An inactivated West Nile Virus vaccine derived from a chemically synthesized cDNA system

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ABSTRACT

A cDNA comprising the complete genome of West Nile Virus (WNV) was generated by chemical synthesis using published sequence data, independent of any preformed viral components. The synthetic WNV, produced by transfection of *in vitro* transcribed RNA into cell culture, exhibited undistinguishable biological properties compared to the corresponding animal-derived wild-type virus. No differences were found concerning viral growth in mammalian and insect cell lines and concerning expression of viral proteins in cells. There were also no significant differences in virulence in mice following intranasal challenge. After immunizations of mice with experimental vaccines derived from the synthetic and wild-type viruses, protection from lethal challenge was achieved with similar amounts of antigen. Both vaccine preparations also induced comparable levels of neutralizing antibodies in mice. In addition, the synthetic approach turned out to be very accurate, since the rescued WNV genome contained no undesired mutations. Thus, the first flavivirus based on chemical gene synthesis was indistinguishable from the parent virus. This demonstrates that virus isolates from animal sources are dispensable to derive seed viruses for vaccine production or research.

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1. Introduction

West Nile Virus (WNV) is a mosquito-borne, neurotropic member of the genus flavivirus, family *Flaviviridae*, and has been identified in Africa, Europe, the middle East, south and central Asia, Oceania (subtype Kunjin), and most recently North America (reviewed in [1]). In the U.S. WNV activity in human, bird, companion animals or mosquito has been reported since 1999 to the Centers for Disease Control (CDC) from almost all states. Besides WNV, the genus flavivirus comprises a number of medically important pathogens including Japanese encephalitis virus (JEV), yellow fever virus (YFV), tick borne encephalitis virus (TBEV) and the four serotypes of dengue virus (DENV) [2]. The flavivirus genome is a positive-polarity, single-stranded RNA molecule of about 11,000 nucleotides (nt), which functions as mRNA for translation of the viral proteins. Genomic RNA is infectious when introduced into susceptible cells by transfection [3]. For replication and pathogenesis studies, reverse genetic systems have been established for several members of the genus [4–20]. These systems comprise one or two plasmids encoding cDNA of viral genomic sequence under control of bacteriophage promoters allowing transcription of full-length

infectious RNA *in vitro*. For YFV [4], DEN-1 [17], DEN-2 [6,8,10], DEN-4 [11], TBEV [13,15], KUN [9], MVE [7] and WNV lineage I [19] and II [21], cDNA comprising the full genome was stably cloned into bacterial expression plasmids, whereas in other reports [5,8,13,18,20] cDNA was split in two fragments, each integrated in individual plasmids, from which cDNA can be fused together before RNA transcription. An alternative approach was applied to construct a JEV infectious clone, in which the viral coding sequence was put under the control of a eukaryotic promoter and split by introns to circumvent instability during propagation in bacteria [22].

Conventional generation of such cDNA clones requires the production of an initial virus stock, viral RNA isolation, reverse transcription, PCR amplification of subfragments and engineering into the final transcription units. These approaches are sometimes hampered by low fidelity of reverse transcriptase or sequence variations in the starting isolate, which may lead to undesired alterations of the genomic sequence. As a consequence, in most reports in which the viral cDNA clones or generated viruses were analyzed by sequence analysis, nucleotide variations were detected compared to the published sequence of the parent virus [6,7,9,13,14,16,19].

In 2002, a landmark publication proved the feasibility of *de novo* synthesis of a poliovirus by biochemical synthesis precluding any preformed components. The viral cDNA encoding the 7.5 kb genome was assembled from overlapping oligonucleotides and yielded infectious virus after transcription of genomic RNA and

inoculation into cell lysates [23]. Taking advantage of the rapid progression of gene synthesis technology (for review [24]), we intended to adopt such a synthetic approach to produce a flavivirus cDNA system for the generation of a synthetic WNV seed virus for use in vaccine development. In this study we report the generation of a fully functional WNV virus from a completely synthetic source. The whole 11,029-nucleotide WNV genomic sequence was generated by gene synthesis without using natural viral templates. The production and characterization of the resulting West Nile Virus, which fully matched the sequence of the *in silico* designed viral genome, confirms the feasibility and accuracy of the synthetic flavivirus reverse genetic system.

2. Materials and methods

2.1. Viruses and cell lines

WNV wild-type virus strain NY99-flamingo 382-99 was obtained from Centers for Disease Control (CDC, Atlanta) corresponding to GenBank accession #AF196835. This sequence information was also used as template for *in silico* design for *de novo* synthesis of the genomic cDNAs. The cell lines Vero (ATCC CCL-81), BHK (ATCC CCL-10) and C6-36 (ECEACC 123.P. #03D016) were obtained from the American Type Culture Collection or European Collection of Cell Cultures and grown in Dubecco's modified Eagle's medium (DMEM) or TC-Vero Media (Baxter). TC-Vero is an animal protein-free medium based on DMEM/Ham's F12 medium.

2.2. Cloning procedures

Six DNA fragments corresponding to WNV strain NY99-flamingo 382-99 (GenBank accession #AF196835) were generated by chemical synthesis (GENEART, Regensburg, Germany). Plasmid p5′TL-AB carried DNA corresponding to WNV genomic sequence nt 1–1792, plasmid p5′TL-CD to nt 1789–3632, plasmid p3′TL-AB to nt 3622–5801, plasmid p3′TL-CD to nt 5792–8028, plasmid p3′TL-EF to nt 8022–10,025 and plasmid p3′TL-GH to nt 10,022–11,029. Three silent mutations at positions 8859, 8862, and 8880 were introduced to knock out an EcoRI site and to create an StyI site as described previously [19].

2.2.1. pWNVsyn-5′TL

For assembly of plasmid pWNVsyn-5′TL (containing the 5′ third of the WNV coding sequence) an AscI and BsaI cut fragment of p5′TL-AB was ligated to a BbsI and PacI cut fragment of p5′TL-CD (Fig. 1b). The ligation product was amplified using high fidelity PCR (KOD, Invitrogen). Following digestion with BamHI and XbaI, the fragment was cloned into the low copy plasmid vector pBR322-PL (derived from plasmid pBR322 engineered to contain a matching polylinker between the unique restriction sites EcoR1 and EagI resulting in the partial deletion of the tet^R gene) yielding pWNVsyn-5′TL.

2.2.2. pWNVsyn-3′TL

This plasmid contains the 3′ two-thirds of the WNV coding sequence and was generated in three steps: (I) An AscI and BsaI cut fragment of p3′TL-AB was ligated to a BsaI and PacI cut fragment of p3′TL-CD. This fragment was amplified by high fidelity PCR and integrated into a commercially available pPCRscript vector (Clontech). (II) A BtgZI and AscI cut fragment of p3′TL-EF was ligated to a PacI and BtgZI cut fragment of p3′TL-GH. The resulting 3′TL-EFGH ligation product was amplified by PCR using 5′ and 3′ flanking primers, digested with SpeI and XbaI and integrated in pBR322-PL, leading to plasmid pBR322-3′TL EFGH (Fig. 1b). (III) The final pWNVsyn-3′TL was generated by introduction of the 3′TL-ABCD

fragment (derived from pPCRscript3′TL-ABCD) into pBR322-3′TL-EFGH, taking advantage of the unique restriction enzymes SpeI and BamHI (Fig. 1c). All plasmids were amplified in bacterial strain HB101 (Promega) and purified with commercially available systems (Omega and Qiagen). Electroporation of bacterial cells was carried out using a GenePulser Apparatus (Bio-Rad) with settings of 1.8 kV, 25 μF and 200 Ω. Sequence analysis was carried out using a 3130xl genetic analyzer (ABI) using BigDye Terminator v3.1 cycle sequencing Kit (ABI).

2.3. RNA transcription and transfection

pWNVsyn-3′TL was linearized with XbaI followed by mung bean nuclease digestion to remove single stranded nucleotide overhangs in order to generate the correct 3′ end of the WNV coding sequence. The plasmids pWNVsyn-3′TL and pWNVsyn-5′TL were then digested with SphI and the full-length sequence was generated by ligation (T4 Ligase; New England Biolabs) via the SphI sequence overhangs of the 5′ and 3′ parts. The ligated DNA fragments were extracted with phenol–chloroform twice, precipitated with ethanol and resuspended in nuclease free water. RNA was transcribed at 37 °C for 3 h from ligated template DNA by T7 polymerase transcription, using T7 MEGAscript Kit (Ambion). The integrity of RNA transcripts was analyzed in 1% agarose gels containing 6% formaldehyde. For RNA transfection, subconfluent vaccine-certified Vero cells were collected with trypsin, washed twice in serum free TC Vero Medium (Baxter) and twice in ice-cold PBS buffer. Aliquots of approximately 2×10^7 cells were resuspended in 800 μl of ice-cold PBS, mixed with transcribed RNA and transferred to 0.4 gene pulser cuvettes. Cells were electroporated with three successive pulses using a GenePulser Apparatus (Bio-Rad) with settings of 0.8 kV, 25 μF and 200 Ω.

2.4. Immunochemical staining of Vero cell

To visualize intracellular expression of WNV proteins, cells were infected or transfected. Two days later, cells were fixed with acetone–methanol (1:1). Cover slips with fixed cells were dried, rehydrated with phosphate-buffered saline and treated with a polyclonal mouse anti-WNV serum (1:50 dilution) obtained after immunization of mice with a formalin-inactivated whole virus vaccine preparation. Bound antibodies were visualized with fluorescein isocyanate-conjugated anti-mouse immunoglobulin (1:100 dilution; Jackson Research Laboratory).

2.5. Virus titrations and growth curves

Vero or C6/36 cells grown in 175 cm² tissue culture flasks were infected with either WNVsyn or WNVwt stock at an MOI of 0.0001. The inoculum was removed after 1 h, and 40 ml of fresh medium was added. At various time points (1, 6, 24, 48, 54, 72 and 96 h) 0.5 ml of medium was removed. The infectious virus titer of WNV containing samples was determined by a $TCID_{50}$ assay. In brief, serial 10-fold dilutions of virus containing supernatant were inoculated in 96-well microtiter plates seeded with Vero cells. After incubation for 7 days at 37 °C and 5% CO_2, the plates were screened under a light microscope for the presence of CPE in individual wells. From the number of CPE positive wells per dilution step, the $TCID_{50}$ was calculated according to the Poisson formula by means of an in house calculation software program.

2.6. cDNA synthesis

Viral RNA was extracted from supernatant containing viral material corresponding to 3×10^7 $TCID_{50}$ by TRIZOL extraction.

RNA was precipitated with ethanol and the RNA pellet was resuspended in $50\,\mu l$ of nuclease-free water. One μl of RNA was used for cDNA transcription using Superscript III cDNA synthesis Kit (Invitrogen) and primers binding in the $3'$ end of the NS5 coding region, the NS2B3 coding region and the $3'$ noncoding region.

2.7. Preparation of experimental vaccines

For the generation of inactivated whole virus vaccines, the WNVsyn and WNVwt stocks were amplified on BHK cells to serve as prime/boost antigen in animal studies. The WNVsyn preparation (designated CAg 4) as well as WNVwt preparation (designated CAg 6) was prepared in the same manner. Ten roller bottles of BHK cells were infected with a MOI of 0.0001. For better virus yields pH was adjusted to 7.5 after 1 h of virus adsorption. After 4 days of growth the supernatant was harvested and cleared through a low spin centrifugation step at 2500 rpm. The cleared supernatant was treated with formalin (final concentration 0.005%) for 48 h. Next, 30 ml of the inactivated virus was loaded on 5 ml of a 20% sucrose cushion per centrifugation tube (Beckman, SW28 tubes). After 2 h centrifugation with $104,000 \times g$ the supernatant was discarded and resulting pellets were pooled in Tris buffered saline (TBS). An aliquot of the resulting vaccine preparations was subjected to a safety assay to exclude any possible remaining infectivity. In order to normalize the administered amounts of antigen given in immunogenicity studies, the antigen content was determined in a WNV-specific antigen ELISA.

2.8. Mouse virulence and protection assays

The lethal dose 50 (LD_{50}) was determined in female 7-week-old Balb/c mice. Groups of six mice were infected intranasally with 1×10^1, 1×10^2, 1×10^3 and 1×10^4 $TCID_{50}$ of WNVsyn or WNVwt, respectively. Survival of mice was recorded for a period of 28 days after infection. The 10-fold virus dilutions were titrated shortly after challenge and were used to calculate the LD_{50} values using the computer program Graph pad Prism 5.

Protection was determined after immunization of female 7-week-old Balb/c mice by subcutaneous injections of formalin-inactivated WNVsyn or WNVwt vaccines in a volume of $100\,\mu l$ in TBS containing 0.2% $Al(OH)_3$. Mice were challenged intranasally with $10\,\mu l$ of PBS (0.01% human serum albumin) containing 2×10^5 $TCID_{50}$ WNVwt virus. Survival was monitored over a period of 28 days after challenge.

2.9. Microneutralization assay

For neutralizing antibody determination, serum samples were serially diluted with cell culture medium in twofold steps. The serum dilutions were mixed at a ratio of 1:1 with a virus stock suspension adjusted to 1×10^2 $TCID_{50}$, incubated for 90 ± 15 min at room temperature and transferred (eight replicates per dilution) to a 96-well microtiter plate seeded with Vero cells. The plates were inspected under a light microscope for the presence of CPE after incubation for 6 days at $37\,^{\circ}C$ and 5% CO_2. The neutralizing titer was calculated by counting CPE negative wells and by usage of the formula $\mu NT\text{-}Titer = (V/2) \times 2E((Nneg/8) + 0.5)$ whereas Nneg is the amount of negative wells and V represents the dilution of the sera in the neutralization mix. For each assay a defined serum positive control was measured and the titer of the viral material was titrated.

2.10. Safety assay

For detecting infectious viral material in formalin-inactivated WNV antigen preparations, Vero and C6/36 cells were seeded in five $175\,cm^2$ tissue culture flasks and inoculated with individual preparations corresponding to 12 ml of the infectious yield from which the preparations were derived. After a 10 day incubation period at $37\,^{\circ}C$ and 5% CO_2, supernatant of each flask was titrated by $TCID_{50}$ and 2 ml supernatant of each flask was carried onto fresh Vero and C6/36 cells. After a 10-day observation period supernatant of each flask was titrated by $TCID_{50}$. The respective antigen preparations were classified as safe, when no CPE was detectable in individual flasks and no viral material was detected in both $TCID_{50}$ assays.

2.11. Antigen ELISA

The amount of WNV antigen in respective samples was determined by means of an ELISA double sandwich system. Briefly, 96-well microtiter plates were coated by overnight incubation at $2–8\,^{\circ}C$ with an anti-WNV IgG polyclonal serum raised in guinea pigs. After subsequent washing steps, serial twofold dilutions of a standard West Nile Virus production material (WNV Peak Pool) with defined antigen amount, a control sample and individual samples were applied to the microtiter plate which was then incubated for 1 h at $37\,^{\circ}C$. After subsequent washing steps a mouse anti-WNV polyclonal serum was applied to the wells and incubated for 1 h at $37\,^{\circ}C$. After washing, the wells were incubated with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Immuno Research Laboratories) for 1 h at $37\,^{\circ}C$. After subsequent washing steps, substrate (o-phenylenediamine/H_2O_2) was added, and the enzyme reaction was stopped after 15 min at $37\,^{\circ}C$ by the addition of 0.25 M H_2SO_4. The absorbance at 490 nm was measured with an ELISA plate reader (BIO-TEK, Winooski, VT, USA) and the antigen content was calculated (KC4 software; BIO-TEK) by means of the standard curve derived from the dilution steps of the WNV Peak Pool standard material.

2.12. Ethics statement

All animal experiments were reviewed by the Institutional Animal Care and Use Committee (IACUC) and approved by the Austrian regulatory authorities and were conducted in accordance with Austrian laws on animal experimentation and guidelines set out by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were housed in facilities accredited by the AAALAC. All experiments with infectious virus were carried out under biosafety level 3 conditions. Experiments were approved by the Baxter internal biosafety committee and by the Austrian Ministry of Health (BMFG-76110/0002-IV/B/12/2005).

3. Results

3.1. Generation of a synthetic bipartite West Nile Virus cDNA system

For the construction of a bipartite infectious clone, six contiguous cDNA fragments encoding the genome of the lineage I WNV strain NY99 were chemically synthesized and integrated in bacterial expression plasmids (see Section 2) according to the cloning strategy outlined in Fig. 1. Three silent marker mutations were introduced (see also [19]) allowing the discrimination of the synthetic virus from the corresponding wild-type isolate (see Table 1). The six synthetically generated WNV subfragments were ligated stepwise, resulting in two plasmids with corresponding parts of the complete genomic WNV sequence. For this purpose, either unique restriction sites in the WNV sequence were used, or – where appropriate – asymmetric restriction sites were generated in the plasmid vector backbone adjacent to the WNV fragments. Cleavage of these asymmetric sites created overhangs in the WNV sequence
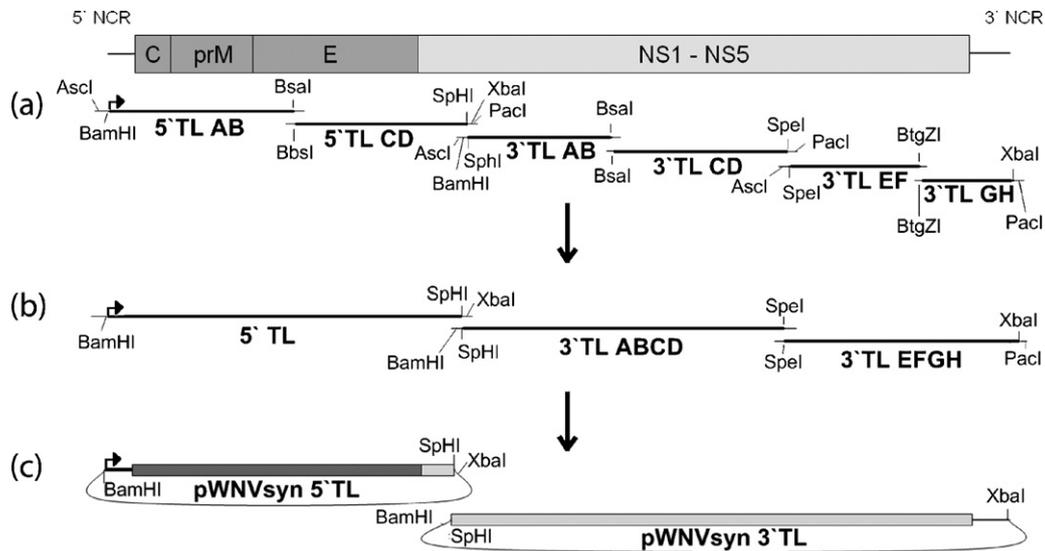
Fig. 1. Construction of the two partial cDNA clones. A schematic drawing of the WNV genome is indicated on the top. Dark gray boxes represent the viral structural and light gray boxes the nonstructural part of the coding sequence. The 5′ and 3′ noncoding regions (NCR) are indicated as thin lines. (a) Six WNV sequence segments were generated by gene synthesis (vector backbones are not shown). The 5′TL AB segment is corresponding to WNV genomic sequence nt 1–1792, 5′TL CD to nt 1789–3632, segment 3′TL AB to nt 3622–5801, 3′TL CD to nt 5792–8028, 3′TL EF to nt 8022–10,025 and 3′TL GH to nt 10,022–11,029. (b) Fragments 5′TL AB and 5′TL CD were ligated yielding the final 5′ partial clone insert (5′TL) and two 3′ part fragments were generated by fusing fragments 3′TL AB with 3′TL CD and 3′TL EF with 3′TL GH. (c) Final partial clones, integrated in pBR322, are indicated. The 3′ partial clone was generated by ligation of the 3′TL ABCD with the 3′TL EFGH insert, taking advantage of the unique SpeI restriction site.

by which corresponding fragments could be fused together. Following this strategy, two plasmids were generated, containing either the 5′ third (nt 1–3632 under control of a T7 promoter) or the 3′ two-thirds (nt 3622–11,029) of the WNV genomic sequence, designated as pWNVsyn-5′TL or pWNVsyn-3′TL, respectively. Each of the cloning steps was evaluated by complete sequencing of the cDNA insert and no undesired sequence alterations were observed. Further, in the final two plasmids no nucleotide alterations were found with the exception of the intended silent marker mutations.

3.2. Generation of synthetic infectious WNV RNA and expression of West Nile Virus proteins in Vero cells

To analyze the functionality of the cDNA system, RNA transcripts corresponding to the entire genome of WNV were generated. Full-length WNV cDNA templates were obtained by ligation of pWNVsyn-3′TL and pWNVsyn-5′TL. Genome length viral RNA was transcribed and the integrity of RNA transcripts was analyzed in 1% agarose gels containing 6% formaldehyde. An RNA band of approximately 11,000 nucleotides was obtained, indicating the presence of WNV full-length RNA (data not shown).

To characterize the ability of the transcribed RNA to replicate and to be translated after introduction in host cells, viral protein expression was examined by immunofluorescence (IF) staining. The WNVsyn RNA was electroporated into Vero cells which were subjected to indirect IF staining 2 days later (Fig. 2). Viral protein expression was monitored with a specific polyclonal mouse anti-WNV antibody and a FITC-conjugated second antibody (see Section 2). Cells infected with MOI 0.0001 of WNVwt and were used as

staining control. WNVsyn-transfected and WNVwt-infected Vero cells exhibited WNV protein expression in approximately 20% of all cells. As expected, viral antigen staining is mainly confined to perinuclear regions of the cells (Fig. 2). Immunofluorescence staining is only detectable from replication- and translation-competent viral templates and could not be shown in replication-deficient mutant viral genomes [19,25] thus proving the replication and protein expression capacity of the synthetic WNV genome.

In order to further analyze the genotypic and phenotypic properties, a stock of the synthetic WNV was produced. Confluent Vero cells were transfected as described above and upon onset of cytopathic effect (CPE) after 3 days, cell culture medium was harvested and the virus titer was determined on Vero cells, yielding a titer of 1.62×10^8 TCID$_{50}$/ml. Overlapping DNA fragments which cover the whole WNVsyn genomic coding region were amplified by PCR after cDNA transcription of isolated viral RNA. Sequencing confirmed that the rescued viral material contained no mutations compared to the *in silico* designed WNV genome and the presence of the engineered nucleotide changes proved the identity of the synthetic virus. In addition, in order to show IF staining behavior in Vero cells not only after transfection of RNA, cells were infected with MOI 0.0001 of WNVsyn and processed for IF as described above. As expected, the WNVsyn virus stock gave rise to a similar staining pattern as seen for the WNVwt stock (Fig. 2d).

3.3. WNVsyn exhibits the same growth kinetics and virulence as WNV wild-type virus

In order to analyze the growth properties of WNVsyn and WNVwt, one step growth curves were carried out. Susceptible mammalian (Vero) and mosquito (C6/36) cells were infected with a MOI of 0.0001. Viral titers, determined at the time points indicated in Fig. 3, demonstrate that in both cell types the growth kinetics of WNVsyn match exactly those of the wild-type virus. In addition, plaque morphology (Fig. 3a and b) and CPE (not shown) were comparable to the wild-type control. Virus grown in Vero cells peaked at 48 h posttransfection but declined from day 2 on correlating

Table 1
Engineered marker mutations of WNVsyn.

Nucleotide exchange	Position[a]	Amino acid change	Genomic location
C → A	8859	Silent	NS5
A → G	8862	Silent	NS5
A → G	8880	Silent	NS5

[a] Positions according to wild-type WNV genomic sequence GenBank accession #AF196835.
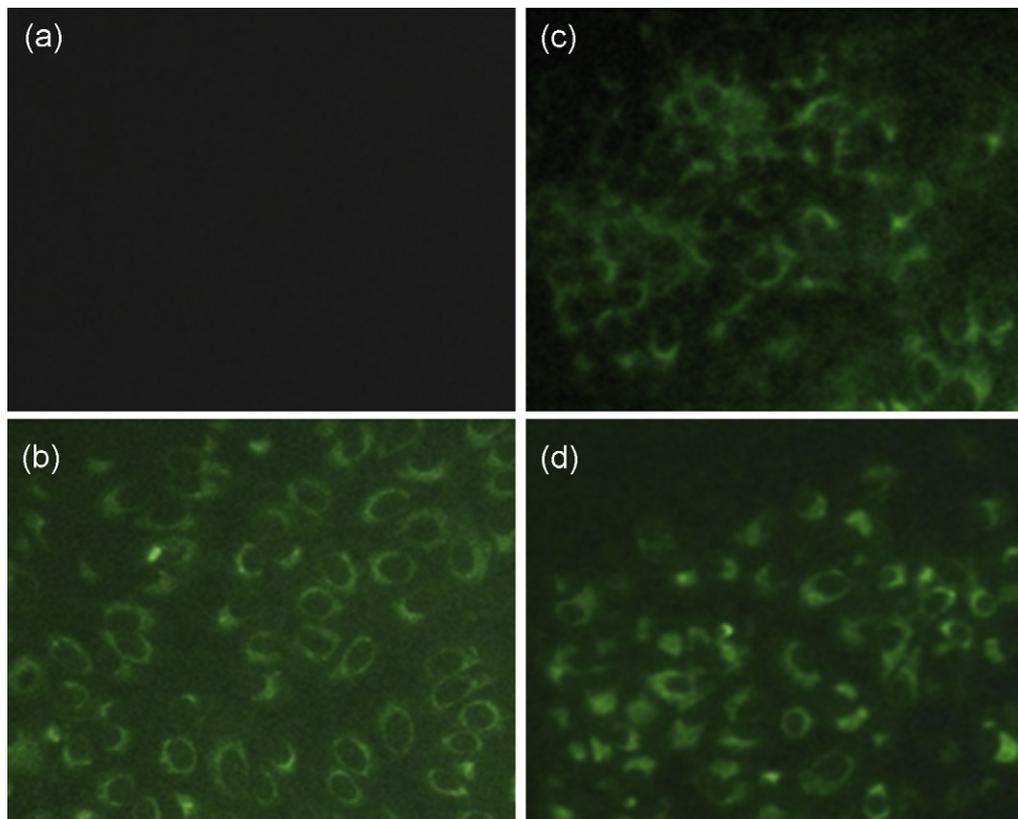
Fig. 2. Detection of WNV antigen in Vero cells by indirect immunofluorescence. Vero cells, uninfected (a), transfected with WNVsyn-transcribed RNA (b), infected with WNV wild-type virus (c), or infected with a WNVsyn virus stock (d) were tested for the expression of WNV proteins by indirect immunofluorescence two days after infections or transfection.

with the onset of CPE of the infected cells from day 3 on (Fig. 3c). Growth kinetics in the mosquito cells was delayed as observed by others [19,25], reaching equal titers compared to Vero cells at day 4 postinfection (Fig. 3d). Taken together, these data indicate that WNVsyn and the corresponding WNVwt isolate are indistinguishable with respect to replication and infectivity in both tested cell lines.

In addition, virulence of WNVsyn and WNVwt were compared in cohorts of 7-week-old Balb/c mice. For this purpose mice were infected intranasally with virus dilutions corresponding to 2×10^5 to 2×10^2 TCID$_{50}$ per animal. Survival was monitored for 21 days postinfection and LD$_{50}$ values were calculated. Similar mortalities of infected mice induced by the two WNV viruses were observed (Table 2). The lethal dose 50 for WNVsyn and WNVwt was 3.6 and

Table 2
Virulence of WNVsyn and WNVwt after intranasal application in Balb/c mice.

Titer [log(10) TCID$_{50}$]	Survival of mice (n/n total)	Average survival time (days ± SD)	LD$_{50}$[a] [log(10) TCID$_{50}$]
(A) WNVsyn			
3.50	3/6	8.0 ± 0.0	3.6
2.34	4/6	9.0 ± 0.0	
1.23	5/6	10	
0.12	5/6	10	
(B) WNVwt			
4.42	0/6	7.0 ± 0.0	3.4
3.71	4/6	9.5 ± 0.7	
2.29	6/6	No deaths	
1.29	4/6	10.5 ± 0.7	

[a] LD$_{50}$, lethal dose 50; results from a representative experiment (see also Section 2).

3.4 log 10 TCID$_{50}$, respectively. The experiment was repeated once and similar results were obtained.

3.4. Generation and analysis of a WNVsyn-based inactivated experimental vaccine

Following the demonstration that WNVsyn exhibits indistinguishable biological properties compared to the WNV wild-type isolate, the protective efficacy of experimental vaccines derived from both viruses was analyzed. For this purpose, groups of ten mice were immunized twice with decreasing doses of formalin-inactivated, alum-adjuvanted whole virus vaccines derived from the viruses (see Section 2). Quantification by ELISA of vaccine preparations prior to formulation and adjuvantation confirmed the presence of equal amounts of antigen in the respective dosage groups. Further, Western blotting confirmed equivalent amounts and protein patterns in the two antigen preparations (Fig. 4b). The predominant band in these preparations is the envelope antigen (E) migrating in the 60 kDa range, the fainter bands representing the pre-membrane (prM) and the dimeric membrane (M) proteins (see also [26]).

Two weeks after the second vaccination WNV-specific neutralizing antibodies were determined by a microneutralization assay. Serum analysis demonstrated high neutralizing antibody levels in both vaccine preparations (see Fig. 4a and Table 3). Mice were then challenged intranasally with a lethal dose (1×10^5 TCID$_{50}$) of WNV wild-type virus. Vaccination with both preparations resulted in a high degree of protection in vaccinated mice. Complete protection was achieved using doses as low as 63 nanograms of the WNV antigens while 95% of the non-vaccinated controls died. The vaccines clearly induced a dose-dependent protection correlating with NT titers (Table 3).

Fig. 3. Plaque morphology of wild-type WNV (a) and WNVsyn (b) at day 4 postinfection of Vero cells seeded in six-well plates with a titer of 100 TCID$_{50}$. Staining was carried out with neutral red for 4 h. Growth of synthetic (solid diamonds) and wild-type WNV (open squares) was analyzed in Vero (c) and C6/36 cells (d) infected with a MOI of 0.0001. The growth curve experiment was repeated once. The titers are means of two determinations ± SD indicated by the error bars.

Table 3
Antibody responses after subcutaneous immunization of Balb/c mice and survival after intranasal challenge with WNVwt virus.

Preparation	Antigen (ng)	Survival[a], n/n total (%)	μNT-Titer, GMT (SD)
WNVwt	1000	20/20 (100)	306 (207)
	250	20/20 (100)	190 (0)
	63	20/20 (100)	98 (52)
	16	14/20 (70)	15 (2.7)
	4	5/20 (25)	9 (2.7)
WNVsyn	1000	19/20 (95)	237 (14.5)
	250	20/20 (100)	155 (28.3)
	63	20/20 (100)	129 (7.8)
	16	19/20 (95)	45 (21.8)
	4	12/20 (60)	15 (2.7)
Mock	0 (PBS)	1/20 (5)	<7.1

[a] Challenge dose 2×10^5 TCID$_{50}$ per animal; results of two independent experiments were combined.

4. Discussion

Reverse genetics systems of positive-sense RNA viruses allow, for instance, for mutagenesis procedures and generation of chimeric viruses and thus are invaluable tools for live vaccine development and for studying the biology of those viruses (see
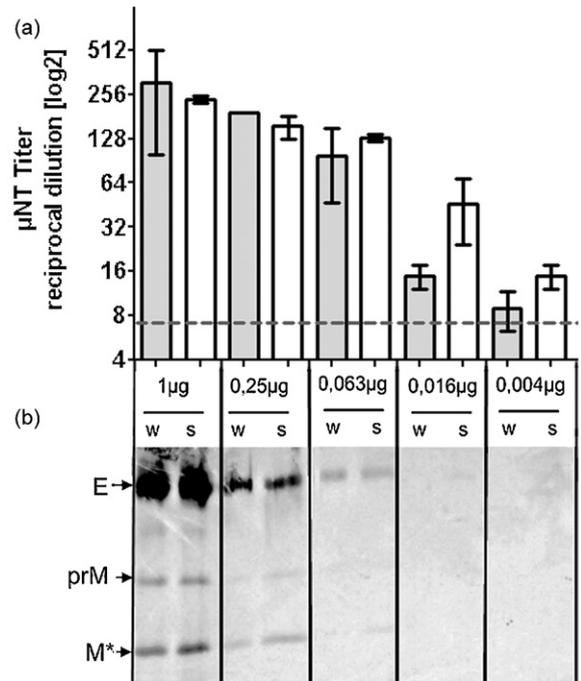
Fig. 4. Neutralizing antibody titers in Balb/c mice after immunization using WNVwt and WNVsyn derived vaccine preparations (a) and characterization of the inactivated vaccine preparations used to immunize mice by Western blotting (b). Gray bars represent neutralizing antibody titers of mice vaccinated with WNVwt antigen and white bars neutralizing antibody titers of mice vaccinated with WNVsyn preparations (upper panel). Western blotting was carried out to analyze the antigen content used in individual formulations (lower panel); w, WNVwt; s, WNVsyn; E, WNV envelope protein; prM, pre-membrane protein; M*, dimeric membrane protein.

e.g. Refs. [27,28]). Usually the starting material for the generation of seed viruses for vaccines or such reverse genetics systems are virus stocks derived from a biological source. This virus is plaque-purified, viral RNA is isolated, transcribed into cDNA which is amplified by PCR and in turn gets assembled into a DNA copy of the viral genome. These approaches bear the risk of introducing mutations selected via plaque purification steps. To minimize this type of mutations we chose to generate a reverse genetics system using a different approach, independent of preformed viral RNA components and animal sources. The feasibility of generating such systems by chemical synthesis of DNA was proven previously, for instance, by the generation of poliovirus [29], bacteriophage φX174 [30] or H1N1 Spanish influenza virus [31], and SARS-like coronavirus [32]. On the basis of these studies, we report for the first time the generation of an 11,000 nucleotide long synthetic genome of a member of the family *Flaviviridae*. Sequence data from GenBank referring to lineage I West Nile Virus strain NY99 were used as template for *in silico* design of the cloning strategy.

RNA viruses replicate their genome with an error prone mechanism (for reviews see [33]), resulting in a multitude of distinct but related nucleic acids forming a quasispecies [34]. Sequencing of a virus genome (usually cloned by plaque purifications prior to sequence analysis) consisting of millions of molecules, results in a 'consensus' sequence, representing the majority genotype having defined biological properties. Biological properties may change, for instance, when pressure imposed by the host selects for changes of the genomic sequence, visible as a new 'consensus sequence' in the sequence analysis. In all of the cloning and propagation steps no mutations changing the wild-type consensus sequence were introduced by PCR using synthetic templates of verified nucleotide sequence proving the accuracy of this approach. Thus the synthetic progeny virus was biologically indistinguishable from its natural

parent. Experimental inactivated vaccines derived from WNVwt and WNVsyn were highly immunogenic in animals. Both vaccine preparations induced comparable levels of neutralizing antibodies and led to similar protection results. Only in the low dosing groups of the protection study differences were observed that can be explained by the experimental conditions and the inherent inaccuracies of the biological system rather than by genetic differences in the two viruses. In addition, both virus stocks were indistinguishable concerning their virulence in mice.

Progress in synthetic biology raises biosecurity concerns. The possibility to synthesize pathogens without need for natural sources, for instance the viruses on the Select Agents List [35], results in the expansion of the potential availability of select agents (defined as biological agents and toxins regulated by the US Select Agent Rules that have the potential to pose a severe threat to public, animal or plant health). The US government has developed guidance that addresses this issue [36]. WNV – although a biosafety level 3 virus – is endemic in large parts of the world and does not belong to the Select Agents. However, flaviviruses belonging to the tick-borne encephalitis virus complex are on this list. Construction of infectious flaviviruses, involving DNA synthesis, cloning, assembly into larger units, *in vitro* transcription and transfection steps, is a complex task and can be done in a professional environment only. A recent review on synthetic viruses discusses the dual use concerns in more detail [24].

For vaccine manufacturing, the most important advantage of using primary seed virus stocks derived by gene synthesis is the exclusion of potential contamination with unknown and known adventitious agents – including the transmissible spongiforme encephalopathy agents – which maybe co-isolated from animal-derived viruses or their host cells. Furthermore, this approach renders passaging, plaque purifications and other steps to achieve satisfactory purity of seed viruses from animal sources unnecessary. Our study demonstrates the feasibility of generating the flavivirus WNV in a completely synthetic approach. Synthetic biology is therefore a valuable alternative to obtain viral seed stocks free from the adventitious agents that might accompany recovery from vertebrate or insect cells.

Acknowledgements

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