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Live Attenuated Influenza Viruses Containing NS1 Truncations as Vaccine Candidates against H5N1 Highly Pathogenic Avian Influenza[▽]

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Due to the high mortality associated with recent, widely circulating strains of H5N1 influenza virus in poultry, the recurring introduction of H5N1 viruses from birds to humans, and the difficulties in H5N1 eradication by elimination of affected flocks, an effective vaccine against HPAI (highly pathogenic avian influenza) is highly desirable. Using reverse genetics, a set of experimental live attenuated vaccine strains based on recombinant H5N1 influenza virus A/Viet Nam/1203/04 was generated. Each virus was attenuated through expression of a hemagglutinin protein in which the polybasic cleavage site had been removed. Viruses were generated which possessed a full-length NS1 or a C-terminally truncated NS1 protein of 73, 99, or 126 amino acids. Viruses with each NS genotype were combined with a PB2 polymerase gene which carried either a lysine or a glutamic acid at position 627. We predicted that glutamic acid at position 627 of PB2 would attenuate the virus in mammalian hosts, thus increasing the safety of the vaccine. All recombinant viruses grew to high titers in 10-day-old embryonated chicken eggs but were attenuated in mammalian cell culture. Induction of high levels of beta interferon by all viruses possessing truncations in the NS1 protein was demonstrated by interferon bioassay. The viruses were each found to be highly attenuated in a mouse model. Vaccination with a single dose of any virus conferred complete protection from death upon challenge with a mouse lethal virus expressing H5N1 hemagglutinin and neuraminidase proteins. In a chicken model, vaccination with a single dose of a selected virus encoding the NS1 1-99 protein completely protected chickens from lethal challenge with homologous HPAI virus A/Viet Nam/1203/04 (H5N1) and provided a high level of protection from a heterologous virus, A/egret/Egypt/01/06 (H5N1). Thus, recombinant influenza A/Viet Nam/1203/04 viruses attenuated through the introduction of mutations in the hemagglutinin, NS1, and PB2 coding regions display characteristics desirable for live attenuated vaccines and hold potential as vaccine candidates in poultry as well as in mammalian hosts.

Influenza viruses are segmented, single-stranded, negative-sense RNA viruses of the family *Orthomyxoviridae*. Influenza viruses of the genus *Influenzavirus A*, which pose the most significant threats to human and animal health, are subdivided into 16 hemagglutinin (HA) subtypes. Aquatic avian species are the main reservoir of all *Influenzavirus A* subtypes, and although each subtype is normally nonpathogenic in waterfowl and poultry, subtypes H5 and H7 may become highly pathogenic in some avian species, including gallinaceous poultry. Since 1996, repeated outbreaks of H5N1 highly pathogenic avian influenza (HPAI) have occurred in poultry. Initially concentrated in Asia, these outbreaks subsequently spread to Africa, the Middle East, and Europe (6) and have caused significant economic and public health difficulties (1, 24). Outbreaks of H5N1 HPAI in domestic poultry have been confirmed in 48 countries as of 25 July 2008 (www.OIE.int) and have resulted in the culling or death of an estimated 500 million birds (25). As a result of this spread, H5N1 HPAI virus is now considered to be endemic in large areas of Asia (28), with significant consequences associated with control of the virus.

Traditionally, the control of HPAI has been achieved through the wholesale culling of infected flocks. Recently, however, this strategy has proven ineffectual, and in response, a multicomponent strategy has been recommended by OIE/FAO in collaboration with WHO (www.OIE.int; OIE/FAO International Scientific Conference on Avian Influenza, Paris, France, 7 to 8 April 2005). The recommended approach comprises the following elements: (i) maintenance of adequate biosecurity in farms and industrial units, (ii) implementation of coordinated regional or national surveillance and diagnostic programs, (iii) stamping out of all infected poultry through culling, and (iv) vaccination of uninfected flocks. Immunization of poultry carries a further, important benefit in that an effective vaccine will limit the transmission of HPAI viruses to humans or other mammalian hosts, thereby mitigating the human pandemic threat at its source. The development of an efficacious vaccine against avian influenza virus is therefore clearly desirable.

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Live attenuated vaccines can be rationally designed by exploiting (i) our understanding of influenza virus pathogenicity at the molecular level (22) and (ii) reverse genetics technology (5, 20). One of the most significant and consistent determinants of HPAI pathogenicity in poultry is the presence of a polybasic cleavage site in the HA protein. The introduction of multiple basic residues at the cleavage site is believed to increase the range of host cell proteases which can effect cleavage, and thus activation, of the HA protein, allowing systemic spread of the virus in birds (32) and mammals (9). A second pathogenicity factor which has been repeatedly identified in avian influenza viruses transmitted to (or experimentally introduced into) mammalian hosts is the amino acid 627 of the PB2 polymerase protein (9). The identity of this residue is almost exclusively glutamic acid in viruses which are adapted to avian species and lysine in viruses which are adapted to humans. This polymorphism can influence the enzymatic activity of the influenza virus polymerase complex (15) and has been proposed to contribute to host adaptation (10, 19, 34). Specifically, the presence of a lysine at amino acid 627 has been reported to allow a higher level of replication to take place at 33°C, the approximate temperature in the upper respiratory tract of mammals, whereas presence of a glutamic acid at this position appears to confer lower activity at 33°C and optimal activity at 41°C, the temperature of the avian intestinal tract. The temperature dependency of polymerase function is believed to drive the adaptive changes observed in the polymerase genes when HPAI viruses are passaged in mice (10).

The NS1 gene product constitutes a further determinant of influenza virus pathogenicity. This nonstructural protein has been shown to counteract the host type I interferon (IFN) response, a key component of innate immunity (7, 13, 17).

The balance between cellular innate immunity and antagonism thereof by the NS1 protein contributes to the virulence of any specific influenza virus. Indeed, a mutant influenza virus lacking NS1 protein (delNS1) was shown to be highly attenuated in interferon-competent substrates (8). Poor replication and lack of disease following delNS1 virus infection were furthermore correlated to increased levels of IFN (35). By contrast, NS1 mutant viruses replicate efficiently in IFN-incompetent systems, such as STAT1 knockout mice (8). Viruses carrying the delNS1 mutation may be too attenuated in animal hosts to constitute a viable live attenuated vaccine. However, more moderate attenuation of influenza viruses can be achieved by incremental truncation of the NS1 protein. This approach has produced effective vaccine viruses as demonstrated in both mice (35) and swine (27). More recently, a naturally occurring strain of avian influenza virus with a truncated NS1 protein (3) was shown to be attenuated in a chicken model, suggesting the strategy of NS1 truncation would also be successful in poultry.

With the aim of developing a live attenuated vaccine to protect avian species against HPAI of the H5 subtype, we have generated and characterized a panel of eight viruses based on A/Viet Nam/1203/04 (VN1203) which incorporate modifications of the HA, NS1, and PB2 proteins. The HA gene of all eight viruses was modified by the removal of the polybasic cleavage site. In addition, viruses encoded either full-length NS1 or C-terminally truncated NS1 proteins of 73, 99, or 126 amino acids. Each NS gene was combined with a PB2 polymerase gene which carried either a lysine or a glutamic acid at position 627. All eight viruses were found to grow to high titers in 10-day-old embryonated chicken eggs while showing strongly attenuated phenotypes in mammalian substrates. Viruses containing NS1 truncations were found to induce more interferon than viruses with full-length NS1 proteins and were correspondingly more attenuated in mice. Although all eight viruses were attenuated, in general, viruses encoding PB2 627K were less attenuated in mice than those encoding PB2 627E. All eight candidate vaccines furthermore protected mice against challenge with a mouse-lethal virus which contained the H5 HA protein. Importantly, one virus encoding a truncated NS1 protein (VN HALo/627E/NS1-99) produced no morbidity following inoculation into chickens and completely protected chickens against challenge with 100 50% chicken lethal doses (CLD_{50}) of the homologous highly pathogenic H5N1 virus.

MATERIALS AND METHODS

Cells. 293T and A549 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone). Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium supplemented with 10% fetal calf serum (Gibco). All cells were maintained at 37°C in 5% CO_2.

Generation of recombinant influenza viruses and virus propagation. Sendai virus (strain Cantell) was propagated in 10-day-old embryonated chicken eggs at 37°C. A/Puerto Rico/8/34 (H1N1; PR8) virus was generated by reverse genetics as previously described (8) and also propagated in 10-day-old embryonated chicken eggs at 37°C.

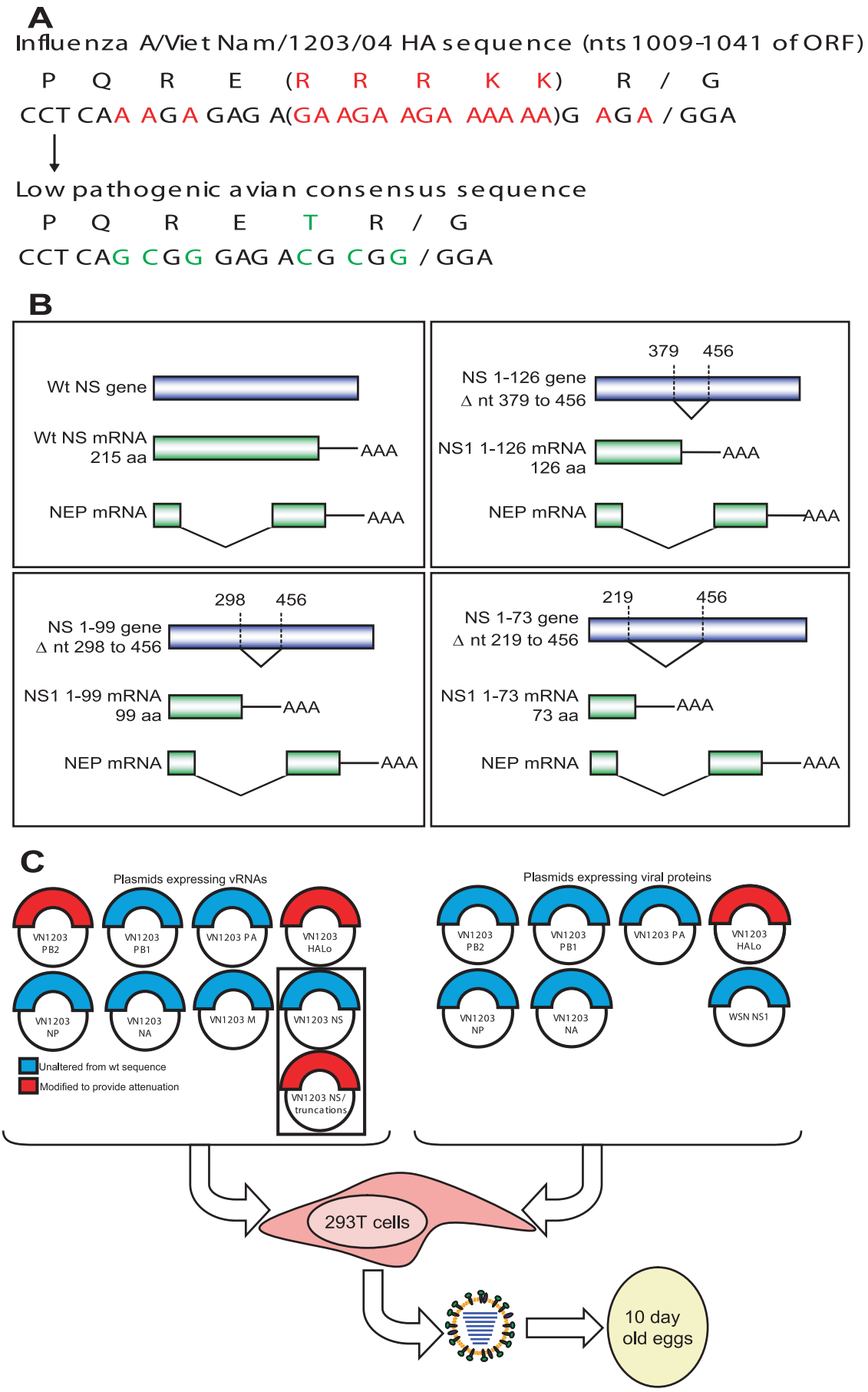
Recombinant A/Viet Nam/1203/04 (VN1203) (H5N1) influenza viruses were generated using reverse genetics techniques as previously described (5). Briefly, 1 μg of each of 15 plasmids was transfected into 293T cells in a monolayer. Each transfection contained viral RNA (vRNA) expression plasmids for the VN1203 PB2, PB1, PA, HALo, NP, NA, M, and NS segments, in addition to protein expression plasmids pCAGGS VN1203-PB2, -PB1, -PA, -NP, -HALo, -NA, and pCAGGS WSN NS1 (the pCAGGS expression plasmid was kindly provided by J. Miyazaki, Osaka University, Osaka, Japan [18]). Twenty-four hours after transfection, 293T cells were resuspended in cell culture supernatant, and cells and supernatant containing transfectant virus were inoculated into 10-day-old embryonated eggs. Viruses were generated which contained one of four VN1203-derived NS segments. The NS segments encoded unmodified NEP protein and either full-length NS1 protein or carboxy-terminal-truncated NS1 protein products comprising amino acids 1 to 126, 1 to 99, or 1 to 73. The HALo plasmid encodes the HA segment from VN1203 which has been modified by the removal of the encoded polybasic cleavage site. Recombinant PR8/VN1203 HALo plus NA virus was obtained as described above, except that the vRNA expression plasmids used encoded A/Puerto Rico/8/34 PB2, PB1, PA, NP, M, and NS segments and the VN1203 HALo and NA segments.

Visualization of RNA segments on a polyacrylamide gel. RNA was extracted from each purified transfectant virus, as previously described (21). In brief, each virus was concentrated by centrifugation though a 30% sucrose cushion. RNA was obtained by phenol-chloroform extraction and ethanol precipitation. The separation of viral RNA segments was achieved by electrophoresis through a 2.8% polyacrylamide gel containing 7 M urea. RNAs were visualized by silver stain.

Preparation of viral stocks. Ten-day-old embryonated chicken eggs (Charles River Laboratories) were inoculated with recombinant viruses and incubated for 48 h at 37°C. Virus present in allantoic fluid was subsequently passaged at limiting dilution in 10-day-old embryonated chicken eggs until maximal growth was obtained. The titer of virus stocks was determined by plaque assay on MDCK cells. Plaque phenotypes were examined in the presence and absence of trypsin.

Growth kinetics of virus in 10-day-old embryonated chicken eggs. Ten-day-old embryonated chicken eggs were inoculated with recombinant VN1203-derived viruses. Allantoic fluid was harvested and subsequently assayed for viral growth at 0, 24, 48, and 72 h postinfection (hpi). The titer of virus present in allantoic fluid was determined by plaque assay on MDCK cells.

Growth kinetics of virus in A549 cells. Cultures of A549 cells were infected singly with each of the VN1203-derived viruses or influenza PR8 virus at a


A
Influenza A/Viet Nam/1203/04 HA sequence (nts 1009-1041 of ORF)
P Q R E (R R R K K) R / G
CCT CAA AGA GAG A(GA AGA AGA AAA AA)G AGA / GGA
Low pathogenic avian consensus sequence
P Q R E T R / G
CCT CAG CGG GAG ACG CGG / GGA
B
Wt NS gene
Wt NS mRNA 215 aa
AAA
NEP mRNA
AAA
379
456
NS 1-126 gene Δ nt 379 to 456
NS1 1-126 mRNA 126 aa
AAA
NEP mRNA
AAA
298
456
NS 1-99 gene Δ nt 298 to 456
NS1 1-99 mRNA 99 aa
AAA
NEP mRNA
AAA
219
456
NS 1-73 gene Δ nt 219 to 456
NS1 1-73 mRNA 73 aa
AAA
NEP mRNA
AAA
C
Plasmids expressing vRNAs
VN1203 PB2
VN1203 PB1
VN1203 PA
VN1203 HALo
VN1203 NP
VN1203 NA
VN1203 M
VN1203 NS
VN1203 NS/ truncations
Unaltered from wt sequence
Modified to provide attenuation
Plasmids expressing viral proteins
VN1203 PB2
VN1203 PB1
VN1203 PA
VN1203 HALo
VN1203 NP
VN1203 NA
WSN NS1
293T cells
10 day old eggs

multiplicity of infection (MOI) of 0.001 in the presence of 1 μg/ml of exogenous tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. Following infection, cell culture supernatant was harvested at 0 h, 18 h, 24 h, 48 h, and 72 h. The titer of virus released into the cell culture supernatant at each time point was determined by plaque assay on MDCK cells.

MDT of 10-day-old embryonated chicken eggs. The pathogenicity of the VN1203-derived viruses was determined by the mean death time (MDT) in 10-day-old embryonated eggs. Groups of six eggs were inoculated with 100 μl of 10-fold serial dilutions of each virus or sham inoculated with phosphate-buffered saline (PBS) by the chorioallantoic sac route. Eggs were incubated at 37°C and candled at 6-h intervals for 5 days. Eggs were scored as alive or dead, and the time of death was recorded. The minimum lethal dose was defined as the highest dilution which killed all embryos. The MDT was calculated as the mean time of death in eggs inoculated with the minimum lethal dose of virus.

Bioassay to measure type I IFN induction. Levels of secreted type I interferon in cell culture were measured by modification of a previously described method (29). Briefly, A549 cells were mock treated or infected with PR8 virus or Sendai virus as controls, or infected with each of the recombinant VN1203 viruses, at an MOI of 3 and incubated at 37°C. At various time points postinfection, supernatant from cell culture samples was harvested and UV inactivated for 10 min. These supernatants were transferred onto fresh A549 cells, incubated for 24 h at 37°C, washed with PBS, and then infected with a recombinant Newcastle disease virus encoding green fluorescent protein (NDV-GFP) at an MOI of 2. Cells were further incubated at 37°C for 18 h. Growth of NDV-GFP virus was assessed by immunofluorescence microscopy.

Determination of the MLD_{50} of challenge virus. Six- to 8-week-old C57BL/6 mice (Taconic) were anesthetized with a ketamine-xylazine mixture and then inoculated intranasally ($n = 4$) with 10^2, 10^3, 10^4, 10^5, or 10^6 PFU of PR8/VN1203 HALo plus NA virus in 50 μl of PBS. Daily monitoring of animals for mortality, weight loss, or other signs of disease over a 2-week period was carried out. Animals were sacrificed and scored as dead if they lost greater than 25% of their body weight or showed obvious signs of distress. Fifty percent mouse lethal dose (MLD_{50}) values were calculated by the method of Reed and Muench (26).

Immunization and infection in mice. Six- to 8-week-old C57BL/6 (Taconic) mice were anesthetized and then inoculated intranasally ($n = 4$) with 10-fold serial dilutions of each VN1203-derived virus within the range of 10^6 to 10^2 PFU in 50 μl of PBS or with a mock PBS inoculum. Mice were monitored daily for weight loss or other signs of disease over a 2-week period. Vaccinated mice were challenged 4 weeks postvaccination with 1,000 MLD_{50} of PR8/VN1203 HALo plus NA virus by the intranasal route and monitored daily for weight loss or other signs of disease over a 2-week period. Additionally, sera were obtained from selected animals at 4 weeks postvaccination and 2 weeks postchallenge. The levels of neutralizing antibody present in sera were assessed by microneutralization assay.

Microneutralization assay. Nonspecific inhibitors of hemagglutination were removed from mouse sera by trypsin-heat-periodate treatment. Briefly, aliquots of sera were mixed with half volumes of 8 mg/ml trypsin (Sigma) in 0.1 M phosphate buffer, pH 8.2, and incubated at 56°C for 30 min. Next, three volumes of 0.011 M potassium metaperiodate were added and the mixture was incubated at room temperature for 15 min. Three volumes of 1% glycerol saline were then added and the mixture incubated at room temperature for 15 min. Finally, two volumes of 85% saline were added to the mixture to give a 1:10 final dilution of the initial sample. Twofold dilutions of treated sera were mixed with 100 PFU of virus (a 6:2 recombinant virus carrying the HALo and NA of VN1203) and incubated for 45 min at 37°C. MDCK cells were inoculated with the virus-antibody mixtures. Following a 45-min incubation, inocula were removed and cells were washed once with PBS. Cells were incubated in the presence of TPCK-treated trypsin at 37°C and, at 72 h postinfection, supernatants were collected and tested for virus growth by hemagglutination assay. Neutralizing titers from quadruplicate samples were used to calculate the geometric mean titer (GMT).

Immunization and infection in chickens. Groups of 2-week-old specific-pathogen-free Leghorn chickens ($n = 6$) were vaccinated with 10^6 PFU of VN HALo/627E/NS1-99 virus in a total of 1 ml of PBS distributed through the intraocular (0.2 ml), intranasal (0.3 ml), or intratracheal (0.5 ml) routes or sham inoculated ($n = 4$) with PBS. At 6 weeks of age, chickens from each group were challenged with either 100 CLD_{50} of influenza A/Viet Nam/1203/04 (H5N1) or with 100 CLD_{50} of A/egret/Egypt/01/06 (H5N1) using the same routes and volumes as described above for the vaccination. All birds were assessed daily for clinical signs of disease and mortality.

Tracheal and cloacal swabs were collected from animals on days 2, 4, and 7 postchallenge and virus present was quantitated by standard 50% tissue culture infectious dose assay using the method of Reed and Muench (26). Additionally, sera were obtained from animals at 4 weeks postvaccination and at 2 weeks postchallenge, at which time the animals were humanely euthanized. The levels of neutralizing antibody present in sera were assessed by both hemagglutination inhibition and microneutralization assay following the recommendations of the World Health Organization.

All work involving HPAI viruses was carried out in USDA- and CDC-approved biosafety level 3+ containment laboratories in accordance with institutional biosafety requirements.

RESULTS

Rescue of eight candidate vaccine viruses. With the aim of developing live attenuated vaccines of the H5 subtype, a panel of eight recombinant viruses based on the highly pathogenic avian influenza virus A/Viet Nam/1203/04 (VN1203; H5N1) was generated. The HA genes of all eight viruses were modified by the removal of the polybasic cleavage site, as previously described (23). As indicated in Fig. 1A, the wild-type amino acid sequence flanking the HA cleavage site, SPQRERRRKK R ↓ G, was changed to that of the low-pathogenicity avian consensus amino acid sequence, PQRETR ↓ G. The VN1203 HA lacking the polybasic cleavage site is hereafter referred to as HALo. To achieve further attenuation, the NS1 genes of six of the eight viruses were modified: deletions and stop codons were introduced into the NS gene segment such that NEP was not affected but the NS1 open reading frame (ORF) encoded carboxy-terminally truncated NS1 proteins of 73, 99, or 126 amino acids (Fig. 1B). Finally, each NS1 gene (full-length or amino acids [aa] 1 to 126, 1 to 99, and 1 to 73) was combined with a PB2 gene which carried either a lysine or a glutamic acid at position 627. The identity of PB2 amino acid 627 represents a well-characterized polymorphism (E627K) associated with adaptation to and pathogenicity in mammalian hosts (10). Thus, by combining stepwise NS1 truncations with alternative PB2 627 genotypes in the VN1203 HALo background, we aimed to produce viruses with a range of attenuation phenotypes; in this way, the candidate most suited for use as a live attenuated vaccine could be selected.

FIG. 1. Schematics of the removal of the polybasic cleavage site from the HA segment, of truncations of the NS segment, and of the virus rescue system. (A) Alteration of the nucleotide sequence of the HA gene of VN1203. The encoded polybasic amino acid sequence, RRRKK, was removed and replaced with the amino acid threonine. Additionally, adenosines were replaced with guanosines or cytosines wherever possible without disrupting the encoded amino acid sequence. Amino acids and nucleotides in the wild-type sequence that were changed are shown in red; introduced amino acids and nucleotides predicted to lower the pathogenicity are shown in green. (B) Schematic diagram of wild-type and truncated NS influenza virus gene segments. The NS gene segment was modified to create NS1 genes encoding 73, 99, and 126 aa, respectively. The nucleotide positions bordering the deletion in each case (nt 379 to 456, 298 to 456, and 219 to 456) are indicated above each NS segment in the schematic. In all truncated segments, stop codons were introduced in each reading frame in place of the deleted sequence. NS1 mutations did not affect the sequence of the NEP. (C) Schematic of the rescue of influenza A/Viet Nam/1203/04-based viruses incorporating truncated NS segments.

All eight recombinant viruses were successfully recovered from cDNA transfection. Reverse transcription followed by PCR and sequencing of the HA gene segment of each of the viruses confirmed that the polybasic cleavage site had been removed in each case. Disruption of the polybasic cleavage site is expected to render an influenza virus unable to grow in cell culture in the absence of exogenous trypsin and, correspondingly, unable to spread systemically in an animal host. To confirm that the eight VN1203 HALo viruses were indeed dependent on trypsin for growth in MDCK cells, we performed plaque assays in the presence and absence of trypsin. All eight viruses required trypsin for plaque formation, in contrast to the laboratory-adapted strain, A/WSN/33 (data not shown).

Confirmation of NS1 truncations at the RNA and protein levels. To confirm that the expected mutations in the NS gene segments were present, biochemical analyses of viral RNA and protein were performed. Viral RNA extracted from purified virus was characterized by acrylamide gel electrophoresis followed by silver staining. The NS gene segments of VN1203 HALo viruses encoding full-length NS1 showed greater mobility than that of PR8 virus (Fig. 2A); this difference is due to a naturally occurring 15-nucleotide (nt) deletion seen in H5N1 viruses of the Z genotype (corresponding to amino acids 80 to 84) (14). Deletions introduced into the NS segments of the NS 1-126, NS 1-99 and NS 1-73 viruses are apparent from the increasing mobilities of the corresponding silver-stained bands. The NA gene segment of VN1203 HALo viruses also showed greater mobility than that of PR8 virus (Fig. 2A); the NA gene of VN1203 contains a 60-nt deletion in the stalk region, which is common to all H5N1 viruses of the Z genotype. The remaining six gene segments exhibited similar mobilities to the corresponding segments of PR8 virus, as expected.

Western blot analyses of infected cell lysates indicated that NS1 was similarly abundant in cells infected with both viruses encoding a full-length NS1 protein (Fig. 2B). Despite the use of a primary antibody which binds to the N-terminal 73 amino acids, NS1 proteins were not detected in cells infected with viruses carrying truncated NS1 genes (data not shown). The reason for the low levels of truncated NS1 gene products is unclear, but not unprecedented. A similar phenomenon was reported by Solorzano et al. in an A/swine/Texas/98 influenza virus background (29). The HA protein, the major protective antigen of influenza virus, was expressed by each virus, consistent with the aim of generating effective vaccine candidates. Levels of HA generated by viruses expressing shortened NS1 protein were in general lower than those of viruses possessing a full-length NS1 protein.

Growth characteristics of recombinant viruses in embryonated eggs and cell culture. An initial estimate of the fitness of each recombinant virus was obtained by evaluating their plaque phenotypes on MDCK cells. All eight viruses formed plaques on this substrate, although the plaques of several viruses were best visualized by following an immunostaining procedure (as opposed to staining with crystal violet dye). As seen in Fig. 3, VN HALo/627K/NS FL produced the largest plaques of the rVN1203 viruses, and these were similar in size to plaques produced by PR8 virus. VN1203 HALo virus encoding a full-length NS1 and PB2 627E also formed distinct and easily visible plaques, but these were markedly smaller than those of VN1203 HALo virus encoding a full-length NS1

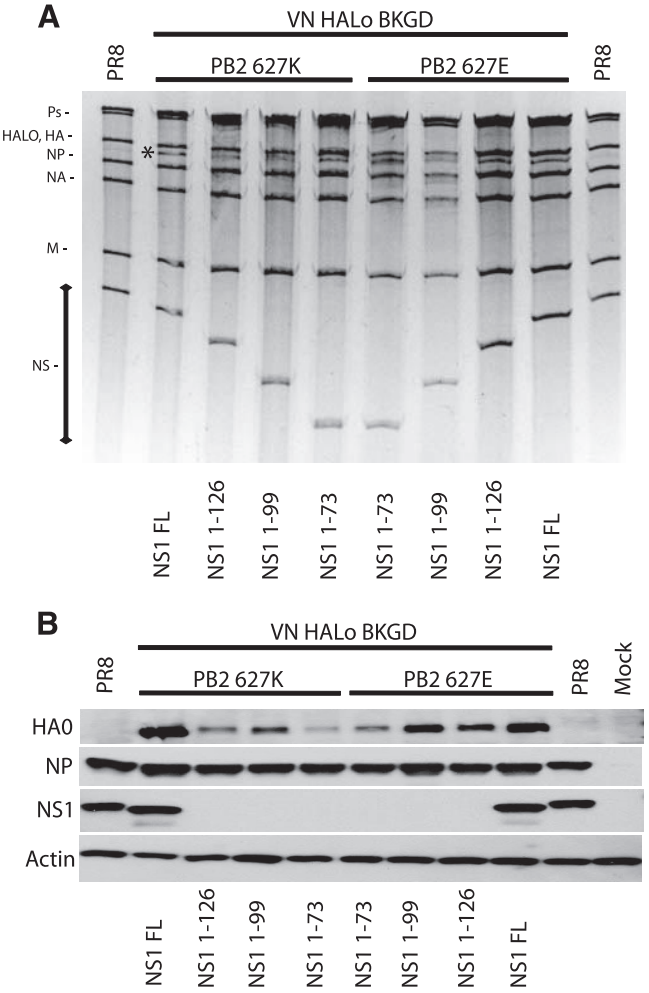


FIG. 2. Biochemical characterization of A/Viet Nam/1203/04-based viruses. (A) vRNAs of purified recombinant A/VN/1203/04-based viruses. RNAs extracted from PR8 and recombinant A/Viet Nam/1203/04-derived viruses were separated by polyacrylamide gel electrophoresis and visualized by silver staining. The positions of the polymerase (Ps), HA (PR8), HALo (A/Viet Nam/1203/04), NP, NA, M, and NS segments are indicated to the left of the image. A contaminating 18S rRNA band is also indicated by an asterisk. Genotypes of viruses are as indicated above and below each lane. (B) Western blot of protein obtained from whole-cell lysates of mock- or virus-infected A549 cells as indicated. Cells were infected at an MOI of 5 PFU/cell and lysates were prepared at 8 h postinfection. Extracts were probed for HA (using HALo monoclonal antibody 9), NP (using an NP specific polyclonal rabbit antiserum), NS1 (using an NS1 1-73-specific polyclonal rabbit antiserum), and actin protein (using an actin-specific monoclonal antibody; Sigma) as indicated. Genotypes of viruses are as indicated above and below each lane.

and PB2 627K. This result confirms the importance of PB2 627 to the phenotype in mammalian cells in this virus background. All viruses containing NS1 truncations produced small plaques, and in general those of viruses encoding PB2 627E were smaller than those of the corresponding viruses encoding PB2 627K.

The growth phenotype of each virus was next assessed in 10-day-old embryonated chicken eggs. Hen eggs are the traditional substrate for the production of influenza vaccines; thus, for production purposes, any candidate vaccine strain must

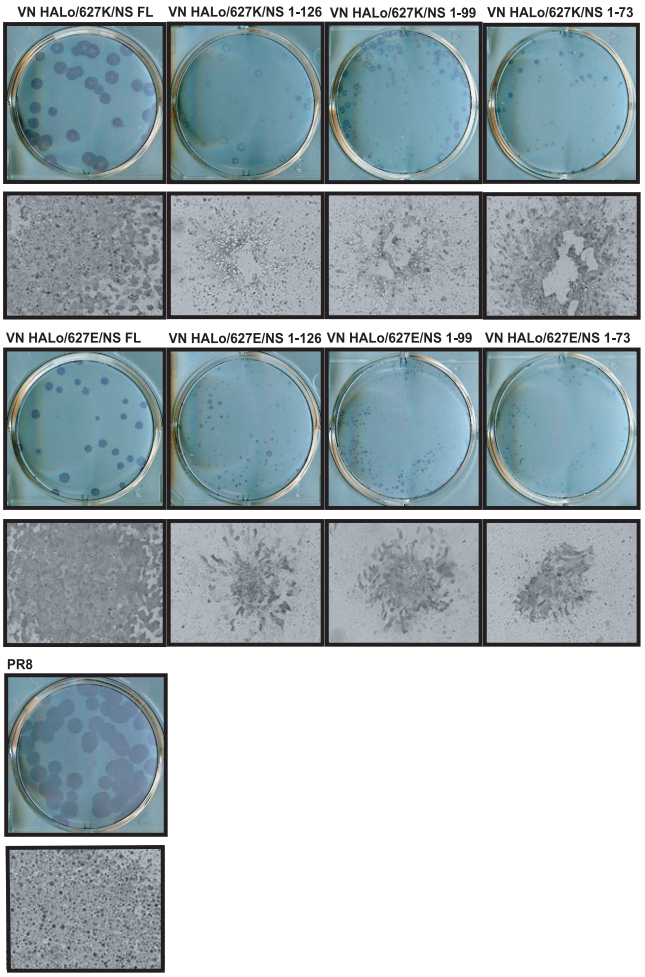


FIG. 3. Plaque phenotypes of viruses on MDCK cells. Plaques were visualized by immunostaining with a polyclonal antibody to NP protein. Macroscopic and microscopic images of plaques formed by each virus are shown, and the identity of each virus is indicated.

reach high titers in this substrate. When working with an avian influenza virus, the lethality for chicken embryos must also be considered: viral titers must reach high levels before embryos succumb to infection. Growth curves revealed that all eight rVN1203 HALo viruses did grow to adequate titers in 10-day-old eggs: both VN1203 HALo viruses encoding full-length NS1 proteins grew to greater than 10^8 PFU/ml by 48 hpi, while the NS1-truncated viruses grew to approximately 10^7 PFU/ml (Fig. 4A and B). An experiment in which the viability of inoculated embryos was monitored every 6 h furthermore indicated that the mean time to death for each virus was ≥50 h (Table 1). Thus, none of the rVN1203 HALo viruses caused death prior to the stage at which allantoic fluid would be harvested for the production of vaccines.

Next, the growth properties of the viruses were assessed in A549 cells, an epithelial cell line derived from human lung. Cells were inoculated at a low multiplicity (0.001 PFU/cell) and supernatants were sampled at 0, 18, 24, 48, and 72 hpi (Fig. 4C and D). In this substrate PR8 virus grew to approximately 3×10^5 PFU/ml. In contrast, both VN1203 HALo viruses with full-length NS1 genes grew to approximately 1,000 PFU/ml. The three VN1203 HALo PB2 627K viruses carrying deletions in the NS1 gene exhibited similar growth kinetics to each other, with peak titers of about 100 PFU/ml (Fig. 4D). The VN1203 HALo PB2 627E NS1 truncation viruses were more attenuated again, yielding titers around the limit of detection (50 PFU/ml per replicate) throughout the time course (Fig. 4C). Thus, in a human cell line, the rVN1203 HALo viruses were highly attenuated relative to PR8, showing very limited or no multicycle growth.

Viruses encoding truncated NS1 proteins induce elevated levels of type I IFN in infected A549 cells. The influenza NS1 protein has been shown to be an interferon antagonist in several in vitro and in vivo systems (27, 35). To determine if reduced growth correlated with an impaired ability to counteract the type I interferon response, a bioassay which measures the ability of NDV-GFP to replicate in pretreated A549 cells was performed. Cell culture supernatant from A549 cells infected at an MOI of 3 PFU/cell with each virus was collected and virus therein was inactivated at various time points postinfection. Fresh A549 cells were pretreated with the supernatants and subsequently infected with NDV-GFP. As shown in Fig. 5, supernatants from mock-infected cells did not inhibit the growth of NDV-GFP. Similarly, following pretreatment with supernatants from cells infected with any virus possessing a full-length NS1 protein, including the control PR8, no inhibition of NDV-GFP was observed until 13 hpi. In contrast, at 7 hpi, IFN had been produced in cells infected with three of the NS1-truncated viruses, while by 10 hpi supernatant from cells infected by any NS1-truncated virus completely inhibited NDV-GFP replication.

All VN1203 HALo viruses are attenuated in mice and protect mice from lethal challenge. To assess the safety of the rVN1203 HALo viruses in a mammalian host and obtain an initial estimate of their vaccine efficacy, we performed vaccine challenge studies in mice. Groups of four animals were vaccinated with 10^2, 10^3, 10^4, 10^5, or 10^6 EID_{50} of each virus through the intranasal route. Body weight was then monitored daily, up to 21 days postvaccination. All four viruses with glutamic acid at PB2 627 were strongly attenuated in mice, with only mice given the highest dose of VN1203 HALo/627E/NS FL showing mild (<10%) weight loss (Table 1). Viruses encoding lysine at PB2 627 were more virulent, but disease was limited to mice inoculated with the highest doses: VN1203 HALo/627K/NS FL induced significant weight loss when 10^5 or 10^6 EID_{50} was administered (22% and 18% weight loss, respectively); VN1203 HALo/627K/NS1-126 and NS1-73 viruses caused disease at 10^6 EID_{50} only, and one mouse given this dose of the NS1-73 virus died; the VN1203 HALo/627K/NS1-99 virus did not induce disease at any dose.

At 4 weeks postvaccination mice were challenged with 1,000 MLD_{50} of a virus carrying the VN1203 HALo and NA genes (a 2:6 reassortant with PR8 virus). Again, body weights were monitored daily, up to 14 days postchallenge (dpc). Vaccination with all eight rVN1203 HALo viruses afforded protection against challenge (Table 1), with the highest doses being most effective. At the highest vaccination dose, 10^6 EID_{50}, the three NS1 truncation viruses carrying PB2 627E completely prevented death but did not protect against transient weight loss. Lethality was also reduced following vaccination with 10^5 EID_{50} of these three viruses or with 10^4 EID_{50} of VN HALo/

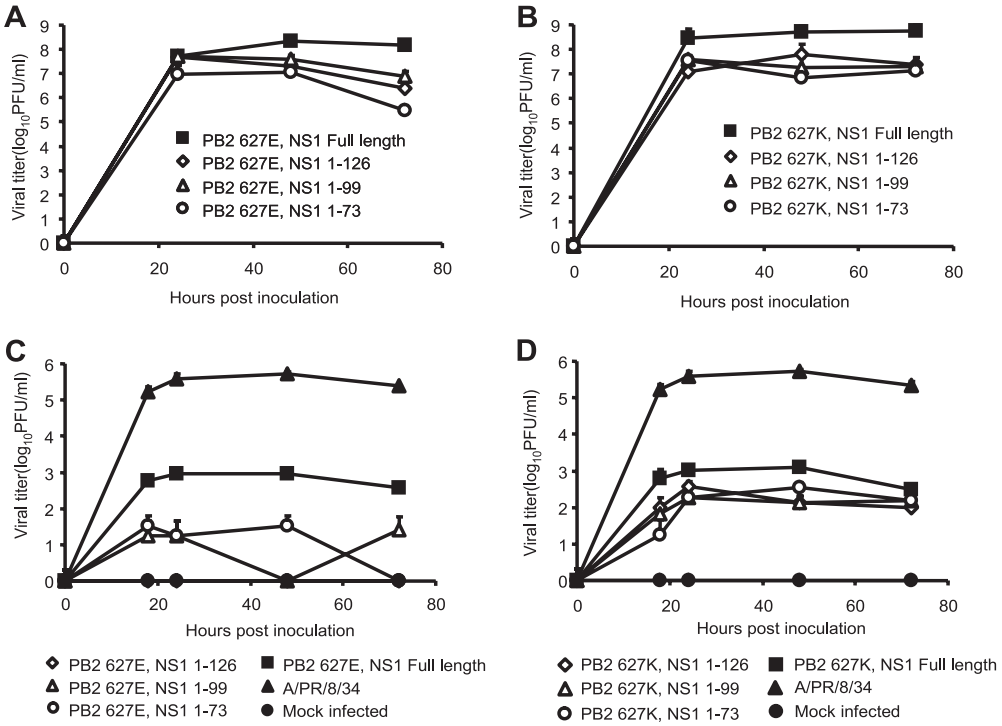


FIG. 4. Growth kinetics of A/Viet Nam/1203/04-derived viruses in embryonated chicken eggs and A549 cells. (A and B) Growth curves of recombinant A/Viet Nam/1203/04-derived viruses containing PB2 627E (A) or PB2 627K (B) in 10-day-old embryonated chicken eggs. Eggs were infected with each recombinant virus at 100 PFU per egg. Virus in allantoic fluid was quantified by plaque assay on MDCK cells. (C and D) Growth curves of recombinant A/VN/1203/04 (H5N1)-derived viruses containing PB2 627E (C) or PB2 627K (D) in A549 cells. In panel C, no open diamonds are visible because no virus was recovered at any time point from cells infected with the NS1-126 mutant. Cells were infected with each recombinant virus at an MOI of 0.001. Virus in cell culture supernatant was quantified by plaque assay on MDCK cells at the time points indicated.

627E/NS1-99. Indeed, VN HALo/627E/NS1-99 appeared to be the most effective of these three vaccine candidates. The VN HALo/627E/FL NS virus was also attenuated in mice: a vaccination dose of 10^3 EID_{50} reduced mortality, doses greater than 10^3 EID_{50} provided complete protection against death, and doses of either 10^5 or 10^6 EID_{50} also prevented signs of disease.

In general, viruses with lysine at PB2 position 627 provided greater protection at lower doses than did the equivalent viruses with PB2 627E. Among the three NS1 truncation mutants with PB2 627K, vaccination with 10^4 EID_{50} or greater gave complete protection from death following challenge. Doses of 10^5 or 10^6 EID_{50} also gave full protection against disease, while mice vaccinated with 10^4 EID_{50} showed mild weight loss (<10%).

Sera were obtained from all animals vaccinated with viruses

TABLE 1. Summary of genotypes and phenotypes in mice of candidate vaccine viruses

Virus name[a]	Genotype[b]	MDT[c] (h)	MLD_{50}[d]	Max % wt loss (SD)[e]	Lowest protective dose (EID_{50})[f]
VN HALo/627K/NS FL	PB2 627K, PB1, PA, HALo, NP, NA, M, NS	64, 61	$>10^6$	18 (4)	10^3
VN HALo/627K/NS 1-126	PB2 627K, PB1, PA, HALo, NP, NA, M, NS 1-126	45, 54	$>10^6$	15 (3)	10^4
VN HALo/627K/NS 1-99	PB2 627K, PB1, PA, HALo, NP, NA, M, NS 1-99	54, 49	$>10^6$	ND	10^4
VN HALo/627K/NS 1-73	PB2 627K, PB1, PA, HALo, NP, NA, M, NS 1-73	62, 63	$>10^6$	19 (8)	10^4
VN HALo/627E/NS FL	PB2 627E, PB1, PA, HALo, NP, NA, M, NS	58, 61	$>10^6$	2 (9)	10^4
VN HALo/627E/NS 1-126	PB2 627E, PB1, PA, HALo, NP, NA, M, NS 1-126	51, 54	$>10^6$	ND	10^6
VN HALo/627E/NS 1-99	PB2 627E, PB1, PA, HALo, NP, NA, M, NS 1-99	52, 47	$>10^6$	ND	10^5
VN HALo/627E/NS 1-73	PB2 627E, PB1, PA, HALo, NP, NA, M, NS 1-73	57, 52	$>10^6$	ND	10^6

[a] The virus name refers to the abbreviated description of the genotype used to identify each virus throughout the paper.

[b] All segments are derived from the A/Viet Nam/1203/04 virus. HALo refers to the HA segment of A/Viet Nam/1203/04 with the polybasic cleavage site removed, as described for Fig. 1. Numbers following the NS segment refer to the number of amino acids present in the NS1 protein starting from the amino-terminal methionine. Numbers following the PB2 segment refer to the identity of amino acid residue 627 in the PB2 protein.

[c] Mean time to death of eggs infected with VN1203 viruses. The results of two independent experiments are shown.

[d] The number of EID_{50} units required to kill 50% of groups of 6- to 8-week-old C57BL/6 mice ($n = 4$).

[e] The maximum average weight loss of groups of mice ($n = 4$) upon vaccination with 10^6 EID_{50} of virus. ND, no weight loss detected. Values in parentheses represent standard deviations from the means.

[f] The lowest dose of vaccination virus which subsequently conferred 100% protection from death following inoculation with 1,000 MLD_{50} of challenge virus.

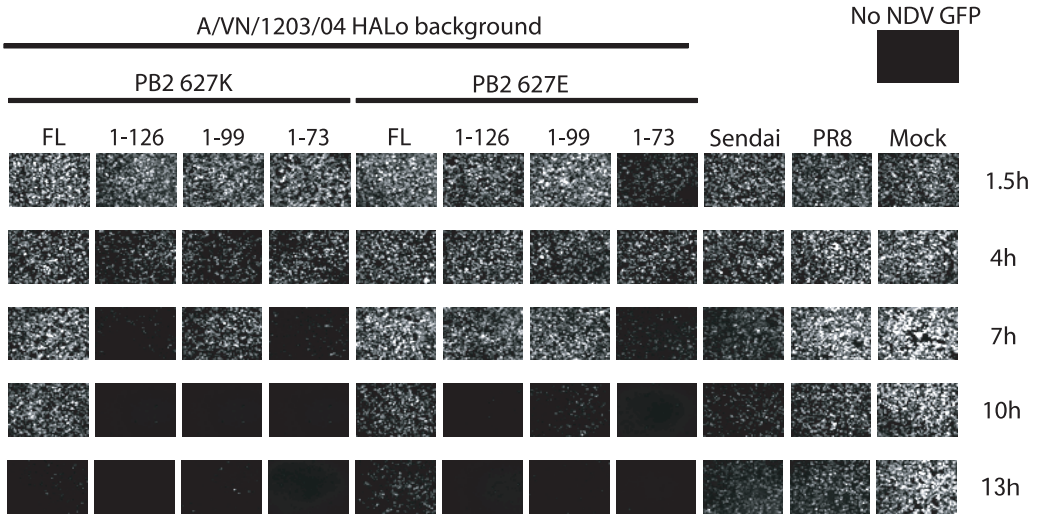


FIG. 5. Induction of IFN-α/β in A549 cells infected with A/Viet Nam/1203/04 (H5N1)-derived viruses. At various time points postinfection (indicated on the right) supernatants were harvested from A549 cells infected with the indicated viruses. Following UV inactivation, supernatants were applied to fresh A549 cells and incubated for 24 h, followed by NDV-GFP infection. At 18 h postinfection, cells expressing GFP were visualized by fluorescence microscopy.

containing PB2 627K, and viruses containing PB2 627E in combination with a full-length NS segment, at 4 weeks postvaccination and at 2 weeks postchallenge. Protection from death generally correlated well with the presence of neutralizing antibodies in postvaccination serum, although mice vaccinated with the lowest protective doses did not always show virus-neutralizing activity above the level of the control sera (Table 2). These mice may have possessed antibodies below the limit of detection of the assay. Mice which did not survive challenge generally did not possess neutralizing antibodies, as determined by microneutralization assay. Mice vaccinated with higher doses of virus tended to have higher neutralizing antibody titers in postvaccination serum (Table 2). Overall, the terminal serum samples yielded higher GMT values than the corresponding prechallenge samples; however, this effect was less pronounced for the VN HALo/627K/NS FL-vaccinated animals and mice that received 10^6 EID_{50} of the VN HALo/627K/NS 1-73 virus. In these latter cases, low terminal GMT values were most likely the result of poor virus replication and therefore little boosting effect upon challenge, due to vaccine-induced immunity. The fact that a low terminal GMT is seen with 10^6 EID_{50} of the HALo/627K/NS 1-73 virus and not 10^6 EID_{50} of the other truncation viruses is most likely due to superior protection by HALo/627K/NS 1-73 in mice. That the HALo/627K/NS 1-73 virus provided greater protection is evidenced by a lower maximum weight loss at the lowest protective dose (10^4 EID_{50}) for mice vaccinated with this strain, relative to results with the NS 1-99 and NS 1-126 equivalents (data not shown).

VN HALo/627E/NS1-99 virus protects chickens from lethal challenge. To examine the efficacy of the VN HALo/627E/NS1-99 virus as a poultry vaccine, 2-week-old specific-pathogen-free leghorn chickens were vaccinated with 10^6 PFU of the candidate vaccine virus. The level of replication of the vaccine strain was assessed through collection of tracheal and cloacal swabs on days 3, 5, and 7 p.i. from 12 animals. All cloacal swabs were negative. Tracheal swabs were negative with the exception of two chickens, which had titers of 4.8×10^3 EID_{50}/ml and 4.8×10^2 EID_{50}/ml on day 3 p.i. and one chicken which had a titer of 4.8×10^1 EID_{50}/ml on day 5 p.i. (Table 3). An

TABLE 2. Neutralizing activities of prechallenge and terminal mouse sera represented as GMTs

Virus (vaccine)	Dose (EID_{50})	GMT[a]	
		Prechallenge	Terminal
VN HALo/627K/NS FL	10^6	18.4	39.4
	10^5	29.9	59.7
	10^4	32.0	55.7
	10^3	≤13.9	128
	10^2	≤10.6	238.9
VN HALo/627K/NS 1-126	10^6	≤11.3	119.4
	10^5	≤8.0	168.9
	10^4	≤7.5	>238.9
	10^3	≤7.5	
VN HALo/627K/NS 1-99	10^6	21.1	>238.9
	10^5	≤9.2	>222.9
	10^4	≤9.2	>119.4
	10^3	≤7.5	
	10^2	BLD	
VN HALo/627K/NS 1-73	10^6	≤10.6	36.8
	10^5	13.9	119.4
	10^4	≤8	137.2
	10^3	≤7.5	207.9
VN HALo/627E/NS FL	10^6	13.9	42.2
	10^5	10.6	111.4
	10^4	11.3	119.4
	10^3	7.5	34.3
	10^2	7.5	

[a] Values represent GMT of sera collected from up to four mice. BLD, below the limit of detection.

TABLE 3. Serological, clinical, and virological outcomes of chicken vaccination and challenge

Group	Bird ID no.	Shedding of vaccine virus in trachea (EID_{50}/ml) (day 3, day 5)	HI/SN titers[a]		Clinical outcome[b]	Time to death (dpc)	Virus shedding after challenge (max. $TCID_{50}$/ml)	
			Homologous	Heterologous			Tracheal	Cloacal
VN HALo/627E/NS 1-99/VN1203 challenge	1	BLD,[c] BLD	160/40	40/40	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/VN1203 challenge	2	BLD, BLD	80/80	20/40	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/VN1203 challenge	3	BLD, BLD	160/160	80/ND	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/VN1203 challenge	4	BLD, BLD	640/320	320/320	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/VN1203 challenge	5	BLD, BLD	20/<40	<20/40	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/VN1203 challenge	6	4.8×10^3, BLD	320/160	80/160	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/Egret06 challenge	7	BLD, 4.8×10^1	320/80	320/80	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/Egret06 challenge	8	BLD, BLD	20/<40	80/40	Dead	11	BLD	BLD
VN HALo/627E/NS 1-99/Egret06 challenge	9	BLD, BLD	320/160	80/40	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/Egret06 challenge	10	4.8×10^2, BLD	640/320	640/160	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/Egret06 challenge	11	BLD, BLD	640/320	640/ND	Protected	ND	BLD	BLD
VN HALo/627E/NS 1-99/Egret06 challenge	12	BLD, BLD	40/<40	160/80	Protected	ND	BLD	BLD
Mock/VN1203 challenge	13	BLD, BLD	<20/<40	<20/40	Dead	4	2.32×10^4	1.08×10^6
Mock/VN1203 challenge	14	BLD, BLD	<20/<40	<20/<40	Dead	1	2.32×10^5	5.00×10^6
Mock/VN1203 challenge	15	BLD, BLD	<20/<40	<20/<40	Dead	3	1.08×10^5	2.32×10^6
Mock/VN1203 challenge	16	BLD, BLD	<20/<40	<20/<40	Dead	1	3.40×10^4	5.00×10^5
Mock/Egret06 challenge	17	BLD, BLD	20/<40	<20/<40	Dead	3	1.08×10^6	1.08×10^4
Mock/Egret06 challenge	18	BLD, BLD	<20/<40	<20/<40	Dead	1	2.32×10^5	2.32×10^4
Mock/Egret06 challenge	19	BLD, BLD	<20/<40	<20/<40	Dead	3	1.58×10^6	5.00×10^4
Mock/Egret06 challenge	20	BLD, BLD	<20/<40	<20/<40	Dead	2	1.58×10^6	2.11×10^5

[a] Hemagglutination inhibition (HI) and serum neutralizing (SN) titers are given as the reciprocal of the highest dilution of serum that showed activity. "Homologous" refers to activity against A/Viet Nam/1203/04 virus, and "heterologous" refers to activity against A/egret/Egypt/01/06 virus. ND, not determined.

[b] Bird number 8 was alert only when personnel were nearby by 7 dpc. Disease progressed thereafter, culminating in the development of neurological signs by 11 dpc, when the animal was euthanized.

[c] BLD, below the limit of detection.

additional two chickens were euthanized on day 3 p.i., and the viral loads in lung and intestinal tissues (cecal tonsils) were assessed. Virus was detected in lung tissues of both animals, with titers of 1.5×10^3 EID_{50}/ml and 3.0×10^4 EID_{50}/ml, but undetectable in intestinal tissue.

Ten out of 12 chickens developed quantifiable hemagglutination inhibition and serum neutralizing titers to both A/Viet Nam/1203/04 and A/egret/Egypt/01/06 influenza virus by 4 weeks postvaccination (0 dpc) (Table 3), whereas sham-inoculated birds did not. Upon challenge with either HPAI virus, all mock-vaccinated birds died, with a mean death time of 2.25 days. Correspondingly, the HPAI viruses replicated to very high titers in both the respiratory and intestinal tracts of all sham-vaccinated birds (Table 3).

In contrast, 100% (6/6) or 83% (5/6) of the vaccinated chickens were fully protected against 100 CLD_{50} of either A/Viet Nam/1203/04 or A/egret/Egypt/01/06 viruses, respectively. One bird (number 8), however, displayed clinical signs of disease by 7 dpc and was euthanized on day 11 when it developed neurological signs (ataxia and torticollis). Nevertheless, the challenge virus was below the limit of detection in either the swab samples or in the several organs taken from this animal. Although we cannot rule out the possibility that this animal died as a consequence of the virus challenge, it did show positive serconversion prior to challenge. Furthermore, the bird did survive longer than any of the mock-vaccinated birds, suggesting at least partial protection.

Virus shedding from the trachea or cloaca was not detected in any of the vaccinated chickens following challenge with either virus (Table 3). Remarkably, none of the A/Viet Nam/1203/04-challenged birds showed significant increases in hemagglutination inhibition/microneutralization titers after challenge (Table 3), suggesting that replication of the homologous virus was abolished at the site of infection. In contrast, at least 80% (5/6) of the A/egret/Egypt/01/06-challenged chickens mounted a quantifiable anamnestic response by 14 dpc. These results imply that although sterile immunity against the heterologous egret virus was not achieved, it was sufficient to both protect animals from clinical disease and to restrict virus shedding to levels below the limit of detection.

Taken together, these results suggest that a single dose of VN HALo/PB2E/NS1-99 vaccine provides sterile immunity against homologous virus and a high level of protection against heterologous HPAI H5N1 challenge in poultry.

DISCUSSION

As a result of the magnitude and severity of recent and ongoing outbreaks of HPAI (H5N1), interest in the development of high-quality vaccines against avian influenza has been heightened. Control programs designed to prevent, manage, or eradicate HPAI which have been developed by OIE/FAO in collaboration with WHO now include the use of vaccines against avian influenza (31). Such a vaccine should ideally meet three criteria: (i) it must be safe in both host and the environment; (ii) its use must be economically feasible; and (iii) it must be efficacious. Here, we have used reverse genetics to generate a panel of eight candidate live attenuated vaccine viruses. The viruses were attenuated in mammalian substrates and grew to high titers in embryonated chicken eggs. Furthermore, all eight vaccine candidates proved effective in protecting mice and one candidate vaccine which was chosen to be tested in chickens was also found to protect this species against challenge with highly pathogenic avian influenza (H5N1) viruses.

The high pathogenicities of currently circulating H5N1 strains present two significant obstacles to vaccine production using conventional methods: (i) high virulence prevents growth of the viruses to maximum titer in 10-day-old embryonated eggs, the industry standard substrate for the production of influenza vaccines, and (ii) high containment facilities are required in order to protect workers and the environment from possible exposure to the virus. Both of these issues have been addressed herein through the design of reverse genetics-based vaccines. The cleavability of the HA protein of avian influenza viruses is a critical determinant of virulence (11, 37); low-pathogenicity influenza A strains of subtypes H5 and H7 have each been reported to gain a high-pathogenicity phenotype in birds following the acquisition of a polybasic cleavage site in the HA protein (12, 33). In order to generate H5 viruses with sufficiently attenuated virulence to allow growth to high titer in 10-day-old embryonated chicken eggs, viruses were reverse engineered to remove the nucleotides encoding the polybasic cleavage site in the HA. Each of the recombinant viruses used in the study grew to maximal titer between 24 and 48 h, a time shorter than the time to death of the embryonated eggs used for growth, demonstrating that 10-day-old eggs can be conveniently used for the production of the vaccine. Furthermore, the use of cDNA to generate the viruses de novo allowed the codon usage at the HA cleavage site to be altered in a way that should reduce the risk of a multibasic cleavage site being reintroduced. Specifically, the number of adenosine nucleotides present at the cleavage site was minimized (Fig. 1A). We reasoned that by removing adenosines we would lower the possibility of a reintroduction of the polybasic cleavage site by limiting the opportunity for polymerase stuttering to occur. Our strategy has proven successful: sequencing of each of the eight recombinant viruses used in this study, following multiple rounds of growth in embryonated eggs (at least seven passages for each virus), has shown no alteration to the engineered sequence introduced into the HA segment. Furthermore, sequencing of three independent clones of the NS 1-73 vaccine virus isolated from vaccinated chickens showed that, in each case, the PB2 627E polymorphism was retained, there were no alterations from the low-pathogenicity cleavage site motif of the HA gene and no reversion from the truncated form of the NS1 gene. In one clonal isolate, there was an amino acid change in the NEP protein (I32T); the significance of this mutation, if any, is currently unknown. Thus, we have shown that the candidate vaccine viruses are stably attenuated in an avian substrate and that the stability of a low-pathogenicity-type cleavage site can be increased through rational engineering of the genome. In addition to attenuating virulence in eggs, we showed that removal of the polybasic cleavage site leads to reduced pathogenicity in a mammalian model. The removal of the polybasic cleavage site from A/Viet Nam/1203/04 virus results in an increase in the mouse LD_{50} value by approximately 10,000-fold (data not shown). A similarly high level of attenuation in a mouse model was observed by Hatta et al. using a strain of H5N1 from the original outbreak of highly pathogenic avian influenza in Hong Kong in 1997 (9). While the potential for human infection with live attenuated poultry vaccines remains a formal possibility, our data suggest that the stable removal of the polybasic cleavage site from the HA of recombinant viruses used in this study greatly reduces the risk they would pose for vaccine production workers or the environment.

In addition to growth to high titers and safety, a basic requirement of a vaccine is adequate protective efficacy. In order to achieve attenuation in virulence while retaining sufficient immunogenicity to induce a protective response, we attempted to cover a broad range of attenuation phenotypes by creating multiple vaccine candidates. We introduced attenuating mutations (in addition to removal of the polybasic cleavage site) into a panel of viruses to produce genotypes which combined (i) incremental truncation of the NS1 protein and (ii) either lysine or glutamic acid at amino acid position 627 of the PB2 protein. The truncations of the NS1 protein were expected to attenuate the recombinant viruses in any host species, while the presence of a glutamic acid in the PB2 protein would attenuate viruses in mammalian hosts only. We hypothesized that a virus could be selected from our panel with the correct balance of attenuation and immunogenicity to provide complete protection from disease in birds, while not inducing pathogenicity on vaccination.

Whether in the background of a virus encoding K or E at PB2 627, truncations of any length in the NS1 open reading frame achieved similar levels of attenuation relative to the corresponding virus encoding a full-length NS1. All viruses encoding NS1 truncations were shown to exhibit attenuated growth in vitro and in ovo and, as expected, the degree of attenuation correlated with increased induction of IFN observed in infected cell cultures. In the mouse model, we saw that vaccination with each of the viruses encoding an NS1 truncation led to protection.

As has been shown before in highly pathogenic influenza virus backgrounds, the presence of E at PB2 627 was found to decrease pathogenicity of the candidate vaccine viruses in a mouse model (9). Attenuating effects of PB2 627E on viral growth would be expected in a mammalian host due to the decreased polymerase activity and viral growth observed at low temperature (33°C) for viruses encoding 627E (15). Indeed, Hatta et al. have reported that viral growth in the upper respiratory tract of mice is restricted by the presence of E at PB2 627 (10). A recently proposed mechanism to explain this dif-

ference in activity involves the presence of an inhibitory factor in mammalian cells which reduces the activity of influenza virus polymerase complexes encoding a PB2 627E polymorphism (16). Toward the aim of increased safety for humans, we introduced a glutamic acid at PB2 position 627. Although this site is subject to rapid mutation upon introduction of avian influenza viruses into mammalian hosts, the initial presence of 627E is desirable and, furthermore, the attenuated replication of the NS1-truncated viruses reduces the probability of reversion.

All eight of our candidate vaccine viruses were protective against lethal challenge in mice. Each virus with a truncated NS1 open reading frame and E or K at PB2 627 was also highly attenuated in mice. Thus, viruses with a desirable balance between attenuation and immunogenicity were obtained. Based on its genotype and its attenuated phenotype in mice, the VN HALo/627E/NS 1-99 virus was chosen to be tested in chickens.

The NS1 1-99 virus also proved to be attenuated in the chicken model. Two-week-old chickens vaccinated with 10^6 PFU of virus exhibited no mortality or morbidity. Crucially, chickens challenged with a homologous clade 1 A/Viet Nam/1203/04 virus subsequent to the single vaccination dose were entirely protected from signs of disease and mortality. We further tested the efficacy of the vaccine by challenging immunized animals with a heterologous clade 2.2 H5N1 virus, A/egret/Egypt/01/06, the HA protein of which possesses approximately 7% amino acid divergence with VN1203 in the HA1 portion. Even after this heterologous challenge, greater than 80% of the vaccinated animals were protected from signs of disease and death. In spite of the increase in antibody titers after the heterologous challenge, none of the vaccinated animals shed detectable amounts of virus, emphasizing the efficacy of the VN HALo/627E/NS 1-99 vaccine in poultry.

Importantly for the feasibility of poultry vaccination, our results show that a single dose of live attenuated vaccine is sufficient to produce a protective immune response in both mouse and chicken models. This observation is consistent with previous results using alternative live attenuated influenza vaccines in chickens (30). In contrast, clinical trials using inactivated H5N1 influenza vaccines have shown that, for the production of a significant immune response, two doses containing large quantities of antigen are needed (4), and furthermore, the use of adjuvants may be required to achieve optimal results (2, 36). Using the live attenuated NS1-truncated H5N1 vaccines, mice and chickens were both shown to be completely protected from disease after a lethal challenge of 1,000 MLD_{50} or 100 CLD_{50} 4 weeks after administration of a single vaccination dose. One consequence of the application of the NS1 truncation strategy to the generation of chicken vaccines is that interferon produced in the substrate (chicken's eggs) will be active in the vaccinated animals. The impact (positive or negative) of interferon present in vaccine preparations has not been evaluated in the present work. Nevertheless, our results suggest that live attenuated vaccines based on truncations of the NS1 protein are an economical and efficient way to achieve immunization.

One current limitation in the use of conventional vaccines against AIV is that vaccinated poultry frequently cannot be differentiated from naturally infected birds based on serology, making surveillance difficult to perform. However, it has been demonstrated that differentiation between vaccinated/exposed animals and vaccinated/unexposed animals is possible, using a strategy known as "differentiating infected from vaccinated animals" (DIVA) (2a). The DIVA strategy requires a vaccine which lacks one or more antigens present in the circulating avian influenza strain. Thus, in principle, a live attenuated virus lacking the C-terminal domain of NS1 could meet the DIVA criterion. Animals vaccinated with an NS1-truncated virus would not produce antibodies against the C terminus of NS1.

In response to the need for effective H5 subtype vaccines in poultry, we have generated and characterized a panel of recombinant viruses encoding attenuating mutations in the HA, PB2, and NS1 proteins. Vaccination of chickens achieved homologous and heterologous protection from challenge with highly pathogenic H5N1 influenza viruses. Safety and protective efficacy in the mouse model furthermore suggested that these vaccines may be suitable for use in mammalian hosts.

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