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Physicochemical and immunological properties of the hepatitis B surface antigen containing the preS2 9 amino acid sequence produced by a recombinant yeast.

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Abstract

The hepatitis B virus surface antigen containing the preS2 nine amino acid sequence produced by a recombinant Saccharomyces cerevisiae (yHBsAg) was purified and its physicochemical properties were determined. Ultrastructurally, the yHBsAg was found to be a homogeneous spherical particle with a diameter of 24 +/- 4 nm. The homogeneity of the yHBsAg particles was also demonstrated by analyses of their buoyant density and isoelectric point. They consisted of protein (53%), lipid (36%) and carbohydrate (11%), and the alpha-helix content was estimated to be 32%, differing from the reported values for human plasma-derived HBsAg (hHBsAg). Immunodiffusion analysis showed that the antigenic specificity of yHBsAg was identical to that of hHBsAg. Immunization of mice demonstrated that the immunogenicity of the yHBsAg was significantly higher than that of hHBsAg.

KEYWORDS: hepatitis B surface antigen, yeast, Pre S2, immunogenicity, recombinant yeast

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Physicochemical and immunologcal Properties of the Hepatitis B Surface Antigen Containing the PreS2 9 Amino Acid Sequence Produced by a Recombinant Yeast

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The hepatitis B virus surface antigen containing the preS2 nine amino acid sequence produced by a recombinant *Saccharomyces cerevisiae* (yHBsAg) was purified and its physicochemical properties were determined. Ultrastructurally, the yHBsAg was found to be a homogeneous spherical particle with a diameter of 24 ± 4 nm. The homogeneity of the yHBsAg particles was also demonstrated by analyses of their buoyant density and isoelectric point. They consisted of protein (53 %), lipid (36 %) and carbohydrate (11 %), and the α-helix content was estimated to be 32 %, differing from the reported values for human plasma-derived HBsAg (hHBsAg). Immunodiffusion analysis showed that the antigenic specificity of yHBsAg was identical to that of hHBsAg. Immunization of mice demonstrated that the immunogenicity of the yHBsAg was significantly higher than that of hHBsAg.

key words : **hepatitis B surface antigen, yeast, Pre S2, immunogenicity, recombinant yeast**

Two different sources of antigens are available for the preparations of hepatitis B vaccine. One is hepatitis B surface antigen (HBsAg) positive human plasma and the other a recombinant yeast carrying a HBsAg gene. Recent work has been directed toward the development and improvement of the yeast-derived antigen (yHBsAg) because of its superiosity over the human plasma-derived antigen (hHBsAg) as to safety and immunogenicity.

In a hepatitis B virus genome there is a preS2 region immediately upstream of the S region encoding for the S domain of the HBsAg (1). Only small amounts of the PreS2 peptide are present in the HBsAg, probably due to proteolysis (2). Evidence accumulated so far suggests that the preS2 peptide plays an important role in immunity to HBV in accordance with the S peptide. We succeeded in the production of a polypeptide consisting of the preS2 nine amino acid sequence and the subsequent S domain with a recombinant yeast (3).

Since HBs polypeptides assemble with host cellular components into particles (4), their immunogenicity seems to be closely related not only to the conformation of the polypeptides, but also

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to the structure and constituents of HBs particles. The yHBsAg that we developed consists of a single polypeptide encoded by the preS2 and S regions. There is a possibility that the preS2 domain may alter the conformation of the polypeptide and thereby affect its association with other cellular components. Therefore, we determined the physicochemical and immunological properties of the yHBsAg, and analyzed its constituents. We also examined its immunopotency in comparison with that of hHBsAg, since the yHBsAg could serve as a useful antigen for a hepatitis B vaccine. In this study we determined the physicochemical properties of the yHBsAg, such as its molecular weight, isoelectric point, buoyant density, ultraviolet absorption spectrum, circular dichroism spectrum and chemical composition. We also describe its antigenic specificity and immunogenicity in mice.

Materials and Methods

Materials. The hHBsAg used as a reference antigen was purified from HBeAg (subtype adr) positive human plasma (3) as described by Gerin *et al.* (5). The yHBsAg containing the preS2 9 amino acid sequence and the subsequent HBsAg was purified from a recombinant yeast, *Saccharomyces cerevisiae* SHY4/pHB103-ME5, as described previously (3). Rabbit anti-yHBsAg serum was obtained by intramuscular injection of the purified yHBsAg in Freund complete adjuvant (Difco Lab., Detroit, USA) into rabbits. Goat anti-hHBsAg serum was purchased from Dako Japan Co., Ltd, (Kyoto, Japan). These antisera were used for immunodiffusion analysis. When the immunopotency of yHBsAg was assayed, the purified yHBsAg was further treated in the same manner as the reference vaccine was. Phosphate buffer (5 mM, pH 9.0) containing yHBsAg was sterilized by filtration through a membrane (Pall Co., Ltd., USA), treated with 0.01 % formalin and then adsorbed on aluminum hydroxide (0.25 mg/0.5 ml). Thimerosal and gelatin were each added at 0.01 % (w/v) as a preservative and a stabilizer, respectively. The hHBsAg reference vaccine used for the immunopotency assay was provided by the National Institute of Health, Japan.

Electron microscopy. The purified yHBsAg was negatively stained with 1 % uranyl acetate on a carbon-coated collodion grid. The grid was then allowed to dry in air. The preparation was examined under a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan)

Determination of molecular weight and density of yHBsAg. The molecular weight of the yHBsAg particle was determined by the sedimentation equilibrium method (6) with an Ultracentrifuge Spinco Model E (Bechman, CA, USA). The yHBsAg (398 μg) was centrifuged at 3995 rpm at 20 °C for 48 h, and then the molecular weight of one particle was calculated from the distribution pattern of the antigen, as described by Yphantis (7). The density of the yHBsAg was determined by cesium chloride density gradient centrifugation. Cesium chloride was added to the purified yHBsAg to give a density of 1.20. Equilibrium density gradient centrifugation was performed at 250,000 x g for 45 h.

Isoelectric focusing. The isoelectric point of the yHBsAg was determined as follows. Pharmalite, pH 3.5–10 (Pharmacia Fine Chemicals, Uppsala, Sweden), was mixed with a sucrose solution in various ratios and then packed into a 110-ml Ampholine Electrofocusing Column (LKB8100-1 Pharmacia). A gradient of pH 3.5 to 10 was formed by supplying an electric current at 400 V for 18 h and 800 V for 7 h. After electrofocusing, 105 fractions were collected. The absorbance at 280 nm and pH were measured for every other fraction. The yHBsAg fraction was identified by radioimmunoassay, which was carried out as described previously (3).

Chemical analysis of yHBsAg. Lipids were extracted from 60 ml of a yHBsAg solution (260 μg protein/ml) by a modified method of Bligh and Dyer (8). The lipid content was determined by weighing the purified lipid after drying under vacuum. The lipid composition was determined with a Iatroscan TH-10 (Iatron Lab., Inc., Tokyo, Japan) equipped with a thin layer chromatography ionization detector (9). Polarized lipids were chromatographed and determined under the following conditions: stationary phase, CHROMAROD-SIII; mobile phase, chloroform-methanol-H_2O (50: 20: 2, v/v/v); gas flow, H_2, 160 ml/min and air, 2 l/min. Egg-sphingomyelin (Avanti Polar-lipids, Inc.) was used as an internal standard, and phosphatidylcholine and phosphatidylethanolamine were determined. Neutral lipids were separated on CHROMAROD-SIII. The 1st, 2nd and 3rd development was performed up to 10 cm with hexane, 3 cm with chloroform-methanol-H_2O (50: 20: 2, v/v/v) and 8 cm with hexane-diethylether-formic acid (60: 10: 0.15, v/v/v), respectively. Sterol esters, free fatty acids and triglycerides were determined using squalene (Wako

Pure Chemicals Ltd., Osaka, Japan) as an internal standard.

The carbohydrate content was determined by the phenol-sulfuric acid method (10) using 0.5 ml of a yHBsAg solution containing 213 μg protein/ml. The carbohydrate components were analyzed with a Shimadzu Gas Chromatograph GC-4CM FID (Shimadzu Seisakusho, Kyoto, Japan) after methanolysis and trimethylsilylation (11). The operating conditions were: column, 2 % silicon OV-17 (0.4 × 200 cm); column temperature, from 110 to 200 °C at 4 °C/min; detection temperature, 200 °C; flow rate, 60 ml/min. Amino sugars were decomposed with 4 N methane sulfuric acid and then assayed with an amino acid autoanalyzer, Model 853 (Hitachi) (12). The protein content was measured by the method of Lowry *et al.* (13).

Other analytical procedures. The UV absorption spectrum was examined with a spectrophotometer, Hitachi 220A (Hitachi). Analysis by means of circular dichroism (CD) spectrometry was performed with a CD spectrometer, JASCO J-40A (Nihon Bunko Co., Ltd., Tokyo, Japan).

Immunogenicity test. Immunodiffusion was performed on agarose (0.7 % in PBS, pH 7.2). A 25-μl sample was added to a well and incubated at 37 °C for 48 h. The immunopotency assay was carried out in the following manner. The yHBsAg and the reference vaccine were two-fold serially diluted with physiological saline. Groups of eight BALB/c mice, 5 weeks of age, were respectively inoculated subcutaneously with 1 ml of each dilution. The anti-HBs antibody titers were determined by means of passive hemagglutination (PHA; Eisai Co., Ltd., Tokyo, Japan) at 5 weeks after immunization. The results were analyzed statistically as to the validity of linearity, parallelism and homoscedasticity (homogeneous antibody response). The linearity and parallelism of regression lines ($\log_2$ antibody titer to $\log_2$ dilution of yHBsAg) were examined by Finney's method (14), and the homoscedasticity by Hartley's method (15). The potency was expressed as a value relative to that for the reference vaccine, as described by Yuasa and Shimojo (16).

Results

yHBsAg consisting of the preS2 domain (nine amino acid sequence) and the S domain was purified and investigated by means of electron microscopy. Fig. 1 shows an electron micrograph of negatively stained yHBsAg. It formed nearly spherical particles with diameters in the range of 24 ± 4 nm. They were very similar to particles of yHBsAg lacking the preS domain (17). Therefore, the preS2 nine amino acid sequence does not seem to affect the structure of the antigen parti-

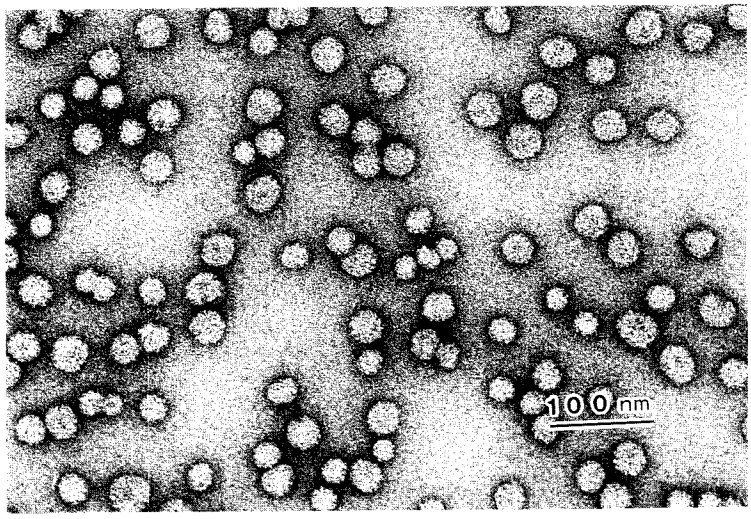

Fig. 1 Electron microscopy of the purified yHBsAg (negatively stained with uranyl acetate).

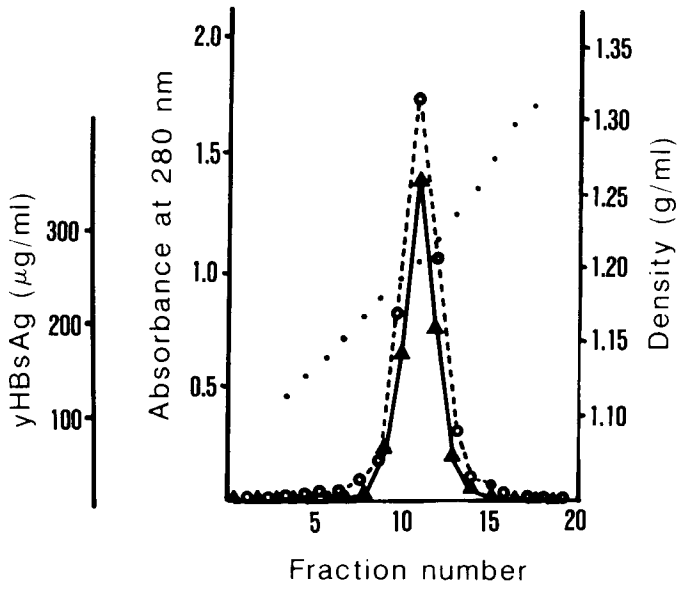

Fig. 2 Isopycnic centrifugation of the purified yHBsAg on a CsCl density gradient. CsCl was added to a solution containing the purified yHBsAg to give a density of 1.20 g/ml. After centrifugation at 250,000 xg at 10°C for 45 h, 20 fractions were collected. The absorbance at 280 nm (○) and yHBsAg content (▲) of each fraction were determined as described in the text. The densities of fractions 3 to 17 (●) were also measured.

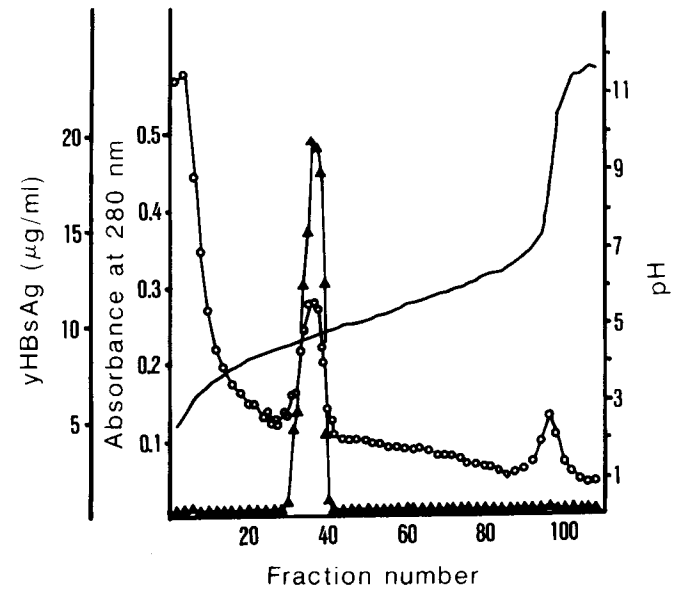

Fig. 3 Isoelectric focusing of yHBsAg. Solutions of various concentrations of sucrose containing carrie ampholite (pH 3.5–10) ation were loaded onto a 110-ml isoelectrofocusig column and the 580 μg of the purified yHBsAg was applied. Electrofocusing and fractionation were performed as described under Materials and Methods. The pH (−), absorbance at 280 nm (○) and yHBsAg content (▲) of each fraction were measured.

cles. The homogeneity of the yHBsAg particles was examined by means of cesium chloride density gradient centrifgation. A symmetric single peak appeared at $\rho = 1.201$, indicating that the yHBsAg particles were of uniform density (Fig. 2).

The surface electric charge of yHBsAg was measured by isoelectric focusing. On a pH gradient of 3.7 to 7.0, it gave a single peak at pH 4.6, indicating that yHBsAg particles are also homogeneous as to the distribution of electric charges (Fig. 3).

The UV absorption spectrum of yHBsAg is an important indication of its identity, because a highly purified hHBsAg shows a characteristic small peak at 290 nm due to the many tryptophan residues present in it (18). Fig. 4 shows the UV absorption spectrum of the purified yHBsAg. A small peak at 290 nm was clearly observed as well as a major peak at 280 nm. The ratio, A_{280}/A_{290}, was 1.227, and was consistent with the value reported for purified hHBsAg (18). The results presented here demonstrate that yHBsAgs are produced as particles uniform in both size and components. The molecular weight of the yHBsAg was determined to be 6.2×10^6 by cesium chloride gradient centrifugation. On the basis of the biochemical composition of the particles (Table 1) and the molecular weight of the polypeptide (3), the yHBsAg particle can be regarded as consisting of about 130 molecules of the yHBsAg monomeric peptide.

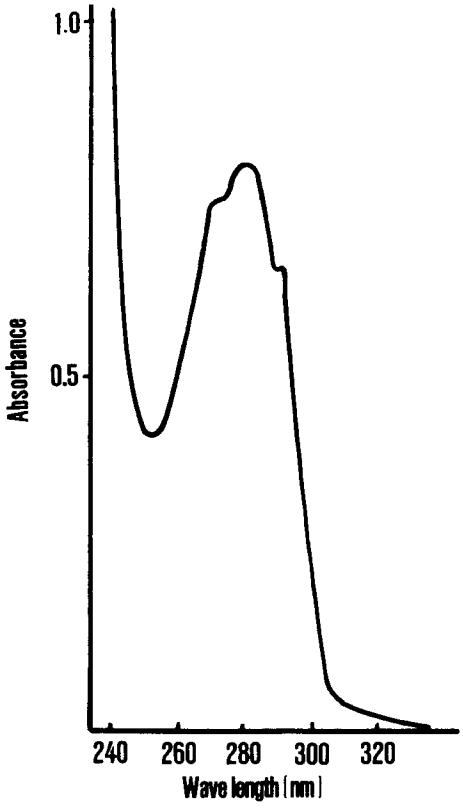

Fig. 4 Ultraviolet absorption spectrum of the purified yHBsAg. Phosphate buffer (10 mM, pH 7.2) containing 190 μg of yHBsAg/ml was used.

Table 1 shows the chemical composition of the yHBsAg. It consists of 53 % protein, 36 % lipid and 11 % carbohydrate. It should be noted that the yHBsAg particle contains less protein and more lipid than a hHBsAg particle. which consists of 70 % protein, 22.5 % lipid and 7.5 % carbohydrate (19). Polar and neutral lipids accounted for 63.7 % and 36.3 % of the total lipids, respectively. The major polar lipids, phosphatidylcholine and phosphatidylethanolamine, amounted to 46.4 and 1.6 % of the total lipids, respectively. Neutral lipids comprised triglycerides (10.5 % of the total lipids) cholesterol esters (3.7 %) and fatty acids (3.6 %). The carbo-

Table 1-A. Composition of yHBsAg.

Protein	Lipid	Carbohydrate
53	36	11 (4.6)

Table 1-B. Lipid composition of yHBsAg.

Neutral lipids				Polar lipids		
TG	SE	FFA	Others	PC	PE	Others
3.8	1.3	1.3	6.7	17	0.58	5.4

Values represent percentages. The value in parenthesis is the mannose content. Abbreviations: TG, triglycerides; SE, sterol esters; FFA, free fatty acids; PC, phosphatidylcholine; and PE, phosphatidylethanolamine.

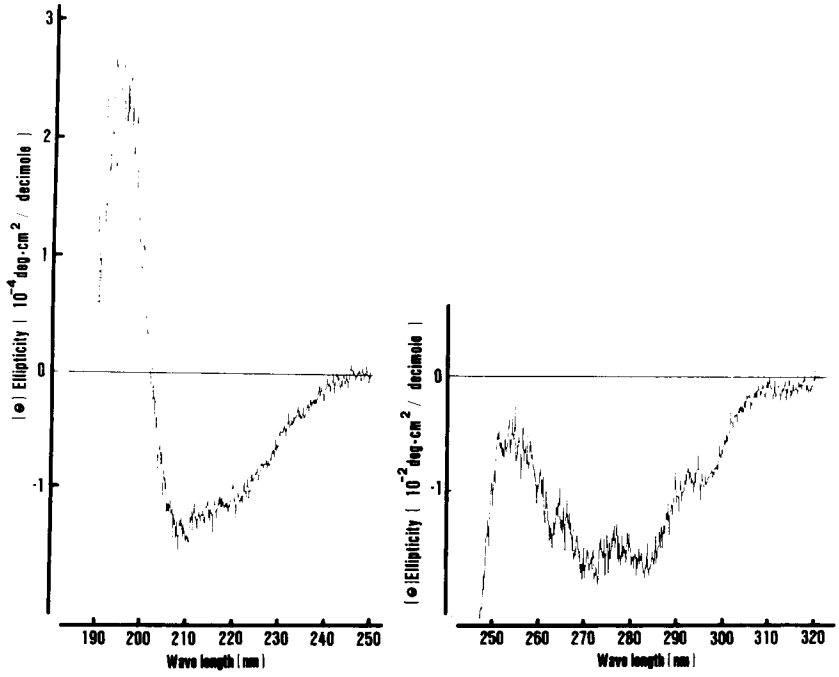

Fig. 5 Circular dichroism of yHBsAg. The purified yHBsAg was dissolved in 10 mM phosphate buffer (pH 7.2) containing 150 mM NaCl to give an absorbance value of 1.098 at 280 nm. Circular dichroism was measured at 26.5 °C using cells of 0.1-and 10-mm pathlength in the ranges of 190 to 250 nm and 245to 320 nm, respectively.

hydrate composition of the yHBsAg was analyzed by gas chromatography using its trimethylsilylated derivatives. Only mannose was detected; other carbohydrates such as fructose, glucose, N-acetyl glucosamine and N-acetyl neuraminic acid were not detectable (less than 0.3 %). Neither glucosamine nor galactosamine was detected when the yHBsAg was treated with methane sulfonic acid and then analyzed with an amino acid autoanalyzer. This clearly indicates that the yHBsAg is not glycosylated, confirming previous results obtained by polyacrylamide gel electrophorsis and PAS-stainig.

The CD spectrum of the yHBsAg was examined to analyze the secondary structure of its polypeptide. As shown in Fig. 5, the CD spectrum of the main chain of yHBsAg had a positive peak at 194 nm, and negative ones at 208 and 222 nm. The crossover point was 202 nm. This

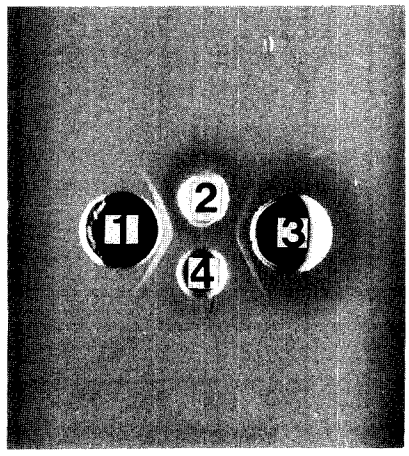

Fig. 6 Ouchterlony double gel immunodiffusion test. Wells: 1, purified yHBsAg; 2 anti-yHBsAg serum; 3, purified hHBsAg; 4, anti-hHBsAg serum.

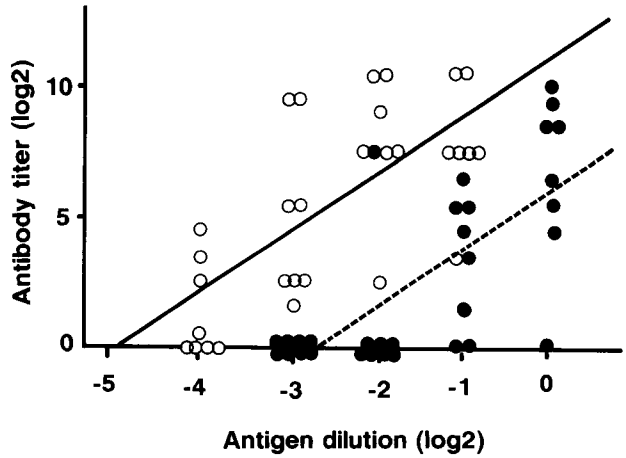

Fig. 7 Comparison of the antibody titers of BALB/c mice immunized with yHBsAg with those with reference hHBsAg vaccine. BALB/c mice were immunized subcutaneously with two-fold serial dilutions of antigens containing 20 μg of protein. The antibody was titrated with serum samples taken 5 weeks after immunization with yHBsAg (○) and the reference vaccine (●). The dose response regression line is depicted by a straight line for the group immunized with yHBsAg and by a dotted line for the group immunized with the reference vaccine.

spectrum is characteristic of the α-helical structure. The α-helix content was approximately 32 % when calculated as described by Greenfield and Fasman (20). When the structure was approximated by means of a computer simulation method (20), the α-helix, β-sheet, irregular structure and β-turn contents were 36.2, 40.9, 14.6 and 8 %, respectively. This α-helix content differed markedly from 70 to 80 % reported by Skeno *et al.* for hHBsAg (18).

The differences in peptide conformation, and the lipid and carbohydrate contents between the yHBsAg and hHBsAg may imply that the two antigens differ in antigenicity or immunogenicity. Immunogenic specificity was examined by two-dimensional immunodifusion test (Fig. 6). The yHBsAg formed a single precipitin band with anti-hHBsAg serum. This band fused with that formed between hHBsAg and anti-hHBsAg serum. This was also the case for anti-yHBsAg serum (Fig. 6). This result indicates that the antigenic determinants of the two antigens are identical.

In order to examine the immunopotency of the yHBsAg, mice were immunized with two-fold serially diluted yHBsAg, and then the anti-HBsAg antibody titer was measured (Fig. 7). The yHBsAg vaccine produced high levels of anti-HBs antibody depending on the dose of the administered antigen. The immunopotency of the reference vaccine was also examined in the same manner and compared with that of the yHBsAg. The relative immunopotency obtained by statistical analysis is shown in Table 2. The result indicates that yHBsAg is highly immunogenic and that its relative potency is greater than that of the reference vaccine. The relative immunopotency as to the reference with 5, 10 and 20 μg of the yHBsAg was determined to be 2.10, 2.99 and 5.21, respectively.

Table 2 Immunopotency of yHBsAg in mice.

yHBsAg/dose (μg)	Potency
5	2.10
10	2.99
20	5.21

The potency was expressed as a value relative to that for the reference vaccine.

Discussion

The yHBsAg used in this work contains a nine amino acid sequence corresponding to a part of the preS2 domain. The presence of such a small peptide did not influence the overall physical properties of the HBsAg particle. However, there was a significant difference in the CD spectrum between yHBsAg and hHBsAg. Therefore, the small peptide can be assumed to affect the secondary structure of the polypeptide. The CD spectrum of yHBsAg did not change even on treatment with 0.1 % sodium dodecyl sulfate or 7.2 M urea (data not shown). Such a stable secondary structure seems to be characteristic of the yHBsAg, and is probably due to intra-and inter-molecular disufide bridges formed by many cysteine residues.

The high immunopotency of the yHBsAg was demonstrated by comparison with the reference hHBsAg vaccine. The preS2 sequence might be involved in the stimulation of anti-HBs antibody production. Milich *et al.* (21) noted that the immunoresponse to the preS2 domain is independent of the S domain and that it can assist S domain nonresponder mice in producing antibodies through the preS2 specific T cell function. This phenomenon may underlie the high immunopotency of the yHBsAg.

The constituents of the yHBsAg significantly differed from those of hHBsAg. This should be due to the differences in cellular components incorporated into antigen particles during their maturation and may be partly responsible for the difference in immunopotency. Another possible explanation for the difference is the presence or absence of carbohydrate side chains in the antigen polypeptides. In general, glycosylated and unglycosylated polypeptides are present in nearly equal amounts in the case of hHBsAg (22). On the contrary, yHBsAg is not glycosylated. The unglycosylated yHBsAg may induce the production of an antibody which is highly reactive with HBsAg. Although the mechanism underlying the stimulation of anti-HBs antibody production remains to be solved, our yHBsAg can be used as a HBs vaccine with high immunogenicity.

The preS2 sequence of the yHBsAg might exert an additional effect on immunity against HBV. Machida *et al.* showed that the preS 55 amino acid sequence bore the receptor for polymerized human and chimpanzee albumins, and suggested that it could be involved in the presumed hepatotropism of HBV (23). Furthermore, Neurath *et al.* (24), and Itoh *et al.* (25) noted that synthetic peptides containing a part of the preS2 region induced the production of antibodies that bound to HBV virions and protected a chimpanzee from HBV infection. Our preliminary experiments showed that an antibody specific to the preS2 nine amino acid sequence was produced in hyperimmune serum of guinea pigs immunized repeatedly with the yHBsAg. The significance of this part of preS2 and its specific antibody in immunity to HBV is still obscure, and to solve this problem further detailed study is necessary.

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