

Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin M Antibody to Hepatitis B Core Antigen

PETER KRYGER,¹* LARS R. MATHIESEN,¹ ANNE MARIE MØLLER,¹ JAN ALDERSHVILE,²
BENGT G. HANSSON,³ AND JENS O. NIELSEN²

Enterovirus Department, Statens Seruminstitut, DK-2300 Copenhagen S.,¹ and Medical Department, Division of Hepatology, Hvidovre Hospital, Copenhagen² Denmark, and Department of Clinical Virology, Allmänna Sjukhuset, Malmö, Sweden³

An enzyme-linked immunosorbent assay for detection of specific immunoglobulin M (IgM) antibodies against the core antigen of the hepatitis B virus (anti-HBc IgM) is described. The interference of IgM rheumatoid factor was evaluated quantitatively. In the anti-HBc IgM test, the rheumatoid factor gave false-positive results when the concentration exceeded 20 IU/ml. The rheumatoid-positive sera were disclosed by a control and retested for anti-HBc IgM after absorption of rheumatoid factor with latex particles aggregated with human IgG. In five of seven selected patients with acute hepatitis B followed to biochemical and clinical recovery, anti-HBc IgM was present transiently until antibodies against hepatitis B surface antigen (anti-HBs) appeared. Two patients had persistent anti-HBc IgM during the follow-up period. Four patients with hepatitis B surface antigenemia and progression to chronic liver disease did not clear their anti-HBc IgM in the period of observation (11 to 24 months). Anti-HBc IgM could not be demonstrated in 223 of 225 Danish blood donors. The two donors found positive for anti-HBc IgM also had anti-HBs. Twenty patients with acute A or non-A non-B hepatitis were negative for anti-HBc IgM. The enzyme-linked immunosorbent assay for anti-HBc IgM described here has a high specificity and sensitivity. The diagnostic relevance needs further evaluation, including quantitation of anti-HBc IgM, but the results presented indicate that anti-HBc IgM may be helpful in differentiating between prior and recent or ongoing hepatitis B infection.

The usual diagnostic technique for identification of acute or chronic hepatitis B virus (HBV) infection has been the demonstration of hepatitis B surface antigen (HBsAg) in serum. Shortly after the appearance of HBsAg, antibody to the core of hepatitis B virus (anti-HBc) becomes detectable, usually several weeks before the appearance of antibody to hepatitis B surface antigen (anti-HBs). The failure of HBsAg testing to identify all cases of ongoing HBV infections has been recognized by the fact that a few blood donors with anti-HBc as the only sign of HBV infection were able to transmit HBV (5, 6). The demonstration of hepatitis B core and surface antigens in liver tissue from patients with anti-HBc, but without HBsAg or anti-HBs in their serum, also points to anti-HBc as a possible marker of continuous replication of HBV (12). However, anti-HBc can also be demonstrated during the convalescent period of HBV infection and may last for several years, possibly a lifetime, and is therefore in itself not useful for differentiating between present and previous HBV infections. The clinical significance of anti-HBc titers in the differentiation between these

two states is also difficult to evaluate because of considerable variation in the individual antibody response.

In other viral infections, demonstration of specific immunoglobulin M (IgM) antibodies has been useful (15, 17). Specific IgM antibodies are present only during the acute phase of infection and usually fall to a subdetectable level shortly after termination of the infection. Recently a novel approach for detection of specific IgM antibodies against hepatitis A virus (anti-HAV IgM) has been described, and several authors have reported results of diagnostic accuracy (2, 7, 10). Using this new approach, we describe in this study an enzyme-linked immunosorbent assay (ELISA) for detection of anti-HBc IgM and the findings in a limited number of patients with HBV infection followed longitudinally.

MATERIALS AND METHODS

Patients. In seven patients with uncomplicated acute type B hepatitis and four patients with persistence of HBsAg and progression to chronic liver disease, serial sera were collected and stored at -20°C until tested. Acute-phase sera, from 10 patients with

acute hepatitis A serologically confirmed by a test for IgM antibodies against hepatitis A virus and from 10 patients with acute non-A non-B hepatitis defined as HBsAg and negative for IgM antibodies against hepatitis A virus, were tested for anti-HBc IgM.

Volunteer blood donors. A total of 225 Danish blood donors previously tested for anti-HAV and IgM antibodies against hepatitis A virus were tested for HBsAg, anti-HBs, total anti-HBc, and anti-HBc IgM.

Sera containing IgM-class RF. To evaluate the interaction of rheumatoid factor (RF) in the ELISA anti-HBc IgM test, 32 sera containing different concentrations of RF were included in the study.

Isolation of core antigen. Hepatitis B core antigen was purified, as described previously (4), from a liver, taken at necropsy, from a dialysis patient who was an HBsAg carrier.

Preparation of conjugated anti-HBc IgG. IgG was isolated by ammonium sulfate precipitation and diethylaminoethyl column chromatography and labeled with horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.), as described previously (9). The anti-HBc IgG fractions were prepared from an HBsAg- and HBeAg-positive serum with an anti-HBc titer of 1:35,000 as determined by radioimmunoassay (RIA) (Corab; Abbott Laboratories, North Chicago, Ill.).

Determination of anti-HBc IgM by ELISA. Microtiter polyvinyl plates were precoated with 75 μ l of rabbit anti-human IgM specific for μ chains (no. 10-091, Dako, Copenhagen, Denmark), diluted 1:25,000 in phosphate-buffered saline (PBS), pH 7.4, and incubated for 24 h at 4°C. The plates were washed three times in PBS-0.05% Tween 20, and each was filled with 1% bovine serum albumin in PBS and incubated overnight at 4°C. After another three washings in PBS-0.05% Tween 20, the test samples were diluted 10-fold from 10^{-1} to 10^{-5} in PBS, and 25 μ l of each dilution was incubated in the coated wells for 4 h at room temperature. The plates were then washed three times in PBS-0.05% Tween 20, and 25 μ l of core antigen, diluted 1:200 in PBS, was added; the plates were then incubated for 24 h at 4°C. After three washings with PBS-0.05% Tween 20, 50 μ l of peroxidase-conjugated anti-HBc diluted in 50% pooled human sera negative for anti-HBc was added. The plates were incubated for 2 h at room temperature and then washed five times in PBS-0.05% Tween 20. A 100- μ l portion of freshly prepared substrate containing 40 mg of *o*-phenylenediamine and 20 μ l of 30% peroxide in 100 ml of citrate buffer, pH 5.0, was added. After 30 min of incubation at room temperature in the dark, 75 μ l of 2 M sulfuric acid was added to stop the reaction. After bringing the total volume to 250 μ l by adding 75 μ l of PBS, the extinction at 493 nm was measured in a spectrophotometer with a rapid sampling microcuvette (Gilford model 250).

Positive and negative controls found negative for RF were included in each experiment. A late convalescent serum, only positive for total anti-HBc by RIA in fractions containing IgG after separation of IgG and IgM by sucrose gradient ultracentrifugation, was used as a negative control.

Anti-HBc IgM was considered to be positive if the

extinction for a sample was more than 2.1 times that of the negative control. To avoid false-positive results caused by binding the RF (anti-IgG of IgM class) to the anti-human IgM-coated plates and the further binding of the RF to the IgG conjugate, each serum was also tested on a plate to which PBS had been added in place of the purified core antigen. Sera giving false-positive results were quantitated for RF. The RF was then removed, and the sera were retested for anti-HBc IgM.

RF quantitation and absorption. IgM RF was quantitated by an ELISA method. The RF concentration was calculated in international units per milliliter from a standard curve by using an international reference preparation (16). RF was removed from the test serum by absorption with latex particles coated with aggregated human IgG (Latex-RF Reagent; Behringwerke, A.G., Marburg/Lahn, West Germany). Before use, soluble IgG was removed from the particles by four centrifugations at 10,000 $\times g$ and resuspension in PBS. The mixture of test serum and the washed latex suspension (diluted 1:200 and 1:5, respectively, in PBS) was incubated for 1 h at 37°C followed by 17 h of incubation at room temperature. The latex particles were partly removed by centrifugation for 30 min at 1,500 $\times g$ (14). The supernatant was then tested in the anti-HBc IgM assay. In each experiment, two controls (negative and positive for anti-HBc IgM, but negative for RF) were included. The RF absorption did not interfere with the results for these RF-negative sera.

Immunoglobulin separation. Serum immunoglobulin separation was done by rate zonal ultracentrifugation on a 10 to 40% (wt/vol) sucrose density gradient in PBS, pH 7.38. A 0.5-ml portion of serum layered on top of 4.5 ml of the gradient was centrifuged for 18.75 h at 32,000 rpm (80,000 $\times g$) in a Beckman L5-65 ultracentrifuge, rotor type SW60. Thirteen fractions of 380 μ l each were collected from the bottom of the tube. The IgG and IgM contents of the serum fractions were measured by single radial immunodiffusion on plates containing commercially available anti-IgM and anti-IgG (8) (Dako).

Reduction of IgM. Reduction of IgM antibodies was obtained by incubating 10 μ l of the test serum with 90 μ l of dithiothreitol for 1 h at 37°C followed by 12 h of incubation at 4°C (13).

RIA procedures. HBsAg, the corresponding antibody (anti-HBs), and anti-HBc were tested by commercial RIA assays (Ausria II-125, Ausab and Corab; Abbott Laboratories).

RESULTS

Specificity. The diagnostic specificity of the ELISA anti-HBc IgM test was established by separating IgG and IgM antibodies by sucrose gradient centrifugation and testing for anti-HBc IgM. Figure 1 shows that the anti-HBc IgM test was positive only in the acute-phase serum and only in fractions which were positive for both IgM by ring diffusion and anti-HBc by RIA. The high sensitivity of the ELISA, as compared with the incomplete separation of IgG and IgM by

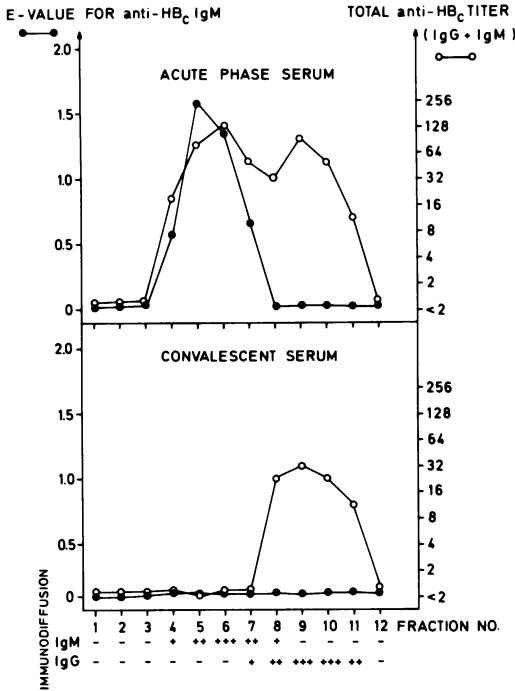


FIG. 1. An acute and a convalescent-phase serum from a patient with uncomplicated hepatitis B tested for anti-HBc IgM by ELISA and for total anti-HBc by RIA after separation by rate zonal ultracentrifugation. -, No precipitate; +, ++, and +++, increasing size of precipitate estimated by the eye.

sucrose gradient centrifugation, made it necessary to dilute the fractions 1,000 times in PBS before testing for anti-HBc IgM. The convalescent serum was negative for anti-HBc IgM in all fractions, regardless of the degree of dilution.

Patients with acute hepatitis. Figure 2 shows the serological findings, including those of anti-HBc IgM in seven patients with uncomplicated hepatitis B and in four patients with persistent HBsAg in their serum and progression to chronic liver disease. In all seven patients with uncomplicated acute hepatitis B, the anti-HBc IgM was detected initially. In five of them, anti-HBc IgM disappeared shortly after the appearance of anti-HBs, whereas the remaining two patients were positive throughout the observation period (12 to 18 months) despite the presence of anti-HBs. The four patients with persistence of HBsAg and development of chronic liver disease showed anti-HBc IgM during the entire observation period (11 to 24 months). None of the acute-phase sera from 20 patients with hepatitis type A or non-A non-B showed anti-HBc IgM.

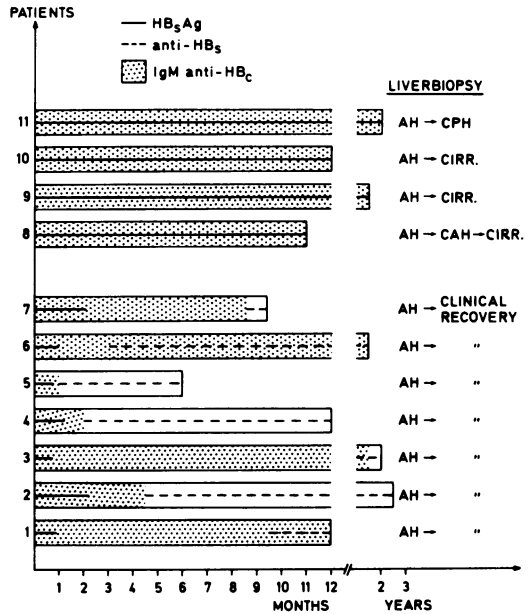


FIG. 2. Relation between anti-HBc IgM, HBsAg, and anti-HBs in seven patients with uncomplicated acute hepatitis B and four patients with persisting HBsAg and progression to chronic liver disease. AH, Acute hepatitis; CAH, chronic active hepatitis; CIRR, cirrhosis hepatis; and CPH, chronic persistent hepatitis.

Volunteer blood donors. Among the 225 Danish blood donors tested, four had detectable anti-HBs and total anti-HBc by RIA. Two of these four, RF-negative donors, were also found positive for anti-HBc IgM, the specificity being confirmed by the disappearance of anti-HBc IgM when the IgM antibodies were inactivated by treatment with dithiothreitol. In two other donors the only sign of previous hepatitis B infection was the presence of anti-HBc of the IgG type, and three donors showed false-positive results in the anti-HBc IgM test, as evidenced by color development in antigen-free wells. After absorption of RF, the sera from these three individuals were negative for anti-HBc IgM.

Sera containing IgM-class RF. To study the interference of IgM-class RF in the anti-HBc IgM test, 32 sera containing RF in concentrations of between 5 to 550 IU/ml were tested (Table 1). Seven of these sera were positive for total anti-HBc by RIA. Of the 32 sera, 18 (60%) gave false-positive results (colored antigen-free wells) in the anti-HBc IgM test, but the activity could in all cases be absorbed with latex particles coated with aggregated IgG. Only sera with RF

TABLE 1. Results of IgM-anti-HBc by ELISA in 32 sera in relation to RF concentrations and presence of total anti-HBc (IgG plus IgM) by RIA^a

IgM-anti-HBc by ELISA	No. of sera negative for total anti-HBc by RIA		No. of sera positive for total anti-HBc by RIA			
	Before RF absorption, RF concn (IU/ml) of:	After RF absorption	Before RF absorption, RF concn (IU/ml) of:		After RF absorption	
			5-20	20-550		5-20
Positive	0	15	0	0	3	0
Negative	6	4	25	4	0	7

^a RF normal value, <3.5 IU/ml.

concentrations higher than 20 IU/ml gave false-positive results.

DISCUSSION

The primary antibody response to immunization consists mainly of production of IgM which is later followed by IgG production. Until recently, assays for determining anti-HBc IgM antibodies have required separation of IgG and IgM by ultracentrifugation or the use of indirect immunofluorescence on liver tissue with IgM-specific fluorescein-isothiocyanate-conjugated anti-human IgG (1, 11). These studies have pointed to anti-HBc IgM as a marker of active virus replication and to its disappearance as a sign of recovery. However, since indirect immunofluorescence is difficult to quantitate, we have developed a specific, sensitive and less time-consuming ELISA for the detection of anti-HBc IgM which is based on the principle first described by Duermeyer et al. (2) and characterized by the use of anti-IgM for coating the solid phase. This technique for the demonstration of anti-HBc IgM was applied by Gerlich and Lüer in 1979 (3).

One problem concerning the specificity of the test is the false-positive results caused by the presence of RF which is an anti-IgG of the IgM class. RF may attach to the anti-IgM-coated microtiter plates, and this may cause false-positive results due to subsequent binding of enzyme-conjugated IgG. To avoid interference by RF, Gerlich and Lüer (3) diluted the test sample in an excess of aggregated IgG, and Duermeyer et al. (2) used conjugates in which the specific reactive site for RF, the Fc part of the IgG molecule, had been removed by pepsin treatment. For removal of RF, we preferred to use a latex-IgG absorption procedure, since this technique is valid, inexpensive, and simple to perform with standardized, easily available re-

agents.

The seven patients with uncomplicated hepatitis B had anti-HBc IgM in all their serum samples until anti-HBs appeared. Five of them cleared their anti-HBc IgM after the appearance of anti-HBs.

The finding of anti-HBc IgM in two donors and in two of the patients with uncomplicated acute hepatitis, despite the presence of anti-HBs, might be explained by individual variation in the IgM antibody response. Although it seems unlikely that the patients should be carriers of HBV, this cannot be excluded. The problems need further investigation, which would include anti-HBc titration and correlation with the presence of core and surface antigens in the liver. Both core and surface antigens have been reported in liver tissue from patients with anti-HBc, as well as with anti-HBc and anti-HBs, in the serum (12).

We believe that the ELISA method described above for the determination of anti-HBc IgM can help to further analyze the significance of anti-HBc IgM.

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