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ENZYME IMMUNOASSAY OF HBeAg EMPLOYING β-D-GALACTOSIDASE

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SUMMARY An enzyme-linked immunosorbent assay (ELISA) system for hepatitis B e antigen (HBeAg) was developed employing β-D-galactosidase conjugated with antibody to HBeAg (anti-HBe) and using m-maleimidobenzoyl-N-hydroxysuccinimide ester as the coupling reagent. The experimental conditions for quantitative assay of HBeAg were determined. The presence of rheumatoid factor in test sera did not affect the results. This assay system is more sensitive than the micro-Ouchterlony method and as sensitive as radioimmunoassay. The use of β-D-galactosidase for ELISA in the field of virology is recommended.

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) is now commonly used in virology, but β-D-galactosidase has seldom been used in virology, although it has often been used for ELISA of hormones (O'Sullivan et al., 1978); in virology, alkaline phosphatase or peroxidase has more often been used for ELISA. The advantage of ELISA over radioimmunoassay (RIA) has been well documented.

HBeAg in human sera is known to be a marker of the communicability of hepatitis B of patients or carriers (Okada et al., 1976; van der Waart et al., 1978; 1980). Thus an exact and sensitive system is needed for its assay. So far, HBeAg has been assayed by RIA (Miyakawa et al., 1979) and the micro-Ouchterlony (MO) method (Blum et al., 1979; Magnius and Espmark., 1972). But the MO method has low sensitivity and often gives ambiguous results. Recently an ELISA system employing peroxidase was reported by Mushahwar and Overby (1981). O'Sullivan et al. (1978) reported a procedure for conjugation of antibody and β-D-galactosidase employing a heterobifunctional reagent, m-maleimidobenzoyl-N-hydroxysuccimide ester (MBS) (Kitagawa and Aikawa, 1976). We followed their procedure for conjugation of β-D-galactosidase with human anti-HBe and developed a fairly sensitive quantitative meth-

od for assay of HBeAg.

MATERIALS AND METHODS

1. *Preparation of antibody-enzyme conjugate and microplates coated with antibody*

Partial purification of anti-HBe was performed by salting out (twice) of anti-HBe-positive and HBsAg-negative human sera with 45% ammonium sulfate followed by Sephadex G-25 column chromatography and DEAE-Cellulose DE-52 column chromatography both with 0.01 *M* phosphate buffer, pH 8.0.

Partially purified anti-HBe (3.5 mg in 78 μl) was mixed with 0.01 *M* phosphate buffer (pH 7.0) to make a total volume of 5.0 ml. Then 50 μl of MBS (Pierce) (730 μg/50 μl dioxane) was added dropwise with continuous agitation and the mixture was incubated at 30 C for 30 min. The anti-HBe-MBS conjugate was separated from free MBS by Sephadex G-25 column chromatography. The conjugate in a volume of 2.5 ml was mixed with 2.5 mg of β-D-galactosidase (Boehringer-Mannheim) in 0.5 ml of 0.01 *M* phosphate buffer (pH 7.0, 0.01 *M* $MgCl_2$), by dropwise addition and the total volume was adjusted to 5.0 ml with 0.01 *M* phosphate buffer (pH 7.0). The mixture was incubated for 30 min at 30 C, and then 25 μl of 2 *M* 2-mercaptoethanol was added and the mixture was applied to a Bio-gel A-5m (Bio-rad) column. Elution was performed with 0.01 *M* phosphate buffer (pH 7.0) containing 1 m*M* $MgCl_2$, 1 m*M* mercaptoethanol and 0.128 *M* NaCl. The eluent was collected in 4 fractions. The third fraction, which showed the lowest non-specific binding, was used as the enzyme-antibody conjugate in experiments.

A preliminary study showed that 80 μg/ml of anti-HBe was optimal for adsorption of antibody to the bottom of the microplate. Samples of 0.1 ml of anti-HBe (80 μg/ml in 0.05 *M* bicarbonate buffer, pH 9.5) were introduced into the wells of a flat polyethlene microplate (96 well flat plate, Cooke) and the plate was stood overnight at 4 C. Then the anti-HBe solution was sucked off and 0.3 ml of 0.05 *M* bicarbonate buffer (pH 9.5) supplemented with 0.1% NaN_3 and 10% normal serum, free of hepatitis B virus related antigens or antibodies and freeze-thawed several times, was added to each well. The plates were stood for 30 min at room temperature and then the wells were washed twice with 300 μl of 0.01 *M* phosphate buffered saline (pH 7.2). The plates were dried at room temperature and stored at 4 C until use. anti-HBe on the solid phase retained a constant titer for at least 6 months.

2. *Detection of hepatitis B surface antigen (HBsAg) and antibody to HBsAg (anti-HBs)*

HBsAg and anti-HBs was detected by reversed passive hemagglutination (R-PHA) and passive hemagglutination (PHA), respectively.

3. *Micro-Ouchterlony (MO) method*

For detections of HBeAg and anti-HBe the method was performed as described by Magnius and Espmark. (1972)

4. *Assay of HBeAg*

Aliquots of 100 μl of test sera and 0.1% bovine serum albumin (BSA) in 0.01 *M* phosphate buffered saline containing 1 m*M* $MgCl_2$ were introduced into the wells of microplates coated with anti-HBe. The plates were stood at room temperature for 2 h, and then each well was washed twice with 300 μl of 0.01 *M* phosphate buffered saline supplemented with 0.1% Tween-20 (Tween-PBS). Then 150 μl of anti-HBe-enzyme conjugate in 0.01 *M* phosphate buffered saline (0.1% BSA, 10% normal frozen and thawed serum, 1 m*M* $MgCl_2$) was added to each well and the plates were stood overnight at room temperature. Each well was washed twice with 300 μl of Tween-PBS, and then 150 μl of 0.1% o-nitrophenyl β-D-galactopyranoside in 0.01 *M* phosphate buffered saline supplemented with 0.1% BSA, 5% methanol and 1 m*M* $MgCl_2$ was added to each well. The plates were stood for 6 h at room temperature. The enzyme action was stopped by adding 150 μl of 3% Na_2CO_3 and the optical density at 410 nm (OD_{410}) was determined with a microplate photometer (Corona MTP-12).

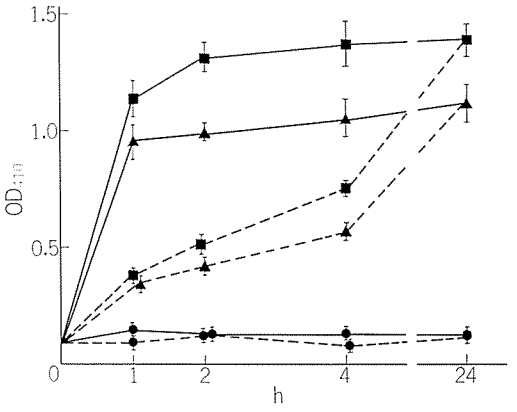
5. *Radioimmunoassay of HBeAg*

Radioimmunoassay of HBeAg was performed with a commercial kit from Abott Laboratories.

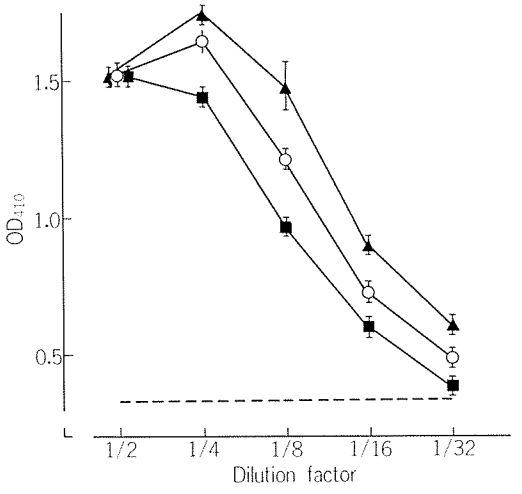
RESULTS

1. *Determination of the optimum incubation period*

The incubation periods indicated in the Materials and Methods were chosen as follows, using two HBeAg positive sera and one HBeAg negative serum. Either the incuba-



FIGURES 1. Determination of optimum incubation periods. Undiluted sera were tested for HBeAg by ELISA using various incubation periods.
■, ▲: HBeAg positive sera by MO
●: HBeAg negative serum
——: I_1, variable; I_2, 24 h
- - - -: I_1, 24 h; I_2, variable
I_1 represents the incubation period of test sera in antibody-coated wells. I_2 represents the incubation period after addition of anti-HBe-Enzyme conjugate to the wells.



FIGURES 2. Dilution curves of the sera for the assay of anti-HBe by ELISA. Serially two-fold diluted sera were used for assay of HBeAg. The dotted line represents the reaction of HBeAg-negative serum. HBeAg-positive sera were diluted with HBeAg negative serum.
Incubation period: I_1, 2 h; I_2, 24 h

tion period of test sera in antibody-coated wells (I_1) or the incubation period after addition of anti-HBe-Enzyme conjugate (I_2) was varied between 0 to 24 h, while the other period was fixed at 24 h. As shown in Fig. 1, 2 h of I_1 was enough for completion of the reaction, while 24 h of I_2 was sufficient for the maximum reaction.

2. *Intraassay variance*

For practical use of this system, intraassay variance was checked. One HBeAg negative (8 wells) and one HBeAg positive (30 wells) serum were used. The wells used in the microplate were selected at random. The OD_{410} for HBeAg positive serum was 0.509 ± 0.040 and that for normal serum was 0.028 ± 0.012. These results show that the intraassay variance for both HBeAg negative and positive sera was negligible.

3. *Test on the dose response*

Serial two-fold dilutions of three HBeAg positive sera were assayed for HBeAg by the present procedure (Fig. 2). At dilutions of 1:4 to 1:32 of the 3 sera, the OD_{410} values decreased linearly and in parallel. This linearity could not be seen at a serum dilution of 1:1 (data not shown) or 1:2. So this assay system can be used for quantitative measurement of HBeAg at appropriate dilutions of sera.

4. *Comparison of this assay system with other systems*

In all, 517 undiluted sera were tested for the presence of HBeAg by this assay system and the results were compared with those obtained with other systems (Table 1). These sera were obtained from individuals free from HBsAg and from asymptomatic hepatitis B virus carriers. The HBeAg positive sera determined by the MO method were all positive for HBeAg with this assay system. Two of 27 anti-HBe positive sera and HBeAg negative sera by the MO method were found to contain HBeAg by this method. Eight of 31

TABLE 1. *Detection of HBeAg by currently used ELISA in sera tested for hepatitis B virus-related antigens and antibodies by other methods*

HBsAg by R-PHA		+		−	
anti-HBs by PHA	−	−	−	−	+
HBeAg by MO	−	+	−	−	−
anti-HBe by MO	−	−	+	−	−
Detection of HBeAg by ELISA[a] positive/total (per cent)	8/31 (25.8)	25/25 (100)	2/27 (7.4)	2/392 (0.5)	0/34 (0)

[a] Cutoff value of 2X the control was used.

TABLE 2. *Comparison of RIA and ELISA for detection of HBeAg*

		ELISA	
	HBeAg	+	−
RIA	+	24	0
	−	0	33

Undiluted sera were tested for the presence of HBeAg.

sera, that were HBeAg- and anti-HBe-negative by the MO method, were HBeAg positive by this method. Of the sera from normal individuals tested, none of 34 anti-HBs positive sera contained HBeAg, while 2 of 397 anti-HBs- and HBsAg-negative sera did contain HBeAg by this method.

This assay system was also compared with the RIA system (Table 2). Undiluted serum specimens were used for the assay. Randomly selected HBeAg positive (24 samples) and HBeAg negative (33 samples) sera from asymptomatic carriers were tested for HBeAg by both the present assay and RIA. The results showed good qualitative concordance between the two assay systems. In these experiments, for judgement of HBeAg positiveness, we adopted a cutoff value of twice the value of control serum.

5. *Effect of rheumatoid factor on this assay system*

We tested the effect of rheumatoid factor on this assay system using 10 sera that contained high levels of rheumatoid factor. The presence of rheumatoid factor did not result in false-positive results. The fact that false-positive results were not obtained might be due to the addition of 10% freeze-thawed serum, free from hepatitis B virus related antigens and antibodies to them, in preparation of anti-HBe-coated microplates; without addition of freeze-thawed serum, 15 of 26 sera positive for rheumatoid factor gave false-positive results. Thus this procedure was essential to avoid false-positive results (Smith and Tedder, 1981).

DISCUSSION

Since β-D-galactosidase is a fairly stable enzyme, it is often used for ELISA in the field of endocrinology, but rarely in virology (Kato et al., 1975; Kitagawa and Aikawa, 1976; Kikutani et al., 1978). The use of this enzyme has several advantages over the use of the enzymes peroxidase and alkaline phosphatase. Unlike alkaline phosphatase, β-D-galactosidase is not present in sera and can easily be conjugated with IgG in a ratio of 1:1 using MBS. So the background level of the reaction is very low. In the case of peroxidase, enzyme activity is lost when NaN_3 is used and hemoglobin has similar enzyme activity to peroxidase. Moreover the method for conjugation of peroxidase with IgG molecule in a ratio of 1:1 is fairly complicated. Futhermore, the substrate of peroxidase, o-phenylenediamine, is a potent carcinogen. Use of a fluorescent substrate for β-galactosidase greatly increases the sensitivity of the reaction.

HBeAg is closely related to the communicability of hepatitis B of patients or healthy carriers. Therefore, we followed the procedure of O'Sullivan et al. (1978) in development of a sensitive and reproducible ELISA system for assay of HBeAg. The small intraassay

variance of this procedure proved its usefulness and reproducibility. The addition of 5% methanol (Shifrin and Hunn, 1969) during the enzyme-substrate reaction increased color development at least two times (data not shown). The results in Fig. 2 show that this assay system is suitable for quantitative analysis of HBeAg. We also found that qualitative assay of HBeAg with our system could be used for detection of anti-HBe by the competition method (data not shown). The finding of HBeAg in 2 of 397 HBsAg- and anti-HBs-negative individuals may reflect cross-reactivity between e antigens of hepatitis B virus and non-A, non-B hepatitis virus (Vitvitski et al., 1980) or may be the result of the low sensitivity of R-PHA for the assay of HBsAg.

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