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Development of a specific enzyme-linked immunosorbent assay for the hepatitis C virus antibody using clone 14

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Summary: The authors isolated a specific cDNA clone (clone 14) for non-A, non-B hepatitis virus infection. In this study, we developed an enzyme-linked immunosorbent assay (ELISA) using a synthetic oligopeptide encoded by clone 14 and examined its usefulness for detecting hepatitis C virus (HCV) antibody in 181 patients with chronic NANB hepatitis, 88 with cirrhosis and 24 with hepatocellular carcinoma associated with NANB hepatitis virus. Anti-clone 14 antibody was detected in 75% of patients with chronic NANB hepatitis, 57% of cirrhotic patients and 58% hepatocellular carcinoma patients. Anti-clone 14 and anti-C-100 antibody assayed using a commercial kit were found in serum from 199 (69%) and 205 (70%) of these 294 patients, respectively. Approximately 85% of the patients showed the presence of anti-clone 14 and/or anti-C-100 antibodies. We compared the presence of these antibodies and the second generation anti-HCV antibody using ELISA and HCV RNA by the polymerase chain reaction assay, in the same blood samples from 49 patients with chronic liver disease who had anti-clone 14 and/or anti-C-100 antibody. HCV RNA was detected in 38 of 40 (95%) plasma samples containing anti-clone 14 antibody, the prevalence of which was similar to that for anti-C-100 antibody (41/42, 98%) and the second generation anti-HCV antibody (46/47, 98%). Furthermore, 6 of 7 plasma samples containing anti-clone 14 antibody and lacking anti-C-100 antibody were positive for the second generation anti-HCV antibody and HCV RNA. These results indicate that anti-clone 14 antibody complements anti-C-100 antibody and that the ELISA of anti-clone 14 antibody is useful for the diagnosis of HCV infection. *Gastroenterol Jpn 1993;28:56-63.*

Key words: *anti-clone 14 antibody; enzyme-linked immunosorbent assay; hepatitis C virus antibody.*

Introduction

Although there is much evidence to suggest the existence of blood-borne non-A, non-B hepatitis (NANB) virus(es), these agents have remained elusive. However, Choo *et al.*[1], recently reported that they succeeded in isolation of a cDNA clone of the etiological agent from the plasma of a chimpanzee infected by the hepatitis non-A, non-B virus, which is now designated as hepatitis C virus (HCV). They also developed an enzyme-linked immunosorbent assay (ELISA) to detect HCV antibody using a fusion protein (C-100) expressed in yeast, which harbors a part of this sequence[2]. HCV antibody was detected in the serum of approximately 80% of patients with chronic NANB liver disease by this ELISA[3-5]. ELISA is thought to be useful in clinical and epidemiological studies and also in screening for HCV infection[6,7]. However, approximately 20% of patients with chronic NANB liver disease lack this anti-C-100 antibody. Furthermore, since the Japanese Red Cross Center has begun screening donated blood for HCV antibody using this ELISA, the number of patients with post-transfusional hepatitis has certainly decreased, though not completely

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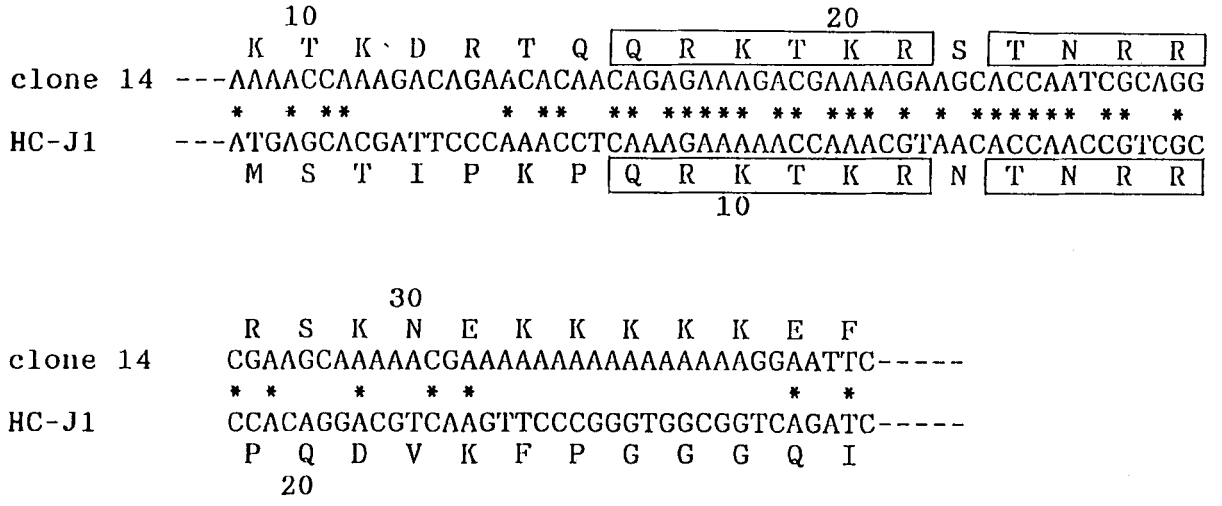

Figure 1. Comparison of the nucleotide and amino acid sequences of clone 14 and HC-J1 which were previously reported in Refs. 10 and 12, respectively. Amino acid numbers are given above and below the sequence. Identical amino acid and base sequence are indicated by a box and an asterisk, respectively.

eliminated.

We also reported that 12 cDNA clones related to NANB hepatitis infection were isolated from a random primed lambda gt11-cDNA library constructed from a donor's plasma presumably infected with the NANB virus[8,9]. One (clone 14) of these clones was determined to be highly specific for HCV infection and consisted of 114 nucleotides and 38 amino acids[10]. Although the nucleotide sequence of clone 14 showed no homology to Chiron's prototype which encodes nearly three-quarters of the HCV genome towards its 3'-terminus[11], the amino acid sequence of clone 14 had a homologous region to that deduced from the HCV genome which has been recently reported by Okamoto et al.[12]. We synthesized a 25-mer oligopeptide deduced from clone 14 and developed an ELISA to detect the antibody against this peptide. In this paper, we report the usefulness of this ELISA for HCV antibody detection in the sera of patients with chronic NANB liver disease.

Materials and Methods

Patients

Frozen serum samples from 293 patients with chronic NANB hepatitis (181 with chronic hepatitis, 88 with cirrhosis, and 24 with hepatocellular carcinoma) referred to Kagoshima University Hospital during the 6 years from January, 1985 to December, 1990 were used for the detection of HCV antibodies. Fresh serum and plasma samples were collected from the blood of another 49 patients with chronic NANB liver disease who were positive for the anti-clone 14- and/or anti-C-100 antibodies. Patients were sero-diagnosed by various tests including virus markers such as hepatitis B surface antigen and antibody to core antigen, as well as imaging examination including abdominal sonogram, liver scintigram and computed tomography. Most patients were diagnosed using histological findings of liver biopsy specimens. Patients with alcoholic liver diseases were excluded from this study. Serum and plasma samples were kept frozen at −20°C and −70°C until use, respectively.

Detection of HCV antibody by ELISA

Anti-clone 14 antibody was assayed by ELISA using a 25-mer synthesized oligopeptide with the sequence KDRTQQRKTKRSTNRRRSKNEKK-KK. Ten out of the 11 amino acids in the sequence QRKTKRSTNRR were identical to that of the prototype 5' structural region of HCV reported by Okamoto et al.[12] (**Figure 1**). The ELISA is a conventional indirect EIA method as follows.

Table 1. Prevalence of anti-clone 14 and anti-C-100 antibody in patients with chronic NANB liver disease

Diagnosis	No of cases	Anti-clone 14/Anti-C-100			
		+/+	+/−	−/+	−/−
Chronic hepatitis	181	101	34	21	25
Cirrhosis	88	44	6	22	16
Hepatocellular carcinoma	24	11	3	6	4
Total	293	156 (53%)	43 (15%)	49 (17%)	45 (15%)

Microtiter plates with 96 wells were coated with the synthesized peptide at a concentration of 1 μg/ml in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, and were allowed to stand at 4°C overnight. An aliquot of 16 mM phosphate buffer (pH 7.2) containing 120 mM NaCl, 0.08% chimerosal, 20% goat serum was added to each plate. The plates were left at 37°C for 120 min. Twenty-microliter aliquots of serum samples were dispensed into each well, followed by 0.2 ml of 20 mM phosphate buffer (pH 7.2) containing 0.45 M NaCl, 10% goat serum, and 0.05% Tween 20. The plates were incubated at 37°C for 1 hr and washed 5 times with 20 mM phosphate buffer (pH 7.2) containing 2% gelatin (wash buffer). After dilution with 20 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl, 0.2% Tween 20, and 10% goat serum, 0.2 ml of goat (anti-human IgG γ chain) polyclonal IgG-horseradish peroxidase conjugate was added to the wells. The plates were again incubated at 37°C for 1 hour and washed 5 times as described above. An aliquot (0.1 ml) of *o*-phenylenediamine (1.7 mg/ml) was added and the plate was allowed to stand for 10 min. The reaction was stopped by adding 50 μl of 4N H_2SO_4. Absorbance was read at 492 nm by an automatic plate reader with a reference wavelength of 690 nm. A cut-off value was tentatively set at 0.480 which represented the mean of normal controls plus 0.400.

Anti-C-100 antibody and the second generation anti-HCV antibody were assayed by commercial ELISA kits of Ortho and Abbott II, respectively.

Preparation of RNA from plasma and detection of HCV RNA by PCR

RNA was extracted from 1 ml of plasma by the acid-guanidinium-phenol-chloroform (AGPC) method originally described by Chomczynski *et al.*[13]. The cDNA was prepared and PCR was performed as described by Enomoto *et al.*[14]. Briefly, oligonucleotide primers deduced from the nonstructual region of the prototype HCV[11] were synthesized on a DNA synthesizer (Applied Biosystems, Model 380B, Tokyo, Japan). The sequences of the PCR primers were 5′-TGGGGATCCCGTATGATACCCGCTGCTTTGA - 3′ (AS-primer). The RNA solution was mixed with AS-primer and the reaction mixture was incubated with reverse transcriptase. The cDNA obtained was amplified in a reaction mixture containing S- and AS-primers and Taq polymerase, by a DNA thermal cycler (Perkin Elmer Cetus, USA) as described previously[15]. After 40 reaction cycles, the PCR products were electrophoresed on 1.5% agarose gel, and stained with ethidium bromide for observation by ultraviolet light to detect the 401 bp fragment. All cases in which HCV RNA was not detected by the PCR method using the NS5 region were further examined by a two-stage PCR method using the 5′ noncoding region described by Okamoto *et al.*[16].

Materials

The ELISA kit for detecting anti-C-100 antibody was purchased from Ortho Diagnostics K.K. (Tokyo, Japan). Goat anti-human IgG-horseradish peroxidase conjugate was obtained from Tago (Burlingame, CA), and the microtiter plates from

Table 2. Detection of anti-clone 14 antibody, anti-C-100 antibody, the second generation anti-HCV antibody and HCV RNA in patients with chronic NANB liver disease

Diagnosis	No of cases	Anti-clone 14	Anti-C-100	Second generation	HCV RNA
Chronic hepatitis	22	18 (82%)	18 (82%)	22 (100%)	22 (100%)
Cirrhosis	12	9 (75%)	11 (92%)	10 (83%)	11 (92%)
Hepatocellular carcinoma	15	13 (87%)	13 (87%)	15 (100%)	14 (93%)
Total	49	40 (82%)	42 (86%)	47 (96%)	47 (96%)

Costar (Cambridge, MA). The t-RNA was purchased from Boehringer Manheim (Mannheim, Germany). RNase inhibitor was obtained from Promega (Madison, WI). Moloney Leukemia Virus reverse transcriptase was purchased from BRL (Gaithersburg, MD). The Hinc II digested ϕx174 DNA was from Nippon gene (Toyama, Japan), and *o*-phenylenediamine was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this study were of analytical grade.

Results

We examined serum samples from patients with chronic NANB liver disease for the presence of anti-clone 14 anti-C-100 antibodies by ELISAs. As shown in **Table 1**, 156 of 293 (53%) with chronic NANB liver disease were positive for both of these antibodies. Only anti-clone 14 antibody or anti-C-100 antibody was present in 43 (15%) or 49 (17%) patients, respectively. Thus, approximately 85% of patients with chronic NANB liver disease had detectable serum levels of anti-clone 14 and/or anti-C-100 antibodies. In the remaining 45 patients (15%) neither antibody was detected. The prevalence of the anti-clone 14 antibody was slightly higher than that of the anti-C-100 antibody in patients with chronic hepatitis, whereas the reverse was true in patients with cirrhosis and hepatocellular carcinoma. The ELISA results with both antigens were generally comparable although some differences were observed in patients with chronic NANB liver disease.

Next, we examined the relationship among the presence/absence of the two HCV antibodies, the second generation anti-HCV antibody and HCV RNA in the plasma of another 49 patients with chronic NANB liver disease (**Table 2**). Anti-clone 14-antibody and/or anti-C-100 antibody was present in all these patients, and the prevalence of these antibodies was very similar as described above. HCV RNA was detected in 43 of 49 plasma samples from these patients by the PCR method using the NS5 region. When a two stage PCR method using the 5′ noncoding region was applied to detect HCV RNA in 6 plasma samples negative for HCV RNA by the PCR method using the NS5 region, 4 of 6 plasma samples were positive for HCV RNA. The final HCV RNA positive rate was 96% in these patients. The presence of HCV RNA often matched that of the antibodies against the clone 14 and C-100 peptides. However, the presence of these antibodies and HCV RNA were somewhat different in the same blood samples from these patients.

Figure 2 shows the relationships among the presence of anti-clone 14 and anti-C-100 and the second generation anti-HCV antibodies in serum, and HCV RNA in the plasma of 49 patients who were suspected to have had HCV. Anti-C-100 antibody was present in 42 of 49 serum samples (86%), and HCV RNA was detected in 41 of 42 (98%) of the plasma samples from the patients with anti-C-100 antibody. Although the prevalence of anti-clone 14 antibody was slightly lower than that of anti-C-100 antibody, HCV RNA was detected in 38 of 40 (95%) plasma samples from patients having anti-clone 14 antibody. Furthermore, 6 of 7 plasma samples containing anti-clone 14 antibody but lacking anti-C-100 antibody were positive for the second generation anti-HCV

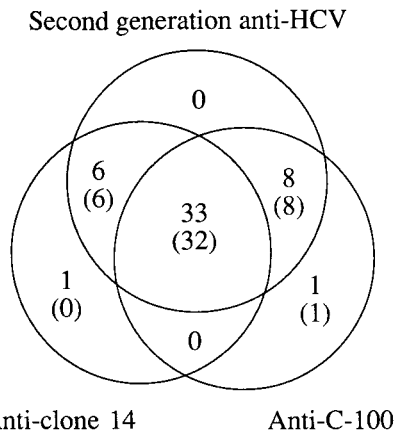

Figure 2. Relationship among the presence of anti-clone 14 antibody, anti-C-100 antibody, the second generation anti-HCV antibody and HCV RNA in patients with chronic NANB liver disease. HCV RNA was assayed by PCR in the plasma of 49 patients with chronic NANB liver disease (22 patients with chronic hepatitis, 12 patients with cirrhosis, 15 patients with hepatocellular carcinoma). All were positive for anti-clone 14 and/or anti-C-100 antibody. Figures in parentheses indicate the number of patients positive for HCV RNA.

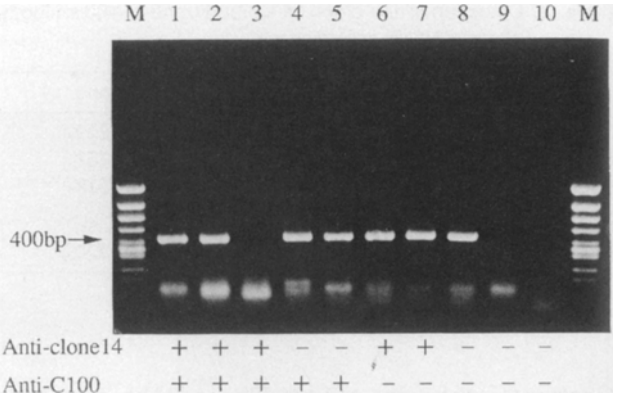

Figure 3. Detection of plasma HCV RNA from 9 patients with chronic hepatitis and a normal subject, by PCR. RNA was extracted from the plasma of 7 patients (lanes 1–7) with chronic NANB hepatitis having anti-clone 14 and/or anti-C-100 antibody, 1 (lane 8) with NANB hepatitis lacking both antibodies, 1 (lane 9) with chronic B hepatitis, and a normal subject (lane 10). PCR products were electrophoresed in a 1.5% agarose gel, and stained with ethidium bromide. HCV cDNA was detected as a 401 bp band. Lane M indicates molecular size marker (ϕx174/Hinc II digest).

antibody and HCV RNA. Thus, the presence of anti-clone 14 antibody in patients with chronic liver diseases was related to HCV infection.

Figure 3 shows an agarose gel electrophoregram of the PCR products from the 7 plasma of these patients using the NS5 region (lanes 1-7), in which the relationship between the presence or absence of HCV antibody and that of HCV RNA were representative. Although 30 of 33 cases with both antibodies had HCV RNA (lane 1 and 2), 3 of them were negative for HCV RNA (lane 3). Two of these 3 cases were finally found to be HCV RNA positive by a two-stage PCR method using the 5′ noncoding region. Eight patient sera having anti-C-100 antibody but lacking anti-clone 14 antibody had HCV RNA (lane 4 and 5), and 6 patients having anti-clone 14 antibody but lacking anti-C-100 antibody also had HCV RNA (lane 6 and 7). HCV RNA was rarely detected in plasma of patients with NANB chronic hepatitis with neither anti-clone 14 nor anti-C-100 antibody (lane 8).

To confirm the specificity of the ELISA using clone 14 peptide for HCV infection, we tested for the presence of HCV antibody in the serum and HCV RNA in plasma samples from a normal subject (lane 10) and 12 patients with chronic hepatitis due to the hepatitis B virus. Although anti-C-100 antibody was found in only 1 of them, neither anti-clone 14 antibody nor HCV RNA was detected in any plasma sample from these patients, as shown in lane 9 in **Figure 3**.

Additionally we further assayed the second generation anti-HCV antibody and compared it with these antibodies (**Figure 2**). The second generation anti-HCV antibody was detected in 47 of 49 (96%) plasma samples, including 6 only anti-clone 14 antibody and 8 only anti-C-100 antibody positive samples. This antibody positive rate matched well with that of HCV RNA.

Because there was some discrepancy between the anti-clone 14 and anti-C-100 antibody as mentioned above, we examined the relationship among the presence of anti-clone 14 antibody, and/or anti-C-100 antibody and the clinical background of the patients. Age, sex, blood transfusion experience and serum AST and ALT activities did not seem to relate to the discrepancy in the presence of the two antibodies (data not shown).

Discussion

Anti-C-100 is highly specific for HCV and useful for clinical and epidemiological studies on liver disease due to the hepatitis virus[3-7]. However, some problems of sensitivity and specificity in the ELISA detecting anti-C-100 antibody have risen[17,18]. In fact, the ELISA for the anti-C-100 peptide failed to detect some HCV-infected patients described here (**Figure 2**). A newly developed ELISA using clone 14 was also specific for HCV infection as follows; (1) Results testing the same serum samples from patients with chronic NANB liver diseases by the two assay systems generally agreed well (**Table 1**); (2) HCV RNA was detected in most plasma samples containing anti-clone 14 antibody, especially in 6 of 7 plasma samples having anti-clone 14 but lacking anti-C-100 antibody (**Figure 2**). The presence of HCV RNA assayed by PCR from the plasma of individual means HCV infection. The anti-clone 14 test detected HCV in 43 of 88 serum samples from patients with chronic NANB liver disease who were negative for the anti-C-100 antibody, thus increasing the detection rate for HCV infection (**Table 1**).

It is important to know whether or not these antibody tests can detect all HCV carriers. As shown in **Table 1**, approximately 15% of patients with NANB liver disease had neither antibody. We randomly tested for HCV RNA in 9 of these patients, and 4 (44%) were positive. Weiner et al.[17] also found some discrepancy between the presence of HCV RNA and that of anti-C-100 antibody in the serum or plasma of patients with NANB liver disease. The results described here indicate that more than 90% of chronic NANB liver disease is caused by HCV infection. However, it is not clear whether those patients negative for both antibodies have any antibody response or not. Because the amount of HCV antigen is very low and the antibody response is frequently poor, there may remain some patients or HCV carriers who fail to have detectable levels of either antibody[19].

The presence of either the anti-clone 14 or anti-C-100 antibody was observed in approximately 15% of patients with chronic liver disease due to NANB or HC virus (**Table 1**). The reason for this discrepancy is of interest. There were no significant differences in background, episodes of blood transfusion, and serum ALT level between patient groups having anti-clone 14 or anti-C-100 antibody in this study. Because HCV is an RNA virus, mutations occur with relatively high frequency[20]. In fact, HCV genomes isolated in Japan have some sequence variations compared to the prototype[21], and there are four major subtypes of the HCV genome in Japan[14]. There may be some HCV subtypes or mutations associated with the disappearance of antigens and the lack of antibody response.

C-100 is a recombinant polypeptide which encodes an epitope of the non-structural region of HCV[6]. As reported previously, clone 14 consists of 114 bases which encode 38 amino acids[10]. Although the overall amino acid sequence of clone 14 peptide is not homologous to that of the HCV recently reported by Okamoto et al.[12], part of the amino acid sequence of clone 14 shows a high degree of homology to that between ^{8}Gln and ^{18}Arg in the HCV core region. Okamoto *et al.*[22] also developed an ELISA system using a synthetic peptide (CP-9) deduced from a prototype core region of the Japanese HCV genome. The amino acid sequence of both clone 14 and CP-9 peptides is found in the core region and is thought to encode different epitopes[12]. An anti-CP-9 test was reported to complement anti-C-100 antibody in the diagnosis of HCV infection as well as the anti-clone 14 test[22]. The prevalence of anti-CP-9 antibody in the serum of patients with chronic NANB liver disease is almost identical to that of the anti-clone 14. The characteristics of these assay systems using peptides encoded in the core region of HCV appear to be similar.

Mishiro *et al.*[23] developed an ELISA system using the GOR 47-1 polypeptide, of which the amino acid sequence did not resemble that of HCV. As GOR 47-1 was detected as a single copy gene in the host cellular genome by Southern blot analysis, they suggested that the anti-GOR 47-1 antibody was an autoantibody. Part of the amino acid sequence of GOR 47-1 polypeptide is homol-

ogous to that of the HCV core region as well as to clone 14. Anti-GOR 47-1 antibody also complements anti-C-100 antibody testing in identifying HCV carriers among blood donors and patients.

The second-generation anti-HCV ELISA has been recently developed by Chiron[24]. Two additional HCV recombinant antigens, one from the NS3 region (C33c) and the other from the core region (pHCV-34), were added to a part (BCD) of the C-100. Therefore, this ELISA would be more sensitive to detect chronic hepatities C infection than the first-generation (anti-C-100) or anti-clone 14. As shown in **Table 2** and **Figure 2**, the second generation anti-HCV antibody was detected in almost all plasma samples, including most samples lacking anti-clone 14 or anti-C-100 antibody. Therefore, the second generation anti-HCV ELISA was more useful for screening chronic hepatitis C virus infection. However, it also means that use of core antigen to detect HCV infection is important. Anti-clone 14 antibody assay is a prototype assay for detecting HCV core antibody, because clone 14 contains only one epitope of core region by the hydrophobicity analysis of the amino acid sequence (Arima T., unpublished observation). Thus, we could improve the sensitivity of anti-clone 14 assay by adding other epitope of core region. In this study we reported that the presence of anti-clone 14 antibody correlates well with that of HCV RHA. However, as shown in **Figure 2**, one case of which was positive only for anti-clone 14 antibody might be false positive because this case was finally negative for HCV RNA.

Recently we have started to examine the usefulness of anti-clone 14 antibody measurement in patients with chronic C hepatitis during interferon therapy. Preliminary results showed that decreased titier of serum anti-clone 14 antibody correlated with the disappearance of HCV RNA. Further clinical applications of clone 14 antibody assay for more than only the diagnosis of the HCV infection are anticipated.

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