DEVELOPMENT OF A RECOMBINANT yHBs VACCINE AND ITS FIELD TRIAL

A Takamizawa, I. Yoshida, H. Gohda, T. Kohnobe, K. Takaku and K. Fukai
Research Foundation of Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan
H. Nagashima*, T. Arima**
The Clinical Trial Group for BR-HB Vaccine.
* Kawatetsu Mizushima Hospital, Kurashiki, Japan
** First Department of Internal Medicine, Okayama University Medical School, Okayama, Japan

Hepatitis B virus (HBV) infection has been one of the most important worldwide problem to be solved. It has been estimated that nearly 200 million people are suffering from this viral infection in the world. In Japan the persistent HBV carrier rate of this virus is estimated to be 2 to 3 percent of the population. Twenty to forty percent of these carriers develop chronic hepatitis and their several percent progress to liver cirrhosis and primary liver cancer. Therefore, intensive efforts have been required to solve this infection, particularly to develop effective vaccines.

Currently, two types of HBV-vaccine, plasma-derived and recombinant ones, are available. However, there are some problems in the plasma-derived vaccine as noted by many authors (1, 2, 3). One of these problems can be unknown contaminants and the other is a limited supply of the carrier plasma, for the production of plasma-derived vaccine. Therefore the recombinant HBV vaccines are promising in these points.

We have developed a yeast recombinant HBV vaccine (BR-HB), and some data regarding purification procedures, physicochemical characterization and immunogenicity of this vaccine will be presented. In addition, preliminary results of our clinical trial in healthy adults will be described in this paper.

Construction of the expression plasmid

The fully double-stranded, complete HBV DNA of HBs antigen subtype adr was prepared by the endogenous DNA polymerase, which was then cloned into charon 28 phage DNA at the XhoI site. Re-cloning of this cloned HBV DNA was performed using E. coli plasmid pBR322 DNA at the BamHI site. The cloned HBV DNA (clone M1B11) consists of 3195 nucleotide pairs which has only one site for both of BamHI and XhoI but has no EcoRI site.

As shown in the Figure 1, we constructed an expression plasmid for HBs gene. DNA fragments containing HBs gene were isolated from the cloned HBV DNA and ligated to the down-stream of yeast repressible acid phosphatase gene promoter region which was isolated from plasmid pPHO5 DNA (4). The resulting DNA was cloned into plasmid pBR325 DNA at the BamHI site and opened with KpnI enzyme

to eliminate the translation initiation codon, ATG, in the PH05 structure gene by digestion with the exonuclease Bal31. After treatment with T4 DNA polymerase and ligase, the plasmid was introduced into **E. coli** and a clone, ME5 was prepared. The BamHI fragment of this clone ME5 DNA was inserted into the BamHI site of a shuttle vector YEp13. The cloned plasmid, pBH103-ME5 DNA was used for transformation of **Saccharomyces cerevisiae.**

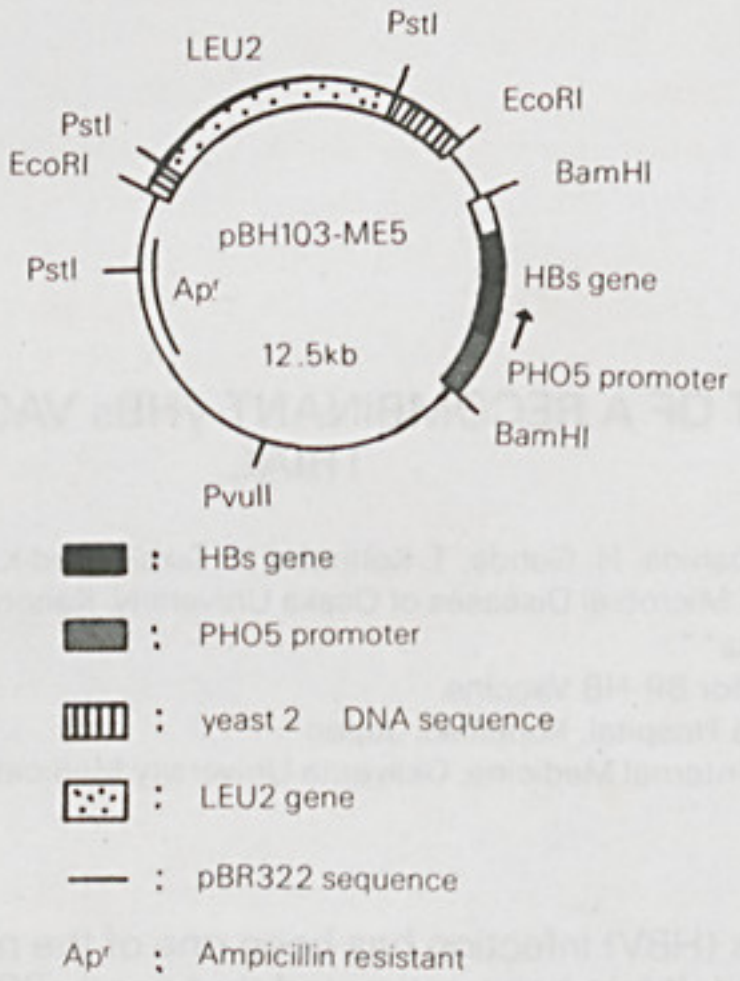

Figure 1 Structure of pBH103-ME5

Nucleotide sequence analysis of this gene indicates that the translation initiation site for HBs gene on this plasmid is presumed to be 82 bp down-stream from Hogness box in the PHO5 promoter region. The methionin codon at this site is estimated to be followed by 9 amino acids of the preS2 peptide and subsequently by 226 amino acids of the HBs antigen. Therefore, 236 amino acids in total were assumed to be synthesized in the yeast transformant.

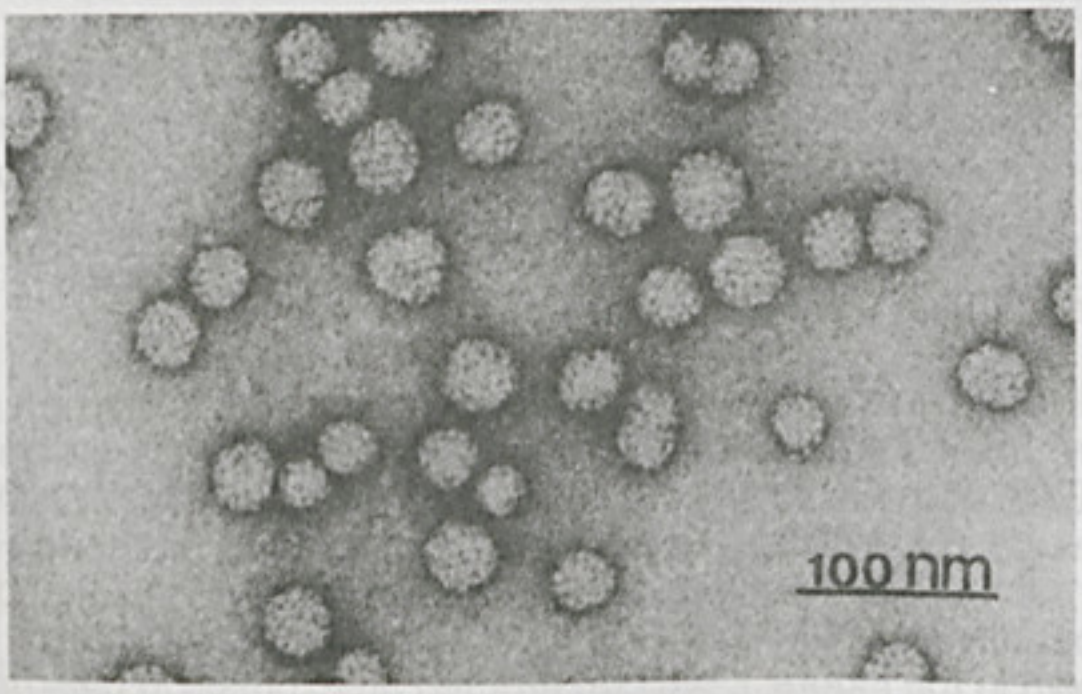

Figure 2 Electron micrograph of yHBsAg particles from recombinant yeast

Preparation of yeast-derived HBs antigen (yHBs antigen) and its physicochemical characterization.

The transformant yeast was cultured in Burkholder medium (5) and the synthesis of yHBs antigen was induced in a low phosphate content medium. The yeast cells were disrupted by a high pressure homogenizer and the crude extract was clarified with silica and charcoal treatments. Those steps were followed by isopycnic banding in KBr and rate zonal centrifugations in sucrose gradients by which the yHBs antigen was highly purified. As shown in Figure 2, the yHBs antigen had a spherical form 24 nm to 34 nm in diameters in electromicrographs which was similar to the HBs antigen particles seen in the patients serum during HBV infection.

An immunodiffusion analysis for the yHBs antigen showed that epitopes on the yHBs antigen were identified by a anti HBs goat serum against human native HBs antigen. The yHBs antigen banded at a density of 1.2 in CsC1 gradient and its molecular weight and isoelectronic point were about 24K and pI4.5, respectively.

The amino acid composition of yHBs antigen was almost identical with the one expected from the nucleotide sequence of the HBV DNA in the yeast transformant.

Circular dichroism analysis of this antigen showed a typical CD-spectrum for - helix protein structure and it did not change in the presence of 0.1% SDS or 7.2 M urea. This results suggested that the yHBs antigen was rigid and stable in the secondary structure.

Chemical composition of the yHBs antigen particles is summarized as follows. The contents of protein, lipid and carbohydrate were 53%, 36%, and 11%, respectively. The protein in the yHBs preparation consists of greater than 99% of HBs antigen determined by ELISA, HPLC, PCA in rats and SDS-PAGE. Carbohydrate was mainly mannose probably originated from host cell. However, any sugar units covalently bound to the yHBs peptide could not be detected. Yeast DNA in the preparation was determined to be less than 10 pg/20 g of yHBs antigen.

Immunogenicity of yHBs vaccine in animals

The immunological potency of the yHBs antigen was tested in animals. The antigens were adsorbed to aluminum hydroxide gel and given subcutaneously to the animals. Five weeks old BALB/C mice were injected once with 1 ml of serially diluted antigen and 5 weeks after immunization, anti-HBs titers were determined by using a commercial kit (PHA, Fujirebio., co., Ltd.) High titer antibodies against yHBs antigen were raised in the mice and our yHBs antigen preparation was determined to be more immunogenic than the plasma-derived HBs antigen.

Figure 3 showed the result of immunization of cynomologus monkeys with yHBs antigen. The monkeys were immunized subcutaneously with 10 μg yHBs antigen adsorbed to aluminum hydroxide 250 μg or 125 μg per dose at 4 weeks interval. The additional inoculum was given at 6 months after the first injection. The monkeys also responsed well.

Clinical trials of yHBs vaccine in healthy adults

The vaccine was prepared for the clinical trial to see side effects, and efficacy in differences of dose, administration route, sex and age in healthy adults who were negative for hepatitis B serological markers. The purified yHBs antigen was treated with formalin to stabilize the immunogenicity of antigen and adsorbed to aluminum hydroxide gel. Each dose in 0.5 ml contained either 10 μg or 20 μg of yHB antigen as well as 0.25 mg of aluminum hydroxide and 0.01% thimerosal as a preservative in phosphate buffer, pH 7.2. The vaccine was given thrice subcutaneously or intramuscularly at 0, 1 month, and 6 months.

None of the 453 vaccinees were reported to have a serious symptom attributable to the vaccine. Mild pain at the injection site was informed in 6% to 22% of the vaccinees. Only mild urticaria noted on the day of injection was reported in a single

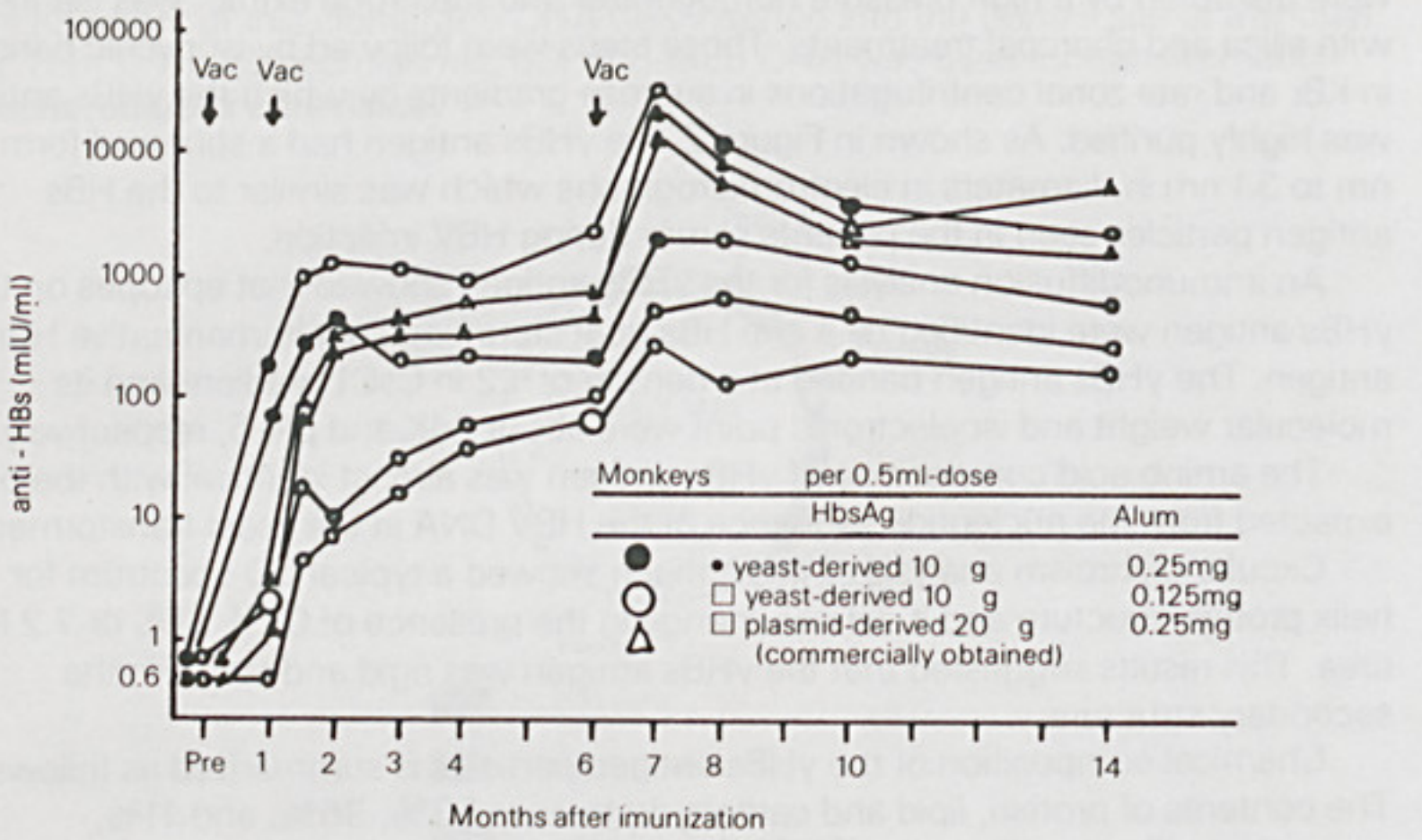

Figure 3 Immune response of cynomolgus monkeys injected with yeast-or human plasma-derived HB vaccine

case. In only several cases, local redness, headache and fatigue were described but these were quite mild and transient. There was no dose-dependency in these side effects, and their incidence did not increase in the time course of vaccination. Furthermore, there is no difference in side effects among the route of administration.

Analysis of serum sample before and after vaccination revealed no increases in yeast antibody titers as measured by ELISA and Western blot.

Table 1 shows the seroconversion rates and anti-HBs titer in the 453 subjects. The seroconversion rates at one month after the second dose were 60% to 80% and were raised to 86% to 94% at 4 months after the second dose. Finally one month after the third dose, 94% to 98% vaccinees responsed to have detectable amount of anti-HBs. When 20 μg of yHBs vaccine was given subcutaneously, at one month and two months, the seroconversion rate was slighly higher than the other groups given 10 μg of the antigen. However, at 6 and 7 months, there is no significant difference of seroconversion rates among these 4 groups.

The geometric mean anti-HBs antibody titer among responders was 141 or 200 mIU/ml at 7 months after subcutaneous injection of 10 ug or 20 ug of the yHBs vaccine, respectively (Table 1). In the group given 10 ug or 20 ug of the vaccine intramuscularly, the antibody titer was 281 or 363 mIU/ml at 7 months, respectively which tended to be higher than the titers determined in subcutaneous injection groups. The female and younger vaccinees responsed better than man or older vaccinees to the yHBs vaccine.

By giving the third dose at 4 months despite of 6 months, anti-HBs seroconversion rate in females one month after the final inoculation was 95.3% which is equivalent to the values observed in the standard inoculation schedule. However in male vaccinees, the seroconversion rates was 80.3% which is slightly lower than these. Again anti-HBs titer in female is higher than male.

Table 1 Immune responses in healthy adults vaccinated with recombinant yHBs vaccine(BR-HB)

Post.vaccination		1 month		2 months		6 months		7 months	
Route	Dose	seroconversion rate (%)	GMT*	seroconversion rate (%)	GMT	seroconversion rate (%)	GMT	seroconversion rate (%)	GMT
S.C	10µg	24/217(11.1)	5	132/214(62.1)	10	201/225(89.3)	25	188/200(94.0)	141
	20µg	11/53 (20.8)	3	42/52 (80.8)	5	46/49 (93.9)	40	49/50 (98.0)	200
i.m	10µg	7/67 (10.4)	3	44/65 (67.7)	13	56/65 (86.2)	32	60/64 (93.8)	281
	20µg	5/44 (11.4)	23	30/42 (71.4)	11	40/43 (93.0)	22	41/42 (97.6)	363

‡ Geometric mean titer of anti-HBs (mIU/ml)

Conclusion

A fragment of a cloned HBV-DNA (subtype adr) containing whole coding region for the HBs Ag was expressed in **S. cerevisiae**.

yHBs Ag thus produced was highly purified and characterized with physicochemical techniques. Amino acid and sugar analysis for the antigen peptides suggested their composition of about 236 residues certaining 9 additional residues of the C-terminus of preS2 region and lacking the carbohydrate units. The morphological appearance and physicochemical properties were similar to those seen in human plasma during HBV infection. HB vaccine was prepared for the clinical trial from purified formalin treated yHBs antigen adsorbed to aluminum hydroxide and given subcutaneously at 0,1 and 6 months to healthy adults volunteers to see side effects and immunogenicity.

It was concluded that our recombinant yHBs vaccine is safe and highly immunogenic. A clinical trial is still continuing to evaluate the efficacy of this recombinant vaccine in general population and babies born to HBV carrier mothers.

REFERENCES

1. J.C. Edman et al. Nature **291**, 503-506(1981)
2. R.A. Hitzeman et al. Nucleic. Acid Res, **11**, 2745-2763 (1983)
3. W.J. Mc Aleer et al. Nature **307**, 178-180(1984)
4. K. Arima et al. Nucleic. Acid Res, **11**, 1657-1672(1983)
5. P.R. Burkholder. J. Bot, **30**, 206-211(1943)