Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5971–5975, July 1992
Medical Sciences

Transient immunoglobulin M antibody response to hepatitis C virus capsid antigen in posttransfusion hepatitis C: Putative serological marker for acute viral infection

(RNA virus/chronic infection)

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Communicated by Saul Krugman, March 27, 1992

ABSTRACT **The development of serological assays for hepatitis C virus (HCV) has made specific diagnosis possible. However, markers useful in indicating acute-phase HCV infection have not been identified. By an immunoblotting method, we characterized the IgM and IgG antibody response against HCV capsid antigen in patients with HCV infection. Among 88% of patients with acute posttransfusion hepatitis C recruited in a prospective study, there was a transient IgM antibody response. The IgM antibody appeared shortly after onset of hepatitis (average 3.7 weeks), persisted for several months (average 18 weeks), and then disappeared. In contrast, the IgG antibody persisted long-term once it appeared. Among patients with chronic hepatitis C with milder disease activities (serum aminotransferase increase above normal levels of <4-fold), the IgM antibody was negative in the majority (72%). In those with acute exacerbations (aminotransferase increase of >10-fold), about 55% were negative for the IgM antibody. The reactivity of the IgM antibody in the rest was weaker or became negative upon further dilution of serum. The results suggest that IgM anti-capsid antibody may serve as a marker indicating acute or active HCV infection.**

Hepatitis C virus (HCV) is the major cause of non-A, non-B hepatitis worldwide (1, 2). Currently, sensitive serological or immunoblot assays are available to identify most HCV carriers (3, 4) and are used in blood screening to reduce effectively the incidence of posttransfusion hepatitis (5). The present assay of anti-HCV antibodies, although useful, cannot differentiate whether the infection is acute or chronic. The distinction between acute and chronic HCV infection is important in management of these patients. As HCV infection frequently becomes chronic and results in significant sequelae (6, 7), therapeutic intervention (such as interferon treatment) should be implemented as soon as the infection occurs (8). Accordingly, a serological marker indicating acute HCV infection is desperately needed.

Such a marker would also be useful for clarifying epidemiological discrepancies of HCV infection. For example, in the clinically acute non-A, non-B hepatitis, the anti-C100-3 seropositive rate in intravenous drug abusers is significantly higher than that in posttransfusion or sporadic cases (9, 10). Given the high likelihood of multiple exposures to HCV in intravenous drug abusers, many may have already contracted hepatitis C. Therefore, it is possible that a portion of such acute hepatitis C victims actually have chronic infection but with acute exacerbations.

One feasible approach for finding an acute HCV infection marker is, by analogy with hepatitis A and B, to look for virus-specific IgM antibody. Indeed, one study to correlate IgM antibody for a viral nonstructural protein (the C100-3 antigen) with acute HCV infection has been performed, but the results are not convincing (11). In the present communication, we show that by immunoblotting assay, the IgM antibody for HCV capsid antigen (anti-HCc IgM) was transiently elevated in most patients who contracted acute, posttransfusion hepatitis C. In contrast, anti-HCc IgM was absent or barely detected in patients with chronic hepatitis C with milder disease activity and was absent in about 55% of those chronic patients with acute exacerbations. The results suggest that the anti-HCc IgM may be a marker in indicating acute HCV infection and perhaps also a marker for active HCV infection.

SUBJECTS AND METHODS

Expression of HCV Capsid (Core) Antigen in *Escherichia coli*. A cDNA fragment of 360 base pairs (bp) encompassing the amino-terminal 120 amino acids of HCV capsid protein was obtained by the procedure described previously (12). Total RNA was extracted from the liver tissue of a patient with acute posttransfusion hepatitis C. After reverse transcription, the cDNA was amplified by a pair of primers (5′-ATGAGCACGAATCCTAAACCTCA-3′ and 5′-TTAACCCAAATTACGCGACCTACGCCG-3′) (an additional termination codon TAA was added at the 3′ end of amplified cDNA) (13). The fragment was cloned into a bacteriophage λ leftward promoter (P_L)-containing expression vector (14), pG408N, which expresses the protein from the authentic initiation codon of the capsid gene (Fig. 1*A*). The resulting plasmid was transformed into host bacteria DG116 that contains a heat-inducible *c*1857 repressor gene. The transformants were then cultured in supplemented M9 medium (M9 containing 0.5% Casamino acids and 0.5% glucose) at 30°C to an optical density of 0.5 at 600 nm. The culture temperature was raised to 42°C for 3 hr to induce the expression of cDNA from the P_L promoter. Before and after induction, cells (1 ml) were harvested and analyzed by electrophoresis on SDS/12.5% polyacrylamide gels. The proteins were visualized by Coomassie brilliant blue staining (Fig. 1*B*).

Immunoblotting Procedures. Aliquots of total bacteria lysate were electrophoresed on SDS/12.5% polyacrylamide gels and then electrotransferred onto nitrocellulose filters

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Abbreviations: HCV, HBV, and HAV, hepatitis C, B, and A viruses; anti-HCc and anti-HBc, antibodies for HCV and HBV capsid antigen; ALT, alanine aminotransferase; P_L, leftward promoter of phage λ; BSA, bovine serum albumin.

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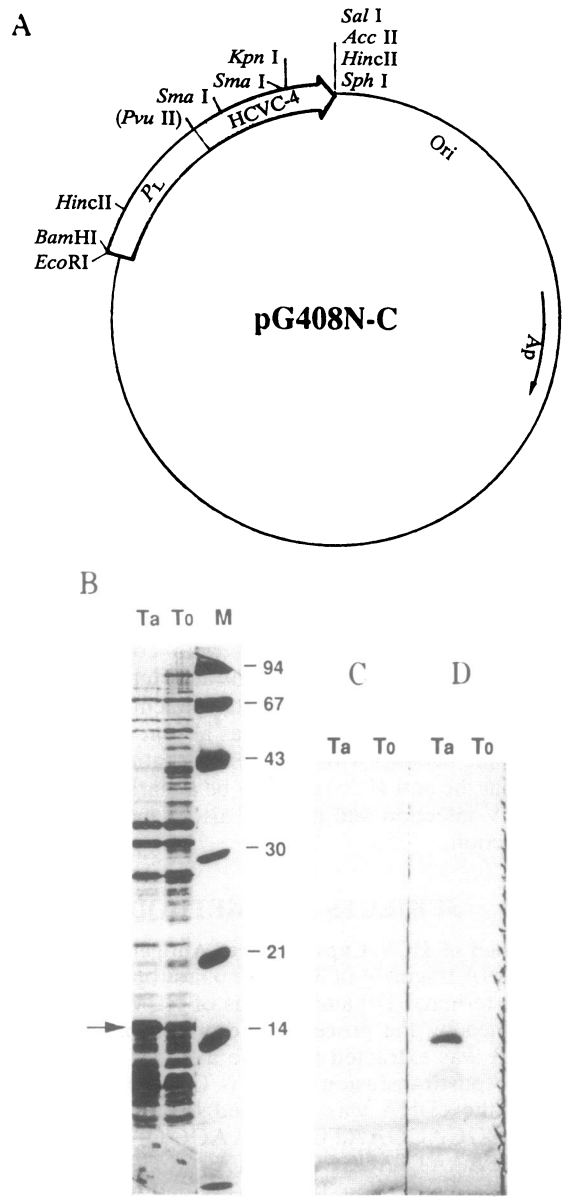

FIG. 1. Expression of HCV capsid antigen in *E. coli*. (*A*) Diagram showing the construct and relevant restriction sites of the HCV capsid-expressing plasmid pG408N-C. HCVC-4 represents the PCR-amplified DNA fragment containing the amino-terminal 120 amino acids of the viral capsid gene. Ap, ampicillin-resistance gene. (*B*) Expression of HCV capsid protein (indicated by an arrow) shown by Coomassie brilliant blue staining. Bacterial proteins are shown without induction (lane T0) and after heat induction (lane Ta). (*C* and *D*) Reaction of the recombinant protein with serum from a control (*C*) or a hepatitis C patient (*D*) is shown.

(15). Filters were first blocked with 5% bovine serum albumin (BSA) in TBS (50 mM Tris, pH 7.6/0.01% NaCl) and then allowed to react with patient serum samples (diluted 1:500 in TBS containing 3% BSA) at room temperature for 2 hr. The blots were washed with TBS containing 0.25% Tween 20 and incubated for 2 hr with alkaline phosphatase-conjugated goat antiserum (diluted 1:3000 in TBS containing 3% BSA) specific for either human IgG or IgM (Boehringer Mannheim). After the second antibody solution was removed, filters were again washed three times with TBS containing 0.25% Tween 20, and color developed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate for 1–2 min.

IgG-Depletion and Antigen-Blocking Experiments. To deplete IgG from serum, 10-μl aliquots of serum were mixed with increasing amounts (40, 80, 160, and 320 μl) of protein A-beaded agarose (Pierce; with IgG binding capacity of >15 mg per ml). Phosphate-buffered saline (PBS) was added into each aliquot to make a final volume of 1 ml. The mixture was incubated at room temperature for 30 min with constant shaking. After centrifugation, the supernatant was collected and diluted with 5 vol of 5% BSA. The IgG-depleted serum sample was then used directly for immunoblotting to examine IgM and IgG anti-HCc reactivity.

The antigen-blocking experiments were conducted in a similar way but with the following changes. Aliquots of serum (10 μl) were mixed with 5, 20, or 40 μl of purified recombinant HCV capsid antigen at 1 mg per ml. PBS was added to make a 1-ml mixture, which was incubated at room temperature for 2 hr until used for immunoblotting.

Patients with Acute Posttransfusion Hepatitis C. All nine patients were arbitrarily recruited from a prospective study of posttransfusion hepatitis conducted at the National Taiwan University Hospital since 1987 (7). Briefly, they had normal liver function tests before blood transfusion and no previous history of liver disease. Pretransfusion sera were negative for both hepatitis B virus (HBV) surface antigen and anti-HCV antibody by sensitive immunoassays (Ausuria-II and anti-HCV/anti-C100-3; Abbott). After transfusion, they were followed every 2–3 weeks during the first 3 months and every 2–4 weeks thereafter. Blood was sampled regularly to determine serum alanine aminotransferase (ALT) activity, anti-HCV antibody, and HCV RNA by reverse transcription–polymerase chain reaction. A *de novo* anti-HCV seroconversion and appearance of serum HCV RNA were demonstrated in each case (16). Serial serum samples from each patient (average, 10 specimens) were used to study anti-HCc IgG and IgM responses simultaneously by the aforementioned immunoblotting procedures.

Chronic Hepatitis C Patients with Milder Disease Activity or Acute Exacerbation. The patients were recruited from our gastroenterology clinic. They had been followed up every 4–8 weeks for more than 1 year because of chronic liver disease and were positive for anti-HCV antibody. None were positive for the HBV surface antigen or had a history of abuse of alcohol or drugs. The 18 patients with milder disease activity had ALT increase of <4-fold over normal levels. The other 9 patients with acute exacerbations were defined as those who had episodes of ALT elevation of >10-fold. For each exacerbation, serial serum samples (average, three specimens) before, during, and after exacerbations of the hepatitis were examined for anti-HCc IgG and IgM.

Patients with Acute Hepatitis A, Acute Hepatitis B, or Primary Biliary Cirrhosis. Acute hepatitis A was diagnosed in patients in the presence of serum anti-hepatitis A virus (HAV) IgM antibody at the symptomatic stage and became IgG antibody positive after recovery. Sera of patients with acute hepatitis B were positive for IgM antibody against HBV core antigen (anti-HBc IgM) at the symptomatic stage and showed subsequent seroconversion to the antibody against HBV surface antigen after recovery. Primary biliary cirrhosis was diagnosed in cholestatic patients who were positive for anti-mitochondria antibody and had histological confirmation.

RESULTS

The amino-terminal 120 amino acids of HCV capsid antigen was successfully expressed as a 15-kDa protein in *E. coli* after heat induction (Fig. 1*B*, lanes Ta vs. lanes T0). The protein was specifically recognized by serum samples positive for anti-HCV antibody (Fig. 1*D*, lane Ta) but not by serum samples negative for anti-HCV antibody (Fig. 1*C*, lane Ta).

The recombinant HCV capsid antigen was used to study the IgM and IgG reactivity among patients with acute posttransfusion hepatitis C. The profile of antibody response and

clinical course of a typical case is illustrated in Fig. 2. A transient response of anti-HCc IgM at the stage of acute hepatitis was noted. However, before this test could be extended to more patients, the specificity of this IgM response has to be demonstrated. Most importantly, the possibility of transient appearance of rheumatoid factor after blood transfusion has to be addressed. The rheumatoid factor or a like factor has been a well-known artifact in sera of non-A, non-B hepatitis patients (17, 18), and the factor may bind the anti-HCc IgG to produce a false-positive IgM reactivity.

Therefore, the test serum was first preincubated with protein A to deplete IgG before examination of the anti-HCc IgM reactivity. After being adsorbed by increasing amounts of protein A, the serum IgG was gradually depleted as demonstrated by the lesser reactivity of anti-HCc IgG (Fig. 3*A Right*). At the end point, the IgG was completely depleted, and there was no anti-HCc reactivity at all (Fig. 3*A Right*, lanes 4 and 5). In contrast, the serum anti-HCc IgM reactivity was not affected by protein A treatment (Fig. 3*A Left*). Even at the points when the serum IgG was completely depleted, the anti-HCc IgM reactivity still remained unchanged (Fig. 3*A Left*, lanes 4 and 5). These results indicated that the serum anti-HCc IgM reactivity in our experiment was independent of IgG and thus was not an artifact produced by rheumatoid factor binding. Aiming at further demonstration of the antibody specificity, we also performed antigen-blocking experiments. Serum was incubated with increasing amounts of HCV capsid antigen before immunoblotting. After this treatment, both anti-HCc IgM and IgG reactivities were blocked

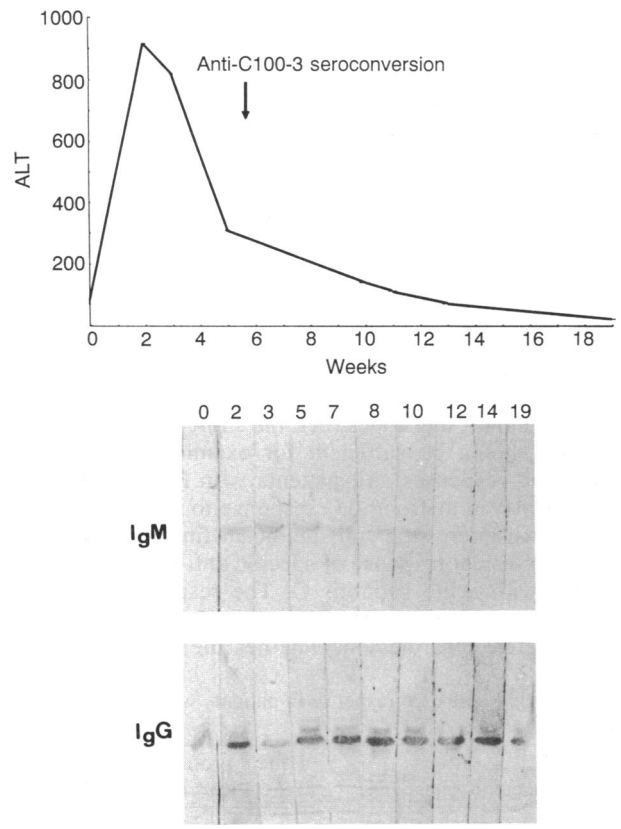

FIG. 2. The clinical course and temporal sequence of the IgM and IgG responses against HCV capsid antigen (anti-HCc) in a case of posttransfusion hepatitis C. Serum ALT levels (international units/liter) are shown in the diagram and the time point of anti-C100-3 seroconversion is indicated by an arrow. Results of immunoblotting to assay the anti-HCc IgM and IgG from serial serum samples from the patient are shown in the middle and bottom. Numbers at top indicate the time after onset of hepatitis in weeks (week 0 indicates the onset of hepatitis and the incubation period is 36 days).

(Fig. 3*B*, lane 1 vs. lane 4). The result indicates that the antibodies could be specifically neutralized by HCV capsid antigen. Finally, serum samples from patients with acute hepatitis A, hepatitis B, and primary biliary cirrhosis (at least two in each group) and patients with rheumatoid arthritis were studied for anti-HCc IgM and IgG reactivities, but so far all were negative (data not shown).

Nine patients recruited from a prospective study of acute posttransfusion hepatitis C were examined for anti-HCc IgM and IgG in serial serum samples. Eight of the nine patients had a transient response of anti-HCc IgM as demonstrated by specific immunoblotting procedures (seven at 1:1000 dilution of serum and six at 1:2000 dilution). The temporal courses of both anti-HCc IgM and IgG in these nine patients are summarized in Fig. 4*A*. In general, the anti-HCc IgM appeared soon after onset of the hepatitis (average, 3.7 weeks), lasted for several months (average, 18 weeks), and then became undetectable (Fig. 4*B*). On the other hand, the anti-HCc IgG persisted once it appeared at week 3 after hepatitis onset (Fig. 4 *A* and *B*). In some patients (nos. 1, 3,

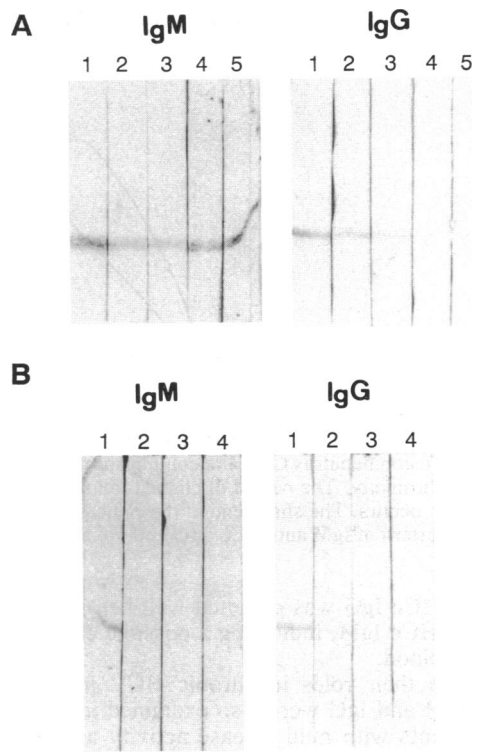

FIG. 3. (*A*) An immunoblot showing that the serum anti-HCc IgM reactivity is independent of IgG. To deplete the IgG, 10-μl aliquots of serum were preincubated with 0 (lane 1), 40 (lane 2), 80 (lane 3), 160 (lane 4), and 320 μl (lane 5) of protein A-beaded agarose in PBS (final volume, 1 ml) at room temperature for 30 min. After centrifugation, the supernatant was diluted with 5 vol of 5% BSA and then used in immunoblotting to examine the anti-HCc IgM (*Left*) or IgG (*Right*) reactivity. After being adsorbed with an increasing amount of protein A, serum IgG was removed, and the anti-HCc IgG activity decreased gradually (*Right*) and then disappeared (lanes 4 and 5). In contrast, even at these end points, the anti-HCc IgM reactivity was not affected (*Left*, lanes 4 and 5). (*B*) Blocking of serum anti-HCc IgM and IgG reactivity by preincubation with HCV capsid antigen before immunoblotting. To demonstrate the antibody specificity, a 10-μl aliquot of serum was preincubated with 0 (lane 1), 5 (lane 2), 20 (lane 3), and 40 μg (lane 4) of purified HCV capsid antigen in PBS buffer (final volume, 1 ml) at room temperature for 2 hr. The mixture was then diluted with 5 vol of 5% BSA and used in immunoblotting to examine any residual anti-HCc IgM (*Left*) or IgG (*Right*) activity. After treatment with increasing amounts of HCV capsid antigen, reactivities of both anti-HCc IgM and IgG in the serum decreased correspondingly (lanes 1–4).

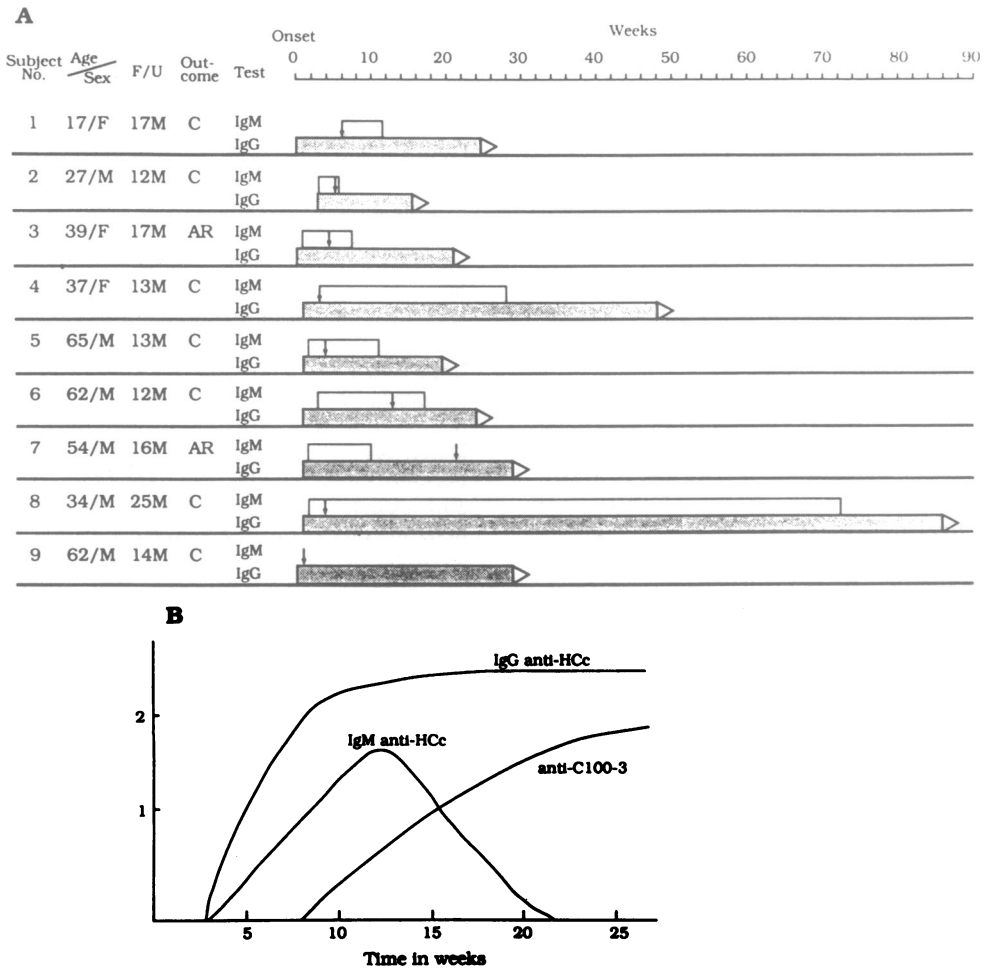

FIG. 4. (*A*) Summary of clinical data and temporal profile of IgM (blank rectangles) and IgG (shaded rectangles) anti-HCc among nine patients with posttransfusion hepatitis C. In the column showing outcome of acute hepatitis C, AR represents hepatitis that is "acute-resolving," while C represents chronicity. The period of clinical follow-up (F/U) in months (M) is shown. The arrow indicates the time point when anti-C100-3 seroconversion occurs. The sharp end at the right side of the shaded rectangle indicates a persistent anti-HCc IgG response. (*B*) Depiction of the temporal pattern of IgM anti-HCc, IgG anti-HCc, and anti-C100-3 after acute hepatitis C as revealed by this study. Units in the vertical axis are arbitrary.

and 9), anti-HCc IgG was detected well before the appearance of anti-HCc IgM, indicating a possible carryover from the donors' blood.

To explore their roles in chronic HCV infection, both anti-HCc IgM and IgG were also examined in chronic hepatitis C patients with mild disease activity and those with acute exacerbations. Among the first group, although the anti-HCc IgG was always present, most of the patients were anti-HCc IgM negative (13/18), and the reactivity of the remaining five was relatively weak. This result is practical and significant in that the majority of patients with chronic hepatitis C have mean ALT levels no more than 3 times the normal upper limit (19) and, thus, corresponds to the patients in the first group we studied.

In the second group (with exacerbations), all were positive for anti-HCc IgG. Anti-HCc IgM was also positive in 55% of them, but the reactivity in 3 of the 16 sera was weak (Table 1). When the tested serum was further diluted to 1:2000, those with a positive anti-HCc IgM response decreased to a mere 14% (Table 1). The results suggest that a significant proportion of patients with chronic hepatitis C do not have anti-HCc IgM or have the IgM antibody at low levels.

DISCUSSION

The HCV capsid antigen used in this study includes only the amino-terminal 120 amino acids of the entire capsid protein (190 amino acids) (20). However, because most of the human antibody epitopes have been localized exclusively to the amino-terminal 70 residues (21), the short capsid antigen we obtained should be sufficient for examining the humoral anti-capsid responses. All patients with hepatitis C in our study displayed a strong IgG response to this antigen.

The most interesting and significant finding of our study was the transient response of specific anti-HCc IgM in most patients with acute hepatitis C. The response usually appeared shortly after onset of hepatitis but subsided several months later, regardless of whether the hepatitis was re-

Table 1. IgM anti-HCc response in patients with chronic type C hepatitis

Chronic hepatitis C patients			Patient profile of IgM anti-HCc reactivity,* no. of patients (%)		
Disease activity	*n*	Serum dilution	Negative	Weakly positive	Positive
Low	18	1:500	13 (72)	5 (28)	0 (0)
Acute episodes	29	1:500	13 (45)	3 (10)	13 (45)
		1:1000	16 (55)	4 (14)	9 (31)
		1:2000	16 (55)	9 (31)	4 (14)

*Negative is defined as no signal in immunoblotting; positive, as a clearly visible signal; and weakly positive, as an equivocal signal.

solved or became chronic (Fig. 4*A*). After evolving into chronicity, the anti-HCc IgM did not reappear, despite persistently elevated ALT levels. Therefore, we further examined anti-HCc IgM as a marker for acute HCV infection.

That this IgM antibody response denotes acute infection is supported by the fact that the anti-HCc IgM seropositive rate among patients with chronic hepatitis C of mild activity was low. However, among chronic patients with acute exacerbations, about 45% displayed definite anti-HCc IgM responses. This raises two possible interpretations regarding the presence of anti-HCc IgM in HCV infection. First, the anti-HCc IgM may actually be present in both acute and chronic infections but with differing titers, as is the case for anti-HBc IgM in HBV infection (22). When serum from chronic hepatitis C patients with exacerbations was screened at 1:2000 dilution, the anti-HCc IgM-negative rate increased from 45% to 55%. These results suggest that the anti-HCc IgM may become a legitimate and feasible marker for acute HCV infection. Unfortunately, the immunoblotting assay we used has the major drawbacks of being neither facile nor quantitative. Development of a convenient radioimmunoassay or enzyme-linked test will allow study of this antibody response in more cases. Whether a quantitative difference in anti-HCc IgM titer distinguishes acute from chronic hepatitis C remains to be answered.

The second interpretation is that anti-HCc IgM is correlated with hepatitis disease activities in HCV infection (due to viral reactivation or reinfection). Accordingly, the anti-HCc IgM response may resemble the elevation of anti-HBc IgM in reactivations of chronic hepatitis B in which active HBV replication has been postulated to stimulate the IgM response (23). Thus, it would be interesting and revealing to correlate the anti-HCc IgM with the serum HCV level and with viral antigens in the liver tissue. Such information will be important in evaluating anti-HCc IgM as a marker for active HCV replication. In turn, anti-HCc IgM might then be used to predict or to follow the responses to antiviral therapy in analogy with the case of chronic hepatitis B (24).

In conclusion, we have found a transient IgM antibody response to the capsid antigen of HCV in acute hepatitis C. Although this anti-HCc IgM can also be documented in some cases of chronic hepatitis C, especially in those with acute exacerbations, the titers are usually lower. These findings form the basis of possibly using the anti-HCc IgM test to distinguish acute from chronic hepatitis C.

Note added in proof: After submission of this manuscript, an article on anti-HCc IgM in acute HCV infection reports results consistent with our findings (25).

We thank Ms. M. S. Chuang and Mr. S. J. Tu for assistance. The work was supported in part by grants from the National Science Council (80-0419-B-002-81 and 81-0419-B-002-19) and from the Department of Health, Taiwan, Republic of China.

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