Analysis of Integrity of Killed *Hantavirus* Vaccine by Antigen-Capture Reverse Transcriptase PCR

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Abstract Hantavax® is one of the killed *Hantavirus* vaccines, and is commercially available in South Korea. This vaccine was developed by inactivation of virus isolated from infected suckling mouse brain with formalin. Although Hantavax® can induce neutralizing antibodies in vaccinees, the strength of this induction and the duration of the humoral immune response are controversial issues. In this study, we studied the native conformation of the killed vaccine by antigen-capture reverse transcriptase polymerase chain reaction with patient and vaccinee sera containing neutralizing antibodies against *Hantavirus*. The results showed that Hantavax® could bind HTNV patient and vaccinee sera like live virus, suggesting that the integrity of the viral epitope is maintained in Hantavax® and induces the protective antibodies, even though the virus was inactivated with formalin.

Key words: Hantavirus, killed vaccine, antigen-capture RT-PCR

Hantaviruses cause two fatal human diseases, hemorrhagic fever with renal syndrome (HFRS) and *Hantavirus* pulmonary syndrome (HPS) [5]. The first *Hantavirus*, named Hantaan virus (HTNV), was isolated from the striped field mouse, *Apodemus agrarius* [1, 7]. The major human pathogenic hantaviruses in Asia are HTNV and Seoul virus (SEOV), and they cause HFRS, which is characterized by symptoms such as high fever, chills, headache, generalized myalgia, abdominal and back pain, and hemorrhagic manifestations [10]. HTNV is estimated to annually cause over 100,000 cases of HFRS in Asia with a mortality rate of 0.1–5% [11].

The first *Hantavirus* vaccine commercially available in Republic of Korea was Hantavax® (Korea Green Cross, Seoul, Korea), which was produced by 0.05% formalin-inactivation of the ROK 84-105 strain passaged through suckling mouse brain [6]. The protective efficacy of this vaccine has been confirmed by several independent field studies [3, 6]. Furthermore, since Hantavax® became available in 1990, the total number of hospitalized HFRS patients in Republic of Korea decreased from 1,234 in 1991 to 750 in 1998 [14]. However, the strength of the induction of virus-neutralizing antibodies and the duration of the humoral immune response still remain controversial, because the native conformation of the viral surface proteins may be compromised during the inactivation procedure or the titer of killed virus in the vaccine preparations may be low [3, 6, 13, 14]. Therefore, it is necessary to investigate from a vaccine potency study whether the integrity of the vaccine protein is maintained to induce the correct neutralizing antibodies.

The objective of this study was to determine the conformational integrity of the viral surface protein of Hantavax® by antigen-capture reverse transcriptase polymerase chain reaction (AC-RT-PCR), in which HFRS patient and vaccinee sera were used as a coating antibody (Fig. 1a).

The killed *Hantavirus* vaccine used in this study was Hantavax®, which was developed by Korea Green Cross Inc. (Seoul, Korea). The detailed manufacturing procedure was described in a previous report [3]. This vaccine has a titer of 1,024 ELISA unit/ml. HTNV ROK-84-105 was used as the positive control virus, and was grown in suckling mouse brain.

The sera of three HFRS patients were collected from the Korea National Institute of Health. Each serum was adjusted to an IFA titer of 1:512 for AC-RT-PCR. A conformation-sensitive monoclonal antibody (HE4) directed against HTNV nucleocapsid (N) protein was produced as

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a

b
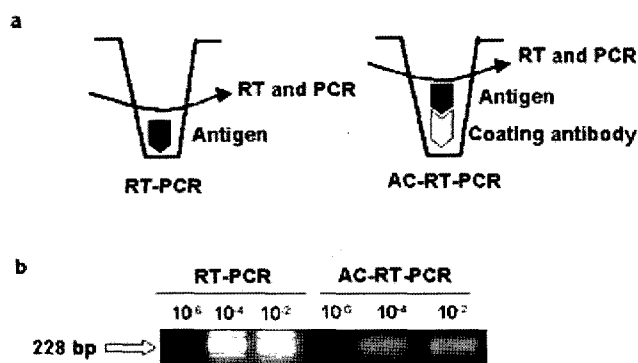
Fig. 1. Design of AC-RT-PCR (a) and comparison of sensitivity of AC-RT-PCR and standard RT-PCR (b).
Control HTN virus as antigen was ROK 84-105 (10^5 pfu/ml), which was serially diluted 10-fold. The coating antibody for AC-RT-PCR was used with patient A serum, of which IFA titer was adjusted at 1:512. The arrow indicates the 228-bp PCR band.

described previously [2], which was the IgG2a isotype with IFA titer of 1:8,192. The sera of two vaccinees, who were vaccinated twice (at an interval of one month) with Hantavax®, were prepared at the Korea National Institute of Health, and these sera were also adjusted to an IFA titer of 1:512 for AC-RT-PCR.

AC-RT-PCR was performed as described previously [15] with minor modifications. Microcentrifuge PCR tubes (Perkin Elmer) were coated with 100 μl each of HFRS patient serum (adjusted as IFA titer 1:512), monoclonal antibody HE4 (adjusted as IFA titer 1:512), and vaccinee sera (adjusted as IFA titer 1:512) at 37°C for 4 h. As an antigen, Hantavax® (80 μl) or ROK 84-105 virus (10^4 pfu/tube) used as a positive control (80 μl) was added to an antibody-coated tube and incubated overnight at 4°C. Each tube was heated at 95°C for 5 min, and the reverse transcriptase reaction (Gibco-BRL, Rockville, U.S.A.) was performed with the HS 398S primer (5'-GCATCATCGT-CTATCTTACATC-3') in the same tube. The first PCR reaction was performed with the HS 398S primer and the HS 1395R primer (5'-GTGCAAATATGATTGATAATG-3'); then nested PCR was performed with the HS 861S primer (5'-TATGGAGACAAAGGAGTCAAAGGC-3') and the HS 1089R primer (5'-AGATGTTCCAACTGTCTTA-GATGCC-3'). The final 228-bp DNA amplicon was detected by 1% agarose gel electrophoresis, followed by ethidium bromide staining.

After the World Health Organization (WHO) recommended the development of an effective killed vaccine against HFRS in 1984, Lee *et al.* [6] developed a killed HTNV vaccine, which is prepared from the HTN strain ROK 84-105, grown in suckling mouse brain and inactivated with formalin. This killed vaccine has commercially been produced since 1990 under the name Hantavax® in Republic of Korea [3]. However, the efficacy of Hantavax® is contentious in terms of the extent and duration of the

humoral immune response, although the vaccine induces a high seroconversion rate [3, 6, 14].

Although formalin is widely used for the inactivation of several killed vaccines, including Hantavax©, the inactivation processing may affect the integrity of the vaccine proteins. The integrity of the neutralizing epitope is especially important for the efficacy of the vaccine [12]. Therefore, it is useful to determine whether the integrity of the viral proteins in Hantavax® is maintained. Recently, AC-RT-PCR has been developed for the diagnosis and typing of viruses, and to determine the integrity of their capsid proteins and genomes [4, 12, 15]. Therefore, AC-RT-PCR may be ideal for use in the above studies.

Initially, the conditions necessary to efficiently coat microcentrifuge PCR tubes with antibody and capture virus were investigated using live ROK 84-105 virus as a positive control and the patient sera raised against it. ROK 84-105 virus (10^5 pfu/ml) was serially diluted 10-fold and used for AC-RT-PCR and standard RT-PCR with the same primer set. The PCR product titers were similar in both experiments, even though the band intensity of RT-PCR was higher than that of AC-RT-PCR, indicating that the sensitivity of AC-RT-PCR is similar to that of standard RT-PCR [8] (Fig. 1b).

We used the sera of three HFRS patients and two vaccinees for AC-RT-PCR. These sera could protect against HTNV infections of Vero-E6 cells in plaque reduction neutralizing tests, indicating that these sera contain specific immunoglobulins directed against the viral neutralizing epitopes. Neutralizing antibody titers (50% reduction) of Patient A, B, and C were 1:40, 1:40, and 1:10, respectively. Neutralizing antibody titers (50% reduction) of vaccinee A and B were 1:20 and 1:10, respectively. The binding of the patient sera to the HTNV vaccine and of the vaccinee sera to the live HTNV suggests that the vaccine has the correct epitope with which to induce the specific antibody. The IFA titers of these sera were adjusted to coat microcentrifuge PCR tubes at 1:512. As shown in Fig. 2, the PCR tubes coated with each serum produced the correct band size (228 bp), representing the HTNV genome. Subsequently, we sequenced some PCR products to confirm the HTNV genome (data not shown). These data indicate that Hantavax® contains the epitope in the correct conformation to induce specific antibodies for successful seroconversion after immunization. It is noteworthy that the intensity of the bands produced in the PCR tubes incubated with HTNV vaccine and those incubated with live virus was similar, indicating that the integrity of the surface proteins of Hantavax® and the live virus were similar (Fig. 2). Therefore, the formalin-inactivation procedure did not affect the conformation of the epitope or the integrity of the HTNV genome. These results are consistent with data from foot-and-mouse disease virus inactivated with formalin [15].
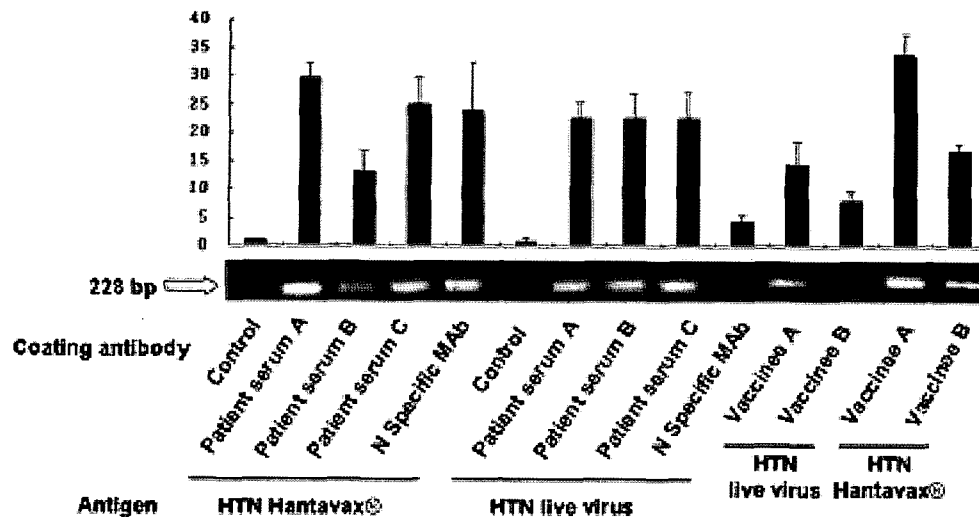
Fig. 2. Agarose gel electrophoresis of amplified 228 bp of the *Hantavirus* genome by AC-RT-PCR.

Results (means±standard errors [SE]; *n*=3) were quantified by densitometric analysis using ImageJ 1.29X, and normalized to the control (None+Hantavax®) arbitrarily set to 1.00. Data are from one of three independent experiments. Control represents normal serum without HTNV-specific antibody. The arrow indicates the 228-bp PCR band.

Interestingly, PCR tubes coated with monoclonal antibody directed against N protein (HE4) and incubated with Hantavax® produced PCR bands of higher intensity than tubes incubated with live virus (Fig. 2, lanes 5 and 10). The N protein is usually inside the virion and is immunodominant [9]. Therefore, these results suggest that N protein may be deposited on the outside of Hantavax® during the inactivation process, and that the formalin-inactivation process may affect the topology of the viral capsid structure. This may explain why Hantavax® usually induces a strong humoral immune response against N protein in the vaccinee.

In summary, although the topology of Hantavax® may be affected by formalin inactivation, this vaccine has the protective conformational epitope to induce neutralizing antibodies against *Hantavirus* and to guarantee a high seroconversion rate following immunization.

Acknowledgments

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