Determination of Immunoglobulin M Antibodies for Hepatitis B Core Antigen with a Capture Enzyme Immunoassay and Biotin-Labeled Core Antigen Produced in *Escherichia coli*

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Received 13 May 1985/Accepted 16 July 1985

A new capture enzyme immunoassay for the determination of immunoglobulin M (IgM) antibodies against hepatitis B core antigen (HBcAg) is described. Core antigen produced in *Escherichia coli* was labeled with biotin and subsequently detected by an avidin-biotin-peroxidase complex. The biotin-labeled core antigen was effective at concentrations as low as 20 ng/ml. Of 561 serum samples from different groups of patients that were tested, 465 samples were negative for other hepatitis B virus markers and also for anti-HBcAg IgM. Sera from the early stages of hepatitis B infection had high levels of anti-HBcAg IgM, and a clear correlation with the acuteness of the disease was observed in 45 follow-up sera from 23 patients with acute or recent hepatitis B. Sera from 21 patients with past hepatitis B were all negative for anti-HBcAg IgM. Twenty serum samples from chronic carriers of hepatitis B surface antigen showed slightly elevated antibody levels for anti-HBcAg IgM. Ten sera which were positive for anti-HBcAg IgG antibodies and had high levels of rheumatoid factor were negative for anti-HBcAg IgM.

The presence of immunoglobulin M (IgM) antibodies against hepatitis B core antigen (HBcAg) is an indication of acute or recent hepatitis B virus infection (2, 4, 7, 8, 10). The test for anti-HBcAg IgM is particularly important in the absence of other hepatitis B markers and in cases in which patients are chronic carriers of hepatitis B surface antigen (HBsAg) without clinical symptoms.

Two problems have been associated with the clinical use of anti-HBcAg IgM determinations. Rheumatoid factor (RF) and other similar substances may seriously interfere with any solid-phase assay for IgM antibodies (1, 6). The frequent occurrence of low levels of anti-HBcAg IgM for prolonged periods after acute hepatitis B has been reported (4). This has been associated with the use of core antigen from human liver. In fact, increased levels of different autoantibodies against liver components, as well as increased levels of RF, have been observed in hepatitis B (2, 4).

We have developed a new modification of enzyme immunoassay for the determination of anti-HBcAg IgM which seems to be able to avoid the undesired effect of RF and other autoantibodies. The core antigen used in this modification was a purified product from *Escherichia coli* produced by recombinant DNA technology and should be naturally free from contaminating human tissue components. The present modification was a capture assay with anti-human μ on the solid phase. Biotin labeling of the core antigen was used, and labeled antigen was detected by an avidin-biotin-peroxidase complex (ABC). In this paper, the optimal conditions for the assay and some clinical experiences with its use are described.

**MATERIALS AND METHODS**

Antigen. HBcAg was produced in *E. coli* (9) and was kindly supplied by Biogen, Geneva, Switzerland, in the form of a lyophilized preparation with a total protein content of 1.75 mg/ml. The final purity of this product was 95% when expressed as densitometric readings of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the antigen preparation.

Biotinylation of HBcAg. HBcAg was labeled with biotin as described by Vilja et al. (12). HBcAg was dialyzed overnight at 4°C against 0.1 M NaHCO₃. The solution was diluted with 0.1 M NaHCO₃ to give a concentration of 1 mg/ml. Biotinyl-N-hydroxysuccinimide ester (BNH; Calbiochem-Behring, La Jolla, Calif.) was dissolved in N,N-dimethylformamide at various concentrations. BNH was added to HBcAg solution at a ratio of 1:15 (vol/vol), and the mixture was incubated for 2 h at room temperature and dialyzed overnight at 4°C against phosphate-buffered saline (PBS; pH 7.2). Biotin-labeled antigen was stored in 20-μl portions at −20°C until use.

Enzyme immunoassay. Microtiter plates (Nunc-Immuno-plate I; Nunc, Roskilde, Denmark) were sensitized with 150 μl of u-chain-specific rabbit anti-human IgM (DAKO, Copenhagen, Denmark) per well diluted 1:3,000 in 0.01 M carbonate-bicarbonate buffer (pH 9.6). The plates were incubated overnight at 4°C and then washed with PBS containing 0.5% (vol/vol) Tween 20. In routine testing, sera were tested at only the predetermined optimal dilution of 1:1,000. Serum samples diluted in PBS containing 1% (wt/vol) bovine serum albumin and 0.1% (vol/vol) Tween 20 were added in duplicate and incubated for 1 h at 37°C. After washing as before, the plates received biotinylated HBcAg diluted 1:1,000 in PBS-bovine serum albumin-Tween 20. The plates were incubated for 1 h at 37°C and washed as before. During the incubation of biotinylated HBcAg we prepared an ABC by mixing equal volumes of avidin-DH and biotinylated horseradish peroxidase (both from Vector Laboratories, Burlingame, Calif.) and diluting this mixture 1:200 in PBS-bovine serum albumin-Tween 20 for 30 min of preincubation.
negative sera which were positive for RF when tested by a latex agglutination test (Rapitex-RF; Behringwerke) were used to study the effect of RF on the assay.

**Positive and negative control sera.** A serum sample from a patient with serologically and clinically confirmed acute hepatitis B was used as a positive reference serum throughout the study. Serum from a healthy laboratory technician who was negative for serological markers of hepatitis B was used as a negative reference.

**Quantitation of anti-HBcAg IgM antibody activity.** The quantitation of antibody activity in samples was based on the use of positive and negative reference sera which were included in the assay. Antibody levels were expressed as arbitrary units, EIA units (EIU), which were determined by the formula (11): antibody levelsample = [(ODsample – ODnegative control) / (ODpositive control – ODnegative control)] x 100 EIU, where OD is the optical density measured at a wavelength of 492 nm. The limit value for positivity was determined as the mean plus three standard deviations (SDs) of the antibody levels for the negative controls.

**RESULTS**

**Biotin labeling of the antigen.** HBsAg was allowed to react with different amounts of BNHS, and the preparations were tested with known anti-HBcAg IgM-positive and -negative reference sera (Fig. 1). The highest positive to negative ratio was achieved when the ratio of BNHS to antigen was 1.5 (wt/wt). With this preparation, the negative reference serum gave reasonably low absorbance readings throughout the dilution range, whereas the positive reference still exceeded the cutoff level at a dilution of 1:100,000, corresponding to a protein concentration of 20 ng/ml (Fig. 2).

**Determination of optimal conditions for the assay.** We determined the optimal dilutions of the reagents and proper times and temperatures for incubations by varying each parameter individually while keeping the other variables constant. A dilution of 1:3,000 of rabbit anti-human IgM and incubation at +4°C overnight was optimal for coating of the solid phase. Dilutions of biotin-labeled HBcAg were tested with different dilutions of positive and negative reference sera (Fig. 3). The highest positive to negative ratio with a linear

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**FIG. 1.** Different biotin-antigen conjugates and serum dilutions in the anti-HBcAg IgM assay. The BNHS-to-HBcAg ratios (wt/wt) were 0.02 (▲), 0.2 (■), and 1.5 (●). Black and white symbols represent the positive and negative reference sera, respectively. Broken line, OD ≥ 2.0.

**FIG. 2.** Serial dilutions of the biotin-labeled HBcAg tested against the P (●) and N (○) reference sera. The serum dilution was 1:1,000. The cutoff level was determined as twice the mean OD value for the HBcAg at the lowest dilution used.
slope for the dose-response curve was obtained when a dilution of 1:1,000 for biotin-labeled HBcAg was used.

A dilution of 1:1,000 for serum samples gave the highest specific reactivity. Incubation for 1 h at +37°C was optimal for both sample and antigen incubations. High specific reactivity with a low background was achieved with 30 min of incubation at +37°C for the ABC.

Application of the assay to clinical samples. First, 465 samples negative for other hepatitis B markers were studied. All of these sera were negative for anti-HBcAg IgM, with a mean antibody level of 2.1 EIU and an SD of 4.3 EIU. The limit value for positivity was thus determined as 15 EIU, corresponding to a limit of confidence of 99% (P < 0.01).

A clear correlation between the EIA antibody levels for anti-HBcAg IgM and the acuteness of disease was observed when 45 follow-up sera from 23 patients with acute or recent hepatitis B were tested (Fig. 4). All of the sera collected during the first month after the onset of symptoms were positive for anti-HBcAg IgM, with a very high mean antibody level of 194 EIU and an SD of 27 EIU. A rapid decline in anti-HBcAg IgM levels was subsequently observed. A mean antibody level of 83 EIU with an SD of 30 EIU was detected in sera from the third month of the follow-up period. However, all of these sera were still positive although two of these five sera had already converted to HBsAg negative. Anti-HBcAg IgM disappeared, in most cases, 6 to 12 months after the onset of disease; a mean antibody level of 11 EIU with an SD of 15 EIU was detected. All of the six serum samples from that period were negative for HBsAg.

Sera from 20 chronic carriers on HBsAg were also tested for anti-HBcAg IgM. A mean antibody level of 10 EIU with an SD of 4 EIU was found. Two of these patients (10%) could be considered positive for anti-HBcAg IgM, although at very low antibody levels.

None of the 21 serum samples from patients with past hepatitis B was positive for anti-HBcAg IgM. The mean antibody level of 2.5 EIU with an SD of 5 EIU was very close to the mean antibody level for negative control sera.

We also tested 10 sera which were positive for anti-HBcAg and RF, as determined by a latex agglutination test, for anti-HBcAg IgM to assay the possible interfering effect of RF on the present assay. However, all of these sera were totally negative, with a mean antibody level of 2.3 EIU and an SD of 4 EIU.

DISCUSSION

This modification of an enzyme immunoassay consists of two main steps. During serum incubation, IgM is separated from IgG by anti-human IgM on the solid phase. In the next step, biotin-labeled HBcAg is allowed to react and to detect specific IgM antibodies.

The key element in the present assay is the use of biotin labeling on HBcAg. The high affinity of avidin for biotin (K_d = 10^{-15} M) (3) guarantees a very high sensitivity for the detection of biotinylated HBcAg by the ABC. Others have reported difficulties in the use of radioactive labeling of core protein of hepatitis B virus (13). However, the biotinylation of HBcAg could be easily performed, so that under optimal conditions the labeled antigen could still be detected at a dilution of 1:100,000, corresponding to a protein concentration of 20 ng/ml. This may be due to the low molecular weight of biotin (244), which should allow efficient labeling with no significant loss of immunological activity of HBcAg. The use of E. coli-derived HBcAg excludes the interfering effect of contaminating polypeptides of human origin which may lead to nonspecific reactions in the assay for anti-HBcAg IgM.

In other assays using capture enzyme immunoassay for the determination of virus-specific IgM antibodies, overnight incubation of the antigen has often been used (2, 4, 5, 7, 8). In the present assay, only 1 h of incubation was needed for optimal reactivity, which might be explained by the small size of the biotin-antigen complex. Only 3 h were needed for the performance of the entire test. This makes the test clinically very useful. Furthermore, the high sensitivity of the assay also leads to low consumption of reagents, especially of valuable HBcAg.

FIG. 3. Different dilutions of the biotin-labeled HBcAg and the serum. The dilutions of the biotinylated antigen were 1:250 ( ), 1:1,000 ( ), 1:2,000 ( ), and 1:4,000 ( ). Black and white symbols represent the P and N reference sera, respectively. Broken line, OD ≤ 2.0.

FIG. 4. Correlation between the antibody level (EUI) in EIA for anti-HBcAg IgM and the onset of clinical symptoms in 45 follow-up sera from 23 patients with acute hepatitis B. Anti-HBcAg IgM antibody levels in sera from 20 chronic carriers of HBsAg and from 21 patients with past hepatitis B infection were also tested. Symbols: , HBsAg positive; , HBsAg negative. Serum dilution, 1:1,000.
Immunossays using the capture principle are usually less sensitive to interference by RF and other autoantibodies (1, 2). In the present assay, direct labeling of antigen was used with no need for secondary antibodies for the indicator system. Thus, no possibility for interference by RF should exist in the present modification of the assay for anti-HBcAg IgM. We confirmed this in the present study by applying the test to RF-positive serum samples containing IgG class antibodies against the core antigen.

Some conclusion about the suitability of anti-HBcAg IgM determinations in the diagnosis of hepatitis B may be drawn. High levels of anti-HBcAg IgM antibodies were consistently found during the first 2 months after the onset of symptoms. A rapid decline of anti-HBcAg IgM antibodies seemed to be typical, so that in most of the cases of acute hepatitis B, anti-HBcAg IgM antibodies had disappeared 6 to 12 months after the onset of disease. The value of anti-HBcAg IgM determinations was further emphasized in cases in which HBcAg became negative but anti-HBcAg IgM antibodies could be easily detected at high levels.

Patients with past hepatitis B infection had essentially the same anti-HBcAg IgM antibody levels as negative controls, emphasizing the high specificity of the assay. Interestingly, chronic carriers of HBsAg did show slightly higher mean antibody levels than did controls. This difference was statistically significant ($P < 0.001$). However, even the highest antibody levels seen among carriers of HBsAg just exceeded the limit value for positivity.

It is hard to see why chronic carriers of HBsAg should have a continuous synthesis of IgM class antibodies against core antigen in a situation in which such antigen is not believed to be synthesized. One possible explanation for slightly elevated anti-HBcAg IgM activity would be a non-specific reactivity, which could be mediated through the presence of HBsAg in these sera. However, further studies are needed to clarify this phenomenon.

The technique described seems to be sensitive and specific and has good clinical applicability. Excellent differentiation among patients with acute and past hepatitis B as well as patients who are chronic carriers of HBsAg was achieved, suggesting that the determination of anti-HBcAg IgM could be used as one of the main indicators of acute hepatitis B infection.

**ACKNOWLEDGMENTS**

We thank Marja Raita for excellent technical assistance.

This work was supported by the KOTURA fund from the Academy of Finland.

**LITERATURE CITED**


