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RESEARCH ARTICLE

Evidence of Association between Epstein Barr Virus Serum Antibodies with HAs and RI as Biomarkers of Active Rheumatoid Arthritis

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Abstract: ***Background:*** Epstein Barr Virus (EBV) infection has been associated with the Rheumatoid Arthritis (RA) etiopathology, thus, considering it a relevant etiological agent in the disease development. We have previously observed the hyaluronic acid (HA) serum increase in active RA patients (DAS 28-4 > 2.6) which might be responsible for the erythrocyte deformability, estimated by the erythrocyte rigidity index (RI).

Objective: In the present study, we analyzed in one hundred RA patients if anti-EBV serum antibodies (TsEBV) and HA and RI levels are related with the disease activity. ***Results:*** [HA] s [[controls]]: 20.0 ± 9; [HA] s [[AR]]: 155.8 ± 44 ($p< 0.00001$ *vs*. controls (n:40). Antibodies anti-EBV (1/ TsEBV) [[controls]]: 2.55 ± 0.49; [[RA]]: 1.85 ± 0.35 ($p< 0.00001$ *vs*. controls). There were significant correlations between DAS 28-4 *vs*. log2 1/TsEBV (r: 0.70, $p < 0.00001$) and DAS 28-4 *vs*. RI (r: 0.75, $p < 0.00001$) in active RA patients. However, we observed a strong positive correlation between [HA] s in active RA patients *vs.* log2 1/TsEBV (r: 0.83, $p < 0.00001$) and [HA] s *vs.* RI (r: 0.91, $p < 0.00001$). ***Conclusion:*** The correlation obtained points out that the TsEBV may be related with the degree of RA activity determined by DAS28-4 and [HA]s in these patients. There was also a decrease in eryth- rocyte deformability estimated as RI, which is correlated with the DAS 28-4 activity index. Therefore, RI can be used as a reliable marker of RA activity. These findings might suggest a role of EBV infec- tion regarding an autoimmune mechanism present in RA.

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1. INTRODUCTION

Rheumatoid arthritis (RA), a systemic inflammatory disorder that causes synovitis leading to joint destruction, is considered an autoimmune disease due to the presence of auto antibodies, such as the rheumatoid factor and anti- citruline protein antibodies (ACPA) [1, 2]. RA has 0.5 to 1.0 % prevalence [3]. Research into its pathogenesis has shown that an abnormal immune response in a genetically predisposed patient is the hallmark of the disease [4].

The development of RA involves genetic and environmental factors with infections being the major risk factors [5]. Among the infectious triggering agents, viruses have long been suspected to promote the development of an autoimmune disease. To be a candidate for a causal role in RA, a suspected virus must be ubiquitous and persistent within the host, since RA is a chronic disease, common throughout the world, with recurrent flares, showing a direct or indirect tropism for the joints and last but not least, the virus must be able to alter the host immune response [6, 7]. The Epstein- EBV is a gamma herpes virus whose genome is a 172-kb double strand of DNA in- fecting almost 98% of the world population. EBV causes infectious mononucleosis and has been linked to the development of several malignant tumors such as Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma andpost-transplant lymphoproliferative disease [14, 15].

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Initially, EBV first replicates in nasopharyngeal epithelial cells and B-lymphocytes The virus reactivates occasionally, switching from latent to lytic cycle, with the production and expression of transactivating proteins, structural viral proteins, and envelope glycoproteins. The typical antigens for the lytic cycle are the viral capsid antigen (VCA), the early antigen (EA), and the membrane antigen (MA) [16]. The lytic and the latent cycles have distinctive serological pro- files: antibodies to VCA, EA, and envelope antigens are pro- duced early during the infection, whereas antibodies to EBNA develop later and persist throughout life [16].

Several arguments suggest a role for molecular mimicry between EBV and self antigens in patients with RA. One of the proteins of EBV, the EBNA-1, contains in its N-terminal region a sequence, which is characterized by glycine- arginine repeats, that are also present in the cytoskeleton proteins including cytokeratin and type 2 collagen [6]. Thus, EBNA-1 could play a role in ACPA production and anti- EBV antibodies may be produced. Besides, the RA suscepti- bility sequence QKRAA located in HLA-DRB1*0401 allele shares homology with a sequence of the gp110 glycoprotein [7, 17], an EBV lytic cycle antigen expressed in the endo- plasmic reticulum and on the cell membrane and the viral capsid [18, 19]. In this scenario, EBV infection may lead to an immune response against HLA-DR molecules containing the shared epitope.

Thus, EBV exhibits many characteristics that make it a likely candidate for a role in the pathophysiology of RA. This virus is extremely common in humans, causes chronic infection, is capable of altering host immune responses, and exhibits some degree of preference for the joints. However, a proof of a direct causal link between EBV and RA is lacking. Instead, the abnormalities in the EBV-directed immune responses observed in RA patients may be related to the immune dysfunction induced by RA [20].

Previous reports dealing with erythrocyte deformability in RA patients [21, 22] are controversial, which could be due to the differences in applied methodologies or in the heterogeneity of patient conditions, among others. Our hypothesis is that RA could modify the red blood cell (RBC) membrane similarly as the alterations produced in the membrane of connective tissue cells (*e.g*., synoviocytes), therefore, turning the erythrocyte into a reliable marker of the patient clinical stage.

In previous studies, we observed that the increase in erythrocyte rigidity index (RI) of patients with RA correlated significantly with serum levels of hyaluronic acid (HA_S). These suggest that HA would be responsible for the impairment of red blood cell membrane and, therefore, lower erythrocyte deformability. Thus, both the RI and HA would be equivalent markers of disease activity [23].

In the present study, we investigate in RA patients if anti-EBV antibodies (TsEBV) serum titers, are related with HA_S and RI, both markers of the degree of disease activity.

2. MATERIALS AND METHODS

2.1. RA Patients

The Ethics Committee of the Faculty of Medical Sciences, National University of Rosario, Argentina approved the study protocol, and all participants signed an informed consent according to the recommendations of the Declaration of Helsinki [25].

One hundred caucasian female RA patients attending an outpatient service at the Department of Rheumatology, Faculty of Medical Sciences, National University of Rosario (Argentina) were included in the present study (mean age 48 ± 17 yr). RA diagnosis was established following the American College of Rheumatology criteria [26, 27]. Exclusion criteria were: patients with cardiovascular or liver disease, cancer, chronic infectious diseases, HIV infection, diabetes mellitus, heavy smokers (>20 cigarettes/day) and patients taking medications altering hemorheological blood properties (*i.e*. anticoagulants, antihypertensives, contraceptives).

RA clinic activity was evaluated by means of the Disease Activity Score (DAS) 28-4 [28]. The cut-off values. used are the following: high disease activity > 5.1, low disease activity < 3.2, remission < 2.6.

Control group: 40 healthy volunteers, non-smokers females, caucasian, age-matched (mean 43 ± 12 yr) were included.

2.2. Blood Sample Collection and Laboratory Assays

RA patients and controls were obtained by venipuncture and separated in 2 aliquots. One of them was collected in tubes containing EDTA and assigned to determine he- matimetric indexes, plasmatic protein concentration and rheological parameters. The other was collected in a dry tube and centrifuged 5 min at 5,000 g in order to determine serum HA and TsEBV concentration. Both patients and controls had previous EBV infection assayed by a commercial EBV indirect immunofluorescence (Bion Enterprises LTD, US). Ten percent of the cells should exhibit solid fluorescent staining of the entire cell, with uninfected cells stained reddish-orange due to the counter stain.

a) Hematimetric indexes: Erythrocyte count was assessed by a hemocytometer and hemoglobin by the cyanmetahemoglobin method. From these values, Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC) were calculated.

b) HA assay: by an ELISA commercial test kit (Japan Biopharmaceutical company CHUGAI quantitative test Kit), using HA Binding Protein as capture molecule [29].

c) Measure of TsEBV titers: TsEBV titers were measured using an indirect immunofluorescence (IIF) assay developed in our lab [30]. Briefly, P3HR1 cells, a Burkitt's lymphoma cell line that is latently infected by EBV, were cultured in RPMI 1640 medium (PAA Laboratories GmbH, Austria) supplemented with 2 mM L-glutamine, 100 U/ml penicillin (Sigma, Argentina), 100 μg/ml streptomycin (Sigma, Argentina), and 10% fetal calf serum (Gibco, US). P3HR1 exponential growth culture cells were treated with 40 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) to induce the EBV lytic cycle in order to overexpressed VCA lytic phase antigen. After 24 hs, cells were collected for slide prepara- tion. Each aliquot was centrifuged at 800 rpm for 10 min (Eppendorf 5415D, rotor F54-24-11, Germany). Pellets were washed twice with phosphate buffered saline (PBS) and cells were suspended with PBS at a concentration of 10^6 cells/ml.

P3HR1 cell suspensions (20 µl) were plated on immunofluorescence slides to obtain 200 cells/slide. Suspensions were dried at 37°C and fixed with ethanol, acetone, ethanol/acetone (1:1, vol/vol), and stored at -20°C until use.

Titers of antibodies to VCA were determined by IIF in RA patients and controls. P3HR1 cells slides were treated with serial dilutions of sera (from 1/20 to 1/1,280) prepared in PBS with glucose (0.12 M NaCl, 0.03 M H_2KPO_4/HNa_2PO_4 with 2 mg/ml glucose), and incubated in the darkness at 37°C in a moist chamber. Then, slides were washed three times in PBS for 5 minutes and incubated with fluorescein isothiocyanate-conjugated antibodies to human immunoglobulin G (IgG) (Bion Enterprises LTD, US) ac- cording to manufacturer instructions. Slides were washed three times in PBS for 5 minutes and mounted in glycerol- PBS (1:1) and observed at 400x magnification of a fluores- cent microscope (Olympus BK50, Zeiss, Germany). A serum dilution was considered positive for anti-VCA antibodies if specific fluorescence in the EBV-VCA infected cells was defined. The EBV-VCA fluorescent staining pattern con- sisted of a solid staining of the entire cell. Approximately 11% of the cells exhibited a specific fluorescent pattern within uninfected cells staining reddish-orange due to coun- terstaining.

d) RI: Whole blood from RA patients was centrifuged at 5,000 rpm for 5 minutes, plasma and buffy coat were separated and the erythrocytes were washed twice with PBS. RBC was suspended (10% hematocrit) in PBS with bovine albumin (0.25%) (Sigma Chemical Co., St.Louis, MO, USA) in order to prevent erythrocyte aggregation. Erythrocyte fil- tration was performed in a computerized instrument using the Reid *et al*. technique [31]. Briefly, a 10% suspension of washed erythrocytes was passed through a polycarbonate filter, 5 µm pore size (Nucleopore Corp. USA), using a nega- tive filtration pressure of 10 cm H_2O. The flow time required for 1 ml of RBC suspension to pass through the filter was measured. Results were expressed as rigidity index (RI) that is an estimation of erythrocyte rigidity, inverse of erythro- cyte deformability, and [32] defined as:

$$RI = (Tb - Ts) / (Ts) \times 100 / Hct,$$

where Tb: time of passage of the cell suspension through the filter

Ts: time of passage of an equal volume of PBS

Htc: hematocrit (10%)

The erythrocyte deformability measurements are in accordance with the International Committee for Standardization of hemorheological methods [33].

2.3. Statistical Analysis

Comparisons between hematimetric index (MCV, CHCM), DAS28-4, RI, serum levels of HA and TsEBV in RA patients and controls groups were analyzed using Stu- dent´s *t* test for unpaired data by the Med Calc Software (version 4.20.021).Correlation coefficient (r) was used to analyze the rela- tionship of DAS28-4 and the following parameters: serum concentration of HA ([HA] s), RI and EBV antibody titer using the base two logarithm of the reciprocal of the dilution as a continuous variable in 100 patients with active RA.

RESULTS

The biochemical values (Hæmatocrit, MCHC,VCM), hemorrheological (RI) values and activity markers (HAs and DAS28-4) and TsEBV of patients with RA and their controls are described and correlated.

Erythrocytes from the active RA patients had RI values significantly higher than those of the control group (Table **1**). Our assays in patients with RA showed that RI was not altered by changes in the MCV, or the MCHC. Besides, active RA patients with DAS 28-4 showed higher values, with a significant increase in [HA]s and 1/ TsEBV *vs.* the control group.

Correlation was performed between disease activity DAS 28-4 and [HA] s in active RA patients (r: 0.87, $p < 0.00001$). The high correlation among these parameters point out the fact that HAs in RA patients is associated with RA activity since serum HA is a feature of certain inflammatory condi- tions.

On the other side, there was a significant correlation between DAS 28-4 *vs.* base log 2 1/TsEBV (r: 0.70, $p < 0.00001$) and DAS 28-4 *vs.* RI (r: 0.75, $p < 0.00001$) in active RA patients (Table **2**). These findings clearly suggest that DAS28-4 in active RA patients is associated with the plasma EBV and the alteration of the erythrocyte deform- ability (RI).

However, we observed strong positive associations between [HA] s in active RA patients *vs.* log base 2 1/TsEBV (r: 0.83, $p < 0.00001$) and [HA] s *vs.* RI (r: 0.91, $p < 0.00001$). In Table **2** the significant correlation between RA activity and the DAS 28-4 score could confirm RI as a probable indicator.

HA is a glycosaminoglycan --a high molecular weight polysaccharide—that showed a considerable increase [HA]s in active RA patients associated with TsEBV (Table **3**).

Consequently, these results indicate that TsEBV may be associated with the degree of RA activity determined by DAS28-4 score and [HA] s in these patients (Table **3**).

Table 1. Biochemical and Hemorheological variables in RA patients and their controls.

	Patients (n =100)	**Controls (n = 40)**	**Probability**
1/ TsEBV	2.55 ± 0.49	1.85 ± 0.35	$p < 0.00001$
[HA] s (µg/ml)	155.8 ± 44	20.0 ± 9	$p < 0.00001$
Hæmatocrit (%)	38.45 ± 3.53	39.6 ± 2.19	NS
RI	14.79 ± 4.71	6.92 ± 1.31	$p < 0.001$
MCV (µm3)	89.7 ± 1.6	93.15 ± 7.7	NS
MCHC (g/dl)	32.5 ± 0.7	31,45 ± 1.32	NS
DAS 28-4	5.53 ± 0.17	2.09 ± 0.05	$p < 0.001$

Table 2. Correlation between Rheumatoid Arthritis degree of activity and EBV antibodies titers and RI in 100 patients.

Parameters	DAS 28-4 r	Probability
[HA]s	0.87	0.00001
base log 2 1/TsEBV	0.70	0.00001
RI	0.75	0.00001

DAS 28-4 degree of Rheumatoid Arthritis activity
RI Erythrocyte Rigidity index,HA serum concentration
base log 2 1 / TsEBV base two logarithm of the reciprocal TsEBV.
r correlation coefficient

Table 3. Correlation between of serum hyaluronic acid concentration with serum Acs anti-VEB titers and RI in 100 patients with active RA.

Parameters	[HA]s r	Probability
RI	0.91	0.00001
log base 2 1/TsEBV	0.83	0.00001

RI Erythrocyte Rigidity index,
HA serum concentration
base log 2 1 / TsEBV base two logarithm of the reciprocal TsEBV.
r correlation coefficient

Data presented as mean ± standard deviation. Comparisons performed by Student´s t test. NS: not significant. Erythrocyte rigidity index (RI), serum concentration of hyaluronic acid ([HA] s), mean corpuscular hemoglobin concentration (MCHC), DAS 28-4 degree of Rheumatoid Arthritis activity and 1/ serum titers anti-VEB (1/ TsEBV)

3. DISCUSSION

For over 20 years, it has been hypothesized that a suspected association of EBV as a viral factor that contributes to the pathogenesis of RA. At the center of this association, there is strong evidence of molecular mimicry between EBV antigens and self proteins in RA [18,19]. A decreased T-cell response to gp110 has been reported and this response was correlated with inflammation parameters and systemic involvement [5].

On the other hand, EBV is involved in modulating the host immune response by encoding a protein homologous to the interleukin 10 (IL-10). The viral IL-10 can suppress the response of T helper type I cells, and act as an autocrine growth factor for B cells. Several studies showed that EBV-infected B cells may secrete metalloproteinases and proinflammatory cytokines that play an important role in the pathogenesis of RA [34-36]. Then, if the increased activity of EBV in RA patients is due to a dysregulation mechanism acting upon the immune response, the virus could have a significant role in the joint damage observed in RA. It is therefore difficult to discern whether the increased levels of TsEBV in RA patients are a cause or an effect of the disease.

RA is a heterogeneous disease that can be aggressive with significant anatomical and functional consequences. Therefore, it is important the initial evaluation in order to properly categorize them and to modify the natural course of the disease. Consequently, it is a must to have reliable prognostic markers that might identify whether a patient has active RA or not at the initial evaluation.

Weyand and Goronzy [37] reported a close relationship between disease activity (DAS28-4) and the presence of shared epitope on HLA-DR, showing that patients with two risk alleles had a more severe disease than those who held one. These findings suggest that HLA-DR genetic markers are more related to the degree of expression than to susceptibility. Based on this and other similar results [38, 39] it seems probable that the host genetic background is of the utmost importance to determine the degree of disease activ- ity.

In our study, the TsEBV was associated with the RA activity degree determined by the DAS28-4 index. TsEBV assessed by IF assay is more easily measured and less expensive than HLA-DR allele detection with the shared epitopes in RA patients and could be used as a reliable laboratory marker to predict disease progression or remission. Moreo- ver, these findings would suggest the contribution of EBV infection in the mechanisms of autoimmunity associated with RA.

As HA is synthesised by synoviocytes and is also a prominent component of articular cartilage, an increased systemic level might act as an early indicator of structural damage and hence provide a useful prognostic marker [40].

Raised serum levels of HA have previously been noted in RA. The HA level has been reported to correlate with indices of joint inflammation and may have some specificity for synovial inflammation. It has been suggested that raised HA level early in disease may also predict future joint destruc- tion [41, 42].

This explains why [HA] s correlates with clinical evaluation (DAS 28-4) and is a more specific indicator of AR activity than standard laboratory parameters [43, 44].

HA is an acidic polypeptide that like albumin could be absorbed to the erythrocyte cell surface [45] reducing the flexibility of the membrane and increasing the RI. This hypothesis is confirmed when there is a significant correlation between RI and the [HA]s in RA patients (Table 3), which impairs the ability of deformed red blood cells in the microcirculation [46].

HA interacts with the erythrocyte surface, giving place to modifications in erythrocyte rheological (IR) and flow properties.

Considering that HA is increased in inflammatory processes, we claim that the effect of HA upon erythrocytes -and thus, on circulatory function-- should not be disregarded; in fact, special attention should be paid to this matter.

In our AR patients, an increase of [HA] s is produced due to chronic inflammatory process estimated by DAS28-4. This increase in [HA]s results in increased erythrocyte IR (loss of erythrocyte deformability) [46]. Activation of the immune system in RA disease can lead to reactivation of

EBV which is latent in B cells, as evidenced by an increase in TsEBV in blood. However, these can act as autoantibodies directed against epitopes present in the synovial membrane proteins causing the destruction of cartilage matrix. We observed that the increase in TsEBV is correlated with the increase in [HA] due to this process present in AR [23, 24].

CONCLUSION

We determined a decrease in erythrocyte deformability measured as an increase in RI which correlated with the activity index DAS 28-4. Therefore, RI can be used as a reliable marker of activity of AR.

The association obtained with the correlation coefficient indicates that increase in TsEBV titers in blood and of [HA] is related with the degree of RA activity determined by DAS28-4 in these patients.

We note that the increase in TsEBV is correlated with the increments in [AH] s due to the inflammatory and destruc- tion process of the cartilage matrix present in AR.

These findings confirm the contribution of EBV infection in the autoimmune mechanism associated with RA.

LIST OF ABBREVIATIONS

[HA]s	=	Serum concentration of hyaluronic acid
ACPA	=	Anti-citrullinated protein antibody
DAS	=	Disease Activity Score
DNA	=	Desoxyribonucleic acid
EBNA-1	=	Epstein-Barr nuclear antigen 1
EBV	=	Epstein -Barr virus
EDTA	=	Ethylenediaminetetraacetic acid
HA	=	Hyaluronic acid
HIV	=	Human Immunodeficiency
IL-10	=	Interleukin 10.
MCHC	=	Mean corpuscular hemoglobin concentration
MCV	=	Mean corpuscular volume
PBS	=	Saline phosphate buffer
RA	=	Rheumatoid arthritis
RBC	=	Red blood cells
RI	=	Erythrocyte Rigidity index
TsEBV	=	Serum titers of anti-EBV antibodies

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare that they have no competing interests. All authors read and approved the final manuscript.

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