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A STUDY ON ANTIGENICITY OF RECOMBINANT HUMAN GROWTH HORMONE (LBD-009) IN MICE AND GUINEA PIGS

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ABSTRACT: *Antigenic potential of recombinant human growth hormone (LBD-009), a newly developed drug for growth hormone deficiency, was investigated in mice and guinea pigs.*

1. *Mice showed production of antibodies against LBD-009 (1.5 IU/kg) 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-009 (0.15 IU/kg~1.5 IU/kg) only and of LBD-009 with complete Freund's adjuvant (CFA) as an adjuvant did produce weak positive reactions in homologous passive cutaneous anaphylaxis (PCA).*

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

3. *In homologous ASA with guinea pigs were negative reaction in 10 times the clinical dose (1.5 IU/kg) included the inoculation of aluminum hydroxide gel.*

   On the other hand, the inoculation of ovalbumin with complete Freund's adjuvant (CFA) produced positive reaction in ASA (Active Systemic Anaphylaxis).

Key words: *Antigenic potential, Human Growth Hormone (LBD-009), Mouse, Guinea Pig*

INTRODUCTION

Recombinant human growth hormone (HGH) is one of which have now been purified, cloned and produced in the biotechnology industry. We received the test

substance from Lucky R & D center, Biotechnology (Daejeon, Korea).

Human GH(hGH) has been available for more than 30 yr for the treatment of children with hypopituitary dwarfism. Until recently the only source of hGH was human pituitary gland. Pituitary-derived hGH was effective in treating dwarfism but was suspected in the occurrence of Jacob-Creutzfeldt disease in some chidren treated with this material. Pituitary derived GH was initially produced by endocrinologists, and later by pharmaceutical companies. Early preparations were often of considerable impurity until intensive purification methods were developed. However, it has come to be able to produce hGH by means of genetic engineering and therefore the problem of impurity has been solved. And it has come to be able to produce the large enough volume of hGH. In this study, the antigenicity of LBD-009 was evaluated in mice and guinea pigs as a part ot its safety research.

This study was carried out according to the Guidelines for Antigenicity Studies for Safety Evaluation of Drugs for Human Use (National Institute of Safety Research, Korea, 1988).

MATERIALS AND METHODS

Test Substances

LBD-009 (for injection, Lot No. JI-014) and vehicle (D-Mannitol 50 g, CH_3COONH_2 200 g, $Na_2HPO_4 \cdot 7H_2O$ 16 g in D.W., 10 *l*) were used. Ovalbumin (OVA, Lot No. 98C8060, Sigma chemical Co., St., Louis, Mo., USA) served as a positive control material (Ogita, T and Mizushima, Y). And clinical dose (MTD) was 0.15 IU/kg.

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 24.3 to 31.9 g, respectively, purchased at 7 to 8 weeks of age, male Sprague Dawley rats of 10 weeks of age, weighing 338.1 to 427.0 g, purchased at 8 weeks of age and male Hartley guinea pigs of 7 weeks of age, weighing 391.9 to 485.7 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 (Hwasung, Korea).

They were fed with solid diet (mice and rats; Jeil Feed Co., Guinea pigs; Purina Korea Co.) and given water 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 analphylaxis (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.

The study was done according to GLP and inspected by the QAU of TRC/KRICT (Toxicology Research Center, Korea Research Institute of Chemical Technology).

Sensitization of Animals

Guinea Pigs

Sensitization schedule is shown in Table 1. LBD-009 was dissolved at a concentration of 1.5 IU/kg and mixed with a equal volume of complete Freund's adjuvant and injected after the calculation of inocula according to body weights (1 ml/kg). All test substances were injected subcutaneously into the animals of all groups. Sensitization were repeated 9 times (A-1 and A-2) at intervals of every other day and repeated 3 times (A-3 and A-4) once in 3 weeks. In gorup A-4, sensitization were repeated 11 times. 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℃.

Mice

Sensitization schedule is shown in Table 2. LBD-009 was dissolved at a concentration of 1.5 IU/kg and mixed with a half volume is aluminum hydroxide gel and injected after the calculation of inocula according to body weights (10 ml/kg). LBD-009 (0.15 IU/kg) and 10-fold of LBD-009 (1.5 IU/kg) were injected subcutaneously into the animals of groups B-1 and B-2. Mixed aluminum hydroxide gel

Table 1. Sensitization groups of guinea pigs

Group	Substance	Dose	times	No. of Animals	Route
A-1	LBD-009	0.15 IU/kg	9[a]	5	s.c.
A-2	LBD-009	1.5 IU/kg	9	5	s.c.
A-3	LBD-009+CFA	1.5 IU/kg	3[b]	5	s.c.
A-4	OVA+CFA	2.5 mg/kg	3	5	s.c.
A-5	Vehicle control	1 ml/kg	11[c]	5	s.c.

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

Table 2. Sensitization groups of mice

Group	Substance	Dose	times	No. of Animals	Route
A-1	LBD-009	0.15 IU/kg	9[a]	5	s.c.
A-2	LBD-009	1.5 IU/kg	9	5	s.c.
A-3	LBD-009+CFA	1.5 IU/kg	3[b]	5	i.p.
A-4	OVA+CFA	330 μg/kg	3	5	i.p.
A-5	Vehicle control	10 ml/kg	11[c]	5	i.p.

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

were injected intraperitoneally into the animals of groups B-3, B-4. Sensitization were repeated 9 times (B-1 and B-2) at intervals of every other day and repeated 3 times (B-3 and B-4) once in 3 weeks. In group B-4, sensitization were repeated 11 times. 6 days after the final sensitization, blood samples were collected from the 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-009 (0.15 IU/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
[++] : Severe; above, piloerection, salivation, nostril discharge, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side positions, 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-009 (0.15 IU/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-009 (0.15 IU/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, thr rats 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.

RESULTS

Active Systemic Anaphylaxis Test in Guinea Pigs

The results are shown in Table 3.

In groups A-1, A-2, A-3 and A-5, urination and/or evacuation were observed

in several animals but these signs were negative reaction. On the other hand, all the animals challenged with ovalbumin in gorup A-4, showed anaphylactic signs which are characterized by coughing, sneezing, salivation, nostril discharge, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side position and flattening.

Homologous PCA Test in Guinea Pigs

The results are shown in Table 4.

Table 3. Active systemic anaphylaxis test in guinea pigs sensitized with LBD-009 or ovalbumin

Group	Sensitizing antigen	Challenging antigen	No. of animals	Severity of Anaphylaxis[a]				
				[+]	[+]	[+]	[++]	[+++]
A-1	LBD-009 (0.15 IU/kg)	LBD-009 (0.15 IU/kg)	5	2	3			
A-2	LBD-009 (1.5 IU/kg)	LBD-009 (0.15 IU/kg)	5	3	2			
A-3	LBD-009+CFA (1.5 IU/kg)	LBD-009 (0.15 IU/kg)	5	4	1			
A-4	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	5			3	2	
A-5	Vehicle control (1 ml/kg))	LBD-009 (0.15 IU/kg)	5	3	2			

[a]: Severity of anaphylaxis was expressed as follows
[−] : Asymptomatic
[+] : Mild; urination, evacuation
[+] : Moderate; above, coughing, sneezing
[++] : Severe; above, piloerection, salivation, nostril discharge, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side position, flattening.
[+++] : Death

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

Group	Sensitizing antigen	Challenging[a] antigen	No. of animals	PCA[b] titer	Positive ratio
A-1	LBD-009 (0.15 IU/kg)	LBD-009 (0.15 IU/kg)	10	×10~×160	4/10
A-2	LBD-009 (1.5 IU/kg)	LBD-009 (0.15 IU/kg)	10	×10~×40	5/10
A-3	LBD-009+CFA (1.5 IU/kg)	LBD-009 (0.15 IU/kg)	10	×20~×640	10/10
A-4	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	10	×640~×1280	10/10
A-5	Vehicle control (1 ml/kg))	LBD-009 (0.15 IU/kg)	10	—[c]	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

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

Group	Sensitizing antigen	Challenging[a] antigen	No. of animals	PCA[b] titer	Positive ratio
B-1	LBD-009 (0.15 IU/kg)	LBD-009 (0.15 IU/kg)	10	—[c]	0/10
B-2	LBD-009 (1.5 IU/kg)	LBD-009 (0.15 IU/kg)	10	—	0/10
B-3	LBD-009+CFA (1.5 IU/kg)	LBD-009 (0.15 IU/kg)	10	×40~×160	10/10
B-4	OVA+CF (330 μg/kg)	OVA (2.86 mg/kg)	10	×320~×640	10/10
B-5	Vehicle control (10 ml/kg))	LBD-009 (0.15 IU/kg)	10	—	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

Test sera of A-1 and A-2 challenged with LBD-009 (0.15 IU/kg~1.5 IU/kg) were observed slightly positive reaction. However, in half volume of A-3 (1.5 IU/kg+CFA) test sera were observed positive reaction in all animals. On the other hand, antibodies were detected from in all 10 guinea pigs in group A-4 (OVA: 1.67 mg/kg) with PCA titer ranging from ×640 to ×1280.

Heterologous PCA Test in Mice

The results are shown in Table 5.

In group B-1, B-2 and B-5, all test sera challenged with LBD-009 (0.15 IU/kg~1.5 IU/kg) were negative. In group B-3 (1.5 IU/kg+alum), IgE antibodies were detected ranging from ×40 to ×160 in all 10 rats. On the other hand, IgE antibodies were detected from all 10 rats in group B-4 (OVA: 330 μg/kg) with PCA titer ranging from ×320 to ×640.

DISCUSSION

Adequate therapy for a patient with failure to thrive due to proven growth hormone deficiency (GHD) constitutes the administration of human grwoth hormone (hGH). Growth hormone (GH) from other species dose not act in the human because there is strong species specificity. The first report of successful therapy in a patient with GHD was given by Raben (1958). Since this initial report, many papers on treatment with hGH have appeared in the literature.

Passive cutaneous anaphylaxis (PCA) reaction utilizes one of the fundamental characteristics of the immediate type hypersensitivity reactions, i.e. the increase of permeability of the post-capillary venules in the skin following antigen-antibody reaction. This local anaphylactic reaction, PCA, corresponds in every respect to systemic anaphylaxis (Ovary, 1964). In this work, antigenicity of recombinant hu-

man growth hormone (LBD-009) was studied with mice and guinea pigs and IgE antibody production in mice was examined by the method of heterologous PCA using rats (Ovary, 1958; Okudaira and Ishizaka, 1973). LBD-009 has a little immunogenicity sensitized with 10 times the clinical dose (1.5 IU/kg+alum). However positive antibody titer was observed in 10 times the clinical dose in relation to the inoculation of OVA+Alum and OVA+CFA. On the other hand, all the mice and guinea pigs sensitized OVA-Alum and OVA-CFA definitely showed positive reaction. Therefore it is considered that LBD-009 showed a little antigenicity not only guinea pigs in the studies of ASA (Active Systemic Anaphylaxis) and PCA but also mice of PCA from the results of the present study and above considerations.

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