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Immunological Properties of Recombinant Hepatitis B Surface Antigen Expressed in Mammalian Cell (C127)

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We examined the immunological properties of the recombinant hepatitis B surface antigen (r-HBsAg) which was expressed in mammalian cell (C127). The cross-immunity of r-HBsAg and plasma-derived hepatitis B surface antigen (p-HBsAg) were tested using Western blotting and ELISA with guinea pig polyclonal antibody and naturally infected human-derived antibody and the both antigens show the same results in their response pattern and intensity, which indicate they have a good cross-immunity. From the measurement of ED_{50} after formalin- or heat-inactivation, both r-HBsAg and p-HBsAg showed ED_{50} of 0.2~0.3 in formalin-inactivation, while r-HBsAg was 0.05~0.09 and p-HBsAg was 0.03~0.07 in heat-inactivation, which means heat-inactivation method is 3~4 times superior in immunogenicity. In the immunopersistency test performed in guinea pig for the period of 3 months with two different adjuvants, antibody titer was 34.2 with muramyl dipeptide adjuvant, which was 1.8 times greater than the antibody titer of 18.9 with $AlPO_4$ adjuvant. The mutagenicity of r-HBsAg, examined by Ames test and micronucleus test, were all negative. In conclusion, r-HBsAg has the same cross-immunity with p-HBsAg, and heat-inactivation method and muramyl dipeptide adjuvant allow development of r-HBsAg vaccine with excellent immunogenicity.

Key words : Recombinant hepatitis B surface antigen (r-HBsAg), Immunological properties, Plasma-derived hepatitis B surface antigen (p-HBsAg), Cross immunity, Heat inactivation

INTRODUCTION

The discovery of Australia antigen, now known as hepatitis B surface antigen, (Blumberg *et al.*, 1965; Blumberg *et al.*, 1969) allowed development of several kinds of vaccine against hepatitis B. Hepatitis B virus, found in the serum of HBV carrier, consists of 42 nm of Dane particles (Dane *et al.*, 1970), 22 nm of spherical particles, and 100 nm of tubular particles. Among these, the 22 nm spherical particle has been used for vaccine preparation, while 42 nm of Dane particle has infectivity. Dane particle is composed of hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg), 3.2 kb of double helix DNA molecule, and DNA polymerase (Tiollais *et al.*, 1985). The first attempt of active immunization was performed with heat-inactivated HBsAg (1 min. at 98°C) from HBsAg positive-carrier sera (Krugman *et al.*, 1970; Krugman *et al.*, 1973). Purell *et al.* (1975) reported the administration of formalin-inactivated HBsAg protects the chimpanzee from infection of virulent challenge. Hilleman *et al.* (1975) found the pepsin-treated, purified and formalin-inactivated HBsAg-positive serum gave high protective efficacy in a chimp test. Reesink *et al.* (1981) prepared vaccine by pooling HBsAg-positive plasma, and treating them with polyethylene glycol and ultracentrifugation. The vaccine was heat-inactivated and treated with aluminum hydroxide, then tested on rabbits, chimpanzees and human volunteers, which all showed the vaccine was safe and had a good immunogenicity. Lee *et al.* (1987) developed a hepatitis B vaccine from HBsAg-positive serum, which had a good immunogenicity and a high level of pre-S by the method of purification and heat-inactivation. The first recombinant vaccine was prepared from *Saccharomyces cerevisaie* by Valenzuela *et al.* but this vaccine had S only without immune-dominant pre-S (Valenzuela *et al.*, 1982). Michel *et al.* could make a pre-S contained vaccine which carry pHSA receptor by expressing HBsAg particles in Chinese hamster ovary cell using DNA transfection with dihydrofolate reductase as a genetic marker (Michel *et al.*, 1984; Michel *et al.*, 1985). Number of clinical results showed pre S antigen is important in preventing HBV infection. Neurath *et al.* reported that antibody against pre-S antigen can completely neu-

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tralize HBV (Neurath *et al.*, 1986). Since pre-S antibody is uprising before S antibody (Neurath *et al.*, 1986; Krone *et al.*, 1990) in human, vaccination with a pre-S contained vaccine can be effective in preventing the infection of high risk personale and new-born babies of potential maternal vertical infection (Chung *et al.*, 1994).

In this study, we prepared a pre-S contained vaccine by expressing r-HBsAg in mouse fibroblast cell (C127) and examined the cross-immunity of r-HBsAg and p-HBsAg. We determined an inactivation method which enforces immunogenicity as well as safety, then selected appropriate adjuvant which confers a good immunopersistency. Also, we performed a mutagenicity study using AMES test and micronucleus test to develop a safe vaccine.

MATERIALS AND METHODS

Preparation of recombinant HBsAg

HV1-4 cell line was generously provided by Dr. Samanta, which was prepared by the transfection of pHPS1 encoding pre-S and S of HBV into C127 cell, followed by the selection of a cell line producing high level of HBsAg (Samanta *et al.*, 1989). The cells were grown in roller bottle (surface area; 490 cm^2) with modified DMEM media and the supernatant was further processed by diafiltration, PEG precipitation, KBr gradient ultracentrifugation, gel filtration, Mono-Q-chromatography for the purification of the recombinant HBsAg.

Cross-immunity test

Antibody preparation: 0.5 ml of HBsAg in solution (1 mg HBsAg/ml) was injected into guinea pig (s.c.) twice at an interval of 4 week. Four weeks after immunization, blood was taken from the heart of guinea pig and the serum was separated and stored in small aliquots at -20°C. The serum of the non-vaccinated but infected persons was also used as positive antibody of human natural immunization.

Western blotting: After SDS-PAGE was performed, the gel was transferred onto nitrocellulose membrane in Tris-glycine transfer buffer (12 mM Tris, 96 mM glycine, 10% methanol, pH 8.3) for 90 minutes at 45 volts. The non-specific binding was blocked by treating the membrane for 30 minutes with 5% skim milk in PBS solution (pH 7.4), followed by incubation with the primary antibody for an hour. Unbound primary antibody was removed by washing the membrane with PBST solution (PBS+0.5% Tween 20) for 3~4 times. The membrane was treated for an hour with HRP-conjugated secondary antibody solution that was diluted 1,000~2,000 times with PBS containing 5% skim milk. After washing out the secondary antibody with 3~4 times of PBST solution for 5~10 minutes, the bands were visualized by treating the membrane with colorizing agent [0.1 M Tris (pH 7.4) 25 ml, 4-chloro-l-naphthol 25 mg (5 mg/ml in methanol), H_2O_2 3 μl/50 ml].

ELISA: 100 μl (200 ng) of p-HBsAg and r-HBsAg were fixed in each well of 96 well plate at 4°C for 16 hour, and non-specific binding was blocked by incubation with 100 μl of BSA at 37°C for 2 hours. After washing with PBS containing 0.05% Tween-20 for 3 times, the plate was incubated for 2 hours with each 100 μl of two fold serial diluted samples which were initially diluted 10,000 times with PBS. The plate was washed 3 times again and then incubated with HRP-conjugated secondary antibody that was diluted 1,000~2,000 fold for 2 hours, followed by washing 3 times. 1 mg/ml of OPD (o-phenylenediamine dihydrochloride) and 100 μl of 0.1 M citrate-phosphate buffer (pH 4.9) containing 0.03% H_2O_2 was added and shielded to protect from light for 20~30 minutes in room temperature, then 1 M sulfuric acid was added to stop the reaction. The absorbance was read at 490 nm using Microplate Reader (Dynatech Product MR580).

Inhibition of immune response

Two fold serial diluted antigens (p-HBsAg or r-HBsAg) were fixed on 96 well plate for 16 hrs at 4°C. Non specific binding was blocked by the incubation with 100 μl of BSA at 37°C for 2 hrs. After washing with PBS containing 0.05% Tween-20, the r-HBsAg or p-HBsAg was reacted with antibodies (anti-p-HBsAg GP Ab, anti-r-HBsAg GP Ab, and natural HBV infected human serum antibody) at the concentration of 2 μg/ml. When the reaction was finished, the residual antibody in the reaction was measured by radioimmuno assay (Abbott, Ausab kit).

Antigen inactivation

Heat treatment: The first heat treatment was performed in water bath of 102°C with antigen (protein concentration of 1~4 mg/ml), followed by the second heat treatment at 65°C for 12 hrs (Reesink *et al.*, 1981).

Formalin treatment: Antigen solution (protein concentration of 1 mg/ml) was inactivated with formalin 0.01% (w/v) for 96 hrs at 4°C, then dialyzed with saline solution.

Adjuvanting

Preparation of $AlPO_4$ gel: 0.1 N Na_3PO_4 solution was slowly added with stirring to 0.1 M $AlCl_3$ solution containing 0.15 N NaCl until the pH of the solution

reach 4.5 to allow the precipitation of $AlPO_4$.

$$AlCl_3+Na_3PO_4 \rightarrow AlPO_4+3NaCl$$

Then the pH of the solution was adjusted to 6.2 with 1 N Na_2CO_3.

Antigen adsorption: 1) $AlPO_4$ gel adsorption. Heat-treated or formalin-treated HBsAg (2 μg/ml, final) were added to the $AlPO_4$ gel with stirring (30~50 rpm) at room temperature for 1 hr. In this final reaction, the concentration of antigen was 1 μg/ml and that of Al was 0.266 mg/ml. 2) Muramyl dipeptide adsorption. 2.5 mg of muramyl dipeptide (Sigma) was dissolved in 20 ml of saline, then 25 μg of HBsAg was added and the final volume was made to 25 ml with saline for use in experiment.

Mouse immunogenicity test (ED_{50})

ICR mice (ca. 20 g, male) were intraperitoneally injected with 0.5 ml of two fold serial diluted HBsAg (1 μg/ml) adjuvant in 10 mice per group. Dilution was made using $AlPO_4$ gel solution. After28 days, mice were bled by heart puncture and individual serum was subjected to anti-HBsAg test by radioimmunoassay using Ausab test kit (Abbott Lab., USA) The cpm value of sample was measured and divided by that of negative control. If it was over 2.1, it was counted as antibody positive. The number of antibody positive mice in each group was counted and the ED_{50} was calculated using Reed-Muench method from the antibody positive rate.

Guinea pig immunopersistency test

1 ml (1 μg/ml) of hepatitis antigen was adjuvanted with $AlPO_4$ or muramyl dipeptide and injected subcutaneously to 10 of 6 to 8 week-old male Hartley guinea pig. 1, 2 and 3 months post-administration, the blood was taken from the heart and antibody titer of the blood against hepatitis antigen was measured using Ausab test kit (Abbott Lab., USA).

Splenocyte proliferation assay

On the 8th day after immunization, spleen was dissected out and homogenized for the suspension of spleen cell, then the cell suspension was centrifuged for 10 minutes at 1,000 rpm. The cell pellet was suspended with ice-cold 0.17 M NH_4Cl red blood cell lysis buffer and washed with RPMI 1640 containing 10% FBS. Each of 2×10^5 cells were cultured in RPMI 1640 and 10% FBS containing either HBsAg on 37°C and four days later treated with [methyl ^3H] thymidine (Amersham TRK120, USA) 1 μCi for 16~18 hrs. After the reaction, the cells were harvested using multiharvester (Inutech, Switzerland) and the incorporation of [methyl-^3H] thymidine was measured using liquid scintillation counter (TRZ-CARB 1600TK, Packard). Pokeweed mitogen was used as a positive control (Gibco, USA) (Ruthman *et al.*, 1989).

RESULTS AND DISCUSSION

Antigenic properties of recombinant hepatitis B surface antigen (r-HBsAg)

In an initial experiment, we asked whether r-HBsAg has similar immunological properties to plasma-derived hepatitis B surface antigen (p-HBsAg). p-HBsAg and r-HBsAg were applied for cross-immunity test by Western blotting method using guinea pig antibody raised against p-HBsAg as well as naturally infected human serum. Both r-HBsAg and p-HBsAg showed the paralleled response pattern and intensity against guinea pig antibody as well as human serum that was raised by HBV infection (Fig. 1). S, pre-S1, and pre-S2 bands were all identified in both p-HBsAg and r-HBsAg, and among which the intensity of S band was greater than those of the other two bands.

The same antigen-antibody reaction was observed with the serial diluted samples of each antibody in ELISA assay (Fig. 2, Fig. 3).

p-HBsAg and r-HBsAg were serially diluted and neutralized in vitro with guinea pig antibody or human antibody against each antigen, then the residual antibody titer was compared. The results showed the same level of antibody inhibition with both antigen in each dilution (Fig. 4).

From the results of the western blotting, ELISA, and Inhibition immune response assays, r-HBsAg and p-HBsAg are believed to have an immune response with

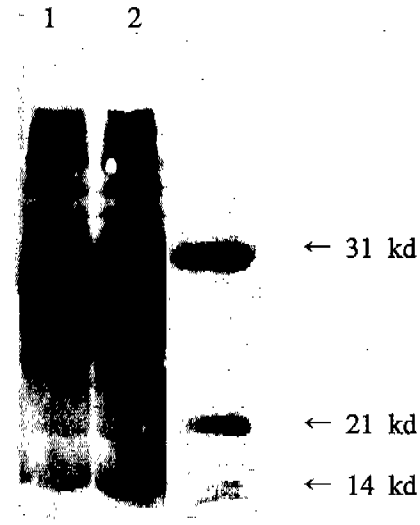

Fig. 1. Western blotting analysis of r-HBsAg and p-HBsAg using guinea pig antiboldy against p-HBsAg. After SDS-PAGE of each HBsAg, blotted on nitrocellulose membrane. Lane 1, r-HBsAg; lane 2, p-HBsAg.

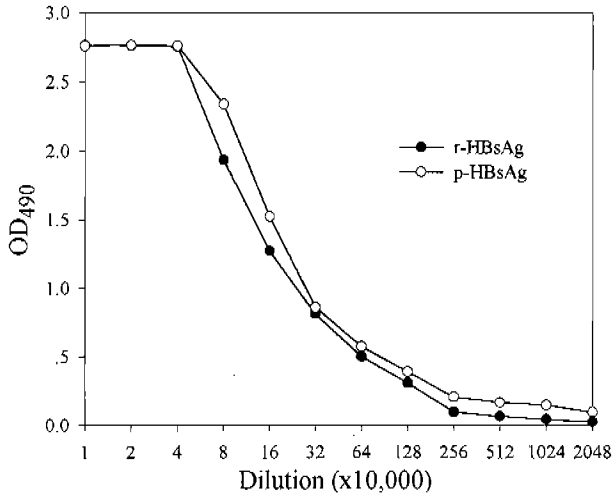

Fig. 2. Enzyme-linked immunosorbent assay of r-HBsAg and p-HBsAg with guinea pig antibody raised p-HBsAg. 100 μl (200 ng) of-HBsAg or r-HBsAg was fixed in each well. After incubating with serially diluted serum of guinea pig immunized with p-HBsAg, followed by washing, secondary Ab incubation, colorization with OPD solution.

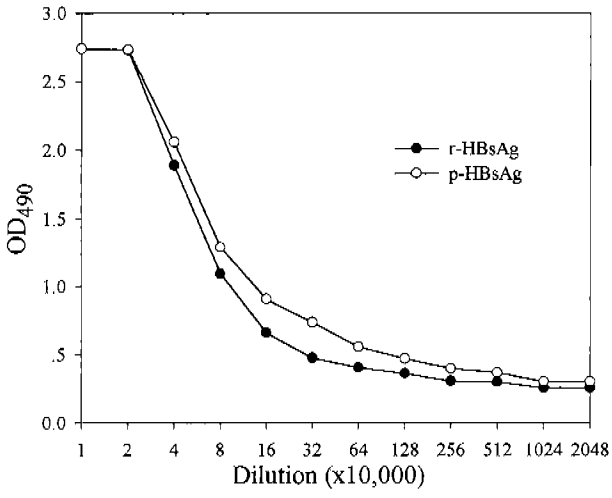

Fig. 3. Enzyme-linked immunosorbent assay of r-HBsAg and p-HBsAg with naturally HBV infected human serum. 100 μl (200 ng0 of p-HBsAg or r-HBsAg was fixed in each well. After incubating with serially diluted serum of naturally HBV infected human, followed by washing, secondary Ab incubation, colorization with OPD solution.

the same epitopes as well as cross-immunity, which suggests r-HBsAg can be useful for the preparation of recombinant hepatitis B vaccines.

Immunogenicity in two inactivation methods

Immunogenicities in two inactivation methods were compared using mice as shown in Table I, which indicates the immunogenicities of r-HBsAg and p-HBsAg in two different inactivation condition were comparable to each other (same level). For both antigens heat inactivation gave 3~4 times higher immunogenicity than formalin inactivation method. The results are consistent with the report of Reesink *et al.* (1981) that the inactivation (101°C, 90 seconds) of HBsAg showed good immunogenicity in rabbits and chimpanzees. This good immunogenicity is thought to come from the activation of host immune system by the adjuvant effect of HBsAg, that was induced from the heat-mediated structural and morphological change as well as molecular size increase.

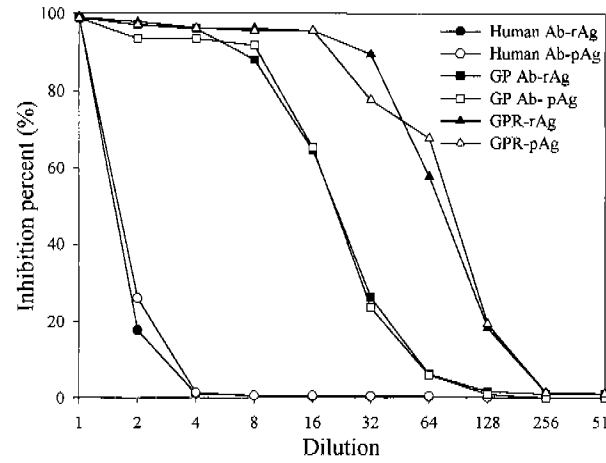

Fig. 4. RIA inhibition pattern of r-HBsAg and p-HBsAg caused by guinea pig antibodies against r-HBsAg and p-HBsAg, and naturally HBV infected human serum. After the preincubation of two fold serial diluted antigen with each antibodies, the amount of residual antibody was measured by radioimmuno assay (Ausab kit, Abbott). HuAb-rAg, Incubation of human antibody form naturally infected HBV serum with p-HBsAg; GP(p)-rAg, Incubation of guinea pig antibody against p-HBsAg with r-HBsAg; GP(p)-pAg, Incubation of guinea pig antibody against p-HBsAg with p-HBsAg; GP(r)-rAg, Incubation of guinea pig antibody against r-HBsAg with r-HBsAg; GP(r)-pAg, Incubation of guinea pig antibody against r-HBsAg with p-HBsAg.

Table I. Comparison of immune response in mice injected with HBsAgs inactivated by two different methods

Inactivation[a]	Immunogenicity (ED_{50}, μg/mouse)	
	r-HBsAg	p-HBsAg
Formalin	0.23±0.05	0.20±0.03
Heat	0.07±0.02	0.05±0.02

[a]Each antigen was inactivated with 0.01% formalin at 4°C for 96 hours or was inactivated with heat treatment at 102°C for 160 sec and pasteurization

Immunogenicity in different pH and protein concentration

In heat treatment as shown in Table II, antigen aggregation were 75% at pH 5 with mouse ED_{50} of 0.02~0.04 and 55% at pH 7 with mouse ED_{50} of 0.04 ~0.08. The results indicates that the lower the pH, the better the antigen aggregation and mouse ED_{50} decreased proportionally with the antigen aggregation.

Table II. Effect of pH on the immunogenicity of r-HBsAg during heat treatment

Activation pH	Aggregation (%)[a]	ED_{50} in mice (μg/mouse)
4	60	0.03±0.01
2	55	0.05±0.01
1	52	0.06±0.02

[a]Aggregation (%)=$(1-\frac{\text{antigenicity after heat treatment}}{\text{antigenicity before heat treatment}})\times 100$

Table III. Effect of protein concentration on the immunogenicity of r-HBsAg during heat treatment

Activation pH	Aggregation (%)[a]	ED_{50} in mice (μg/mouse)
4	60	0.05±0.02
2	55	0.04±0.03
1	52	0.04±0.02

[a]Aggregation (%)=$(1-\frac{\text{antigenicity after heat treatment}}{\text{antigenicity before heat treatment}})\times 100$

However, the antigen aggregation was not significantly affected by different protein concentration (1~4 mg/ml) in the heat-inactivation method (Table III).

Immunopersistency in different adjuvanting method

When general adjuvant $AlPO_4$ and mycobacterial cell wall component muramyl dipeptide were compared in immunopersistency, antibody titer in the 3rd month was about 1.8 times greater in using muramyl dipeptide than in using $AlPO_4$ (Table IV). The use of $AlPO_4$ as adjuvant gave similar antibody titers in both antigens. Przewlocki *et al.* (1986) reported that murabutide adjuvanting method gave 2~3 times greater antibody production for the conjugated synthetic polypeptide of pre-S (of hepatitis antigen) and tetanus toxoid than alum adjuvanting method did. *et al.* (1990) also reported muramyl dipeptide adjuvanting method of HBsAg gave a good early phase antibody titer and persistency. The high immuno-persistency in the muramyl dipeptide treatment shows the possibility of development of more advanced and convenient vaccine in terms of the required number of inoculation for protection comparing alum adjuvant vaccine, that needs 3 times of inoculation and boosting immunity.

Table IV. Persistency of immune response in guinea pigs immunized with HBsAg plus two different adjuvants

Adjuvant[a]	Average RU (relative unit)[b]		
	1 mo	2 mo	3 mo
$AlPO_4$			
r-HBsAg	15.0	11.0	18.9
p-HBsAg	8.6	16.5	22.7
Muramyl dipeptide			
r-HBsAg	17.6	18.6	34.2

[a]One ml of HBsAg adjuvanted with $AlPO_4$ or muramyl dipeptide was injected subcutaneously into guinea pig. The blood was taken from heart at 1, 2 and 3 month post-immunization, the Ab titer was measured using Ausab kit (Abbott).
[b]RU (relative unit) means the cpm of sample divided by the cpm of negative control.

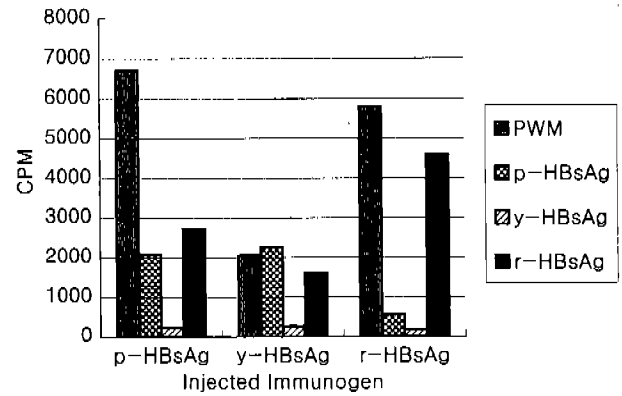

Fig. 5. Murine splenocyte proliferation by p-HBsAg, y-HBsAg and r-HBsAg.

Cellular immune response of r-HBsAg

The splenocyte proliferation by r-HBsAg and p-HBsAg in p-HBsAg-primed murine was elicited about 10 times higher than that by y-HBsAg. Also, in the y-HBsAg-primed murine, the splenocyte proliferation by r-HBsAg and p-HBsAg were elicited 6 to 8 times higher than that by y-HBsAg. Furthermore, in the r-HBsAg-primed murine, the splenocyte proliferations were elicited 3 times higher by p-HBsAg and 26 times higher by r-HBsAg, comparing with that by y-HBsAg (Fig. 5). The results suggest both r-HBsAg and p-HBsAg containing pre-S region have a stronger splenocyte proliferation activities than y-HBsAg that does not contain pre-S region.

In summary, our r-HBsAg shows an equivalent cross-immunity with p-HBsAg. Furthermore, the use of heat inactivation method and muramyl dipeptide adjuvant confers this DNA recombinant Hepatitis B vaccine as immunogenitically prominent as well as clinically convenient in its dosing schedule.

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