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Immunogenicity of Hepatitis B Surface Antigen Derived from the Baculovirus Expression Vector System: a Mouse Potency Study

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Abstract. A standard mouse potency test was performed to evaluate the immunogenicity of recombinant hepatitis B surface antigen (HBsAg) produced in the baculovirus/insect cell expression system Groups of NIH Swiss mice were immunized with serial four-fold amounts of either baculovirus-derived HBsAg adsorbed to aluminum sulfate or a commercially available yeast-derived recombinant HBsAg vaccine preparation Results from these experiments showed that the effective dose of baculovirus- and yeast-derived HBsAg vaccine preparations necessary to seroconvert 50% of the animals were similar The duration of the antibody response to HBsAg was studied in mice immunized with the highest doses of the two recombinant vaccine preparations 3 and 6 months after injection No decrease in the anti-HBs response was observed 6 months after injection. No decrease in the anti-HBs response was observed 6 months after immunization with either of the two vaccine preparations These results indicate that the baculovirus-derived recombinant HBsAg could serve as an alternative vaccine candidate for hepatitis B virus

Introduction

Hepatitis B virus (HBV) is a major etiologic agent of human liver diseases, including acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. More than 200 million individuals are chronically infected with HBV worldwide.[1] The control of HBV infection represents a high priority public health objective, especially in developing nations.

HBV is a 42 nm enveloped virus containing a partially double-stranded circular DNA genome that encodes for four groups of proteins: the hepatitis surface antigen (HBsAg), the hepatitis core and e antigens (HBcAg/HBeAg), a DNA polymerase and the X protein.[2] HBsAg consists of three protein components based on initiation of translation at three distinct inframe start codons. The major protein or S protein (S), the middle protein ($preS_2$ + S), and the large protein ($preS_1$ + me $preS_2$ + S), constitute the three distinct products. The S protein and the large protein are present in both unglycosylated and glycosylated forms. The middle protein occurs in two distinct glycosylated forms. Since antibodies specific for HBsAg neutralize HBV and protein against HBV infection, HBsAg represents the major component of the two currently licensed HBV vaccines.[3,4,5] The first of such vaccines consisted of HBsAg purified from the plasma of chronically infected individuals. This preparation contained predominantly the S protein and was commercially available by the early 1980s. This subunit vaccine was both safe and efficacious; however, the relatively high cost of this subunit vaccine preparation and the limited availability of suitable chronic HBV carrier donors have led to the use of recombinant DNA technology to express HBsAg in hosts such as bacteria, yeast, insect and mammalian cells. A yeast-derived recombinant HBsAg, which is composed exclusively of the S protein, has been commercially available since 1987 Currently licensed HBV vaccines are either yeast-derived recombinant S protein preparations or plasma-derived HBsAg that is predominantly the S protein. In the United States, the yeast-derived recombinant HBsAg vaccine preparations have replaced the plasma-derived counterparts.

In this study, we have examined the immunogenicity in mice of a recombinant HBsAg derived from the insect cell/baculovirus expression vector system. The immunogenicity of this preparation has been

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compared to that of the commercially available yeast-derived HBsAg. The results of this investigation indicate that baculovirus-derived recombinant HBsAg might represent a useful candidate vaccine for HBV.

Materials and methods

Vaccine preparations

HBsAg containing exclusively the S gene product was produced using the baculovirus vector expression system and was purified by equilibrium sedimentation in CsCl gradients as previously described.[6] Baculovirus-derived HBsAg was adsorbed to aluminum potassium sulfate by methods described below.

The commercially available yeast-derived recombinant HBsAg vaccine, adsorbed onto aluminum hydroxide, was obtained from Merck, Sharp & Dohme (West Point, Pennsylvannia).[7]

Alum precipitation

Ten per cent aluminum potassium sulfate (w/v) was dissolved in 0·05 M phosphate buffered saline (PBS), pH 6·2, resulting in an alumina solution of 5·7 mg/ml. Eight milligrams of alumina were added slowly to 1 mg of baculovirus-derived HBsAg. The pH was adjusted to 5 with 1 N NaOH. The mixture was allowed to stir for 2 h at room temperature and was then centrifuged at 3000 rpm for 10 min. The supernatant was examined spectrophotometrically at 280 nm to calculate the approximate amount of unadsorbed proteins. The extinction coefficient used was 37 for 1% preparation. The amount of protein present in the supernatant was subtracted from the total amount of protein present in the starting material. The pellet was washed once in 0·85% NaCl, resuspended in the same solution to a final concentration of 10 μg/ml, and stored at 4°C with 0·01% Thimerosal (Sigma Chemical Co., St. Louis, Missouri).

Evaluation of the protein concentration in the alum precipitate

To ascertain whether equivalent concentrations of HBsAg were present in the baculovirus- and yeast-derived vaccine preparations, 500 μl of each suspension were dialysed against 3% (w/v) sodium citrate for 2 h at room temperature to remove the alum. The protein solutions were then dialysed against 0·05 M PBS, pH 7·2, for 2 h at room temperature. Serial amounts of free baculovirus- and yeast-derived recombinant HBsAg were disrupted in electrophoresis sample buffer containing 2% SDS and 2% 2-mercaptoethanol, heated at 100°C for 5 min, and subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli.[8] Polyacrylamide gels were either silver stained, following the procedure described by Blum *et al.*[9] or processed for Western blot analysis as previously reported.[10] The presence of HBsAg was determined using rabbit antiserum specific for HBsAg and ^{125}I-protein A. The intensity of the bands obtained with baculovirus-derived HBsAg was compared with that of the bands obtained with the yeast-derived HBsAg and known protein concentration standards. The concentration of the two HBsAg recombinant preparations was verified spectrophotometrically by utilizing an extinction coefficient of 37·26 for a 1% preparation at 280 nm.[11]

Immunization of mice

Groups of 10 female NIH/Swiss mice (National Cancer Institute, Frederick Cancer Research Facility, Bethesda, Maryland), approximately 5 weeks old, were immunized intraperitoneally with 0·5 ml of four serial four-fold dilutions (2·5, 0·625, 0·156, 0·039 μg) in 0·85% NaCl of each vaccine preparation. Twenty-eight days after this inoculation, sera were obtained from all mice. In addition, sera were also obtained from those groups of mice immunized with 2·5 μg of either baculovirus- or yeast-derived recombinant HBsAg, 3 and 6 months after inoculation.

Radioimmunoassay and enzyme-linked immunosorbent assay

Individual serum samples obtained 28 days after immunization from all mice were assayed for production of antibodies specific for HBsAg (anti-HBs) by a commercially available radioimmunoassay (RIA) (AUSAB; Abbott Laboratories, North Chicago, Illinois). This RIA was performed according to the manufacturer's instructions. Anti-HBs titers were expressed as RIA units/ml of serum. The negative control included pooled sera obtained from the mice prior to immunization. In addition, individual serum samples obtained 28 days, 3 months and 6 months after inoculation from the mice immunized with 2·5 μg of either baculovirus- or yeast-derived HBsAg were assayed for anti-HBs by an enzyme-linked immunosorbent assay (ELISA). Briefly, 100 ng of plasma purified HBsAg was diluted in 50 μl of 0·05 M borate buffered saline (BBS), pH 8·2, added to each well of a 96-well polystyrene microtiter plate (Corning Glass Works, Corning, New York), and allowed to adsorb to the solid phase overnight at 4°C. After blocking on non-specific binding sites with 10%

normal goat serum (NGS) in BBS for 30 min at 37°C, the plate was washed with 0·01% Tween-20 PBS (T-PBS), pH 7·2. Serial four-fold dilutions of the various mouse sera were added to duplicate wells for 1 h at 37°C and then the plate was washed with T-PBS. To detect specific binding of the mouse antibodies, horseradish peroxidase conjugated goat anti-mouse immunoglobulin (KPL, Gaithersburg, Maryland) was diluted in 10% NGS and added to each well for 1 h at 37°C. The plate was then washed with T-PBS. The substrate was 15 mg/ml of 2,2′-azino-di (3-ethyl-benzthiazole sulfonic acid) (Sigma) in 0·1 M citrate adjusted to pH 4·0 with 1 N NaOH. Immediately before use, 0·01% H_2O_2 v/v was added. The addition of 5% SDS was used to stop the reaction. Optical density (OD) of each well was determined on an automatic ELISA plate reader (MR600; Dynatech Laboratories, Inc., Alexandria, Virginia) at 410 nm (OD 410). Background absorbance levels, obtained by using each of the reagents except for the mouse antisera, were subtracted from each microtiter well.

Statistical analyses

Arithmetic mean titers were obtained by summing all values within a group and dividing the sum by the number of values contributing to the sum. Geometric mean titers were obtained by calculating the product of the n values within a group (substituting a value of 1 for any zero values) and taking the nth root of the product.

The calculation of the effective dose necessary to seroconvert 50% of the animals (ED_{50}) was performed by first calculating the percent of seroconversions for each reported dose. The smallest 100% dose and all intervening doses to the largest 0% dose were subjected to a linear regression analysis, and the regression equation was used to predict the 50% seroconversion dose.

Statistical significance of the differences in the arithmetic and geometric mean titers was determined using the t-distribution. The null hypothesis stated that the appropriate mean titers of the baculovirus- and yeast-derived HBsAg were actually identical, and subtracting one from the other would produce a difference of zero. The alternative hypothesis was constructed for either a one-tailed test (difference of the appropriate means was greater or less than zero) or a two-tailed test (difference of the appropriate means was unequal to zero) as appropriate from an examination of the mean titers. P-values were calculated using the statistics functions of a Hewlett-Packard 28S Advanced Scientific Calculator.

Results

Vaccine preparations

Baculovirus-derived recombinant HBsAg was adsorbed to aluminum potassium sulfate. The approximate amount of protein present in the precipitate was determined by subtracting the amount of HBsAg present in the supernatant as calculated by adsorption at 280 nm from the initial amount of protein used for the alum precipitation. Proteins were diluted to a final concentration of 10 μl/ml to approximate the commercially available yeast-derived recombinant HBsAg vaccine. The alum was removed from aliquots of each vaccine preparation and the resulting free proteins were subjected to SDS–PAGE, silver staining and Western blot analysis. The baculovirus-derived recombinant HBsAg was present in the unglycosylated (p25) and glycosylated (gp29) forms based on SDS–PAGE analysis and silver staining (Fig. 1). The yeast-derived HBsAg migrated as a single band at about 25 KD, consistent with the lack of HBsAg glycosylation in the yeast expression system.[4] Comparison of the two recombinant prepara-

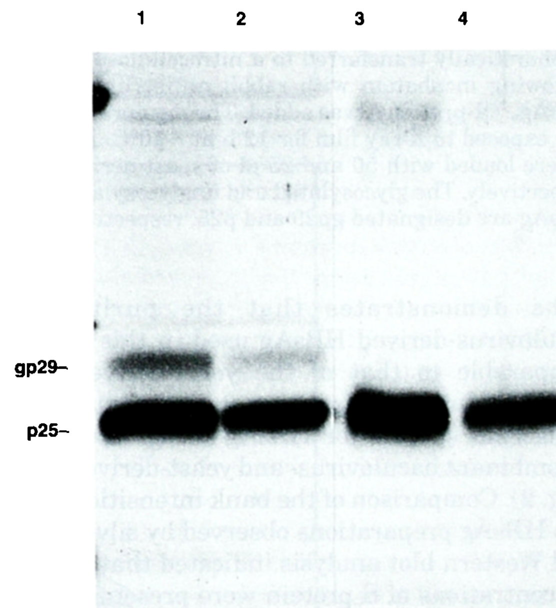

Figure 1. Comparison of baculovirus- and yeast-derived HBsAg by SDS–PAGE and silver staining. Aliquots of the two vaccine preparations (10 μg/ml) were analysed by SDS–PAGE after dialysis against 3% sodium citrate. Lanes 1 and 2 were loaded with 50 and 25 μg of baculovirus-derived HBsAg, respectively. Lanes 3 and 4 were loaded with 50 and 25 μl of yeast-derived HBsAg, respectively Baculovirus HBsAg is present in both glycosylated (gp29) and unglycosylated (p25) forms. Only p25 is present in the yeast-derived HBsAg preparation, consistent with the lack of HBsAg glycosylation in the yeast expression system.

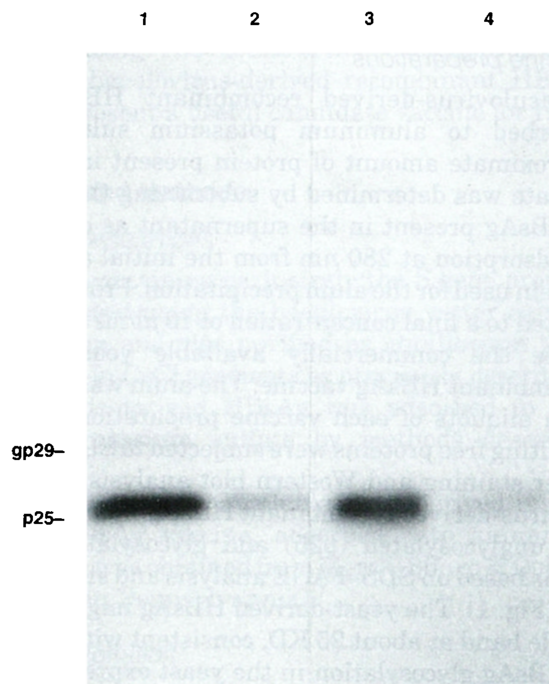

Figure 2. Comparison of baculovirus- and yeast-derived HBsAg by Western blot analysis. Aliquots of the two vaccine preparations (10 μg/ml) were subjected to SDS–PAGE after dialysis against 3% sodium citrate. Proteins were electrophoretically transferred to a nitrocellulose membrane Following incubation with rabbit antiserum specific for HBsAg, ^{125}I-protein A was added. The membrane was dried and exposed to X-ray film for 12 h at −70°C Lanes 1 and 2 were loaded with 50 and 25 μl of yeast-derived HBsAg, respectively. The glycosylated and unglycosylated forms of HBsAg are designated gp29 and p25, respectively

tions demonstrates that the purity of the baculovirus-derived HBsAg used in this study was comparable to that of the yeast-derived HBsAg. Western blot analysis demonstrated that the rabbit antiserum specific for HBsAg recognized both the recombinant baculovirus- and yeast-derived proteins (Fig. 2). Comparison of the bank intensities for these two HBsAg preparations observed by silver staining and Western blot analysis indicated that equivalent concentrations of S protein were present in the two vaccine preparations.

Mouse potency test

A standard mouse potency test was performed by immunizing groups of 10 NIH Swiss mice with various doses of either baculovirus- or yeast-derived recombinant HBsAg administered as an alum precipitate. Mice were bled 28 days after a single inoculation. Sera from individual mice were tested for the presence of anti-HBs by a commercial RIA and the results are summarized in Table 1. Immunization with concentrations of 2·5 μg and 0·625 μg of either baculovirus- or yeast-derived recombinant HBsAg elicited a specific anti-HBs response in the majority of the individuals within these groups. Two of the 10 mice immunized with 0·156 μg of yeast-derived

Table 1. Comparison of anti-HBs responses induced in NIH/Swiss mice immunized with different doses of two recombinant HBsAg preparations

	Baculovirus-derived HBsAg Dose (μg)			
	2·5	0·625	0·156	0·039
	5400*	160	0	0
	51 200	160	0	0
	5400	540	0	0
	1600	160	0	0
	1600	360	0	0
	9200	0	0	0
	15 800	5120	0	0
	26 200	360	0	0
	3600	360	0	0
	5400	80	0	0
AMT†	12 540	730	0	0
GMT‡	7001	183	0	0
ED—50§	0·83			
	Yeast-derived HBsAg Dose (μg)			
	2·5	0·625	0·156	0·039
	18 300*	0	0	0
	95 400	720	720	0
	800	360	0	0
	7200	160	0	0
	1600	160	0	0
	3600	2920	160	0
	13 500	540	0	0
	20 800	360	0	0
	23 500	0	0	0
	82 400	160	0	0
AMT†	26 710	538	88	0
GMT‡	11 216	120	3	0
ED—50§	0·89			

* Numbers represent anti-HBs titers of individual mice expressed in RIA units per millilitre.
† Arithemtic mean titers.
‡ Geometric mean titers.
§ Effective dose necessary to seroconvert 50% of the animals.

HBsAg developed a low anti HBs response, whereas none of the mice immunized with 0·156 μg of baculovirus-derived HBsAg produced detectable anti-HBs. The lowest dose of vaccine (0·039 μg) used in this study was unable to elicit an anti-HBs response in mice immunized with either baculovirus- or yeast-derived recombinant HBsAg.

A comparison of the arithmetic and geometric mean titers obtained in mice immunized with the 2·5 μg dose of the two vaccine preparations indicated that the yeast-derived HBsAg resulted in higher anti-HBs titers compared to baculovirus-derived HBsAg. However, this difference was not statistically significant ($P > 0{\cdot}05$). The two mean anti-HBs titers obtained by immunizing mice with 0·625 μg of yeast-derived HBsAg were slightly lower than those obtained in mice immunized with the same dose of baculovirus-derived HBsAg. This difference in titers

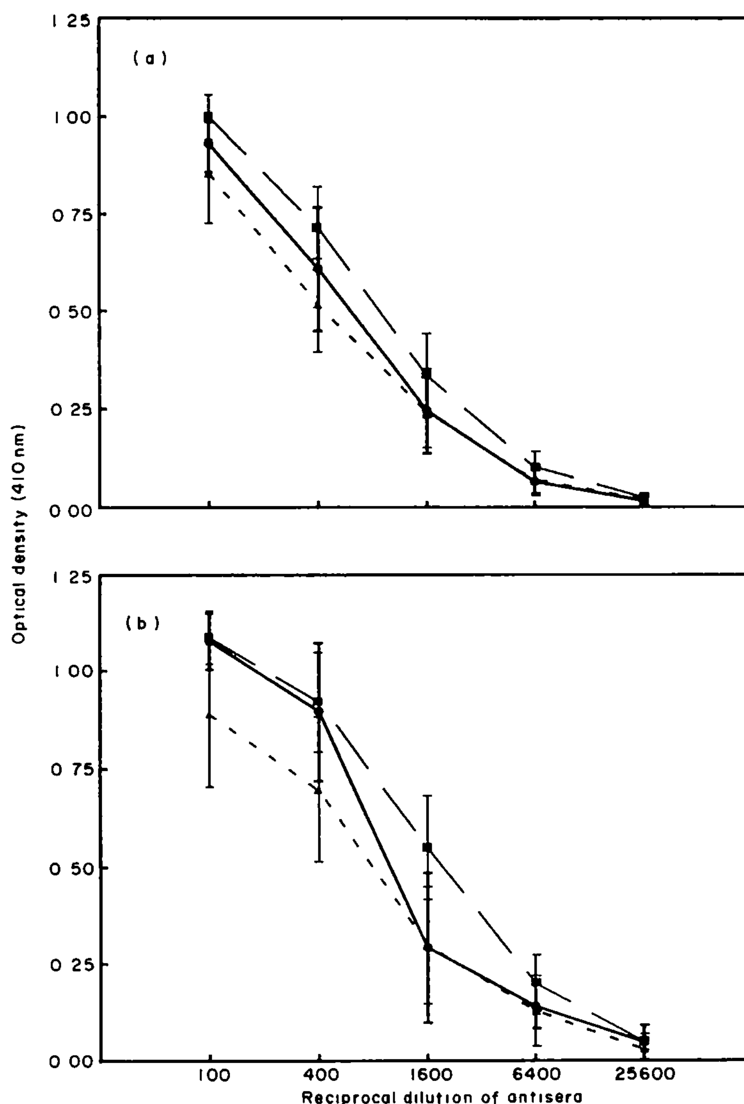

Figure 3. Reactivity of sera from mice immunized with either baculovirus- or yeast-derived HBsAg with plasma purified HBsAg. The binding curves were obtained by ELISA. Each point represents the mean of the OD values observed by testing sera from 10 individual mice. The vertical bars represent the range of values. Sera were obtained 28 days (- - - -), 3 months (– – – –) or 6 months (———) after immunization. Groups of 10 mice were immunized with 2·5 μg of baculovirus (a) or yeast (b) derived HBsAg vaccine preparation

was not statistically significant ($P > 0.05$). The ED_{50} values of the baculovirus- and yeast-derived HBsAg vaccine preparations were determined to be similar (0·83 and 0·89, respectively). These results indicate that the overall anti-HBs responses obtained by immunizing with the two vaccine preparations are comparable.

Duration of the anti-HBs response

Sera from mice injected with 2·5 μg (the only dose able to elicit sero conversion in 100% of mice) of either baculovirus- or yeast-derived HBsAg obtained 3 and 6 months after immunization, along with sera obtained 28 days after immunization, were screened for anti-HBs activity by ELISA. Anti-HBs binding curves were determined for the sera obtained from sequential bleeds from mice immunized with either baculovirus- or yeast-derived HBsAg (Fig. 3). The anti-HBs activity observed 3 and 6 months after immunization increased slightly compared to that obtained 28 days after immunization for each group of mice These data indicate that both baculovirus- and yeast-derived HBsAg vaccine preparations are able to elicit a long-term anti-HBs response.

Discussion

The three basic criteria used in the selection of an appropriate expression system for the production of a recombinant-derived vaccine preparation are scale-up, safety and efficacy. The baculovirus/insect cell expression system provides a potential advantage over the bacterial, yeast and mammalian expression systems in that a number of studies have indicated that this former expression system is highly efficient at producing large quantities of recombinant proteins.[12] Previous studies have demonstrated that the levels of HBsAg synthesis obtained by using the baculovirus expression system are exceedingly high compared to those in other systems.[6] In addition, this expression system may be one of the most cost-effective means for producing recombinant antigens.[12] A low cost protein-free medium that supports insect cell growth, virus replication and recombinant protein expression at levels equivalent to those observed in serum-containing medium has been developed.[13] The use of fermenters for large-scale insect cell propagation might increase the cost related advantages of the baculovirus recombinant technology.[14] The baculovirus expression system appears therefore to satisfy the scale-up criterion.

The safety of a vaccine preparation refers to the degree of possible undesirable effects that may occur following its use. Safety factors of a given vaccine preparation are mainly dependent on the purity of the preparation. Antigen preparations produced in the insect cell/baculovirus expression system are devoid of some of the potential risks associated with the use of recombinant antigens obtained from mammalian expression systems. These include the possible presence of dangerous residual cellular DNA of mammalian origin that may contaminate the recombinant antigen expression system. In addition, baculoviruses are not pathogenic to vertebrates. Although the yeast-derived HBsAg vaccine is highly purified, allergic reactions to residual yeast contaminants have been described.[15]

The efficacy of a vaccine preparation refers to the ability to elicit a protective immune response and constitutes the most important of the selection criteria. With HBV, the chimpanzee represents the relevant animal model for assessing protective immunity from an infectious viral challenge inoculum. However, the high cost and limited availability of this animal model precludes its use prior to examining the immunogenicity of a putative HBV vaccine in smaller and less expensive animal models. The present study represents a first step in addressing the immunogenicity issue for the evaluation of baculovirus-derived HBsAg as a vaccine preparation for potential use in humans.

To assess the immunogencity of the baculovirus-derived HBsAg vaccine preparation, a standard mouse potency study was performed The immune response elicited by the baculovirus-derived HBsAg preparation was compared with that obtained by immunization with a commercially available yeast-derived HBsAg vaccine preparation. Although the yeast-derived HBsAg appeared to be more immunogenic than the baculovirus-derived HBsAg when a dose of 2·5 μg was used, the arithmetic and geometric mean anti-HBs titers obtained by injection of 0·625 μg of the vaccine preparations were slightly higher in baculovirus-derived HBsAg immunized mice. The ED_{50} of the two vaccine preparations was similar and the antibody responses elicited by both vaccine preparations were stable for 6 months after a single injection. These results suggest that the immunogenicity in mice of the baculovirus-derived HBsAg vaccine preparation is comparable to that of the commercially available yeast-derived recombinant HBsAg and indicate that the baculovirus-derived HBsAg might satisfy the efficacy criterion required for evaluation of a vaccine preparation. However, conclusively to address this point, further studies to demonstrate the ability of this vaccine

preparation to induce protective immune response in chimpanzees are required.

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