PORTAL CD4+ AND CD8+ T LYMPHOCYTE CORRELATE TO INTENSITY OF INTERFACE HEPATITIS IN CHRONIC HEPATITIS C

Ana Teresa Rodriguez VISO(1), Thaís de Castro BARBOSA(2), Lídia YAMAMOTO(3), Carla PAGLIARI(4), Elaine Raniero FERNANDES(5), Roosecelis Araújo BRASIL(5), Heitor Franco de ANDRADE JUNIOR(6), Maria Irma Seixas DUARTE(6) & Antônio Alci BARONE(1)

SUMMARY

Background. The pathogenesis of chronic hepatitis C is still a matter of debate. CD4+ and CD8+ T lymphocytes (TL) are typically observed within the portal and periportal spaces of affected livers, but their functional role in hepatitis C progression has not been fully elucidated. Methods. CD4+ and CD8+ TL were quantified by immunohistochemistry in portal and periportal spaces of 39 liver biopsies from patients with chronic hepatitis C. They were associated to demographic data, histological parameters, laboratory findings of patients and hepatitis C genotypes. Results. There was high numbers of CD4+ and CD8+ TL from which the density of CD4+ T was higher than CD8+ TL in portal and periportal spaces. CD4+ and CD8+ TL were directly correlated to intensity of interface hepatitis. CD8+ TL correlated to serum enzyme levels. Conclusion. The high numbers of CD4+ and CD8+ TL in portal and periportal spaces and their correlation to interface hepatitis suggest that hepatitis C evolution depends on the action of intrahepatic T lymphocytes, lending support to the notion of an immune-mediated mechanism in the pathogenesis of chronic hepatitis C.

KEYWORDS: CD4+ lymphocytes; CD8+ lymphocytes; Chronic hepatitis C; Piecemeal necrosis; Immunohistochemistry.

INTRODUCTION

Chronic hepatitis C (CHC) is a Public Health problem that involves about 170 million people infected worldwide, being the main cause of liver transplants in developed countries. In Brazil, it is estimated that there are at least three million subjects with CHC, making up 2.5 to 4.9% of the country’s population.

Human hepatitis C virus (HCV) infection results in chronic disease in almost three quarters of infected individuals. Most patients present blood chemistry and histology compatible with mild or moderate necro-inflammatory activity and 20% of them can progress to cirrhosis.

Early reports considered a direct cytopathic effect of HCV as the primary form of liver injury caused by the virus itself, similarly to the findings with other flaviviruses and subsequent studies raised the possibility of genotype 1a being more pathogenic. Other studies on the chronic phase of HCV infection did not favor the cytopathic hypothesis and showed that the hepatic lesions were mediated by a hyper reactive immune response. This issue is still controversial and, in some situations, one or the other mechanism might predominate in the HCV infected patient.

The severity of hepatitis and the perpetuation of chronic HCV infection were related to T cell activation in the liver and cytokine production by activated CD4+ or CD8+ T lymphocytes. Those cells could be viewed only as markers of HCV infection or to be involved in the progression of liver disease. The reason for the persistence of HCV in most cases of acute infection by hepatitis C virus that progress to chronic infection is uncertain but viral clearance in acute infection has been attributed to the reactivity of cytotoxic TL, with an inefficient and poor humoral response to HCV.

This work compares the quantitative in situ expression of CD4+ and CD8+ TL in the portal and periportal spaces of liver tissue from patients with CHC. In addition, the cell density of these two TL subpopulations were associated to demographic, histology and biochemical data as well to HCV genotypes.

PATIENTS AND METHODS

Patients: From August 2001 through August 2002, thirty-nine CHC patients were selected during their sequential attendance at an Outpatients Hepatitis Centre in the General Hospital of the School of Medicine of the University of São Paulo (HC FMUSP). All patients

(1) Departamento de Moléstias Infecciosas e Parasitárias da Faculdade de Medicina da Universidade de São Paulo (FMUSP), São Paulo, SP, Brasil.
(2) Laboratório de Regulação Hormonal e Expressão Gênica do Instituto de Ciências Biológicas da USP, São Paulo, SP, Brasil.
(3) Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brasil.
(4) Departamento de Fisiopatologia Experimental da FMUSP, São Paulo, SP, Brasil.
(5) Disciplina de Patologia de Moléstias Transmissíveis, Departamento de Patologia, FMUSP, São Paulo, SP, Brasil.
(6) Departamento de Patologia da FMUSP, São Paulo, SP, Brasil.
Correspondence to: Laboratório de Hepatites (LIM 47) da Universidade de São Paulo, Av. Dr. Enéas C. Aguiar 470, 1º andar, sala 12, 05403-000 São Paulo, SP, Brasil. Tel.: +55.11.3061-7031; Tel./Fax: +55.11.3085-1601. E-mail: ar-viso@uol.com.br
selected were between 18 and 60 years old, of both genders. The race or ethnicity was classified according to IBGE (Brazilian Institute of Geography and Statistics)\textsuperscript{23}, a Brazilian measurement of the skin color\textsuperscript{8}, defined as: white, black and “pardo” - translated earlier as “mulatto”\textsuperscript{9}.

The inclusion criteria for the selection of patients were: positive HCV serology (second or third generation ELISA) confirmed by positive polymerase chain reaction (PCR) for HCV, elevate ALT (alanine aminotransferase) levels at least 1.5 times the upper normal limit (UNL) collected until two months before the hepatic biopsy (nearest date) and liver biopsy indicative of CHC according to Ishak classification\textsuperscript{25}. The quantification of HCV RNA was not available in the “Hospital das Clinicas” in the period of selection of the patients.

The exclusion criteria for the selection of patients were: alcohol abuse in the last six months before the liver biopsy; previous infection by HIV or HTLV; use of corticosteroids in the last six months before the liver biopsy; previous HCV treatment (use of interferon or ribavirin); presence of other liver diseases such as hepatitis B infection, drug hepatotoxicity, alpha 1 antitrypsin deficiency, Wilson’s disease, hemochromatosis, autoimmune hepatitis and liver neoplasms.

The procedures used were approved by the Research Ethics Committee of “Hospital das Clinicas”. All the selected patients gave their signed informed consent.

**Liver biopsies:** Percutaneous liver biopsies were performed for patient diagnosis using a tru-cut needle under local anesthesia. Biopsy liver fragments should be at least 1 cm long or 10 portal spaces. They were submerged in 10% buffered formalin and sent to the Laboratory of Pathology of Transmissible Diseases for paraffin embedding and were processed in an automatic ABI Prism DNA Sequencer, using the ABI Prism Big Dye\textsuperscript{TM} Kit Terminator Cycle Sequencing Ready Reaction, version 2.0 (Applied Biosystems). The obtained sequences were also analyzed and compared with the HCV Data Base, referenced to Basic Local Assignment Tool (BLAST), and the genotypes were compatible with HCV\textsuperscript{7}.

**Statistical analysis:** Quantitative and semi-quantitative data were initially related using Pearson’s parametric correlation (expressed as r) for ordinal or quantitative data, and Spearman rank correlation (expressed as rs) for proportions. The quantitative data were separated according to the ordinal classification, compared using variance analysis (ANOVA) and confirmed by the Kruskal Wallis test. Event difference was considered significant when the probability of equality was lower than 0.05 (p < 0.05). Events were considered related when the probability of absence of relationship was lower than 0.05 (p < 0.05).

**RESULTS**

The sample comprises 39 hepatic biopsies from patients with CHC. All of the patients were selected according to inclusion and exclusion criteria.

In Table 1, patient’s age varied between 25 and 53 years; gender and ethnic distributions were: 25 (64%) males, 14 (36%) females; 30 (77%) white, 4 (10%) black and 4 (13%) “pardo” (according to IBGE classification\textsuperscript{25}).

Serum enzymes ALT, AST (aspartate transaminase) and GGT (gamma-glutamyl transferase), collected the newest before liver biopsy, ranged from one to seven times the UNL., with mean range 2.1 for AST, 2.8 for ALT and 2.2 for GGT. The distribution of HCV genotypes was: 22 (56.4%) patients with genotype 1b, six (15.3%) with genotype...
la, two (5.1%) with genotype 2a, one (2.5%) with genotype 2b and eight (20.5%) with genotype 3a (Table 1).

All patients presented architectural changes and portal inflammation in the hepatic biopsy according to Ishak classification\(^2\), with four (10%) patients architectural stage 5 and 6, six (16%) patients architectural stage 3 and 4 and 29 (74%) patients architectural stage 1 and 2 (Table 1). Interface hepatitis was absent in 12 (30%) patients, with grade 1 in 21 (55%) patients, grade 2 in four (10%) patients and two (5%) patients grade 3. No confluent necrosis was found in this sample (Table 1).

Figure 1 shows CD4+ and CD8+ TL stained in brown in immunohistochemistry reactions (1A and 1B respectively). The number of lymphocyte subpopulations in portal areas is shown in Table 1, with mean of CD4+ TL 186.8 (varying from 57 to 2195.2 cells/sq mm\(^2\)) and CD8+ TL mean 48.7 (varying from 44.8 to 504 cells/sq mm\(^2\)).

Interface hepatitis correlated to CD4+ TL (p 0.018) and CD8+ TL (p 0.027) in portal and periportal areas (Table 2) and this relationship was directly proportional as shown in Figures 2A and 2B. There was no correlation between the CD4/CD8 ratio and interface hepatitis (Fig. 2C). The inflammatory activity in liver was grouped in the lower and

Table 1
Demographic and laboratory characteristics of 39 patients with CHC

| Case | Sex/age | Race* | AST** | ALT** | GGT** | VHC genotype | Interface hepatitis | Portal inflam. | Focal necrosis | Staging | TL CD4+ | TL CD8+ | CD4/CD8 |
|------|---------|-------|-------|-------|-------|---------------|-------------------|---------------|---------------|---------|---------|---------|---------|
| 1    | F/27    | white | 7     | 1     | 1     | 1b            | 1                 | 2             | 1             | 2       | 686.4   | 300.8   | 2.3     |
| 2    | M/25    | white | 2     | 1     | 1     | 0              | 2                 | 1             | 2             | 438     | 332.8   | 1       |
| 3    | M/45    | black | 2     | 1     | 1     | 1b            | 3                 | 3             | 2             | 1110.4  | 360     |
| 4    | M/45    | white | 4     | 1     | 1     | 1b            | 1                 | 2             | 1             | 700.8   | 304     |
| 5    | M/22    | white | 1     | 1     | 1     | 1b            | 0                 | 1             | 1             | 57      | 204.8   |
| 6    | F/23    | white | 1     | 2     | 1     | 1b            | 1                 | 2             | 1             | 822.4   | 347.2   |
| 7    | F/52    | white | 1     | 2     | 1     | 0              | 1                 | 1             | 1             | 432     | 262.8   |
| 8    | M/47    | black | 1     | 2     | 1     | 1b            | 1                 | 2             | 1             | 441.6   | 244.8   |
| 9    | M/24    | white | 1     | 2     | 1     | 1b            | 1                 | 1             | 2             | 702.4   | 115.2   |
| 10   | M/44    | pardo | 2     | 2     | 1     | 1b            | 1                 | 2             | 1             | 292.8   | 361.6   |
| 11   | F/22    | white | 1     | 2     | 1     | 0              | 2                 | 3             | 1             | 395.2   | 134.4   |
| 12   | M/25    | “pardo”| 2     | 2     | 1     | 0              | 2                 | 1             | 2             | 603.2   | 214.4   |
| 13   | M/39    | “pardo”| 2     | 2     | 1     | 0              | 2                 | 2             | 2             | 1162.7  | 474.7   |
| 14   | M/38    | white | 1     | 2     | 1     | 1b            | 1                 | 2             | 2             | 1020.8  | 70.4    |
| 15   | M/23    | white | 2     | 2     | 1     | 1b            | 1                 | 2             | 1             | 672     | 244.8   |
| 16   | M/48    | black | 4     | 2     | 1     | 3a            | 2                 | 2             | 0             | 731.2   | 300.8   |
| 17   | F/47    | white | 1     | 2     | 1     | 3a            | 0                 | 2             | 0             | 304     | 198     |
| 18   | M/29    | white | 1     | 2     | 1     | 1b            | 1                 | 2             | 2             | 532.8   | 56      |
| 19   | M/37    | white | 1     | 2     | 1     | 3a            | 1                 | 2             | 1             | 651.2   | 148.8   |
| 20   | M/30    | “pardo”| 1     | 2     | 1     | 1b            | 1                 | 2             | 1             | 396.8   | 188.8   |
| 21   | F/41    | black | 1     | 2     | 1     | 1a            | 1                 | 3             | 1             | 283.2   | 44.8    |
| 22   | M/58    | white | 2     | 3     | 1     | 1b            | 1                 | 2             | 1             | 148.8   | 81.8    |
| 23   | M/36    | white | 1     | 3     | 1     | 3a            | 1                 | 2             | 1             | 448     | 75.2    |
| 24   | M/41    | white | 1     | 3     | 1     | 3a            | 1                 | 2             | 1             | 592     | 265.6   |
| 25   | M/37    | white | 1     | 3     | 2     | 2a            | 0                 | 1             | 1             | 593.6   | 128     |
| 26   | F/52    | white | 6     | 3     | 2     | 1a            | 2                 | 2             | 3             | 723.2   | 395.2   |
| 27   | M/48    | white | 3     | 3     | 2     | 3a            | 2                 | 2             | 1             | 1264    | 302.4   |
| 28   | M/25    | white | 1     | 3     | 3     | 1a            | 0                 | 1             | 1             | 617.6   | 168     |
| 29   | F/59    | white | 5     | 3     | 3     | 1b            | 0                 | 2             | 1             | 1139.2  | 355.2   |
| 30   | M/48    | “pardo”| 5     | 3     | 3     | 1b            | 0                 | 1             | 1             | 1048    | 169.6   |
| 31   | M/20    | white | 1     | 4     | 4     | 1b            | 0                 | 2             | 1             | 275.2   | 56      |
| 32   | M/25    | white | 1     | 4     | 4     | 1a            | 1                 | 2             | 1             | 811.2   | 102.4   |
| 33   | F/49    | white | 1     | 4     | 4     | 1a            | 1                 | 3             | 1             | 1276.8  | 118.8   |
| 34   | M/27    | white | 2     | 4     | 4     | 2b            | 3                 | 4             | 1             | 1408    | 419.2   |
| 35   | M/35    | white | 4     | 4     | 4     | 1a            | 0                 | 1             | 1             | 624     | 189     |
| 36   | F/46    | white | 2     | 5     | 5     | 1b            | 1                 | 2             | 1             | 534.4   | 228.8   |
| 37   | F/49    | white | 4     | 6     | 6     | 3a            | 2                 | 4             | 2             | 558.4   | 252.8   |
| 38   | F/56    | white | 1     | 6     | 7     | 2a            | 1                 | 2             | 1             | 2195.2  | 230.4   |
| 39   | F/24    | white | 2     | 7     | 7     | 1b            | 1                 | 1             | 1             | 510.4   | 70.4    |

*Race or ethnicity according to IBGE; **number of times of ULN*
higher grades of activity of interface hepatitis, which means, the 33 (85%) patients with interface hepatitis grade 0 and 1 of Ishak classification were grouped in the category of low activity and the six (15%) patients with grade 2 and 3 of Ishak classification were classified as high activity (Fig. 2D). The higher interface activity was correlated to CD4+ TL (p < 0.05) and CD8+ TL (p < 0.01) expression, but not to the CD4/CD8 ratio (Fig. 2 D).

CD8+ TL correlated significantly to AST (p = 0.003) and GGT (p = 0.004). Such correlation was not observed for CD4+ TL (Table 2).

There was no significant association between lymphocyte populations and age, gender, ethnicity or HCV genotype (Table 2).

There was no correlation between HCV genotypes and the histological characteristics by Ishak classification in this casuistic. AST was correlated significantly to CD8+ TL (p = 0.012) and interface hepatitis (p = 0.052). The same correlation was not noticed for ALT. GGT correlated to staging (p = 0.001), portal inflammation (p = 0.006) and interface hepatitis (p = 0.013).

DISCUSSION

This study shows the expression of CD4+ and CD8+ TL by immunohistochemistry in the portal and periportal areas of hepatic biopsies from patients with CHC, and the correlation between CD4+ and CD8+ TL densities and intensity of interface hepatitis. These results suggest that intrahepatic TL have an active role in the pathogenesis of CHC.

Most of the patients of this sample presented blood chemistry and histology compatible with mild inflammatory lesions (Table 1), similarly to what is generally described in CHC.

We found a high prevalence of TL in the portal and periportal liver areas, with CD4+ TL prevailing over CD8+ TL (Table 1). Many studies show higher number of CD4+ than CD8+ LT and argue that the first have a function similar to CD8+TL, a cytotoxic action in CHC besides an indirect action by cytokines production. In this research we did not analyzed the function of these cells, but we can infer that the CD4+TL have an important participation in pathogenesis of chronic hepatitis C. The expression of CD8+ TL in these patients corroborate the notion of a cytotoxic mechanism participating in the hepatic lesions in CHC because these lymphocytes are considered the main effector cells in the hepatic lesion in chronic hepatitis C and capable of limiting viral replication in patients with CHC. The close relationship between tissue CD8+ TL and both biochemical markers of hepatocyte injury as the increase of ALT and GGT and histological findings of interface hepatitis (Table 2) are other evidences of hepatic lesion by these cells, similarly to the findings of others, who analyzed the gene expression of CD8β in chronic hepatitis C patients.

Two patients (cases 20 and 34) presented cirrhosis (grade 6 of Ishak), with CD4+ TL densities 396.8 and 1408 cells/sq mm and CD8+ TL densities 419.2 and 188.8 cells/sq mm. CD4+ and CD8+ and two other patients (cases 21 and 27) had pre-cirrhotic lesions (grade 5 of Ishak), with CD4+ TL densities 283.2 and 1264 cells/sq mm and CD8+ TL cell densities 44.8 and 302.4 cells/sq mm (Table 1). In these four cases there were marked changes in the hepatic architecture but, despite the observational difficulties imposed by such alterations, it has a tendency to higher numbers of the CD4+ and CD8+ TL subpopulations. This analysis was limited by the small number of advanced fibrosis in our patients.

Among the alterations that take place in the liver during chronic disease caused by hepatotropic viruses, the most important is interface hepatitis, also known as periportal or periportal hepatitis, previously, “piecemeal necrosis”, due to the greater chance of progression of fibrosis as seen in chronic hepatitis C. Then, the patients were grouped according to interface hepatitis into lower activity (grade 0 and 1 of Ishak classification) and higher activity (grade 2 and 3 of Ishak classification) and there was a significant and direct correlation between interface hepatitis and the liver expression of CD4+ and CD8+ TL (Fig. 2D). Although it did not analyze whether these TL were HCV specific, as shown in other studies, the present observation correlates the TL expression and interface hepatitis in patients with CHC strongly suggesting a relationship between these cells and the progression of CHC, advocating in favour of an intrahepatic immune response, as argued by many researchers.
The significant positive correlation between portal and periportal CD8+ LT expression and interface hepatitis (Fig. 2B) can be seen as an evidence of the occurrence of a cytotoxic immune-mediated lesion. It is possible that these lymphocytes are not able to destroy HCV completely and the hepatic lesions subsequent to their action are enough to cause interface hepatitis. Studies using cytotoxic assays with HCV synthetic peptides have shown specific class I TL epitopes against HCV in the inflammatory infiltrates of chronic hepatitis C livers, suggesting viral persistence despite the presence of immune T cell cytotoxicity. Some authors criticize data from cells specifically stimulated in vitro with viral CD8+ antigens, arguing that there is in fact a biased selection of responsive cells in culture that would thus not reflect what actually happens in vivo. Quantitative assays similar to ours would contribute greatly to the elucidation of CHC pathogenesis because they show the actual intrahepatic response, within the natural environment of cellular participants. Some studies found a correlation between AST and interface hepatitis or AST and progression of fibrosis in CHC. In this research the AST was correlated significantly to CD8+ and interface hepatitis, besides the GGT correlated to staging, portal inflammation and interface hepatitis. These pieces of evidence support the notion that AST results from an hepatic lesion and that this is probably due to CD8+ TL action.

Many host factors have been observed to increase the risk of progression of liver disease, such as the later age of acquisition of hepatitis C, male gender and afro-descendants, but we could not...
Table 2
Quantitative data of TL in portal areas related to demographic, blood chemistry, viral genotype and histological scores

| Event                          | Mean score | Relation scores with |
|-------------------------------|------------|----------------------|
|                               | CD4+ cells/mm² | CD8+ cells/mm² | CD4/CD8 ratio |
| Age (years)                   | 37.64      | rs = 0.2963          | rs = 0.2465   | rs = -0.0284 |
| SD                            | ± 11.73    | p = .067             | p = .130      | p = .864     |
| Serum AST                     | 2.08       | r = 0.1562           | r = 0.4624    | r = -0.2604  |
| SD                            | ± 1.59     | p = .342             | p = .003*     | p = .109     |
| Serum ALT                     | 2.77       | r = 0.0204           | r = 0.3214    | r = -0.1939  |
| SD                            | ± 1.42     | p = .902             | p = .046*     | p = .237     |
| Serum GGT                     | 2.21       | r = 0.2154           | r = 0.4555    | r = -0.2279  |
| SD                            | ± 1.78     | p = .188             | p = .004*     | p = .163     |
| No. patients with genotype 1  | 28/39      | rs = 0.0810          | rs = 0.9806   | rs = 0.1468  |
| %                             | 71.79%     | p = .624             | p = .963      | p = .372     |
| No. patients with genotype 3  | 8/39       | rs = -0.1072         | rs = -0.0649  | rs = -.0671  |
| %                             | 20.51%     | p = .516             | p = .649      | p = .685     |
| Histological staging (Ishak)  | 2.05       | r = 0.2527           | r = 0.2330    | r = -0.0673  |
| SD                            | ± 1.43     | p = .121             | p = .153      | p = .684     |
| Portal Inflammation           | 1.97       | r = 0.2499           | r = 0.2398    | r = 0.0282   |
| SD                            | ± 0.71     | p = .125             | p = .142      | p = .865     |
| Piecemeal necrosis            | 0.90       | r = 0.3777           | r = 0.3545    | r = 0.0361   |
| SD                            | ± 0.79     | p = .018*            | p = .027*     | p = .827     |
| Focal necrosis                | 1.0513     | r = 0.1587           | r = -0.0810   | r = 0.3013   |
| SD                            | ± 0.39     | p = .335             | p = .624      | p = .062     |

r represents Pearson correlation, rs Spearman correlation and * p < 0.05

find any relationship between these demographic data and the expression of intrahepatic TL (Table 2), possibly because of the small number of individuals within each category.

The clinical significance of genotyping is still under investigation3,11,28, and in this study, unlike previous studies, there was no correlation between HVC genotypes and the CD4+ or CD8+ TL expression in the liver23,34, possibly because of the small number of genotypes 2 and 3 in this sample.

This study shows an important correlation between CD4+ and CD8+ TL and interface hepatitis in patients with chronic hepatitis C, thus suggesting that these cells take part in the progression of the chronic disease in liver, and it is a significant additional contribution for the knowledge of action of TL in chronic hepatitis C.

RESUMO
A quantificação de CD4+ e CD8+ portal em hepatite C crônica está relacionada com a intensidade da hepatite de interface

Introdução: A patogênese da hepatite C crônica ainda está em discussão. Sabe-se que linfócitos T (LT) CD4+ e CD8+ são tipicamente observados no espaço portal e peri-portal de pacientes com hepatite C crônica, mas o conhecimento exato de suas ações no fígado, bem como a sua influência na progressão da doença hepática ainda estão em discussão. Métodos: Os LT CD4+ e T CD8+ foram quantificados por imunohistoquímica nos espaços porta e peri-portais em 39 biópsias hepáticas de pacientes cronicamente infectados pelo vírus da hepatite C. Esses dados foram associados com os dados demográficos, as alterações histológicas, os achados laboratoriais dos pacientes com hepatite C e com os genótipos do vírus da hepatite C. Resultados: Houve grande quantidade tanto de LT CD4+ como de CD8+, sendo que houve maior densidade de LTCD4+ do que CD8+ nos espaços portal e peri-portal. Tanto o número de linfócitos T CD4+ como de CD8+ foram diretamente relacionados com a intensidade da hepatite de interface. Os linfócitos T CD8+ foram estatisticamente relacionados às enzimas hepáticas. Conclusão: O encontro de numerosos linfócitos T CD4+ e linfócitos T CD8+ no espaço-portal e peri-portal e sua correlação com a hepatite de interface sugerem que a evolução da hepatite C dependa da ação dos linfócitos T intra-hepáticos, ou seja, há um mecanismo imuno-mediado na patogênese da hepatite C crônica.

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