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Bacterial DNA Induces the *Complement* System Activation in Serum and Ascitic Fluid from Patients with Advanced Cirrhosis

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Translocation of intestinal bacteria to ascitic fluid is, probably, the first step in the development of spontaneous bacterial peritonitis in patients with cirrhosis. Proteins of the complement system are soluble mediators implicated in the host immune response to bacterial infections and its activation has been traditionally considered to be an endotoxin-induced phenomenon. The aim of this study was to compare the modulation of these proteins in response to the presence of bacterial DNA and/or endotoxin in patients with advanced cirrhosis and ascites in different clinical conditions. Groups I and II consisted of patients without/with bacterial DNA. Group III included patients with spontaneous bacterial peritonitis and Group IV with patients receiving norfloxacin as secondary long-term prophylaxis of spontaneous bacterial peritonitis. Serum and ascitic fluid levels of endotoxin and truncated residues of the complement system were measured by ELISA. The complement system is triggered in response to bacterial DNA, as evidenced by significantly increased levels of C3b, membrane attack complex, and C5a in patients from Groups II and III compared with patients without bacterial DNA (Group I) and those receiving norfloxacin (Group IV). Gram classification did not further differentiate the immune response between patients within groups II and III, even though endotoxin levels were, as expected, significantly higher in patients with bacterial DNA from gram-negative microorganisms. The complement protein activation observed in patients with bacterial DNA in blood and ascitic fluid is indistinguishable from that observed in patients with spontaneous bacterial peritonitis and may occur in an endotoxin-independent manner.

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is probably the most relevant infectious complication developing in patients with advanced cirrhosis and ascites (1, 2). Bacterial translocation (BT) is considered to be the main pathogenic mechanism through which bacteria may cross the intestinal wall from the intestinal lumen reaching mesenteric lymph nodes (MLNs), and then reaching blood and ascitic fluid (AF) (3, 4). The classical diagnostic criteria of BT requires the growth of a certain bacteria *spp* in mesenteric lymph nodes (5), which is obviously difficult to assess in patients except in infrequent circumstances (6). This fact has traditionally circumscribed the investigation of BT to animal models of cirrhosis (7) and has allowed the correlation of BT with the development of ascitic fluid (AF) infection only in an experimental setting (8).

The arrival of bacteria or endotoxin to AF is followed by the activation of an alternative pathway of the complement system (9), which constitutes a humoral, unspecific bactericidal mechanism aimed at opsonizing bacteria and allowing ulterior lysis by macrophages. The bactericidal activity of human peritoneal fluid is similar to ascitic fluid of tumoral origin (9, 10), but significantly higher than that observed in AF of cirrhotic origin (11). This is relevant, since a poor bactericidal activity is associated with an increased risk of developing SBP in patients with advanced cirrhosis (12). From a different standpoint, patients with lower concentration of total protein or C3 in AF, who

ABBREVIATIONS. BT: bacterial translocation; AF: ascitic fluid; SBP: spontaneous bacterial peritonitis; SID: selective intestinal decontamination; bactDNA: bacterial DNA

maintain a close relationship with bactericidal activity, are indeed predisposed to develop episodes of SBP (13, 14).

A recent study in an experimental model of CCl_4-induced cirrhosis with ascites has positively correlated the simultaneous presence of bacterial DNA (bactDNA) in blood and AF with the identification of bactDNA in MLNs (15). These results strongly suggest that bactDNA identification in serum and AF may be considered as a surrogate marker of BT in patients with advanced cirrhosis (15). The availability of this new diagnostic tool allows the investigation of the real incidence of BT in patients, the type of translocating bacteria, and the immune consequences induced by this fact.

The host innate immune response to bactDNA initiates a broad range of activities, including the secretion of soluble mediators implicated in the regulation of the inflammatory process (16–18). Immunoregulatory proteins such as proteins of the complement system give rise to an unspecific response starting with bacterial opsonization, followed by phagocytic recruitment by chemotaxis. One of the major components of the complement system is C3, a factor that is cleaved into C3b, leading to the formation of the membrane attack complex (MAC) and the excision of the small C5a peptidic fragment. However, no information is available regarding the complement behavior induced by the presence of bactDNA in patients with advanced cirrhosis.

The aim of this study was to evaluate the consequences of BT, as demonstrated by the presence of bactDNA, on serum and AF levels of proteins of the complement system in patients with advanced cirrhosis and AF, and its comparison with patients without BT, those with an ongoing bacterial infection and those receiving norfloxacin as secondary prophylaxis of SBP.

PATIENTS AND METHODS

Patients and Study Design

We conducted a prospective study in serum and AF of patients with cirrhosis in different clinical conditions as described next. Inclusion criteria for patients were the presence of cirrhosis and AF requiring a large volume paracentesis. Cirrhosis was diagnosed by histology or by clinical, laboratory, and/or ultrasonographic findings. Exclusion criteria for patients were the upper gastrointestinal bleeding or intake of antibiotics in the preceding 2 weeks with the exception of norfloxacin for secondary prophylaxis of SBP, hepatocellular carcinoma (one nodule bigger than 5 cm in diameter, or three or more nodules 3 cm in diameter each), and/or portal thrombosis, previous liver transplantation, transjugular intrahepatic portosystemic shunt (TIPS), alcoholic hepatitis, age more than 80 or less than 18 years, and the refusal to participate in the study. SBP was defined as the presence of more than 250 PMN/μL in AF and signs and symptoms compatible with this complication. Since it has been shown that the inflammatory reaction observed in patients with SBP is different according to the result of the AF microbiological culture (19), in order to reduce the variability among this group, only patients with positive AF culture were considered. The Ethics committee of the hospital approved the study protocol and all patients gave informed consent for inclusion in the study.

Blood was obtained for routine hematological, biochemical, and coagulation studies. Simultaneously, a paracentesis was performed on all patients during admission in aseptic conditions following the usual procedures (20), and samples for routine biochemical study and PMN count were obtained. Total protein, albumin, leukocyte count, and polymorphonuclear count were performed on all AF specimens. Both blood and AF were inoculated at bedside in aerobic and anaerobic blood culture bottles, 10 mL each (21). Finally, separate blood and AF samples were inoculated under aseptic conditions in rubber-sealed sterile Vacutainer SST II tubes (BD Diagnostics, Belgium) that were never exposed to free air.

Identification of Bacterial DNA Fragments

To detect and identify the presence of bactDNA fragments in both blood and AF, a broad-range polymerase chain reaction (PCR) and a nucleotide sequencing analysis were performed according to the methodology previously described (22). Briefly, DNA was isolated with QIAmp DNA Blood Mini kit (QIAgen, Hilden, Germany) and a broad-range PCR amplification of the bacterial 16SrRNA-gene-conserved region was performed using the following primers: 5′-TTCCGGTTGATCCTGCCGGA-3′ as forward and 5′-GGTTACCTTGTTACGACTT-3′ as reverse. BactDNA fragments were purified with QIAquick Purification kit (QIAgen) and the purified amplicons were used for the sequencing reactions with Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, US). The same reverse oligonucleotide used for PCR amplification was used as the sequencing primer. The final product was purified by precipitation with ethanol acetate and analyzed in the ABI PRISM 310 automated sequencer (Applied Biosystems). Sequences obtained were compared with the database from the National Center for Biotechnology Information (NCBI, www.ncbi.nih.gov) using the advanced BLAST search tool.

Quantification of Proteins of the Complement

During the activation of the complement system, iC3b is generated. The C3b molecule has a very short half-life

and is rapidly cleaved into iC3b, which can serve as a marker for complement activation. The membrane attack complex (MAC) is a stable complex generated by the assembly of C5–C9, which mediates the irreversible cell-membrane damage associated with complement activation, giving an indication of the status of the terminal complement pathway. Serum and AF levels of iC3b and sC5b-C9 were measured by Quidel EIA kits (Quidel Corporation, San Diego, CA). C5a is a multifunctional proinflammatory mediator, which is rapidly cleaved to the C5a-desArg form by the endogenous serum carboxypeptidase N enzyme. C5a-desArg was evaluated by handling BD OptEIA Human C5a (BD Biosciences, Belgium). Lower limits of detection for all the complement protein assays were between 5 and 8 pg/mL and for the endotoxin test was 0.05 UE/mL. The quantitative chromogenic limulus amebocyte lisate test (BioWhittaker, Nottingham, UK) was performed, according to the manufacturer's instructions, to evaluate the LPS levels in serum and ascitic fluid samples from all patients included in the study. All samples were tested in triplicate and read at 405 nm (iC3b, SC5b-C9, and endotoxin) and 450 nm (C5a-desArg) in a Thermomax microplate reader (Molecular Devices). Standard curves were generated for each plate needed and the average zero standard optical densities were subtracted from the rest of the standards, controls, and samples to obtain a correct concentration of each protein.

Statistical Analysis

Continuous variables are reported as mean ± standard deviation and categorical variables as frequencies or percentages. Statistical differences of basal characteristics between groups were analyzed using the Fisher test for categorical data and the Mann–Whitney *U* test for quantitative data. Bivariate correlations between continuous variables were calculated using the Spearman test. Statistical differences between cytokines were analyzed using the ANOVA test with Bonferroni correction for multiple comparisons. All reported *P* values are two-sided, and *P* values lower than 0.05 were considered to indicate significance. All calculations were performed using the SPSS 12.0 software.

RESULTS

Characteristics of Patients

A consecutive series of 141 patients fulfilling the aforementioned characteristics were initially considered for inclusion in the study. The first 21 patients showing absence or presence of bactDNA comprised Groups I and II, respectively. All patients in this case series showing a culture-positive SBP at admission and those patients receiving norfloxacin as secondary prophylaxis of SBP were included in Groups III ($n = 9$) and IV ($n = 9$), respectively. The inclusion of patients in this study was consecutive and no process of selection was performed, with the exemptions of diagnosis for distribution into groups as described earlier.

The clinical and analytical characteristics of this series of 60 patients with cirrhosis and ascites, fulfilling the inclusion and exclusion criteria as defined earlier are detailed in Table I. Among baseline clinical, basic haemodynamic, serum, and AF analytical characteristics, WBC, PMNs, and percentPMNs in AF were statistically increased, as expected, in patients from Group III (SBP) when compared with the rest of the groups. The mean age of patients included in Group III (SBP) was lower than the rest of the groups. Meld and Child–Pugh scores were not statistically different between groups.

Bacteria identifications in serum and AF from patients in Groups II (bactDNA+) and III (SBP), and the percentage of sequence alignment with the NCBI database are resumed in Table II. AF positive cultures for patients in Group III (SBP) included: *Escherichia coli* (7), *Staphylococcus aureus* (1) and *Enterococcus faecalis* (1).

BactDNA Triggers the Complement System in Decompensated Patients with Cirrhosis and AF

Several cleaved proteins throughout the complement-system-activation pathway provide information about the engagement of this nonspecific immune response. Representative activation products of this process are clearly increased in patients from Group II (bactDNA+) in comparison with the values present in patients from Group I (bactDNA−). All differences between these two groups regarding this set of molecules are statistically significant, except for C5a, which did not reach significance despite the evident increment in its value (111.28 ± 25.35 versus 88.31 ± 35.25 ng/mL). When analyzing the rest of the patients, parallel Group I (bactDNA−)/Group IV (SID) and Group II (bactDNA+)/Group III (SBP) behaviors were observed. As previously seen for the other sets of mediators (23), AF levels were generally higher than the values observed in serum. Detailed information to this extent is provided in Table III. Complement-system-activation proteins in bactDNA + patients (Group II) were also analyzed according to gram classification but no significant differences were observed, either in serum or in AF (Table IV). No correlations between any of the experimental variables included, either in serum or AF, were found for any of the study groups.

Table I. Basic Clinical and Analytical Characteristics of Patients Included in the Study

Variable	Group I (DNAbact−) ($n = 21$)	Group II (DNAbact+) ($n = 21$)	Group III (SBP) ($n = 9$)	Group IV (SID) ($n = 9$)
Age mean ± SD (range)	60.05 ± 11.60 (35–78)	64.37 ± 11.28 (40–80)$^{\$}$	52.77 ± 7.17 (41–62)	64.62 ± 4.47 (56–68)
Male sex *n* (%)	14 (66.6)	11 (52.4)	7 (77.8)	6 (66.6)
Etiology *n* (%)				
Alcohol	14 (66.6)	12 (57.1)	6 (66.6)	6 (66.6)
HCV	4 (19.0)	5 (23.8)	2 (22.2)	3 (33.3)
Alcohol + HCV	2 (9.5)	2 (9.5)	1 (11.1)	0 (0)
Other	1 (4.7)	2 (9.5)	0 (0)	0 (0)
Previous episodes of ascites *n* (%)	5 (24.0)	4 (19.0)	2 (22.2)	2 (22.2)
Child–Pugh mean score	9.6 ± 1.5	9.1 ± 1.5	10.2 ± 2.4	8.2 ± 1.8
Child–Pugh (A/B/C) %	0.0/63.1/36.8	0.0/61.9/38.	0.0/55.5/44.4	22.2/55.5/22.2
Meld mean score	12.87 ± 4.5	11.5 ± 6.2	19.8 ± 12.0	12.7 ± 5.1
Mean arterial pressure (mmHg)	86.5 ± 15.9	83.0 ± 11.0	85.9 ± 13.6	77.5 ± 14.8
Bilirubin (mg/dL)	3.3 ± 2.1	3.4 ± 3.1	5.5 ± 6.4	2.5 ± 1.3
Albumin (g/dL)	2.8 ± 0.6	2.6 ± 0.4	2.9 ± 0.3	3.2 ± 0.4
Quick (%)	61.2 ± 14.6	57.8 ± 14.0	42.5 ± 20.8	60.0 ± 10.9
Serum creatinine (mg/dL)	0.8 ± 0.4	0.8 ± 0.3	1.5 ± 1.4	0.8 ± 0.2
Serum sodium (mEq/L)	135.0 ± 5.1	134.1 ± 5.0	130.8 ± 9.8	133.2 ± 4.1
Blood WBC/mm^3	5576.4 ± 2389.3	8079.3 ± 4770.5	6115.5 ± 3560.6	4883.7 ± 2210.6
AF WBC/mm^3	285.0 ± 255.9	123.6 ± 70.5	4662.7 ± 5027.1*	135.0 ± 123.7
Percent AF PMNs	24.20 ± 17.73	30.26 ± 31.81	72.87 ± 18.05*	15.0 ± 17.60
AF Total protein (g/dL)	1.7 ± 0.8	1.4 ± 0.5	1.3 ± 0.8	1.1 ± 0.4
AF albumin (g/dL)	0.7 ± 0.5	0.6 ± 0.3	1.2 ± 1.2	0.5 ± 0.3

Note. SD: standard deviation; WBC: white blood cells; AF: ascitic fluid.
*$p < 0.05$ compared with all groups. $^{\$}p < 0.05$ compared with Group III.

Table II. Bacteria Identifications by Broad-Range PCR and Partial Sequencing Analysis of 16SrRNA Gene in Serum and AF of Patients with Cirrhosis and Ascites Compared with Patients with Spontaneous Bacterial Peritonitis (SBP)

	Group II (DNAbact+)		Group III (SBP)		
	Nucleotide sequence identification	Percent sequence alignment[a]	Nucleotide sequence identification	Percent sequence alignment[a]	AF culture
Gram −	*E. coli* (14)	99.4–99.8	*E. coli* (7)	99.5–99.8	*E. coli* (7)
	Klebsiella (1)	99.8			
Gram +	*Staphylococcus spp* (2)	99.6–99.7	*Staphylococcus spp* (1)	99.7	*S. aureus* (1)
	S aureus (4)	99.6–99.8	*Enterococcus spp* (1)	99.4	*E. faecalis* (1)

[a]BLAST nucleotide sequence alignment at www.ncbi.org.

Table III. Complement-System-Cleaved Protein Levels Shown in Serum and Ascitic Fluid Distributed by Groups of Patients

	Group I (DNAbact−) ($n = 21$)	Group II (DNAbact +) ($n = 21$)	Group III (SBP) ($n = 9$)	Group IV (SID) ($n = 9$)
Serum				
C3b (μg/mL)	81.61 ± 24.11	115.64 ± 21.06*	132.28 ± 41.24*	83.18 ± 15.39
MAC (ng/mL)	99.57 ± 25.83	152.42 ± 37.76*	150.70 ± 51.56*	96.88 ± 13.55
C5a (ng/mL)	80.83 ± 24.34	99.81 ± 24.02	118.15 ± 28.77	78.67 ± 21.58
AF				
C3b (μg/mL)	89.47 ± 5.19	127.98 ± 22.81*	143.55 ± 37.73*	94.97 ± 17.78
MAC (ng/mL)	98.71 ± 15.44	141.82 ± 34.54*	141.70 ± 53.34*	93.18 ± 20.32
C5a (ng/mL)	87.97 ± 36.15	110.18 ± 25.76	118.58 ± 31.25	85.54 ± 22.11

Note. Values expressed as mean ± SD. MAC: membrane attack complex; AF: ascitic fluid; SBP: spontaneous bacterial peritonitis; SID: selective intestinal decontamination.
*$p < 0.05$ compared with Group I.

Table IV. Complement System Cleaved Protein Levels in Serum and Ascitic Fluid of Patients from Group II (bactDNA+) and III (SBP), According to Gram Classification

	Group II (DNAbact+)		Group III (SBP)	
	Gram − (15)	*Gram* + (6)	*Gram* − (7)	*Gram* + (2)
Serum C3b (μg/mL)	115.26 ± 22.98	116.68 ± 17.53	123.57 ± 37.50	162.80 ± 52.75
Serum MAC (ng/mL)	148.51 ± 36.26	164.18 ± 45.42	144.98 ± 53.20	170.75 ± 57.06
Serum C5a (ng/mL)	96.43 ± 23.87	109.98 ± 24.74	114.93 ± 29.30	129.45 ± 33.87
AF C3b (μg/mL)	126.91 ± 22.82	130.95 ± 26.04	143.41 ± 36.27	142.30 ± 59.11
AF MAC (ng/mL)	147.32 ± 34.03	128.35 ± 37.41	156.35 ± 51.43	90.44 ± 11.79
AF C5a (ng/mL)	106.15 ± 19.12	122.28 ± 41.46	126.09 ± 31.18	92.32 ± 14.45

Note. Values expressed as mean ± SD; MAC: membrane attack complex; AF: ascitic fluid; SBP: spontaneous bacterial peritonitis.

Endotoxin and Bacterial DNA Do Not Synergize in the Complement System Activation in Patients with Cirrhosis and Ascites

Endotoxin levels in study groups were analyzed and patients from groups II and III showed higher values both in serum and AF than the patients included in the rest of the groups, although differences did not reach statistical significance (Fig. 1A). Further distribution of patients from groups II and III according to the gram classification shows, as expected, significant differences in serum and AF endotoxin levels between patients with bacterial DNA from gram-negative and gram-positive microorganisms (Fig. 1B). In the first group, significant correlations were found between endotoxin and the different complement residues studied, both in serum and AF (data not shown), whereas these correlations were lost when endotoxin was compared with complement residues in patients from Groups I and IV and in patients from Groups II and III with bacterial DNA from gram-positive cocci.

As shown in Table IV, the presence of endotoxin in serum and AF of patients with bacterial DNA from gram-negative microorganisms did not increase the complement activation products further, suggesting a nonsynergistic effect of both the immune stimulators in this setting.

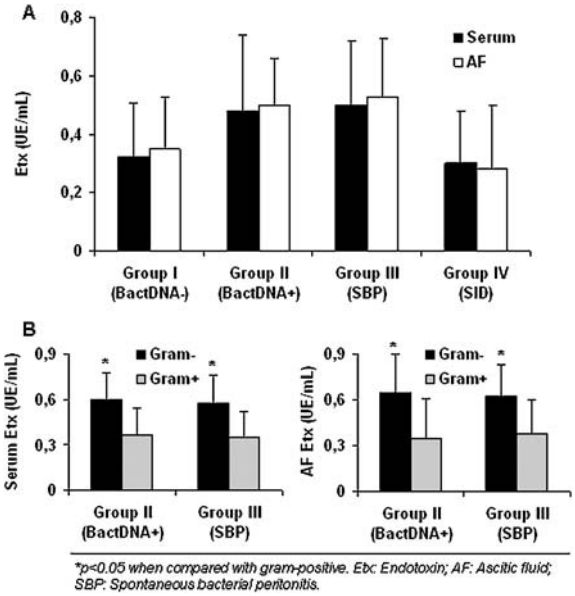

Fig. 1. (A) Endotoxin levels shown as mean ± SD in serum and ascitic fluid of patients, according to group distribution. (B) Endotoxin levels shown as mean ± SD in serum and ascitic fluid of patients from groups II and III, according to Gram classification. $^{*}p < 0.05$ when compared with gram-positive. Etx: Endotoxin; AF: Ascitic fluid; SBP: Spontaneous bacterial peritonitis; SID: Selective intestinal decontamination.

DISCUSSION

In this paper, we report that BT identified through the detection of bactDNA in blood and AF in patients with advanced cirrhosis, is associated with the secretion of activation products of the complement system, and that the genera of translocating bacteria seems to have no influence in the complement activation status. This situation is similar to that observed in patients with SBP.

The complement system constitutes a first-line, unspecific, and rapid humoral defense mechanism against bacterial invasion. The existence of an effective bactericidal activity, mediated by the complement system in normal peritoneal fluid (24) and in tumoral AF, has been previously demonstrated. This activity is significantly higher than that observed in AF of cirrhotic origin (11). The complement is activated through its alternative pathway by the lipopolisaccharide present in gram-negative bacteria, although the presence of capsule (25) and of certain subtypes of *E. coli* may offer resistance to an effective opsonization (26), allowing the growth of bacteria and impairing the clinical evolution of patients (25). The effi-

cacy of this system inversely correlates to the likelihood of the development of SBP (26, 27). However, it is difficult to evaluate the efficacy of this system, and so, the measurement of total protein or complement components in AF has been considered. Patients with lower than 1 g/dL (13) or reduced concentration of C3 in AF have been shown to be predisposed to the development of SBP (13).

The present theory of BT suggests that the arrival of bacteria in AF activates the complement system which allows the opsonization of the invading bacteria. This activation is associated with the simultaneous consumption of the complement factors involved, which is decreased as a consequence of the efficacy of this system. Consequently, the abrogation of BT by means of SID with norfloxacin allows the amelioration of this bactericidal system through a reduced consumption (28). However, no methodology was available to routinely detect BT in patients with cirrhosis. The recent demonstration that bactDNA detection constitutes a surrogated marker of BT (15), allows a deeper investigation of the consequences of BT in patients, not solely related to the development of SBP episodes, but also to the inflammatory reaction induced by bactDNA.

In this investigation, we show that BT (as considered when bactDNA is present) induces the activation of the complement system. The increased serum and AF levels observed in the three products representative of the complement system activation in patients with BT compared with those without BT are clear-cut evidence of this fact. In addition, these same protein residues are also increased in patients with SBP, and no significant differences were observed throughout this study for any of the three proteins among patients with presence of bacterial DNA and those with SBP. The very similar unspecific immune response established in both clinical settings suggests that patients with bacterial DNA merge into those with SBP, at least from an immunological point of view.

Although most of the community-acquired episodes of SBP are due to gram-negative bacteria, an increased incidence of gram-positive cocci in nosocomial episodes has been recently reported. A minor but relevant portion of SBP episodes are due to gram-positive microorganisms (29), and gram-positive cocci have been described in mesenteric lymph nodes in patients with cirrhosis undergoing abdominal surgery (6). When an independent analysis of complement activation products was undertaken among patients from groups II and III according to their gram classification, no significant differences were observed, even though the endotoxin levels are evidently different (Fig. 1). Despite the shortness of the series, these data point out that bacterial DNA, a well-known immunogenic bacterial product, is capable of activating the complement system in an LPS-independent manner.

Patients receiving norfloxacin for secondary prophylaxis of SBP showed decreased levels of all studied activation products of the complement system in serum and AF, reaching similar levels to those shown by patients without bactDNA, that is, without BT. To ascertain whether norfloxacin acts by suppressing the gram-negative bacterial population in the intestinal lumen, thereby reducing BT, is an issue that remains to be addressed.

In summary, bactDNA triggers the complement system activation in patients with cirrhosis facing the translocation of bacterial genomic fragments and showing the same unspecific immune profile as patients with fully established SBP.

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