Generation of E3-deleted canine adenovirus type 2 expressing the Gc glycoprotein of Seoul virus by gene insertion or deletion of related terminal region sequences

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Seoul virus (SEOV) is one of the four hantaviruses known to cause haemorrhagic fever with renal syndrome. The medium genome segment encodes the Gn/Gc glycoproteins of SEOV, which form the major structural part of the virus envelope. Gc and/or Gn are the candidate antigens of hantavirus for induction of a highly immunogenic response for hantavirus vaccine. In this study, the immune response induced by a replication-competent recombinant canine adenovirus type 2 expressing the Gc protein of SEOV was evaluated 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 an intensive infectious challenge with ~1000 50% infective doses for SEOV strain CC-2. Protective-level neutralizing antibodies were maintained for at least 20 weeks. This recombinant virus is therefore a potential alternative to the inactivated vaccine.

INTRODUCTION

Haemorrhagic fever with renal syndrome (HFRS) is an acute and often fatal disease caused by viruses belonging to the genus Hantavirus. There are at least four hantaviruses that can cause HFRS: Hantaan virus, Seoul virus (SEOV), Dobrava virus and Puumala virus (Kariwa et al., 2007). Because distinct hantaviruses are usually carried by only one principal rodent host species, their distribution is generally limited to the range of that host (Schmaljohn & Hjelle, 1997). SEOV is more widely disseminated than any other recognized hantavirus because its host, the common urban rat (Rattus norvegicus), is found throughout the world. The total number of HFRS patients is about 60 000–150 000 annually worldwide, and more than 90% of these cases occur in Asian countries, including China, Russia and Korea (Kariwa et al., 2007).

The genus Hantavirus is one of the five genera in the family Bunyaviridae. Hantaviruses are enveloped viruses with a negative-sense RNA genome organized in three segments: S (small), M (medium) and L (large) (Elliott, 1990; Jonsson & Schmaljohn, 2001; Schmaljohn & Le Duc, 1998). The S segment encodes the nucleocapsid (N) protein; the M segment encodes two envelope glycoproteins, Gn (70 kDa) and Gc (55 kDa); and the L segment encodes the RNA-dependent RNA polymerase protein. The glycoproteins are the major structural protein and the major antigen of hantaviruses, and induce virus-neutralizing antibodies known to provide protection against hantavirus challenge and to prevent infection in mice (Hooper et al., 1999).

With developments in biotechnology and molecular virology, the use of viruses as vectors for the delivery of vaccine antigens has potential for the development of improved vaccines. Recombinant canine adenovirus type 2 (rCAV-2) is one of the preferred virus vectors used in...
vaccine production (Ferreira et al., 2005). 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 use in humans and other animals (Randrianarison-Jewtoukoff & Perricaudet, 1995). Studies on the structure, replication and transcription of adenoviruses have 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 titres and high-level gene expression (Hu et al., 2006, 2007).

In this trial, we selected Gc (the smaller glycoprotein) as the gene of interest, taking into account the capacity of CAV-2. The purpose of the study was to construct a rCAV-2 that could express the Gc protein of SEOV, and to evaluate the efficacy of this vaccine formulation in mice.

RESULTS

Recovery and identification of rCAV-2-Gc

To generate rCAV-2 containing the Gc gene of SEOV (rCAV-2-Gc), a Gc expression cassette, driven by the cytomegalovirus immediate-early promoter with the simian virus 40 (SV40) polyadenylation signals at the end of the gene, was constructed. The expression cassette was subcloned into the E3 region of the pPolyII-CAV-2 plasmid (Zhang et al., 2002), generating the recombinant plasmid pPolyII-CAV-ΔE3-Gc (Fig. 1). Seven days after transfection of the recombinant genome into Madin–Darby canine kidney (MDCK) cells, a typical cytopathic effect (CPE) of grape-cluster-like cells was observed under a microscope (data not shown). Adenovirus-like particles were observed under the electron microscope after negative staining of the cell culture with potassium phosphotungstate (data not shown). The growth characteristics of the recombinant virus were similar to those of the CAV-2 vaccine strain YCA18 (data not shown). Identification of the recombinant virus genome by restriction endonuclease digestion and PCR amplification confirmed that the SEOV Gc cDNA and its expression cassette were present in the recombinant virus (data not shown).

Identification of the expressed product

An indirect immunofluorescence assay (IFA) was used to detect the Gc protein expressed by rCAV-2-Gc in MDCK cells. A large amount of green fluorescence was seen in the MDCK cells infected by rCAV-2-Gc (Fig. 2a, left panel), but none was seen in cells infected by CAV-2 (Fig. 2a, right panel), indicating that SEOV Gc protein was expressed by the recombinant adenovirus in the inoculated MDCK cells. The whole-cell lysates harvested from CAV-2- and rCAV-2-Gc-infected cells were subjected to SDS-PAGE followed by Western blotting. Mouse anti-SEOV-positive serum was used as the primary antibody and horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (Sigma) was used as the secondary antibody. As shown in Fig. 2(b), the results indicated that rCAV-2-Gc-infected cells expressed the Gc protein (55 kDa) of SEOV. The results also indicated that the expressed foreign gene retained its immunological reactivity.

Clinical observations and CAV-2 isolation

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

Antibody responses

Antibodies against Gc in the serum of rCAV-2-Gc-vaccinated mice were detectable 3 weeks after vaccination. The Gc antibody level produced by rCAV-2-Gc was lower than that induced by inactivated HFRS vaccine, with the appearance of Gc antibody stimulated by rCAV-2-Gc approximately 1 week later than that of the inactivated vaccine (Fig. 3). There was a significant difference in IgG antibody response between the groups immunized with the inactivated vaccine and rCAV-2-Gc (P<0.05). The titre was higher in rCAV-2-Gc-vaccinated mice compared with the 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 SEOV Gc.

CAV-2 antibody

Haemagglutination-inhibiting (HI) antibodies against CAV-2 increased from 3 weeks after immunization in all mice vaccinated with rCAV-2-Gc and CAV-2. The antibody levels peaked at 8–10 weeks and persisted for approximately 20 weeks (Table 1). The highest HI titre of the mice sera was 1 : 28 in the mice immunized with rCAV-2-Gc and CAV-2 at 8–10 weeks. In mice vaccinated with inactivated HFRS vaccine and PBS, specific antibodies to CAV-2 were not detected.

Lymphoproliferative responses

We also analysed the lymphoproliferative responses of immunized mice. As shown in Table 2, the groups immunized with inactivated HFRS vaccine and rCAV-2-Gc produced vigorous lymphocyte responses, and splenocyte proliferation was approximately threefold higher than in the control groups immunized with CAV-2 and PBS (P<0.05), but there was no significant difference between mice immunized with inactivated HFRS vaccine and rCAV-2-Gc (P>0.05). Splenocytes from all experimental
and control groups proliferated to comparable levels in response to the mitogen concanavalin A (data not shown).

**SEOV-specific neutralizing antibodies in vaccinated mice**

The neutralizing activity of Gc-specific antibody was measured using an 80% focus-reduction neutralization test (FRNT). As summarized in Table 3, all mice immunized with inactivated HFRS vaccine and rCAV-2-Gc developed FRNT antibodies, with mean titres ranging from 1:40 to 1:640 within 2 weeks of immunization. Control mice inoculated with CAV-2 or PBS alone were negative for FRNT antibodies.

**Evaluation of protective immunity induced by rCAV-2-Gc for prevention of SEOV challenge in mice**

To assess protective immunity, mice were challenged with SEOV at 20 weeks after immunization. Although exceptional fatal infection models in adult mice have been reported (Asada et al., 1989; Wichmann et al., 2002), hantaviruses are generally non-pathogenic to mature rodents. Therefore, it is difficult to evaluate protective immunity. In this study, we used the general SEOV strain CC-2. To differentiate the antibody response induced by immunization from that induced by SEOV infection, seroconversion against N protein accompanied by the establishment of infection was used as an index for
protection (Table 4). All mice immunized with inactivated HFRS vaccine and rCAV-2-Gc 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 indicated that protective immunity was not induced in these mice, confirming that immunization with inactivated HFRS vaccine and rCAV-2-Gc conferred protective immunity.

**DISCUSSION**

In this trial, we tested the efficacy of rCAV-2-Gc by means of intramuscular vaccination. Our results showed that intramuscular administration in mice conferred a protective immune response.

It remains unclear whether or not Gn alone, Gc alone or fragments of the glycoproteins can elicit neutralizing antibodies and protect against infection (Hooper et al., 2001). Vaccination with recombinant baculovirus-infected cell lysates containing Gn or Gc alone, and recombinant vaccinia viruses expressing Gn or Gc alone, failed to elicit neutralizing antibody and exhibited incomplete protection in a hamster infection model (Schmaljohn et al., 1990). These data suggested that a full-length M gene capable of expressing Gn and Gc may be required for protective immunity. In contrast, Bharadwaj et al. (1999) reported low levels of neutralizing antibodies (1:10–1:20) after intramuscular needle injection of mice with DNA vaccine plasmids containing short (~166 aa) sections of the M gene of Sin Nombre virus, a virus associated with hantavirus pulmonary syndrome (Bharadwaj et al., 1999). This finding suggests that eliciting a neutralizing antibody response not only does not require a full-length M gene but also occurs when only fragments of Gn or Gc are expressed. Here, we demonstrated that a single dose of rCAV-2-Gc administered intramuscularly is sufficient to completely protect mice from SEOV.

Replication-competent recombinant adenoviruses of several different species have been developed by homologous recombination, with insertion of foreign genes into the non-essential E3 region (Morrison et al., 2002; Zakhartchouk et al., 1998). The present study, however, used a different approach. The recombinant CAV-2 genome carrying the Gc expression cassette was first constructed, and the recombinant adenovirus was then generated by transfecting MDCK cells with the recombinant genome. This method eliminated the need for plaque screening and purification of recombinant virus, as the appearance of CPE was the evidence that recombinant virus had been generated, and this was further verified by identification of the SEOV gene in the recombinant virus. This method substantially simplifies the procedure for the development of recombinant virus.

The 50% tissue culture infective dose (TCID_{50}) of rCAV-2-Gc was assayed and calculated to be 10^{7.8} ml^{-1}. This virus...
haemagglutinated human red blood cells and had a haemagglutination titre of 1 : 29. This titre is similar to that of the CAV-2 SY vaccine virus, which was isolated from Shenyang City in Liaoning province and attenuated by serial passages in MDCK cell lines (Fan et al., 1982). This indicated that the E3 region in CAV-2 is dispensable for growth in tissue culture, as has been demonstrated for human adenovirus (Shenk, 1996), and that the E3 locus is a suitable site for the insertion of transgenes. The fact that growth of the recombinant virus is not impaired by the recombination event is also an important feature. The haemagglutination of 4 U of the recombinant virus could be completely inhibited by CAV-2-positive serum (at a dilution of 1 : 40).

The packaging limit of the vector was a key factor in producing recombinant virus in our experiment. Studies on human adenovirus have indicated that a maximum of 105 % wild-type genome size can be packaged, above which DNA is deleted and rearranged to reduce the genome size below this limit (Bett et al., 1993). This may not be true for animal adenoviruses, as an ovine adenovirus has been demonstrated to package up to 114 % of its genome size (Xu et al., 1997). The rCAV-2-Gc had a genome size of 107.4 % of the wild-type, suggesting that the maximum packaging size of CAV-2 falls between these limits. A series of constructs containing different lengths of glycoprotein cDNA and different promoters is under development in our laboratory.

The genome of rCAV-2-Gc was analysed for genetic stability by enzymic digestion and was found to retain the correct genome structure at serial passage 32 (data not shown). It was confirmed by RT-PCR that the Gc-coding sequence was successfully cloned into the CAV-2 vector. Expression of Gc in MDCK cells infected with rCAV-2-Gc was confirmed by IFA assay and Western blotting. Moreover, the results of IFA and Western blot analysis of Gc expressed by recombinant virus were the same as those of passage 32 virus (data not shown), indicating that the recombinant virus was stable enough to maintain its genetic integrity and immunogenicity.

We investigated the cell-mediated immune response after rCAV-2-Gc vaccination by using a lymphoproliferative assay. Mice inoculated with rCAV-2-Gc and inactivated HFRS vaccine produced high-level responses similar to the control groups (Table 2). However, cellular immunity did 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) (Christensen et al., 1994; Langeveld et al., 1994). It appears, therefore, that SEOV Gc predominantly stimulates an antibody response, and that the appearance of neutralizing antibodies provides the most valuable evidence of the immunogenicity of the recombinant virus.

Following immunization, neutralizing antibody levels induced by rCAV-2-Gc were relatively low compared with those induced by the inactivated HFRS vaccine. However, as SEOV neutralizing antibody titres of 1 : 40 or higher are considered satisfactory for protection against SEOV, the titres induced by rCAV-2-Gc, which reached 1 : 40–1 : 320, surpassed the minimum required levels for SEOV by a considerable margin. Our results clearly showed that rCAV-2-Gc is safe and efficacious for mice, a conclusion further borne out by the challenge results. After challenge, the control group mice produced anti-N antibody, but the mice inoculated with rCAV-2-Gc and inactivated HFRS vaccine did not. Hence, they could be considered fully protected, with complete protection against SEOV challenge.

In contrast to the other genera in the family Bunyaviridae, hantaviruses are rodent-borne and infect humans via small-particle aerosols of contaminated excreta. Vaccinating stray mice/rats in urban and rural areas using conventional vaccines is always difficult and is not cost-effective for use in most countries including China. Development of an oral vaccine will facilitate HFRS control in these areas. Attenuated CAV-2 has proved to be an effective oral vaccine in dogs (Zhang et al., 2008), and

Table 1. CAV-2 HI antibody titres in mice immunized with inactivated HFRS vaccine, rCAV-2-Gc, CAV-2 or PBS

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks post-vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Inactivated HFRS vaccine</td>
<td>1 : 2</td>
</tr>
<tr>
<td>rCAV-2-Gc</td>
<td>1 : 2</td>
</tr>
<tr>
<td>CAV-2</td>
<td>1 : 2</td>
</tr>
<tr>
<td>PBS</td>
<td>1 : 2</td>
</tr>
</tbody>
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ab, P<0.05; ac, P<0.05; ad, P>0.05; bc, P>0.05; bd, P<0.05; cd, P<0.05.

Table 2. Proliferative responses of peripheral blood lymphocytes of all mice immunized with inactivated HFRS vaccine, rCAV-2-Gc, CAV-2 or PBS

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean stimulation index ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated HFRS vaccine</td>
<td>3.04 ± 0.42</td>
</tr>
<tr>
<td>rCAV-2-Gc</td>
<td>2.98 ± 0.26</td>
</tr>
<tr>
<td>CAV-2</td>
<td>1.04 ± 0.19</td>
</tr>
<tr>
<td>PBS</td>
<td>1.07 ± 0.12</td>
</tr>
</tbody>
</table>

ab, P>0.05; ac, P>0.05; ad, P<0.05; bc, P>0.00; bd, P<0.05; cd, P<0.05.
Table 3. Mean neutralizing antibody titre against SEOV of all mice immunized with inactivated HFRS vaccine, rCAV-2-Gc, CAV-2 or PBS

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Weeks post-vaccination</th>
<th>Weeks post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Inactivated HFRS vaccine a</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>rCAV-2-Gc b</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>CAV-2 c</td>
<td>20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>PBS d</td>
<td>20</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

a, P < 0.05; b, P < 0.05; c, P < 0.05; d, P < 0.05; e, P > 0.05.

Table 4. Antibody titres against SEOV N protein after challenge inoculation in all mice immunized with inactivated HFRS vaccine, rCAV-2-Gc, CAV-2 or PBS

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-N ELISA titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-challenge</td>
</tr>
<tr>
<td>Inactivated HFRS vaccine</td>
<td>&gt;1:100</td>
</tr>
<tr>
<td>rCAV-2-Gc</td>
<td>&lt;1:100</td>
</tr>
<tr>
<td>CAV-2</td>
<td>1:100</td>
</tr>
<tr>
<td>PBS</td>
<td>1:100</td>
</tr>
</tbody>
</table>

is stable at room temperature and atmospheric pressure for several months (Yamamoto, 1966). We have recently begun to extend the concepts of inoculation routes, immune doses and strategies (e.g. prime–boost vaccines, combined immunization with adjuvants) to improve the immune response to SEOV in our laboratory.

METHODS

Cells, virus and plasmid. MDCK cells (Chinese Institute of Veterinary Drug Control, Beijing, China) were grown and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco Life Technologies), 100 mg streptomycin ml⁻¹ and 100 IU penicillin ml⁻¹ at 37 °C in 5% CO₂.

Hantavirus was isolated from the lungs of an infected albino rat with clinical symptoms in a P3 laboratory (Veterinary Institute, Academy of Military Medical Sciences, Changchau, PR China). The isolated virus belonged to the SEOV serotype of hantavirus, which was genetically stable, as demonstrated by serial passages and nucleic acid sequence analysis (data not shown), and was used for further studies. CAV-2 strain YCA18 was isolated by Xia et al. (2000).

The plasmid pPolyII-CAV-2 containing the whole genome of CAV-2 was constructed by Zhang et al. (2002).

RT-PCR for amplification of SEOV Gc. Based on the sequence of the SEOV strain 80-39 segment M (GenBank accession no. NC_005237), a pair of PCR primers was designed as follows: P1 (5'-GCTAGC-GCAGAAAATCCCCCTTG-3', nt 1984–1999) and P2 (5'-GGTACC- TTACGACTTCTTGTGCTTTC-3', nt 3428–3447), which included Nhel and Kpn1 restriction enzyme sites (underlined), respectively, at their 5'-terminal. Viral RNA was extracted from SEOV-infected Vero E6 cells by using TRIzol reagent (Invitrogen) following the manufacturer’s protocol (Reid et al., 2001). The Gc PCR product was cloned into the pMD18-T vector (TaKaRa), giving pMD18-T-Gc, and was sequenced by the UN Corporation (Shanghai, China).

The construction of pPolyII-CAV-ΔE3-Gc. The flow chart of construction (Fig. 1) shows that the 4.8 kb Kpn1 fragment containing the E3 region from pPolyII-CAV-2 was first cloned into pVAX1 (Invitrogen), forming pVAX-E3. The Gc sequence was released with Nhel and Kpn1 from pMD18-T-Gc and cloned into pEGFP-C1 (Clontech Laboratories), forming pEGFP-Gc. The Mlu1/Ase1 fragment of pEGFP-Gc containing the Gc cDNA expression cassette was filled in and cloned into pVAX-E3 digested with Sfl and blunted with Klenow/dNTPs, forming pVAXE3-Gc. The 6.1 kb fragment of Nra1 and Sall double-digested pVAXE3-Gc, containing the Gc expression cassette flanked by residual E3 sequences, was cloned back into pPolyII-CAV-2 by replacing the fragment between the Nra1 and Sall enzyme sites, forming pPolyII-CAV-ΔE3-Gc.

Transfection of recombinant genome in MDCK cells and recovery of recombinant virus. Five micrograms of pPolyII-CAV-ΔE3-Gc was digested with Ascl and Pme1 to release the linear recombinant genome. After extraction with chloroform and precipitation with ethanol, the recombinant genome was used to transfect MDCK cells at 70–80% confluency with Lipofectamine 2000 (Invitrogen). The transfected MDCK cells were passaged routinely until a typical CAV-2 CPE was observed.

Titration of the recombinant virus. Serial tenfold dilutions of the recombinant virus were inoculated onto MDCK cells in 96-well culture plates, using four wells per dilution, and cultured at 37 °C in 5% CO₂ for 6–9 days. Titres (TCID₅₀) were determined as described by Reed & Münch (1938). Haemagglutination and HI of the recombinant virus were assayed by a method described previously (Schmidt et al., 1966). The anti-CAV-2-positive serum used in HI was prepared by intramuscular vaccination with the vaccine strain YCA18. The HI titre of this positive serum against 4 U of CAV-2 was 1:10¹¹.

Identification of Gc expression from rCAV-2-Gc. The Gc protein expressed by rCAV-2-Gc in MDCK cells was evaluated by a SEOV-specific indirect IFA. MDCK cells grown on 15 mm glass coverslips in culture plates, using four wells per dilution, and cultured at 37 °C in 5% CO₂ for 6–9 days. Titres (TCID₅₀) were determined as described by Reed & Münch (1938). Haemagglutination and HI of the recombinant virus were assayed by a method described previously (Schmidt et al., 1966). The anti-CAV-2-positive serum used in HI was prepared by intramuscular vaccination with the vaccine strain YCA18. The HI titre of this positive serum against 4 U of CAV-2 was 1:10¹¹.
nitrocellulose membrane (Pall Corporation) and probed with positive serum against SEOV and HRP-labelled goat anti-mouse IgG antibody (Sigma) using protocols described elsewhere (Li et al., 2006).

**Vaccination procedure.** Six- to eight-week-old specific-pathogen-free BALB/c female mice (Changchun Animal Breeding Center for Medicine, Changchun, PR 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 rCAV-2-Gc (10^8 p.f.u. ml^-1); group II received 0.1 ml CAV-2 (10^8 p.f.u. ml^-1) intramuscularly as a negative control; group III were inoculated intramuscularly with one dose of HFRS bivalent purified vaccine Youerjian (0.5 ml per dose; Guangdong HongMing Biological Science and Technology Co.) as a positive-control; and group IV were injected with 0.1 ml PBS as a negative control. Blood was collected from the tail vein of each mouse 1 day prior to each immunization and at intervals of 2 weeks after inoculation. Sera were separated for detection of specific antibody against SEOV. All sera were separated and stored at -20 °C.

**Clinical observation and collection of samples for virus isolation.** After vaccination, all mice were monitored for clinical signs daily for 3 weeks. Urine and facial samples of mice inoculated with rCAV-2-Gc and CAV-2 were collected on days 2, 4, 6, 10, 14 and 21 after vaccination. The presence or absence of virus was determined in MDCK cells according to a protocol described elsewhere (Tham et al., 1998). If no viral CPE was observed, the samples were considered to be virus free.

**Antibody assays.** Serum samples were taken at intervals of 2 weeks up to 20 weeks. The level of serum SEOV Gc-specific IgG was determined by ELISA based on purified Gc expressed from *Escherichia coli*. Microtitration plates (Nunc) were coated overnight at 4 °C with an optimized concentration of E. coli-expressed SEOV Gc (300 ng per well) in PBS. The plates were blocked with 5% skimmed milk in PBS for 1 h and incubated with mouse sera for 1 h at 37 °C. After washing with PBS/0.05% Tween 20, HRP-conjugated goat anti-mouse IgG (diluted 1: 3000; Sigma) was added to each well and the plates were incubated at 37 °C for 1 h. The plates were then washed and A_{492} was determined after 15–25 min development with TMB Turbo reagent (Pierce).

The *E. coli* expression and purification of Gc and N antigens were performed according to a protocol described previously (Geldmacher et al., 2004). Briefly, cells of *E. coli* strain DE3 were transformed with plasmid pET-32a-Gc or pET-32a-N encoding the respective proteins. Plasmid pET-32a-Gc was prepared by digestion of the Gc fragment of pMD18-T-Gc with *KpnI* and *XhoI*, followed by cloning in the expression vector pET-32a, resulting in pET-32a-Gc. The construction of pET-32a-N has been reported previously by us (Yuan et al., 2008). After sedimentation, the cells were lysed and soluble protein was extracted. The two proteins were precipitated and loaded onto a sucrose gradient. Fractions containing the Gc or N protein were identified and characterized by SDS-PAGE and Western blot analysis, concentrated and stored in glycerol at -20 °C until further use.

**CAV-2 HI antibody assay.** CAV-2 HI antibody titres were determined by a micromethod (Yuan et al., 2009) with a slight modification. Serial twofold dilutions of sera were mixed with 25 μl CAV-2 (4 HA units), with PBS as a control, and incubated at 37 °C for 30 min. Next, 25 μl 1% human group O red blood cells suspended in PBS was added to each well and incubated at 37 °C for 2 h. Titres were determined as the highest dilution showing at least partial agglutination.

**Lymphoproliferative responses.** A lymphoproliferative assay was performed using peripheral blood mononuclear cells (PBMCs) of immunized mice. Mice PBMCs were isolated, as described previously, at week 20 after initial vaccination (Hong et al., 2002; Xiao et al., 2004). The cells were plated in 96-well flat-bottomed plates at 2 x 10^5 cells per well (100 μl) in triplicate in RPMI 1640. The cultures were then stimulated with 10 μg bacterial purified Gc ml^-1, 50 μl concanavalin A (5 μg ml^-1; Sigma) or left unstimulated for 48 h at 37 °C in 5% CO2. Proliferative activity was measured using a methyl thiazolyl tetrazolium (Sigma) dye assay, according to the method described by Bounous et al. (1992). The stimulation index was calculated as the ratio of the mean OD_{570} value of wells containing antigen-stimulated cells to the mean OD_{570} value of wells containing only cells with medium.

**FRNT.** An FRNT using SEOV strain CC-2 and Vero E6 cells was performed to evaluate the level of neutralizing antibody as described previously (Araki et al., 2001). The FRNT titre was expressed as the reciprocal of the highest dilution that gave a reduction of greater than 80% in the number of infected-cell foci.

**SEOV challenge.** At the end of the trial, all mice were injected intramuscularly with SEOV strain CC-2 diluted in 0.2 ml PBS. The challenge dose for each virus was 2000 p.f.u. This dose is ~1000 50% infective doses for SEOV. At 14 days after challenge, the mice were sacrificed by CO2 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.

**Data analysis.** All data were processed and analysed using the SPSS version 13.0 Data Editor (SPSS Inc.). The results of comparisons between groups were considered different if P<0.05.

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