응용약물학회지, The Journal of Applied Pharmacology 5, 174-178(1997)

Immunotoxicity Study of Combined Vaccine (KGCC-95VI) against Japanese Encephalitis and Hantaan Virus Infection in Guinea Pigs

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(Received April 3, 1997; accepted May 20, 1997)

Abstract – The immunogenicity of the possible non-essential component of the combined vaccine (KGCC-95VI) for the prophylaxis against Japanese encephalitis and Hantaan virus infection recently developed by Korea Green Cross Corporation was investigated using the Hartley guinea pigs. The KGCC-95VI was administered to the guinea pigs subcutaneously to sensitize the animals. The guinea pigs did not induce any anaphylactic immune responses which could be detectable by the active systemic anaphylaxis (ASA), the passive systemic anaphylaxis (PSA), and the passive cutaneous anaphylaxis (PCA) tests. The KGCC-95VI is considered not to induce any anaphylactic immune responses except the prophylatic immune effects of the vaccine.

Keywords□ Japanese encephalitis, Hantaan virus infection, vaccine, ASA, PSA, PCA

Japanese encephalitis virus (JEV) is widely distributed in Asia, including Korea, Japan, China, Taiwan, Philippines, far-eastern Russia, all of Southeast Asia and India (Hoke *et al.*, 1988). Mitamura et al. in 1938 isolated the virus from the mosquito, *Culex tritaeniorhynchus*. It is established that pigs and birds are the principal viremic hosts and that *Culex tritaeniorhynchus* is responsible for transmission between these vertebrates and from them to humans (Buescher *et al.*, 1959). The inactivated vaccine using the virus propagated in the brains of the suckling mice has been available (Cho *et al.*, 1994).

Hantaan virus was originally isolated from the Korean striped field mouse, *Apodemus agarius* corea. The virus is one of the etiologic agents of hemorrhagic fever with renal syndrome (HFRS) such as Hantaan virus infection, leptospirosis, rickettsial infection. The inactivated vaccine using the virus propagated in the brains of the newborn mice is being used (Shin, 1992, Lee *et al.*, 1988).

Recently Korea Green Cross Corporation developed, for the convenience in practical immunization, the combined vaccine for the prophylaxis against Japanese encephalitis and Hantaan virus infection. The efficacy of the combined vaccine was confirmed. In this study the antigenicity of the combined vaccine was investigated using the guinea pigs and rats in accordance with the guidelines on the safety tests of the drugs (Guidelines 1996) provided by the Food and Drug Administration, Korea.

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MATERIALS AND METHODS

The test material, the combined inactivated virus vaccine for Japanese encephalitis and Hantaan virus-caused hemorrhagic fever with renal syndrome (referred to as KGCC-95VI hereinafter for convenience), was produced and supplied by Korea Green Cross Corporation based in Korea. Phosphate buffered saline, 1/60 M, pH 7.2, prepared and autoclaved at the laboratory was used as the diluent for the test material. Turkey ovalbumin (Sigma, USA), complete Freund's adjuvant (Sigma, USA) and Evans blue dye were purchased. Aluminum hydroxide

gel (Alhydrogel™, Superfos Biosector, Denmark) was prepared in our laboratory.

The Hartley male guinea pigs (Laboratory of Experimental Animals, Korea) were obtained at the age of 5 weeks. The Sprague-Dawley rats (Laboratory of Experimental Animals, Korea) were obtained at the age of 4 weeks. All the animals were acclimatized for 1 week prior to the tests under the barrier-sustained animal room maintained at a temperature of 23±3℃, a relative humidity of 50±10% and illumination cycle of 12 hours light and 12 hours dark (light during 07:00-19:00). The guinea pigs were housed in the automatic washing cages and fed with new-born calf pellets (Jeil Feed Co., Korea) and tap water ad libitum.

ASA in Guinea Pigs

Following the guidelines on the safety tests of the drugs (Guidelines 1996) provided by the Food and Drug Administration, Korea, the possibility of inducing the anaphylatic immune responses of the KGCC-95VI was investigated. Thirty male guinea pigs entered in this test. The guinea pigs were divided into 6 groups (5 guinea pigs a group). Two groups of the guinea pigs were inoculated subcutaneously with a low (8.3 ml/kg) and a high (83.0 ml/kg) doses of the KGCC-95VI respectively. Other two groups of the guinea pigs were inoculated with a low (8.3 ml/kg) and a high (83.0 ml/kg) of the complete Freund's adjuvant-adjuvanted vaccine material whose protein concentration was exactly same with that of the final formulated vaccine respectively. For the positive control, turkey ovalbumin solution was used. One volume of turkey ovalbumin solution (13.2 mg/ml) was emulsified with equal volume of the complete Freund's adjuvant. One ml of this emulsion was inoculated to each animal of the positive control group subcutaneously. For the negative control, the plain saline was used. One ml of plain saline was inoculated to each animal of the negative control group. The adjuvanted test materials were inoculated three times at 3-week intervals. The plain test materials were inoculated twice a week for four weeks.

The raw vaccine material which was not adjuvanted was used to evoke the anaphylaxis from the sensitized animals. The viral protein concentrations of both Japanese encephalitis and Hantaan virus were adjusted to those in the expected clinical dose. Ten human dose per kg body weight was diluted in 0.5 ml of diluent. Three weeks after the last inoculation of the test materials, the guinea pigs were administered with 0.5 ml of the diluted plain vaccine materials intravenously via the leg vein. The guinea pigs for the positive control were treated with 0.5 ml of the turkey ovalbumin solution. After 30 minutes of the administration, any anaphylactic signs of the guinea pigs were examined and evaluated according to the criteria specified in the guidelines on the safety tests of the drugs (Guidelines 1996).

PSA in Guinea Pigs

The sera (0.5 ml) obtained from the guinea pigs which were inoculated with the KGCC-95VI and the controls as described in active systemic anaphylaxis of the materials and methods were administered into the guinea pigs via the leg veins. After 18 hours of the serum administration, the raw vaccine materials and the control materials were administered into the leg veins of the guinea pigs. Any anaphylactic signs of the guinea pigs were examined and evaluated according to the criteria specified in the guidelines on the safety tests of the drugs (Guidelines 1996).

Homologous Passive Cutaneous Anaphylaxis in Guinea Pigs

In order to prepare the antisera against the test materials, thirty male guinea pigs were used. The guinea pigs were divided into 6 groups (5 guinea pigs a group). The guinea pigs were immunized as the same methods described above in the active systemic anaphylaxis test. Three weeks after the inoculation of the test materials, the sera were collected and stored at -20℃ until use.

In order to produce the passive cutaneous anaphylaxis, two guinea pigs were allocated for the individual sera of the guinea pigs. The backs of the guinea pigs were clipped free of hairs. The guinea pig sera were diluted 10 times in diluent and 2-fold serially diluted to get 20 to 5120 times dilution. The antiserum dilutions (0.1 ml) were administered into the separate sites of the back on each animal. After four hours the guinea pigs were administered intravenously 0.1 ml of the mixture of the one volume of the raw vaccine material and an equal volume of 2% Evans blue solution. After thirty minutes, the guinea pigs were anesthetized with ether and killed to open the skin. The reaction (blue spot) on the subcutaneous surface of the skin was observed. The average of the long and the short diameters over 5mm was considered reactive. The reciprocal of the dilution showing the endpoint of the reaction was defined as the PCA titer.

Heterologous Passive Cutaneous Anaphylaxis in Guinea Pigs and Rats

The guinea pig sera prepared in the homologous PCA were used in this test. In order to produce the passive cutaneous anaphylaxis in the rats with the guinea pig sera, two rats were allocated for the individual sera of the guinea pigs. The backs of the rats were clipped free of hairs. The guinea pig sera were diluted 10 times in diluent and 2-fold serially diluted to get 20 to 5120 times dilution. The antiserum dilutions (0.1 ml) were administered into the separate sites of the back on each rat. After four hours the rats were administered intravenously 0.1 ml of the mixture of the one volume of the raw vaccine material and an equal volume of 2% Evans blue solution. After thirty minutes, the rats were anesthetized with ether and killed to open the skin. The reaction (blue spot) on the subcutaneous surface of the skin was observed. The average of the long and the short diameters over 5 mm was considered reactive. The reciprocal of the dilution showing the end-point of the reaction was defined as the PCA titer.

RESULTS

In order to investigate the possibility to induce the anaphylaxis of the KGCC-95VI the antigenicity test was performed following the guidelines on the safety tests of the drugs provided by the Food and Drug Administration, Korea. The guinea pigs were inoculated with the KGCC-95VI and challenged to produce the active systemic anaphylaxis. To examine the possibility of the passive cutaneous anaphylaxis, the antiserum was administered subcutaneously into the guinea pigs. The guinea pigs were challenged with the raw vaccine material intravenously.

Active Systemic Anaphylaxis

The guinea pigs of the test groups were inoculated as described in the Materials and Methods. The symptoms of the guinea pigs observed after the challenge were summerized in Table I. The guinea pigs administered and challenged with the positive control, the turkey ovalbumin, showed coughing, restlessness, rhochus and staggering gaits. The guinea pigs administered and challenged with the raw vaccine material did not show any symptoms which might be considered as one of anaphylatic responses.

Passive Systemic Anaphylaxis

The guinea pigs of the test groups were administered intravenously with 0.5 ml of serum which were immunized as described in active systemic anaphylaxis of the materials and methods. After 18 hours, the guinea pigs were challenged with the raw vaccine materials and the controls via the leg veins. The symptoms of the guinea pigs observed after the challenge were summarized in Table II. The guinea pigs administered and challenged with the positive control, the turkey ovalbumin, showed restlessness, tremor and rubbing. The guinea pigs administered and challenged with the raw vaccine material did not show any symptoms which might be considered as one of anaphylactic responses.

Table I. Symptoms of Guinea Pigs after Challenge, ASA

Symptoms	Low	High	Low-FCA	High-FCA	Diluent	TOA-FCA
Restlessness	-	-	-	-	-	+
Piloerection	-	-	-	-	-	+
Tremor	-	-	-	-	-	±
Rubbing nose	-	-	-	-	-	++
Sneezing	-	-	-	-	-	+
Coughing	-	-	-	-	-	+
Hyperpnea	-	-	-	-	-	±
Urination	-	-	-	-	-	±
Evacuation	-	-	-	-	-	+
Lacrimation	-	-	-	-	-	-
Dyspnea	-	-	-	-	-	-
Rhonchus	-	-	-	-	-	-
Cyanosis	-	-	-	-	-	-
Staggering gait	-	-	-	-	-	-
Jumping						
Gasping	-	-	-	-	-	-
Convulsion	-	-	-	-	-	-
Side position	-	-	-	-	-	-
Cheyne-Strokes respiration	-	-	-	-	-	-
Death	-	-	-	-	-	-
Evaluation	normal	normal	normal	normal	normal	moderate

Low, treated with low dosage (8.3 ml/kg) of KGCC-95VI; High, treated with high dosage (83.0 ml/kg) of KGCC-95VI; Low-FCA, treated with low dosage of vaccine materials adjuvanted with Freund's complete adjuvant(FCA); High-FCA, treated with high dosage of vaccine material adjuvanted with FCA; TOA-FCA; treated with turkey ovalbumin adjuvanted with FCA; -, negative; +,positive.

Homologous Passive Cutaneous Anaphylaxis

In order to investigate the possibility of the KGCC-95VI to produce humoral immune response to evoke the passive cutaneous anaphylaxis, to the backs of the guinea pigs, the diluted antisera of the guinea pigs which were treated with the vaccine materials and the controls were administered intradermally. After challenging with the

Table II. Symptoms of Guinea Pigs after Challenge, PSA

Symptoms	Low	High	Low-FCA	High-FCA	Diluent	TOA-FCA
Restlessness	-	-	-	-	-	+
Piloerection	-	-	-	-	-	+
Tremor	-	-	-	-	-	±
Rubbing nose	-	-	-	-	-	+
Sneezing	-	-	-	-	-	-
Coughing	-	-	-	-	-	-
Hyperpnea	-	-	-	-	-	-
Urination	-	-	-	-	-	-
Evacuation	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-
Dyspnea	-	-	-	-	-	-
Rhonchus	-	-	-	-	-	-
Cyanosis	-	-	-	-	-	-
Staggering gait	-	-	-	-	-	-
Jumping	-	-	-	-	-	-
Gasping	-	-	-	-	-	-
Convulsion	-	-	-	-	-	-
Side position	-	-	-	-	-	-
Cheyne-Strokes respiration	-	-	-	-	-	-
Death	-	-	-	-	-	-
Evaluation	normal	normal	normal	normal	normal	moderate

Low, treated with low dosage (8.3 ml/kg) of KGCC-95VI; High, treated with high dosage (83.0 ml/kg) of KGCC-95VI; Low-FCA, treated with low dosage of vaccine materials adjuvanted with Freund's complete adjuvant(FCA); High-FCA, treated with high dosage of vaccine material adjuvanted with FCA; TOA-FCA; treated with turkey ovalbumin adjuvanted with FCA; -, negative; +,positive.

raw vaccine materials and the controls, the backs of the guinea pigs were examined. The PCA titer of the positive control was 640 to 1280. The PCA titers of the test material and the negative control were not accessible because there were no detectable blue spots.

Heterologous Passive Cutaneous Anaphylaxis

In order to investigate the possibility of the KGCC-95VI to produce humoral immune response to evoke the passive cutaneous anaphylaxis, to the backs of the rats, the diluted antisera of the guinea pigs which were treated with the vaccine materials and the controls were administered intradermally. After challenging with the raw vaccine materials and the controls, the backs of the rats were examined. The PCA titer of the positive control was 320 to 640. The PCA titers of the test material and the negative control were not accessible because there were no detectable blue spots.

DISCUSSION

The practical use of Japanese encephalitis vaccine purified from infected mouse brains started in 1966 in Japan, led to a rapid increase in the number of the vaccinated people and a rapid reduction in the incidence of this disease (Oya, 1987). At the beginning of the use of the vaccine, the virus was purified by alcohol-protamine precipitation and centrifugation. The vaccine contained a high level of impurities and its potency was very low. Recently the manufacturing procedures employ many sophisticated methods such as ultracentrifugation and ultrafiltration to reduce the impurities and improve the potency (Umenai *et al.*, 1985).

In 1988, Lee and Ahn (Lee *et al.*, 1988) and Yamanishi et al. (Yamanishi *et al.*, 1988) reported the development of inactivated vaccines against HFRS with Hantaan virus infection. Lee and Ahn inoculated Hantaan virus isolated from an HFRS patient into the suckling rat brains and purified and inactivated with the methods to prepare Japanese encephalitis virus mouse brain vaccine with a slight modification. Yamanishi et al. inoculated Seoul virus isolated from a rat tumor into the suckling mouse brains. The available evidences showed that these vaccines induced protective immunity in mice.

Currently some combined vaccines are available. The toxoid vaccines against tetanus and diphtheria and inactivated pertussis whole cell vaccine were combined. The live attenuated virus vaccine against measles, mumps and rubella were combined. The combined vaccines have many advantages over the corresponding monovalent vaccines in the practical use such as manufacturing costs, transportation, storage and administration. The combined vaccines may result in a substantially reduced number of contacts with health care workers to immunize against those diseases. The cost of administration of a vaccine is at least 10 times the cost of the vaccine (Douglas, 1993). Although it is unlikely that this ratio will hold for many other vaccines, reducing the number of visits of health care workers for vaccine administration could clearly result in great savings.

In this study the possibility of inducing the anaphylactic immune response of the combined vaccine against Japanese encephalitis and Hantaan virus infection was investigated by ASA, PSA and PCA using the guinea pigs. In PCA test, Baik *et al.* reported the PCA titer of the positive control in guinea pig and mouse was 512. The

PCA titer of the positive control in this study was 640 to 1280. Paik et al. inoculated the guinea pigs with 1200 times the expected clinical dose adjuvanted with aluminum hydroxide gel and found that their test material did not contain any impurities to cause the unwanted anaphylaxis. In this study, we inoculated the guinea pigs with 200 times the expected clinical dose adjuvanted with Freund's complete adjuvant (FCA). The guinea pigs were inoculated with the test material which were adjuvanted with the FCA, the most potential adjuvant known upto now. However, with these three tests any symptoms which might be considered as one of the anaphylactic symptoms could not be observed. As the viral proteins from both Japanese encephalitis virus and Hantaan virus were purified by the recently developed sophisticated technology, the KGCC-95VI may not contain any impurities which may have the immunogenicity to provoke the unwanted immune responses.

In conclusion, the combined vaccine (KGCC-95VI) against Japanese encephalitis and Hantaan virus infection showed no signs of the immunogenic effects except the vaccine efficacy and is considered not to contain the impurities which may be immunogenic to induce the unwanted immune responses in the guinea pigs.

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