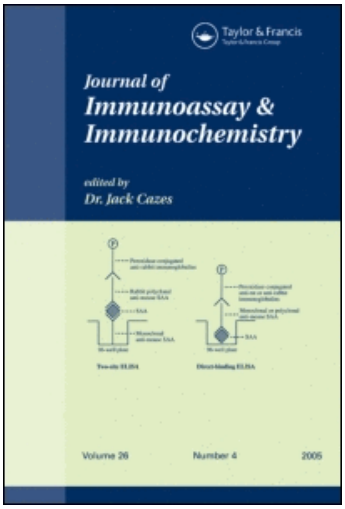

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A DOUBLE SANDWICH MONOCLONAL ENZYME IMMUNOASSAY FOR DETECTION OF HEPATITIS B SURFACE ANTIGEN.

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ABSTRACT

An enzyme immunoassay for detection of hepatitis B surface antigen based on the use of 3 monoclonal antibodies (mAbs) was developed: an IgM as capture and 2 IgG_1 for detection. The system biotin-streptavidin was compared with direct conjugation of mAbs to peroxidase and was preferred because of its higher signal to noise ratio. The possibility of simultaneous addition of human serum and biotin-mAb was discarded because of an evident prozone effect with some sera containing high HBsAg levels. The conjugation of biotin to IgG_1 mAbs through a spacer arm (amidocaproil) and the use of a highly sensitive substrate (tetramethylbenzydine) improved the assay detection limit by about 10 times. (KEY WORDS: Hepatitis B surface antigen, sandwich ELISA, Diagnosis, biotin-streptavidin).

INTRODUCTION

Epidemiologic studies of hepatitis B virus infection in developing countries have been limited by the high cost of the commercial kits for its diagnosis. Nevertheless, this viral agent infects about 300 million persons worldwide and 1-5% of the Latin

American population (1,2), with sequelae of chronicity, cirrhosis and primary hepatocellular carcinoma (1).

A number of plasma-extracted or recombinant vaccines, which contain hepatitis B surface antigen (HBsAg), are now available for the prevention of hepatitis B infection (1). A better understanding of hepatitis B epidemiology would be useful in the development of immunoprophylaxis programs.

In a previous communication we reported the production and characterization of monoclonal antibodies (mAbs) against HBsAg (3). In this paper we describe the standardization of a biotin-streptavidin based enzyme immunoassay for the detection of HBsAg in sera or plasma of infected patients.

MATERIALS AND METHODS

Materials. Unless otherwise mentioned, all reagents were from Sigma Chemical Co (MO, USA). Human sera HBsAg negative or reactive by Hepanostika HBsAg (Organon Teknika B.V.,Boxtel, Holland) or by Ortho HBsAg ELISA Test System (Ortho Diagnostic System, Beerse, Belgium) were provided by Departamento de Hemoterapia, Banco Municipal de Sangre, hospitals Miguel Pérez Carreño, Domingo Luciani and Herrera Vega, and from Instituto de Inmunología Clínica, Caracas, Venezuela. An international standard with known HBsAg concentration was obtained from Paul Erhlich Institute (Germany). Serum panel nº 6 with different levels of HBsAg was provided by the Food and Drug Administration (FDA Office of Biologics, MD, USA). MAbs anti-HBsAg were obtained after fusion with splenocytes of a mouse immunized with vaccine antigen (3).

MAbs purification. MAb 6F4 was purified by protein A-sepharose affinity chromatography (Protein A MAP), 6G10 was purified by DEAE Blue Affigel chromatography (Bio-Rad Laboratories, CA, USA) and 5E8 on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) (4).

HBsAg ELISA. MAb 5E8 (10 μg/ml) was adsorbed on polystyrene plates for 2 hours at 37°C. Different concentrations of HBsAg diluted in neonatal bovine serum (Gibco Laboratories, NY, USA) were added to each well for 1 hour at 37°C; HBsAg concentration in serum was evaluated by titration in parallel with an international standard (300 U/ml). MAbs used for detection were directly conjugated to peroxidase (ActiZyme-HRP Kit, Zymed Laboratories, CA, USA), conjugated to N-hydroxy-succimidobiotin (biotin-mAb), at an optimal ratio of 70 μg reagent / mg protein (4) or conjugated to amidocaproil-N-hydroxysuccimidobiotin (biotin-amidocaproil-mAb), at an optimal ratio of 100 μg reagent/mg protein (5). Biotin-mAbs were diluted in phosphate buffer saline, pH 7.4, containing 20% neonatal bovine serum, 1% bovine serum albumin and 0,05% (v/v) Tween 20 (PBS-Tween-BSA-serum) and incubated 1 hour at 37°C. After each step, 4 washings with PBS-Tween were performed. Streptavidin-peroxidase in PBS-Tween-BSA was then added to each well and incubated for 1 hour at 37°C.

Two substrates were used: *o*-phenyl-enediamine (OPD) in citrate perborate buffer (Sigma) or tetrametilbenzidine (TMB, Kirkegaard & Perry Laboratories Inc, MD, USA) (5). Enzymatic reaction was performed for 30 minutes and stopped with H_2SO_4, 1 mol/L; optical

densities (O.D.) were determined at 492 nm (OPD) or 450 nm (TMB) in a Multiskan II spectrophotometer (Flow Laboratories Inc., VA, USA).

RESULTS

Three mAbs produced against HBsAg (3) were used in a sandwich ELISA for detection of this viral antigen: 5E8, an IgM antibody, as capture, and 6F4, alone or in combination with 6G10, both IgG_1 antibodies, for detection.

The sensitivity of the direct conjugation method compared to the use of biotin-streptavidin system was evaluated. The latter system showed higher O.D. values for a same HBsAg concentration with similar background values (Fig. 1) and was then adopted for the following experiments.

In order to reduce the multiple steps involved in using biotin-streptavidin system, the simultaneous addition of human serum and biotin-6F4 was evaluated; false negative results were obtained with some HBsAg positive sera which were positive when used diluted 1/10 (data not shown). The sequential incubation format was then adopted, but the biotin-mAb and streptavidin-peroxidase incubation time were reduced from 1 hour to 30 minutes, without loss of sensitivity (data not shown).

Different conditions were assessed to improve the detection level of the HBsAg immunoassay, based on the use of the 2 IgG_1 mAbs for detection: conjugation of detection mAbs to amidocaproil-biotin instead of biotin and TMB substrate instead of OPD. An increase in the signal to noise ratio was observed for the same HBsAg concentration, when using biotin-amidocaproil-mAbs and/or TMB (Fig. 2).

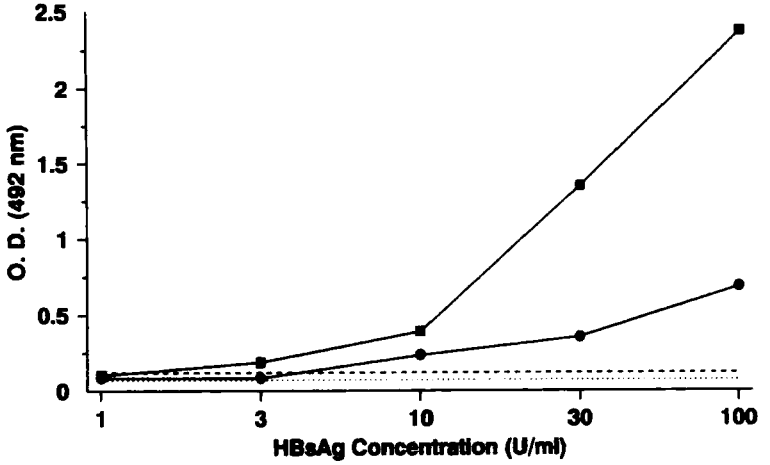

FIGURE 1: Direct peroxidase conjugation HBsAg ELISA versus biotin-streptavidin system. MAb 5E8 was used as capture; different dilutions of HBsAg positive serum were then added. MAb 6F4 conjugated to peroxidase (—●—) or to biotin (—■—) was incubated for one hour; in the latter case, an additional incubation step with streptavidin-peroxidase was performed. Enzymatic reaction was revealed with OPD and read at 492 nm. .....: cutoff value for direct peroxidase conjugation. - - - - -: cutoff value for biotin-strepatvidin system (O.D. value of negative serum).

Each of these two modifications allowed a reduction of the detection limit of about 3 times (Fig. 2). By using both modifications the sensitivity of the assay was then improved almost 10 times.

The suitability of this sandwich ELISA for detection of HBsAg in human sera was evaluated against the FDA reference serum panel. The cutoff value used was the midpoint between a negative control serum and a low HBsAg containing serum (3 U/ml); in previous experiments it was determined that this cutoff was equivalent to the mean of 20 negative sera values plus 3 standard deviations (data not

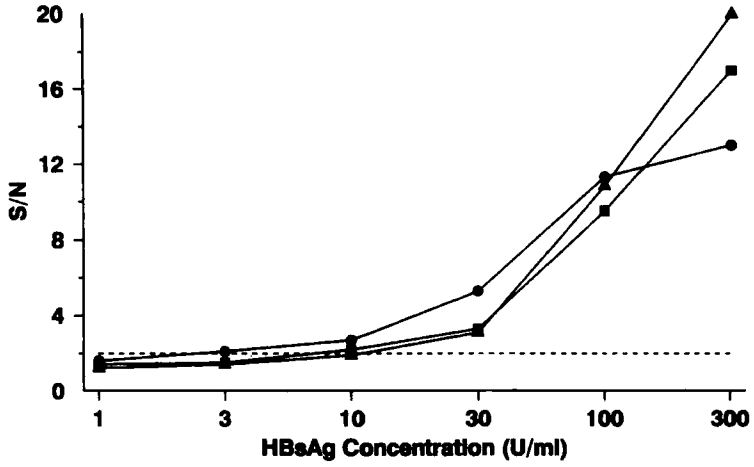

FIGURE 2: Standardization of the double sandwich ELISA for HBsAg detection. MAb 5E8 was used as capture; different concentrations of HBsAg positive serum were then added. MAbs 6F4 and 6G10 conjugated to biotin or to amidocaproil-biotin were then added and incubated for 30 minutes. Streptavidin-peroxidase was incubated for 30 minutes. —▲—:biotin-amidocaproil-mAbs and OPD as substrate. —■—: biotin-mAbs and TMB as substrate. —●—: biotin-amidocaproil-mAbs and TMB as substrate. S/N: signal to noise ratio (positive value / negative value) is reported. – – – – –: the cutoff was established as S/N of 2.

shown). The performance of the ELISA met the requirements of a third-generation test against the FDA panel (Table 1).

The assay was also evaluated with blood bank sera and sera from a clinical institute, tested with Ortho HBsAg ELISA Test System and Hepanostika HBsAg kit respectively. Two out of the 25 positive sera from the clinical institute were not detected by our ELISA; for sera from blood banks correlation was 100% (Table 2).

DISCUSSION

The unique feature of hepatitis B infection, where a high number of surface antigen particles circulate in serum of infected

TABLE 1

Evaluation of HBsAg ELISA against the FDA HBsAg Panel nº 6.

Sample	Subtype	ELISA HBsAg
604 B	ay	2.724 +
607 C	ay	1.075 +
611 C	ay	0.959 +
606 C	ad	0.647 +
602 C	ay	0.513 +
608 C	ad	0.352 +
605(C)	ad	0.221 -
610(C)	ay	0.219 -
613(C)	ay	0.198 -
612 D	ad	0.196 -
601 D	ad	0.185 -
603 N		0.256 -
609 N		0.247 -
Cutoff		0.276

ELISA HBsAg was performed using mAb 5E8 as capture; the different sera were then incubated for 1 hour at 37°C. MAbs 6F4 and 6G10 conjugated to amidocaproil-biotin were incubated for 30 minutes and then streptavidin-peroxidase was incubated for 30 minutes. Enzymatic reaction was revealed with TMB.

B serum is a high HBsAg containing serum which is recognized by second generation tests. C sera are HbsAg positive sera that must be recognized by third generation tests. (C) and D sera contain HBsAg but are allowed not to be detected by diagnostic tests. N sera are HBsAg negative sera.

Symbol + means reactive and - non-reactive. Cutoff value was established as the mean between N = O.D. average value of negative control and P = O.D. average value of positive control serum, 3 U/ml.

hosts, has lead to the development of several sandwich ELISAs (2,6-10). We described in a previous communication the production and characterization of hybridoma cell lines which secrete mAbs against HBsAg; three of them were chosen for the development of a sandwich

ELISA to detect HBsAg, on the basis of sensitivity, specificity and recognition of the different HBsAg antigenic diversities (3).

Direct peroxidase mAb conjugation method was compared to the biotin-streptavidin system (11). Biotin-streptavidin resulted in a double sandwich ELISA with superior sensitivity (Fig. 1), confirming the suitability of the biotin-streptavidin system, which has been previously described for HBsAg detection, using avidin instead of streptavidin (7). Streptavidin yields lower background values (11). A reduction in assay steps has been described by simultaneously incubating sera and biotin-mAb (7), but in our system, simultaneous incubation led to false negative results when using sera with high HBsAg levels. This hook effect was not observed by Liu and Green (7), and only at very high HBsAg concentrations by Palomäki (8), but is a well recognized hazard of ELISA's. Possibly the capture and/or detection capacity of our system is rapidly saturated with sera containing a high antigen concentration.

An increase in sensitivity was obtained when using spacer arm linkage to biotin (5,12) instead of direct linkage and by use of TMB instead of OPD as substrate. Spacer arm linkage for biotin has been reported to increase reactivity with streptavidin conjugates, probably by reducing steric hindrance with the streptavidin (5,13). The performance of this double sandwich ELISA was adequate when evaluated against the FDA sera panel. Both d and y subtypes were recognized (Table 2), along with the recognition of w and r subtypes (3).

Four hundred out of the 400 negative sera tested in local blood banks and a clinical institute were negative by our assay, and 40 out of 42 positive sera were positive by our ELISA (Table 2). In

TABLE 2

ELISA HBsAg Evaluation in Blood Banks and in a Clinical Institute.

Origin	Sera	Number	ELISA HBsAg	Correlation
Blood banks	HBsAg +	17	17	100%
	HBsAg -	376	376	100%
Clinic Institute	HBsAg +	25	23	92%
	HBsAg -	30	30	100%

Sera were provided by different blood banks and a clinic institute from Caracas, Venezuela. Sera were classified as HBsAg reactive (+) or non-reactive (-) according to evaluation by the commercial kit used in each laboratory (Ortho HBsAg ELISA Test System and Hepanostika HBsAg). The cutoff value was determined as in Table 1.

HBsAg testing, there has been always the problem of false positive results, which has lead to the development of confirmatory assays, based upon the neutralization of the ELISA signal by a polyclonal anti-HBsAg positive serum (14,15). One of the two discrepant serum seems however to be true positive, as it was positive for antibody against hepatitis B "e" antigen; the other serum was not tested for other marker. A possible explanation is that the HBsAg concentration in these sera was below the detection level of our assay, as could occur in acute phase of illness. The sensitivity of this double sandwich ELISA was suitable when evaluated against the FDA panel. The detection limit is similar to other third-generation assays (1-10 U/ml) (16,17), with a high specificity, characteristic of ELISAs based entirely on mAbs.

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