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Serodiagnosis of hepatitis C virus (HCV) infection with an HCV core protein molecularly expressed by a recombinant baculovirus

(non-A, non-B hepatitis/ELISA/early diagnosis/blood screening)

JOE CHIBA*, HIROYOSHI OHBA*, YOSHIHARU MATSUURA†, YUSHIRO WATANABE‡§, TOHRU KATAYAMA¶, SHU KIKUCHI‖, IZUMU SAITO‡**, AND TATSUO MIYAMURA‡

*Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278, Japan; Departments of †Veterinary Science and ‡Enteroviruses, National Institute of Health, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan; §Second Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216, Japan; ¶Department of Surgery, Tokyo National Chest Hospital, 3-1-1 Takegaoka, Kiyose-shi, Tokyo 204, Japan; and ‖Department of Surgery, National Sendai Hospital, 2-8-8 Miyagino, Sendai 983, Japan

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ABSTRACT **An enzyme-linked immunosorbent assay (ELISA) was developed for serological diagnosis of hepatitis C virus (HCV) infection, using HCV core protein (p22) synthesized by a recombinant baculovirus. Among 58 clinically well-defined chronic non-A, non-B hepatitis (NANBH) patients, 49 (84.5%) were positive for p22 antibody (anti-p22), whereas 42 (72.4%) were positive for C100-3 antibody (anti-C100-3), as measured by the present assay using the HCV nonstructural protein as antigen. Thirty-nine patients (67.2%) had both antibodies. No significant level of anti-p22 was detected in sera of chronic hepatitis B patients or normal blood donors. In typical post-transfusion NANBH patients, anti-p22 could be detected at, or even before, the first alanine aminotransferase peak. Anti-p22 was also detected in blood donors who were previously shown to be involved in transmitting HCV but in whose serum anti-C100-3 was not detectable. The ELISA detecting antibody to the HCV core protein expressed and properly processed in animal cells will be useful for mass screening of donor blood as well as for early diagnosis of hepatitis C.**

Non-A, non-B hepatitis (NANBH) remains the most common and serious sequel of blood transfusion. The etiological agent(s) of NANBH has long been sought by many research groups (reviewed in refs. 1 and 2), and finally an agent, termed hepatitis C virus (HCV), was identified by molecular cloning and characterization of its RNA genome (3). By using an HCV antigen, C100-3, synthesized by the recombinant DNA method in yeast, a specific assay was developed for detection of the antibody to HCV (4). Worldwide application of this assay has demonstrated that HCV is the major causative agent of transfusion-associated NANBH (4–6). This assay has also detected both HCV carriers and individuals who have had a previous, but clinically resolved, infection with HCV (6, 7). Retrospective testing of stored sera from prospective post-transfusion hepatitis studies indicates that screening of blood donors for anti-HCV antibodies will prevent many cases of NANBH (4–7). However, C100-3 antibody (anti-C100-3) was not detected in all post-transfusion NANBH cases, probably because of the delayed response to C100-3 antigen (4–8) or the existence of different types of HCV (9, 10). Although seroconversion occurs during the acute phase of the infection in about a third of the cases, in more than half of the patients anti-C100-3 is first detected 4–6 months after transfusion and, in some cases, the antibody response takes place considerably later (5, 6, 8). Blood donors with infectious virus but negative for anti-C100-3 for prolonged periods have been reported (11).

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Such delayed response was considered to be due to the fact that the C100-3 antigen is a protein encoded by part of the nonstructural 3-4 region of the HCV genome. The purpose of this study was to establish a more sensitive HCV antibody assay, which could be used for early diagnosis of HCV infection. The entire HCV structural gene was inserted into a baculovirus vector and insect cells were infected with the recombinant virus. The expressed protein was an unglycosylated 22-kDa nucleocapsid (core) protein. By using this protein, an antibody detection system was established to develop a specific sensitive method for diagnosing HCV infection (12).

MATERIALS AND METHODS

Construction of Recombinant Baculovirus. First, to express the HCV core protein in insect (*Spodoptera frugiperda*) cells, a baculovirus transfer vector, pAc316, was constructed (Fig. 1). The HCV cDNA fragment from nt 308 (*Acc* I site) to nt 1662 (*Eco*RI site created by linker ligation) was cleaved out from the plasmid pS7/1-216, which contains the whole structural coding region of the HCV cDNA derived from healthy Japanese HCV carriers (15). The fragment, starting from 12 nt upstream of the polyprotein initiation codon, was treated with the Klenow fragment of DNA polymerase and then inserted into a cassette plasmid, pAcYM1 (16), downstream of the polyhedrin promoter by blunt-end ligation. The construct was then transferred into *Autographa californica* nuclear polyhedrosis virus (AcNPV), a baculovirus, by homologous recombination to make a recombinant virus, Ac316. The recombinant baculovirus was isolated after cotransfection of *S. frugiperda* cells with mixtures of AcNPV DNA and pAc316 DNA as described previously (16).

Expression of Nucleocapsid Protein in Insect Cells. Monolayers of *S. frugiperda* cells (1×10^7) were infected with the recombinant, Ac316, or wild-type AcNPV at a multiplicity of about 10 plaque-forming units/cell. Whole-cell extracts of infected or uninfected cells were prepared 72 hr after the infection and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). For immunoblotting analyses, the method described by Towbin *et al.* (17) was employed.

Establishment of ELISA. *S. frugiperda* cells infected with Ac316 (10^7 cells) were washed with phosphate-buffered saline (PBS) and lysed by ultrasonication with 2 ml of 50 mM Tris·HCl, pH 8.0/2 mM ethylenediaminetetraacetate

Abbreviations: NANBH, non-A, non-B hepatitis; HCV, hepatitis C virus; nt, nucleotide(s); AcNPV, *Autographa californica* nuclear polyhedrosis virus; ALT, alanine aminotransferase (transaminase).

**Present address: Laboratory of Molecular Genetics, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108, Japan.

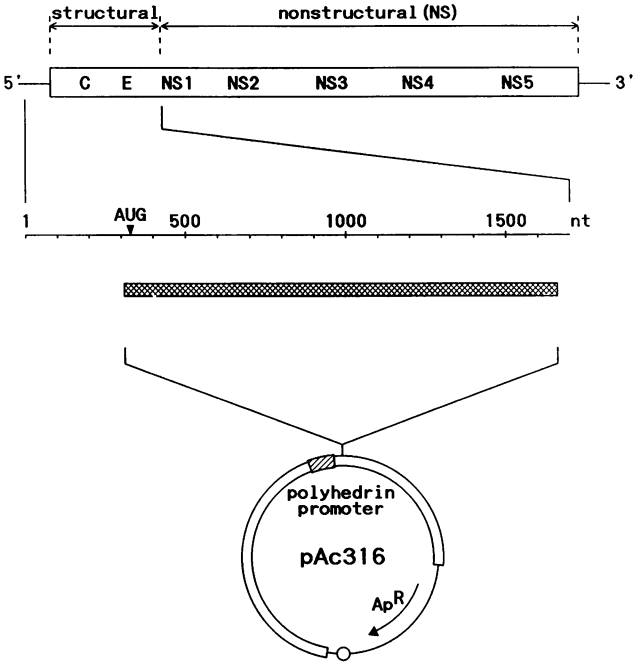

FIG. 1. Construction of a transfer vector plasmid to make a recombinant baculovirus. (*Top*) HCV genome structure (13, 14). The coding frame of the polyprotein is represented by the open box, in which each protein region is shown: C, core; E, envelope; NS, nonstructural protein. (*Middle*) Map of the structural region. Nucleotide (nt) 1 is the 5′ end of the HCV genome. AUG, initiation codon of the polyprotein. cDNA is shown as a cross-hatched box under the genome map. (*Bottom*) Plasmid pAc316. Ap^R, ampicillin-resistance gene; open circle, plasmid replication origin.

(EDTA)/0.1 mM dithiothreitol with 100 μg of phenylmethylsulfonyl fluoride per ml. After centrifugation at 10,000 × *g*, the supernatants were fractionated by adding ammonium sulfate to produce a 33% saturated solution. Precipitates were collected by centrifugation and dissolved in 2 ml of PBS with the help of ultrasonication to prepare partially purified HCV core protein. Wells of ELISA plates (Costar 3590) were then coated with 100 μl of the above solution, which was diluted 1:50 with PBS. After blocking with PBS containing 25% Block Ace (Dainippon Pharmaceutical, Osaka), 100 μl of serum was added to each well and the plates were incubated for 2 hr at room temperature. Bound antibody was detected by a second incubation with alkaline phosphatase-conjugated $F(ab')_2$ fraction of goat anti-human immunoglobulins (Tago 4603) for 1 hr at room temperature. The enzyme activity was measured by using *p*-nitrophenyl phosphate as substrate. The absorbance of each well was measured at 410 nm. The optimal dilution of the patient's serum was usually 1:400 to 1:800, which resulted in the highest ratio of specific to nonspecific signals. Anti-C100-3 was measured by the HCV antibody ELISA test (Ortho Diagnostics, Tokyo).

Determination of Cut-off Value. The cut-off value was calculated from the negative and positive control absorbance values, following the method described in the instruction manual for a commercial ELISA kit for hepatitis A antibody assay (HAVAB-M EIA, Abbott). Samples were determined as positive or negative by (*i*) testing for immunoblotting assay to the p22 antigen expressed in insect cells (described in this paper) or monkey and human cells (12, 31), and (*ii*) assay results of the 1365 blood samples transfused into recipients whose outcomes were analyzed (6, 7).

Patient Sera. Series of sera from well-characterized post-transfusion NANBH patients used in this study were part of collected sera that had been previously analyzed and described (6, 7). Basically, blood samples were taken from all patients who underwent surgery and blood transfusion, before as well as periodically after the operation. Serum samples derived from blood transfused into these patients were also kept. At that time, it was impossible to prevent post-transfusion NANBH because there was no available assay system that could detect the antibody to HCV in the blood. Patients in whom NANBH unexpectedly developed were placed on long-term follow-up. Alanine aminotransferase (alanine transaminase; ALT) levels were expressed in Karmen units (18).

RESULTS

To determine whether HCV core proteins were synthesized in the recombinant virus (Ac316)-infected *S. frugiperda* cells, the lysate of the infected cells was analyzed by SDS/PAGE with dye staining and immunoblotting techniques. In Ac316-infected but not in wild-type AcNPV-infected or uninfected cells, a band stained with Coomassie brilliant blue at a molecular mass of about 22 kDa was detected (Fig. 2). The serum from a patient with chronic NANBH, in whose serum anti-C100-3 had been demonstrated, reacted specifically with this 22-kDa protein, whereas there was no reaction with the lysate of AcNPV-infected or uninfected cells. In addition to the 22-kDa band, broad and less abundant bands above 50 kDa were detected in Ac316-infected cells. These 22- and 50-kDa bands were not detected when sera from normal blood donors were used as primary antibodies (data not shown). The 22-kDa protein is called p22 hereafter. When the infected-cell lysate was treated with glucanase (19), the broad band of 50 kDa converged to a single narrow one at the lowest position (data not shown), suggesting that the 50-kDa protein was glycosylated heterogeneously. In contrast, p22 did not show any evidence of glycosylation.

As the HCV cDNA inserted into pAc316 can potentially express amino-terminal parts of the viral polyprotein (441 amino acids long) which would compose a protein larger than 22 kDa, we thought it was probable that p22 was generated from precursor proteins of about 50 kDa by processing or cleavage at a specific site. The core protein of a flavivirus distantly related to HCV (13, 20) has been demonstrated to be

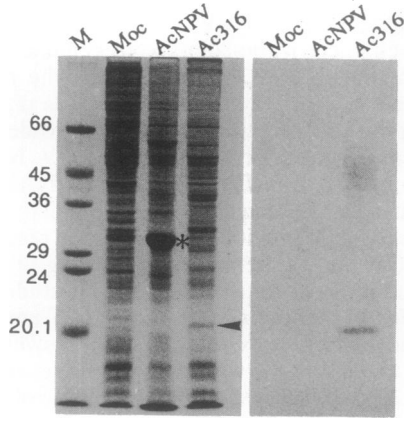

FIG. 2. Expression of HCV core proteins by the recombinant baculovirus. *S. frugiperda* cells were infected with the recombinant Ac316 or wild-type AcNPV and whole-cell extracts of infected or uninfected (Moc) cells were analyzed by SDS/PAGE with Coomassie brilliant blue staining (left) or immunoblotting (right). Sizes of molecular mass markers (lane M) are shown in kDa. The arrowhead in the Ac316 lane indicates p22 protein. The asterisk in the AcNPV lane indicates polyhedrin. The serum from a patient with chronic NANBH positive for anti-C100-3 was reactive with the p22 protein and probable precursor proteins which showed broad bands of about 50 kDa. So far, we have failed to detect envelope protein in this expression system by using this serum.

processed properly in the baculovirus expression system (16). The size of 22 kDa determined by this immunoblotting assay and lack of N-glycosylation are consistent with our predicted character of the HCV core protein from the deduced amino acid sequence (13, 15). Similar results were obtained when the same coding sequences of the HCV structural region were expressed in monkey and human cells (12). Reactivities of NANBH patients' sera against HCV core proteins expressed in monkey and human cells or in insect cells were the same (data not shown). These results suggest that the HCV core protein expressed in insect cells is properly processed to have antigenic structures that are recognized by antibodies raised in patients infected with HCV. However, we have not been able so far to detect a processed protein of the HCV envelope, possibly because of the lack of adequate antibodies against the HCV envelope protein.

To test whether this core protein produced in insect cells could be utilized for diagnosis of HCV infection, p22 was partially purified and an ELISA was established. In this partially purified preparation, both p22 and the 50-kDa precursor protein were detected (data not shown). Antibodies detected by the ELISA were provisionally termed p22 antibody (anti-p22). It should be noted that the antibody to the HCV envelope protein may potentially be detected by the ELISA, although we cannot, at present, detect the envelope protein or the antibody to it. For the current study, we first selected stored serum samples from 58 clinically well-defined chronic NANBH patients, from 10 chronic hepatitis B patients, and from 35 normal blood donors. All of the chronic NANBH patients demonstrated typical clinical courses and fulfilled the criteria of post-transfusion NANBH proposed by the Japanese Society of Gastroenterology in 1985. The absence of infectious NANBH virus in all of the normal donor blood tested had been proven by the fact that none of the recipients had developed post-transfusion hepatitis either clinically or revealed by ALT monitoring for over 2 years.

By the ELISA using the HCV core protein as an antigen, anti-p22 was detected in sera from 49 of the 58 chronic NANBH patients collected during the chronic phases of their disease (Table 1). Anti-C100-3 was detected in 42 patients. Thirty-nine (67.2%) of the patients had both antibodies, but 10 were positive only for anti-p22 and 3 were positive only for anti-C100-3. No significant level of p22 antibody was detected in the 10 chronic hepatitis B patients. Furthermore, anti-p22 was not detected in any of the 35 normal donors. Thus the ELISA using the HCV core protein promises to be a specific assay for HCV infection.

To establish the cut-off value of anti-p22 ELISA assay, we examined 1365 blood specimens, all of which were actually transfused and the clinical outcomes of their recipients were known. Among these, 700 samples were considered to be "normal" because they were transfused into recipients who did not show any signs of post-transfusion NANBH. Seven

Table 1. Anti-p22 and anti-C100-3 in 58 NANBH patients

Anti-p22	Anti-C100-3 +	Anti-C100-3 −	Total
+	39 (67.2%)	10 (17.2%)	49 (84.5%)
−	3 (5.2%)	6 (10.3%)	9 (15.5%)
Total	42 (72.4%)	16 (27.6%)	58 (100%)

Detection of anti-p22 and anti-C100-3 in well-characterized NANBH patients. The cut-off level for anti-p22 was taken as $\frac{1}{10}(A_{410}$ with positive control $- A_{410}$ with negative control) $+ A_{410}$ with negative control. A_{410} with positive control was 1.440 and A_{410} with negative control was 0.097 in this experiment. All of the serum samples were diluted to 1:800 with PBS containing 10% Block Ace and 0.05% Tween 20. C100-3 antibody was measured by the HCV antibody ELISA assay.

of these 700 specimens from "normal" blood donors just exceeded the cut-off value. Since none of these 7 samples were positive for anti-p22 by immunoblotting assay, we consider these 7 samples to be false positive. Alternatively, these recipients might have been infected with HCV but did not develop hepatitis C. We could not detect either C100-3 or p22 antibodies in these recipients. The cut-off value was determined as described in *Materials and Methods* and further tests were performed.

The specificity of the anti-p22 ELISA assay was confirmed by the following findings: (*i*) Among 16 blood samples that were anti-C100-3 positive and consequently could not be used for blood transfusion, 5 were positive for HCV cDNA by the reverse polymerase chain reaction method using primers derived from the HCV core region. All of these 5, but none of the other 11, were positive for anti-p22 (data not shown). (*ii*) Anti-p22 was detected in the blood of 22 of 30 implicated donors transfused into recipients who developed typical chronic NANBH after the transfusion (T.K., T. Matsuda, S. Harada, Y.M., J.C., H.O., I.S., and T.M., unpublished data). All of the recipients who received the 22 blood samples seroconverted to anti-p22. These findings indicate that the presence of anti-p22 was not a false-positive reaction but was closely associated with the existence of infectious HCV. The ELISA would, therefore, be useful for mass screening of donor blood for transfusion.

To test the usefulness of the ELISA using the p22 protein antigen for early diagnosis of hepatitis C, we then retrospectively looked for anti-p22 in two series of well-documented post-transfusion NANBH patients' sera (6, 7), which were collected for over 4 years, starting even before the blood transfusion. The first case (Fig. 3*a*) showed a typical clinical course of chronic NANBH with multiple peaks of ALT, a marker of liver cell damage caused by hepatitis. Anti-C100-3 appeared 57 weeks after the transfusion (6), and hence this patient was diagnosed as having hepatitis C. Surprisingly, a significant level of anti-p22 was already detectable in the serum taken only 7 weeks after the transfusion, when the first increase of ALT was observed. Then the level of the antibody reached an early peak just before the first ALT peak. The antibody level decreased once slightly but then increased gradually, linked to the following multiple ALT elevations. Finally, the antibody reached the maximum level at the time anti-C100-3 first appeared. After that, both antibodies maintained their high levels. We also assayed samples from donor blood that was actually transfused into this particular patient and found that one of 28 units was positive for both p22 and C100-3 antibodies. These results indicate that diagnosis at an early stage, such as at the time even before the first ALT peak, could be achieved by this ELISA using the HCV core protein antigen.

The second case (Fig. 3*b*) showed another typical clinical course of chronic NANBH, having a single ALT peak and maintaining low but abnormal ALT levels for years. Anti-C100-3 was consistently absent for over 4 years and all the donor blood samples transfused into this patient were also negative for anti-C100-3 (6). Therefore this patient's hepatitis had not yet been diagnosed as hepatitis C but, rather as unidentified post-transfusion NANBH. Surprisingly, again, just after elevation of the ALT but before the first ALT peak, the presence of anti-p22 was clearly demonstrated. The antibody reached its first peak at the time of the ALT peak, and then it decreased with the decline of the ALT level. Thereafter, a relatively low but significant level of the antibody was detected until 57 weeks after the transfusion. Then the antibody level increased gradually and reached its highest level at about 20 months after the transfusion. Interestingly, one of the seven units of donor blood was positive for anti-p22. These results suggest that, at least in this particular case, the ELISA using the HCV core protein not only

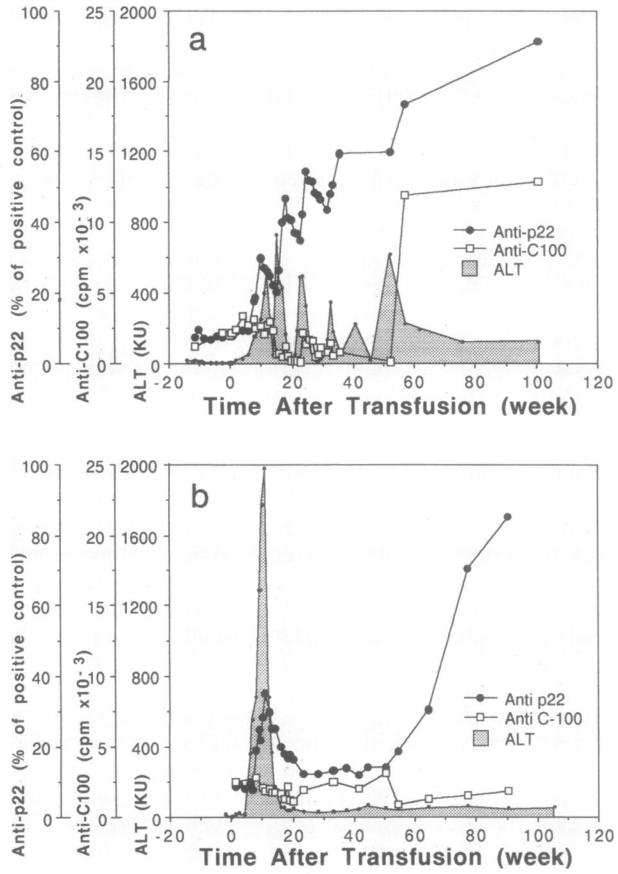

FIG. 3. Detection of anti-p22 in post-transfusion NANBH patients. The first case (*a*) shows a typical course of chronic NANBH with multiple peaks of ALT. The second case (*b*) showed another typical course of chronic NANBH, having a single ALT peak but maintaining a low but significant abnormal ALT value for years. ALT levels and anti-C100-3 levels are shown in Karmen units (KU) and cpm in a radioimmunoassay, respectively. Anti-p22 activity is shown as percentage of the positive control to minimize the difference of A_{410} values in different plates. The positive control sample was from donor blood that was positive for anti-C100-3 and induced NANBH in the recipient.

diagnosed HCV infection at an early stage but also identified the implicated donor blood, which could not be identified by the present anti-C100-3 ELISA test.

The usefulness of the anti-p22 assay for early diagnosis of HCV infection was further examined by comparing the times of seroconversion to p22 and C100-3 antibodies during the course of NANBH. Sequentially collected sera (7–30 months after the transfusion) from 23 patients who had developed typical post-transfusion NANBH were tested. We detected the seroconversion to anti-p22 at an early stage, 1–3 months after the transfusion, in 13 patients. At this stage, seroconversion to anti-C100-3 was observed in 4 patients, in all of whom anti-p22 was detected simultaneously. It was also noteworthy, however, that in 2 patients only anti-C100-3 was detected and in 5 patients neither antibody was detectable for 30 months after the transfusion.

DISCUSSION

HCV is a major causative agent of post-transfusion as well as sporadic parentally transmitted NANBH throughout the world. About half of the acute hepatitis C cases become chronic and many of them eventually develop to hepatocellular carcinoma (21–24). However, accumulating data have suggested that prompt interferon treatment can prevent the acute hepatitis C from becoming chronic (25, 26). For this reason, developing methods for the early diagnosis of hepatitis C is an important objective.

Previously we have cloned cDNA covering the entire structure region of the genome of a Japanese HCV isolate (9, 13). HCV has a positive-strand RNA genome of about 10 kilobases (3), and the HCV core protein is encoded in the amino-terminal domain of the structural region of the HCV genome (9, 13, 15). Amino acid identity of the HCV core protein region is 97% between the Japanese HCV isolate and the original HCV serially passaged in chimpanzees (13). To obtain sufficient HCV core protein, an insect baculovirus expression system was chosen in view of the reported high level of expression of foreign genes (16, 27) and proper processing of the core protein of flaviviruses, which are considered to be distantly related to HCV (13, 14, 20).

We have demonstrated the expression of the HCV core protein by recombinant baculovirus with an HCV cDNA covering the whole structural gene in animal cells. The 22-kDa protein expressed was indistinguishable from that in monkey COS cells transfected with the same cDNA fragment driven by the SRα promotor (12, 31) in size and reactivity with patients' sera. Since there are no potential glycosylation sites indicated by the nucleotide sequence of the core region (13), the HCV core protein expressed in *Escherichia coli* may have antigenic properties similar to those of the protein expressed in insect cells described in this study. A recent short report supports this possibility (28); Western blotting with the core protein expressed in *E. coli* could be useful for detection of HCV infection.

By using the core protein expressed by recombinant baculovirus, an ELISA was established to detect the antibody in NANBH patients' sera. This assay could detect antibody to HCV among patients who had not been diagnosed as having hepatitis C because of the absence of C100-3 antibody. It is noteworthy that diagnosis became possible at an early stage of HCV infection by a simple ELISA method. Our study also suggests that assay for anti-p22 should be used for screening blood. Since C100-3 antibody and p22 antibody react with different gene products of HCV and there are patients positive only for anti-C100-3 (Table 1), it is appropriate to use both assays or combined ones for screening blood. The more efficient detection of HCV by anti-p22 than by anti-C100-3 in this study may be partly due to the fact that the nucleotide sequence of the core region of the HCV genome is more conserved among different HCV isolates than is the nonstructural region 3-4 (13, 29, 30). Worldwide anti-p22 assays of a large number of patients with various liver diseases are now being conducted.

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