Determination of Immunoglobulin M Antibodies Against Hepatitis B Core Antigen and Hepatitis A Virus by Reorienting Sucrose Gradient High-Speed Centrifugation for Diagnosis of Acute Viral Hepatitis

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Immunoglobulin M (IgM) antibodies against hepatitis B core antigen (anti-HBc) and hepatitis A virus (anti-HAV) were determined in 41 cases of acute viral hepatitis. In sera positive for anti-HBc or anti-HAV, IgM was separated from IgG by reorienting sucrose gradient high-speed centrifugation, and the IgG- and IgM-containing serum fractions were tested for the presence of specific antibody by radioimmunoassay. At the onset of illness, 4 of the 41 cases were classified as hepatitis A, 31 were hepatitis B, and 6 were non-A, non-B hepatitis, based on the results of these tests and of assays for hepatitis B surface antigen and antibody and hepatitis B e antigen and antibody. Fourteen of these 41 patients (34%) required IgM anti-HBc or IgM anti-HAV testing or both for appropriate classification. IgM anti-HBc persisted for at least 7 weeks after onset but no longer than 17 weeks in all patients tested with transient hepatitis B surface antigen-positive acute hepatitis. IgM anti-HAV persisted up to but not longer than 62 days in the patients with hepatitis A. Therefore, IgM anti-HBc and IgM anti-HAV determinations are valuable tools for the differential diagnosis of acute A, B, and non-A, non-B hepatitis.

Sensitive radioimmunoassay (RIA) procedures are commercially available for the detection of hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to HBeAg (anti-HBe), each of which is associated with hepatitis B virus (HBV) infection. In most patients convalescing from acute hepatitis B, there is a period of several weeks to several months between the disappearance of HBsAg and the appearance of anti-HBs, when anti-HBc is the only detectable serological marker other than anti-HBe, which may or may not be present. Anti-HBc, alone or together with anti-HBe, may also be observed in some individuals who have recovered from a past HBV infection, with the subsequent loss of detectable anti-HBs. Since a patient with detectable anti-HBc in the absence of HBsAg and anti-HBs is potentially infectious during convalescence (5), it is important to determine whether this serological pattern is due to recent or past HBV infection. Several studies have shown that immunoglobulin M (IgM) anti-HBc is almost always present at the onset of acute hepatitis B and persists for several months to more than 2 years, depending on the sensitivity of the assay (4, 10–12). The development of an assay with the sensitivity to detect IgM anti-HBc only during the acute and convalescent phases of the disease is described in this report, permitting a diagnosis of recent or past HBV infection to be made when HBsAg and anti-HBs are undetectable.

With regard to hepatitis A virus (HAV), a sensitive RIA technique is commercially available for the detection of specific antibody (anti-HAV), but this test does not distinguish between IgM anti-HAV and IgG anti-HAV. While this investigation was being conducted, a commercial RIA procedure for the demonstration of specific IgM anti-HAV was marketed. Detection of IgM anti-HAV has been well documented as a powerful laboratory tool for the recognition of current or recent hepatitis A (2, 3, 6–9, 13–16).

The identification of non-A, non-B (NANB) hepatitis currently depends on the exclusion of hepatitis A and B in clinically typical cases of viral hepatitis, since the agent or agents of this type of hepatitis have yet to be identified. When
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anti-HAV or anti-HBc (in the absence of HBsAg and anti-HBs) is detectable in suspected cases of NANB hepatitis, it is difficult to exclude hepatitis A or hepatitis B, respectively. Proof of the absence of detectable IgM anti-HAV or IgM anti-HBc in these cases would permit a diagnosis of NANB hepatitis to be made (12, 17).

Solid-phase test systems are not commercially available for the detection of IgM anti-HBc and have only recently become available commercially for the determination of IgM anti-HAV. Separation of IgM from IgG, followed by testing the IgM-containing fractions for anti-HBc or anti-HAV by using commercially available non-class-specific RIA procedures, is a practical alternative for IgM anti-HBc and IgM anti-HAV determinations. Sucrose gradient ultracentrifugation is a widely established technique for demonstrating these specific IgM antibodies (4, 9, 12, 15, 17), but the requirement for an ultracentrifuge may be prohibitory in many clinical virology laboratories.

In this study, we describe a reorienting sucrose gradient centrifugation method for the demonstration of IgM anti-HBc and IgM anti-HAV which utilizes commercially available non-class-specific RIA procedures and does not require an ultracentrifuge, but employs a more practical high-speed centrifuge. The determination of IgM anti-HBc and IgM anti-HAV by this technique was evaluated as a laboratory tool for the differential diagnosis of acute A, B, and NANB hepatitis. In addition, the persistence of IgM anti-HBc was determined in relation to HBsAg, non-class-specific anti-HBc, anti-HBs, HBeAg, and anti-HBe from the onset of acute hepatitis B through recovery.

MATERIALS AND METHODS

Sera. Serum specimens were collected from 41 patients at the onset of acute viral hepatitis. The diagnosis of acute viral hepatitis was made on the basis of the patient's history, general clinical findings, clinical chemistry data, lack of exposure to hepatotoxic drugs, and, in 38 patients, histology of liver biopsy tissue. The sera were tested for the presence of anti-HAV, HBsAg, anti-HBc, HBeAg, and anti-HBe. Determination of IgM anti-HAV was carried out on sera with detectable anti-HAV. Sera with detectable anti-HBc in the absence of HBsAg and anti-HBs were tested for the presence of IgM anti-HBc. Cases were classified as hepatitis A when IgM anti-HAV was present, hepatitis B when HBsAg or IgM anti-HBc were detectable, and NANB hepatitis when IgM anti-HAV, HBsAg, and IgM anti-HBc were undetectable. Only acute-phase sera were available for 10 of 31 cases classified as hepatitis B. A total of 125 serial serum specimens were collected 4 to 177 days after the onset of symptoms from 20 of the remaining hepatitis B cases with HBsAg persisting in the serum for less than 5 months. Ten serial serum specimens were also collected 7 to 234 days after the onset of clinical hepatitis from one hepatitis B case with HBsAg persisting in the serum for more than 7 months. The 135 serial serum specimens from these 21 patients were tested for the presence of IgM anti-HBC as well as HBsAg, non-class-specific anti-HBc, anti-HBs, HBeAg, and anti-HBe. Convalescent-phase serum specimens were available from three of four cases classified as hepatitis A and were tested for the presence of IgM anti-HAV.

Tests for HBsAg, anti-HBc, anti-HBs, HBeAg, anti-HBe, and anti-HAV. Sera were tested by RIA for the presence of HBsAg (AUSRIA II, Abbott Laboratories, North Chicago, Ill.), anti-HBc (CORAB, Abbott Laboratories), anti-HBs (AUSAB, Abbott Laboratories), HBeAg and anti-HBe (Abbott Laboratories), and anti-HAV (HAVAB, Abbott Laboratories).

Tests for IgM anti-HBc and IgM anti-HAV. IgM was separated from IgG by reorienting sucrose gradient high-speed centrifugation using an SV-288 vertical rotor (DuPont Instruments, Newton, Conn.) and an RC-5B superspeed centrifuge with automatic rate controller (DuPont Instruments). A 0.25-ml amount of anti-HBc or anti-HAV positive serum was diluted in 0.75 ml of phosphate-buffered saline (pH 7.2), layered on top of a 34-ml linear 5 to 20% (wt/wt) sucrose gradient, and covered with a 1-ml mineral oil overlay. The 5 and 20% gradient solutions were prepared with sucrose for density gradient centrifugation (Beckman Instruments, Inc., Palo Alto, Calif.) dissolved in phosphate-buffered saline. The gradient solutions were mixed in a custom-made gradient former with two interconnected 14 by 110-mm cylindrical chambers. A 17-ml volume of 5% sucrose was placed in the mixing chamber, and 17 ml of 20% sucrose was placed in the reservoir so that the light solutions poured into the 25- by 90-mm polylamellar centrifuge tube (DuPont Instruments) first, through a piece of tubing reaching to the bottom of the tube. Up to eight gradients were centrifuged at a time for 15 h at 20,000 rpm and 5°C with the setting on slow cycle and the automatic rate controller dial on 45. The tubes were bottom- punctured after centrifugation, and 11 3-ml fractions were collected per gradient. A 5-µl amount of each fraction was tested on low-level IgM and IgG radial immunodiffusion plates (Meloy Laboratories, Inc., Springfield, Va.). The concentration of IgM and IgG in each fraction was determined according to the test instructions supplied with the plates. Each fraction was also tested for the presence of anti-HBc or anti-HAV by RIA to demonstrate specific IgM antibodies in the IgM-containing fractions. Only fraction 2 was tested for anti-HBc or anti-HAV reactivity once it was established that this fraction consistently contained the peak concentration of IgM.

Confirmation of specific IgM antibody reactivity. IgM- and IgG-containing fractions reactive for anti-HBc or anti-HAV were treated with 2-mercaptopropionoethanol (Eastman Kodak, Rochester, N.Y.) to confirm the presence or absence of specific IgM antibody. A 10-µl amount of 1 M 2-mercaptopropionoethanol was added to 90 µl of each fraction, and the mixture was incubated at room temperature for 1 h. A 10-µl amount of dis-
tilled water was added to 90 μl of each fraction in the same manner as a control. The 2-mercaptoethanol-treated and untreated fractions were then tested for the presence of anti-HBc or anti-HAV by RIA.

RESULTS

IgM anti-HBc and IgM anti-HAV determinations. Figure 1 shows the typical results of the fractionation of four serum specimens collected from a patient 22, 66, 94, and 131 days after the onset of acute hepatitis B. Peak anti-HBc activity was found in fractions 2 and 3 on days 22 (serum I) and 66 (serum II). This corresponds with the peak concentration of IgM shown for serum II. Fractions 1 and 3 occasionally showed low IgM levels (not shown). No anti-HBc activity was detected in the IgM-containing fractions on days 94 (serum III) and 131 (serum IV). IgM anti-HBc persisted in the serum longer than HBsAg or HBeAg and became undetectable after the appearance of anti-HBe but before the appearance of anti-HBs. Peak anti-HBc activity was also found in fractions 7, 8, and 9 for all four sera. This corresponds with the peak concentration of IgG shown for serum II. Fractions 5, 6, 10, and 11 frequently showed low anti-HBc activity and low IgG levels.

Figure 2 shows similar results following the fractionation of two serum specimens collected from a patient 19 and 134 days after the onset of acute hepatitis A. Peak anti-HAV activity was detected in the IgM-containing fractions on day 19 (serum I) but not on day 134 (serum II). Peak anti-HAV activity was also found in the IgG-containing fractions for both sera.

When anti-HBc or anti-HAV activity was present in the IgM-containing fractions, treatment with 2-mercaptoethanol reduced the antibody activity to undetectable levels. Similar treatment of the IgG-containing fractions showed no reduction in antibody activity. These data suggest that antibody found in the IgM-containing fractions belongs to the IgM class, whereas the antibody in the IgG-containing fractions is of the IgG class.

Classification of acute viral hepatitis.

Four of the 41 cases of acute viral hepatitis were classified as hepatitis A (Table I). Non-class-specific anti-HAV and IgM anti-HAV were present in the serum of all four patients. HBsAg, anti-HBc, and HBeAg were also detectable in
TABLE 1. Classification of acute viral hepatitis as A, B, or NANB by the distribution of serological markers at onset of illness

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<th>Classification (no. of cases studied)</th>
<th>No. with indicated serological pattern</th>
<th>HBeAg</th>
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a NA, Not applicable. IgM anti-HBc determinations are only shown for sera with detectable anti-HBc in the absence of HBsAg and anti-HBs.

one patient with a history of chronic HBV infection together with a current acute hepatitis episode and anti-HAV seroconversion. Anti-HBc was present in the absence of HBsAg and anti-HBs in one patient, but recent HBV infection was considered to be unlikely since IgM anti-HBc was undetectable.

Thirty-one of the 41 cases of acute viral hepatitis were classified as hepatitis B (Table 1). HBsAg and anti-HBc were present in the serum of all 31 patients. HBeAg was detectable in 25 of the 31 patients (81%), anti-HBs was detectable in 5 (16%), and anti-HBe was found in 3 (10%). Anti-HAV was present in 8 of the 31 cases classified as acute hepatitis B, but recent HAV infection was ruled out since IgM anti-HAV was undetectable.

Six of the 41 cases of acute viral hepatitis were classified as NANB hepatitis (Table 1). There was no serological evidence of previous HAV or HBV infection in two of these patients. Anti-HBc, anti-HBs, and anti-HBe were detectable...
in two cases, indicating recovery from past HBV infection. Anti-HBc was present in the absence of HBsAg and anti-HBs in two patients, one of whom also had detectable anti-HBe, but recent HBV infection was considered to be unlikely since IgM anti-HBc was undetectable. Anti-HAV was also detectable in these two patients, but recent HAV infection was also ruled out since IgM anti-HAV was absent.

**Persistence of IgM anti-HBc in acute hepatitis B.** Figure 3 shows the persistence of IgM anti-HBc after the onset of symptoms in relation to the other HBV-associated serological markers in 20 of the 31 cases classified as acute hepatitis B (Table 1). All 20 patients had detectable HBSAg and non-class-specific anti-HBc within 1 week after onset of disease. HBsAg remained detectable in all patients tested for at least 4 weeks and became undetectable within 17 weeks in all but two patients, where it persisted for up to 19 weeks. Non-class-specific anti-HBc remained detectable during acute illness, convalescence, and recovery in every case. All 20 patients had IgM anti-HBc within 1 week after onset, and this marker was present for at least 7 weeks in each patient tested. The incidence of IgM anti-HBc positivity declined from 94% at 8 weeks to 33% at 12 weeks after onset. Only 25% remained IgM anti-HBc positive by 13 weeks, and this marker was not detected beyond 17 weeks. IgM anti-HBc persisted for a period equal to or greater than that for HBsAg in 58% of patients. HBeAg was present in all patients tested within 2 weeks after onset. The incidence of HBeAg positivity declined from 73% at 3 weeks to 40% at 4 weeks after onset. Only 20% remained HBeAg positive by 6 weeks, and this marker was not detected beyond 8 weeks. IgM anti-HBc remained detectable for as long as or longer than HBeAg in all 20 patients. In 13 of 20 (65%) patients, there was a period of several weeks to several months between the disappearance of HBsAg and the appearance of anti-HBs, when anti-HBc was the only detectable serological marker other than anti-HBe, which was present in all 13. IgM anti-HBc persisted for several weeks beyond the disappearance of HBsAg in 6 of these 13 patients (46%).

In addition to the 20 patients shown in Fig. 3, 1 of the 11 remaining cases classified as acute hepatitis B developed chronic HBV infection with HBsAg, non-class-specific anti-HBc, and HBeAg persisting for more than 7 months after onset of acute illness. This patient had undetectable IgM anti-HBc at 15, 70, 141, and 234 days after onset.

**Persistence of IgM anti-HAV in acute hepatitis A.** A single convalescent-phase serum specimen was tested for the presence of IgM anti-HAV in three of the four cases classified as acute hepatitis A in Table 1. IgM anti-HAV was detectable 62 days after the onset of disease in one case and was undetectable on days 86 and 134 in the remaining two cases.

**DISCUSSION**

This study confirms the observations of others that IgM anti-HBc and IgM anti-HAV determinations are valuable tools for the diagnosis of current or recent acute hepatitis B and A, respectively, as well as NANB hepatitis by exclusion. IgM anti-HBc is invariably present at the onset of acute hepatitis B and is detectable for several months to more than 2 years in transient and chronic HBV infections when a sensitive enzyme immunoassay technique is employed (10, 11). This procedure requires exact quantitation of IgM anti-HBc to distinguish between either recent and past acute hepatitis B, or transient and chronic HBV infections. A previous study (12) described a sucrose gradient ultracentrifugation technique for the detection of IgM anti-HBc which also employed the CORAB RIA procedure for detection of anti-HBc in IgM-containing serum fractions, where IgM anti-HBc persisted for up to 15 months. The shorter period of IgM anti-HBc positivity observed in the present report is almost certainly due to the initial 1:4 dilution of serum as well as to differences in the sucrose gradient centrifugation technique used. This study (Fig. 3) and a previous report (4) have found that when less sensitive methods are used, IgM anti-HBc is always present at the onset of symptoms in transient HBsAg-positive acute hepatitis B and becomes undetectable within 8 to 17 weeks. By these methods, IgM anti-HBc is undetectable from the onset when HBsAg persists for more than 6 months with the development of chronic HBV infection. In this study, we were also able to rule out recent acute hepatitis B in three acute A and NANB hepatitis patients by demonstrating the absence of IgM anti-HBc when non-class-specific anti-HBc was present and HBsAg and anti-HBs were undetectable (Table 1). Thus, the procedure for IgM anti-HBc determination described in this report permits an accurate diagnosis of current or recent acute hepatitis B to be made after testing only one serum sample and without the need for exact quantitation of IgM anti-HBc. This technique may also be useful in distinguishing at the onset of acute illness between patients with transient HBsAg-positive acute hepatitis B and those with developing or preexisting chronic HBV infection.

The results of our tests for HBeAg and anti-
HBe (Fig. 3) are consistent with those of a recent study which reported that HBeAg was regularly present in early acute hepatitis B, usually became undetectable within 10 weeks after onset of symptoms, and was followed by the development of anti-HBe in nearly all cases (1). The same study suggested that persistence of HBeAg for more than 10 weeks predicts the development of a chronic HBsAg carrier state (HBsAg positive for more than 6 months). In our study, a chronic HBsAg carrier state was eventually documented in the single acute hepatitis B patient with HBeAg persisting for more than 10 weeks.

IgM anti-HAV is always present at the onset of acute hepatitis A and persists for several months to at most a year, depending upon the sensitivity of the assay. When solid-phase RIA (8, 13, 16) or enzyme immunoassay (6, 7, 14, 16) procedures are used for the detection of IgM anti-HAV, dilution of sera or titration of IgM anti-HAV is necessary for diagnosis of recent infection since low titers of this antibody may be present long after the acute illness. The sensitivity of the HAVAB-M RIA procedure (Abbott Laboratories), which has recently become commercially available for the detection of specific IgM antibody against HAV, has been adjusted to detect levels of IgM anti-HAV only in the first 4 to 6 months after onset of illness. This is accomplished by first diluting patient sera more than 1:4,000, permitting a diagnosis to be made after testing only one serum sample. In this study, we were able to demonstrate IgM anti-HAV in four patients at the onset of acute hepatitis A which became undetectable within 3 to 4 months in three patients with postrecovery serum available. IgM anti-HAV was also absent in 10 acute hepatitis B and NANB hepatitis patients with detectable non-class-specific anti-HAV. Therefore, the sensitivity of the assay for IgM anti-HAV described in this study is similar to that reported by others (2, 9) and appears to be appropriate for diagnosis of recent HAV infection without requiring additional serum dilution or IgM anti-HAV titration.

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LITERATURE CITED


