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A STUDY ON ANTIGENICITY OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR(LBD-005) IN MICE AND GUINEA PIGS

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ABSTRACT: *Antigenicity of Recombinant Granulocyte macrophage colony stimulating factor(LBD-005), a newly developed drug for hematopoietic growth, was investigated in mice and guinea pigs.*

1. *Mice showed production of antibodies against LBD-005 (100 μg/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-005 only and of LBD-005 with complete Freund's adjuvant(CFA) as an adjuvant did not produce 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 (Active systemic Anaphylaxis) with guinea pigs positive reaction was found in 10 times the clinical dose (10 μg/kg).*

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

Key Words: *Antigenicity, Recombinant Granulocyte Macrophage Colony Stimulating Factor(LBD-005), Mouse, Guinea pig*

INTRODUCTION

Granulocyte-macrophage colony stimulating factor(GM-CSF) is one of several hematopoietic growth factors 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). It is now clear that the factor is able not only to stimulate the proliferation of granulocyte and macrophage colonies (Burgess & Metcalf, 1980; Sieff *et al.*, 1985; Mecalf *et al.*, 1986) but also to regulate some of the functional activities of stem cells(Metcalf, 1986). The possible relationships of GM-CSF in combating myeloid leukemias and other leukocyte deficiency diseases(Gasson, 1984; Barlogie *et al.*, 1990) have stimulated cellular and molecular biologists to carry out a great deal of research. Demands in the clinical field have also accelerated the researchers to produce many kinds of recombinant GM-CSF.

Human clinical trials, using different recombinant form of GM-CSF, are still continuing, with varing degrees of success(Goldstone and Khwaja, 1990; Lieschke *et al.*, 1939). In this study, the antigenicity of LBD-005 was evaluated in mice and guinea pigs as a part of its safety research(掘内淑彦, 1976).

MATERIALS AND METHODS

Test Substances

LBD-005(for injection, Lot No. CI-016) and PBS(Phosphate Buffered Saline, Lot No. CV-007) were used. Ovalbumin(OVA, Lot No. 98C8060, Sigma Chemical Co., St., Louis, Mo., USA) served as a positive control material(萩田 忠厚, 水島 裕, 1977). And Clinical Dose(MTD) was 10 μg/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

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

Twenty five male mice and guinea pigs were divided into six groups according to immune response.

Environmental Conditions

The animals were housed in controlled semi-barrier rooms at a temperature of 23±3°C and at a humidity of 50±10%. The lighting cycle was 12 hours of alternating light and dark. Commercial feed (mice and rats: Jeil Feed Co., guinea pigs: Purina Korea Co.) and water were made available for guinea pigs and *ad libitum* (1 g/*l*: water).

Sensitization of Animals

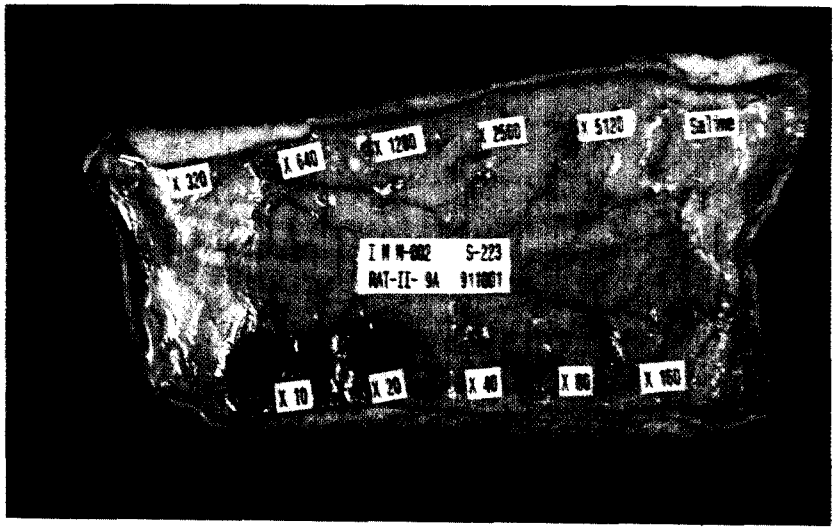

Photo 1. Passive cutaneous anaphylaxis of LBD-005 (100 μg/kg) in mice. The endpoint antibody titer was $\times$20. Mice were immunized with LBD-005(100 μg/kg). Antisera of mice were obtained 6 days after final immunization. 24 hrs intradermal injections of antisera, rat was challenged by LBD-005 (10 μg/kg).

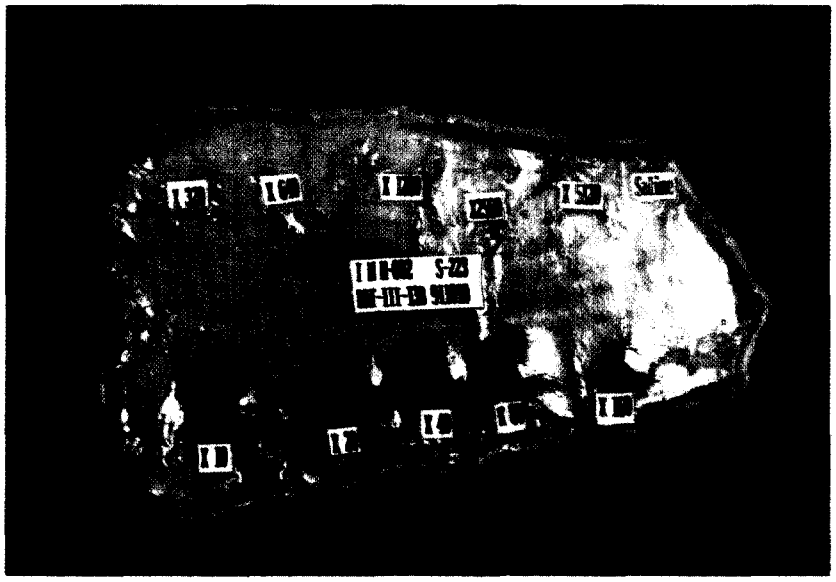

Photo 2. Passive cutaneous anaphylaxis of LBd-005 (100 μg/kg) in mice. The endpoint antibody titer was $\times$160. Mice were immunized with LBD-005 (100 μg/kg) + hydroxy aluminum gel. Antisera of mice were obtained 6 days after final immunization. 24 hrs after intradermal injections of antisera, rat was challenged by LBD-005 (10μg/kg).

Mice

Sensitization schedule is shown in Table 1. LBD-005 was dissolved at a concentration of 100 μg/kg in PBS 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-005(10 μg/kg) and 10-fold of LBD-005(100 μg/kg) were injected subcutaneously into the animals of groups A-1 and A-2. Mixed aluminum hydroxide gel 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 under ether anesthesia, and obtained antisera were stored at −80°C.

Guinea Pigs

Sensitization schedule is shown in Table 2. LBD-005 was dissolved at a concentration of 100 μg/kg in PBS 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(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°C.

Active Systemic Anaphylaxis(ASA) Test in Guinea Pigs

2 weeks after the final sensitization, LBD-005(10 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	No. of Shots	No. of Animals	Route
A-1	LBD−005	10 μg/kg	9[a]	5	s.c
A-2	LBD−005	100 μg/kg	9[a]	5	s.c
A-3	LBD−005+Alum	100 μg/kg	3[b]	5	i.p
A-4	OVA+Alum	330 μg/kg	3[b]	5	i.p
A-5	PBS	10 μg/kg	3[b]	5	i.p

[a]: Three times in a week (every other day), [b]: Once in three weeks

Table 2. Sensitization schedule of guinea pigs

Group	Substance	Dose	No. of Shots	No. of Animals	Route
B-1	LBD−005	10 μg/kg	9[a]	5	s.c
B-2	LBD−005	100 μg/kg	9[a]	5	s.c
B-3	LBD−005+CFA	100 mg/kg	3[b]	5	s.c
B-4	OVA+CFA	2.5 μg/kg	3[b]	5	s.c
B-5	PBS	1 μg/kg	3[b]	5	s.c

[a]: Three times in week (every other day), [b]: Once in three weeks

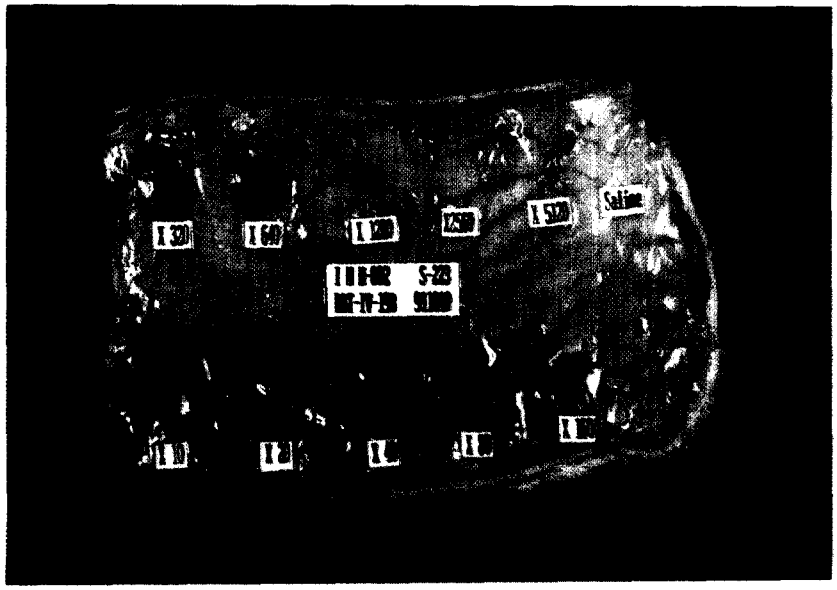

Photo 3. Passive cutanous anaphylaxis of Ovalbumin in mice. The endpoint antibody titer was × 640. Mice were immunized with OVA(330 μg/kg)+hydroxy aluminum gel. Antisera of mice were obtained 6 days after final immunization. 24 hrs after intradermal injections of antisera, rat was challenged by OVA(2.86 mg/kg).

[++] : Severe; Above, Nostril discharge, Lacrimation, Nasal bleeding, Convulsion, Dyspnea, Staggering gait, Rhonchus, 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-005(10 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, 19 58).

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-005(10 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 death,

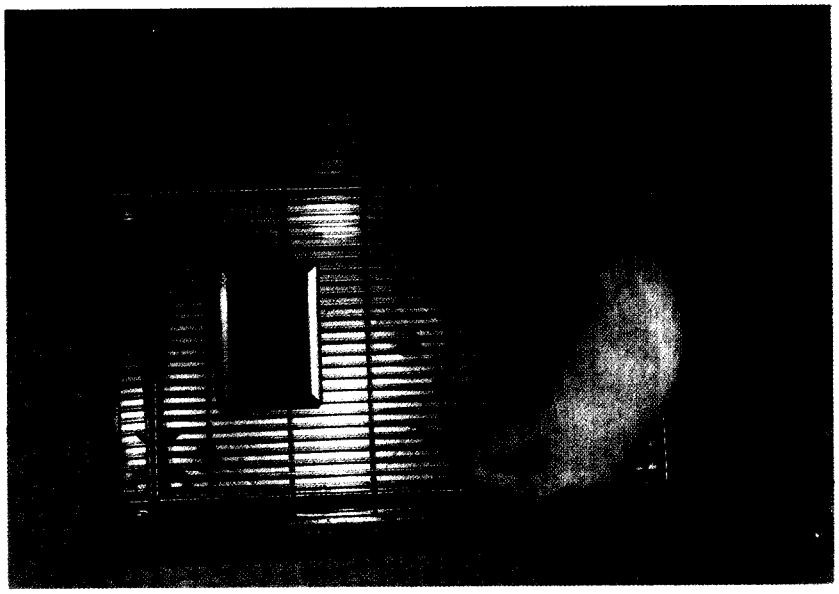

Photo 4. Active systemic anaphylaxis of LBD-005 in guinea pigs sensitized with LBD-005 (100 μg/kg): Urination, Evacuation, Sneezing, Rhonchus, Side posiiton (Arrowhead).

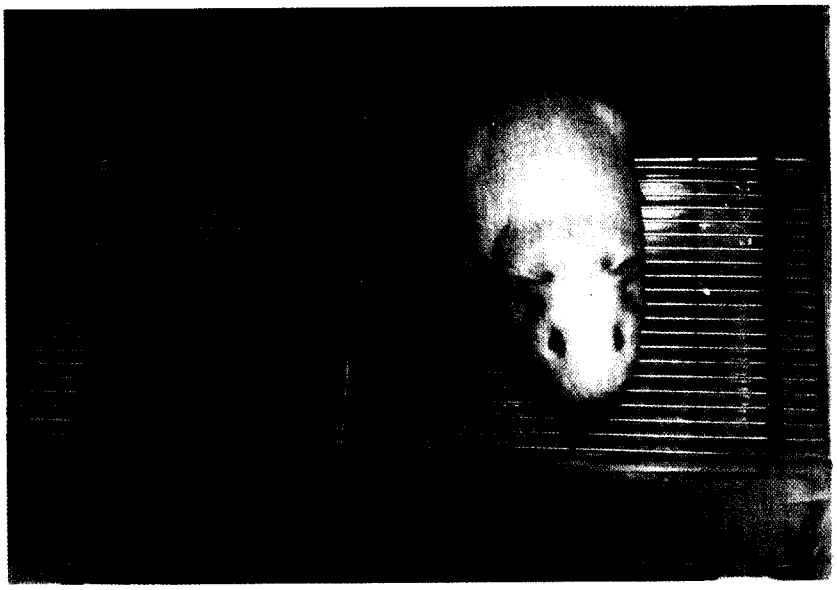

Photo 5. Active systemic anaphylaxis of LBD-005 in guinea pigs sensitized with OVA(2.5 mg/kg) +CFA. Urination, Evacuation, Coughing, Nostril discharge, Nasal bleeding, Lacrimation, Staggering gait, Rhonchus (Arrowhead).

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

Active Systemic Anaphylaxis Test in Guinea Pigs

The results are shown in Table 3.

In groups B-1, B-3 and B-5, urination and/or evacuation were observed in several animals but these signs were negative reaction. However, anaphylactic symptoms(sneezing, rhonchus, side position) were observed in one animl in group B-2 which have included urination and evacuation. On the other hand, all the animals challenged with ovalbumin in group B-4, showed anaphylactic signs which are characterized by coughing, nostril discharge, nasal bleeding, lacrimation, staggering gait, rhonchus, side position and flattening.

Homologous PCA Test in Guinea Pigs

The results are shown in Table 4.

All test sera challenged with LBD-005(10 μg/kg) were negative except those from group B-4. On the other hand, antibodies were detected from in all 10 guinea pigs in group B-4(OVA: 1.67 mg/kg) with PCA titer ranging from ×80 to ×1280.

Heterologous PCA Test in Mice

The results are shown in Table 5.

In group A-1 and A-5, all test sera challenged with LBD-005(10 μ/kg) were negative. In group A-2(100 μg/kg), IgE antibodies were detected ranging from ×10 to ×20 in 7 rats. However, IgE antibodies were detected 9 rats in group A-3(100 μg/kg+alum) with PCA titer ranging from ×20 to ×160. On the other hand, IgE antibodies were detected from all 10 rats in group A-4(OVA: 330 μg/kg)

Table 3. Active systemic anaphylaxis in guinea pigs

Group	Sensitizing antigen	Challenging antigen	No. of animals	Severity of Anaphylaxis[b]				
				[−]	[±]	[+]	[++]	[+++]
B-1	LBD-005 (10 μg/kg)	LBD-005 (10 μg/kg)	5	4	1			
B-2	LBD-005 (100 μg/kg)	LBD-005 (10 μg/kg)	5	3	2		1	
B-3	LBD-005+CFA (100 μg/kg)	LBD-005 (10 μg/kg)	5	3	2			
B-4	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	5				4	1
B-5	PBS (1 ml/kg)	LBD-005 (10 μg/kg)	5	1	4			

[a] Severity of anaphylaxis was expressed as follows.

[−] : Asymptomatic
[±] : Mild: Urination, Evacuation
[+] : Moderate: Above, Coughing, Sneezing
[++] : Severe: Above, Nostril discharge, Nasal bleeding, Lacrimation, Staggering gait, Rhonchus, 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	PCA[b] Titer	Positive Ratio
B-1	LBD−005 (10 μg/kg)	LBD-005 (10 μg/kg)	−[c]	0/10
B-2	LBD−005 (100 μg/kg)	LBD-005 (10 μg/kg)	−	0/10
B-3	LBD−005+CFA (100 μg/kg)	LBD-005 (10 μg/kg)	−	0/10
B-4	OVA+CFA (2.5 mg/kg)	OVA (1.67 mg/kg)	×80~1280	10/10
B-5	PBS (1 ml/kg)	LBD-005 (10 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.

Table 5. Four-hour homologous 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−005 (10 μg/kg)	LBD-005 (10 μg/kg)	−[c]	0/10
A-2	LBD−005 (100 μg/kg)	LBD-005 (10 μg/kg)	×10~×20	7/10
A-3	LBD−005+alum (100 μg/kg)	LBD-005 (10 μg/kg)	×20~×160	9/10
A-4	OVA+alum (330 μg/kg)	OVA (2.86 mg/kg)	×160~640	10/10
B-5	PBS (10 ml/kg)	LBD-005 (10 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.

with PCA titer ranging from ×160 to ×640.

DISCUSSION

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 anaphylatic reaction, PCA, corresponds in every respect to systemic anaphylaxis(Ovary, 1964). In this work, antigenicity of recombinant granulocyte-macrophage colony stimulating factor(LBD-005) was studied with

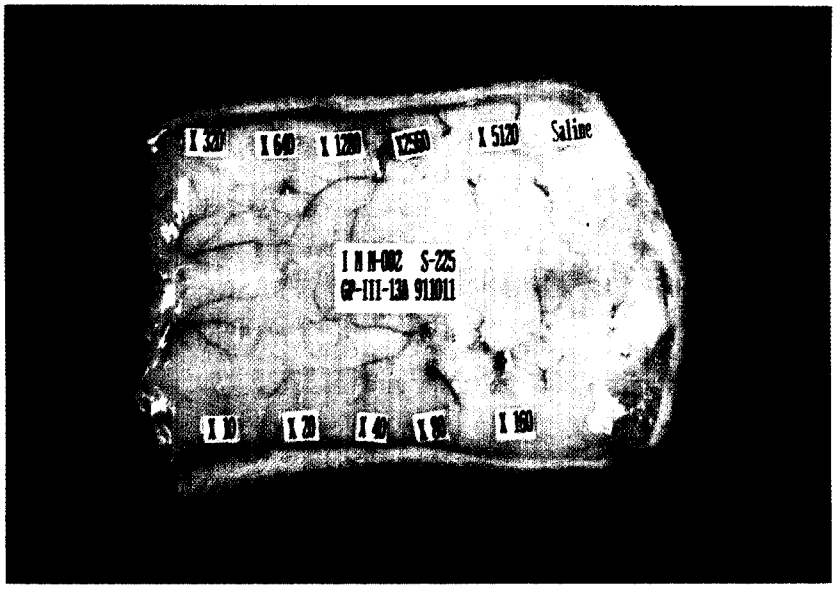

Photo 6. Passive cutaneous anaphylaxis of LBD-005 in guinea pigs. Passive cutaneous anaphylaxis reaction was not detected. Guinea pigs were immunized with LBD-005(100 μg/kg)+CFA. Antisera of guinea pigs were obtained 12 days after final immunization. 4 hrs after intradermal injections of antisera, guinea pigs were challenged by LBD-005 (10 mg/kg).

Photo 7. Passive cutaneous anaphylaxis of LBD-005 in guinea pigs The endpoint antibody titer was $\times$1280. Guinea pigs were immunized with OVA(2.5 mg/kg)+CFA. Antisera of guinea pigs were obtained 12 days after final immunization. 4 hrs after intradermal injections of antisera, guinea pig was challenged by OVA (1.67 mg/kg).

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-005 has a little immunogenicity sensitized with clinical dose(10 μg /kg). However positive antibody titer was observed in 10 times the clinical dose in relation to OVA-Alum inoculation. On the other hand, all the mice sensitized with OVA-Alum inoculation. On the other hand, all the mice sensitized OVA-Alum definitely showed production of IgE antibody. From the results mentioned above, it is considered that LBD-005 showed a little antigenicity not only in guinea pigs (ASA) but also in mice PCA. The human and murine genes have been molecularly cloned and although the products showed about 50% homology at the amino acid level, their activities are species-specific(Dexter, 1989). In consideration of this problem, LBD-005 deserves further safety research.

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