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A Study on Antigenicity of Recombinant Human Interferon β (LB00013) in Mice and Guinea Pigs

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ABSTRACT : *Antigenicity of recombinant human interferon β (LB00013), a newly developed drug for anti-cancer and anti-viral therapeutic use, was investigated in mice and guinea pigs. The following results were obtained:*

1. Mice showed no production of antibodies against LB00013 sensitized with aluminum hydroxide gel (Alum) as an adjuvant, when judged by the heterologous passive cutaneous anaphylaxis (PCA) test in rats. Meanwhile, antibodies against ovalbumin (OVA) sensitized with Alum were clearly detected.

2. In guinea pigs, the sensitization of neither LB00013 only nor LB00013 with complete Freund's adjuvant (CFA) produced positive reactions in the homologous active systemic anaphylaxis (ASA) and the PCA tests. Meanwhile, the sensitization of OVA with CFA produced positive reactions in both PCA and ASA.

3. A LB00013-specific reaction was not observed in an indirect hemagglutination(IHA) assay using sera isolated from LB00013 sensitized mice.

The present results suggested that LB00013 may have no antigenic potential in mice and guinea pigs.

Key Words : *Antigenicity, Recombinant human interferon β, PCA, ASA, IHA, Mice, Guinea pigs*

I. INTRODUCTION

Antigenicity of recombinant proteins has been concerned in many preclinical and clinical situations, because the production of antibodies against a particular protein may cause not only a dramatic alteration of its pharmacokinetics in plasma, but also a loss of its pharmacological action by neutralization. For example, based on the studies by Konrad *et al.* (1987), a recombinant human interferon-beta induced antibody production in 11% of populations receiving intravenous administration, of which 4% had neutradizing antibody, while subcutaneous exposure resulted in a 31% incidence of antibody of which 70% was neutralizing.

It has been characterized that the structures of individual types interferons (α, β, γ) are different and that marked dissimilarities in amino acid sequence exist among species indicating that antibodies may be readily raised in heterologous species (Taniguchi *et al.*, 1980). IFNs are a family of proteins characterized by a potent ability to confer a virus-resisitant state in their target cells (Kobayashi, 1981). In addition, IFNs can inhibit cell proliferation and modulate immune responses. These properties have led into the clinical use of IFN for the treatment of viral infections and malignancies.

To date, interferon alpha has been approved by the Food and Drug Administration of the United States for the treatment of patients with hairy cell leukemia, acquried immune deficiency syndrome-related Kaposi's sarcoma, and condylomata acuminata (Itri, 1992).

Human IFN-β is secreted by fibroblasts in response to viral infection or exposure to synthetic double stranded RNA (Stewart, 1981). IFN-β is glycosylated and has an apparent molecular weight of about 22,000 daltons. However, genetically engineered recombinant human IFN-β expressed in *E. coli* is not glycosylated and it's molecular weight is 19 KD. Currently, IFN-β has also been characterized as a drug for anti-cancer and anti-virus therapeutic

use in hairy cell leukemia, Kaposi's sarcoma, blood cancers, multiple myeloma and chronic myeloid leukemia. In addition, IFN-β also contributes to the treatment of hepatitis B and C. LB00013 is a recombinant human interferon (IFN) β produced from genetically engineered *E. coli* and composed of 166 amino acids containing disulfide bonds.

In this study, the antigenicity of LB00013 was evaluated in mice and guinea pigs because of its modified structure when compared to the natural β-interferon. The studies were done according to the Korean GLP guideline.

II. MATERIALS AND METHODS

1. Test Substances

LB00013 (for injection, Lot No. BI002) with a titer of 1.7×10^7 IU/ml of a diluent solution (10 ml acetate mixed with 5% dextrose) was supplied by LG Chemicals Ltd. (104-1, Moonji-Dong, Yusong-Ku, Taejon, Korea). Ovalbumin (OVA, Lot No. 98C8060, Sigma Chemical Co., St., Louis, Mo., USA) was used as a positive antigen (Ogita and Mizushima, 1977).

2. 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 as previously described (Levine and Vaz, 1970).

3. Animals

In this study, mice and rats were obtained from the Laboratory of Experimental Animal Science, Korea Research Institute of Chemical Technology (KRICT), and guinea pigs were purchased from Samyuk Experimental Animal Breeding Center (O-San, Kyunggi-Do, Korea). 8-week old male BALB/c mice, 8-week old male SD rats and 5-week old male Hartley guinea pigs were received, quarantined and acclimatized for 2 weeks.

4. Environmental Conditions

The animals were housed at a temperature of $23\pm3^{\circ}C$ and a humidity of $50\pm10\%$. The lighting cycle was 12 hours of alternating light and dark. Commercial feed (for mice and rats, Jeil Feed Co.; for guinea pigs, Purina Korea Co.) and tap water were given *ad libitum*. Vitamin C at 1 g/l was dissolved into water for maintaining guinea pigs.

5. Sensitization of Animals:

1) Mice

The sensitization schedule is shown in Table 1. LB00013 was dissolved at a concentration of 5 x 10^5 IU/kg in diluent solution, mixed with a half volume of Alum, and injected intraperitoneally according to body weights (10 ml/kg). The sensitization was repeated 9 times (for groups I, II and V) at intervals of every other day and repeated 3 times (for groups III and IV) once in 3 weeks. Six days after the final sensitization, blood samples were collected from the retro-orbital venous plexus of the animals under ether anesthesia, and prepared antisera were stored at -80°C until use.

2) Guinea pigs

Sensitization schedule is shown in Table 2. LB 00013 was dissolved at a concentration of 5×10^5

Table 1. Sensitization of mice for heterologous passive cutaneous anaphylaxis and indirect hemagglutination test

Group	Substance	Sex	No. of animals	No. of treatment	Dose (IU/kg)	Route
I	LB00013	♂	5	9[a]	5×10^4	i.p.
II	LB00013	♂	5	9[a]	5×10^5	i.p.
III	LB00013+Alum	♂	5	3[b]	5×10^5	i.p.
IV	OVA+Alum	♂	5	3[b]	330 μg/kg	i.p.
V	Vehicle[c]	♂	5	9[a]	-	i.p.

[a] 3 times in a week (every other day).
[b] Once in 3 weeks.
[c] Diluent solution (10 ml/kg).

IU/kg in diluent solution, mixed with an equal volume of CFA, and injected subcutaneously according to body weights (1 ml/kg). The sensitization was repeated 9 times (for groups VI, VII and X) at intervals of every other day and 3 times (for groups VIII and IX) once in 3 weeks. Twelve days after the final sensitization, blood samples were collected from retro-orbital venous plexus of the animals under ether anesthesia, and prepared antisera were stored at -80°C until use.

6. Active Systemic Anaphylaxis (ASA) Test in Guinea Pigs

2 weeks after the final sensitization, either LB 00013 (5×10^5 IU/kg) or OVA (1.67 mg/kg) was injected into the leg vein of the animals. Signs of anaphylaxis were evaluated for 30 min according to the following criteria:

[-] : asymptomatic
[±] : mild; restlessness, piloerection, tremor, rubbing or licking nose
[+] : moderate; sneezing, coughing, hyperpnea, urination, evacuation, lacrimation
[++] : severe; dyspnea, rhonchus, cyanosis, staggering gait, jumping, gasping and writhing, convulsion, side position, Cheyne-Strokes respiration
[+++] : death

7. Homologous PCA Test in Guinea Pigs

The test was performed according to the method of Ovary (1958). Guinea pig sera (0.1 ml) diluted from 10 to 5120-fold in saline were injected intradermally into the back of guinea pigs which had been clipped their back hair short. Four hours after the initial injection, 50 μl of 1:1 mixture of either LB00013 (5×10^5 IU/kg) or OVA (1.67 mg/kg) solution and a 1% solution of Evans blue were injected into the leg vein. Thirty minutes after the final injection, 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 from the formula, (major diameter + minor diameter)/2 (Ovary, 1958).

8. Heterologous PCA Test in Rats

This test was performed according to the method of Mota and Wong (1969), in which 0.1 ml 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 injection, 50 μl of 1:1 mixture of LB00013 (5×10^4 IU/kg) or OVA (2.5 mg/kg) solution and a 1% solution of Evans blue were injected into the tail vein. Thirty minutes after the final injection, the 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 from the formula, (major diameter + minor diameter)/2 (Mota *et al.*, 1968).

9. Indirect Hemagglutination(IHA) Test in Mice

In the IHA test, the sera prepared in the heterologous PCA test was used. Fresh sheep red blood cells (SRBCs) were incubated with 0.005% tannic acid at 37°C for 1 hr. The tanned SRBCs were suspended in phosphate-buffered saline and incubated with antigens to be coated at 37°C for 2

Table 2. Sensitization of guinea pigs for active systemic anaphylaxis and homologous passive cutaneous anaphylaxis

Group	Substance	Sex	No. of animals	No. of treatment	Dose (IU/kg)	Route
VI	LB00013	♂	5	9[a]	5×10^4	s.c.
VII	LB00013	♂	5	9[a]	5×10^5	s.c.
VIII	LB00013+CFA	♂	5	3[b]	5×10^5	s.c.
IX	OVA+CFA	♂	5	3[b]	2.5 mg/kg	s.c.
X	Vehicle[c]	♂	5	9[a]	-	s.c.

[a]3 times in a week (every other day).
[b]Once in 3 weeks.
[c]Diluent solution (1 ml/kg).

hr. Finally, the antigen-coated SRBCs were suspended in phosphate-buffered saline to a 1% solution. The sera were diluted with phophate-buffered saline from 10 to 20,480 fold in 96-well hemagglutination plate. Then 1% solution of antigen-coated SRBCs were added into each well and the plates were placed at room temperature for at least 2 hr to determine the positive hemagglutination. Uncoated SRBCs were also used to determine antigen-specific hemagglutinations.

III. RESULTS

1. Active Systemic Anaphylaxis Test in Guinea Pigs

The results were shown in Table 3. In group VI, any anaphylactic signs were not observed. However, urination and/or evacuation were observed in 2 animals in groups VII, VIII and X. Meanwhile, 4 animals challenged with OVA in group IX, showed anaphylactic signs which were characterized by restlessness, piloerection, rubbing or licking nose, sneezing, hyperpnea, urination, evacuation, lacrimation, rhonchus, staggering gait, jumping, gasping and writhing, convulsion, and side position.

2. Homologous PCA Test in Guinea Pigs

The results were shown in Table 4. All tested sera challenged with LB00013 (5×10^5 IU/kg) were negative. Meanwhile, antibodies were detected from all 10 guinea pigs in group IX (OVA: 1.67 mg/kg) with PCA titer ranging from $\times40$ to $\times1280$.

3. Heterologous PCA Test in Mice

The results were shown in Table 5. All tested sera challenged with LB00013 (5×10^5 IU/kg) were negative. On the other hand, IgE antibodies were

Table 3. Active systemic anaphylaxis in guinea pigs

Groups	Sensitizing antigen	Challenging antigen	No. of animals	Severity of anaphylaxis[a]				
				[-]	[±]	[+]	[++]	[+++]
VI	LB00013 (5×10^4 IU/kg)	LB00013 (5×10^5 IU/kg)	4	4				
VII	LB00013 (5×10^5 IU/kg)	LB00013 (5×10^5 IU/kg)	4	2	2			
VIII	LB00013+CFA (5×10^5 IU/kg)	LB00013 (5×10^5 IU/kg)	5	3	2			
IX	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	5		1		4	
X	Vehicle (1 ml/kg)	LB00013 (5×10^5 IU/kg)	5	2	2			

[a]Severity of anaphylaxis was expressed as follows;

1. restlessness	2. piloerection	3. tremor	4. rubbing or licking nose
5. sneezing	6. coughing	7. hyperpnea	8. urination
9. evacuation	10. lacrimation	11. dyspnea	12. rhonchus
13. cyanosis	14. staggering gait	15. jumping	16. gasping and writhing
17. convulsion	18. side position	19. Cheyne-Strokes respiration	20. death

[-]: Asymptomatic.
[±]: Mild; symptoms of 1 to 4.
[+]: Moderate; symptoms of 1 to 10.
[++]: Severe; symptoms of 1 to 19.
[+++]: Death.

Table 4. Four-hours passive cutaneous anaphylaxis test in guinea pigs with sera from sensitized guinea pigs

Groups	Sensitizing antigen	Challenging antigen[a]	PCA titer[b]	Positive ratio
VI	LB00013 (5×10^4 IU/kg)	LB00013 (5×10^5 IU/kg)	-[c]	0/8
VII	LB00013 (5×10^5 IU/kg)	LB00013 (5×10^5 IU/kg)	-	0/8
VIII	LB00013+CFA (5×10^5 IU/kg)	LB00013 (5×10^5 IU/kg)	-	0/10
IX	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	$\times1280$~$\times2560$	10/10
X	Vehicle (1 ml/kg)	LB00013 (5×10^5 IU/kg)	-	0/10

[a]Challenging antigen was intravenously injected 4 hours after sensitization of guinea pigs with sera.
[b]PCA titer represents the maximum dilution fold of original serum which showed positive reaction.
[c]Specific antibodies were not detected in 10-fold dilution of original sera.

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

Groups	Sensitizing antigen	Challenging antigen[a]	PCA titer[b]	Positive ratio
I	LB00013 (5×10^4 IU/kg)	LB00013 (5×10^5 IU/kg)	-[c]	0/10
II	LB00013 (5×10^5 IU/kg)	LB00013 (5×10^5 IU/kg)	-	0/10
III	LB00013+Alum (5×10^5 IU/kg)	LB00013 (5×10^5 IU/kg)	-	0/8
IV	OVA+Alum (330 μg/kg)	OVA (2.5 mg/kg)	×640~×1280	10/10
V	Vehicle (10 ml/kg)	LB00013 (5×10^5 ml/kg)	-	0/10

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

Table 6. Indirect hemagglutination test with sera isolated from sensitized mice

Groups	Sensitized antigen	IHA with uncoated SRBC		IHA with LB00013-coated SRBC		IHA with OVA-coated SRBC	
		IHA titer[a]	Positive ratio	IHA titer	Positive ratio	IHA titer	Positive ratio
I	LB00013 (5×10^4 IU/kg)	-[b]	0/5	-	0/5	-	0/5
II	LB00013 (5×10^5 IU/kg)	-	0/5	-	0/5	-	0/5
III	LB00013+Alum (5×10^5 IU/kg)	-	0/5	-	0/5	-	0/5
II	OVA+Alum (330 μg/kg)	-	0/4	-	0/4	640~1280	4/4
V	Saline (10 ml/kg)	-	0/5	-	0/5	-	0/5

[a]IHA titer represents the maximum dilution fold of original serum which showed the positive hemagglutination.
[b]Specific antibodies were not detected in 10-fold dilution of original sera.

detected from all 10 rats in group IV (OVA: 330 μg/kg) with PCA titer ranging from ×640 to ×1280.

4. Indirect Hemagglutination(IHA) Test in Mice

The results were shown in Table 6. No antibody was detected in sera isolated from LB00013-injected mice. Meanwhile, antibodies appeared to be produced in all animals of group IV with the titer ranging from ×640 to ×1280.

IV. DISCUSSION

Interferons are immunologically active modifier of biological response in humans and animals. In case of recombinant products, the identity and purity has to be defined with the naturally active agent. In toxicological testing of recombinant human interferon β through animal experiment, careful attention must be paid not only to the antigenicity, but also to the reaction, which results from pharmacological profile of the testing agent (Hohbach and Koss, 1987).

The objective of the present study was to evaluate a possible antigenicity of LB00013, a recombinant human interferon-beta in guinea pigs and mice. The passive cutaneous anaphylaxis, active systemic anaphylaxis and indirect hemagglutination were used in the present study. The guinea pigs and mice were chosen in this study, because these two species have greatly been used in evaluating antigenic potential of testing compounds (Levine and Vaz, 1970; Mota and Wong, 1969). Using the same animal models, the antigenicity of recombinant human interferon-alpha was also investigated earlier (Park *et al*, 1993).

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 human IFN β (LB00013) was studied in mice and guinea pigs and IgE antibody production in mice was ex-

amined by the method of heterologous PCA using rats (Ovary, 1958; Okudaira and Ishizaka, 1973). Only the mice sensitized with OVA-Alum showed production of IgE antibodies. Similar results were also obtained in the indirect hemagglutination tests. No antibody was produced by the administration of LB00013 in mice, while the specific antibody was clearly produced when OVA, a positive control, was administered with the same dosing schedule. From the results mentioned above, it is considered that LB00013 has no immunogenicity in mice. LB00013 also showed no antigenicity in PCA and ASA (Active Systemic Anaphylaxis) test in guinea pigs.

Therefore it might be considered that LB00013 may be free of antigenicity.

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