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Evaluation of Allergenic Potency of an Inactivated Combination Vaccine against Hantaan and Puumala Viruses Using Mice and Guinea Pigs

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Abstract – Hantaan (HTN) and Puumala (PUU) viruses are major etiological agents of hemorrhagic fever with renal syndrome (HFRS), an important public health problem in Korea after the Korean War. The objective of present study was to determine allergenic potency of an inactivated combination vaccine against HTN and PUU viruses infection. As a series of allergenicity assessment, a homologous active systemic anaphylaxis (ASA) and homologous/heterologous passive cutaneous anaphylaxis (PCA) tests using the mice and guinea pigs were carried out. In the ASA test, no anaphylactic symptoms were observed in the guinea pigs sensitized with the vaccine alone as well as the vaccine emulsified with an adjuvant. By homologous PCA test, the vaccine did not induced the potential IgE antibody production in the sera obtained from the sensitized guinea pigs. In addition, IgE against the vaccine was not significantly enhanced from the mice inoculated with the vaccine, which was judged by the heterologous PCA test in rats. On the other hand, the inoculation of ovalbumin appeared to allergenic reactions both in the ASA and PCA tests. The results suggest that a combination vaccine against HTN and PUU viruses have no allergenic potential in mice or guinea pigs.

Key words □ allergenicity, hantaan virus, puumala virus, inactivated combination vaccine

The causative agents of hemorrhagic fever with renal syndrome (HFRS), Hantaan (HTNV), Seoul (SEO), Puumala (PUUV) and Belgrade/Dobrava (BEL/DOB) viruses, are serologically related viruses of the family Bunyaviridae and have a worldwide distribution. The primary hosts of hantaviruses are rodents, and human is mainly infected by inhalation of viral particles from the infected rodent excreta such as feces, urine and saliva. All hantaviruses are carried by specific rodent species; the bank vole *Clethrionomys glareolus* being the host for PUUV, and the striped field mouse *Apodemus agrarius* for HTNV. HFRS is severe epidemic and endemic disease in Asia, Europe and in the Far East Russia (Kanerva *et al.*, 1998; Lyudmila *et al.*, 2000).

HTNV was the cause of 3,000 cases of HFRS during the Korean War, and there are currently reported 500~900 cases per year (Peters *et al.*, 1999). A formalin- inactivated HTNV vaccine originated from suckling mouse brain has been available in Korea (Lee *et al.*, 1990). The HTNV vaccine is prepared from the HTNV strain ROK 84-105 and has been shown to induce protective immunity in mice and humans. Recently, Lee et al. (1997), have developed a formalin inactivated PUUV vaccine derived from suckling hamster brain, and an effective HTNV-PUUV combination vaccine to prevent HFRS caused by HTN and PUU viruses infection. The PUUV K27 strain isolated from an HFRS patient in Ufa, Bashikiria in Russia, was used as the seed virus. Only a few studies have reported to the adverse side effects of HTNV, PUUV and HTN/PUUV vaccine, although these vaccines have demonstrated to the preventive efficacy in humans. Therefore, the present study, as a part of safety assessment, was carried out to evaluate the hypersensitivity of an HTNV-PUUV combination vaccine in the mice and guinea pigs under the guidelines for the safety tests of drugs provided by the Food and Drug Administration, Korea (KFDA, 1999).

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MATERIALS AND METHODS

Test substance

The inactivated HTNV-PUUV vaccine (Lot No. VI-

005) against HTN and PUU viruses was supplied by Green Cross Co. Ltd. (Yongin, Korea). The test substance was acceptable to the quality assurance criteria and stored at 4°C in the dark before use. Ovalbumin (OVA) served as a positive control material and other reagents were purchased from Sigma or Aldrich Chemical Company (St. Louis, U.S.A.)

Animals and maintenance

For this experiment, five-week-old male BALB/c mice (23 to 28 g body weight), nine-week-old male Sprague-Dawley rats (250 to 350 g body weight) and six week-old male Hartley guinea pigs (280 to 380 g body weight) were purchased from Sam Yuk Experimental Animals Breeding Center (Seoul, Korea). Animals were housed in the animal room maintained with 12 hrs (07:00~19:00) light-dark cycle, at a constant temperature of 23±2°C, and a relative humidity of 50±5%. The animals were observed daily, and were used after 1 week acclimation period for the experiment detailed below. Commercial pellet diet (mice and rats: Jeil Feed Co., Daejeon, Korea; guinea pigs: Purina Korea Co.), and tap water were available *ad libitum* throughout the study. Animals were selected on the basis of their weight at the start of dosing and randomly assigned to each group.

Sensitization of animals

Guinea pigs: Sensitization schedule is shown in Table I. One dose of HTNV-PUUV combination vaccine contains both 5,120 units/ELISA of HTNV and PUUV antigen in 1.0 ml. The vaccine was dissolved at the dosages of 0.034 and 0.34 dose/kg in sterile saline. The low dose was equivalent to 2 fold of a clinical dosage (0.017 dose/kg) given to a man (60 kg). The vaccine (0.34 dose/kg) and OVA (5 mg/kg) emulsified with an equal volume of FCA were subcutaneously injected into guinea pigs based on body weight (1 ml/kg). The sensitization was repeated 7 times (A-I, A-II and A-III) at the intervals of 4 days or repeated 4 times (A-IV and A-V) once a week. On ten day after final sensitization, blood samples were collected from the abdominal vein of the animals under ether anesthesia, and collected antisera were stored at −80°C before use.

Mice: Sensitization was carried out by the method described previously (Ogita and Mizushima, 1977). The vaccine at a dosage 0.034 dose/kg and its 10-fold 0.34 dose/kg was subcutaneously injected into the animals of groups B-II and B-III. The vaccine, at the dosage 0.34 dose/kg and OVA emulsified with an equal volume of FCA were subcutaneously injected into the animals of groups B-IV and B-V. The

Table I. Sensitization of the guinea pigs used in the active systemic anaphylaxis assay

Groups	Substance	Dose (dose/kg)	No. of treatment	No. of animals	Route
A-I	Saline	1 mg/kg	7[a]	5	s.c.
A-II	HPV	0.034	7	5	s.c.
A-III	HPV	0.34	7	5	s.c.
A-IV	HPV+FCA	0.34	4[b]	5	s.c.
A-V	OVA+FCA	5 mg/kg	4	5	s.c.

HPV: HTNV-PUUV combination vaccine; FCA: Freund's Complete Adjuvant; OVA: Ovalbumin; s.c.: subcutaneous.
[a]once at intervals of 4 days.
[b]once a week.

Table II. Sensitization of the mice used in the passive cutaneous anaphylaxis assay

Groups	Substance	Dose (dose/kg)	No. of treatment	No. of animals	Route
B-I	Saline	10 mg/kg	7[a]	5	s.c.
B-II	HPV	0.034	7	5	s.c.
B-III	HPV	0.34	7	5	s.c.
B-IV	HPV+CFA	0.34	4[b]	5	s.c.
B-V	OVA+CFA	5 mg/kg	4	5	s.c.

HPV: HTNV-PUUV combination vaccine; FCA: Freund's Complete Adjuvant; OVA: Ovalbumin; s.c.: subcutaneous.
[a]once at intervals of 4 days.
[b]once a week.

vaccine was injected after the calculation of inocular according to body weight (10 ml/kg). The sensitization was repeated seven times (B-I, B-II and B-III) at the intervals of 4 days or repeated four times (B-IV and B-V) once a week (Table II). After final sensitization, the antisera were obtained from the orbital venous plexus of the animals under ether anesthesia, and stored at −80°C.

ASA test in guinea pigs

On ten day after the final sensitization, the vaccine (0.34 dose/kg) or OVA (50 mg/kg) was injected into the metatarsal vein of each animals. The signs of anaphylaxis shock were evaluated according to the criteria of Table III.

Homologous PCA test in guinea pigs

The test was performed according to the method described previously (Ovary, 1958). Each sera of the sensitized guinea pigs diluted from 4 to 1024-fold was intradermally injected into the back of guinea pigs which had been clipped before a day. At 24 hours after the initial inoculation, 1 ml of 1 : 1 mixture (v/v) of the vaccine (0.34 dose/kg) or OVA (50 mg/kg) solution, and a 1% solution of Evans blue was injected into the metatarsal vein of each guinea pigs. At 30 min after the inoculation, the guinea pigs were bled to death, and the leakage of dye at the serum injected site was examined to determine the PCA titer.

Table III. Scoring criteria for the active systemic anaphylaxis

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.	Syaggering gait
15.	Jumping
16.	Gasping and writhing
17.	Convulsion
18.	Side position
19.	Cheyne-Stokes Respiration
20.	Death

Evaluation of the intensity: [−] Asymptomatic
[[±] Mild: symptoms 1~4
[+] Moderate: symptoms 1~10
[++] Severe: symptoms 1~19
[+++] Death

Heterologous PCA test in rats

The assay was conducted by the literature methods (Mota *et al.*, 1968; Mota and Wong, 1969). Each sera of the sensitized mice diluted from 4 to 1024-fold was intradermally injected into the back of rats which had been clipped before a day. At 24 hours after the inoculation, 1 ml of 1 : 1 mixture of the vaccine (0.34 dose/kg) or OVA (50 mg/kg) solution, and 1% solution of Evans blue were injected into the tail vein of rats. After 30 min, the PCA titer was determined by the leakage of dye at the serum injected site of rats.

RESULTS AND DISCUSSION

Drug and food allergies are a major problem in the clinic and the development of new products. Hypersensitivity refers to an increased reactivity or sensitivity to an antigen which has been exposed previously, and divide into four categories; immunoglobulin-mediated (immediate) hypersensitivity reaction as type I, II and III and cell (delayed-type) mediated immunity as a type IV reaction. Immediate hypersensitivity is a reaction mediated by IgE antibodies reactive with specific allergens attached to basophil or mast cell Fc receptors. Cross-linking of the cell-bound IgE antibodies by antigen is followed by mast cell or basophil degranulation, with release of pharmacological mediators. These mediators include vasoactive amines as histamine, which causes the increase of vascular permeability, vasodilation, bronchial spasm and mucous secretion (Cruse and Lewis, 1999). Anaphylaxis is a shock reaction that occurs within minutes following the injection of an antigen, which the susceptible subject has IgE specific antibodies. Cutaneous anaphylaxis is a local reaction specifically elicited in the skin of an actively or passively sensitized animals, which involve the *in vivo* passive transfer of IgE antibodies that mediate immediate type hypersensitivity.

The present studies were evaluated the allergenic potency of an HTNV-PUUV combination vaccine at a series of immune toxicity assessment; an ASA, and a

Table IV. Active systemic anaphylaxis of an HTNV-PUUV combination vaccine in guinea pigs

Groups	Sensitization	Challenge (dose/kg)	No. of animals	Intensity of anaphylaxis					Positive ratio
				–	±	+	++	+++	
A-I	Saline	HPV 0.34	5	–	–	–	–	–	0/5
A-II	HPV	HPV 0.34	5	–	–	–	–	–	0/5
A-III	HPV	HPV 0.34	5	–	–	–	–	–	0/5
A-IV	HPV+FCA	HPV 0.34	5	–	–	–	–	–	0/5
A-V	OVA+FCA	OVA 50 mg/kg	5	–	–	–	–	5	5/5

HPV: HTNV-PUUV combination vaccine; FCA: Freund's Complete Adjuvant; OVA: Ovalbumin.

Table V. Homologous passive cutaneous anaphylaxis in guinea pigs with the sera of guinea pigs sensitized an HTNV-PUUV combination vaccine

Groups	Sensitization	Challenge (dose/kg)	PCA titer[a]	Positive ratio
A-I	Saline	HPV 0.34	–[b]	0/10
A-II	HPV	HPV 0.34	–	0/10
A-III	HPV	HPV 0.34	–	0/10
A-IV	HPV+FCA	HPV 0.34	–	0/10
A-V	OVA+FCA	OVA 50 mg/kg	×4~×1024	10/10

HPV: HTNV-PUUV combination vaccine; FCA: Freund's Complete Adjuvant; OVA: Ovalbumin.
[a]PCA titer represents the maximun dilution value of orignal sera which reveal positive reaction.
[b]Allergonic potency was not detected in 4-fold dilution of the sera.

Table VI. Heterologous passive cutaneous anaphylaxis in rats with the sera of mice sensitized an HTNV-PUUV combina-tion vaccine

Groups	Sensitization	Challenge (dose/kg)	PCA titer[a]	Positive ratio
A-I	Saline	HPV 0.34	–[b]	0/10
A-II	HPV	HPV 0.34	–	0/10
A-III	HPV	HPV 0.34	–	0/10
A-IV	HPV+FCA	HPV 0.34	–	0/10
A-V	OVA+FCA	OVA 50 /kg	4×1024	10/10

HPV: HTNV-PUUV combination vaccine; CFA: Freund's Complete Adjuvant; OVA: Ovalbumin.
[a]PCA titer represents the maximun dilution value of orignal sera which reveal positive reaction.
[b]Allergonic potency was not detected in 4-fold dilution of the sera.

homologous/heterologous PCA assay. Both clinical signs and body weight changes of guinea pigs, mice and rats were not observed the noticeable changes during experiment period (data not shown). In the ASA assay, no anaphylactic symptoms were observed in the groups except the A-V group challeneged OVA (Table IV). The animals challenged with OVA in the A-V group revealed the severe anaphylactic signs which are characterized by restlessness, piloerection, tremor, rubbing and licking nose, sneezing, coughing, hyperpnea, urination, evaculation, lacrimation, dyspnea, rhonchus, etc. Therefore, the results indicate that the vaccine has no any signs of immediate hypersensitivity mediated by IgE antibodies.

The PCA reaction was also conducted to investigate the immediate-type hypersensitivity, since the permeability of the post-capillary venules in skin is increased by vasoactive mediators of which antigen induces crosslinkage of the cell-fixed IgE receptor bound to mast cells and basophils. Vascular permeability factors act on the vessels to permit plasma and dye to leak into the extravascular space forming a blue area. In the homologous PCA assay using the sera obtained from sensitized guinea pigs, which challenged with the vaccine (0.34 dose/kg) did not appeared to the positive reaction at any dilution values. On the other hand, the sera of guinea pigs sensitized with OVA (50 mg/kg) produced the positive reaction, which the PCA titer ranged from ×4 to ×1024 (Table V). In addition, in the heterologous PCA assay using rats, the sera of the mice except its sera challenged with OVA revealed the negative reaction. In the rats challenged with OVA, the positive response of OVA was detected with PCA titer ranging from ×4 to ×1,024 fold (Table VI). The figures of positive reaction challenged with OVA were not presented in this study.

From the results, it suggests that the HTNV-PUUV combination vaccine has no any kind of allergenic potency in mice and guinea pigs. Therefore, it is expected to be useful vaccine without immune toxic effects to prevent HFRS. However, it must be consider to the potential of cell mediated hypersensitivity, since it is not possible to predict the delayed-type hypersensitiv-

ity of the vaccine under the present study.

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