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Correlation of Serum Antibody Titers against Hepatitis C Virus Core Protein with Clinical Features by Western Blot (Immunoblot) Analysis Using a Recombinant Vaccinia Virus Expression System

AKIRA NISHIZONO,[1]* MASAHARU HIRAGA,[1] KUMATO MIFUNE,[1] HIDEO TERAO,[2] TOSHIO FUJIOKA,[2] MASARU NASU,[2] TOSHIO GOTO,[3] JUN-ICHI MISUMI,[3] MITSUHIKO MORIYAMA,[4] YASUYUKI ARAKAWA,[4] NAKANOBU HAYASHI,[5] MARIKO ESUMI,[5] AND TOSHIO SHIKATA[5]

Department of Microbiology,[1] Second Department of Internal Medicine,[2] and Department of Public Health and Hygeine,[3] Oita Medical University, Hasama-machi, Oita 879-55, and Third Department of Internal Medicine[4] and First Department of Pathology,[5] Nihon University School of Medicine, Ooyaguchi, Itabashi-ku, Tokyo 173, Japan

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In order to study the relationships among the clinical features of hepatitis C patients, the presence of hepatitis C virus (HCV) RNA in their blood, and their serum antibody titers against the core protein of virus and to study the antibody levels in asymptomatic HCV carriers, a recombinant vaccinia virus containing a core protein gene was constructed. The recombinant virus expressed a protein with a molecular mass of 22 kDa in RK-13 cells as determined by Western blot (immunoblot) analysis. By using the cell lysate of virus-infected cells and serially diluted serum samples, core antibody titers in the groups of patients in the chronic hepatitis phase and in the convalescent phase as well as in asymptomatic carriers were determined by enhanced chemiluminescence Western blot analysis. Almost all patients in the chronic phase were shown to have high antibody titers of more than 1:500,000 and with no exception had of HCV RNA in their sera. On the other hand, patients who had recovered naturally and were in the convalescent phase were shown to have significantly lower antibody titers, and the antibody was not detected in the lowest serum dilution of 1:500 in 43% of these patients (three of seven total patients). Antibody levels of patients who showed a good response to interferon treatment decreased to intermediate levels between those of patients in the chronic phase and those of patients in convalescent phase. The antibody titers in asymptomatic carriers varied considerably from 1:500,000 to 1:500, and 41% (11 of 27 total individuals) of these carriers showed a high titer equivalent to that of those in the chronic phase. Core antibody was detected consistently in the individuals in whom HCV RNA was detected. This system for core antibody might be useful for identifying the stage of an apparent HCV infection.

The hepatitis C virus (HCV) genome is a positive-stranded RNA approximately 9,400 nucleotides (nt) in length with a single open reading frame and analyses of the deduced amino acid sequences have suggested that HCV is closely related to the *Flaviviridae* (6, 14, 24). The infectivity of HCV in blood has been reported to be 10^2 to 10^3 chimpanzee infectious doses, or 10^6 times lower than that of hepatitis B virus (1, 2, 27). Polymerase chain reaction (PCR) has been utilized to detect the HCV genome in patient sera or liver specimens (8, 13, 26). However, there have been no means to demonstrate the presence of virus particles or viral antigens in the patients' sera except for the detection of core protein in the plasma by concentration using buoyant density ultracentrifugation (23). Consequently, it is difficult at this moment to analyze the direct relationship between the clinical features and the amount of virus in HCV infection, which compels us to study it only by antibody tests.

Artificial expression of a partial protein of HCV has enabled us to develop the serological diagnosis system for HCV infection which is known as the anti-C100-3 assay (7). As a result, HCV has been identified as a major causative agent of posttransfusional and sporadic non-A, non-B hepatitis (15). More recent developments of the enzyme-linked immunosorbent assay (ELISA) system using synthetic peptides or structural proteins expressed in *Escherichia coli* and by baculovirus or a eukaryotic expression system have provided more sensitive and earlier diagnosis of HCV infection (4, 10, 12, 17, 18).

In the present study, we have constructed a recombinant vaccinia virus to express the putative core protein of HCV in eukaryotic cells and an attempt has been made by enhanced chemiluminescence Western blot (immunoblot) (ECL-WB) to search for possible relationships among stages of HCV infection, the presence of HCV RNA in the blood, and the serum antibody titers against core protein.

MATERIALS AND METHODS

Construction of recombinant vaccinia virus expressing HCV core protein. The HCV cDNA clone used in the present study was from our previous study and was isolated from a single Japanese chronic hepatitis patient (11). Our cDNA clone has 96% nucleotide sequence homology (and the protein has 95% amino acid homology) to the core protein region (14, 19, 20, 24) of the previously reported Japanese clone (J4) which is the most common Japanese type. A cDNA fragment 576 nt in length from authentic ATG start codon, which encodes the core protein, was amplified by the PCR method using synthetic oligonucleotide sense and antisense primers and the HCV cDNA as a template. The sequence of the antisense primer was designed to include a

* Corresponding author.

nonsense stop codon (TAA) just downstream of the last (no. 191) amino acid (alanine) of the protein. The cDNA fragment thus obtained was inserted into the unique *Sma*I site of the pSC11 vaccinia virus coexpression vector plasmid, which was provided by C.-J. Lai, National Institute of Health, Bethesda, Md. This recombinant pSC11 plasmid was transfected by the calcium phosphate method to RK-13 cells which had been infected with wild-type vaccinia virus as described elsewhere (16). The vaccinia virus used in the present study was the LC16m8 strain (22) obtained from S. Hashizume, Chiba University, Chibashi, Japan. Vaccinia virus recombinants expressing HCV core protein (rVacC) were selected by β-galactosidase assay (3) on thymidine kinase mutant L929 cells in the presence of bromodeoxyuridine (25 μg/ml). After three consecutive plaque purifications, recombinant virus stocks were prepared in RK-13 cells. The infectivity titer of the stock virus was 5×10^8 PFU/ml. Recombinant vaccinia virus vSC8 (10^7-PFU/ml infectivity titer) containing the *lacZ* gene but no HCV sequence was also provided by C.-J. Lai and used for negative control virus.

Human blood samples. In this study, we analyzed 54 consecutive cases diagnosed as posttransfusional or sporadic HCV infection. Of the 54 patients, 20 persistently had both elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (at least twice the upper limit of the normal ranges, which are <35 and <40 IU/liter, respectively) for at least 1 year and upon histological examination exhibited chronic active hepatitis. These 20 patients were all positive for anti-C100-3 antibody (Ortho Diagnostic Systems, Raritan, N.J.) and/or for HCV RNA. This group was defined as the chronic hepatitis group. Of these 20 patients, 16 received interferon therapy (8 patients received 504 mega international units [MIU] [total doses] of natural alpha human interferon, 6 patients received 348 MIU [total doses] of recombinant alpha-2a human interferon, and 2 patients received natural 270 MIU [total doses] of beta interferon). Blood samplings were performed during a period of 10 to 24 months after the end of interferon therapy. Simultaneously, the levels of transaminase and the presence of HCV RNA were determined in these cases. The remaining 4 patients did not receive interferon treatment.

Seven patients who had recovered naturally from posttransfusional or sporadic acute HCV infection were in the convalescent state. Acute hepatitis C was determined from the presence of HCV RNA in the elevated-transaminase phase and typical histological findings of acute hepatitis. In these patients, transaminase levels remained within a normal range for more than 6 months after the acute phase (at this point, these patients were judged to be in the convalescent phase) and afterward the transaminase levels did not show any fluctuation. Blood samples were taken during a period of 6 to 12 months after the patients were determined to be in convalescence.

Blood samples from another 27 individuals were collected from asymptomatic carriers of HCV identified by an annual epidemiological investigation in a certain district of Oita Prefecture, Japan. None of them had a history of acute hepatitis, chronic hepatitis, or other liver diseases. All in this group were positive for anti-C100-3 antibody and/or HCV-RNA but had not exhibited abnormal ALT and AST levels for 3 to 7 years. Liver biopies were not performed in these cases, but liver function tests were performed once a year.

Western blot analyses (ECL-WB). Recombinant vaccinia virus (rVacC)-infected cell lysates were used as antigens. Antigen preparation and the standardization of Western blot were as follows. RK-13 cells (10^7) were infected with rVacC at a multiplicity of infection of 1 PFU per cell and incubated for 24 h at 37°C. The infected cells were washed with phosphate-buffered saline (PBS) twice and then lysed with 1 ml of lysing buffer (25 mM Tris-HCl, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol), and the supernatant was collected after ultracentrifugation at 13,000 × *g* for 10 min. The protein concentration of the supernatant was adjusted to 700 μg/ml with lysing buffer, and 10 μl of the supernatant was applied per lane after boiling for 5 min. Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide minigel electrophoresis, electroblotted onto polyvinylidene difluoride membranes, and blocked with 5% skim milk (Difco, Detroit, Mich.) in PBS containing 0.1% Tween 20. Test sera were serially diluted 10-fold from 1:500 to 1:5,000,000 with 1% skim milk in TBS (20 mM Tris, 137 mM NaCl, pH 7.6)–0.1% Tween 20) and incubated with polyvinylidene difluoride membranes for 2 h at room temperature. After three washings with TBS–0.1% Tween 20, blotted membranes were reacted with 1:5,000-diluted peroxidase-conjugated goat anti-human immunoglobulin γ or μ chain (Cappel, Malvern, Pa.) for 1 h at room temperature. After the membranes were washed three times with TBS–0.1% Tween 20, the ECL-WB detection system (Amersham, Tokyo, Japan) was added to the membranes as described by the supplier and reacted for 1 min. Membranes were then covered with plastic wrap and exposed to X-ray film (RX; Fuji Film, Tokyo, Japan) for 1 min.

HCV serological tests. Anti-HCV antibody was measured by a first-generation ELISA (EIA-1; Ortho Diagnostic Systems) according to the manufacturer's directions. This assay kit utilizes a recombinant HCV antigen, designated C100-3, which is a fusion protein of an HCV nonstructural gene product combined with yeast superoxide dismutase (15). In a group which was believed to consist of asymptomatic carriers, all samples were further examined by the immunoblot assay RIBA II (Ortho Diagnostic Systems) (25). In RIBA II, the antigens expressed in yeast cells by both the core region (C22-3) and the nonstructural NS3 region (C33c) genes were added to the 5-1-1, C100-3, and superoxide dismutase antigens present in the first-generation assay. The antigens were immobilized in bands on nitrocellulose strips, and the antigen band intensities after reaction with serum samples were compared with those of weakly positive (level 1) and moderately positive (level 2) immunoglobulin G control bands. A response of 1+ or greater (up to 4+) to any two or more HCV antigens was interpreted as a positive result, a response to one antigen was interpreted as intermediate, and no response to any antigen or response only to the superoxide dismutase band was interpreted as a negative result.

Detection of HCV RNA. HCV RNA in patient sera was detected by a reverse transcription nested PCR performed by a slight modification of the procedure described by Garson et al. (8), using synthetic oligonucleotide primers to detect a cDNA fragment of HCV 5′-noncoding region (20). In this assay, the external primer pair consists of a sense primer of 5′-GGCGACACTCCACCATAGAT-3′ (nt 18 to 37) and an antisense primer of 5′-ACCCTATCAGGCAGTA CCAC-3′ (nt 283 to 302) (20). The internal primer pair consists of a sense primer of 5′-TCCCGGGAGAGCCATGG TGG-3′ (nt 127 to 146) and an antisense primer of 5′-AAG GCCTTTCGCGACCCAAC-3′ (nt 263 to 282). Numbering of the HCV sequence is based on that of Han et al. (9).

Total RNA was extracted from 100 μl of serum by the guanidium thiocyanate-phenol-chloroform procedure (5).

Reverse transcription was performed on half of the RNA volume with a random hexaprimer (50 ng/ml) (TAKARA Shuzo, Kyoto, Japan). The first round of amplification (35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min was performed with the external primer pair. In the second round, 1 μl of the first reaction product was amplified through 35 cycles with the internal primer pair by the same protocol as above. The PCR products (10 μl) were analyzed by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Expression of HCV core protein. Indirect immunofluorescence and Western blot techniques were used to analyze the expression of HCV core protein in the rVacC-infected RK-13 cells. A serum sample from a chronic non-A, non-B hepatitis patient who was positive for both the C100-3 antibody and HCV RNA was used as the first antibody for immunofluorescence study. Strong fluorescence with a granular pattern was observed in the cytoplasm of the infected cells (Fig. 1A). There was no obvious fluorescence in the vSC8-infected cells or in the rVacC-infected cells stained with normal human serum (Fig. 1B).

In the Western blot analysis, rabbit antiserum directed against the 120 amino acids of HCV core region expressed as a fusion protein with β-galactosidase in *E. coli* (kindly supplied by C. Nozaki and K. Mizuno, Chemo-Sero-Therapeutic Co., Kumamoto, Japan) was used as the first antibody. By using this antibody, a significant 22-kDa band was detected (Fig. 2A, lane 1). A band with the same mobility was also observed when the sample was reacted with the same patient serum used in the immunofluorescence study (Fig. 2A, lanes 3 and 4). A band in lane 3 was visualized by using peroxidase labeled-anti-human μ chain as the second antibody instead of anti-human γ chain (Fig. 2A, lane 4), suggesting that immunoglobulin M antibody against HCV core protein could be detected in some cases of chronic hepatitis. No significant signal was detected with normal human serum (Fig. 2A, lane 5) or in vSC8-infected cell lysates reacted with rabbit antiserum and the patient serum (Fig. 2A, lanes 2 and 6). Several faint bands of higher molecular weight might be the background derived from the proteins coded for by vaccinia virus genes.

To determine the antibody titer by ECL-WB, we usually used human serum samples serially diluted 10-fold from 1:500, and the titer was expressed as the reciprocal of the maximum dilution with which the 22-kDa band can be clearly recognized. In the case of Fig. 2B, the 22-kDa band could be distinctly recognized until a dilution of 1:50,000 was reached but not clearly recognized in a dilution of 1:500,000, and therefore, the core antibody titer was expressed as 1:50,000, though a serum sample of chronic hepatitis diluted to 1:500 showed high background (Fig. 2B, lane 2), probably from excess antibodies.

Core antibody titers in blood samples from patients with chronic hepatitis, with good response to interferon treatment, and in the convalescent phase after acute HCV infection. As shown in Fig. 3, all of the chronic hepatitis C patients possessed core antibody titers of more than 1:50,000 and most of them showed core antibody titers of 1:500,000. HCV RNA was present in all members of this chronic hepatitis group. On the other hand, the titers were apparently lower in convalescent-phase patients, and three of these patients (43%) showed a titer of less than 1:500. ALT levels of all seven patients in this group had normalized directly from the acute phase without progressing into the chronic phase. HCV RNA was not detected in six patients (86%) of this group but was still present in the patient having the highest antibody titer (1:5,000) in this group.

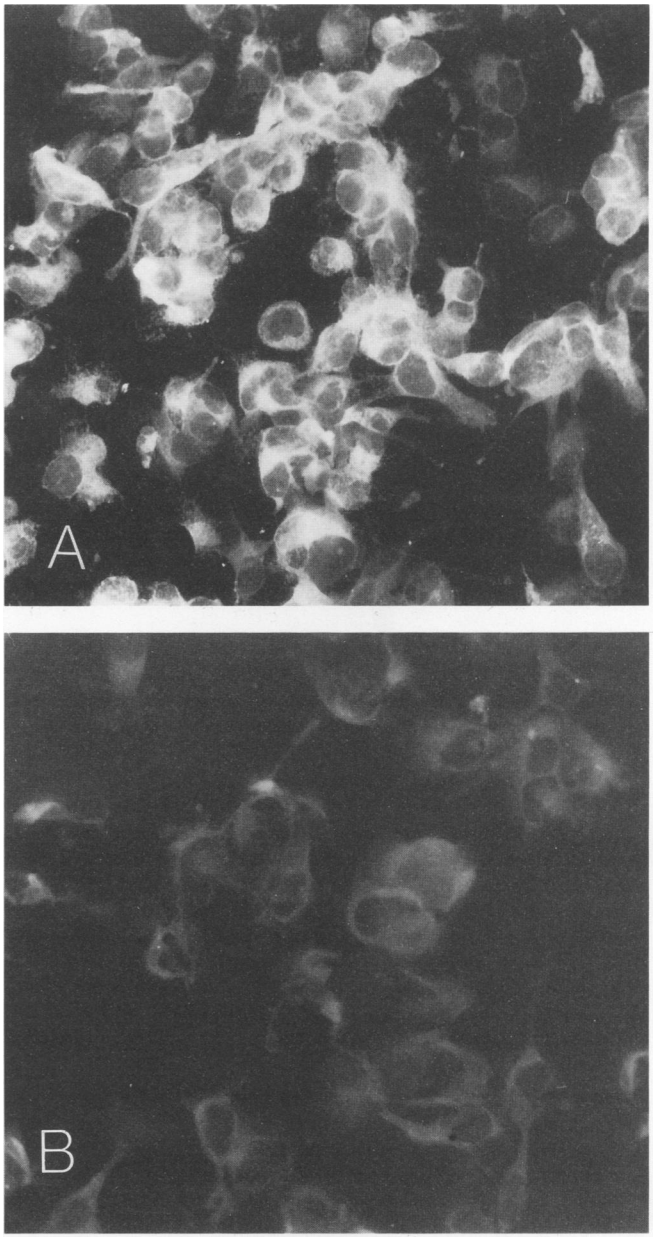

FIG. 1. Expression of HCV core protein by indirect immunofluorescence with a serum sample from a chronic hepatitis C patient in rVacC-infected (A) and vSC8-infected (B) RK-13 cells.

Sixteen patients with chronic hepatitis received interferon treatment, and core antibody levels were determined in the blood samples obtained from 10 to 24 months after the end of interferon therapy as described in Materials and Methods. Nine of sixteen cases showed good response to interferon treatment; that is, the ALT and AST levels and other liver functions decreased at the end of therapy, and afterward the liver functions remained in the normal range continuously for 10 to 24 months at least. As shown in Fig. 3, core antibody titers of good responders to interferon treatment

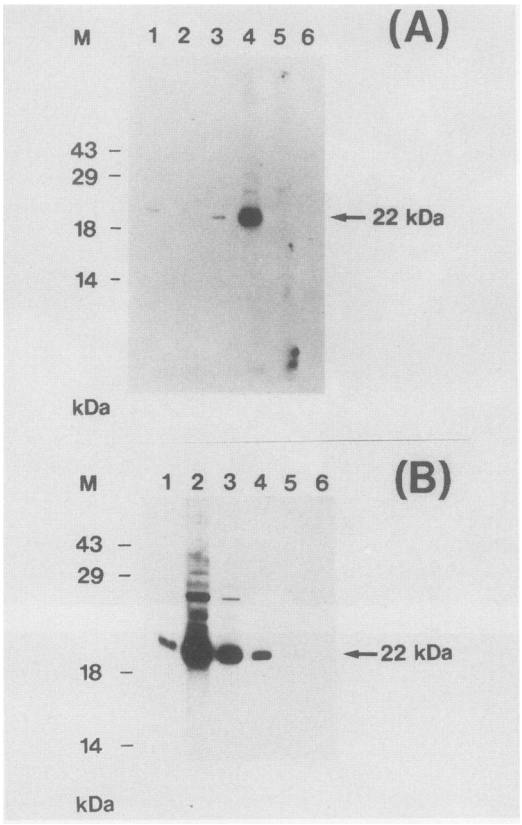

FIG. 2. (A) Western blot analysis of the proteins expressed in rVacC-infected and vSC8-infected RK-13 cells. Cell lysates of rVacC-infected (lane 1) and vSC8-infected (lane 2) RK-13 cells were reacted with rabbit serum raised against the 120 amino acids of HCV core protein. Lanes 3 and 4, lysates of rVacC-infected cells reacted with a serum sample (1:50,000 dilution) from a chronic hepatitis C patient and with peroxidase-labeled anti-human μ chain and γ chain; lane 5, lysate of rVacC-infected cells reacted with similarly diluted normal human serum; lane 6, lysate of vSC8-infected cells reacted with serum from a chronic hepatitis C patient and with peroxidase-labeled anti-human γ chain. (B) Immunoreactivity of serially diluted patient serum against HCV core protein expressed in rVacC-infected cells. Lane 1, infected-cell lysate reacted with 1:5,000-diluted rabbit serum against the 120 amino acids of HCV core protein; lanes 2 to 6, infected-cell lysates reacted with 1:500-, 1:5,000-, 1:50,000-, 1:500,000-, and 1:5,000,000-diluted serum from a chronic hepatitis patient.

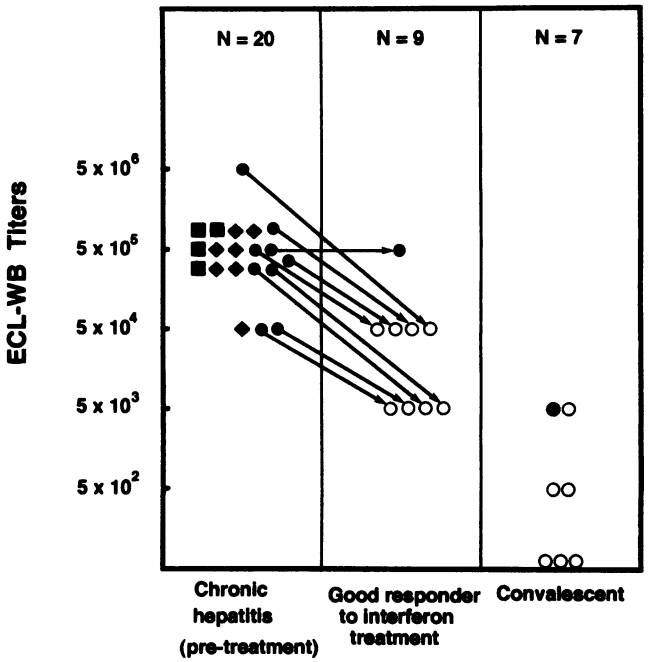

FIG. 3. Distribution of Western blot antibody titers against HCV core protein in the sera from individuals with chronic hepatitis C, with good responses to interferon treatment, and in the convalescent phase. Symbols: ●, positive for HCV RNA; ○, negative for HCV RNA; ◆, nonresponder to interferon treatment and positive for HCV RNA; ■, no interferon treatment and positive for HCV RNA.

decreased to the intermediate levels between those of the chronic hepatitis group and the convalescent group. Moreover, HCV RNA disappeared in all cases except one, and this case showed no change in core antibody titer after interferon treatment. By contrast, no improvement was observed in the liver functions and core antibody titers in seven other cases (◆ in Fig. 3) after interferon treatment and HCV RNA remained present (data not shown).

Distribution of core antibody titers in HCV asymptomatic carriers. Table 1 summarizes the relationships among core antibody titers, HCV RNA, anti-C100-3 antibody, and RIBA II in 27 individuals who were suspected to be HCV asymptomatic carriers. Eleven (41%) of the group showed core antibody titers of more than 1:500,000, and all individuals were positive for HCV RNA with one exception. But this individual was positive both for anti-C100-3 antibody and by RIBA II. Another was negative for anti-C100-3 antibody, but HCV infection was confirmed by RIBA II. Six (22%) of this group showed core antibody titers of 1:50,000, and all of these were positive for HCV RNA. There was no discrepancy in positivity between anti-C100-3 antibody and RIBA II.

However, the lower the core antibody titers were, the greater the appearance of discrepancy in positivity among anti-C100-3 antibody, RIBA II, and HCV RNA was, although there was no essential discrepancy between the positivity of HCV RNA and core antibody. In the cases of an antibody titer of 1:5,000, five of six individuals were positive for HCV RNA and four of six were positive for anti-C100-3 antibody, but by RIBA II only two of six were positive, and these two cases were intermediate and negative. However, there was no case that was negative in all of these tests. Four (4%) of the group showed core antibody titer of 1:500, and all

TABLE 1. Characterization of suspected HCV asymptomatic carriers by determination of several parameters of HCV infection

Core antibody titer	No. of individuals[a]							
	Total (%)	HCV RNA		Anti-C100-3		RIBA II		
		P	N	P	N	P	I	N
≥500,000	11 (41)	10	1	10	1	11	0	0
50,000	6 (22)	6	0	6	0	5	1	0
5,000	6 (22)	5	1	4	2	2	2	2
500	4 (15)	4	0	1	3	0	0	4
Total (%)	27 (100)	25 (93)	2 (7)	21 (78)	6 (22)	18 (67)	3 (11)	6 (22)

[a] P, positive; N, negative; I, intermediate.

of these individuals were positive for HCV RNA. On the contrary, only one individual was positive for anti-C100-3 antibody, and all showed negative results with RIBA II. This inconsistency in the results of these tests might be largely due to a different sensitivity between the HCV RNA test and the other two anti-C100-3 antibody and RIBA II tests. These results suggest that our ECL-WB system for core antibody is as good as reverse transcription nested PCR methods in its sensitivity for the detection of HCV infection.

DISCUSSION

In the present study, we analyzed the relationships between the serum core antibody levels and the clinical features of hepatitis C patients by Western blot analysis using recombinant vaccinia virus. By the use of artificially expressed or synthesized polypeptides, core antibody detection systems such as ELISA have been developed and proposed to be useful for rapid and sensitive diagnosis of acute and chronic hepatitis C (4, 10, 17). Although Western blotting is semiquantitative and time-consuming and can probably detect only the antibodies recognizing the primary structures of the protein and therefore further improvement is necessary for its routine use, the advantages of this method are that we can quantitate the antibodies much more easily than by other methods, because of its high sensitivity, and consequently can monitor the changes of antibody titers during a long clinical course and test the effectiveness of interferon therapy.

The present study demonstrated that core antibody titers were significantly higher in the chronic hepatitis phase than in the convalescent phase of those who recovered naturally from acute HCV infection. Antibody titers of good responders to interferon treatment were at levels intermediate between those of the above two groups. These observations suggest that the level of core antibody is closely correlated with the stage of HCV infection. However, this hypothesis does not appear to be applicable to asymptomatic carriers as described below. Its usefulness in determining the stage of HCV infection seems to be limited to apparent infection until the nature of asymptomatic carriers is clarified.

In the present study, asymptomatic carriers were defined to be the patients having a normal range of ALT and/or AST levels and at least one positive result either to anti-C100-3 antibody or to HCV RNA. This group was further examined for their core antibody titers and RIBA II.

Of interest was the finding that core antibody titers in asymptomatic carriers were considerably varied and 17 individuals exhibited high titers of antibodies equivalent to those of chronic hepatitis patients, indicating that even though ALT and AST levels are in the normal range, the core antibody levels are not necessarily low but consist of a variety of antibody titers from extremely high to very low. The wide variety of the antibody levels might suggest that asymptomatic carriers are not a uniform group but rather comprise a variety of individuals. While the majority of HCV RNA-positive patients has been thought to be associated with liver dysfunctions, there were many asymptomatic individuals having normal ALT and/or AST levels but positive for HCV RNA. Whether this finding represents subclinical hepatic damage or possible HCV replication in a secondary site (other than the liver) remains obscure.

Recent retrospective histological studies with liver biopsy specimens have shown that the presence of lymphoid aggregates and/or follicles in the tissue and a relatively weak necroinflammatory finding in the parenchyma but severe inflammation in the portal area are characteristic of chronic hepatitis C (21). Thus, an examination to determine whether asymptomatic HCV carriers have subclinical or cryptic hepatic damage would be of great interest in further characterizing or classifying asymptomatic carriers and in searching for an analogy between the HCV carrier and the hepatitis B virus carrier states. In hepatitis B virus infection, asymptomatic healthy carriers are defined as being continuously positive for viral antigen and anti-core antibody (HBc) in the blood but without any finding of hepatitis, including histological features.

The present study demonstrated that our system for core antibody determination is as sensitive as the reverse transcription nested PCR method for HCV RNA in the diagnosis of HCV infection. Core antibody was consistently detected in individuals whose blood was positive for HCV RNA; however, the reverse was not necessarily true. Almost all individuals who were good responders to interferon treatment were shown to be HCV RNA negative in spite of having intermediate levels of core antibody. A few similar cases were also noticed in asymptomatic carriers. The presence of core antibody in HCV RNA-negative cases might be explained either by the strong memory of core antibody production or by the long half-life of antibodies.

The recombinant vaccinia expression system might be useful in performing similar studies with HCV envelope and nonstructural 1 (NS1) proteins with carbohydrate chains and also in clarifying the possible involvement of immunopathological events in the pathogenesis of HCV infection.

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