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Two lambda gt11 cDNA clones derived from non-B non-C hepatitis

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Abstract

Objective: In Japan about 10% of patients with a histologic diagnosis of chronic active hepatitis and with liver cirrhosis and/or hepatocellular carcinoma are negative for all known serologic hepatitis viral markers, however, their histological features are compatible to viral hepatitis.

Design: We studied two lambda gt11 random-primed cDNA clones derived from patients with histologically confirmed chronic active non-B non-C hepatitis and without sufficient evidence of alcohol-, drug- or obesity-etiology in the presence of antibodies against the clones.

Methods: Two clones (669 and 990) were prepared by a plaque immuno-enzyme assay to detect antibodies in healthy controls and in patient serum.

Results: Antibody against clone 669 was detected in 0%, 27.5%, 9.7%, and 6.2% of healthy controls, and patients with non-B non-C hepatitis, chronic hepatitis B, and chronic hepatitis C, respectively. Antibody against clone 990 was present in 0%, 15%, 3.2%, and 0.9% of those respective groups. Among 57 blood-transfused patients, sera from 4 patients contained antibodies to one of the clones. In 40 patients with non-B non-C hepatitis, whose histologic intensity of the hepatic inflammation had been assessed, antibody against clone 669 was detected in 11%, 29%, and 50% of patients with acute hepatitis, mild to moderate chronic active hepatitis, and severe chronic active hepatitis, respectively; antibody against clone 990 was detected in 11%, 21%, and 10% of those groups, respectively.

Conclusions: These results suggested that the peptides encoded by the isolated clones might be a part of the antigenic epitope involved in non-B non-C chronic active hepatitis agent(s).

Key words: Non-B non-C hepatitis ; Lambda gt11 cDNA clone ; Plaque Immunoscreening

Introduction

The development of screening assays for hepatitis C virus was most significant step in the prevention of post-transfusion non-A non-B hepatitis[1-3]. Following discovery of the hepatitis C virus[1, 4-6], about 10% of cases in acute and chronic hepatitis still remained negative for all known viral markers, including hepatitis B virus surface antigen (HBsAg), HB core-antibody (HBcAb), hepatitis C virus antibody (HCVAb), HBV DNA, and HCV RNA.

The search for other infectious agents responsible for these residual cases of post-transfusion non-B non-C, in cryptogenic fulminant hepatic failure[7-9], and in chronic hepatitis[10-13] has been ongoing. Recent findings indicate that although infection with hepatitis G virus (HGV) is widespread, HGV is not responsible for most cases of chronic non-B non-C hepatitis. In addition to viral markers as above, non-B non-C hepatitis cases are

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negative for antinuclear and antimitochondrial antibodies. Moreover, their histopathologic findings are consistent with chronic active hepatitis (CAH).

In our department, about 10%, 10% and 70% of the inpatient cases are non-B non-C hepatitis, hepatitis B, and hepatitis C respectively. In this study two independent λ gt11 clones for chronic non-B non-C hepatitis were studied by plaque immuno-enzyme assay in patients with chronic hepatitis B, chronic hepatitis C or non-B non-C hepatitis. Furthermore, their grades of histologically evidenced inflammatory activity and clinical records of blood transfusion were reviewed.

Materials and methods

1. Serum sampling

After obtaining informed consent from 184 patients, serum samples were obtained from January 1992 to September 1999 and frozen for storage. As healthy controls, 60 blood donors from the Japanese Red Cross Society with normal transaminase activity and negative for HBsAg, HBV DNA, HCVAb, HCV RNA and anti Human T lymphotrophic virus type 1 Ab were tested. This study was approved by the Japanese Redese Cross Society Human Investivation Committee. To protect rights to privacy, we did not get identifying information from Japanese Red Cross Society. So we achieved complete anonymity. The patients included 31 with chronic hepatitis B, 113 with chronic hepatitis C, and 40 with non-B non-C hepatitis (Table 1). Hepatitis B patients have elevated transaminase activities and positive for HBsAg or HBV DNA. And hepatitis C patients have elevated transaminase activities and positive for HCVAb or HCV RNA. We defined non-B non-C hepatitis as manifesting elevated transaminase activities but no established viral markers (HBsAg, HBcAb, HBV DNA, HCVAb, and HCV RNA) and histologically confirmed as viral hepatitis[7, 12]. Patients with autoimmune hepatitis, primary biliary cirrhosis, or alcoholic liver injury were excluded, as were cases where a drug or toxin was implicated. Careful efforts were made in each case to exclude known hepatotoxins.

Table 1. Characteristics of Patients.

	Control	Hepatitis B	Hepatitis C	Non-B non-C hepatitis
Male/Female	51／9	18／13	62／51	16／24
Age (yrs): Mean	36	51	64	58
Range	16－61	18－71	24－88	16－85
ALT (IU/L): Mean	19	59	54	67
Range	8－40	15－198	11－223	16－135
		HBeAg+/Ab－ 7		
		HBeAg－/Ab+ 24		

2. Synthesis of cDNA

A λgt11-cDNA library was constructed using sera from patients with non-B non-C hepatitis. RNA was extracted from serum by Trizol LS Reagent (Invitrogen, Carlsbad, California) according to the manufactures' instructions. The λgt11-cDNA library was constructed using a commercially available kit, the Super Script Choice System for cDNA Synthesis (Invitrogen). Translation product was immunoscreened using serum pooled from patients with acute and chronic non-B non-C hepatitis as the primary antibody and horseradish peroxidase (HRPO)-conjugated goat anti-human IgG (ICN Biomedicals, Aurora, Ohio) as the secondary antibody (section 3 and 4 below).

3. Plating and blotting

Plating and blotting were carried out as previously reported[14], with minor modifications. Tests were performed on replicas of plaques formed by the cloned recombinant phage on Y1090 cells using a serum panel including hepatitis B, hepatitis C, and non-B non-C hepatitis. Phage λgt11 cDNA clones were plated on LB agarose plates. After expression of plaques, nitrocellulose membranes with the addition of Isopropyl β-D-thiogalactopyranoside (Sigma, St.Louis, Missouri) were used for transfer blotting of peptides encoded by the clones.

4. Immunoscreening

Membranes were used for immunoscreening according to a method previously reported[14], with minor modifications. After the membranes were blocked against nonspecific binding, they were incubated with primary antibody solution. Anti-human IgG conjugated with HRPO diluted 1:200 with dilution buffer was applied as the secondary antibody. The membranes were washed and stained by 4-chloro-1-naphthol dissolved in substrate buffer. Results in all positive samples were confirmed by retesting.

5. DNA and amino acid sequence encoded by the cloned DNA

The recombinant DNA that was inserted in phage

clones selected by immunoscreening was amplified by PCR using λgt11 forward and reverse primers. The amplified double strands DNA was labeled by the λgt11 forward primed with cycle sequencing using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). The labeled DNA was electrophoresed and analyzed by ABI PRISM 310 and Sequence Navigator Software (PE Applied Biosystems, Foster City, CA, USA). Homology search was conducted with DNASIS Version 3.5 of GenBank R 83.O. DNASIS also gave the amino acid sequences and the sequences were compared by homology search. Each clone sequence was analyzed for hydrophobicity and protein 2D structure by the methods of Kyte and Doollittle, and Chou and Fasman respectively.

6. Statistical analysis

Differences between groups were evaluated by the chi-squared test. Statistical analyses were performed using the Stat View J-4.5 software package (Abacus Concepts, Berkeley, CA). A P value less than 0.05 were considered to indicate statistical significance.

Results

1. The cDNA library

The recombinant bacteriophages were amplified by growth in E.coli and the resulting library of 10^7 cDNA clones were plaque immunoscreened for the presence of chronic non-B non-C hepatitis related antigens.

2. Antibody against clone 669 products in healthy controls and patients with chronic hepatitis

Healthy controls were all negative for an antibody against the clone 669 product. And patients with non-B non-C hepatitis, hepatitis B, and hepatitis C were positive for an antibody against the clone 669 product in 11 of 40 sera (27.5%), 3 of 31 sera (9.7%) and 7 of 113 sera (6.2%), respectively. Significant difference was demonstrated between hepatitis C and non-B non-C hepatitis patients (Table 2).

Table 2. Positive rate of antibodies against clone 669 and 990.

Subjects		Number of positive cases (%)			
Category	Number	Clone 669		Clone 990	
Control	60	0		0	
Hepatitis B	31	3 (9.7)		1 (3.2)	
Hepatitis C	113	7 (6.2)		1 (0.9)	
Non-B non-C hepatitis	40	11 (27.5)	P=0.0003	6 (15)	P=0.0003
Total	244	21		8	

3. Antibody against clone 990 products in healthy controls and patients with chronic hepatitis

Healthy controls were all negative for antibody against the clone 990 product. Non-B non-C hepatitis, hepatitis B and hepatitis C patients were positive for antibody against the clone 990 product in 6 of 40 (15%), 1 of 31 (3.2%), and 1 of 113 sera (0.9%),respectively, showing significant difference between hepatitis C and non-B non-C hepatitis patients (Table 2).

4. Antibody against clone 669 products and histologic grade of inflammation activity

When 40 patients with non-B non-C hepatitis were grouped by histologic findings, 1 of 9 (11%), 4 of 14 (28.6)%, and 5 of 10 (50%) were positive for antibody against clone 669 product in acute hepatitis, mild to moderate chronic active hepatitis, and severe chronic active hepatitis, respectively (no significant difference between the pathologic diagnoses, Table 3).

5. Antibody against clone 990 products and histologic grade of inflammation activity

Sera from 1 of 9 (11%), 3 of 14 (21%), and 1 of 10 (10%) patients were positive for antibodies against clone 990 product in acute hepatitis, mild to moderate chronic active hepatitis, and severe chronic active hepatitis, respectively (no significant difference between the pathologic diagnoses, Table 3).

Table 3. Presence of antibodies against clone 669 and 990 histologic findings.

Subjects	Number	Clone 669	Clone 990
Acute hepatitis	9	1	1
Chronic active hepatitis			
Mild	7	2	2
Moderate	7	2	1
Severe	10	5	1
Chronic persistent hepatitis	2	0	0
Hepatocellular carcinoma	5	1	1
Total	40	11	6

6. Antibodies against clone products and blood transfusion

Among 57 transfused patients, 3 HCV patients (5.3%) were positive with respect to clone 669, and one non-B non-C hepatitis patient (1.8%) was positive with respect to

Table 4. Antibodies against both clones and blood transfusion.

Clone	Positive	Negative
669	3*	54
990	1†	56

Total: 57 patients

*All represented hepatitis C, †Non-B non-C hepatitis

the clone 990 (Table 4).

7. DNA and predicted epitope

Analysis of each DNA and their encoded amino acid sequence shows no homology with sequences related to HAV, HBV, HCV, HEV and HGV as reported in available scientific literature, or with any other sequences entered in the GenBank databases. Clone 669 has a length of 669

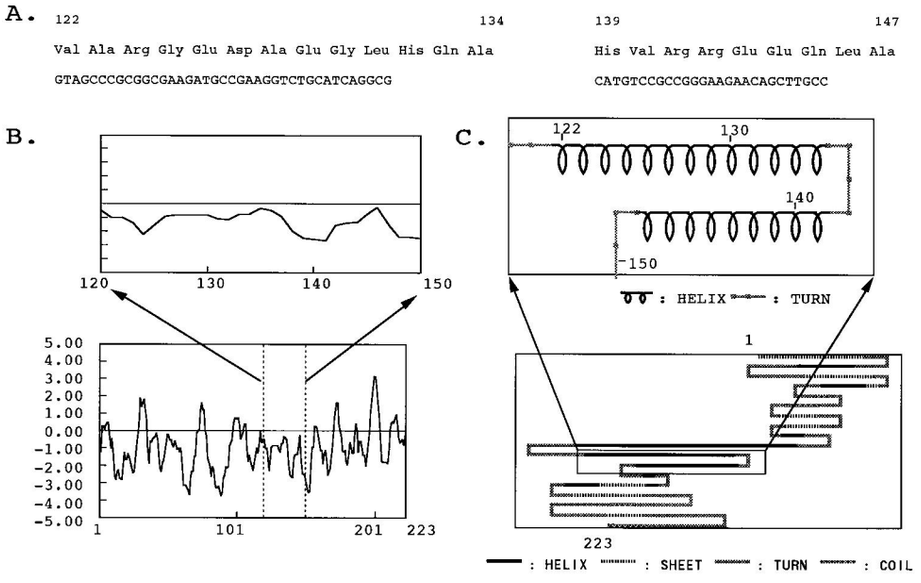

Figure 1. Clone 669.
A: Amino acid sequence and DNA, B: Hydrophobicity, Kyte & Doolittle, Window: 6 Average: -0.94 Threshold Line: 0.0, C: 2D structure, Function: Chou and Fasman.

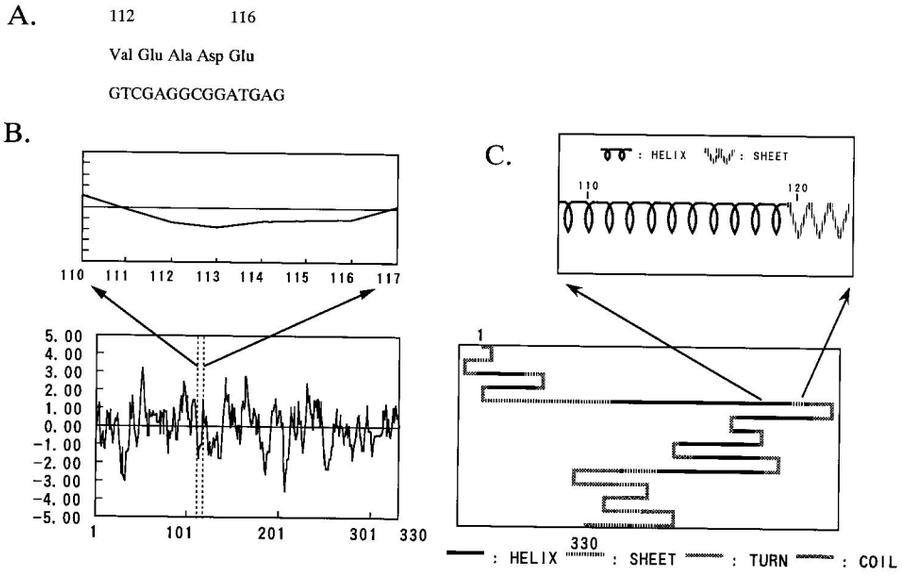

Figure 2. Clone 990.
A: Amino acid sequence and DNA, B: Hydrophobicity, Kyte & Doolittle, Window: 6 Average: 0.01 Threshold Line: 0.0, C: 2D structure, Function: Chou and Fasman.

base pairs, and clone 990 has 990. DNASIS analysis indicated that each hydrophilic portion of the constructed helix structure in the sequences were candidates for epitopes. The predicted epitope sequence was 13 and 9 amino acids for clone 669, and 5 amino acids for clone 990 (Figs. 1 and 2).

Discussion

In this study, we found that two peptides encoded by clones isolated using λgt11 may represent antigenic epitopes of the agent or agents responsible for non-B non-C hepatitis and/or chronic active hepatitis.

The clinical features of chronic non-B non-C hepatitis are incompletely defined. Most cases of chronic hepatitis, cirrhosis, and hepatocellular carcinoma are caused by HBV or HCV infections associated with heavy alcohol intake. A small proportion of liver diseases, however, are of unknown aetiology.

Non-B non-C hepatitis may represent an aggregate of several forms of liver injury, and may be caused by more than one infectious agent. Sequential or coexisting infection of HCV or HBV with non-B non-C hepatitis agents might occur. Further, a mutant form of a known virus such as HBV or HCV could cause non-B non-C hepatitis. To exclude occult HBV infection in the cases studied here, HBcAb was tested for, and HBV-DNA was ruled out by polymerase chain reaction.

The clones studied here encode parts of a new, previously unrecognized agent or agents, but it is uncertain whether these clones are derived from a part of a viral genome sequence. But for healthy controls being negative and the prevalence among hepatitis cases with active inflammatory conditions is suggestive of the clones being at least related to chronic hepatitis. A chimpanzee transmission study, for examples, would further support the hepatotropic, pathogenic potential of any new agent[15].

TTV and HGV were discovered by the method of representational difference analysis, a method that detects not only viral agents but also other possibly agents. HGV and TTV appear to lack any pathogenetic role in causing acute or chronic liver disease[15,16]. Such findings serve to emphasise the importance of causation in interpretating the role of new agents discovered by molecular biologic techniques[17].

We found immunoscreening to be useful in investigating HCV. Although clones discovered by immunoscreening are not always viral clones, they may still be important for blood screening if the clones are expressed specifically in non-B non-C hepatitis.

When we examine serum from recovered patients, antibodies against the viral surface antigen can be obtained, but the titre of the antibody may be low. We cannot always differentiate low antibody titre from an absence of antibody. When we examine serum from a chronic hepatitis patient, we can expect to obtain antibodies against the non-neutralizing viral antigen.

Chronic hepatitis is the most common causes of cirrhosis and hepatocellular carcinoma. The pathogen from which our two clones were derived can cause acute and chronic hepatitis, since each clone was positive in some cases of histologicaly acute or chronic hepatitis and negative in healthy controls.

Studies involving strict selection of patients and controls, large numbers of patients, sequential samples before and after infection, coded sample panels, and animal models of establishment of liver disease will be required before any newly discovered agent will warrant classification as a hepatitis virus[16]. Based on just the data in this study, the two clones examined have obviously not reached this level. Further, the mode of transmission needs to be studied if the clones are derived from hepatitis virus.

Each of the clones studied here showed positivity not only in some non-B non-C hepatitis cases but also in some sera from HBV- or HCV-infected patients. In some instances, the clone sequences and HBV or HCV might have undergone blood-borne transmission together. Immunoscreening is an antigen-antibody reaction method, so there is the possibility that a non-specific reaction may be observed in hepatitis B, or hepatitis C patients.

To clarify the transmission of the clones we intend to study the status of each of the clones in their serum, and their prevalence in patients having had blood transfusions or those who have undergone maintenance hemodialysis, and who might be thought of to be at high risk for blood transmitted diseases.

Most core protein consists of a tertiary structure with helix or sheet structures. Exposing parts of these structures requires mostly hydrophilic amino acids. So we selected each sequence with DNASIS analysis as the epitope. But at this time, there is no definitely evidence

that those are the epitope truly. Hydrophilic portion is not always epitope, and hydrophobic portion is also epitope some times. But another study in line with the other screenings that we intend to carry out in the future will decide definitely.

The probes synthesized from polynucleotides derived from these cDNAs have an immunodiagnostic utility, such as Enzyme linked immunosorbent assay does.

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非B非C型肝炎患者から得られた，二つのラムダgt11 cDNAクローン

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目　　的：日本では，慢性活動性肝炎患者や，肝細胞癌の有無に関わらず肝硬変患者の内で，約10％はその原因が不明である。それらの患者は，組織所見はウイルス肝炎に合致し，今まで知られている肝炎ウイルスマーカーはすべて陰性である事を確認して，非Ｂ非Ｃ型慢性肝炎と除外診断されている。

デザイン：非Ｂ非Ｃ患者の血清RNAに由来するcDNAライブラリーから得た，二つのラムダgt11クローンに対する抗体検査を行った。これらのクローンは，組織学的には慢性活動性で，臨床的にアルコール性・薬剤性・脂肪肝を十分に除外した患者から得られた。

方　　法：免疫スクリーニング法を用いて，二つのクローン（669と990）に対する抗体の有無の検査を，健常人60人・Ｂ型慢性肝炎患者31人・Ｃ型慢性肝炎患者113人・非Ｂ非Ｃ型慢性肝炎患者40人で行った。

結　　果：クローン669に対する抗体は，健常人で０％，非B非C患者で27.5％，Ｂ型慢性肝炎患者で9.7％，Ｃ型慢性肝炎患者で6.2％の陽性率であった。クローン990に対しては，健常人で０％，非Ｂ非Ｃ患者で15％，Ｂ型慢性肝炎患者で3.2％，Ｃ型慢性肝炎患者で0.9％の陽性率であった。
57人の輸血歴を有する患者では，クローン669に対しては，３人のC型慢性肝炎患者で陽性（5.3％）であった。クローン990に対しては，１人の非Ｂ非Ｃ型肝炎患者で陽性（1.8％）であった。40人の非Ｂ非Ｃ肝炎患者を組織学的分類で分けて検討すると，クローン669では，急性肝炎で11％，軽度から中等度慢性活動性肝炎で29％，高度慢性活動性肝炎で50％の陽性率であった。クローン990では，急性肝炎で11％，軽度から中等度慢性活動性肝炎で21％，高度慢性活動性肝炎で10％の陽性率であった。

結　　語：分離されたクローンにより規定されるペプチドが，非Ｂ非Ｃ型慢性活動性肝炎の抗原決定基の一部分である可能性を，これらの結果は示唆する。