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Studies on Hypersensitivity of Recombinant Hepatitis B Vaccine (LBD-008) in Mice and Guinea pigs

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Abstract—Toxicity study of recombinant hepatitis B vaccine (LBD-008), a newly developed drug for acute and chronic hepatitis, was investigated in mice and guinea pigs.

1. Mice showed no production of antibodies against LBD-008 inoculated with aluminum hydroxide gel (Alum) as an adjuvant, judged by the heterologous anaphylaxis (PCA) test using rats.

On the other hand, antibodies against ovalbumin (OVA) inoculated with alum were definitely detected.

2. In the studies with guinea pigs, both the inoculation of LBD-008 only and of LBD-008 with complete Freund's adjuvant (CFA) as an adjuvant did not produce positive reactions in any of homologous active systemic anaphylaxis (ASA).

On the other hand, the inoculation of ovalbumin with complete Freund's adjuvant (CFA) produced positive reaction in both of PCA and ASA.

3. These findings suggested that LBD-008 has no antigenic potential in mice or guinea pigs.

Keywords □ toxicity, recombinant hepatitis B vaccine, mouse, guinea pig.

Hepatitis B virus (HBV) has been shown to be a causative agent for acute and chronic hepatitis and primary hepatocellular carcinoma. HBV infection is a serious health problem, especially in Southeast Asia, the Middle East, and Africa. To date, hepatitis B virus surface antigen (HBsAg) small particles derived from the plasma of HBV carriers (h-HBsAg) have been used as an immunogen for hepatitis B (HB) vaccine (first generation vaccine). The h-HBsAg particles consist mainly of P25 (25 kDa). Recently, the S gene product produced in *saccharomyces cerevisiae* (y-HBsAg) by using the recombinant DNA technique (Miyanohara *et al.*, 1983; Valenzuela *et al.*, 1982) has been developed as another immunogen for HB vaccine (second generation vaccine) (Mcaleer, *et al.*, 1984). It is very difficult to produce the large enough volume of hepatitis B vaccine for commercial purposes. This problem has been solved by Lucky R & D Center, Biotechnology (84 Jang Dong, Daejeon, Korea).

LBD-008 is a recombinant hepatitis B vaccine with pre-s region (3rd generation).

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As a part of toxicological safety research hypersensitivity test of LBD-008 in mice and guinea pigs was studied. This study was performed to assess the potential sources of cutaneous anaphylaxis and carried out according to the guidelines for antigenicity study for Safety Evaluation of Drugs for Human Use (National Institute of Safety Research, Korea, 1988). Also, the study was done according to GLP and inspected by the QAU of STRC/KRICT (Screening and Toxicology Research Center, Korea Research Institute of Chemical Technology).

Materials and Methods

Test Substances

LBD-008 (for injection, Lot No. VI-005) was supplied from the Lucky R & D Center, Biotechnology with a protein content of 20 μg/ml/vial, pH of 7.4 and osmotic pressure of 279 mOsm.

On the other hand, LBD-008 was obtained by means of genetic engineering from yeast (*Saccharomyces cerevisiae*). Phosphate buffered saline (PBS, Lot No. VV-002) was used as a vehicle. Ovalbumin (OVA, Lot No.

98C8060, Sigma Chemical Co., St., Louis, Mo., USA) served as a positive control material (Ogita and Mizushima, 1977).

Adjuvants

Complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, MI., USA) and aluminum hydroxide gel (Alum) were used as adjuvants. Aluminum hydroxide gel was prepared (Levine and Vaz, 1970) in our own laboratory.

Animals and Environmental Conditions

Male BALB/c mice of 10 weeks of age, weighing 26.8 to 31.9 g, respectively, purchased at 8 weeks of age, male Sprague Dawley rats of 10 weeks of age, weighing 352.9 to 418.0 g, purchased at 8 weeks of age and male Hartley guinea pigs of 7 weeks of age, weighing 355.6 to 477.6 g, purchased at 5 weeks of age were used. Mice and rats were supplied from Laboratory of Experimental Animal Science, Korea Research Institute of Chemical Technology (KRICT) and guinea pigs were supplied from Sam-yuk Experimental Animal Breeding Center (77-1 Surangdong, Osan, Kyunggido, Korea).

They were fed with solid diet (mice and rats; Jeil Feed Co., guinea pigs; Purina Korea Co.) and given water and were made available ad libitum under conditions 23± 3℃ room temperature, 55± 10% relative humidity, and with a 7:00 A.M to 7:00 P.M light period.

Mice and rats were identified with the number marked on their tails with oil color paints and guinea pigs used for immunization were identified with ear punching and recipient guinea pigs used for passive cutaneous anaphylaxis (PCA) test were identified with the number marked on their ears with oil color paints. Animals were selected on the basis of their weight at the start of dosing and randomly assigned to each group.

Sensitization of Animals

Mice : Sensitization schedule is shown in Table 1. LBD-008 was dissolved at a concentration of 2 μg/kg in PBS and mixed with a half volume of aluminum hydroxide gel, and injected after the calculation of inocula according to body weights (10 ml/kg). LBD-008 (0.2 μg/kg) and 10-fold of LBD-008 (2 μg/kg) were injected subcutaneously into the animals of groups A-1 and A-2. Mixed aluminum hydroxide gel and were injected intraperitoneally into the animals of groups A-3, A-4 and A-5. Sensitization were repeated 9 times (A-1 and A-2) at intervals of every other day and repeated 3 times (A-3, A-4 and A-5) once in 3 weeks. Six days after the final sensitization, blood samples were collected from the retro-orbital venous plexus of the animals (Ogita and Mizushima, 1977) under ether anesthesia, and obtained antisera were stored at −80℃.

Guinea pigs : Sensitization schedule is shown in Table 2. LBD-008 was dissolved at a concentration of 2 μg/kg in PBS and mixed with an equal volume of complete Freund's adjuvant and injected after the calculation of inocula according to body weights (1 ml/kg). All animals were injected subcutaneously into the animals of all groups. Sensitization were repeated 9 times (B-1 and B-2) at intervals of every other day and repeated 3 times (B-3, B-4 and B-5) once in 3 weeks. 12 days after the final sensitization, blood samples were collected from retro-orbital venous plexus of the animals under ether anesthesia, and obtained antisera were stored at −80℃.

Active Systemic Anaphylaxis (ASA) Test in Guinea pigs

2 weeks after the final sensitization, LBD-008 (0.2 μg/kg) or OVA (1.67 mg/kg) was injected into the leg vein of the animals. Signs of anaphylaxis were evaluated according to the following criteria:

[−]: Asymptomatic
[±]: Mild; urination, evacuation
[+]: Moderate; above, coughing, sneezing

Table 1. Sensitization schedule of mice

Group	Substance	Dose (μg/kg)	No. of treatment	No. of animals	Route
A-1	LBD-008	0.2	9[a]	5	*s.c.*
A-2	LBD-008	2	9	5	*s.c.*
A-3	LBD-008+Alum	2	3[b]	5	*i.p.*
A-4	OVA+Alum	330	3	5	*i.p.*
A-5	PBS	10 ml/kg	3	5	*i.p.*

[a]: three times in a week (every other day).
[b]: once in three weeks.
s.c.: subcutaneously.
i.p.: intraperitoneally.

Table 2. Sensitization schedule of guinea pigs

Group	Substance	Dose (μg/kg)	No. of treatment	No. of animals	Route
B-1	LBD-008	0.2	9[a]	5	*s.c.*
B-2	LBD-008	2	9	4	*s.c.*
B-3	LBD-008+CFA	2	3[b]	5	*s.c.*
B-4	OVA+CFA	2.5 mg/kg	3	5	*s.c.*
B-5	PBS	1 ml/kg	3	5	*s.c.*

[a]: three times in a week (every other day).
[b]: once in three weeks.
s.c.: subcutaneously.

[++]: Severe; above, piloerection, salivation, nostril discharge, lacrimation, nasal bleeding, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side position, flattening

[+++]: Death

Homologous PCA Test in Guinea pigs

This test was performed according to the method of Ovary (1958). Each 0.1 m*l* of the guinea pig sera diluted from 10 to 5120-fold was injected intradermally into the back of guinea pigs which had been clipped their back hair short. Four hours after the initial inoculation, 1 m*l* of 1 : 1 mixture of LBD-008 (0.2 μg/kg) or OVA (1.67 mg/kg) solution and a 1% solution of Evans blue were injected the leg vein. Thirty minutes after the final inoculation, the guinea pigs were bled to death, and leakage of the dye at the serum-injected site was examined to determine the PCA titer. The endpoint of the positive PCA reaction was set at a diameter of 5 mm or more (major diameter+minor diameter)/2 (Ovary, 1958).

Heterologous PCA Test in Rats

This test was performed according to the method of Mota and Wong (1969), each 0.1 m*l* of the mouse serum diluted from 10 to 5120-fold was injected intradermally into the back of rats which had been clipped their back hair short. Twenty-four hours after the initial inoculation, 1 m*l* of 1 : 1 mixture of LBD-008 (0.2 μg/kg) or OVA (330 μg/kg) solution and a 1% solution of Evans blue were injected the tail vein. Thirty minutes after the final inoculation, the rats were bled to

Table 3. Active systemic anaphylaxis in guinea pigs

Group	B-1				B-2				B-3					B-4				B-5					
Sensitization	LBD-008 (0.2 μg/kg)				LBD-008 (2μg/kg)				LBD-008+CFA (2 μg/kg)					OVA+CFA (2.5mg/kg)				PBS (1 m*l*/kg)					
Challenge	LBD-008 (0.2 μg/kg)				LBD-008 (0.2 μg/kg)				LBC-008 (0.2 μg/kg)					OVA (1.67 mg/kg)				LBD-008 (0.2 μg/kg)					
Sensitiation period	3 times/wk ×3				3 times/wk ×3				once/3wk ×3					once/3wk ×3				once/3wk ×3					
Animal No.	1	2	3	4	6	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Symptoms on challenge																							
Urination	−	+	+	+	+	−	+	−	−	−	−	+	−	−	+	+	+	+	−	−	−	−	−
Evacuation	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	+	+	−	−	−	−	−
Coughing	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sneezing	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Piloerection	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salivation	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Nostril discharge	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
Lacrimation	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−
Nasal bleeding	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Convulsion	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Dyspnea	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−
Staggering gait	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Rhonchus	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−
Cyanosis	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Side position	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−
Flattening	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Death	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Evaluation of the intensity	[−]	[±]	[±]	[±]	[±]	[−]	[±]	[−]	[−]	[−]	[−]	[±]	[−]	[++]	[++]	[++]	[++]	[++]	[−]	[−]	[−]	[−]	[−]

[−]Asymptomatic.
[±]: Mild; urination, evacuation.
[+]: Moderate; above, coughing, sneezing
[++]: Severe; above, piloerection, salivation, nostril discharge, lacrimation, nasal bleeding, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side position, flattening.
[+++]: Death.

death, and leakage of the dye at the serum-injected site was examined to determine the PCA titer. The endpoint of the positive PCA reaction was set at a diameter of 5 mm or more (major diameter+minor diameter)/2 (Mota *et al.*, 1968).

Results and Discussion

The infection by human hepatitis B virus (HBV) has been considered to be one of the most popular viral infections and appeared to induce a spectrum of clinical manifestations, such as mild inapparent disease, fulminant hepatitis, severe chronic liver diseases and cirrhosis (Ganem, 1982). The HBV also has been implicated to develop primary hepatocellular carcinoma (Beasley, 1988). To overcome these problems, many laboratories have developed to produce potent vaccines to this virus, using recombinant DNA techniques in prokaryotic and eukaryotic cells, particularly in the yeast cells.

The primary objective of our present study was to examine if LBD-008 (Recombinant hepatitis-B vaccine) possesses hypersensitivity in mice and guinea pigs.

In Table 3, the active systemic anaphylaxis was tested in guinea pigs. In groups B-1 and B-2, urination (mild symptom) was observed from two or three animals of each group. However, urination and/or evacuation were observed only one animal in group B-3. On the other hand, all the animals challenged with ovalbumin in group B-4, showed anaphylactic signs which are characterized by coughing, sneezing, piloerection, salivation, nostril discharge, lacrimation, nasal bleeding, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side position and flattening. In group B-5, was not observed any anaphylactic symptoms. The results indicate that the vaccine showed no sign of hypersensitivity.

In Table 4, the homologous PCA was examined in guinea pigs.

Table 4. Four-hour homologous passive cutaneous anaphylaxis test in guinea pigs with sera of sensitized guinea pigs

Group	Sensitizing antigen	Challenging[a] antigen	PCA[b] titer	Positive ratio
B-1	LBD-008 (0.2 μg/kg)	LBD-008 (0.2 μg/kg)	−[c]	0/10
B-2	LBD-008 (2 μg/kg)	LBD-008 (0.2 μg/kg)	−	0/10
B-3	LBD-008+CFA (2 μg/kg)	LBD-008 (0.2 μg/kg)	−	0/10
B-4	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	×320 ~×640	10/10
B-5	PBS (1 ml/kg)	LBD-008 (0.2 μg/kg)	−	0/10

[a]: Challenging antigen was intravenously injected 24 hours after sensitization of rats with sera.
[b]: PCA titer represents the maximum dilution factor of original serum which gives positive reaction.
[c]: Specific antibodies were not detected in 10-fold dilution of original sera.

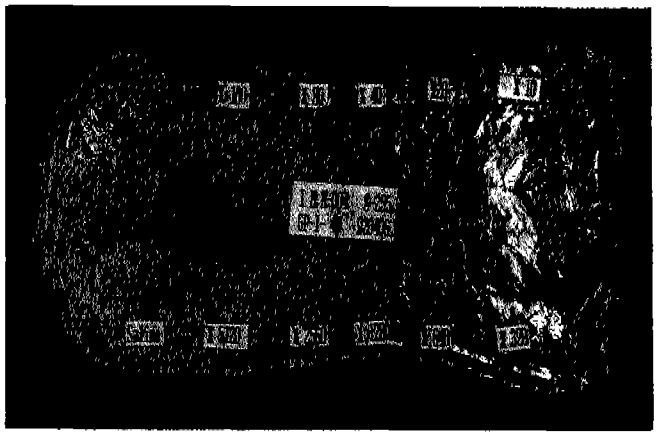

Fig. 1. Passive cutaneous anaphylaxis of LBD-008 (2 μg/kg) in guinea pigs.
Passive cutaneous anaphylaxis reaction was not detected. Guinea pigs were immunized with LBD-008 (2 μg/kg)+CFA. Antisera of guinea pig were obtained at 12 days after the final immunization. Four hrs after intradermal injections of antisera, quinea pig was challenged by LBD-008 (0.2 μg/kg).

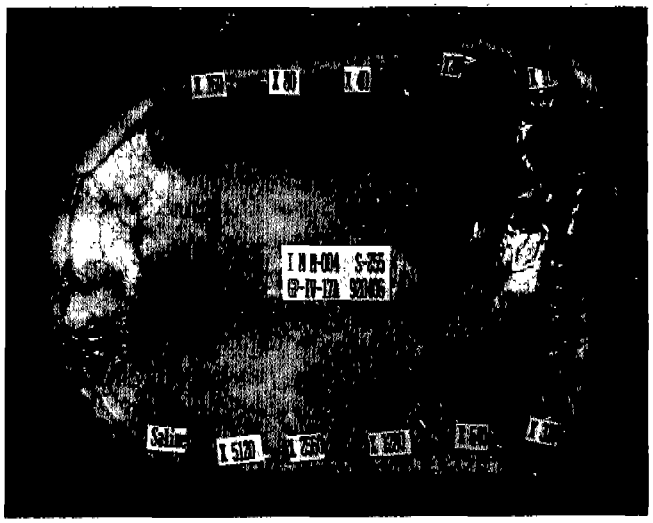

Fig. 2. Passive cutaneous anaphylaxis of OVA (2.5 mg/kg) in guinea pigs.
The endpoint antibody titer was ×640. Guinea pigs were immunized with OVA (2.5 mg/kg)+CFA.
Antisera of guinea pigs were obtained at 12 days after the final immunization. Four hrs after intradermal injections of antisera, guinea pig was challenged by OVA (1.67 mg/kg).

Table 5. 24-hour heterologous passive cutaneous anaphylaxis test in rats with sera of sensitized mice

Group	Sensitizing antigen	Challenging[a] antigen	PCA[b] titer	Positive ratio
A-1	LBD-008 (0.2 μg/kg)	LBD-008 (0.2 μg/kg)	−[c]	0/10
A-2	LBD-008 (2 μg/kg)	LBD-008 (0.2 μg/kg)	−	0/10
A-3	LBD-008+Alum (2 μg/kg)	LBD-008 (0.2 μg/kg)	−	0/10
A-4	OVA+Alum (330 μg/kg)	OVA (2.86 mg/kg)	×10	10/10
A-5	PBS (10 ml/kg)	LBD-008 (0.2 μg/kg)	−	0/10

[a]: Challenging antigen was intravenously injected 24 hours after sensitization of rats with sera.
[b]: PCA titer represents the maximum dilution factor of original serum which gives positive reaction.
[c]: Specific antibodies were not detected in 10-fold dilution of original sera

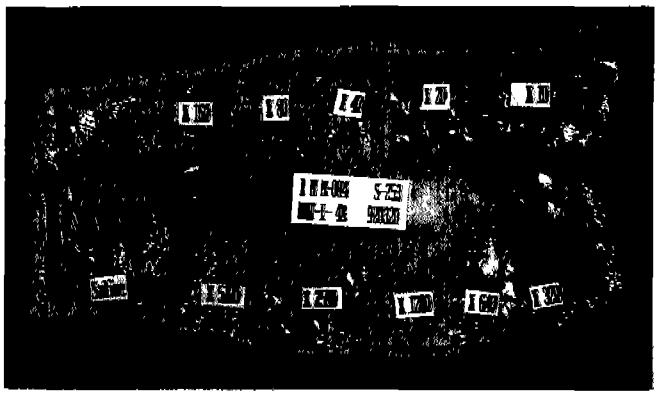

Fig. 3. Passive cutaneous anaphylaxis of LBD-008 (2 μg/kg) in mice Passive cutaneous anaphylaxis reaction was not detected. Mice were immunized with LBD-008 (2 μg/kg)+hydroxy aluminum gel. Antisera of mice were obtained at 6 days after the final immunization. Twenty-four hrs after intradermal injections of antisera, rat was challenged by LBD-008 (0.2 μg/kg).

All test sera challenged with LBD-008 (0.2 μg/kg) were negative (Fig. 1) except those from group B-4 (Fig. 2). On the other hand, antibodies were detected from all 10 guinea pigs in group B-4 (OVA; 1.67 mg/kg) with PCA titer ranging from ×320 to ×640.

In Table 5, the heterologous PCA was performed in mice and rats. All test sera challenged with LBD-008 (0.2 μg/kg) were negative (Fig. 3) except those from A-4 (Fig. 4). On the other hand, IgE antibodies were detected from all 10 rats in group A-4 (OVA; 330 μg/kg) with PCA titer ranging from ×10 to ×1280.

The PCA reaction has been reported to determine

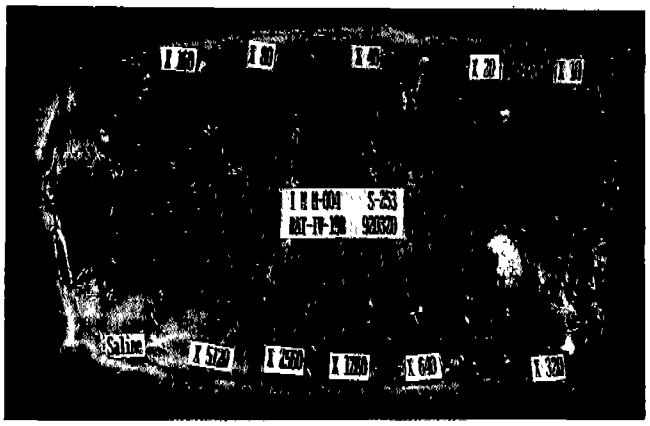

Fig. 4. Passive cutaneous anaphylaxis of OVA (330 μg/kg) in mice The endpoint antibody titer was ×320.
Mice were immunized with OVA (330 μg/kg)+hydroxy aluminum gel. Antisera of mice were obtained at 6 days after the final immunization. Twenty-four hrs after intradermal injections of antisera, rat was challenged by OVA (2.86 mg /kg).

the immediate type hypersensitivity, since the permeability of the post-capillary venules in skin is increased following antigen-antibody reaction. In addition, the PCA reaction has been known to share similar mechanism with the systemic anaphylaxis (Ovary, 1964). Since the PCA reaction was originally designed to detect IgE antibody in sensitized sera *in vivo* (Ovary, 1958; Okudaira and Ishizaka, 1973). Our present results indicate that LBD-008 has no hypersensitivity in mice and guinea pigs.

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