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Amplification Immunoassay for the Determination of Hepatitis B Surface Antigen

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ABSTRACT

A sensitive sandwich immunoassay for the determination of Hepatitis B surface antigen (HBs) was developed, using a cascade system of Limulus amebocyte lysate as a signal amplification system. Lipopolysaccharide (LPS) was conjugated to anti-HBs antibody. Anti-HBs antibody was adsorbed to polystyrene beads. First, HBs were reacted to solid phase anti-HBs antibody (ā-HBs). After the reaction, the beads were rinsed, and were then reacted with ā-HBs-LPS. Then, LPS activity specifically bound to the beads was measured. HBs could be measured in the range of 10^{-10}–10^{-12} g/mL.

Index Entries: Enzyme immunoassay; cascade amplification; hepatitis B surface antigen; lipopolysaccharide; limulus amebocyte lysate.

INTRODUCTION

Various kinds of enzyme immunoassay (EIA) have been widely applied in the field of clinical analysis (*1–5*). In EIA, enzyme activity is used as the marker. Enzymes are covalently bound to antigens or antibodies, and are used to detect antigen–antibody complex. The sensitivity of EIA depends on both the turnover number of the enzyme used, and the sensitivity of the product detection in the enzyme reaction. In normal EIA, chromogenic

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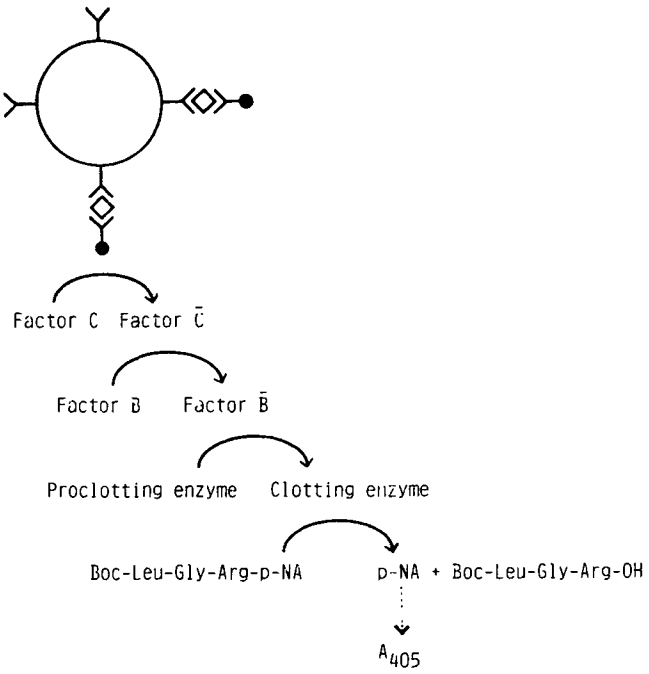

Fig. 1. Schematic diagram of sandwich immunoassay–cascade amplification system.

substrates for the enzymes are used (*6–8*). A number of approaches have been used to enhance the sensitivity of EIA, including the use of fluorescent and luminescent substrates for some enzymes (*9–12*). In other approaches to enhance the sensitivity, the use of enzyme cycling was reported (*13–15*).

On the other hand, we noted that the cascade amplification system can be useful to increase the signal and to enhance the sensitivity. We have recently developed a novel immunoassay, using Limulus amebocyte lysate as a cascade amplification system (*16*). Furthermore, chromogenic Limulus test commercialized by Seikagaku Kogyo Co., Ltd. was employed as a cascade system, and IgG and anti-IgG were measured, using solid phase competitive immunoassay (*17*), and this system could be applied to the sandwich immunoassay.

In this report, we demonstrate the application of the cascade system to the sandwich immunoassay, and Hepatitis B surface antigen (HBs) was measured. HBs is regarded as a specific marker in an infectious disease of Hepatitis B virus (HBV). It is important, therefore, to develop a sensitive and specific detection method for HBs in screening of HBV infection. Figure 1 shows the schematic diagram of sandwich immunoassay and chromogenic Limulus test commercialized by Seikagaku kogyo Co., Ltd. (*18*) as cascade amplification system. In this cascade system, four kinds of proenzymes and synthesized substrate for clotting enzyme, Boc-Leu-Gly-

Arg-pNA are contained. LPS can activate factor C to factor $\bar{C}$. Factor $\bar{C}$ activates factor B to factor $\bar{B}$. Factor $\bar{B}$ activates proclotting enzyme to clotting enzyme. Clotting enzyme catalyzes the hydrolysis reaction of the synthesized substrate, and *p*-nitroaniline is produced. By the measurement of absorbance of *p*-nitroaniline at 405 nm, LPS can be detected quantitatively.

MATERIALS AND METHODS

Reagents

HBs, antiHBs antibody, and human serum albumin were purchased from The Green Cross Corporation (Osaka, Japan). Lipopolysaccharide (*E. coli*, 0111:4B W) was purchased from DIFCO (Detroit, MI). Polystyrene beads were purchased from Sekisui Kagaku Kogyo Co. Ltd. Toxicolor LS-1 set, that is a chromogenic limulus test kit, was purchased from Seikagku Kogyo Co., Ltd. Sepharose CL-4B and NAP-25 column were purchased from Pharmacia. Distilled water for injection (WFI) was purchased from Otsuka Pharmaceutical Corp., and was used in the preparation of serial dilutions of samples, antiHBs bound beads, and buffer solutions. All glass instruments were heated at 250°C for 2 h to inactivate the contaminated pyrogen.

Preparation of LPS-Labeled Anti-HBs Antibody

LPS (100 mg) was dissolved in 5 mL of water, pH was adjusted at 5.0 by acetic acid. 15 mg of $NaIO_4$ was added to the solution and reacted for 1 h at room temperature. The reaction mixture was then desalted by using NAP-25 column. 2.5 mL of anti-HBs solution (200 U/mL) and 12.5 mL of carbonate buffer (0.2*M*, pH 9.8) were added to the desalted sample solution, and was reacted for 3 h at room temperature under gentle stirring. 0.2 mL of $NaBH_4$ solution (6 mg/mL) was added to the reaction mixture at 4°C for 2 h under gentle stirring. Anti-HBS-LPS conjugate was then purified by Sepharose CL-4B column.

Assay of ā-HBs-LPS

ā-HBs-LPS was measured using Toxicolor LS-1 according to the reagent manual. 20 μL of sample solution was added to 0.8 mL of Toxicolor solution and incubated at 37°C for 30 min, followed by the addition of 2.0 mL of 0.8*N* acetic acid solution, in order to stop the reaction. Then, the absorbance at 405 nm was measured.

Preparation of Anti-HBs Antibody Adsorbed Polystyrene Beads

Anti-HBs antibody (ā-HBs) adsorbed polystyrene beads were prepared as follows: polystyrene beads were washed by 0.1% dcn 90 (Decon Labo. Ltd) solution with sonication for 2 h. The beads were then rinsed with distilled water and simultaneously sonicated. The beads were immersed in ethanol and simultaneously sonicated for 2 h, followed by rinsing with distilled water. Again, 80% ethanol solution containing 0.2*N* KOH was used to wash the beads and followed by sonication for 2 h, and an overnight immersion in its solution. The same beads were rinsed with WFI with sonication. The rinsed beads were immersed in 40 U/mL of anti-HBs antibody solution (0.02*M* PBS, pH 7.4) at 4°C overnight. The same beads were then rinsed with 0.02*M* PBS and immersed in 1% HSA solution (0.02*M* PBS, pH 7.4) at 4°C overnight in order to inhibit nonspecific adsorption of HBs and/or LPS labeled anti-HBs antibody. Finally, the beads were rinsed with 0.02*M* PBS (pH 7.4), and were stored in 0.1% HSA solution (0.02*M* PBS pH 7.4).

Assay of HBs

Serial dilutions of HBs were made in 0.02 *M* PBS (pH 7.4). To the standard solution, one of the ā-HBs antibody absorbed polystyrene bead was added and incubated for 1 h at 37°C, and the bead was rinsed four times with PBS. The bead was then immersed in various concentration of ā-HBs-LPS solution and was incubated for 1 h at 37°C. After incubation, the bead was rinsed four times with PBS. Finally, the bead was then immersed in the 0.8 mL of Toxicolor LS-1 solution, and the reaction was allowed to proceed for 30 min at 37°C, after which 2.4 mL of 0.8*N* acetic acid was added to stop the reaction, and absorbance at 405 nm was measured.

RESULTS AND DISCUSSIONS

Preparation of ā-HBs-LPS

Figure 2 shows the chromatography pattern of the ā-HBs-LPS on Sepharose CL-4B column. In our previous report (*17*), it was reported that LPS-labeled IgG and LPS-labeled anti-IgG were prepared and applied to Sepharose CL–4B column chromatography. The existance of protein and polysaccharide were tested in each fraction, and it was shown that the first peak contained protein and polysaccharide. The first peak, therefore, considered as a LPB-labeled IgG fraction. In this case, therefore, the first peak was considered as ā-HPs-LPS fraction.

Figure 3 shows the calibration curve for the determination of ā-HBs-LPS using Toxicolor LS-1. ā-HBs-LPS could be measured in the range of 1.04×10^{-10}–10^{-9} g/mL.

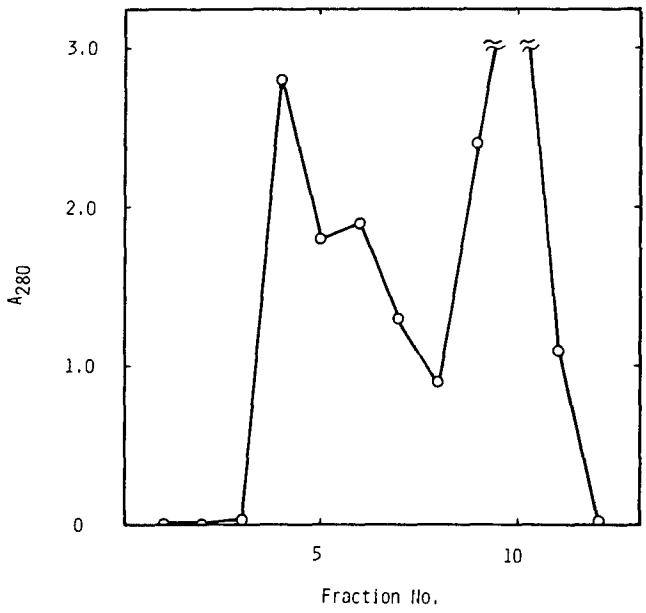

Fig. 2. Chromatography pattern of ā-HBs-LPS on Sepharose CL-4B column. (Column size: $\phi 1.2 \times 35$ cm)

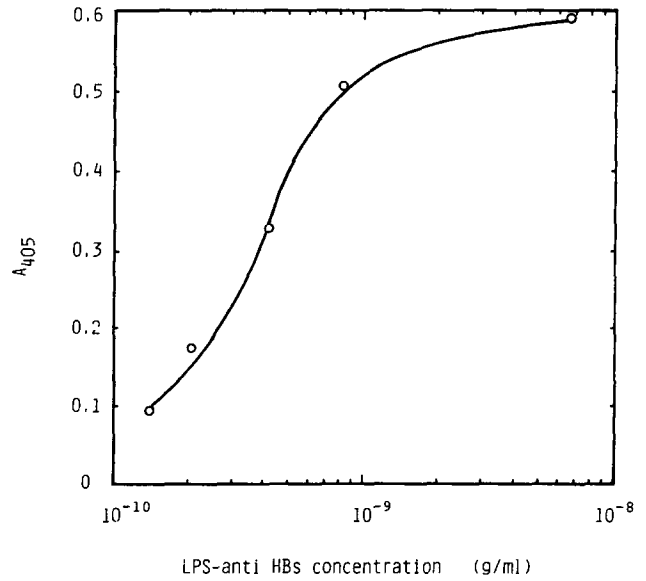

Fig. 3. Calibration curve for the determination of ā-HBs-LPS using chromogenic limulus test.

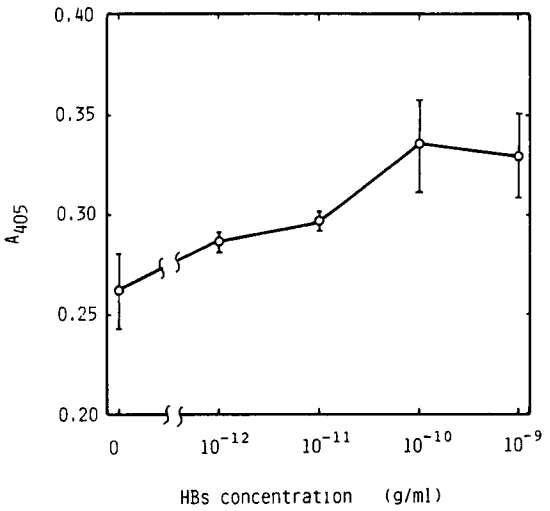

Fig. 4. Calibration curve for the determination of HBs using a combination of a sandwich immunoassay and enzyme cascade reaction. (ā-HBs-LPS concentration was 0.25×10^{-6} g/mL).

Assay of HBs

Figure 4 shows the calibration curve for the determination of HBs. In the solid-phase sandwich immunoassay, according to the increase of antigen concentration, the amount of antigen binding to antibody immobilized on solid-phase increases. The amount of labeled-antibody specifically binding to antigen, therefore, increases. In this case, according to the increase of HBs concentration, the LPS activity on polystyrene bead increased. When ā-HBs-LPS concentration was 0.5×10^{-6} g/mL, HBs was measured in the range of 10^{-12}–10^{-10} g/mL. However, when ā-HBs-LPS concentration was 10^{-7} g/mL, HBs could not be measured because ā-HBs-LPS concentration was too low to be used in immunoassay.

On the other hand, according to the increase of ā-HBs-LPS concentration, the amount of nonspecific adsorption of the conjugate increases. In solid phase immunoassay, nonspecific adsorption brings a decrease of the sensitivity and the reduction of the measurement range. Therefore, in solid immunoassay, it is important to determine the adequate conjugate concentration. it is to be noted that a sensitive sandwich immunoassay for the determination of HBs antigen has been established by the use of lipopolysaccharide as a label compound, and chromogenic Limulus test as a cascade amplification system.

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