Genetic vaccines protect against Sin Nombre hantavirus challenge in the deer mouse (Peromyscus maniculatus)

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We used a deer mouse (Peromyscus maniculatus) infection model to test the protective efficacy of genetic vaccine candidates for Sin Nombre (SN) virus that were known to provoke immunological responses in BALB/c mice (Bharadwaj et al., Vaccine 17, 2836–2843, 1999). Protective epitopes were localized in each of four overlapping cDNA fragments that encoded portions of the SN virus G1 glycoprotein antigen; the nucleocapsid gene also was protective. The protective efficacy of glycoprotein gene fragments correlated with splenocyte proliferation in the presence of cognate antigen, but none induced neutralizing antibodies. Genetic vaccines against SN virus can protect outbred deer mice from infection even in the absence of a neutralizing antibody response.

Introduction

Hantaviruses are negative-stranded RNA viruses that constitute a genus of the family Bunyaviridae (Schmaljohn et al., 1985). The genomes of hantaviruses are divided into three RNA segments: a large (L) segment encoding the viral transcriptase, a middle (M) segment encoding the envelope glycoproteins G1 and G2, and a small (S) segment encoding the nucleocapsid core protein, N. Hantaviruses have long been associated with epidemics of haemorrhagic fever with renal syndrome in Korea, China, Russia and Europe (Lee & van der Groen, 1989; Plyusnin et al., 1996). Sin Nombre (SN) hantavirus, which is endemic in the western United States, is the prototypical aetiological agent for hantavirus cardiopulmonary syndrome (HCPS) (Nichol et al., 1993). Approximately 1000 cases of HCPS have been recorded in nine countries since its discovery, with an overall case-fatality ratio of about 40–45% (Schmaljohn & Hjelle, 1997). All cases have occurred in the western hemisphere. While at least six distinct hantaviruses have been implicated as aetiological agents for HCPS, SN virus remains the most important in the northern hemisphere.

SN virus, like all pathogenic hantaviruses, is carried by a rodent reservoir host. The reservoir for SN virus is the deer mouse Peromyscus maniculatus (Childs et al., 1994). Although it is not uncommon to find populations of wild deer mice with 30–50% prevalence for SN virus, and seropositive deer mice are persistently infected, deer mice are not adversely affected by the infection. Humans contract the infection through inhalation of virus-contaminated excreta. Hantavirus outbreaks can involve significant numbers of people, and for at least one species, interpersonal transmission of infection has been recognized (Enria et al., 1996). About 50–100 000 cases of HFRS occur every year in China (Lee & van der Groen, 1989). In the western hemisphere, HCPS has a much greater economic importance than would be suggested by the sheer number of cases. HCPS outbreaks in New Mexico in 1993 and in Bariloche, Argentina in 1996 caused considerable economic damage and stigma to affected communities. Despite years of effort, to date there are no vaccines proven to be highly efficacious against hantavirus diseases. Potential recipients for a vaccine against SN virus could number in the millions, since virtually anyone living in a rural area in the western United States and Canadian provinces is at risk for infection (Khan et al., 1996).

The envelope glycoproteins of SN virus are expressed as a precursor molecule of 1140 amino acids. At least for the prototypical hantavirus Hantaan, the precursor polypeptide is cotranslationally cleaved into G1 and G2 by a cellular protease immediately after a hydrophobic region that ends in the conserved sequence WAASA (Schmaljohn et al., 1987; Arikawa et al., 1990; Lober et al., 2001). The two glycoproteins, which are both transmembrane proteins, assemble into heterodimers and for most members of the family Bunyaviridae they
are retained efficiently in the Golgi (Schmaljohn, 1996). For unclear reasons, it has been difficult to obtain good expression of recombinant SN virus glycoproteins in eukaryotic systems except when the genes are fragmented into small pieces (unpublished data of the authors).

To overcome the problem of poor glycoprotein expression, we previously prepared a series of 10 overlapping clones of the SN virus envelope glycoproteins G1 and G2, each 500 nt in length and sharing 100 nt of overlap with the more 5’ clone (Bharadwaj et al., 1999). We immunized groups of BALB/c mice with these individual clones, and separately with a full-length clone of the viral N gene, and measured the immunological responses. Several clones induced strong splenocyte proliferative response, especially those at the 5’ and middle portion of the G1 glycoprotein. Neutralizing and non-neutralizing antibody responses against the G1 and G2 antigens were present in some cases, but titres were very low. We noted that these vaccine candidates would be worthy of evaluation upon the development of an animal challenge model for SN virus (Bharadwaj et al., 1999). Recently, we developed such a model by using a novel outdoor containment laboratory in which we could conduct the challenge experiments (Botten et al., 2000a, b). The challenge experiments may be conducted safely in an outdoor setting but would require a biosafety level 4 (BSL-4) facility if conducted indoors (Centers for Disease Control & Prevention, 1994; Mills et al., 1995).

Methods

■ Deer mice and virus. Six-to-ten week-old outbred P. maniculatus rufus were obtained from the University of New Mexico’s colony in Albuquerque, NM, USA. The colony was founded from wild specimens collected in 1998 (Botten et al., 1995). We immunized groups of BALB/c mice with these individual clones, and separately with a full-length clone of the viral N gene, and measured the immunological responses. Several clones induced strong splenocyte proliferative response, especially those at the 5’ and middle portion of the G1 glycoprotein. Neutralizing and non-neutralizing antibody responses against the G1 and G2 antigens were present in some cases, but titres were very low. We noted that these vaccine candidates would be worthy of evaluation upon the development of an animal challenge model for SN virus (Bharadwaj et al., 1999). Recently, we developed such a model by using a novel outdoor containment laboratory in which we could conduct the challenge experiments (Botten et al., 2000a, b). The challenge experiments may be conducted safely in an outdoor setting but would require a biosafety level 4 (BSL-4) facility if conducted indoors (Centers for Disease Control & Prevention, 1994; Mills et al., 1995).

Table 1. Splenocyte proliferation responses in the presence of cognate antigen in deer mice that were not challenged with SN virus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coordinates</th>
<th>Coordinates</th>
<th>[3H]Thymidine incorporation (c.p.m.) (mean)</th>
<th>Stimulation index (WA – NA)/NA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleotide</td>
<td>Amino acid</td>
<td>No antigen (NA)</td>
<td>With antigen (WA)</td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>49–549</td>
<td>1–167</td>
<td>4708</td>
<td>67892</td>
</tr>
<tr>
<td>B</td>
<td>448–948</td>
<td>132–299</td>
<td>4480</td>
<td>52406</td>
</tr>
<tr>
<td>C</td>
<td>847–1347</td>
<td>265–432</td>
<td>4220</td>
<td>65732</td>
</tr>
<tr>
<td>D</td>
<td>1246–1746</td>
<td>399–565</td>
<td>4715</td>
<td>48644</td>
</tr>
<tr>
<td>E</td>
<td>1507–2007</td>
<td>486–652</td>
<td>4922</td>
<td>22984</td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>2008–2508</td>
<td>1–167</td>
<td>4088</td>
<td>15666</td>
</tr>
<tr>
<td>G</td>
<td>2392–2007</td>
<td>134–300</td>
<td>4447</td>
<td>18632</td>
</tr>
<tr>
<td>H</td>
<td>2509–3006</td>
<td>266–433</td>
<td>3480</td>
<td>18274</td>
</tr>
<tr>
<td>I</td>
<td>2902–3474</td>
<td>400–488</td>
<td>3900</td>
<td>34162</td>
</tr>
<tr>
<td>N</td>
<td>43–1330</td>
<td>1–428</td>
<td>3980</td>
<td>74010</td>
</tr>
<tr>
<td>–</td>
<td>pCMVi</td>
<td>–</td>
<td>4855</td>
<td>15128</td>
</tr>
</tbody>
</table>

* Construct identical to construct described in Bharadwaj et al. (1999).
challenged with SN virus. We prepared strip immunoblot assays (SIA) as previously described (Bharadwaj et al., 2000; Botten et al., 2000a). Briefly, we suctioned the following materials onto separate lanes of an 8 x 10 cm nitrocellulose membrane using a ‘slot blot’ maker, from top to bottom: (1) a Coomassie blue solution to allow orientation of the strips; (2) a dilution of deer mouse serum that contained sufficient IgG antibodies to produce a dark grey (‘3 +’) reactivity with the alkaline phosphatase-conjugated anti-Peromyscus leucopus conjugate upon development; (3) 1:8 µg of affinity-purified recombinant SN virus N antigen; and (4) a more dilute deer mouse serum solution to show a light grey (‘1 +’) reaction with the conjugate antibody upon development (Botten et al., 2000a). We then cut the nitrocellulose membrane lengthwise into 1 cm wide strips with a paper shredder. We incubated each strip with a deer mouse serum (1:200 dilution) in a Western blot tray overnight, and then detected the bound antibody with the anti-P. leucopus IgG conjugate, followed by an alkaline phosphatase substrate. In a few cases where antibody reactivity was slight, we used a Western blot test to verify the results of the SIA (Yamada et al., 1995).

Genetic immunization and virus challenge. We purified plasmid DNA with an endotoxin-free kit (Endofree, Qiagen), and dissolved DNA to a concentration of 1 mg/ml in 0.9% NaCl. Five to twelve deer mice were immunized with each construct three times at 4 week intervals, using 50 µg of plasmid into each set of quadriceps muscles for a total of 100 µg. No adjuvants were used.

To compare immunological responses to vaccination as well as protective efficacy of each vaccine construct, we used two experimental groups: an immunology replicate and a challenge replicate. Both of the experimental groups were subjected to immunization with glycoprotein gene fragments, the intact N gene or vector alone, followed by two identical boosts. In the immunology replicate, we did not subsequently challenge with SN virus, but we instead sacrificed the mice for examination of splenocyte proliferative responses to each bacterially expressed antigen and neutralizing antibody responses to the vaccine. In the challenge replicate, we subsequently subjected the mice to virus challenge. Mice in the immunology replicate were killed 2 weeks after the second booster vaccination and spleens and blood samples were collected. All of the blood samples from the immunology replicate were tested for neutralizing antibodies, whereas splenocytes were used for assays for proliferation in the presence of bacterially expressed cognate antigen.

After moving the immunized mice to the outdoor biocontainment laboratory, we challenged the mice in the challenge replicate with 5 ID₅₀ of SN77734 by the i.m. route 2 weeks after the third vaccination, a dose that corresponds roughly to 50–200 focus-forming units (Botten et al., 2000a,b). We use animal ID₅₀ to measure virus infectivity rather than plaque-forming units because it is the most relevant unit for infectivity for the studies in question, and because we have established that animal-passaged SN virus is qualitatively different from virus that has been passed in tissue culture (unpublished data). This regimen led to infection of 100% of unimmunized deer mice. Four weeks after challenge, we killed the mice with tribromethanol. We tested blood samples for antibodies using the SIA (Bharadwaj et al., 2000). In addition, we prepared RNA from 10–50 mg of heart tissue for nested RT–PCR assays using primers either in the N gene (for mice immunized with M segment fragments or control mice) or in the G1 gene for mice immunized with the N gene (Botten et al., 2000a; Hjelle et al., 1994). The purified RNAs were diluted with 2 µl water for each mg of tissue that was used in its preparation, and 5 µl of RNA was used in nested RT–PCR reactions. The nested PCR assay we used has been an extremely sensitive measure of infection in our hands, with no false positive assays in more than 5 years. The sensitivity is under 10 copies of RNA template per reaction (unpublished data).

Antibody testing. SIA and FRNT tests were as described (Bharadwaj et al., 1999; Botten et al., 2000a). The SIA was conducted at a 1:200 serum dilution and the FRNT screen at 1:40. The SIA was considered positive if the N antigen band became darkened after development, whereas a serum was considered to be positive in the FRNT if it demonstrated a reduction of 80% or more of foci.

For the FRNT, we screened sera from the immunology replicate animals at a 1:40 dilution, as described previously (Bharadwaj et al., 1999). Briefly, we mixed 5 µl of deer mouse serum that had been diluted with 95 µl of medium with an equal volume of medium containing 45–80 focus-forming units of SN virus strain CC 107 (a generous gift of C. Schmaljohn) and incubated for 1 h at 37 °C, and then used the mixture to infect a confluent monolayer of Vero E6 cells in duplicate wells of a 48-well dish, using a 1:2% methylcellulose overlay in the medium to confine the virus to foci. After 1 week, we detected the viral foci with polyclonal rabbit anti-N antibody followed by peroxidase-conjugated goat anti-rabbit IgG.

Cellular proliferation assay. Spleen cell preparation and splenocyte proliferation assays were performed by analysis of [³H]thymidine uptake as described (Bharadwaj et al., 1999). We used 10 µg/ml of cognate antigen for in vitro stimulation, based upon our previous dose optimization data from the BALB/c model. The proliferative capacity of splenocytes used in our experiments was verified by stimulation with 10 µg/ml of concanavalin A. When we developed this assay for BALB/c mice, we demonstrated that splenocytes derived from a mouse that was immunized with a particular construct did not proliferate in the presence of the cognate antigen expressed from another non-overlapping vaccine construct, indicating that the proliferation was specific for a particular peptide that was indeed expressed in the immunized deer mouse. We stimulated splenocytes from control mice immunized with vector alone with the amino-terminal glycoprotein peptide ‘A’.

Statistical analyses. We compared the splenocyte proliferation indices of vaccinated mice with those of mice that received the CMV vector alone by two-way ANOVA, and the protective efficacy of the various constructs using Fisher’s exact test.

Results

Two experimental groups of rodents were used as described in Methods. One group was used solely to determine the immunological responses to vaccination, another to determine the degree of protection afforded by a particular vaccine construct.

Immunology replicate

Ten groups of five deer mice were examined for in vitro immunological responses to inoculation with SN virus gene fragments, in part to allow comparison with previous studies with the inbred Mus musculus strain BALB/c (Bharadwaj et al., 1999). The vaccination regimen was identical to that used in the BALB/c model: after we immunized the deer mice, we collected spleens and serum from each animal 2 weeks after the last boost.

Splenocyte proliferative responses induced by cognate antigen differed markedly from construct to construct. Both N antigen and G1 or G2 peptides induced a similar degree of
Table 2. Fraction of deer mice infected by SN virus challenge

<table>
<thead>
<tr>
<th>Gene</th>
<th>Construct</th>
<th>Fraction infected*</th>
<th>P value (Fisher’s exact)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>A</td>
<td>1/9 (11%)</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3/12 (25%)</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0/6 (0%)</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1/6 (17%)</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>7/10 (70%)</td>
<td>0.2105</td>
</tr>
<tr>
<td>G2</td>
<td>F</td>
<td>3/5 (60%)</td>
<td>0.1099</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>9/9 (100%)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>9/10 (90%)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>6/7 (66%)</td>
<td>0.4400</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>1/5 (20%)†</td>
<td>0.04995</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>9/9 (100%)</td>
<td>–</td>
</tr>
</tbody>
</table>

* Infectious status determined by strip immunoblot assay (SIA) and reverse transcription–PCR.
† Four out of five positive by SIA, but negative by PCR.

Splenocyte proliferation from genetically immunized deer mice in comparison to BALB/c mice (Table 1). The comparisons between the results of Bharadwaj et al. (1999) and the present study indicate that inbred BALB/c M. musculus and outbred P. maniculatus do not differ dramatically in splenocyte proliferative responses to SN virus antigens delivered through genetic immunizations.

Four of the subjects from group N were examined for antibodies to SN virus N antigen by SIA (data not shown). Like the results of Bharadwaj et al. (1999), some (four) deer mice produced an antibody response against N antigen in response to the genetic vaccine, with an endpoint titre of at least 1:10000. No neutralizing antibody response to immunization with glycoprotein segments was evident by FRNT, probably reflecting the inability of the short peptide products to fold into conformations that mimic the intact glycoproteins of the virus.

We were unable to examine the antibody responses produced by the immunized deer mice against the partially purified G1 and G2 fragments, as described in our previous work, because we encountered much higher background and lower signal with the immunized deer mice than with the BALB/c mice (Bharadwaj et al., 1999).

**Challenge replicate**

The results of the challenge experiments are summarized in Table 2, and typical data are depicted in Fig. 1. We assessed infection by (1) seroconversion to N antigen by SIA, and (2) RT–PCR detection of SN virus RNA in the heart. With the glycoprotein constructs, there was a close correspondence between seroconversion in the SIA and the detection of SN virus S segment RNA in the heart of challenged mice. In a few cases, anti-N antibody reactivities were slight by the SIA but their presence could be confirmed in a Western blot test (data not shown) using N antigen as target (Table 2, groups G and I). SN virus S segment RNA could be detected in the hearts of those specimens, confirming that they had become infected. Ultimately, there were no discrepancies in the comparisons between the SIA test and RT–PCR analysis.

Constructs designed to express glycoprotein antigens differed markedly in their ability to protect deer mice from subsequent viral challenge, with protective efficacies ranging from 0 to 100%. The most active constructs were A through D (Table 2), which protected 75% to 100% of deer mice from infection, as well as the N gene. For the glycoprotein vaccines and the N vaccine, there was a strong correlation between the splenocyte proliferation in the presence of cognate antigen and the fraction of deer mice that were protected from subsequent SN virus challenge (Fig. 2).

**Protective efficacy of N gene**

Since some mice immunized with the N gene construct produced an antibody response to N antigen, the SIA was not...
useful in assessing infection. For this group, we assessed infection by RT–PCR with primers from the G1 gene of SN virus. (Hjelle et al., 1994). In this group, one of the five mice was positive by RT–PCR using primers in G1, indicating that the N construct vaccine was 80% protective against subsequent challenge.

Discussion

Vaccines against SN virus

A vaccine prepared against any single hantavirus might not be able to provide protection against every member of the antigenically diverse genus Hantavirus. Early vaccines were produced from inactivated virus produced in rodent brains, but immune responses have been short-lived (Cho & Howard, 1999). Recent efforts to develop hantavirus vaccines have included genetic vaccine approaches as well as subunit vaccines utilizing purified proteins or vaccinia delivery systems (Chu et al., 1995; Hooper et al., 1999; Schmaljohn et al., 1992). Clinical trials using recombinant vaccinia virus vaccines did not consistently produce immune responses in vaccinia-immune volunteers (McClain et al., 2000). Recently, investigations using hamster and other rodent challenge models have shown that genetic vaccines can be effective against the agents of HFRS, but no similar progress has been noted with agents of HCPS. In a hamster model, however, it has recently been possible to demonstrate that hantaviruses that are agathogenic in that species, including Hantaan and SN viruses, confer protection from disease by a virus that produces pulmonary oedema, Andes virus (Hooper et al., 2001). The slow progress with vaccines against the agents of HCPS could be related to the need for biosafety level 4 containment for challenge experiments, to difficulties establishing animal models, and/or to difficulties in obtaining high-level expression of the envelope glycoproteins of SN virus and other agents of HCPS.

Efficacious hantavirus vaccines can be prepared from short genomic segments

Most studies of hantavirus vaccines have associated protective efficacy with the induction of neutralizing antibodies. However, some investigations have shown partial or complete protection using the viral nucleocapsid antigen as immunogen, which does not elicit neutralizing antibodies (Lundkvist et al., 1996; Schmaljohn et al., 1990; Ulrich et al., 1998). By contrast, a role for T cells in protective immunity has been little-studied, but adoptive transfer studies have suggested that T cells can confer protection to a naïve host (Yoshimatsu et al., 1993). Previously, it has been possible to localize the protective epitopes within a peptide vaccine through deletion analysis (Lundkvist et al., 1996). Since we have previously experienced difficulties obtaining adequate expression of the envelope glycoproteins of SN virus in eukaryotic systems, we chose to scan the G1 and G2 genes for protective epitopes by introducing the genes in small fragments that were known to be competent for expression (Bharadwaj et al., 1999). That the glycoprotein gene segments were in fact being expressed was demonstrated by splenocyte proliferation in the presence of peptide after vaccination, an effect that was specific for those mice that had received the genetic vaccine encoding that cognate antigen.

The deer mouse system was favoured over the better-characterized M. musculus for several reasons. First, hantavirus models using M. musculus are generally unsatisfactory, allowing only transient and generally fatal infections of the central nervous system in neonates (Tsai et al., 1982; Ebihara et al., 2000). Second, outbred deer mice are the natural reservoir host for SN virus and thus were taken as the model that is more reflective of the normal host–pathogen relationship. Furthermore, use of the deer mouse model allows the possibility for later studies of the modes of transmission, persistence/reactivation, tissue and host tropism, and clearance of the virus in its natural reservoir. It is possible that the recent advent of the hamster infection model for SN and Andes viruses will overcome some of these difficulties (Hooper et al., 2001).

Our data show that SN virus glycoprotein and N gene segments induce similar proliferative responses of splenocytes and that the N gene segment elicits similar anti-N antibody responses in outbred deer mice and M. musculus, although a few of the less immunogenic segments differed slightly between the species (Bharadwaj et al., 1999). As judged by polyclonal splenocyte proliferation, some strongly immunogenic gene segments are capable of eliciting immune responses in disparate species and in disparate MHC backgrounds. For this series of experiments, we elected to establish a higher cut-off dilution (1:40 with an 80% reduction of foci) for detection of neutralizing antibody responses than we did with our
previous experiments with BALB/c mice, because we wanted to have greater confidence that the neutralization responses we might detect were important in protection. Using the 1:40 screening dilution, no evidence for such (neutralizing) response was detected, nor would such a response be evident using that standard in the BALB/c model.

The N gene conveyed protection in deer mice, as did several small peptides derived from G1 and G2. The basis for protection by the small glycoprotein fragments has yet to be determined. The induction of neutralizing and non-neutralizing antibodies by these small gene segments is slight, as one might expect given their likely poor reproduction of the native folding of the intact G1/G2 complex (Wang et al., 1992). Because in preliminary data (not shown) non-neutralizing antibodies to the glycoproteins were difficult to distinguish from nonspecific reactivity in deer mice we could not study those antibodies in this model. There appears to be a good correlation between the splenocyte proliferation responses and those antibodies in this model. There appears to be a good correlation.

The basis of protection by the short segments utilized herein is due to the protective efficacy of each vaccine construct. It is possible that the glycoprotein segments are processed by the class I pathway and elicit CD8 cell immunity, through either production of antiviral mediators or via cytotoxic mechanisms. The basis of protection by the short segments utilized herein will require additional work to clarify, and may require the development of a cytotoxic T cell assay involving inbred strains of deer mice, and/or reagents capable of detecting deer mouse cytokines such as interferon-γ.

By identifying multiple apparently independent targets for sterilizing immune responses, we may be able to develop synergistic or additive polyvalent vaccine preparations, which may also allow the incorporation of homologous segments from other hantaviruses. To test such cocktails it may be necessary to use a higher challenge dose of virus to increase the failure rate in the protective gene segments from G1 and N.

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