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Original Article

No Definite Association between Human Parvovirus B19 Infection and Behçet Disease

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This article has Continuous Medical Education (CME) credit for Iranian physicians and paramedics. They may earn CME credit by reading this article and answering the questions on page 552.

Abstract

Background: The etiology of the Behçet disease (BD) has remained obscured. There have been studies to show the association of BD to infections like herpes simplex, hepatitis, and parvovirus B19 however, the findings are rather controversial.

Materials and Methods: We selected 55 patients with the best matched symptoms of BD and measured the loads of B19 DNA in their plasma by quantitative real time PCR and verified their seropositivity by ELISA. All findings were compared to the results from 42 healthy persons.

Results: Patients showed a wide spectrum of BD symptoms. Serologic studies showed high prevalence of B19 IgG among the tested patients which was not statistically different with the healthy population (72.7% vs. 85.7%, respectively). Similarly, the prevalence of B19 IgM between patients and controls was not different (18% vs. 11.9%, respectively). No correlation was found between the presence of anti-B19 antibodies and the clinical observations. Only one person from the patient and control groups had detectable levels of B19 DNA without any difference or correlation with the disease symptoms.

Conclusion: Our data could not establish an association between B19 parvovirus infection and Behçet disease, although there have been reports of such correlation. Nevertheless, there might be indirect relation in genetically susceptible individuals after viral infections. More studies on designed animal models and surveys on patients should be done to resolve this controversy.

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Introduction

Behçet disease (BD) is a complex multi-organ inflammatory disease recognized with debilitating severe systemic vasculitis but with unknown etiology. The triad of recurrent oral aphthosis, genital ulcer, and uveitis are the main classic presentations of the BD. However, the International Study Group for Behçet Disease (ISGB) suggested the diagnostic criteria of oral ulcers in association with any two of the genitalia, eye, and skin involvement or a positive pathergy test to standardize the diagnosis.[1,2] Behçet appears to be endemic along the ancient Silk Road, in the Middle East, countries bordering the Mediterranean Sea, Europe, Korea, and Japan.[3] With such geographical distribution, the reported prevalence of the disease could vary widely depending on the target region, ethnicity of the population and the methodology

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used for the study. In general, BD can affect all age groups of males and females equally, although the disease onset is most common between the second through the fourth decades of life.[4]

Iran is also among the geographical regions with incidence of Behçet Disease. The incidence of the disease was estimated around 68 per 100,000 individuals.[5] In a detailed study, Davatchi et al. showed that most clinical features including mucocutaneous, ocular and joint manifestations of the Iranian people with BD are similar to findings from other regions.[6] Due to extreme variations in pathogenesis of BD, more than one mechanism has been suggested to be involved in the formation of the disease. In fact, evidence shows that genetics and environmental factors should both be responsible to modulate prevalence and expression of BD.[7-9] Among environmental factors, there have been observations in the past suggesting the role of infectious agents as etiologic cause. It was shown that positive pathergy decreases when the skin lesion is surgically cleaned. Similarly, others showed the effectiveness of penicillin on mucocutaneous lesion of patients diagnosed with BD.[10,11] Most importantly, Sezer et al. reported the isolation of a kind of virus from ocular fluid of a BD patient. He considered the virus as the BD causative agent when the inoculation of the virus in animal models caused symptoms resembling BD disease. However, these results could not be confirmed by others later on.[12] Since that time, with the availability of new techniques, more investigators searched to find clues to link BD with viral or other infectious agents. In this regard, researchers claimed finding viral DNA or RNA strands complementary to herpes simplex virus 1 (HSV-1) in peripheral blood cells or saliva from BD patients more frequently than in healthy people.[13,14] More recent data from animal model may support such findings.[15] However, many others could not reproduce those findings and instead detected HSV genome in clinical samples from oral lesions or blood leucocytes of patients with BD.[16] Hepatitis viruses have also been considered as etiologic cause for BD[17] as they have been shown to be associated with several rheumatologic diseases.[18] Many others have examined the association of BD with other viruses like human immunodeficiency virus, varicella zoster, or cytomegalovirus however; no one could show convincing evidence to consider those viruses as definite causative infectious factor for the disease.

Parvovirus B19 is a non-enveloped icosahedra single stranded DNA virus, infecting human with a wide spectrum of clinical presentations. The virus selectively targets the early erythroid precursor cells, megakaryocytes, endothelial cells, placental cells, and many other cells through binding to blood group P antigen on the cell surfaces. Infection with the virus can cause mild self-limiting erythema infectiosum in immunocompetent children, but produce lethal cytopenias in immunocompromised patients. Moreover, B19 infection could persist for a long time and triggers autoimmune inflammatory disorders.[19] B19 can be found worldwide and its seroprevalence increases with age. The virus is normally transmitted through the respiratory route; however, vertical transmission and transmission via transfused blood products have been reported.[20] B19 infection has been associated with some autoimmune disorders such as forms of collagen vascular diseases or conditions that mimic systemic lupus erythematosus (SLE).[21,22] Moreover, it was shown that patients with recent B19 infection may develop leukocytoclastic vasculitis.[23,24] Therefore, in this study in the southwest of Iran, we investigated whether there is an association between the presence of parvovirus B19 DNA in peripheral blood of BD patients as shown by other investigators. In addition, we examined whether the B19 serologic prevalence in the patients is higher than what exist in healthy individuals.

Materials and Methods

Patients and Sampling

To assess the prevalence of parvovirus B19 viral infection, a cross-sectional case control study was accomplished during 2010-2012 in Fars province, southwest Iran.

Sampling of the participants (based on a confidence level of 95% and the margin of error of 15%) comprised of 42 healthy individuals and 55 patients with BD diagnosis classified according to the criteria proposed by the International Study Group for Behçet's disease.[1] The patients and age and sex matched controls were from similar ethnicities of south west Iran. The selected patients were divided into two groups of patients with active and inactive disease according to their clinical presentations. Active phase of the disease was defined if one of the symptoms of uveitis, subcutaneous venous thrombosis, skin lesions (such as erythema nodosum), genital ulcers, arthritis, intestinal ulceration, progressive central nervous system lesions, progressive vasculitis, and epididymitis were found to be persistent for more than two weeks in screening. Patients in the active group were subdivided into mild, moderate, and severe conditions based on

the disease severity. The total clinical severity score (CSS) of the disease was determined as described in the literature.[25] In this way, patients with more than 6 points were grouped as severe, patients with scores between 4 and 6 grouped in the moderate group and those scored less than 4 were grouped as mild. Additionally, patients should have at least two weeks history of persisting oral aphthous, skin or genital lesion and uveitis to be included in the active phase of the disease. Most patients were receiving standard chemotherapeutic medications at the time of sampling. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences and performed in the department of Immunology and Internal Medicine of Shiraz University of Medical Sciences, Shiraz, Iran in accordance with the 1964 Declaration of Helsinki. All participants gave their informed consent before being enrolled in the study.

Blood samples from patients and age/sex matched healthy controls were drawn by venipuncture. Plasma samples were subjected to nucleic acid extraction with spin column; designed for viral nucleic acid extraction and done based on manufacturer's instruction (Invitek, Germany). The eluted viral nucleic acid samples were kept at -80°C until the next step of real time PCR. Aliquots of plasma samples were also kept frozen for the serological studies.

Serologic Study

Anti-parvovirus B19 IgG and IgM were measured in the plasma samples by a commercial enzyme linked immunosorbent assay (Euroimmun, Germany). The procedure and interpretation of the results were done according to the manufacturer's instruction.

Detection and Quantitation of Human Parvovirus B19 by Quantitative Real Time PCR

To detect traces of human parvovirus (B19) virus in the plasma samples, commercial virus specific real time PCR Kit (Shanghai ZJ Bio-Tech, China) was used. The primers in the kit allow specific amplification of a 76 bp region of the parvovirus B19 genome. An internal control was added in the amplification reactions to identify possible PCR inhibition by measuring the VIC/JOE fluorescence of the internal control amplification. Moreover, the presence of an external positive control with definite copy numbers of target sequence (1×10^7 copies/ml) was used for the quantitative measurement of the viral loads. Multiple serial dilutions (up to 10 copies/ml) of the positive control were prepared for each run and quantitative standard curve was plotted against the Ct values to extrapolate the actual viral loads in each sample. PCR reactions of 40 µl were set with the following protocol on ABI 7500 thermocycler device (ABI, USA) for amplification at 94°C for 2-5 min followed by 40 cycles of 93°C for 15 sec → 60°C for 60 sec.

The amplification of B19 specific primers was detected through FAM with the fluorescent quencher BHQ1 at the end of the extended time at 60°C. Melting curve was used to confirm fluorescent emission from the target sequence.

Statistical Analyses

To evaluate the normal distribution of the collected samples, one sample Kolmogorov-Smirnov test was used. Significance of differences of numeric variables was evaluated by the student t-test and the nonparametric Kruskal–Wallis test was used to evaluate non-numeric variables. Ordinal variables were compared by chi-square test (linear by linear association) and nominal variables were compared by chi-square or Fisher's exact test. Correlations were tested by Spearman rank test. Data were analyzed by SPSS (version 16.0) and P values less than 0.05 were considered as significant.

Results

Patients and Clinical Findings

Fifty-five patients (22 males and 33 females, M/F=0.66) with Behçet's disease, diagnosed according to the ISG criteria, and an additional 42 healthy controls (16 males and 26 females, M/F=0.61) were enrolled in this study. The male/female sex ratio in the studied groups were not significantly different (P>0.8). The mean age of the patients and the controls was 37.40 and 37.69 years, respectively. The mean age of the patients at the time of the disease onset was 28.92±7.15 years, with a mean duration of 7±5.906 years. The disease duration of more than 10 years was detected in 29.1% of the patients.

Detailed clinical and paraclinical workouts were performed for all patients. All patients had normal values of white blood cell (WBC) count, rheumatoid factor (RF), anti-nuclear antibody (ANA), anti-double-stranded DNA, and anti-neutrophil cytoplasmic antibody (ANCA); although the results of the erythrocyte sedimentation rate (ESR) test and C-reactive protein (CRP) test in some patients (12.7% and 11.8%, respectively) showed some abnormalities. At the time of the study, only 8 patients (14.5%) showed inactive disease. Only one male patient with inactive disease was among them while there was no significant difference between males and females in the active disease groups.

The most common clinical manifestations of the patients were mucocutaneous lesions, including oral and genital aphthous and skin rashes (89.1%). Intestinal and pulmonary vasculitis were the least common clinical manifestations observed in the patients (N=1, 1.8%). Table 1 summarizes the distribution of the clinical findings among patients with different disease severities.

The majority of the patients (N=50, 90.1%) were receiving systemic immunosuppressive drugs. Almost half of them (N=24) had cytotoxic regimen while the rest (N=26) had combinations of colchicine and/or steroid and DMARD. The rest of the patients (N=5) were treated with herbal medications.

Obviously, there were significant correlations between the disease severity score and ocular lesions (P<0.0001), CNS involvement (P<0.0003), and mucocutaneous manifestations (P<0.005). However, there was no statistically significant correlation between the disease severity score and other variables such as age, gender, age at the onset of the disease, duration of the disease, CRP, vascular and articular manifestations or pulmonary and intestinal vasculitis (P>0.05). Younger patients in our study showed more obvious mucocutaneous manifestations compared with elderly patients. In fact, the mean age of the patients with mucocutaneous involvement was 36.44±8.48 years and in patients without mucocutaneous involvement was 45.16±6.33 years (P=0.019). In the study of association between mucocutaneous presentation and other serious manifestations of BD including ocular, CNS, peripheral vascular thrombosis and arthritis, we did not find a significant statistical correlation (P>0.4) although all patients with CNS involvement and vascular thrombosis had apparent mucocutaneous lesions (P>0.048). Fatigue was also not in association with either of ocular, CNS, vascular and articular manifestations (P>0.2). However, there was no significant correlation between having ocular lesions, CNS, vascular and articular manifestations and age of patients (P>0.08). Similarly, there was no correlation of the clinical manifestations to either sex. Peripheral vascular thrombosis was reported in only three patients and all were male.

Table 1: Distribution of patients with different clinical manifestations among sub-groups of the disease activity

Clinical presentation	No %	Inactive	Mild	Moderate	Severe
Mucocutaneous	-	4	1	1	0
		7.28	1.82	1.82	0
	+	4	17	15	13
		7.28	30.9	27.3	23.6
Ocular	-	8	18	8	5
		14.6	32.5	14.6	9.1
	+	0	0	8	8
		0	0	14.6	14.6
Vascular	-	8	16	16	12
		14.6	29.1	29.1	21.85
	+	0	2	0	1
		0	3.65	0	1.82
Articular	-	8	17	13	11
		14.6	30.9	23.7	20
	+	0	1	3	2
		0	1.82	5.46	3.65
CNS	-	8	18	15	8
		14.6	32.76	27.3	14.6
	+	0	0	1	5
		0	0	1.82	9.1
Intestinal	-	8	18	16	12
		14.6	32.76	29.1	21.85
	+	0	0	0	1
		0	0	0	1.82
Pulmonary	-	8	18	16	12
		14.6	32.76	29.1	21.85
	+	0	0	0	1
		0	0	0	1.82
Fatigue	-	6	4	7	2
		10.92	7.28	12.74	3.64
	+	2	14	9	11
		3.64	25.5	16.4	20

Thirty-eight of the patients had known HLA type. Of those, 33 patients (86.8%) were HLA-B51 type. Data showed that, ocular lesions were detectable only in the HLA-B51 positive patients and neither of the patients with negative HLA-B51 showed ocular disease (P<0.0001).

Anti-B19 Seroprevalence

Anti-B19 IgG antibody was found in 72.7% (N=40, 16 male and 24 female) of the patients and 85.7% (N=36, 13 male and 23 female) of the healthy individuals. The rate of IgG seroprevalence was not significantly different between the patients and the healthy participants (P>0.1). Ten patients (18%) and five healthy persons (11.9%) had detectable B19-specific IgM antibody in their plasma. Comparison of B19 IgM prevalence did not show significant difference between the patients and the healthy group (P=0.3). Figure 1 demonstrates the distribution of IgG and IgM immunoglobulin concentrations of the patients and controls. Interestingly, in both studied groups, individuals with B19 IgM simultaneously had detectable amounts of IgG antibody in their plasma. Among the studied

patients, no statistically significant correlation was found between B19 seroconversion and disease severity, disease onset history or other studied clinical findings (P>0.1). Figure 2 shows the distribution of the seropositive and seronegative patients with different disease severity. There was no statistically significant difference in the frequency of patients with B19 antibodies among patients with different therapy regimens (P>0.3).

B19 Viral DNA Findings

Using virus specific primers and probes adapted in the commercial kit, we tested plasma samples of all participants for the presence of parvovirus B19 genome traces. Only one patient (1.8%) and one of the healthy individuals (2.4%) had detectable loads of the parvovirus B19 DNA. The viral loads, was not significantly different in both groups (54.34 p/ml vs. 57.21 p/ml, respectively). The patient with detectable B19 DNA had positive B19-IgM and IgG results, however the healthy person was found negative for both antibodies. No significant correlation was found between B19 DNA load and Behçet disease severity or other clinical findings (P>0.1).

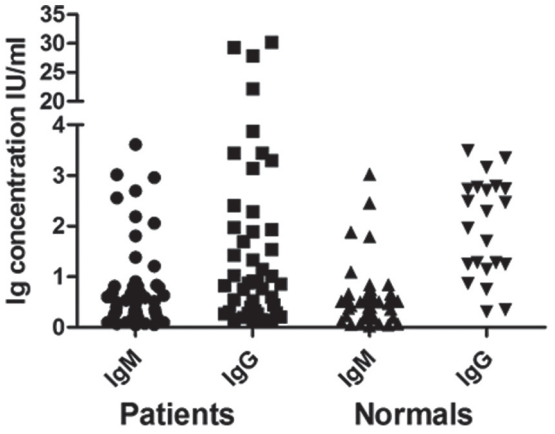

Figure 1: Shows the distribution of B19 specific IgG and IgM concentration in the patient and control groups.

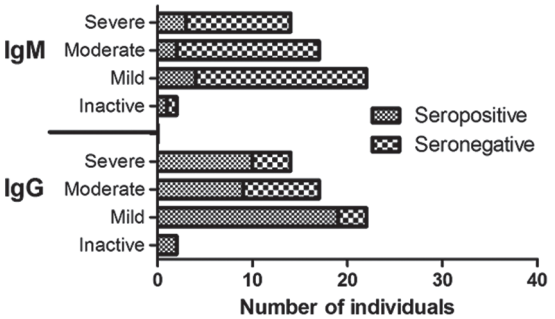

Figure 2: Shows the number of seropositive and seronegative patients in the disease severity sub-groups.

Discussion

Although strong association of B19 infection and rheumatoid arthritis (RA) has been shown by some evidence,[26-28] the relationship between this viral infection and Behçet disease remained obscure. BD is a complex and multi-organ inflammatory disease with severe systemic vasculitis. In fact, the complexity of the disease and variations in the diagnosis could be one of the major hindrances in this regard. Therefore, in this study, we selected a series of 55 patients with best matched criteria to the ISG guidelines and explicated their clinical manifestations as possible. Further to the clinical manifestations, we analyzed the plasma samples of the patients for the presence of B19 viral DNA plus IgG and IgM antibodies against the virus and compared the results to the findings from the healthy individuals of the same geographical region. Commercial high sensitivity quantitative real time PCR and ELISA kits were used for the analysis. With a similar prevalence of B19 specific IgG (72.7% and 85.4%, respectively) both groups of the patients and the controls showed comparable history of B19 infection. Results from previous studies in the same geographical region detected anti B19 IgG in 61% of tested women,[29] which is slightly lower than our findings. In our study, patients showed slightly higher rate B19 IgM than controls (18% vs. only 11.9%). All those individuals were positive for IgG too, which can show mounting a potent immune response against the virus in those people. Other researchers also have shown such observation in their study. Kozireva et al. showed that most tested patients (83.3%) with rheumatoid arthritis and healthy individuals had detectable levels of both IgG and IgM anti-B19 antibodies in their sera.[30]

When investigating the presence of viral DNA, only one person of each group of the patients or the controls (1.8% vs. 2.4%, respectively) was found to have detectable amounts of B19 viral DNA in his/her plasma. The patient with positive real time PCR result had high titers of both IgG and IgM; however, the person from the control group was negative for both antibody classes. Lack of antibody in this person could be explained by possible chronic viral infection at the time of sampling. Alternatively, he might have been in the pre-seroconversion period of the infection or may have failed to mount a strong detectable humoral response. The lack of anti-B19 antibody has also been reported in patients with systemic lupus erythematosis with confirmed B19 DNA in their sera[31] or in RA patients that had B19 DNA in their peripheral blood cells[30] or in thalassemic

patients that showed B19 virus in their blood.[32] Therefore, the parvovirus B19 seroprevalence patterns of our patients and controls were similar and followed the typical pattern of reaction.

It has been shown that B19 viremia occurs 1 week after exposure and lasts up to 5 days. Anti-B19 antibodies can be detected later about day 10 to day 12, which can persist for a long time.[33] Reports show that viral DNA can be detected in plasma after the infection even longer than it was previously thought. In fact, Lindblom et al. could detect B19 DNA 128 weeks after the development of anti-B19 IgG antibodies.[34] This is in spite that we failed to detect viral DNA in plasma samples of all individuals with IgM or IgG anti-B19 antibodies. We used a commercial probe based real time PCR system to detect B19 DNA, which showed reasonably low background and linear results in detection range in all experiments. However, still there might be the possibility of false negative results due to variation in sequences and different genotypes in different regions. Similar to our findings, some other investigators also were not able to detect B19 DNA from significant numbers of the patients they tested, although they were found seropositive for the virus. In a report by Irschick et al., B19 viral DNA was not found in BD patients.[35] All these data could be translated to a big controversy in findings about the direct role of parvovirus B19 infection in BD. Moreover, beside B19, other infectious particles, including HSV-1, HBV, Chlamydophila pneumoniae or hepatitis viruses have also been suspected by researchers, where others failed to prove their direct role as infectious etiologic agent of BD.[17,35-37] Here, in spite of earlier findings but similar to others, our findings could not support the assumption of parvovirus B19 as the etiologic agent for Behçet disease. Nevertheless, factors such as limited number of patients (due to stringent clinical criteria in the selection process), potential technical limitation for detection of the virus (due to different genotype) and possible overlapping diseases with Behçet might have affected the result of the study.

Conclusion

All controversies about the role of infections in BD plus a diverse spectrum of symptoms of the disease may demonstrate a more complex etiology of the disease instead of simple accusation of an infectious organism as etiologic agent. It is possible that in patients with genetic predispositions, like HLA-B51, infections with certain viruses or bacteria trigger some unwanted or pathologic cascade of immune responses. These reactions could eventually lead to symptoms of BD. More information is necessary to find the exact relation between infections and BD in such scenario. Animal models[36] should be exploited in experimental studies in conjunction with detailed evidence from patients to answer questions in this regards.

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Conflict of interest: None declared.

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