A single immunization with a recombinant canine adenovirus type 2 expressing the seoul virus Gn glycoprotein confers protective immunity against seoul virus in mice

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\section*{A B S T R A C T}

Seoul virus (SEOV), a member of hantavirus genus, is one of the causative agents of hemorrhagic fever with renal syndrome (HFRS) and afflicts tens of thousands of people annually. In this paper, we evaluate the immune response induced by a replication-competent recombinant canine adenovirus type 2 expressing the Gn protein of SEOV (rCAV-2-Gn) in BALB/c mice. Sera from immunized mice contained neutralizing antibodies that could specifically recognize SEOV and neutralize its infectivity in vitro. Moreover, the recombinant virus induced complete protection against a lethal challenge with the highly virulent SEOV strain CC-2. Protective level neutralizing antibodies were maintained for at least 20 weeks. The efficacy of the recombinant was similar to that induced by a currently available inactivated HFRS vaccine. This recombinant virus is therefore a potential alternative to the inactivated vaccine.

1. Introduction

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are rodent-borne viral zoonoses caused by viruses in the Hantavirus genus of the Bunyaviridae family. Currently, four genotypes are associated with HFRS, Hantaan (HTNV), Dobrava (DOBV), Seoul (SEOV) and Puumala virus (PUUV) with HTNV and SEOV responsible for the majority of the Chinese HFRS cases. The total number of HFRS patients globally is approximately 60,000–150,000 annually. More than 90% of these cases occur in Asian countries, including China, Russia, and Korea [1]. The incidence and mortality associated with HFRS clearly indicates the need for developing more effective vaccines.

Hantavirus virions are enveloped particles that contain a tripartite genome consisting of three negative-sense RNA segments [2]. The large (L) segment encodes the RNA-dependent RNA polymerase, the small (S) segment encodes the nucleocapsid protein (N) and the medium (M) segment encodes a polypeptide that is cotranslationally cleaved to yield two membrane-associated glycoproteins, Gn and Gc. Gn and Gc form oligomers that comprise the surface morphologic units of the virion and are the targets of neutralizing antibodies [3–5].

Inactivated HTNV, SEOV, or PUUV vaccines prepared from the viruses grown in suckling mouse brain or tissue culture cells have been developed in Korea and China [6,7]. Due to the biohazards associated with vaccine production and the poor efficacy of the current vaccines attempts have been made to develop efficacious recombinant protein-based vaccines. However, immunizations with recombinant Gn and Gc proteins expressed by baculovirus or vaccinia virus induce very low or negligible amounts of neutralizing antibody [8–10]. Therefore, there remains an important opportunity to develop more efficacious and safe vaccines.
Avirulent canine adenovirus type-2 (CAV-2) is an effective tool for gene delivery and expression [11–13]. The biological features of adenoviruses show that they are able to infect a broad range of cell types, but their genomes do not become integrated into the host genome, therefore adenoviruses are considered to be safe vaccine vectors for humans and other animals [14]. Research on the structure, replication and transcription of adenoviruses has been extensive. When foreign genes are inserted into the non-essential E3 region of CAV-2, replication of the recombinant adenovirus is not impacted, resulting in high virus titers and high-level gene expression [12,13]. We previously constructed a recombinant canine adenovirus expressing the Seoul virus Gn glycoprotein (rCAV-2-Gn). Here, we report the efficacy of this prototype vaccine in mice.

2. Materials and methods

2.1. Cells and viruses

The Vero E6 cells (Africa green monkey kidney cell lines, Vero C1008; Americal Type Culture Collection CRL 1586) purchased from Chinese Institute of Veterinary Drug Control, Beijing, China, and baby hamster kidney (BHK-21) and Madin–Darby canine kidney (MDCK) cells, were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) during growth phase, Eagle’s medium (DMEM, Invitrogen, USA) supplemented with 2% FBS during stationary phase.

2.2. Vaccines

rCAV-2-Gn was previously maintained in MDCK cells, and then passaged in BHK-21 cells inoculated with 100 multiplicity of infection (MOI) for 20 generations of adaptation before it was used in BALB/c mice. Cytotoxic effect (CPE) was observed, and after the cells were collected, titration of plaque forming unit (PFU) of the recombinant virus was assayed according to the method described elsewhere [16].

2.3. Vaccination of mice and bleeding

Six to eight weeks old SPF BALB/c female mice (Changchun Animal Breeding Center for Medicine, Changchun, China) were used in the immunization and challenge experiments and maintained in pathogen free conditions. The mice were randomly assigned to four experimental groups (20 mice per group). Group I was intramuscularly inoculated once with 0.1 ml of the recombinant virus rCAV-2-Gn (10^6.0 PFU/ml); group II received 0.1 ml of CAV-2 (10^6.2 PFU/ml) intramuscularly as negative control; group III were intramuscularly inoculated with one dose of HFRS Bivalent Purified Vaccine as a positive control; group IV were injected 0.1 ml with PBS (pH 7.4) as blank control. Blood was collected from the tail vein of each mouse 1 day prior to each immunization and at intervals of 2 weeks after the inoculation. Sera were separated for detection of specific antibody against SEOV. All sera were separated and stored at −20 °C.

2.4. Clinical observation and collection of samples for virus isolation

After vaccination, all mice were monitored for clinical signs daily for 3 weeks. Urine and fecal samples of mice inoculated with rCAV-2-Gn and CAV-2 were collected at days 2, 4, 6, 10, 14 and 21 after vaccination. Presence or absence of canine adenovirus was determined in MDCK cells according to the protocol described elsewhere [18]. If no viral CPE was observed, the samples were considered to be virus free.

2.5. ELISA

Serum samples were taken at intervals of 2 weeks until 22 weeks. The level of serum SEOV Gn-specific IgG was determined by ELISA based on the purified Gn expressed from *Escherichia coli* (E. coli). Microtitation plates (Nunc a/s, Rosakilde, Denmark) were coated overnight at 4 °C with optimized concentration of E. coli-expressed SEOV Gn (300 ng/well) in PBS. The plates were blocked with 5% dried skim milk (DSM) for 1 h and incubated with mouse sera for 1 h at 37 °C. After washing with PBS-T, HRP-conjugated goat anti-mouse IgG (1:3000; Sigma, USA) was added to each well and plates were incubated at 37 °C for 1 h. The plates were then washed and *A. albino* was determined after15–25 min development with TMB turbo reagent (Pierce).

2.6. Canine adenovirus hemagglutination inhibition (HI) antibody assay

CAV-2 HI antibody titers were determined by a micromethod [19] with a slight modification. Serial twofold dilutions of sera were mixed with 25 μl CAV-2, titer 4 HA units, with PBS (0.01 M, pH 7.2) as control, and incubated at 37 °C for 30 min. Then 25 μl 1% human group O red blood cells suspended in PBS (pH 7.2) was added to each well and incubated at 37 °C for 2 h. Titers were determined as the highest dilution showing at least partial agglutination.

2.7. Lymphoproliferative responses

Lymphoproliferative assay was performed using the peripheral blood mononuclear cells (PBMCs) of immunized mice. Mice PBMCs were isolated as described previously at week 20 post-initial vaccination [20,21]. The PBMCs were plated in 96-well flat-bottom plates at 100 μl/well (2 × 10^5 cells/well) in triplicate in RPMI-1640. Subsequently, these cultures were then stimulated with 10 μg/ml bacterial purified Gn, 50 μl Concanavalin A (5 μg/ml; Sigma), or left unstimulated, for 48 h at 37 °C in 5% CO₂. The proliferative activity was measured using a methyl thiazolyl tetrazolium (MTT, Sigma) dye assay, according to the method described by Bounous et al. [22]. The stimulation index (SI) was calculated as the ratio of the average OD₅₇₀ value of wells containing antigen-stimulated cells to the average OD₅₇₀ value of wells containing only cells with medium.

2.8. Focus reduction neutralization test

The focus reduction neutralization test (FRNT) using SEOV strain CC-2 and Vero E6 cells was performed to evaluate the level of neutralization antibody (NA) as previously described [23]. The FRNT titer was expressed as the reciprocal of the highest dilution that gave a reduction of greater than 80% in the number of infected cell foci.

2.9. SEOV challenge

At the end of the trial, all mice were injected intramuscularly with the indicated SEOV strain CC-2 diluted in 0.2 ml PBS. The chal-
length dose for each virus was 2000 PFU. This dose is ~1000 50% infective doses (ID_{50}) for SEOV. At 14 days after challenge, the mice were sacrificed by carbon dioxide asphyxiation, as approved by the China Small Animal Protection Association. Pre- and post-challenge sera were evaluated for the presence of N-specific antibodies by ELISA and for the presence of neutralizing antibodies by FRNT. Detecting post-challenge N-specific antibody indicated that the mice were infected with the challenge virus.

2.10. Data analysis

All data were processed and analyzed by SPSS13.0 Data Editor (SPSS Inc., Chicago, IL, USA). The results in comparisons between groups were considered different if $P < 0.05$.

3. Results

3.1. Clinical observation and CAV-2 isolation

No vaccine-related adverse effects or other abnormal behavior was observed in any vaccinated group. Canine adenovirus was not isolated from MDCK cells incubated with samples of urine or feces from mice vaccinated with rCAV-2-Gn or CAV-2, at days 2, 4, 6, 10, 14, 21 and 42 after vaccination.

3.2. Strong antibody responses induced by rCAV-2-Gn in mice

Antibodies against Gn in the sera of rCAV-2-Gn vaccinated mice were detectable 3 weeks after the vaccination. The Gn antibody level produced by the rCAV-2-Gn was lower than that induced by the inactivated HFRS vaccine, with the appearance of Gn antibody stimulated by rCAV-2-Gn about 1 week later than that of the inactivated vaccine (Fig. 1). There was a significant difference in lgG antibody responses between the groups immunized with the inactivated vaccines and the rCAV-2-Gn ($P > 0.05$). The titer was higher in rCAV-2-Gn-vaccinated mice, compared with two groups vaccinated with CAV-2 and PBS ($P < 0.05$). After challenge with SEOV strain CC-2, the antibody responses increased significantly in all groups including CAV-2 and PBS-vaccinated mice. In contrast, mice vaccinated with CAV-2 and PBS did not generate antibody responses against Gn of SEOV.

3.3. CAV-2 antibody

The HI antibodies against CAV-2 increased from 3 weeks after immunization in all mice vaccinated with rCAV-2-Gn and CAV-2. The antibody peaked at 8–10 weeks and persisted for approximately 22 weeks (Table 1). The highest HI titer of the mice sera were $1:2^b$ in the mice immunized with rCAV-2-Gn and CAV-2 at 8–10 weeks. In mice vaccinated with inactivated HFRS vaccine and PBS, specific antibodies to CAV-2 were not detected.

3.4. Lymphoproliferative responses

We also analyzed the lymphoproliferative responses of immunized mice. As shown in Table 2, the three groups produced vigorous lymphocyte responses, compared to the blank control ($P < 0.05$), but there was no significant difference between mice immunized with inactivated HFRS vaccine, rCAV-2-Gn or CAV-2 ($P > 0.05$).

3.5. SEOV-specific neutralizing antibodies in vaccinated mice

The neutralizing activity of the Gn-specific antibody was measured using the 80% FRNT. As summarized in Table 3, all mice immunized with inactivated HFRS vaccine and rCAV-2-Gn developed FRNT antibody with mean titers ranging from 1:40 to 1:640 and 1:40 to 1:320 within 2 weeks after immunization, respectively. All control mice inoculated with CAV-2 or PBS alone were negative for FRNT antibody.

3.6. Evaluation of protective immunity induced by rCAV-2-Gn for preventing SEOV challenge in mice

To assess the protective immunity, mice were challenged with SEOV at 20 weeks after immunization. Although exceptional fatal infection models in adult mice have been reported [24, 25], hantaviruses are generally non-pathogenic to mature rodents. In this study, we used a general SEOV strain CC-2. Therefore, it is difficult to evaluate protective immunity. To differentiate the antibody response induced by immunization from that induced by SEOV infection, seroconversion against NP accompanied with establishment of infection was used as an index for protection (Table 4).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks post-vaccination</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Inactivated HFRS Vaccine</td>
<td>1:2</td>
</tr>
<tr>
<td>rCAV-2-Gn</td>
<td>1:2</td>
</tr>
<tr>
<td>CAV-2</td>
<td>1:2</td>
</tr>
<tr>
<td>PBS</td>
<td>1:2</td>
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### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean stimulation index ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated HFRS vaccine</td>
<td>3.04 ± 0.42</td>
</tr>
<tr>
<td>rCAV-2-Gn</td>
<td>2.82 ± 0.34</td>
</tr>
<tr>
<td>CAV-2</td>
<td>2.93 ± 0.31</td>
</tr>
<tr>
<td>Blank control (PBS)</td>
<td>1.07 ± 0.12</td>
</tr>
</tbody>
</table>

Lymphoproliferative assay was performed on peripheral blood lymphocytes collected at week 20 post-initial vaccination, and peripheral blood lymphocytes before vaccination were used as negative control. Data represent the mean ± S.D. $P < 0.05$; $P > 0.05$; $P < 0.05$; $P > 0.05$; $P < 0.05$; $P > 0.05$; $P < 0.05$; $P > 0.05$; $P < 0.05$; $P > 0.05$.
All mice immunized with inactivated HFRS vaccine and rCAV-2-Gn remained negative for anti-N antibody in ELISA, indicating that the mice were protected from SEOV infection. In contrast, seroconversion was detected in CAV-2-immunized mice and in PBS control mice. These results indicate that protective immunity was not induced in these mice, confirming that immunization with inactivated HFRS vaccine and rCAV-2-Gn conferred protective immunity.

4. Discussion

In this report, we demonstrated that CAV-2 could be used as a live vector for vaccine development against HFRS caused by SEOV in mice. Although an inactivated hantavirus vaccine has been developed and a protective efficacy comparable to those of other virus vaccines has been reported, low efficacy for the induction of NA remains an important aspect to be overcome [7,26]. As observed in this study, the rCAV-2-Gn was able to induce NA with titers comparable to those reported for inactivated vaccines in humans as well as for recombinant proteins in the mouse model [7,26]. Meanwhile, the presence of a NA response in mice vaccinated with rCAV-2-Gn, is consistent with published data that monoclonal antibodies to G1 and G2, but not N, have neutralizing activity [27–30] and that vaccination with vaccinia recombinants expressing G1 and/or G2, but not N, elicits a neutralizing response [31–34]. Furthermore, the rCAV-2-Gn conferred protective immunity for hantavirus challenge in the mouse model.

Moreover, safety of the vaccine was evaluated primarily in this experimental immunization. The recombinant virus could not be isolated from the urine and feces of vaccinated mice from the inoculating day to 42 days. This means that the recombinant virus could not be shed through urine and feces to the environment during this period. More information regarding where the virus replicates and how it persists in the body is required. The histological changes and any immunosuppressive effects caused by the recombinant vaccine also need to be checked.

We investigated the cell-mediated immune response after rCAV-2-Gn vaccination by lymphoproliferative assay. All 3 test groups (inactivated HFRS vaccine, rCAV-2-Gn, and CAV-2) produced similar high responses, compared with the blank control (Table 2). But cellular immunity does not appear to be an important component of the protection afforded by the recombinant virus. This has also been observed with other experimental parvovirus vaccines (peptide vaccines and baculovirus systems) [35,36]. It appears, therefore, that SEOV Gn stimulates predominantly an antibody response, and appearance of neutralizing antibodies provides the most valuable evidence of the immunogenicity and their measurement is widely used to assess the ability of a vaccine to protect against virus challenge [37]. To determine the protective efficacy of rCAV-2-Gn, all vaccinated mice were challenged with SEOV strain CC-2, after 14 days, using serological assays to detect evidence of infection. Specifically, if a challenged mouse developed antibodies to SEOV N protein (which is not a component of the vaccine), then that mouse was considered to be infected. On the other hand, if a challenged mouse failed to develop a N-specific antibody response, then that mouse was considered non-infected (i.e., protected against infection). As shown in Table 4, the mice vaccinated with commercial inactivated vaccine and rCAV-2-Gn did not produce anti-N-specific antibodies (which could provide the protection against SEOV challenge), and the mice of the other two groups vaccinated with CAV-2 and PBS produced anti-N-specific antibodies (which could not provide the protection against SEOV challenge). A 4-fold increase in the NA response after challenge also served as a marker for evidence of infection (Table 3). This is the first successful application of CAV-2 as a vector of hantavirus for the induction of protective immunity.

Table 3

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Titer for 80% FRNT</th>
<th>Weeks post-vaccination</th>
<th>Weeks post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Inactivated HFRS vaccine</td>
<td>&lt;20</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>rCAV-2-Gn</td>
<td>&lt;20</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>CAV-2</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

| A FRNT values are the reciprocal serum dilution that reduces plaque number by 80%.<sup>a</sup>b c d e f g h i j k l m n o p q r s t u v w x y z |

In order to enhance the level of NA of rCAV-2-Gn in mice, another group of mice was injected with rCAV-2-Gn twice with the same dose at 30-day intervals, and challenged intramuscularly with 1000 ID<sub>50</sub> of the virulent SEOV strain CC-2 at day 30 after the last vaccination, but only 15% protection was obtained in the vaccinated mice (data not shown). This may be caused by the antigenicity of CAV-2, and repetitive administration can decrease the immune response [38]. In addition, we are also testing the effect of different administration routes on immunization (including oral, intranasal and intraperitoneal inoculation), and hope to exploit a novel vaccine, especially develop oral vaccine to the stray mice to control or decrease the spread of SOEV.

In conclusion, we have shown that rCAV-2-Gn provides long-term, safe and effective immunization against SEOV in mice. Our work also suggests that CAV-2 might be used as vector to develop new types of vaccines against infectious diseases affecting mice.

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References