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Enzyme-Linked Immunosorbent Assay for Detection of Antibody to the Hepatitis B Surface Antigen-Associated Delta Antigen

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A blocking enzyme-linked immunosorbent assay (ELISA) was developed for specific detection of antibody to the hepatitis B surface antigen-associated δ antigen. The sensitivity of ELISA was intermediate between that of previously described immunofluorescence and radioimmunological assays for anti-δ. Performance of ELISA was simple and required only ordinary and inexpensive laboratory equipment.

The hepatitis B surface antigen (HBsAg)-associated δ antigen (δ Ag) (9) appears to represent a marker of a transmissible defective agent distinct from the hepatitis B virus (HBV) yet requiring HBV replication for its synthesis (10, 11).

Despite its intrahepatic synthesis (9), δ Ag is rarely detectable in the blood (14), and the epidemiology of δ infection was assessed from the prevalence of antibody to δ (anti-δ) (12, 14).

In different geographical areas anti-δ predominates in HBsAg carriers with chronic hepatitis (12), and its detection permits, therefore, the identification of a subgroup of HBsAg carriers coinfected by HBV and δ agent who presumably have liver disease.

Anti-δ has been measured by a δ immunofluorescence (IFL) blocking test of low sensitivity (2) and by a solid-phase radioimmunoassay (RIA) (14); the latter assay is sensitive but requires expensive equipment and repeated preparation of a radioiodinated probe.

We developed, therefore, a simple enzyme-linked immunosorbent assay (ELISA) technique and compared its diagnostic usefulness with that of the RIA.

MATERIALS AND METHODS

Reagents. The δ reagent used in this study was obtained from the autopsy liver of an HBsAg seropositive patient with chronic hepatitis and intrahepatic δ; the immunopathological characterization of this liver was previously reported (13). A piece of liver (50 g) was minced and washed in cold saline until the supernatant fluid was clear. One part of tissue was suspended in 9 parts of 0.5 M sucrose in TKM buffer [2 mM $MgCl_2$, 25 mM KCl, and 5 mM tris-(hydroxymethyl)aminomethane-hydrochloride, pH 7.4] (4), and the suspension was homogenized with an Ultra Turrax and a Dounce homogenizer. Dry guanidine-hydrochloride was then added to the homogenate to a final molarity of 6, and the preparation was stirred at room temperature for 4 h. The 6 M guanidine-hydrochloride-extracted liver was diluted fivefold with phosphate-buffered saline (PBS; 0.85% NaCl and 0.01 M phosphate buffer, pH 7.4) to a final 1.2 guanidine-hydrochloride molarity and then centrifuged for 20 min at 4,000 rpm. The supernatant fluid was collected and used as stock δ reagent. Partially purified δ Ag prepared from the δ-positive nuclei of the same liver (13) was used as reference δ reagent. Liver from an HBsAg-seronegative patient who died from myocardial infarction, and liver from an HBsAg-seropositive patient with cirrhosis and intrahepatic hepatitis B core antigen (HBcAg) and without tissue or serological expression of δ/anti-δ, were submitted to 6 M guanidine-hydrochloride extraction as described above and used as negative antigen controls.

The 22-nm form of HBsAg was purified from the plasma of an HBsAg carrier (3). A Dane particle-associated HBcAg reagent was prepared (15) and used in ELISA with the addition of 0.1% Nonidet P-40 and 0.1% β-mercaptoethanol. An HBsAg-positive serum characterized as containing hepatitis B e_1 antigen (HBe_1Ag) and hepatitis B e_2 antigen (HBe_2Ag) by munodiffusion and reacting in HBeAg-RIA at 1:2,560 dilutions (1) was used as HBeAg reagent; it contained antibody to HBcAg detectable at 1:50,000 dilution and no anti-HBs or anti-δ.

The anti-δ reagent used in this study was an HBsAg-positive serum from a patient with chronic persistent hepatitis which contained a high titer of anti-δ (between $1:10^4$ and $1:10^5$ by RIA) and a low titer of anti-HBc (1:500 by RIA); the immunological characterization of this serum was previously reported (13). Im-

munoglobulin G (IgG) was isolated from the anti-δ reagent by saturated ammonium sulfate precipitation and diethylaminoethyl cellulose column chromatography and labeled with horseradish peroxidase (HRPO) by the method of Nakane and Kawaoi (6) as described by Mathiesen et al. (5). Purified IgG was labeled with ^{125}I as described previously (13). The anti-HBs reagent was a guinea pig antiserum to HBsAg subtype *ad* (Research Resources Branch, National Institute of Allergy and Infectious Diseases, catalog no. V801-503-558). The anti-HBc and anti-HBe reagents were human antisera whose immunological characteristics were previously reported (1, 13); neither contained δ Ag or anti-δ by RIA. HRPO anti-HBs was obtained from a commercial source (Auszyme; Abbott Laboratories, N. Chicago, Ill.); HRPO anti-HBc and anti-HBe were prepared as described above from the IgG purified from the respective reagents. Sera devoid of HBV markers and containing antinuclear, antimitochondrial, or antimicrosomal autoantibodies by indrect IFL were used as negative antibody controls.

Assays. ELISA for anti-δ was performed according to conditions previously described for the anti-δ RIA (14). Wells of polyvinyl microtiter plates (Cooke Laboratories, Inc., Alexandria, Va.) were coated with 150 μl of anti-δ reagent (diluted 1:1,000) and incubated for 4 h at room temperature. After repeated washings with PBS, wells were filled with 200 μl of PBS containing 1% bovine serum albumin and incubated at 4°C overnight. Plates were washed, dried, and then inoculated with 25 μl of an optimal dilution of standard δ reagent, established by ELISA titration, and incubated overnight at 4°C. After washing, duplicate wells of the plates containing δ were inoculated with 100-μl dilutions of test sera. After 2 h of incubation at 37°C in a humid chamber, plates were washed, filled with standard HRPO anti-δ IgG, incubated for 2 h at 37°C, and washed, and the HRPO reaction was developed. A positive control (anti-δ reagent diluted 1:20), a negative control (HBsAg serum devoid of anti-δ in RIA, diluted 1:20), and a blank control (PBS + 1% bovine serum albumin) were included in each antibody plate. Dilutions of sera and reagents other than HRPO anti-δ were made in PBS + 1% bovine serum albumin. HRPO anti-δ was diluted in 50% fetal bovine serum in PBS. The HRPO reaction was developed by the addition of 100 μl of substrate (ortho-phenylenediamine, 0.4 mg/ml, with 0.0006% hydrogen peroxide in citrate buffer, pH 5.0) freshly prepared according to Mathiesen et al. (5). After 30 min in the dark at room temperature, the reaction was stopped by adding 50 μl of 2 M H_2SO_4. Results were evaluated by eye inspection and graded according to the brown color developed by the HRPO reaction. A test sample was considered negative for anti-δ when the brown color intensity of the HRPO reaction was equal to that of the negative antibody control; lack of brown coloration corresponded to a +++ result, and intermediate intensities were scored as + + and +. HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe were measured by commercial radioimmunoassays (Ausria II, Ausab, Corab, and HBe Kit; Abbott Laboratories). HBcAg was assayed by the microtiter RIA of Purcell et al. (8). Titers in ELISA and RIA were expressed as that dilution resulting in a 50% decrease in the optical density at 493 nm or radioactivity (counts per minute) as compared to the negative control.

Clinical specimens. One thousand sera were assayed for anti-δ by ELISA and RIA. They included 300 HBsAg-negative sera and 700 sera from chronic HBsAg carriers with and without hepatitis, collected in different regions of Italy; they represent part of a study to be published elsewhere.

RESULTS

The δ reagent from crude liver homogenate; comparison with reference δ prepared from δ-positive nuclei. The stock δ reagent contained δ Ag activity (sample/negative ratio by RIA, 120), HBsAg (sample/negative ratio by RIA, 8) and no HBcAg, HBeAg, anti-HBs, anti-HBc, or anti-HBe activity by RIA. The δ reagent from the crude liver homogenate and the reference preparation of δ Ag obtained from δ-positive nuclei were diluted to contain in the same volume (5 ml) the total activity extracted from 1 g of original liver tissue. Titers of duplicate 25-μl samples of each of the two preparations were determined in twofold dilutions by RIA. The RIA titration curve of δ Ag in the crude homogenate is shown in Fig. 1; δ Ag activity was detected up to a 1:16 dilution in the preparation of the crude liver extract and a 1:4 dilution in the preparation from the purified nuclei.

Titration of HRPO anti-δ IgG and δ reagent in ELISA. The titers of HRPO anti-δ IgG were determined in twofold dilutions on microtiter plates coated sequentially with anti-δ reagent and the reference antigen. In this system, the optimal dilution of HRPO anti-δ for ELISA was 1:800 (12.5 μg of protein per ml), and this dilution was designated as the standard HRPO anti-δ IgG (HRPO anti-δ IgG was diluted in 50% fetal bovine serum in PBS). The stock δ Ag reagent was titrated in ELISA in twofold dilutions with standard HRPO anti-δ IgG; the titration curve is shown in Fig. 1. In ELISA δ Ag was detected up to the 1:64 dilution. The δ dilution (1:16) corresponding to the highest optical density on the linear portion of the titration curve was used as standard δ reagent; it contained no HBsAg detectable by RIA.

Specificity of ELISA for δ and anti-δ and titration of anti-δ reagent by blocking IFL, ELISA, and RIA. Plates coated with anti-δ reagent were incubated with either the standard δ Ag reagent, the HBsAg, HBcAg or HBeAg reagents, or extracts from the HBsAg-negative liver or the HBsAg-positive, δ-negative liver; each combination was tested with HRPO anti-δ and with the anti-HBs, anti-HBc, and anti-HBe

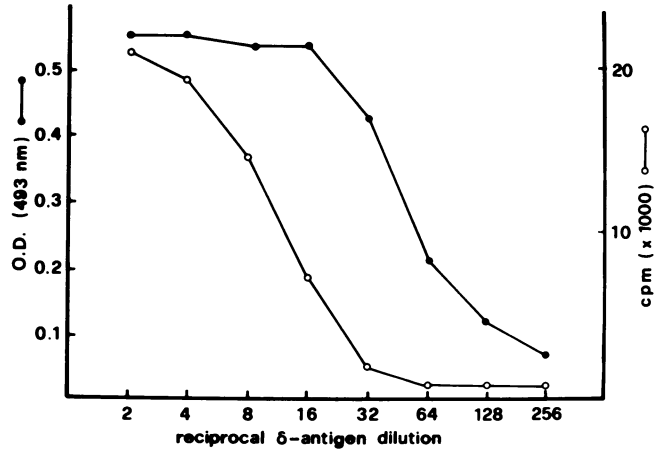

FIG. 1. *Titration curve of δ antigen in ELISA (●) and in RIA (○). Duplicate wells of microtiter plates coated with a 1:1,000 dilution of anti-δ serum were incubated with 25 μl of serial twofold dilutions of stock δ Ag reagent. For ELISA, 50 μl of standard HRPO anti-δ IgG was added, and the wells were analyzed for optical density at 493 nm after development of the HRPO reaction. For RIA, 50 μl of ^{125}I-labeled anti-δ IgG was added, and wells were counted for residual radioactivity.*

HRPO conjugates. Only HRPO anti-δ was bound and only in wells containing δ Ag reagent. No other combination bound the HRPO anti-δ reagent or other HRPO conjugate. The δ Ag plates were inoculated with anti-δ reagent diluted 1:10 and with comparable dilutions of five sera containing anti-δ by RIA; dilutions of HBsAg-negative sera with autoantibodies and reference anti-HBs, anti-HBc, and anti-HBe sera served as controls. Wells were also inoculated with sera obtained from a chimpanzee chronic carrier of HBsAg before (preserum, negative for anti-δ in RIA) and after (postinfection serum, positive for anti-δ in RIA) an episode of δ Ag-positive hepatitis (10).

The anti-δ reagent, the five positive sera, and the postinfection champanzee serum inhibited the binding of HRPO anti-δ; no inhibition was observed with any of the various negative control sera or the chimpanzee preserum. Titers of the anti-δ reagent were determined in serial 10-fold dilutions by ELISA and RIA on δ Ag plates and by blocking IFL. In different experiments, the blocking IFL test was positive at serum dilutions between 1:10 and 1:100. The anti-δ titers in ELISA and RIA were 1:700 and 1:10,000, respectively (Fig. 2).

Comparison of anti-δ detection by ELISA and RIA in HBsAg-negative and -positive sera. None of 300 HBsAg-negative control sera was positive by either assay. Of 700 positive specimens, 120 were positive for anti-δ by both assays at titers between 1:10 and 1:10^6; 10 additional sera were positive by RIA only at a titer of 1:10. The ELISA was repeated, and the HRPO reaction was read with a spectrophotometer at 493 nm; none of the 10 sera, however, decreased significantly (by more than 50%) the optical density measured in the negative control. The 10 sera negative in ELISA and reactive in RIA were from five asymptomatic carriers, four patients with inactive cirrhosis, and a patient with chronic persistent hepatitis.

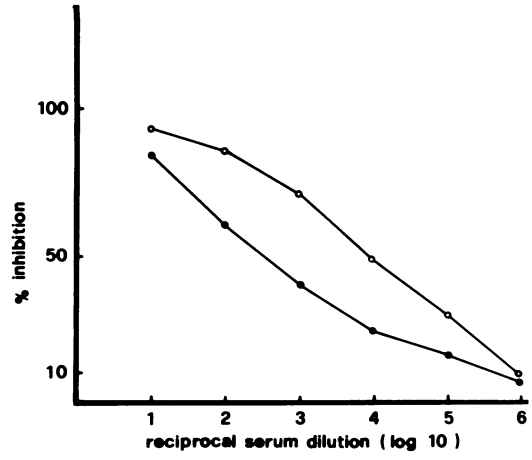

FIG. 2. *Titration curve of the standard anti-δ by RIA (○) and ELISA (●). Duplicate wells of microtiter plates covered with standard δ reagent were inoculated with 100 μl of serial ^{125}I-labeled anti-δ IgG or standard HRPO anti-δ IgG. The titer is expressed as the dilution which caused a 50% decrease in the radioactivity or optical density as compared to the negative control and was 1:700 by ELISA and 1:10,000 by RIA.*

DISCUSSION

We report an ELISA for antibody to δ. A whole-liver homogenate, extracted with 6 M guanidine-hydrochloride, was used as the standard δ Ag reagent instead of the isolated nuclei previously used to develop the RIA (13). The crude extract yielded a higher amount of δ Ag activity per gram of tissue than the purified nuclear preparation. The specificity of the ELISA was shown by the lack of cross-reactivity when autoantigens or HBV determinants were linked to the solid phase instead of the δ Ag reagent, or when autoantibodies or antibodies other than anti-δ were used to block the reaction. No cross-reactions were observed between the δ system and the HBV markers present in the standard reagents. The HBsAg activity in the δ Ag stock was not detectable at the working dilution of the standard δ Ag reagent and, in addition, the standard anti-δ reagent had no anti-HBs activity. Whereas the anti-δ reagent contained a low titer of anti-HBc, no HBcAg was detectable by IFL or electron microscopic examination of the liver source of δ Ag, and HBcAg is unstable to treatment with denaturing agents (7).

Detection of anti-δ in ELISA was more sensitive than the blocking IFL test, but 10- to 15-fold less sensitive than RIA. ELISA, however, appeared more sensitive than RIA in detecting δ Ag. Since δ Ag is revealed by development of the HPRO staining, the enzymatic nature of the reaction may presumably entail an amplifying effect compared with the RIA. Conversely, the enzymatic effect may diminish the sensitivity of the anti-δ ELISA since it is based on inhibition of anti-δ binding. Because of its lower sensitivity, ELISA may be of limited usefulness in epidemiological surveys, as acute δ infections often elicit only borderline and transient anti-δ reactions in RIA (14). The clinical purpose of detecting anti-δ, however, is the identification of a subgroup of HBsAg carriers with chronic active and progressive hepatitis, and in this situation the antibody was found in RIA at titers between 1:500 and $1:10^6$ (14). Similar titers are well within the range of detection by ELISA, and comparison of the prevalence of anti-δ in chronic HBsAg carriers by both assays showed a 92% agreement of results; this included all the HBsAg carriers with chronic active δ infection and disease. The 8% variation was due to low titers of anti-δ not detectable by ELISA in sera from patients with inactive liver disease or nonpathogenic infection. Performance of the anti-δ ELISA for routine clinical screening required only minimal and inexpensive equipment and avoided the inherent hazards associated with handling of radiolabeled reagents. Evaluation of the results was performed by visual grading of the HRPO reaction. This proved to be accurate; none of the HBsAg-positive sera that had borderline activity by RIA and were negative by visual inspection of ELISA was different from negative control sera when retested in ELISA and read with the spectrophotometer.

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