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Comparison of the Protective Efficacy of Naked DNA, DNA-based Sindbis Replicon, and Packaged Sindbis Replicon Vectors Expressing Hantavirus Structural Genes in Hamsters

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Seoul virus (SEOV) is a member of the *Hantavirus* genus (family Bunyaviridae) and an etiological agent of hemorrhagic fever with renal syndrome. The medium (M) and small (S) gene segments of SEOV encode the viral envelope glycoproteins and nucleocapsid protein, respectively. We compared the immunogenicity and protective efficacy of naked DNA (pWRG7077), DNA-based Sindbis replicon (pSIN2.5), and packaged Sindbis replicon vectors (pSINrep5), containing either the M or S gene segment of SEOV in Syrian hamsters. All of the vectors elicited an anti-SEOV immune response to the expressed SEOV gene products. Vaccinated hamsters were challenged with SEOV and monitored for evidence of infection. Protection from infection was strongly associated with M-gene vaccination. A small number of S-gene-vaccinated animals also were protected. Hamsters vaccinated with the pWRG7077 vector expressing the M gene demonstrated the most consistent protection from SEOV infection and also were protected from heterologous hantavirus (Hantaan virus) infection.

INTRODUCTION

Hantaviruses are rodent-borne viruses in the Bunyaviridae family. Hantaan and Seoul viruses (HTNV and SEOV) are etiological agents of hemorrhagic fever with renal syndrome (HFRS). Hantaviruses are transmitted by aerosolization of infected rodent urine and excreta, and each virus is carried principally by one rodent host (HTNV: *Apodemus agrarius*, SEOV: *Rattus norvegicus*). Because of the rodent host specificity of hantaviruses, their distribution is generally limited to the range of the rodent that carries them (HTNV: Asia, SEOV: worldwide) (reviewed in Schmaljohn *et al.,* 1997).

Because hantaviruses cause disease worldwide, there is a need for development of hantavirus vaccines (reviewed in Gonzalez-Scarano and Nathonson, 1996). Several approaches have been used to develop candidate hantavirus vaccines. These approaches include cell-culture or rodent-brain-derived inactivated virus vaccines (Lee *et al.,* 1990; Song *et al.,* 1992; Zhu *et al.,* 1994; Lu *et al.,* 1996), protein subunit vaccines expressed in insect cells (Schmaljohn *et al.,* 1990; Yoshimatsu *et al.,* 1993; Lundkvist *et al.,* 1996), a chimeric hepatitis B virus core particle subunit vaccine (Ulrich *et al.,* 1998), recombinant vaccinia-vectored vaccines (Schmaljohn *et al.,* 1990, 1992; Xu *et al.,* 1992; Chu *et al.,* 1995), and most recently naked DNA-based vaccines (Hooper *et al.,* 1999). One approach that has not been examined is the use of recombinant alphavirus-based expression systems for hantavirus vaccine development.

Alphaviruses (family: Togaviridae, genus: *Alphavirus*) are enveloped viruses that possess a single-strand, positive-sense RNA genome (Strauss *et al.,* 1994). The development of infectious clones for a number of alphaviruses has led to the description of several powerful expression systems. Expression systems based on a self-replicating RNA (replicon) that can be packaged into viral particles were developed for Semliki Forest virus (SFV), Venezuelan equine encephalitis (VEE) virus, and Sindbis virus (SINV) (Xiong *et al.,* 1989; Geigenmuller-Gnirke *et al.,* 1991; Liljestrom and Garoff, 1991; Bredenbeek *et al.,* 1993; Pushko *et al.,* 1997). A complete description of alphavirus replication can be found in Strauss *et al.* (1994). The SFV and SINV systems also were engineered into DNA-based vectors (Dubensky *et al.,* 1996; Berglund *et al.,* 1998; DiCiommo *et al.,* 1998; Hariharan *et al.,* 1998; Kohno *et al.,* 1998). The DNA-based alphavirus vectors drive transcription of the replicon RNA from a RNA polymerase II-dependent promoter (cytomegalovirus early promoter). This replicon RNA then becomes self replicating and drives expression of a heterologous gene of interest. Both packaged replicon vectors and DNA-based replicon vectors have been used in vaccine studies for a variety of infectious diseases (London *et al.,* 1992; Pugachev *et al.,* 1995; Zhou *et al.,* 1995; Mossman *et al.,* 1996; Pushko *et al.,* 1997; Berglund *et al.,* 1998; Colombage *et al.,* 1998; Fleeton *et al.,* 1999; Hariharan *et al.,* 1998; Hevey *et al.,* 1998; Tsuji *et al.,* 1998). Packaged replicon expression vectors have sev-

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eral potential advantages as vaccine vectors: (1) transient, high-level, protein expression; (2) cytoplasmic mRNA transcription, eliminating potential mRNA splicing events that may be associated with nuclear transcription; and (3) a broad range of susceptible cells (Frolov *et al.,* 1996). Although the DNA-based replicon vectors do not share all of these advantages, the combination of DNA stability and alphavirus expression capability make them attractive vaccine delivery vehicles. For these reasons, we were interested in testing alphavirus-based expression vectors as possible hantavirus vaccine vehicles.

In this report we compared the immunogenicity and protective efficacy of three different vaccine vectors (naked DNA, DNA-based SINV replicons, and packaged SINV replicons) expressing the SEOV M gene, encoding the G1 and G2 envelope proteins, or S gene, encoding the nucleocapsid protein, in a hantavirus hamster infection model.

RESULTS

Transient expression

All vaccine constructs were analyzed by radio-immunoprecipitation (RIP) to assess expression of the SEOV proteins. DNA vaccine constructs were transfected into COS cells, and replicon viruses were used to infect BHK-21 cells. The expressed proteins were immunoprecipitated with SEOV-specific polyclonal rabbit antisera and then analyzed by SDS–PAGE. Proteins of the expected molecular weight were immunoprecipitated for SEOV G1, G2, and N by using all three expression vectors (Fig. 1). As observed previously, truncated forms of the nucleocapsid protein were precipitated after transfection of COS cells with pWRG/SEOV-S DNA (Fig. 1C, lane 2) (Hooper *et al.,* 1999). The truncated forms of N were not detected in pSIN2.5/SEOV-S DNA transfected or Rep5/SEOV-S virus-infected cells.

Immunogenicity and protective efficacy of SEOV-S vaccines

Groups of hamsters were vaccinated with control constructs or constructs expressing the SEOV S gene. To determine whether vaccinated animals had developed an antibody response to N, prechallenge sera were analyzed by ELISA. All but one animal vaccinated with a SEOV S gene expressing construct developed anti-N antibodies (Table 1). The prechallenge anti-N antibody geometric mean titers (GMT) for each vector are shown in parentheses in Table 1. Animals vaccinated with the pSIN2.5/SEOV-S DNA construct had higher anti-N antibody responses than either pWRG/SEOV-S DNA or Rep5/SEOV-S virus-vaccinated animals (Table 1). Only the difference in anti-N antibody titer between pSIN2.5/SEOV-S and pWRG/SEOV-S DNA constructs approached significance (GMT: 6400 and 864, respectively, $P = 0.059$).

We also analyzed negative control-vaccinated animals that received a lacZ-expressing construct for the development of anti-betagalactosidase (βgal) antibodies by ELISA. All animals vaccinated with lacZ-expressing constructs developed anti-βgal antibodies before challenge (data not shown). To determine whether animals vaccinated with replicon viruses developed an anti-SINV antibody response, prechallenge sera were analyzed by anti-SINV ELISA. All of the SIN replicon virus-vaccinated animals developed anti-SINV-specific antibodies before hantavirus challenge (data not shown).

To compare the protective efficacy of the SEOV-S vaccines, vaccinated animals were challenged with 1000 plaque-forming units (PFU) of SEOV 3 weeks after the final boost. Twenty-eight days postchallenge the hamsters were evaluated for evidence of SEOV infection by testing sera for the development of anti-G1-G2 antibodies with a plaque reduction-neutralization test (PRNT). All but one of the animals vaccinated with the pWRG/SEOV-S DNA construct developed SEOV-neutralizing antibodies, indicating that only one pWRG/SEOV-S DNA vaccinated hamster was protected from SEOV infection (Table 1). Similar results were noted with pSIN2.5 DNA construct vaccinated hamsters. Two of eight animals vaccinated with pSIN2.5/SEOV-S DNA appear to have been protected from SEOV infection as they did not have detectable SEOV-neutralizing antibodies (Table 1). None of the Rep5/SEOV-S virus-vaccinated animals was protected from SEOV infection as all of the animals had neutralizing antibodies after challenge (Table 1). All of the negative control animals developed high levels of both anti-N and neutralizing antibodies after SEOV challenge, indicating that the animals were infected (Table 1).

Immunogenicity and protective efficacy of SEOV-M vaccines

Groups of hamsters were vaccinated with constructs expressing the SEOV M gene. To determine whether vaccinated animals had developed antibody responses to the expressed SEOV envelope proteins, prechallenge sera were analyzed by PRNT. All but one animal vaccinated with an M gene-expressing construct developed SEOV neutralizing antibodies before challenge (Table 2). The neutralizing antibody GMT for animals vaccinated with pWRG/SEOV-M DNA was significantly higher than for animals vaccinated with pSIN2.5/SEOV-M DNA (3225 and 211, respectively. $P = 0.04$), but not for Rep5/SEOV-M virus-vaccinated animals (Table 2).

Comparing the kinetics of neutralizing antibody responses between DNA and replicon virus-vaccinated animals revealed that hamsters vaccinated with pWRG/SEOV-M DNA developed PRNT titers after a single vaccination, whereas none of the animals vaccinated with Rep5/SEOV-M virus developed neutralizing antibodies until after the second vaccination (Fig. 2). Moreover,

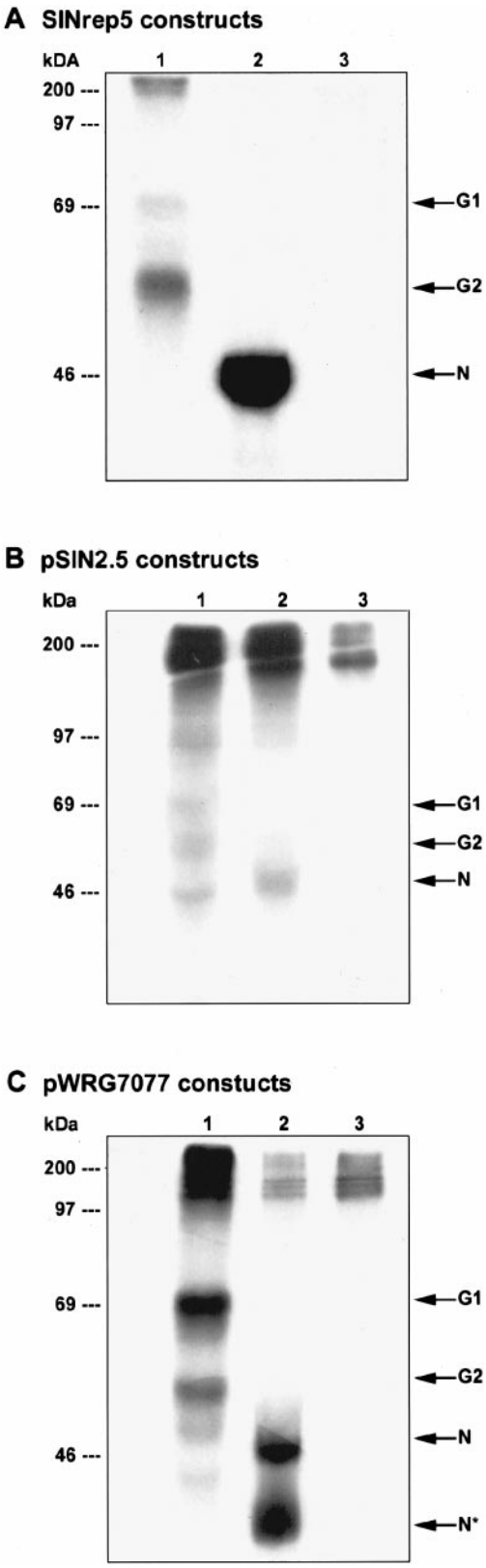

FIG. 1. Radio-immunoprecipitation of SEOV structural proteins expressed *in vitro* from SINrep5 (A), pSIN2.5 (B), and pWRG7077 (C) expression vectors. Lane 1, SEOV M gene expression products. Lane 2, SEOV S gene expression product. Lane 3, antibody negative control. Molecular mass markers (kDa) are indicated on the left, and the positions of the SEOV G1, G2, and N proteins are shown on the right of each gel. N*, truncated N expression product. Anti-SEOV polyclonal rabbit sera was used to precipitate SEOV proteins.

pWRG/SEOV-M DNA vaccinated animals had higher neutralizing antibody titers than did Rep5/SEOV-M virus vaccinated animals at all the time points analyzed (Fig. 2).

To compare the protective efficacy of the SEOV-M vaccines, vaccinated animals were challenged with SEOV, and 28 days postchallenge the hamsters were evaluated for evidence of SEOV infection by testing their sera for anti-N antibodies by ELISA. None of the pWRG/SEOV-M gene-vaccinated animals developed detectable anti-N antibodies after challenge (Table 2). Thus all of these animals were protected from SEOV infection. Two of five animals vaccinated with pSIN2.5/SEOV-M DNA developed anti-N antibodies after challenge, whereas the remaining three animals did not and thus were protected from infection (Table 2). A postchallenge ELISA of Rep5/SEOV-M virus vaccinated hamster sera indicated that four of five animals developed anti-N antibodies, indicating that only one Rep5/SEOV-M virus-vaccinated animal was protected from infection (Table 2).

Because animals vaccinated with pWRG/SEOV-M DNA were completely protected from homologous viral infection (Table 2), this vaccine was used in a heterologous hantavirus challenge experiment. Hamsters received a priming vaccination with either the negative control plasmid pWRG7077 or pWRG/SEOV-M DNA followed by two boosts at 3-week intervals. Vaccinated animals then were challenged with HTNV 3 weeks after the final boost. Twenty-eight days postchallenge, the hamsters were evaluated for evidence of HTNV infection.

All of the pWRG/SEOV-M DNA vaccinated animals developed neutralizing antibodies specific for SEOV before HTNV challenge, with PRNT end-point titers ranging from 160 to 5120 (data not shown). In addition, three of the four pWRG/SEOV-M DNA vaccinated animals had detectable levels of cross-reactive antibodies capable of neutralizing HTNV before challenge (Fig. 3). All of the negative control vaccinated animals developed high anti-N and neutralizing antibodies after challenge, indicating that all of the animals became infected with HTNV (Fig. 3). In contrast, three of four animals vaccinated with pWRG/SEOV-M DNA had no detectable anti-N antibodies after HTNV challenge. One pWRG/SEOV-M DNA vaccinated animal developed a detectable anti-N antibody response after HTNV challenge, thus three of four pWRG/SEOV-M DNA vaccinated animals were protected from HTNV infection (Fig. 3).

Sequential vaccination with Rep5/SEOV-S and Rep5/lacZ viruses

To determine whether hamsters vaccinated with one replicon virus could be revaccinated with a second rep-

TABLE 1

Serological Responses and Protection of Hamsters Vaccinated with SEOV S Gene Constructs

Vaccine	Hamster	Anti-N ELISA[a]		PRNT[b]		Infection[d]
		Pre[c]	Post	Pre	Post	
pWRG/SEOV-S DNA[e] (GMT 864)[f]	1	200	25,600	<20	40,960	+
	2	100	6,400	<20	40,960	+
	3	<100	1,600	<20	20,480	+
	4	3,200	12,800	<20	5,120	+
	5	200	3,200	<20	81,920	+
	6	400	12,800	<20	5,210	+
	7	25,600	12,800	<20	<20	−
	8	25,600	12,800	<20	40,960	+
	9	3,200	1,600	<20	320	+
pSIN2.5/SEOV-S DNA[e] (GMT 6,400)[f]	1	6,400	25,600	<20	5,120	+
	2	6,400	3,200	<20	1,280	+
	3	6,400	25,600	<20	10,240	+
	4	6,400	51,200	<20	40,960	+
	5	6,400	6,400	<20	40	+
	6	12,800	51,200	<20	20,480	+
	7	6,400	6,400	<20	<20	−
	8	3,200	3,200	<20	<20	−
Rep5/SEOV-S virus[e] (GMT 1,600)[f]	1	1,600	1,600	<20	160	+
	2	1,600	6,400	<20	1,280	+
	3	800	1,600	<20	2,560	+
	4	3,200	6,400	<20	2,560	+
pWRG7077 DNA[e]	n = 9[g]	<100	2,962[h] (1,600–6,400)[i]	<20	10,240[h] (1,280–40,960)[i]	+
pSIN2.5/lacZ DNA[e]	n = 5[g]	<100	3,675[h] (800–51,200)[i]	<20	8,914[h] (5,120–20,480)[i]	+
Rep5/lacZ virus[j]	n = 6[g]	<100	1,269[h] (800–3,200)[i]	<20	3,225[h] (1,280–10,240)[i]	+

[a] ELISA endpoint titers are the reciprocal serum dilution that had OD 450 nm values greater than control serum + 3 standard deviations.
[b] PRNT values are the reciprocal serum dilution that reduces plaque number by 80%.
[c] pre: prechallenge serum sample. post: postchallenge serum sample.
[d] −, animal was protected from SEOV infection; +, animal was not protected from SEOV infection.
[e] Hamsters were vaccinated three times at 3-week intervals.
[f] Prechallenge anti-N antibody geometric mean titers for each vector.
[g] Number of animals vaccinated with negative control construct.
[h] Geometric mean antibody titer for negative control animals.
[i] Range of negative control ELISA or PRNT endpoint titers.
[j] Hamsters were vaccinated three times at 4-week intervals.

licon virus, a selected group of animals were sequentially vaccinated with two different recombinant replicon viruses. Hamsters vaccinated with Rep5/SEOV-S virus and then challenged with SEOV were inoculated with 1×10^6 IFU of Rep5/lacZ virus ~8 weeks after the SEOV challenge. Sera were collected 4 weeks after Rep5/lacZ virus vaccination and analyzed for anti-βgal antibodies by ELISA. The anti-N, anti-SINV, and anti-βgal ELISA responses for individual animals before and after sequential vaccination with Rep5/lacZ virus are shown in Table 3. For comparison, the same antibody responses were determined for control animals (SINV naive) after one vaccination with Rep5/lacZ virus (Table 3). Three of the four animals that received a secondary vaccination with Rep5/lacZ virus developed anti-βgal antibodies, and all of the control animals vaccinated with Rep5/lacZ virus developed anti-βgal antibodies (Table 3). An anti-βgal antibody response was elicited even though the animals had preexisting antibody to SINV, indicating that sequential vaccination with two different SINV replicons was possible.

DISCUSSION

Previously, we showed that hamsters vaccinated with a naked DNA vector expressing the SEOV M gene were protected from infection with SEOV, whereas those expressing the SEOV S segment were not (Hooper *et al.,*

TABLE 2

Serological Responses and Protection of Hamsters Vaccinated with SEOV M Gene Constructs

Vaccine	Hamster	Anti-N ELISA[a]		PRNT[b]		Infection[d]
		Pre[c]	Post	Pre	Post	
pWRG/SEOV-M DNA[d] (GMT 3,225)[f]	1	<100	<100	5,120	10,240	−
	2	<100	<100	2,560	2,560	−
	3	<100	<100	2,560	5,120	−
	4	<100	<100	5,120	10,240	−
	5	<100	<100	2,560	2,560	−
	6	<100	<100	2,560	2,560	−
pSIN2.5/SEOV-M DNA[e] (GMT 211)[f]	1	<100	200	320	1,280	+
	2	<100	800	20	2,560	+
	3	<100	<100	640	320	−
	4	<100	<100	80	320	−
	5	<100	<100	1,280	1,280	−
Rep5/SEOV-M virus[g] (GMT 1,270)[f]	1	<100	3,200	<20	2,560	+
	2	<100	400	5,120	2,560	+
	3	<100	200	2,560	640	+
	4	<100	200	640	1,280	+
	5	<100	<100	1,280	320	−

[a] ELISA endpoint titers are the reciprocal serum dilution that had OD 450 nm values greater than control serum + 3 standard deviations.
[b] PRNT values are the reciprocal serum dilution that reduced plaque number by 80%.
[c] pre, prechallenge serum sample; post, postchallenge serum sample.
[d] −, Animal was protected from SEOV infection. +: Animal was not protected from SEOV infection.
[e] Hamsters were vaccinated three times at 3-week intervals.
[f] Prechallenge neutralizing antibody geometric mean titers for each vector.
[g] Hamsters were vaccinated three times at 4-week intervals.

1999). The data presented in this report confirm and extend those results and further demonstrate the potential of two other nucleic-acid-based vectors as hantavirus vaccines.

Although all three systems expressed hantavirus proteins and were immunogenic in hamsters, we noted some qualitative and quantitative differences. Comparing gene expression levels between the two DNA-based vectors, pWRG7077 and pSIN2.5 *in vitro* revealed that the pWRG7077 constructs consistently expressed higher levels of protein than pSIN2.5 constructs in cell culture. This result was unexpected based on the self-amplifying nature of SIN replicon RNAs (Dubensky *et al.,* 1996; Hariharan *et al.,* 1998). In addition, the difference in expression levels between the two vectors was not due to the pSIN2.5 vector not functioning as a replicon in tissue culture, as we were able to demonstrate that a DNA-based SINV replicon vector with a deletion in the SINV nonstructural gene region was unable to express SEOV genes (data not shown). However, reduced expression *in vitro* did not always correlate with reduced immune response *in vivo*. This was best illustrated by a comparison of the pWRG/SEOV-S and pSIN2.5/SEOV-S DNA constructs. Assay of N expression revealed that pWRG/SEOV-S DNA transfected COS cells expressed more N than did pSIN2.5/SEOV-S DNA transfected cells, even when plasmid copy number (pSIN2.5 is about three times as large as pWRG7077) and transfection efficiency were controlled for (data not shown). However, despite the relatively low *in vitro* expression level, hamsters vaccinated with pSIN2.5/SEOV-S DNA developed higher GMTs to N than did animals vaccinated with pWRG/SEOV-S DNA (6309 and 857, respectively $P = 0.059$). Berglund *et al.* (1998) noted a similar phenomenon (low *in vitro* expression, high *in vivo* immune response) when comparing a conventional RNA-polymerase-II-dependent, promoter-based plasmid and a DNA-based SFV vector, both expressing influenza A virus genes. Others suggest that the enhanced immune response elicited by DNA-based alphavirus vectors expressing certain genes may be due to vector-mediated cell death or induction of interferons as a result of alphavirus replication within transfected cells (Berglund *et al.,* 1998; Hariharan *et al.,* 1998). In contrast to the results with the S segment constructs, pSIN2.5/SEOV-M DNA constructs failed to elicit higher antibody responses than pWRG/SEOV-M DNA constructs (PRNT GMT 211, and 3225, respectively $P = 0.04$). These data suggest that the level of the antibody response elicited from a DNA-based alphavirus vector may vary dependent on the gene expressed.

Three of four animals vaccinated with Rep5/SEOV-S virus and subsequently vaccinated with Rep5/lacZ virus

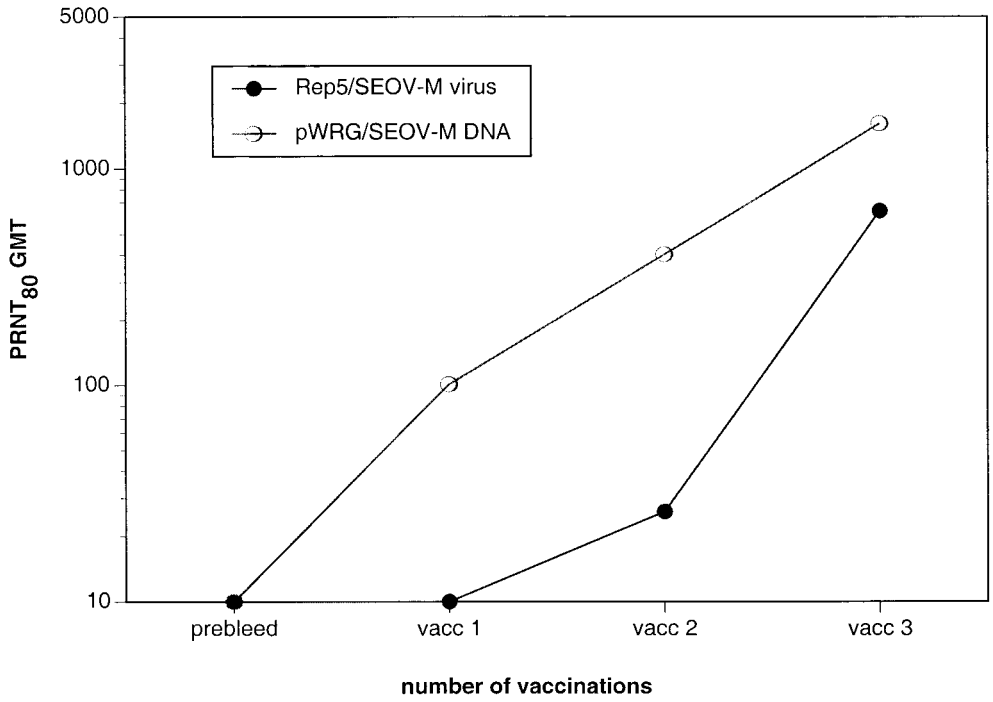

FIG. 2. Analysis of the development of neutralizing antibodies over time in pWRG/SEOV-M DNA and Rep5/SEOV-M virus-vaccinated hamsters. Hamster serum was collected before vaccination (prebleed) and 3 (pWRG/SEOV-M) or 4 (Rep5/SEOV-M) weeks after each vaccination (vacc) and analyzed by PRNT. Each time point represents the PRNT geometric mean titer (GMT) for either six pWRG/SEOV-M DNA vaccinated or five Rep5/SEOV-M virus-vaccinated animals.

developed anti-βgal antibodies even in the presence of anti-SINV antibodies. The anti-SINV antibody response elicited in replicon virus-vaccinated animals was most likely due to the presence of replication competent virus in the replicon virus stocks (data not shown). Mice sequentially vaccinated with VEE replicons expressing influenza virus hemagglutinin and Lassa virus nucleocapsid also were able to develop antibodies to both proteins, although these animals either had no or very low levels of preexisting anti-VEE antibodies (Pushko *et al.,* 1997). Animals with no preexisting SINV immunity developed much higher end-point anti-βgal antibody responses after a single Rep5/lacZ virus vaccination than did the Rep5/lacZ virus sequentially vaccinated animals, suggesting that preexisting SINV immunity may have limited the anti-βgal response in the sequentially vaccinated animals.

Because no animal model of disease is known for hantaviruses, our protection model was based on detection of antibodies to non-vaccine-derived viral proteins after challenge. This is an extremely rigorous definition of protection because it is based on sterile immunity. For example, one Rep5/SEOV-M virus-vaccinated animal had low but detectable anti-N antibodies after challenge despite having a high prechallenge neutralizing antibody titer (5120) and demonstrating a drop in neutralizing antibody after challenge (2560). It is possible that this animal developed an immune response to the protein present in the challenge inoculum even though replication may not have occurred. Therefore it is difficult to definitively assess whether animals that display this type of immune profile are productively infected with SEOV.

Previously, we were unable to demonstrate that hamsters vaccinated with pWRG/SEOV-S DNA alone could be protected from SEOV infection (Hooper *et al.,* 1999). In that study, pWRG/SEOV-S DNA vaccination data did reveal an about fivefold reduction in postchallenge PRNT titer in SEOV S gene-vaccinated animals as compared with negative control-vaccinated/SEOV-challenged animals (Hooper *et al.,* 1999). These data suggest that, although SEOV S-vaccinated animals became infected, the infection may have been limited as compared with the infection in negative control animals (Hooper *et al.,* 1999). The data presented here confirm those results in that one of nine hamsters vaccinated with pWRG/SEOV-S DNA and two of eight hamsters vaccinated with pSIN2.5/SEOV-S DNA were protected from SEOV infection. It may be that the larger number of animals tested in this study allowed us to detect individuals that had developed a protective immune response. It is also possible that these animals did not receive an infectious dose of SEOV at challenge, thus resulting in a false-positive appearance of protection. This possibility is unlikely considering that all of the control animals became infected after receiving the same SEOV challenge dose. There was no clear correlation of prechallenge anti-N antibody titer

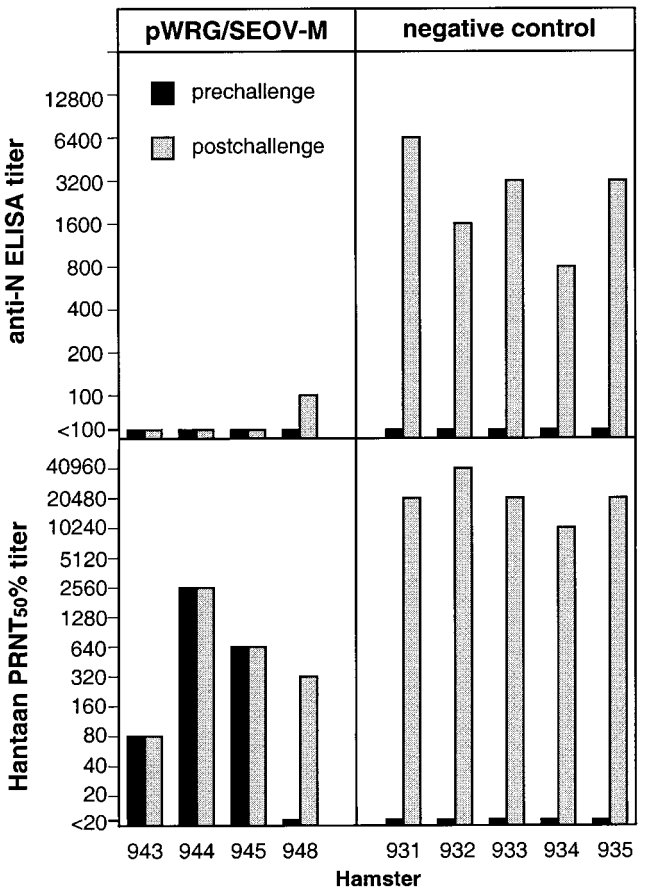

FIG. 3. Protection from HTNV challenge. Prechallenge and postchallenge anti-N ELISA and PRNT titers are shown for control (pWRG7077) and pWRG/SEOV-M vaccinated animals. ELISA values are reciprocal serum dilution end-point titers. PRNT values are the reciprocal serum dilution that reduced plaque number by 50%.

and protection. That is, some animals with anti-N ELISA titers as high as 25,600 were not protected from infection. These data suggest that anti-N antibody levels are not good markers of a protective immune response. Others have demonstrated the protective capacity of hantavirus N (Schmaljohn *et al.,* 1990; Xu *et al.,* 1992; Yoshimatsu *et al.,* 1993; Lundkvist *et al.,* 1996; Ulrich *et al.,* 1998); however, the most successful approaches have been with recombinant baculovirus-infected cell lysates containing N or chimeric hepatitis B virus core particles that display N epitopes. (Schmaljohn *et al.,* 1990; Lundkvist *et al.,* 1996; Ulrich *et al.,* 1998). Vaccination with vaccinia virus recombinants expressing N only partially protected animals from infection (Schmaljohn *et al.,* 1990; Xu *et al.,* 1992). Cell-mediated immunity is believed to be important for clearing a hantavirus infection (Nakamura *et al.,* 1985; Asada *et al.,* 1987, 1989) and cell-mediated immunity to N as well as the envelope proteins have been demonstrated (Yoshimatsu *et al.,* 1993; Ennis *et al.,* 1997). Analysis of the immune response elicited in animals vaccinated with recombinant N compared with the immune response in animals vaccinated with vectors that produce N endogenously will be required to determine what the critical factors are that result in N protein-specific protection.

All of the SEOV M gene-expressing vectors were capable of eliciting a neutralizing antibody response in hamsters but only the pWRG/SEOV-M DNA vector was capable eliciting sterile immunity in all SEOV-challenged animals. In addition, a comparison of when animals developed neutralizing antibodies after DNA-based or SIN replicon virus-based vaccination with the M gene revealed that the DNA-vaccinated animals developed neutralizing antibodies not only earlier but also to higher end-point titers. Based on these results, the pWRG/SEOV-M DNA vector was used in a HTNV cross-protection study.

These results show for the first time, that DNA vaccination with the SEOV M gene can elicit a cross-protective immune response against HTNV infection. These data agree with protection data derived from gerbils vaccinated with a vaccinia-vectored SEOV M gene and then challenged with HTNV (Xu *et al.,* 1992) and with data derived from live hantavirus cross-protection studies in rodents (Yamanishi *et al.,* 1988; Yoshimatsu *et al.,* 1993; Lu *et al.,* 1996). Three of four pWRG/SEOV-M DNA vaccinated animals had detectable HTNV-neutralizing antibodies before challenge and were protected from infection with HTNV, although all had prechallenge SEOV-neutralizing antibodies. The animal that was not protected from heterologous viral infection had no detectable prechallenge HTNV-neutralizing antibodies and also had the lowest prechallenge neutralizing antibody titer to SEOV (PRNT = 160). The level of anti-SEOV neutralizing antibodies in the animal that was not protected from HTNV challenge was less than that required to induce sterile immunity but it appeared to be high enough to limit HTNV replication as indicated by a much lower anti-N antibody response (1:100) than those of negative controls (range 800–6400). Nevertheless our findings demonstrate the feasibility of a DNA-based hantavirus vaccine that can afford cross-protective potential.

Although our studies using recombinant DNA-based vaccines demonstrated cross-protection in hamsters between SEOV and HTNV, a vaccinia-vectored vaccine for HTNV did not protect against Puumala virus (Chu *et al.,* 1995). Consequently, these data suggest that a multivalent vaccine will be required for protection from all of the pathogenic members of the *Hantavirus* genus. An important first step in this process is determining what vector system to use in hantavirus vaccine studies. Based on the data presented here, we believe that a DNA-based approach shows great promise for multivalent hantavirus vaccine development. In addition, these data begin to define the minimal complement of hantavirus genes required in a multivalent DNA-based vaccine.

TABLE 3

Sequential SINV Replicon Vaccination of Individual Hamsters

ELISA[a]	Hamster 1	Hamster 2	Hamster 3	Hamster 4	GMT[b]
Three vaccinations with Rep5/SEOV-S virus[c]					
N	1,600	1,600	400	800	951
SINV	3,200	6,400	400	1,600	1,903
βgal	<50	<50	<50	<50	25
Single vaccination with Rep5/lacZ virus in Rep5/SEOV-S virus vaccinated animals[d]					
N	400	800	3,200	800	951
SINV	400	3,200	200	1,600	800
βgal	100	800	200	<50	141
Single vaccination with Rep5/lacZ virus (naive hamsters)[e]					
ELISA	Hamster 5	Hamster 6	Hamster 7	Hamster 8	GMT
N	<50	<50	<50	<50	25
SINV	800	6,400	800	100	800
βgal	3,200	800	3,200	3,200	2,263

[a] Antigen-specific ELISA.
[b] GMT: geometric mean ELISA titer specific for each antigen.
[c] ELISA titers for the indicated antigens were determined 3 weeks after the final Rep5/SEOV-S virus vaccination.
[d] ELISA titers for the indicated antigens were determined 4 weeks after a single Rep5/lacZ virus vaccination in animals previously vaccinated with Rep5/SEOV-S virus.
[e] ELISA titers for the indicated antigens were determined 4 weeks after a single Rep5/lacZ virus vaccination in SINV naive hamsters.

METHODS

Viruses, cells, and medium

SEOV, strain SR-11 (Kitamura *et al.,* 1983), and HTNV, strain 76-118 (Lee *et al.,* 1978), were propagated in Vero E6 cells (VERO C1008;ATCC CRL 1586). SINV (strain HRsp) (Strauss *et al.,* 1984), and SIN replicon viruses were produced in BHK-21 cells (ATCC CCL-10). Transient expression experiments were performed in either BHK-21 cells or COS cells (COS-7; ATCC CRL1651). Cells were maintained in Eagle's minimal essential medium with Earle's salts (EMEM) containing 10% fetal bovine serum, 10 mM HEPES pH 7.4; and antibiotics [penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml)] (cEMEM).

Plasmid constructs

RT–PCR and cloning of the M and S genome segments of SEOV were described previously (Arikawa *et al.,* 1990).

*Naked DNA vectors.*The pWRG/SEOV-M and SEOV-S DNA vectors were described previously (Hooper *et al.,* 1999) (Fig. 4A).

DNA-based SINV vector. Construction of the pSIN2.5 DNA vector was described elsewhere (Hariharan *et al.,* 1998) (Fig. 4B). The pSIN2.5/lacZ DNA construct was provided by Chiron Technologies, San Diego, CA. PCR primers were designed to amplify the SEOV M and S gene open reading frames (ORFs) with unique *Xho*I and *Not*I restriction sites at the 5′ and 3′ ends, respectively. The *Xho*I/*Not*I-digested SEOV PCR products then were ligated into *Xho*I/*Not*I-linearized pSIN2.5 DNA to produce the pSIN2.5/SEOV-M and pSIN2.5/SEOV-S DNA constructs.

SINV replicon vector. The pSINrep5 vector (Invitrogen, Carlsbad, CA) (Fig. 4C) and pSINrep5/lacZ construct were described previously (Bredenbeek *et al.,* 1993). The SEOV M and S gene ORFs were subcloned into the *Xba*I site of pSINrep5 to produce pSINrep5/SEOV-M and pSINrep5/SEOV-S. Defective helper plasmid pDH-BB (Bredenbeek *et al.,* 1993) was used to supply the SINV structural proteins in *trans* to generate packaged replicons.

Replicon virus production

The SINV replicon vectors and defective helper DNAs were linearized to allow run-off transcription. Transcription reactions were carried out with an InvitroScript CAP SP6 *in vitro* transcription kit (Invitrogen) as described by the manufacturer. The transcribed RNAs were used without treatment with DNase or further purification. A BioRad (Hercules, CA) gene pulser was used for all electroporations. BHK-21 cells were suspended in phosphate-buffered saline, (pH 7.4) (PBS) at a density of 1×10^7 cells/ml, and 0.8

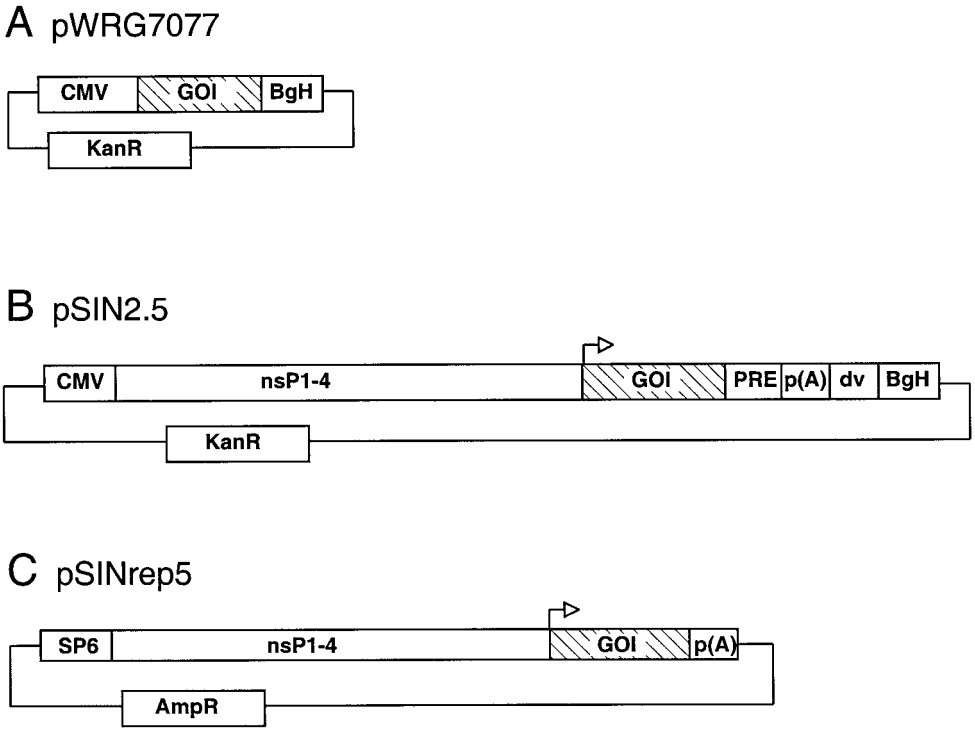

FIG. 4. Vaccine expression vectors. (A) pWRG7077, (B) pSIN2.5, (C) pSINrep5. CMV, cytomegalovirus immediate early promoter sequence; GOI, gene of interest; BgH, bovine growth hormone transcription terminator; PRE, hepatitis B virus posttranscriptional regulatory element; p(A), poly (A) tract; dv, hepatitis delta virus ribozyme sequence; nsP1–4, Sindbis nonstructural protein genes; SP6, SP6 RNA polymerase promoter sequence; KanR, kanamycin resistance gene; AmpR, ampicillin resistance gene. Open arrow represents Sindbis virus 26S subgenomic promoter.

ml of the cell suspension combined with ~10 μg each of replicon and helper RNAs was used per electroporation. Each cell/RNA mixture was transferred to 0.4-mm gap cuvettes (BioRad) and pulsed three times with the electroporator conditions set at 1.1 kV and 25uF. Packaged replicon viruses were harvested 36–48 h postelectroporation and purified by centrifugation through a 20% sucrose cushion as described previously (Kamrud *et al.,* 1995). Replicon viruses generated were designated Rep5/X virus where X represented the SEOV gene cloned into the pSINrep5 construct. Purified replicon viruses were resuspended in sterile PBS and titrated by immunofluorescent antibody (IFA) assay as described previously (Kamrud *et al.,* 1995).

Animal vaccination

Outbred, 6- to 8-week-old, golden Syrian hamsters (Charles River) received a priming vaccination followed by two boosts at 3- or 4-week intervals. Animals vaccinated with DNA constructs received ~3.0 μg DNA delivered by gene gun per vaccination as described previously (Hooper *et al.,* 1999). Animals vaccinated with replicon viruses received ~1 $\times$ 10^6/infectious units (IFU) of virus, diluted in sterile PBS, by subcutaneous inoculation per vaccination.

ELISA

The method for detecting hantavirus N-specific antibodies was described previously (Elgh *et al.,* 1997; Hooper *et al.,* 1999). Briefly, amino acids 1–117 of SEOV N were expressed as a histidine-tagged fusion protein in *Escherichia coli*. Affinity-purified protein diluted in carbonate buffer (pH 9.6) was coated onto 96-well ELISA plates (Costar, Cambridge, MA) overnight at 4°C. Hamster sera then were diluted in PBS containing 3% goat serum, 5% skim milk, 1% *E. coli* lysates, and 0.05% Tween 20 and analyzed for the presence of anti-N specific antibodies by ELISA. Sera from hamsters vaccinated with control constructs that expressed the lacZ gene were analyzed for the development of anti-βgal antibodies. For these studies, ELISA plates were coated with 500 ηg/well of purified βgal protein (Boehringer Mannheim, Indianapolis, IN) in carbonate buffer overnight at 4°C. Animals vaccinated with recombinant SIN replicon viruses were analyzed for the development of anti-SINV antibodies. SINV (strain HRsp) purified on a 20–60% sucrose gradient was diluted 1:500 in carbonate buffer and coated onto ELISA plates overnight at 4°C. Horseradish peroxidase-conjugated goat, anti-hamster secondary antibody (diluted 1:10,000) [Kirkegaard & Perry Laboratories (KPL), Gaithersburg, MD] and tetra-methylbenzidine substrate (KPL) were used in all ELISA exper-

iments as described previously (Hooper *et al.,* 1999). The colorimetric reaction was stopped by adding stop solution (KPL), and the optical density (OD), at 450 nm, was measured. End-point titers were determined as the highest dilution with an OD greater than the mean OD value of negative control serum samples (pWRG7077 DNA, pSIN2.5/lacZ DNA, or Rep5/lacZ virus-vaccinated animals) (diluted 1:100) plus 3 standard deviations.

Radio-immunoprecipitation (RIP)

RIP analysis of naked DNA constructs (pWRG7077 or pSIN2.5) was conducted in COS cells. COS cells grown in 25 cm^2 flasks were transfected with 8 μg of plasmid DNA with FuGENE 6 (Boehringer Mannheim) as described by the manufacturer. RIP analysis of SINV replicon constructs was conducted in BHK-21 cells. BHK-21 cells grown in 25 cm^2 flasks were infected with replicon viruses at a m.o.i. of 3. After 24 h (48 h for pSIN2.5 DNA constructs), the cells were radiolabeled with 200 μCi ^{35}S Promix (Amersham, Arlington Heights, IL) per flask. The cells were labeled for 3 h, cell lysates were produced with Zwittergent (Calbiochem-Novabiochem Corp., La Jolla, CA) lysis buffer, and SEOV proteins were immunoprecipitated with rabbit anti-SEOV polyclonal sera as described previously (Schmaljohn *et al.,* 1997; Kamrud *et al.,* 1998).

Plaque-reduction neutralization test (PRNT)

Neutralization assays were performed as previously described (Hooper *et al.,* 1999). All serum samples were heat inactivated (56°C, 30 min), and neutralization assays were carried out in the presence of (5%) guinea pig complement (Accurate Chemical and Scientific Corp., Westbury, NY).

Hantavirus challenge

Hamsters were inoculated with 1000 PFU of either HTNV or SEOV intramuscularly as previously described (Hooper *et al.,* 1999). Twenty-eight days after challenge, serum samples were evaluated for the presence of anti-HTNV or anti-SEOV antibodies. Because hantavirus neutralizing antibodies are directed at the envelope proteins (G1 and G2) (Dantas *et al.,* 1986; Arikawa *et al.,* 1989), sera were analyzed by PRNT to follow the anti-glycoprotein antibody response. Anti-N protein-specific ELISA was performed to analyze the anti-nucleocapsid immune response in vaccinated animals. Because there is no disease model available for hantaviruses, we evaluated instead whether our vaccines could protect against infection. An animal was considered infected if antibodies to viral proteins other than the vaccine immunogen were detected after viral challenge.

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