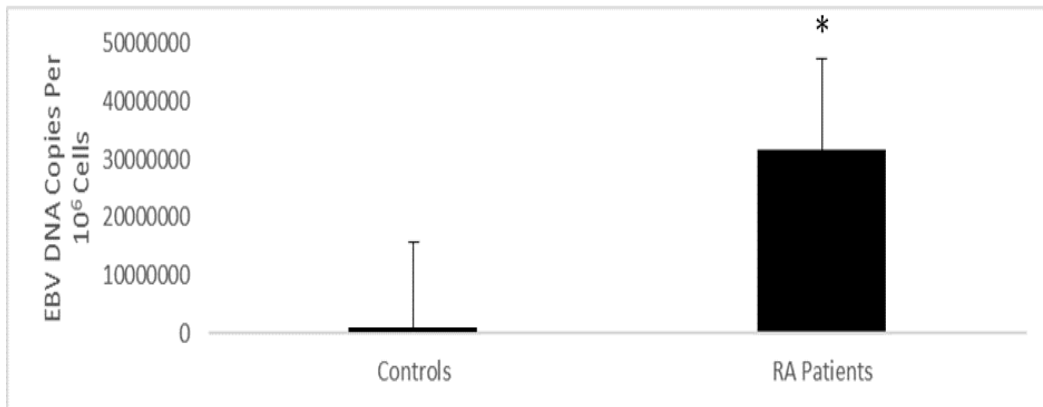


Figure 1: Average levels of EBV DNA in RA subjects and non-RA controls (* indicates $p < 0.05$ compared to controls).

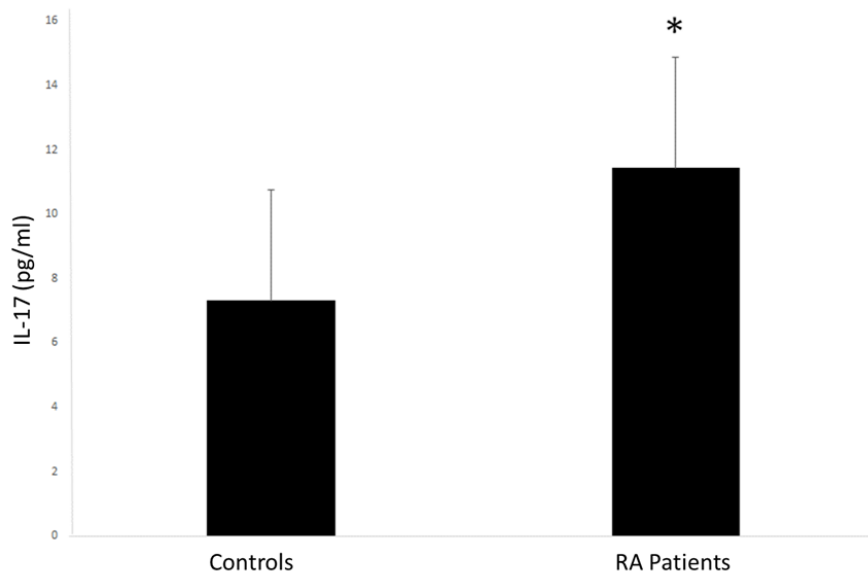


Figure 2: Average levels of IL-17 in RA subjects and non-RA controls (* indicates $p < 0.05$ compared to controls).

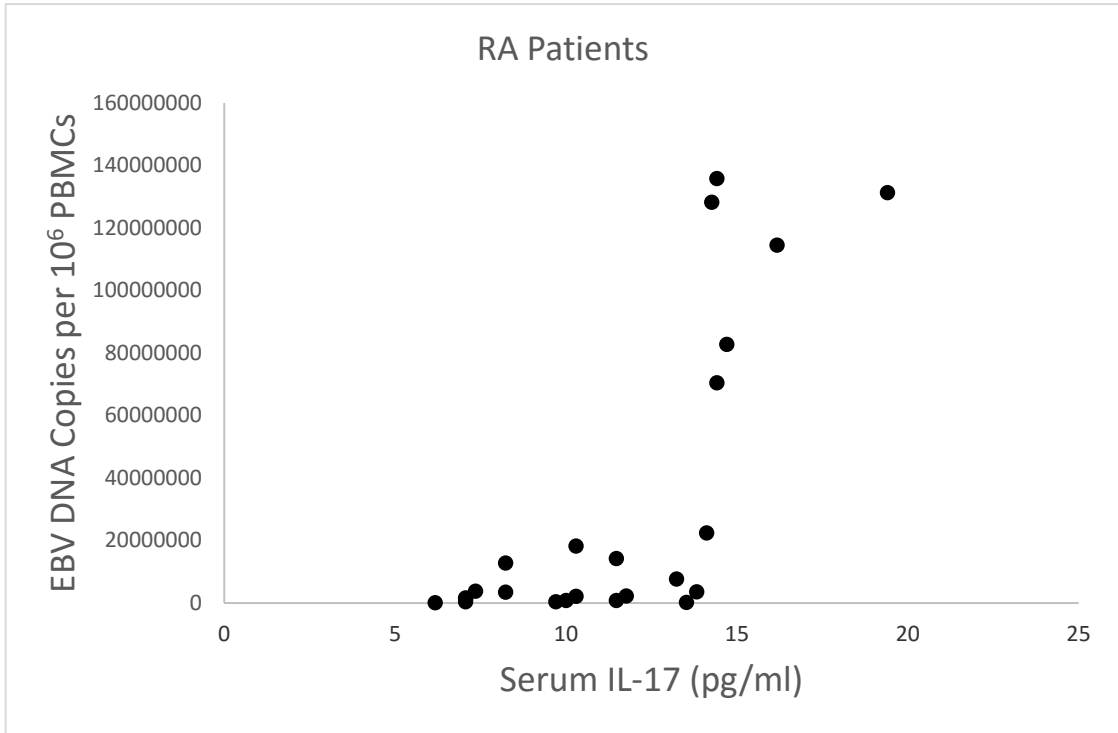


Figure 3: EBV DNA copy numbers and IL-17 serum levels in RA subjects. Spearman's Rho Coefficient=0.732, p<0.001

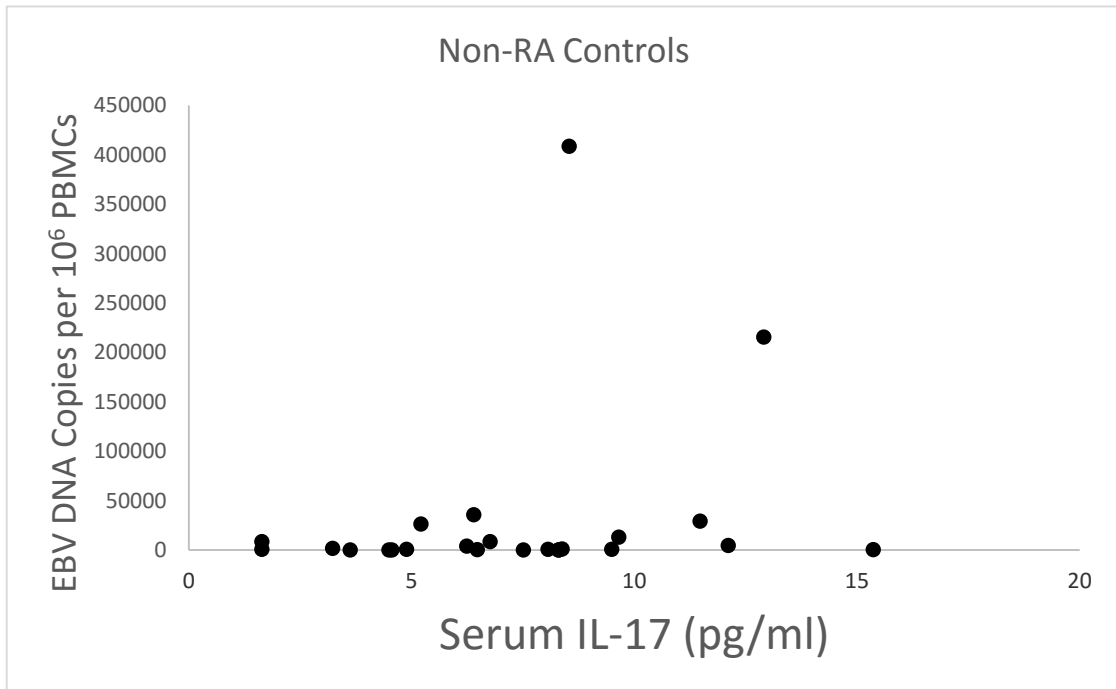


Figure 4: EBV DNA copy numbers and IL-17 serum levels in non-RA controls. Spearman's Rho Coefficient=0.236, p=0.267

CHAPTER V

DISCUSSION

A previous study conducted at the Department of Experimental Pathology, Immunology, and Microbiology showed that the risk of multiple sclerosis (MS) increases with a pre-established HHV-6 infection. A higher frequency of HHV-6 DNA positivity was detectable in MS patients compared to controls (118). Another study conducted at the Department of Experimental Pathology, Immunology, and Microbiology aimed at assessing the effects of whole genomic HHV-6A and EBV DNA on IL-17 production in BALB/c mice. This study showed that mice injected with HHV-6A or EBV DNA had higher levels of IL-17 (2).

EBV, a herpes virus, is capable of establishing latency and reactivation. Moreover, this leads to a continuous shedding of its viral antigens and DNA. There is increased evidence that EBV might be a possible trigger of many autoimmune diseases in humans; some of these autoimmune diseases are MS, RA, and SLE (115,119). Previous studies concluded that RA patients have higher levels of EBV DNA (107,108). EBV antigens possibly play a role in contributing to autoimmune mechanisms such as enhancing IL-17 production (120). IL-17 is primarily produced by Th17 cells and is a pro-inflammatory molecules believed to contribute to various allergy and autoimmune associated diseases such as and RA (121).

Therefore, in the study at hand we examined whether EBV DNA levels correlate with those of IL-17 in both RA subjects and non-RA controls. Our study showed that RA patients have higher EBV DNA levels. Serum IL-17 levels were also significantly increased in RA patients when compared to controls.

Our results are consistent with several studies that have reported higher levels of EBV viral load in RA patients than controls (122,123). Moreover, our data is also consistent with previous studies that have reported a relative increase in serum IL-17 in rheumatoid arthritis (124-126).

Our analysis indicates that in RA subjects, a propensity for a linear correlation exists between EBV DNA levels and serum IL-17; however, such a linearity was not detectable in non-RA controls. This may indicate that RA subjects become more prone to IL-17 production in the presence of EBV DNA due to preexistent rather over-active proinflammatory pathways. Alternatively, this may indicate the presence of a particular genetic factor in RA subjects that makes them more prone to IL-17 production due to EBV DNA. Another possibility is that a common genetic factor may result in both increased IL-17 levels and EBV replication. Whether reactivation of EBV replication would result in a flare up or enhanced severity of disease remains to be examined. Any association between disease status and EBV copy numbers should be considered in future studies.

In our study, the increase in IL-17 levels may be due to the binding of EBV DNA to cellular receptors. Possible receptors that may bind viral nucleic acids include NOD-like receptors, RIG1-like receptors, DAI, AIM2 and some Toll-like receptor family members (127,128). Whether particular alleles or variations of these receptors are more responsive to EBV DNA and may thus be associated with RA is another factor that may be examined by future studies.

In conclusion, our data indicates a potential causal relationship between EBV DNA copy numbers and serum IL-17 levels in RA subjects but not in controls. Further research is required to examine the mechanism by which EBV increases IL-17. A better

understanding may contribute to drug design, development and implementation in RA. Our findings implicate EBV DNA in exacerbating the disease. Alternatively, particular genetic factors may underlie both RA and enhanced EBV replication.

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