DNA vaccine coding for the full-length infectious Kunjin virus RNA protects mice against the New York strain of West Nile virus

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A plasmid DNA directing transcription of the infectious full-length RNA genome of Kunjin (KUN) virus *in vivo* from a mammalian expression promoter was used to vaccinate mice intramuscularly. The KUN viral cDNA encoded in the plasmid contained the mutation in the NS1 protein (Pro-250 to Leu) previously shown to attenuate KUN virus in weanling mice. KUN virus was isolated from the blood of immunized mice 3–4 days after DNA inoculation, demonstrating that infectious RNA was being transcribed *in vivo*; however, no symptoms of virus-induced disease were observed. By 19 days postimmunization, neutralizing antibody was detected in the serum of immunized animals. On challenge with lethal doses of the virulent New York strain of West Nile (WN) or wild-type KUN virus intracerebrally or intraperitoneally, mice immunized with as little as 0.1–1 μg of KUN plasmid DNA were solidly protected against disease. This finding correlated with neutralization data *in vitro* showing that serum from KUN DNA-immunized mice neutralized KUN and WN viruses with similar efficiencies. The results demonstrate that delivery of an attenuated but replicating KUN virus via a plasmid DNA vector may provide an effective vaccination strategy against virulent strains of WN virus.

West Nile (WN) virus is a mosquito-transmitted flavivirus that produces a potentially fatal disease in humans and horses and has traditionally been associated with viral outbreaks in Europe and Africa (1). In 1999, WN virus appeared for the first time in the New World, associated with an outbreak of a fatal or debilitating disease in humans and equines and extremely high levels of morbidity and mortality in several species of native birds in New York (2). Since its introduction into North America, WN virus has spread to most of the United States and to parts of Canada and Mexico via mosquito–bird transmission cycles (ref. 3; www.cdc.gov/ncidod/dvbid/westnile/index.htm). There is now an urgent need for an effective vaccine for veterinary and medical prophylaxis.

Kunjin (KUN) virus is a genetically stable Australian flavivirus originally shown to be very closely related antigenically (by virus neutralization tests and monoclonal antibody binding studies) to the Sarafend strain of WN virus (4, 5) and genetically to the prototype Ugandan strain (6, 7). When the North American WN virus strain NY99 was isolated, the data provided by Lanciotti *et al.* (2, 7) revealed 98–99% amino acid homology with KUN virus throughout the coding sequence, compared with 93% homology for the Ugandan strain. Because of the homology with WN NY99 and other lineage 1 strains of WN virus (8), KUN virus was arbitrarily reclassified as a subtype of WN virus by the International Committee on Taxonomy of Viruses (9). However, unlike WN virus, KUN produces only rare nonfatal cases of human and equine disease (10). Indeed, comparative studies of the New York WN strain and KUN virus have revealed that 1,000- to 10,000-fold more infectious virus of the latter is required to produce disease in adult mice by peripheral inoculation (11).

Recently we succeeded in generating a plasmid DNA (pKUN1) encoding full-length KUN cDNA under the control of the cytomegalovirus (CMV) promoter and demonstrated that transfection of this DNA into BHK cells resulted in the recovery of an infectious KUN virus (12). Inclusion in pKUN1 DNA of a mutation in the KUN NS1 gene, which we have previously shown to reduce KUN virulence in mice (13), ensured significant attenuation of pKUN1-derived virus. Therefore, we predicted that injection of pKUN1 DNA into selected tissues of mice would produce limited virus amplification in the animals and induce protective immunity against KUN virus and the closely related New York strain of WN virus without causing disease. Using plasmid DNA encoding a functional viral genome as a vaccine eliminates a number of concerns associated with preparations of live viral vaccines, including possible contaminations with endogenous viruses during preparation of vaccine in cell cultures and the instability of enveloped viruses at ambient temperatures. Only one report of using plasmid DNA encoding infectious full-length genomes of an RNA virus as a vaccine has been published to date. Ward *et al.* (14) demonstrated partial protection against foot-and-mouth disease virus (FMDV) in swine immunized with DNA coding for a full-length infectious FMDV genome. Here we describe the use of plasmid DNA directing *in vivo* transcription of the full-length infectious KUN viral RNA for vaccination of mice to assess the induction of a protective immune response against the wild-type KUN virus and the virulent New York strain of WN virus.

Materials and Methods

Cell Culture and Virus Preparations. Vero cells were grown in Hepes-buffered Medium 199 (GIBCO) supplemented with antibiotics and 10% FBS and incubated at 37°C. C6/36 cells were cultured in Medium 199 supplemented with antibiotics and 10% FBS and incubated at 28°C and 5% CO_2. For virus stock production, Vero cells were infected with KUN virus (MRM61C strain; ref. 4), WN virus (NY99-4132 strain, obtained from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, CO), or FLSD KUN virus (derived from KUN cDNA clone FLSD; refs. 13 and 15) at a multiplicity of infection of 0.1–1 and cultured in medium supplemented with 2% FBS. Culture supernatant was harvested and clarified at 72–96 h postinfection when 50–70% of cells showed cytopathic effects (CPE). The concentration of infectious virus in stocks was determined by titration on Vero cells in 96-well plates and calculated as ID_{50} per ml (13). One ID_{50} is equivalent to 1 infectious unit (i.u.).

Plasmid DNA Constructs. Construction of plasmid DNAs pKUN1, coding for the infectious full-length KUN RNA, and

Abbreviations: KUN, Kunjin; WN, West Nile; i.c., intracerebral(ly); i.u., infectious unit(s).

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pKUN1dGDD, coding for the nonreplicating full-length KUN RNA, was described previously (12). In these plasmids, the KUN cDNA sequence is placed under the control of cytomegalovirus early promoter-enhancer region (CMV) to allow *in vivo* transcription of KUN RNA by the cellular RNA polymerase II. The plasmids also have the hepatitis delta virus ribozyme sequence (HDVr) inserted immediately after the last nucleotide of KUN cDNA sequence to ensure production of KUN RNAs with the precise 3′ terminus, which was shown to be beneficial for more efficient RNA replication (16). The KUN genome in pKUN1 and pKUN1dGDD plasmids is derived from the full-length cDNA clone FSDX, which has recently been fully sequenced (ref. 17; GenBank accession no. AY274504). The sequence of FLSDX and FLSD cDNA clones is the same and both contain a Pro to Leu substitution at amino acid 250 in the NS1 gene. The FLSDX clone has six other conservative amino acids substitutions compared with the published sequence of MRM61C strain of KUN virus (17) that did not appear to affect the growth properties of recovered virus.

Mouse Immunization and Challenge. Groups of 5–12 BALB/c mice 4–5 weeks of age were immunized with a total of 0.1, 1, or 10 μg of pKUN1 DNA injected i.m. in two sites of the thigh muscles. Groups of mice were similarly immunized with 1 or 10 μg of defective DNA (pKUN1dGDD). An additional group was immunized i.p. with a sublethal dose of 10^3 i.u. of attenuated FLSD virus (contains the same genomic sequence as pKUN1 progeny virus) and one group remained unimmunized. At 21 days postimmunization, mice from each group were challenged intracerebrally (i.c.) with 10^3 i.u. of the wild-type KUN virus (MRM61C strain). Similarly, immunized mice were also challenged with 20 i.u. of WN virus (NY99-4132 strain) i.c. or i.p.

The appropriate challenge doses for KUN and WN viruses in 7- to 8-week-old BALB/c mice were previously determined in a pilot study (results not shown). It was observed that i.p. inoculation of 20 i.u. of WN virus was sufficient to produce clear disease symptoms (severely ruffled fur and lethargy) in all animals by 7–8 days postinfection. Of these, ≈60–70% progressed to a more severe form of disease (flaccid hind leg paralysis, hunched posture, moribundity) and were killed. In contrast, relatively high doses of the wild-type KUN virus inoculated i.p. did not produce consistent rates of morbidity or mortality in these mice. Intracerebrally, 20 i.u. of WN and 1,000 i.u. of KUN viruses were sufficient to produce 80–100% mortality in inoculated mice.

Detection of KUN Virus in Immunized Mice. For virus detection, three mice from each group were tail bled on days 3, 4, 5, 6, and 7 postimmunization under aseptic conditions, and blood was allowed to clot overnight at 4°C. The serum was then separated, snap frozen, and stored at −70°C until tested. On the day of analysis sera were rapidly thawed then kept on ice. Twenty-five microliters of pooled undiluted sera was inoculated in duplicate onto fresh monolayers of C6/36 cells in 96-well plates. After 7 days inoculation the culture supernatant was then carefully removed and replaced with an equal volume of 20% acetone in PBS and the cells were allowed to fix for 2 h at 4°C. The fixative was then removed and the plates were dried at 37°C. Viral antigens were then detected by ELISA (18) using anti-KUN rabbit antiserum (19). Supernatants from inoculated C6/36 cells were further passaged onto fresh monolayers of C6/36 cells and the process was repeated to detect progeny virus.

Measurement of Serum Antibody Responses. For detection of virus-specific antibody in sera, mouse blood was collected by tail bleed at 19 days postinoculation and held at 4°C overnight to clot, and serum was separated and snap frozen at −70°C until tested. Sera from each mouse group were pooled and titrated in doubling dilutions in fixed-cell ELISA against the viral proteins of wild-type KUN virus and WN virus as described (20). The reciprocal of the serum dilution that produced an OD of at least 0.3 on viral antigen and at least 0.2 higher than that produced on control antigen (fixed uninfected cells) was deemed the ELISA titer of each sample.

Table 1. Isolation of KUN virus from mouse sera collected at various times postinjection with KUN DNAs or with attenuated FLSD virus

	Days postimmunization*				
Immunogen	3	4	5	6	7
FLSD virus	++[†]	++	+	−	−
0.1 μg of pKUN1	−	++	−	−	−
1 μg of pKUN1	+	−	−	−	−
10 μg of pKUN1	−	−	−	−	−
10 μg of pKUN1dGDD	−	−	−	−	−
Unimmunized	−	−	−	−	−

*Attenuated FLSD virus (1,000 i.u.) was injected i.p., and DNAs were injected i.m.
[†]Virus was detected in both (++) or one (+) of duplicate wells of C6/36 cells after secondary passage from cultures inoculated with pooled serum from three mice.

Microneutralization Assays. Sera samples were tested for neutralization of KUN and WN viruses by microneutralization assay as described (21). Briefly, sera from each mouse group were pooled, heat-inactivated at 56°C, and serially diluted 2-fold in cell growth medium. Twenty-five microliters of each dilution was then added in duplicate to wells of a 96-well culture plate. An equal volume of growth medium containing ≈100 i.u. of virus was then added to each well, and plates were allowed to incubate at 37°C with occasional gentle agitation. Fifty microliters of growth media containing ≈10^4 Vero cells was then added to each well and plates incubated at 37°C for 5 days. The reciprocal of the serum dilution that inhibited the formation of viral cytopathic effects was deemed the neutralization titer.

Results

Injection of pKUN1 DNA Induces Low-Level Viremia in Mice. We showed previously that transfection of pKUN1 DNA directing transcription of replication-competent full-length KUN RNA but not of pKUN1dGDD DNA directing transcription of replication-deficient full-length KUN RNA into BHK cells resulted in production of secreted infectious KUN virus (12). In this section we examined whether injection of pKUN1 DNA into mice would lead to production of infectious virus *in vivo*. Groups of mice were injected i.m. with different doses of pKUN1 DNA or injected i.p. with a sublethal dose (1,000 i.u.) of attenuated KUN FLSD virus. FLSD virus was prepared in BHK cells transfected with FLSD RNA (13) that has a genomic sequence identical to that expected to be present in the pKUN1-derived virus. The sera from injected mice were tested for the presence of infectious KUN virus between 3 and 7 days postinjection, by inoculation of C6/36 and Vero cell cultures.

KUN virus was isolated after the second passage of culture supernatant from mosquito cells initially inoculated with undiluted mouse serum collected at 4 days postinjection with 0.1 μg of pKUN1 DNA and at 3 days postinjection with 1 μg of pKUN1 DNA (Table 1). In the group injected i.p. with FLSD, progeny virus was detected after secondary passage of mouse serum samples collected 3, 4, and 5 days after FLSD injection. No virus was isolated from mice that received 10 μg of pKUN1 DNA or 10 μg of pKUN1dGDD (defective) DNA. Although serum samples were not titrated, the observation that virus was not detected in the first passage on C6/36 cells of undiluted mouse sera collected from each experimental group indicated that only

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Table 2. Detection of antibodies to KUN and WN viruses in sera from immunized mice by ELISA and virus neutralization assays

	Neutralization titers		ELISA titers	
Mouse group	KUN	WN	KUN	WN
Unimmunized	<10	<10	<40	<40
10 μg of pKUN1dGDD	<10	<10	<40	<40
0.1 μg of pKUN1	10	10	160	80
1 μg of pKUN1	20	20	320	80
10 μg of pKUN1	20	20	320	80
1,000 i.u. of FLSD	40	80	1,280	640

Mice were bled 19 days after a single immunization.

trace amounts of virus were present in samples positive on the second passage.

Analysis of virus recovered from animals vaccinated i.m. with pKUN1 DNA confirmed that the attenuating mutation at NS1 residue 250 was retained, along with the monomeric NS1 phenotype (results not shown). In addition, the nucleotide sequence spanning the entire NS1 gene (≈1,000 nucleotides) was identical with the corresponding sequence of the pKUN1 DNA used to immunize the animals, indicating that the viral RNA was transcribed from the plasmid with high fidelity *in vivo* (results not shown).

The data presented in this section demonstrate that i.m. injection of as little as 0.1 μg of pKUN1 DNA resulted in production of infectious, but nonlethal KUN virus in adult mice.

Immunization with pKUN1 DNA Induces a Neutralizing Antibody to the New York Strain of WN Virus. Nineteen days after immunization, mice were bled and sera from each group were pooled and tested for antibody to wild-type KUN virus in ELISA and by microneutralization assay. Sera from mice immunized i.p. with 1,000 i.u. of KUN FLSD virus showed the highest titer to KUN antigens in ELISA (1,280), whereas sera from mice receiving i.m. injection of 0.1, 1, or 10 μg of pKUN 1 DNA displayed ELISA titers of 160, 320, and 320, respectively (Table 2). KUN-specific antibody responses in unimmunized mice or mice receiving defective DNA could not be detected (ELISA titer <40). Neutralizing antibody to KUN virus was also detected in sera of mice immunized with pKUN1 DNA (titers of 10–20) or FLSD virus (titers of 40). Slightly higher levels of antibody as measured by ELISA and virus neutralization correlated with a longer period of viremia in FLSD-immunized mice (Table 1).

Sera from mice immunized with FLSD virus and with KUN plasmid DNAs were then tested for reactivity with viral antigens of the New York strain of WN virus in ELISA and for neutralization of the New York strain of WN virus *in vitro*. Slightly lower ELISA titers to WN antigen compared with that of KUN antigen were recorded for mice immunized with each dose of pKUN1 DNA (80) or FLSD virus (640). However, similar neutralizing titers were observed (Table 2).

Thus, the results presented in this section clearly demonstrate that immunization with pKUN1 plasmid DNA induces cross-reactive antibodies that are able to neutralize the New York strain of WN virus with efficiency similar to that observed for neutralization of KUN virus.

Immunization with pKUN1 DNA Protects Mice Against the New York Strain of WN Virus. In the first series of protection experiments we wished to establish whether immunization with pKUN1 DNA would protect mice against i.c. challenge with the wild-type strain of KUN virus. Three weeks after a single immunization with pKUN1 or pKUN1dGDD DNAs or with attenuated FLSD virus, mice were challenged i.c. with 1,000 i.u. of the wild-type KUN virus (MRM61C strain). Animals immunized with 1 or 10 μg of pKUN1 DNA or with 1,000 i.u. of FLSD virus were solidly protected from challenge (0–20% mortality), whereas unimmunized mice or those receiving defective pKUN1dGDD DNA were highly susceptible to the challenge (80% mortality) (Table 3). Survival of two-fifths (60% mortality) of mice immunized with 0.1 μg of pKUN1 DNA indicated partial protection, but this result was not statistically significant.

To determine whether immunization with KUN DNA and attenuated FLSD virus would also protect mice from WN virus, immunized animals were challenged i.p. or i.c. with a lethal dose (20 i.u.) of the virulent New York strain (NY99-4132). Mice immunized with FLSD or 1 μg of pKUN 1 DNA were completely protected (0% mortality) against i.c. challenge with WN virus, whereas 0.1 μg of pKUN1 DNA induced partial protection (Table 3). On the other hand, unimmunized mice or those immunized with the defective KUN DNA were highly susceptible (90% mortality). Similarly, mice immunized with FLSD virus or pKUN 1 DNA were solidly protected from morbidity or mortality compared with controls when challenged i.p. with WN virus (Table 3).

Discussion

In a previous report we showed that immunization of mice with plasmid DNA coding for a self-replicating subgenomic (replicon) KUN RNA expressing heterologous cytotoxic T lympho-

Table 3. Protection of immunized mice from lethal challenge with NY99 WN and KUN viruses

	% Morbidity and mortality after i.p. challenge with NY99 WN virus (20 i.u.)*		% Mortality after i.c. challenge with KUN or NY99 WN viruses*	
Mouse group	% Morbidity† (sick/challenged)	% Mortality‡ (dead/challenged)	WN, 20 i.u. (dead/challenged)	KUN, 10^3 i.u. (dead/challenged)
Control (unimmunized)	100 (11/11)	64 (7/11)	89 (8/9)	80 (4/5)
10 μg of pKUN1dGDD DNA§	ND	ND	ND	80 (4/5)
1 μg of pKUN1dGDD DNA§	75 (9/12)	58 (7/12)	90 (9/10)	ND
0.1 μg of pKUN1 DNA§	16 (2/12)	8 (1/12)	33 (2/6)	60 (3/5)
1 μg of pKUN1 DNA§	8 (1/12)	8 (1/12)	0 (0/10)	0 (0/5)
10 μg of pKUN1 DNA§	ND	ND	ND	20 (1/5)
1,000 i.u. of FLSD virus¶	0 (0/12)	0 (0/12)	0 (0/10)	20 (1/5)

ND, not done.

*Mice were challenged i.p. or i.c. 3 weeks after a single immunization.

†Percentage of mice that developed symptoms of fever (severely ruffled fur and lethargy).

‡Percentage of mice that progressed to symptoms of encephalitis (hunched posture, flaccid hindleg paralysis, and moribundity).

§Mice were immunized i.m. with varying doses of pKUN1 or pKUN1dGDD DNA at 4–5 weeks of age.

¶Mice were immunized i.p. at 4–5 weeks of age.

cyte (CTL) epitopes resulted in induction of epitope-specific CTL responses (22). Here we demonstrate that immunization of mice with plasmid DNA encoding the attenuated but infectious full-length KUN RNA provides protective immunity against challenge with the wild-type KUN virus and, most importantly, against challenge with the highly pathogenic NY99 strain of WN virus. Apparently, replication of viral RNA produced in pKUN1 DNA-transfected cells and spread of the infectious progeny virus to nearby cells allow sufficient virus amplification in the inoculated animal to produce a protective immune response that is similar in magnitude to that observed during natural virus infection. However, the limited replication of the attenuated pKUN1 progeny virus and the low amounts of infectious virus observed in mouse serum would likely preclude virus transmission to arthropod vectors, thus making the vaccine safer.

Our inability to detect virus in serum between 3 and 7 days postimmunization with the highest dose (10 μg) of pKUN1 DNA suggests that a rapid, transient viremia may have occurred during the first 72 h before the testing period. This finding is consistent with our detection of virus at only a single time point in serum after immunization with 1 μg (day 3) and 0.1 μg (day 4) doses. In contrast, i.p. injection of FLSD virus resulted in a viremia of at least 3 days duration (days 3–5). The difference between the routes of injection for plasmid DNA (i.m) and virions (i.p.) may account for the observed differences in the longevity of viremia.

Although as little as 0.1–1 μg of pKUN1 DNA was sufficient to induce protective immunity, mice immunized with 1–10 μg of the defective plasmid DNA construct (pKUN1dGDD) failed to generate a detectable humoral response. This finding suggests that the amounts of viral proteins produced from a messenger KUN RNA transcribed under direction of a cytomegalovirus (CMV) promoter are insufficient to induce protective immunity, consistent with previous reports using DNA-based subunit flavivirus vaccines expressing the prM-E structural genes of Japanese encephalitis virus (23), Murray Valley encephalitis virus (24), and WN virus (25). These studies reported that 50–100 μg doses were required to elicit protective immunity by a single injection i.m. The efficacy of the WN subunit vaccine could be significantly enhanced by performing coelectrotransfer on the inoculated muscle tissue immediately after the injection (25). This procedure reduced the effective dose in mice to 1 or 0.1 μg, similar to the efficacy of the pKUN 1 DNA (injected into muscle without coelectrotransfer) observed in our study. It is also worth noting that a significant advantage of a vaccine expressing the full-length flavivirus genome over the prM-E subunit is that humoral responses to the NS1 protein and cell-mediated responses to NS3 epitopes may also contribute to more efficient viral clearance and protection (20, 26, 27).

In Australia, KUN virus is widespread and endemic in some areas. However, overt infection in man is extremely rare and nonfatal (10) demonstrating natural attenuation of this virus in humans. KUN virus has been extensively studied for over 30 years, and these studies, particularly in molecular biology of virus replication, provided major benchmarks for other flaviviruses (28). The neutralizing antibody responses to KUN virus and their cross-reactivity are well documented (4). Moreover, we have recently demonstrated that KUN virus is genetically and antigenically very closely related to the North American strain of WN virus (2, 8). Indeed, reactions of a panel of monoclonal antibodies produced to KUN virus have revealed that the majority of antibody binding sites on the viral E protein are conserved between the two viruses, including those determinants that elicit neutralizing antibodies (refs. 2 and 8, and R.A.H. and D.J.N., unpublished results). These data support the presented results showing that pKUN1- and FLSD-immunized mice were protected from both KUN and WN virus challenge and developed an antibody response that neutralized both viruses with similar efficiency *in vitro*.

Despite the high degree of homology between the two viruses, studies by Beasley *et al.* (11) have demonstrated that wild-type KUN virus is significantly less virulent in adult NIH Swiss mice than the New York strain of WN by peripheral inoculation. These findings agree with our inability to produce consistent mortality rates in adult BALB/c mice after inoculation with KUN virus i.p. (results not shown). The inclusion of a stable proline to leucine mutation at residue 250 in the NS1 protein, shown to attenuate KUN virus in mice (13), into the pKUN1 DNA vaccine backbone should result in further attenuation of the KUN virus-based vaccine, while retaining highly efficient protective immunity

Conclusions

The rapid spread of WN virus across North America during 2002 has resulted in numerous fatal cases of human and equine disease (ref. 3; www.cdc.gov/ncidod/dvbid/westnile/index.htm). Consequently, an effective and safe vaccination strategy against the virus is urgently being sought for both medical and veterinary purposes. The very close antigenic relationship that KUN virus shares with the North American strain of WN virus makes it an attractive vaccine candidate. The naturally attenuated phenotype, stable genetics, and well studied ecology, epidemiology, and molecular biology of KUN virus provide a solid background for vaccine derivation. The safety of a KUN-based vaccine is further enhanced by the inclusion of a well characterized, stable attenuative mutation. The presented results demonstrating that mice vaccinated with pKUN1 DNA were protected from a challenge with the lethal doses of the New York strain of WN virus suggest that immunization with KUN virus-based plasmid DNA may provide a rapid, convenient, and effective vaccination strategy against further outbreaks of WN virus.

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