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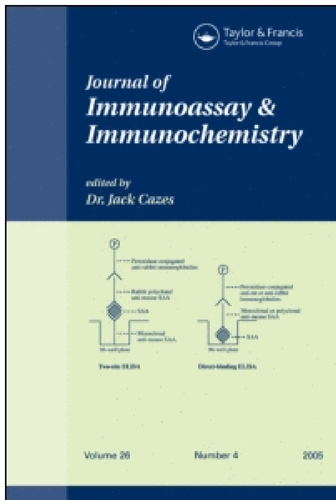

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A Monoclonal Inhibition Enzyme Immunoassay for Detection of Antibodies Against Hepatitis B Core Antigen: Confirmation of an Immunodominant Epitope

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A MONOCLONAL INHIBITION ENZYME IMMUNOASSAY FOR DETECTION OF ANTIBODIES AGAINST HEPATITIS B CORE ANTIGEN: CONFIRMATION OF AN IMMUNODOMINANT EPITOPE.

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ABSTRACT

Monoclonal antibodies (mAbs) were raised against hepatitis B virus core produced by a recombinant clone of Escherichia coli (rHBc). The three mAbs recognized rHBc by Western blot, suggesting that they reacted with non-conformational epitopes. Competition experiments between mAbs and human anti-HBc sera confirmed the existence of an immunodominant HBc epitope within the viral antigen. A monoclonal competition enzyme immunoassay using an IgM mAb conjugated to biotin and streptavidin-peroxidase as the detection system yielded 99% sensitivity and 100% specificity, when compared to other commercial assays. (KEYWORDS: Hepatitis B core antigen, monoclonal inhibition EIA).

INTRODUCTION

Antibody to hepatitis B core antigen (anti-HBc) is an excellent epidemiological indicator of active and past infection (1). Testing for anti-HBc often has been included as a mandatory test for blood banks in some countries, because it has been shown that hepatitis B virus circulates in some surface antigen negative sera (1-6).

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The first generation of anti-HBc detection systems were radio- or enzyme-immunoassays (RIA or EIA) based on the inhibition of labeled human anti-HBc by human anti-HBc in the unknown specimen for interaction with immobilized HBc (7,8). More recently, a monoclonal EIA has been developed (9). This kind of assay can be performed since the majority of anti-HBc activity seems to be directed to a single immunodominant epitope (10-13). In the present communication, we report the development of a monoclonal antibody (mAb) based competition EIA for detection of anti-HBc antibodies.

MATERIALS AND METHODS

Patient sera. A total of 91 human anti-HBc positive plasma or sera, tested either by CORAB RIA (Abbott Laboratories, IL) or by Enzygnost anti-HBc monoclonal (Behringwerke, Marburg, Germany) and 53 anti-HBc negative plasma or sera were used. Hepatitis B reference serum with anti-HBc activity and a HBe antigen positive serum was acquired from the Paul Ehrlich Institut (Langen, Germany).

MAb production. A Balb/c mouse was immunized with purified rHBc produced in Escherichia coli (14,15). The first dose was administered i.p. with complete Freund Adjuvant. Fifteen days later a second dose was administered i.p. with incomplete Freund Adjuvant. Four days before the fusion an i.v. dose was administered; each dose consisted of 15 µg of rHBc. Fusion of NS1 myeloma cells with spleen cells was performed according to previously reported procedures (16). Hybridoma cell lines secreting specific antibodies against rHBc were selected by EIA (17). Microtiter wells were sensitized with 50 µl of rHBc for 2 hours at 37°C. After 3 washes with phosphate buffered saline pH 7.4 containing 0.05% Tween 20 (PBS-Tween), 50 µl of hybridoma suppernatant were added and incubated for 1 hour at 37°C. After 3 washes with PBS-Tween, 50 µl of anti- mouse Ig-peroxidase (1/500 in PBS with 1% bovine serum albumin,

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PBS-BSA) was incubated for 1 hour at 37°C. After 3 additional washes, enzymatic reaction was revealed by a 30 min. incubation with 50 µl of o-phenylenediamine (OPD) in citrate perborate buffer (Sigma) and stopped with 50 µl of 1M H_2SO_4. O.D. values were determined at 492 nm in a Multiskan II spectrophotometer (Flow Laboratories Inc., VA, USA). Isotype analysis was done by the same EIA described above, using an isotyping kit instead of anti-mouse Ig-peroxidase (Bio-Rad Laboratories, CA, USA).

To test the ability of human anti-HBc positive sera to inhibit the binding of mAbs, 50 µl of human anti-HBc positive serum (diluted 1/10 in neonatal bovine serum, NBS) were added in microtiter plates previously sensitized with rHBc., and incubated overnight at 4°C. After 3 washes with PBS-Tween, 50 µl of mAb supernatant were incubated for 1 hour at 37°C, and the immunoassay was performed as described above. To test the ability of mAbs to inhibit the binding of human anti-HBc, 50 µl of mAb ascitic fluids (1/100) were incubated for 1 hour at 37°C in microtiter plates previously sensitized with rHBc, followed by incubation with 50 µl of human anti-HBc serum (1/100 in PBS-BSA) for an additional hour. Subsequently 50 µl of anti-human-IgG-peroxidase (1/5000 in PBS-BSA, Sigma) were added and incubated for an additional hour at 37°C. Enzymatic reaction was revealed with OPD as described above.

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Western blot. rHBc (10 µg/lane) was subjected to electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS/PAGE) and then transferred to nitrocellulose membranes (18). Following incubation with mAb ascitic fluids (1/100 in PBS-BSA), immunological reactions were revealed after sequential incubations with anti-mouse polyvalent immunoglobulins conjugated to peroxidase (1/100 in PBS-BSA) and diaminobenzidine as substrate.

Monoclonal competition EIA. MAbs were purified from ascitic fluid by chromatography on G-200, and conjugated to amidocaproil-N-hydroxy-succimidobiotin according to

previously reported procedures (19). One hundred μl of rHBc (100 ng/ml in 0.05 M carbonate-bicarbonate buffer pH 9.5) were passively adsorbed to polystyrene microtiter plates (Immulon 2, Dynatech, VA) by incubation at 37°C for 2 hours. After washing 4 times with PBS-Tween, 100 μl of human serum were added and incubated for 1 hour at 37°C or overnight a 4°C. After washing 4 times with PBS-Tween, 100 μl of biotin-mAb (250 ng/ml) diluted in PBS-Tween containing 1% BSA and 20% NBS (19) were incubated for 1 hour at 37°C. Simultaneous incubation of human serum and biotin-mAb was also evaluated. After washing 4 times with PBS-Tween, 100 μl of streptavidin-peroxidase (Sigma, 1/1000 in PBS-Tween-BSA) were incubated for 30 min. at 37°C. After 4 more washes, 100 μl of OPD were added and incubated for 30 min.; reaction was stopped with 100 μl of 1M H_2SO_4. O.D. values were determined at 492 nm. In two ways competition experiments, mAbs ascitic fluids (diluted in PBS-BSA) were added instead of human serum to test their ability to inhibit the binding of biotin-mAbs as described before.

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RESULTS

Out of 104 wells with hybridomas, 2 IgM and 1 IgG_1 mAbs recognizing rHBc were obtained from a fusion of an mouse immunized with rHBc. The 3 mAbs reacted with rHBc by Western blot analysis (data not shown). The 2 IgM mAbs, 2E11 and 6D10 were more efficiently inhibited by human polyclonal antibodies for binding to rHBc (data not shown) and were used for all subsequent experiments. MAb 2E11 was also able to mediate a partial inhibition (25-55%) of binding of human anti-HBc to rHBc (Fig. 1). A two ways inhibition assay between mAbs 2E11 and 6D10 showed that they seemed to be directed against the same antigenic region (Fig. 2).

In order to evaluate their utility in a competitive EIA format for the detection of human anti-HBc activity, mAbs 2E11 and 6D10 were conjugated to amidocaproil-biotin. Human sera were tested for their ability to inhibit the binding of these biotin-mAbs to

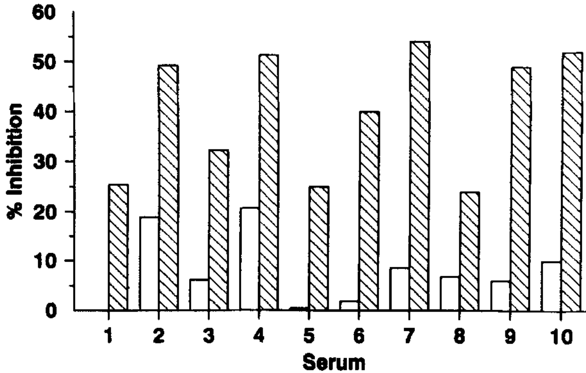

Figure 1: Inhibition of binding of human anti-HBc antibodies by mAb 2E11. After coating with rHBc, mAb 2E11 (▧) or an irrelevant mAb (□) were added before adding human serum.

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adsorbed rHBc. The inhibition of binding of mAb 2E11 was more easily achieved when human serum was incubated prior to the addition of biotin-mAb (sequential format, Fig.3). For mAb 6D10, simultaneous incubation of human sera and biotin-mAb was more efficient than the sequential incubation format (Fig. 3).

Seventy two human sera were evaluated by the competitive monoclonal EIA using 2E11 in a sequential format and 6D10 in a simultaneous format. A higher sensitivity and specificity was obtained with 2E11 in a sequential overnight incubation format (Table 1).

A total of 91 anti-HBc positive sera and 53 anti-HBc negative sera were tested by this monoclonal EIA, yielding a 99% sensitivity (90/91 positive) and 100% specificity compared to the results obtained with commercial immunoassays. A serum standard positive for HBeAg also tested positive by this monoclonal EIA; thus eliminating the possibility that 2E11 was inhibited by anti-HBe antibodies and not by anti-HBc ones. To further evaluate the sensitivity of the assay, a standard of anti-HBc was titrated: the assay detection limit was 1-2 PEIU / ml (Fig. 4).

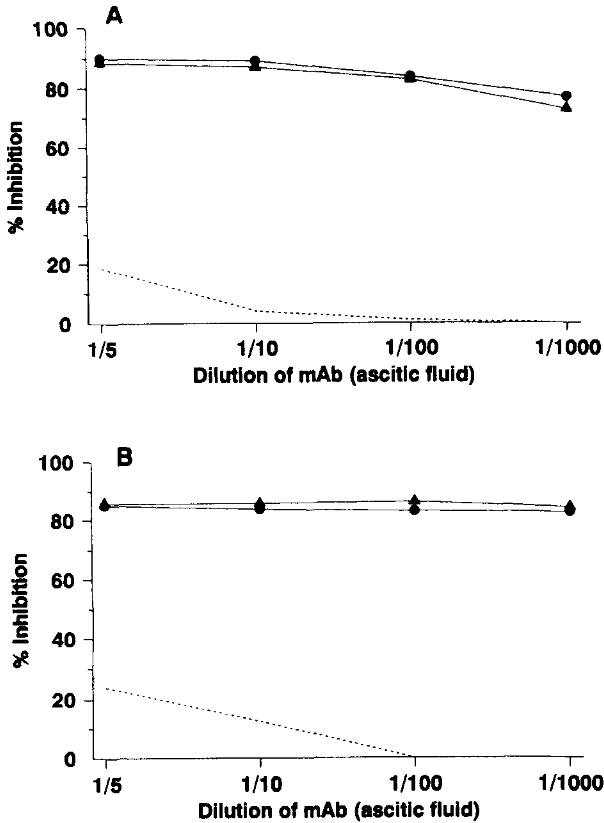

Figure 2: Two ways competition assay between mAbs. After coating with rHBc, mAb 2E11 (●), mAb 6D10 (▲), or an irrelevant mAb (----) were added before adding biotin-mAb 2E11 (A) or 6D10 (B).

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DISCUSSION

Since the cloning in E. coli of hepatitis B core (HBc) (20), a number of mAbs against this viral diagnostic antigen has been produced (12,13,21,22), allowing the development of monoclonal competition EIA for anti-HBc detection. The mAbs obtained in this study were able to recognize denatured HBc on Western blot, suggesting that the

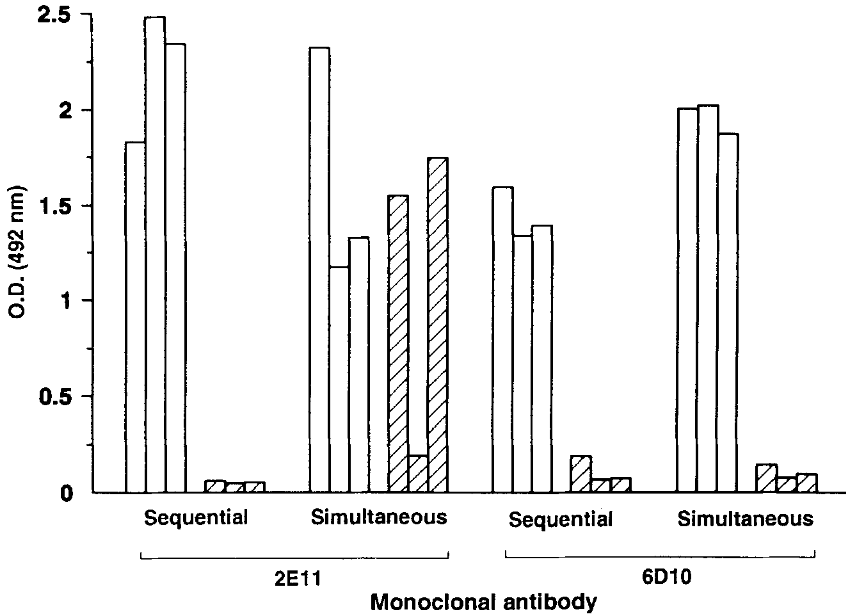

Figure 3: Evaluation of monoclonal inhibition EIA in a sequential or simultaneous format. After coating with rHBc, human negative (□) or positive (▨) serum were added before adding biotin-mAb in a sequential or simultaneous format.

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epitope(s) recognized by them are not strictly conformation dependent, as has been reported previously for HBc antigenicity (13,21). More recently, different linear binding sites inside the complete discontinuous HBc antigenic region have been described (10,22). The inhibition by a HBeAg positive serum confirmed that the mAbs used in this study were directed against HBc epitope and not against the HBe determinant present in the core molecule (11). Further studies using synthetic peptides are needed, however, to precisely define the exact location of the epitopes recognized by these mAbs.

Inhibition of binding between monoclonal and human polyclonal anti-HBc confirms previous observations regarding the presence of an immunodominant epitope in this antigen (10,12,21). However, mAb 2E11 was only able to mediate a partial inhibition

Table 1

Evaluation of mAbs for monoclonal inhibition EIA.

Plasma[1]	Monoclonal antibody		
	2E11 (sequential)[2]		6D10
	1 hour	overnight	simultaneous
62 +	59 + (95%)	61 + (98%)	48 + (77%)
10 -	10 - (100%)	10 - (100%)	8 - (80%)

1: Seventy-two plasma were tested for anti-HBcAg by RIA, + means positive by the assay, - means negative. 2: mAb 2E11 was tested in a monoclonal inhibition EIA in a sequential format (preincubation of human plasma for 1 hour or overnight) and 6D10 by a simultaneous incubation of human plasma and mAb. Cut off value was determined as the mean O.D. of a strong control positive serum (more than 90% inhibition) and a control negative serum.

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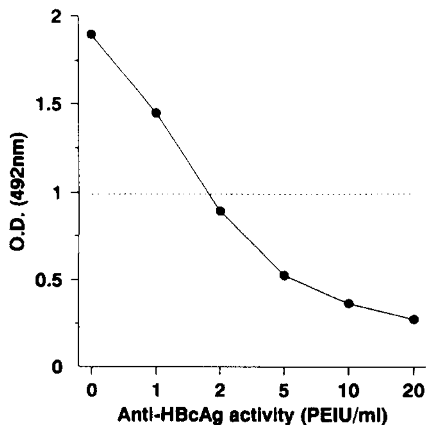

Figure 4: Evaluation of the anti-HBc assay detection limit. A standard reference serum (100 Paul Ehrlich Institut Units / ml) was diluted in neonatal bovine serum and tested by the monoclonal inhibition assay (●). Cut off (----) was determined as in table 1.

of binding of human antibodies to HBcAg (Fig. 1), although these same human sera inhibited more than 90% binding of this mAb when tested in the monoclonal inhibition EIA. Even if the two inhibition assays are not strictly comparable and that differences in affinity could explain the different behaviors in this two ways competition, the difficulty of the mAb to mediate a total inhibition suggests that there may be other antigenic epitopes within the HBcAg, different from the immunodominant one. Nelles et al. (12) reported an efficient inhibition of binding of human antibodies to HBcAg using a single mAb and suggested a single immunodominant epitope; another possible explanation could be a strong steric interference to any secondary epitope(s) by the mAb used by Nelles et al. (12).

The performance of the competition EIA was dependent on the mAb used. When using 6D10, a simultaneous incubation of human serum and biotin-mAb was more efficient; however, an overnight preincubation of human serum before adding 2E11 was preferred (Fig. 3). Possible explanations for this different behavior may be due to differences in avidity between the 2 mAbs, or interfering substances in the patient serum that can affect the binding of 2E11 more than the binding of 6D10. Although more time consuming, a sequential incubation procedure was adopted, because of it high sensitivity (99%) and specificity (100%) compared to the simultaneous incubation format (Table 1); no false-positive results were obtained with this monoclonal EIA. We speculate that this format reduces the possibility of false positive results with interfering substances present in the serum. It is important to note that problems in specificity in EIA has been described by others for anti-HBc detection (7). The use of biotin-streptavidin yielded a high difference between positive and negative O.D. values, as has been reported previously in other EIA (19). This amplification procedure allowed to reach low detection limit (Fig. 4), assuring high sensitivity. In conclusion, a monoclonal anti-HBc competition EIA was developed and standardized for the detection of anti-HBc activity in human sera.

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