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Experimental Allergic Encephalomyelitis Study of Combined Vaccine (KGCC-95VI) Against Japanese Encephalitis and Hantaan Virus Infection

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ABSTRACT *: The possibility of the allergic encephalomyelitis caused by the combined vaccine (KGCC-95VI) for the prophylaxis against Japanese encephalitis and Hantaan virus infection, recently developed by Korea Green Cross Corporation, was investigated in the Hartley guinea pigs. The KGCC-95VI was administered to the guinea pigs subcutaneously to sensitize the animals three times at one month intervals. There were no clinical signs or gross pathological findings. There were no abnormal histopathological findings at cerebrums, cerebellums, brain stems and the spinal cords. The concentration of myelin basic protein was 1.10 ng/dose quantified by ELISA, which met the guide-line of below 2 ng/ml/dose recommended by American Society of Health -System Pharmacists(AHPS) Drug Information. Accordingly, the KGCC-95VI is considered not to induce any allergic immune responses which may lead to the experimental allergic encephalomyelitis.*

Key Words *: Japanese encephalitis, Hantaan virus infection, Vaccine, Experimental allergic encephalomyelitis, MBP concentration*

I. INTRODUCTION

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.* (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 humans (Buescher and Scherer, 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) (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 and Ahn, 1988).

Recently, Korea Green Cross Corporation(KGCC) 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 possibility to induce the allergic encephalomyelitis of the combined vaccine was investigated using the guinea pigs. Additionally, myelin is a specialized membrane that surrounds and electrically insulates axons, facilitating the conduction of nerve impulses. In the central nervous system (CNS), myelin is produced by oligodendrocytes. Myelin basic protein (MBP) is one of the major CNS myelin proteins and represents as much as 30% of the

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total myelin proteins. Kitaka(1972) reported that MBP might cause the autoimmune encephalitis when administered excessively into human. In this study, the MBP contained in the combined vaccine (KGCC-95VI) against Japanese encephalitis and Hantaan virus infection was quantified by ELISA

II. 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(KGCC) 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 purchased.

The Hartley male guinea pigs (Laboratory of Experimental Animals, Korea) were obtained at the age of 5 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°C, a relative humidity of 50±10% and illumination cycle of 12 hr light and 12 hr dark (light during 07:00-19:00). The guinea pigs were housed in the automatic washing cages (Dae-Jong, Korea) and fed with new born calf pellets (Jeil Feed Co., Korea) and tap water ad libitum.

Following the protocols suggested by Huntingdon Research Centre, the possibility of inducing the allergic encephalitis of the KGCC-95VI was investigated. Twenty five guinea pigs were used in this test. The guinea pigs were divided into 5 groups (5 guinea pigs per a group). Group one was administered with the diluent containing the same amount of aluminum hydroxide gel as that of the KGCC-95VI as the negative control. Group two was administered with the mock vaccine (protein contents corresponded to the expected clinical dose of KGCC-95VI) which was prepared in the exactly same way from the mouse brains without virus inoculation. Group three was administered with one tenth of human dosage subcutaneously. Group four was administered with one human dosage. Group five was administered with the homogenate of the rabbit spinal cord as the positive control.

Following the first inoculation, the animals were observed daily for ataxic gait, paralysis of the hind limbs and loss of lighting reflex. At the day 30, all the animal were inoculated in the same way as described above. The animals were observed for further 7 days and sacrificed. During the observation period, any allergic signs of the guinea pigs were examined and evaluated according to the criteria specified in the minimum requirements and test methods on the safety tests of the drugs(Guidlines for Safety Tests of Drugs, 1996). The brains and spinal cords were dissected out and immersed in Bouins solution for fixation. Sections obtained from cerebrums, cerebellums, brain stems, and spinal cords were stained with Hematoxylin and Eosin, Luxol Fast Blue for myelin, and Glees Marsfand for axon and examined microscopically.

The quantification of the MBP was performed following the methods of Laviada *et al.*, (1992) with a slight modification. The 96-well microplates (Nunc-Immuno, Denmark) were coated with the monoclonal antibody against MBP (Serotec, USA). The aliquots of the MBP purified and quantified from the mouse brains in the previous experiment (AHPS, 1996) was used as the MBP standard. The 100 μl of vaccine material before formulation as the 10 x concentrate (KGCC, Korea) was applied to the well. Separately, 100 μl of the MBP standard solution (1 μg/ml) was diluted 2-fold serially up to 211. The microplates were incubated for 1 hr at 37°C and washed three times with the washing buffer (PBS, pH 7.2). The diluted anti-MBP from the rabbit (100 μl) was added to each well. The microplates were incubated for 1 hr at 37°C and washed three times with the washing buffer. The peroxidase-conjugated anti-rabbit IgG from the goat (100 μl/well, Sigma, USA) was added to each well and incubated at 37°C for 30 min. The microplates were washed five times with the washing buffer. The substrate (100 μl/well, 0.02% H_2O_2 and 0.01% 2,2'-Azino-bis-(3-ethylbenzodiazolin sul-

fonate, pH 4.0) was added to each well. The microplates were incubated for 30 min for the color development and added 50 μl of the stop solution (1N H_2SO_4). The light absorbance of each well was measured at the wavelength 405 nm by an ELISA reader (SLT 340ATC, Austria). With the diluted MBP standard solutions the standard curve was plotted. The amount of MBP contained in one human dose of the KGCC-95VI was calculated against the standard curve.

III. RESULTS

In order to investigate the possibility to induce the allergic encephalomyelitis of the KGCC-95VI, the experimental allergic encephalomyelitis study was performed following the protocol suggested by Huntingdon Research Centre. During the observation period, there were no death or clinical findings such as ataxic gait, paralysis of the hind limbs and loss of lighting reflex. Even at the second inoculation and during the following 7-day observation, there were no death and no clinical findings in the groups inoculated with the test materials (Table 1).

At the termination of the observation period all the animals were sacrificed. Cerebrums, cerebellums, brain stems, and spinal cords were examined histopathologically. There were no abnormal histopathological findings (Table 2).

The MBP concentration of the combined vaccine (KGCC-95VI) was quantified by ELISA. The vaccine material (10 x concentrate) before formulation was used for the quantification. The amount of MBP contained in one human dose of the KGCC- 95VI was 1.10 ng/dose.

Table 1. Symptoms of guinea pigs

Symptoms	Diluent with Al $(OH)_3$	Mock vaccine	1/10 human dose	One human dose	Rabbit spinal cord
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-Stokes respiration	–	–	–	–	+
Death	–	–	–	–	–
Evaluation	–	–	–	–	++

[-] asymptomatic, [ ] mild : 1~4 symptom, [+] moderate : 1~10 symptom, [++] severe : 1~19 symptom, [+++] death

Table 2. Histopathological findings of brain and spinal cord sections

Sections examined	Diluent with $Al(OH)_3$	Mock vaccine	1/10 human dose	One human dose	Rabbit spinal cord
Cerebrum	No findings	No findings	No findings	No findings	Inflammation
Cerebellum	No findings	No findings	No findings	No findings	Inflammation
Brain stem	No findings	No findings	No findings	No findings	Inflammation
Spinal cords	No findings	No findings	No findings	No findings	No findings

IV. DISCUSSION

The practical use of Japanese encephalitis vaccine purified from infected mouse brains started in 1966 in Japan has 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 sophisticate methods such as ultracentrifugation and ultrafiltration to reduce the impurities and improve the potency(Umenai *et al.*, 1985).

Lee, Ahn and Yamanishi *et al.*, (1988) reported the development of inactivated vaccines against HFRS with Hantaan virus infection. They inoculated Hantaan virus isolated from an HFRS patient into the suckling rat brains and purified and inactivated with the methods used in preparing Japanese encephalitis virus mouse brain vaccine with a slight modification. Yamanishi *et al.*, (1988) inoculated Seoul virus isolated from a rat tumor into the suckling mouse brains. The available evidences appeared 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 individual 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 individual vaccine is at least 10 times the cost of the combined 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.

The confirmed human dosage in practice is 0.5 *ml* (8.3 μl/kg body weight). In this study one tenth and one human dose respectively were inoculated in order to understand that the immune response is evoked in these dose range. During the observation period there occurred no death and allergic signs in the groups inoculated with the test materials. During the observation period the body weight changes were monitored (data not shown). There was no abnormality in the body weight gains in the test material groups comparing to those in the groups inoculated with the homogenate of the rabbit spinal cords and the diluent with $Al(OH)_3$ gel.

There were no histopathological findings in the sections of cerebrums, cerebellums, brain stems, and spinal cords of the groups, diluent with Al(OH)3, mock vaccine, one dose, and 1/10 dose. Whereas there were some findings of inflammation in the sections of cerebrum, cerebellum, and brain stem.

MBP is present in five forms of different molecular weights which are encoded by five different mRNA species. MBP synthesis reaches its maximum level 18 days after birth(Roch *et al.*, 1987).

The experimental allergic encephalitis was observed when Pasteur inoculated the rabbit attenuated rabies virus vaccine into the patient. The patient showed a transient paralysis with encephalopathy. At that time it was thought that the paralysis was due to the incomplete attenuation of the rabies virus. In 1930's and 1940's, it was reported that the brain extracts adjuvanted with the Freund's complete adjuvant caused the severe encephalopathy in many experimental animals. Ever since this encephalopathy was referred to as the experimental allergic encephalitis, on which many researchers have been concentrated(Kim *et al.*, 1987). The MBP concentration of the combined vaccine (KGCC-95VI) was quantified by ELISA. The vaccine material (10x concentrate) before formulation was used for the quantification. The concentration of MBP contained in one human dose of the KGCC-95VI was 1.10 ng. This concentration of MBP met the guide-line of below 2 ng/ml/dose recommended by AHFS Drug Information(AHFS, 1996).

In conclusion, the combined vaccine (KGCC-95VI) against Japanese encephalitis and Hantaan

virus infection showed no signs of the experimental allergic encephalomyelitis and is considered not to induce any experimental allergic encephalomyelitis.

REFERENCES

American Society of Health-System Pharmacists (AHPS) (1996) : Japanese encephalitis virus vaccine. AHPS Drug Information, **2**, 2470.

Buescher, E.L., Scherer, W.F. (1959) : Ecological studies of Japanese encephalitis in *Japan. Am. J. Trop. Med. Hyg.*, **8**, 719-722.

Cho, H.W., Kim, C.L., Ban, Nam, J.H. *et al.*(1994) : Antigenic analysis of JE virus isolated from mosquitoes (C. t) in Korea and the development of a rapid diagnosis for JE by using monoclonal antibody. Report of NIH, Kor., **31**, 130-138.

Douglas, R.G. (1993) : The children's vaccine initiative-will it work? *J. Inf. Dis.*, **168**, 269-274.

Guidelines for Safety Tests of Drugs (1996) : Food and Drug Administration, Korea, Provision #1996-8 (April 16, 1996).

Hoke, C.H., Nisalak, A., Sagawhipa, N. *et al.*(1988) : Protection against Japanese encephalitis by inactivated vaccine. *N. Engl. J. Med.*, **319**, 608-613.

Huntingdon Research Centre (1995) : To assess the potential of a test compound to induce allergic encephalomyelitis in the guinea pig, personal communication.

Kim, H., Jang, Y.S., An, C.N., Kim, H.C., Lee, H.W. (1995) : The quantitation of myelin basic protein using ELISA in the mouse brain-derived Vaccine: Hantavax and Japanese encephalitis vaccine. *J. Korean Soc. Microbiol.*, **30**(5), 563-571.

Kitaka M. (1972) : Follow-up on the use of vaccine in children in Japan. Immunization for Japanese encephalitis. Amsterdam. Excepta Medica, pp 275-277.

Laviada, M.D., Dominguez, M.B., Sanchez-Vizcaino, J. M. (1992) : Detection of african horse-sickness virus in infected spleens by a sandwich ELISA using two monoclonal antibodies specific for VP 7. *J. Viol. Meth.*, **38**, 229-242.

Lee, H.W. and An, C.N. (1988) : Development of Vaccine against Hemorrhagic Fever with Renal Syndrome. *J. Korean. Soc. Virol.*, **18**, 143-148.

Oya, A. (1987) : Guidelines for mass immunization using Japanese encephalitis vaccines. WHO report, 24 August.

Roch J.M., Cooper, B.J., Ramirez, M., Matthieu, J.M. (1987) : Expression of only one myelin basic protein allele in mouse is compatible with normal myelination. *Mol. Brain Res.*, **3**, 61-68.

Shin, K.S. (1992) : Study on the ELISA method using monoclonal antibody for the detection of Hantaan virus. *J. Korean Soc. Microbiol.*, **27**, 297-305.

Umenai, T., Krzyko, R., Bektimirov, T.A. *et al.*(1985) : Japanese encephalitis: current world-wide status. Bull WHO, **63**, 625-631.

Yamanishi, K., Tomishita, O. and Tamura, M. (1988) : Development of inactivated vaccine against virus causing hemorrhagic fever with renal syndrome. *Vaccine*, **6**, 278-282.