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Increased expression of Epstein-Barr virus in primary biliary cirrhosis patients

Syed Ahmed MORSHED[1], Mikio NISHIOKA[1], Ichiro SAITO[2], Kazuo KOMIYAMA[3], and Itaru MORO[3]
[1]Third Department of Internal Medicine, Kagawa Medical School, Kagawa, Japan; [2]Medical Research Institute, Department of Virology and Immunology, Tokyo Medical and Dental University, Tokyo, Japan; and [3]Department of Pathology, Nihon University School of Dentistry, Tokyo, Japan

Summary: Peripheral blood mononuclear cells (PBMC, n = 26), formalin-fixed paraffin-embedded liver tissues (n = 11) and saliva (n = 15) of primary biliary cirrhosis (PBC) patients were used for the detection of Epstein-Barr virus (EBV) sequences by polymerase chain reaction (PCR) assay. The semiquantitative analysis of EBV-DNA was also carried out in a reconstructive experiment using an EBV-infected cell line. The PBMCs of PBC patients showed increased levels of EBV-DNA (61%) in contrast to chronic active hepatitis patients (19%), liver cirrhosis patients (14%) and healthy individuals (11%). Furthermore, formalin-fixed paraffin-embedded liver tissues, as well as saliva from PBC patients, also demonstrated increased levels of EBV-DNA when compared to healthy individuals and those with other liver diseases. The increased levels of EBV-DNA in the PBMC, liver tissue and saliva of the PBC patients suggest that those patients may have a depressed immune function against EBV infection. *Gastroenterol Jpn 1992;27:751-758.*

Key words: *Epstein-Barr virus; polymerase chain reaction; primary biliary cirrhosis.*

Introduction

Epstein-Barr virus (EBV), a human lymphotropic virus, is a member of the herpes viridae family. It is a ubiquitous virus that infects the B cells and the epithelial cells of the oropharynx, the salivary gland duct and the cervix[1]. Infection has been related to benign diseases such as infectious mononucleosis and chronic EBV infection[2]. EBV has been associated with Burkitt's lymphoma, nasopharyngeal carcinoma, thymic carcinoma, Kawasaki-like disease and lymphomas in immunocompromized individuals[3-5]. EBV can exist, in some host cells, in a "latent" or "restringent" form. Therefore, intermittent viral reactivation can occur at a subclinical level. Reactivation has been observed most frequently in the immunocompromized host[6]. Furthermore, it is also related to various autoimmune diseases such as Sjögren's syndrome (SS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[7-9].

Primary biliary cirrhosis (PBC) is characterized by the destruction of intrahepatic bile ducts, the eventual development of cirrhosis and ultimately liver failure. The large number of immunologic abnormalities in patients with the disease suggests that it is caused by some abnormality of immune regulation. Thus, PBC is regarded as an autoimmune disorder[10]. PBC is usually associated with other autoimmune disorders, which include autoimmune thyroiditis, scleroderma, rheumatoid arthritis and SS[11]. The factors responsible for initiating and perpetuating PBC remain unknown. However, environmental agents are likely to play a role for an autoimmune response in PBC patients. Retrospective observations of increased levels of EBV-DNA in SS patients[7], along with a frequent association of SS patients with PBC[12], has prompted us to detect EBV sequences in PBC patients. We have, therefore, investigated DNA of

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Address for correspondence: Mikio Nishioka, M.D., Professor of Internal Medicine, The Third Department of Internal Medicine, Kagawa Medical School, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-07, Japan.

Table 1. Clinical characteristics of primary biliary cirrhosis patients

Patient*	Sex	Age	Clinical findings	Complications	RA factor[a]	ANA[b]	Clinical staging[c]
1.	F	47	AS[d]	Gallstone	+	20	II
2.	F	40	Itching Jaundice	–	+	–	I
3.	F	40	Itching Jaundice	–	–	–	II
4.	F	52	AS	Diabetes Mellitus	–	80	II
5.	F	60	AS	Gallstone	–	–	II
6.	F	48	Itching	Myoma uteri	+	20	I
7.	M	53	AS	Monoclonal gammopathy	–	1280	II
8.	M	62	AS	–	–	160	III
9.	F	67	AS	–	–	–	I
10.	F	41	Jaundice	–	+	20	IV
11.	F	57	AS	–	+	320	II
12.	F	53	Itching	–	–	1280	II
13.	M	46	Itching	–	–	–	II
14.	F	46	AS	Sjögren's syndrome	+	320	II
15.	F	63	AS	–	–	40	I
16.	F	65	AS	–	+	40	II
17.	F	42	Itching	–	+	640	II
18.	F	50	Itching	–	–	20	II
19.	F	51	AS	–	–	20	I
20.	F	49	AS	–	+	40	III
21.	F	41	AS	–	–	320	N.T.[e]
22.	F	61	AS	–	–	320	II
23.	F	81	AS	–	+	40	N.T.
24.	F	76	AS	–	–	80	N.T.
25.	F	32	Itching Jaundice	Sjögren's syndrome	–	160	III
26.	F	45	Itching	–	–	–	I

*All patients were positive for antimitochondrial antibody (AMA). a: Rheumatoid factor; b: Antinuclear antibody; c: Scheuer's classification; d: Asymptomatic; e: Not tested.

peripheral blood mononuclear cells (PBMCs), liver biopsy specimens (formalin-fixed paraffin-embedded tissues) and saliva from patients with PBC for evidence of EBV-DNA by using a semi-quantitative polymerase chain reaction (PCR) technique.

The increased levels of EBV-DNA in PBMCs, liver tissues and saliva from patients with PBC provide one of the first examples that a specific viral agent may reflect the depressed immune regulation against EBV infection. Whether this increased expression of EBV is due to a primary defect or a secondary defect of immune function still remains to be elucidated.

Materials and Methods

Patients

Twenty-six PBC patients (23 female and 3 male, ranging in age from 32 to 81 years, mean±SD: 52.61±11.66 years) and 35 healthy controls (25 female and 10 male, ranging in age from 24 to 79 years, mean±SD: 46.28±16.41 years) were examined for increased levels of EBV-DNA in their PBMCs. Paraffin-embedded liver tissues (9 female and 2 male, mean±SD: 53.18±9.21 years) and saliva (12 female and 3 male, mean±SD: 57.66±12.12 years) from those PBC patients were also used for the detection of EBV-DNA. The cases of PBC were diagnosed on the basis of clinical, biochemical, serological and histological criteria[13]. They were either asymptomatic or with symptoms such as itching and jaundice (**Table 1**). None of the patients received steroids or immunosuppressive medications for at least one year before the beginning of our study. Patients with chronic active hepatitis (CAH, n = 36) and liver cirrhosis (LC, n = 15) were also included as a comparative study. Liver tissues (n = 5) from autopsies with normal liver histology were consid-

Table 2. Sequence of primers and probes

Region	Sequence	Location in genome of EBV
I. Bam W region		
TC60 primer	CCAGAGGTAAGTGGACTT	1396-1416
TC61 primer	GACCGGTGCCTTCTTAGG	1520-1503
TC62 probe	TTCTGCTAAGCCCAAC	1424-1439
II. BMRF1		
TC67 primer	CAGGCTTCCCTGCAATTTTACAAGCGG	80220-80246
TC69 primer	CCCAGAAGTATACGTGGTGACGTAGA	80507-80482
TC68 probe	GATGATAAGGTGTCCAA	80295-80311
III. BMLF1		
TC70 primer	CTTGGAGACAGGCTTAACCAGACTCA	83520-83545
TC72 primer	CCATGGCTGCACCGATGAAAGTTAT	83874-83850
TC71 probe	TCTGGAGCCACGAGAT	83679-83694

ered as normal liver. Informed consent was obtained from all patients included in this study.

Preparation of DNA

Genomic DNA was purified from PBMC, formalin-fixed paraffin-embedded liver tissue and saliva of PBC patients. PBMC from 5 ml blood was separated using Leuco-PREP™ cell separation tubes (Becton Dickinson Labware, Becton Dickinson and Company, Lincoln Park, N. J.) with three washes in phosphate buffered saline (PBS, pH 7.2). DNA was extracted in a lysis buffer (100 mM NaCl, 20 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS). After RNase (10 mg/ml for 2 hours at room temperature) and proteinase K digestion (1 mg/ml for 16 hours at 37°C), DNA was extracted with phenol-chloroform and precipitated with ethanol. DNA from formalin-fixed paraffin-embedded liver tissue was purified by a method previously described[14]. In the saliva cases, each 100 μl sample was mixed with 900 μl of lysis buffer containing 1% SDS, then DNA was extracted as described above. Immediately before use, the samples were resuspended in 10 mM Tris and 1 mM EDTA. All necessary measures were taken in order to avoid cross contamination or "carry over" of trace amounts of DNA from prior samplings until Southern blot hybridization[7].

PCR assay

Based on the published sequences of Baer et al.[15], upstream primers, downstream primers and internal probes corresponding to the *Bam*H1-W and *Bam*-M regions of the EBV genome (**Table 2**), were synthesized using a DNA synthesizer (Model 380; Applied Biosystems, Inc., Foster City, CA). The PCR amplification was performed according to the method of Saito et al.[7] using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). Primers for HLA-DQ α (a single copy human gene) were also used as an internal control in each reaction mixture[16].

Southern Blot Hybridization

PCR products were electrophoresed in 1.7% agarose gel stained with ethidium bromide (PCR-EB). Then, Southern blot hybridization was carried out using the method previously described[7]. Autoradiography was taken at −80°C for 3 hours with a single intensifying screen. The ^{32}P-reactivity detected in the autoradiograph was further quantitated by densitometry tracing.

Reconstruction experiments to quantitate viral genomes

The sensitivity of PCR in detecting EBV-infected cells among uninfected cells, along with the quantitation of EBV genomes by PCR, were performed using a method developed by one of the authors[7,17,18]. Since the EBV and the HLA-DQ α signal comparison provides a better estimate of the viral DNA content per total number of cellular genome equivalents, HLA-DQ α was also used to quantitate the viral genomes (**Figure 1**). Varying

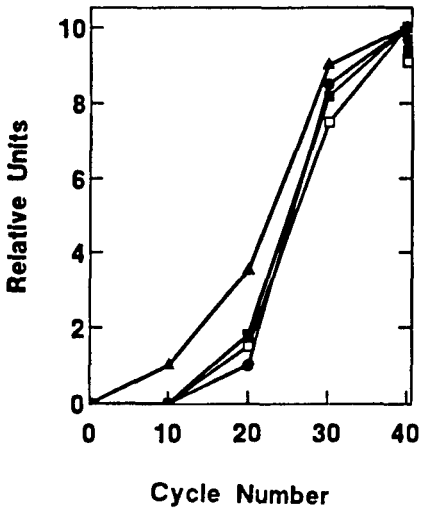

Figure 1. After a varying number of PCR amplification cycles using primers specific for the Epstein-Barr virus (EBV)-encoded genes, *Bam*-W (▲), BMRF-1 (■), or BMLF-1 (□) (**Table 2**) and primers specific for the HLA-DQ α (●) gene (internal control), Southern blot hybridization was performed with ^{32}P-labeled EBV or DQ α-specific oligonucleotides. The intensity of reaction was quantitated using a densitometer. Peak areas are given as relative units. 1 μg DNA from an EBV-transformed cell line (Novako/EBV 1) served as DNA template.

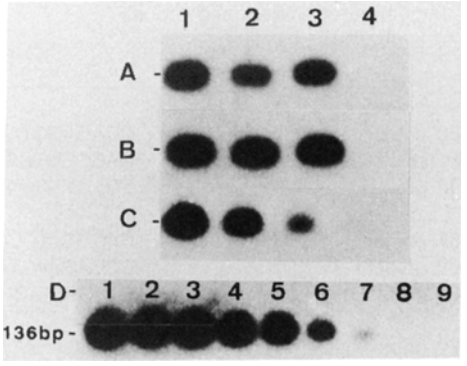

Figure 2. Autoradiograph of Southern blot using Epstein-Barr virus (EBV)-specific *Bam*-W-encoded primers and probe of amplified DNA. EBV-DNA positive samples (lane 1-3) from patients with primary biliary cirrhosis and healthy individuals (lane 4) are shown in **Figure A** (Peripheral blood mononuclear cells), **B** (Saliva), and **C** (Liver). **Figure D**: A mixture of EBV-uninfected cell DNA (RPMI-8402) and EBV-infected cell DNA (Novako/EBV1) was made in a manner that kept the total amount of DNA in each tube at a constant (1 μg). The amount of EBV-infected cell DNA was adjusted, beginning with 10 EBV copies/cell (lane 1) and decreased through 10-fold serial dilutions until 0.000001 EBV copy/cell (lane 8). The last lane (lane 9) shows EBV-uninfected cell DNA as a negative control. After 35 cycles of PCR amplification using *Bam*H1-W primers, an aliquot was removed and subjected to Southern blot analysis using a ^{32}P-labeled oligonucleotide probe.

concentrations of EBV-DNA from an EBV-transformed cell line (Novako/EBV1, a diploid cell line that containing 2-3 copies of EBV-DNA per cell[19]), were mixed with DNA from an EBV-uninfected cell (RPMI 8402, a T cell line) under conditions that kept the total amount of DNA (1 μg) at a constant. After a varying number of PCR cycles using EBV-specific primers (**Table 2**), an aliquot was removed and subjected to Southern blot analysis using a ^{32}P-labeled oligonucleotide probe. Primers specific for HLA-DQ α gene[16] were also included as an internal control in each reaction mixture. Finally, the intensity of the reaction was quantitated using a densitometry tracing of the autoradiograph (data not shown).

Statistical Analysis

Results were expressed as means±SD. Statistical analysis was performed on Macintosh Classic Stat-Work software. The Wilcoxon signed rank test for paired data between various groups was used to examine the significance of differences, with $P = 0.05$ as the minimum level of significance.

Results

Increased level of EBV-DNA in patient samples

After 35 cycles of PCR amplification using *Bam*H1-W-encoded primers and a ^{32}P-end-labeled *Bam*H1-W oligonucleotide internal probe, most of the PBC patients demonstrated increased levels of EBV-DNA when compared to other liver diseases and healthy individuals (examples shown in **Figure 2**). Compared to 4 of 35 (11%) normal PBMC, 7 of 36 (19%) CAH-PBMC, and 2 of 14 (14%) LC-PBMC, 16 out of 26 (61%) patients demonstrated significant reactivity in PBMC-DNA from PBC patients. A significant difference was found on EBV copies between PBC-PBMC and CAH-PBMC ($P<0.009$, **Table 3**). Formalin-fixed paraffin-embedded liver tissues from PBC patients showed increased levels of EBV-DNA in 8 out of 11 (72%) cases. In contrast, no positive

Table 3. Summary of polymerase chain reaction (PCR) detection of Epstein-Barr virus (EBV) DNA in clinical samples

Samples¶		Bam-W primers					BMRF-1 primers				
	n	+++	++	+	−	% positive	+++	++	+	−	% positive
PBC-PBMC	26	9	3	4	10	61	9	2	5	10	61
CAH-PBMC	36	1	2	4	29	19	1	3	3	29	19
LC-PBMC	14	0	0	2	12	14	0	0	2	12	14
Normal-PBMC	35	0	2	2	31	11	0	1	3	31	11
PBC-Liver	11	4	2	2	3	72	3	3	1	4	63
CAH-Liver	12	0	0	1	12	8	0	0	1	12	8
LC-Liver	10	0	0	0	10	0	0	0	0	10	0
Normal-Liver	5	0	0	0	5	0	0	0	0	5	0
PBC-Saliva	15	7	3	2	3	80	7	2	3	4	80
CAH-Saliva	21	0	1	2	18	14	0	1	3	17	19
LC-Saliva	15	0	0	2	13	13	0	0	2	13	13
Normal-Saliva	25	1	0	2	22	12	0	1	2	22	12

The density of autoradiographic band after a limited number of PCR cycles corresponded to 10-20 copies of DNA per 10^5 cells (+++); 5-10 copies EBV genomes per 10^5 cells (++); 1-5 copies EBV per 10^5 cells (+); or less than 1 copy EBV per 10^5 cells (−). ¶PBC: Primary biliary cirrhosis; CAH: Chronic active hepatitis; LC: Liver cirrhosis; PBMC: Peripheral blood mononuclear cells; *$P<0.009$; **$P<0.014$; ***$P<0.022$. (All P values were determined on the relative number of viral copies among groups and were considered significant when $P<0.05$, based on the Wilcoxon signed rank test).

signals were detected in either the 5 cases of normal liver tissues (autopsy) or the 10 cases of LC liver tissues. However, one case among 12 cases (8%) from CAH demonstrated a weak but positive signal for EBV-DNA in its liver tissues.

We have estimated that saliva from normal healthy individuals contained less than one copy of EBV-DNA per 100 μl equivalents. In contrast, saliva from 12 out of the 15 (80%) PBC patients contained 1-10 or more copy equivalents. Saliva from CAH and LC patients showed similar results to that of the normal healthy individuals. PBMC, saliva and liver tissues from the patients with CAH demonstrated a slight increase in EBV-DNA, but no significant differences when compared to the LC cases and the normal healthy individuals. EBV-DNA in PBC patients also showed increased levels compared to EBV-DNA in CAH patients. Again, a significant increase of EBV-DNA was also seen in the saliva of PBC patients.

The number of copies of EBV-DNA in PBC patients was increased in all samples, even in DNA purified from the liver tissues. The results are summarized in **Table 3**. The frequency of EBV-DNA in liver tissues (72%) and saliva (80%) was higher than the frequency of PBMC (61%) in the PBC patients ($P<0.014$). In these DNA samples, specific amplifications using both BMRF-1 and BMLF-1 regions of EBV primers (**Table 2**) were also done (**Figure 1**).

Detection of EBV-DNA by PCR

In order to confirm that the EBV *Bam*-W (reiterated gene) and *Bam*-M (BMRF1 and BMLF1, a single copy gene) were specific for the EBV-DNA, PCR amplifications of those regions were performed with DNA extracted from the EBV-infected cell line (i.e., Novako/EBV1). Positive

signals were detected using both primers. In contrast, the DNA from EBV-uninfected cells showed no density by Southern hybridization. The reiterated viral DNA sequence (*Bam*-W) was detected at a lower number of cycles than the single copy viral genes or the single copy HLA-DQ α gene. Based on our reconstruction experiments, it was possible to detect approximately one EBV transformed cell per 100,000 uninfected cell DNA equivalents (**Figure 2D**). As an internal control, the content of a single copy human gene (DQα) in each sample was also measured. Since the total amount of human genomic DNA remained constant in each sample, an identical pattern of DQα amplification was noted for each dilution of viral DNA. This pattern of DQα amplification was identical to that described in **Figure 1**. Thus, comparison of reactivity with viral-specific probes and single copy human gene probes makes it possible to approximate the content of viral DNA in an unknown sample.

Discussion

In this study, we have extended and analyzed our preliminary data regarding the detection of EBV-DNA in PBC patients[20]. Our findings have clearly shown the presence of increased levels of EBV-DNA in PBC-PBMCs and PBC saliva, as well as in liver tissues from patients with PBC (**Table 3**). The presence of EBV-DNA with increased levels (61%) in PBMC of PBC patients when compared with healthy individuals (11%), suggests an elevated number of circulating EBV-infected B cells in PBC patients. Increased levels of EBV-infected lymphocytes, as well as EBV-DNA, have been observed in patients with RA, SLE, and SS[7,8,21]. Most of their results on EBV in PBMC correspond to our findings. We have detected EBV sequences in the saliva from the patients with PBC with increased levels when compared to the normal individuals (80% vs. 12%). EBV has been demonstrated in the saliva and oropharyngeal epithelial cells of infectious mononucleosis patients. Furthermore, EBV could be cultured from the saliva of up to 20% of EBV-seropositive individuals[1]. Increased levels of EBV-DNA in saliva have also been demonstrated in SS, AIDS, AIDS-related complex, acute infectious mononucleosis patients and renal transplant recipients[22-24]. Our findings in the saliva are concurrent to those described previously[22-24]. These findings suggest that these patients have a depressed immune function against EBV.

We have also detected EBV-DNA in liver tissues of PBC patients. The source of this virus in these tissues is still unresolved. Varying degrees of B cell infiltrate have been noted to surround the bile ducts of PBC livers[25]. Thus, the EBV source may be that of the B cells observable in the portal area in the liver tissue. Blood contamination during the biopsy may be a second possible source of the EBV that was found in the EBV-positive samples. The presence of EBV-DNA in various autoimmune patients is also very intriguing. Study of this virus in chronic autoimmune diseases may be important in understanding the interaction of genetic and environmental factors in those diseases. Furthermore, PBC may serve as a prototype human disease concerning genetic and environmental interaction.

The presence of EBV-DNA in liver tissues of PBC patients by PCR is indeed interesting and exciting. However, we were unable to demonstrate the exact locations of the virus in those tissues. Further studies, with the help of in situ hybridization techniques and immunohistochemical analysis, are essential to elucidate viral locations in liver tissues of PBC patients. The prospective study of EBV serologic responses and EBV-DNA levels in PBC patients may help to determine the temporal association of EBV reactivation with the development and subsequent course of PBC. Furthermore, the clinical characteristics of the PBC patients studied did not correlate with the EBV copies present in the samples. Due to the lack of appropriate samples from the same patients, it is difficult to predict the clinical significance of increased expression of EBV found in these PBC patients. However, a strong tendency of increased copies of EBV genome had been seen in saliva and liver tissues when EBV genome with an increased copies was present in PBMCs of PBC patients. Increased numbers of viral copies were more

frequent in stages III and IV but not in all cases. In the case of EBV, virtually all adults harbor the virus at low levels and only increased levels are likely to be associated with disease[26,27]. Our finding of increased levels of EBV-DNA in saliva, as well as PBMC, could reflect reactivation of the virus.

The abnormality of lymphocyte functions, as well as circulating activated B cells, has previously been identified in patients with PBC[28]. T cells from these patients have a diminished capacity to suppress immunoglobulin synthesis in vitro, a diminished T cell proliferative response in the autologous MLR and diminished suppressor function when activated with concanavalin A. Thus, the existence of elevated numbers of EBV-infected B cells in patients with PBC may contribute to the persistent state of enhanced B cell activation observed in this disease. Although the mechanisms of increased levels of EBV genomes in PBC patients are not clearly understood, T cell abnormality[28] may be a key to understanding the increased level of EBV through B cell infiltration.

In our study, the detection of EBV-DNA, with increased levels in blood, liver tissues and saliva of PBC patients, suggests that these patients may have a depressed immune suppression of EBV infection. While the mechanism of immune suppression of EBV infection has not been completely elucidated, laboratory and clinical studies have implicated T-lymphocytes and natural killer cells as contributing to this process[1]. By either autostimulatory growth factor induction, or other mechanisms, EBV could promote lymphocyte proliferation and thereby contribute to the maintenance and progression of PBC. However, our data with increased levels of EBV-DNA in PBC patient samples does not suggest this specific viral agent as the etiologic agent for PBC. Whether EBV has any role in the pathogenesis of PBC is unproven at this point and certainly requires further investigation. There is a constant debate about the relevance of EBV to various diseases since not all individuals infected with EBV develop the disease. The triggering factor(s) behind the mechanism of "autoimmune" attack on bile duct seems obscure, perhaps owing to its inappropriate isolation technique or because its presence is at a level too low for our detection systems.

From our results, it is evident that formalin-fixed paraffin-embedded tissue allows sensitive and specific detection of viral DNA by PCR, which is in accordance with the observations of others[14,29]. The extraction of nucleic acids from routinely fixed and processed tissue is particularly important, as it permits the use of archival material for the retrospective investigation of the disease[30]. Since only a small amount of tissue is required for the PCR, this technique can be used for the detection of other compounds, such as the expression of cytokines.

In conclusion, we have demonstrated an increased expression of EBV genomes in PBMCs, saliva and liver tissues from patients with PBC when compared to other liver diseases and normal healthy individuals. The increased expression of EBV genomes in PBC patients may represent a primary defect (i.e., an inherited inability to eliminate EBV-infected cells, as occurs in some immunodeficiency diseases)[26] or a secondary defect (i.e., the decreased ability to respond effectively to EBV as an acquired consequence of immune dysfunction)[27]. Further characterization of the tissue involvement of EBV and the immune response to EBV in PBC patients is required to evaluate these possibilities.

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