Feasibility of reconstructed ancestral H5N1 influenza viruses for cross-clade protective vaccine development

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Since the reemergence of highly pathogenic H5N1 influenza viruses in humans in 2003, these viruses have spread throughout avian species in Asia, Europe, and Africa. Their sustained circulation has resulted in the evolution of phylogenetically diverse lineages. Viruses from these lineages show considerable antigenic variation, which has confounded vaccine planning efforts. We reconstructed ancestral protein sequences at several nodes of the hemagglutinin (HA) and neuraminidase (NA) gene phylogenies that represent ancestors to diverse H5N1 virus clades. By using the same methods that have been used to generate currently licensed inactivated H5N1 vaccines, we were able to produce a panel of replication competent influenza viruses containing synthesized HA and NA genes representing the reconstructed ancestral proteins. We identified two of these viruses that showed promising in vitro cross-reactivity with clade 1, 2.1, 2.2, 2.3.4, and 4 viruses. To confirm that vaccine antigens derived from these viruses were able to elicit functional antibodies following immunization, we created whole-virus vaccines and compared their protective efficacy versus that of antigens from positive control, naturally occurring, and broadly reactive H5N1 viruses. The ancestral viruses' vaccines provided robust protection against morbidity and mortality in ferrets challenged with H5N1 strains from clades 1, 2.1, and 2.2 in a manner similar to those based on the control strains. These findings provide proof of principle that viable, computationally derived vaccine seed viruses can be constructed within the context of currently licensed vaccine platforms. Such technologies should be explored to enhance the cross reactivity and availability of H5N1 influenza vaccines.

universal | cross-reactive | pandemic

Highly pathogenic avian influenza (HPAI) A H5N1 remains a significant pandemic threat (1). The precursor of these viruses, A/goose/Guangdong/1/1996, was first isolated from a goose in Guangdong, China (2), and its derivatives have since spread throughout Southeast Asia, Eurasia, and Africa. As of June 17, 2010, the World Organization for Animal Health updates of HPAI H5N1 outbreaks highlighted 6,731 outbreaks in poultry in 51 countries (3). Reported human cases caused by this virus as of August 3, 2010, numbered 505, of which 300 were fatal (4).

In conjunction with their spread, H5N1 viruses have diversified into multiple lineages, many of which continue to concurrently circulate and cause human infections. These lineages have been classified into clades (0 to 9) and subclades on the basis of their hemagglutinin (HA) gene genealogy (5). Viruses from these different clades also show considerable antigenic variation, which confounds pandemic preparedness efforts. Additional complexities have emerged as a result of the difficulty in handling and shipping HPAI viruses and sovereignty issues surrounding isolates. Antiviral drugs are effective against HPAI H5N1 infection if they are administered early enough (6). However, acquired resistance to these drugs can develop. Therefore, significant research efforts have been directed toward developing broadly reactive and alternative reagents, including vaccines, for pandemic preparedness.

Several approaches for alternative, broadly reactive vaccines have been proposed. M2e peptide vaccines, based on the conserved ectodomain of matrix protein 2, have shown promise to provide broad protection against different influenza viruses when used in murine models (7, 8). Another potential solution presented has been to mix antigens from multiple viruses in a single vaccine. This approach was previously tested in animal models with a mixture of inactivated whole viruses from different HPAI H5N1 clades, which successfully protected ferrets against challenge with clades 1 and 2 (6). However, it requires the production of multiple antigens, which will limit the number of doses produced. Consensus HA and neuraminidase (NA) synthetic DNAs have also been explored as an approach to enhance H5N1 vaccine efficacies. With such DNA vaccines, only partial protection was observed in mice challenged with HPAI H5N1 (9–11). Although promising, the consensus DNA methods underestimate the true diversity of the circulating viruses and are based on influenza vaccine platforms not yet approved for human use.

Here we propose an ancestral sequence reconstruction method based on a resolved phylogenetic topology to produce vaccine candidate viruses. Ancestral sequences are computationally derived sequences that represent the most recent common ancestor of a pool of viruses (i.e., the internal nodes of a phylogenetic tree) (12). Despite their potential benefits, it is unknown if these ancestral HA and NA proteins could be used to construct viable, antigenically representative viruses able to fit within the limitations of currently licensed inactivated influenza vaccine platforms (13, 14). To address these issues we generated the sequence of putative ancestral HA and NA genes and created a panel of replication competent attenuated influenza strains. The ability of these viruses to elicit functionally useful antibodies in the context of inactivated vaccines was then assessed in vitro and in vivo in the ferret model alongside two of the broadly reactive vaccine seed strains recommended by the World Health Organization (WHO). This study provides the proof of concept that viable vaccine seed viruses can be generated by the synthesis of putative ancestral sequences and that further evaluation and optimization of such approaches is warranted.

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Results

Phylogenetic Reconstruction and Calculation of Ancestral Sequences. To generate the putative ancestral viral antigens, phylogenetic trees were produced from the H5 and N1 sequences available in public databases. The phylogeny generated for the HA gene was consistent with current H5N1 clade nomenclature (Fig. 1*A*). For the NA gene, the phylogeny indicated that there were two major groups, with and without the 20-aa deletion in the NA stalk (Fig. 1*B*). The putative HA ancestral aa sequences were calculated for four nodes (Fig. 1*A*) and differed by only 3 to 9 aa (3 aa difference between B and C and between B and D; 5 aa between C and D; 6 aa between A and B; and 9 aa between A and C and between A and D). Putative NA ancestral amino acid sequences were predicted for the root of the phylogeny (r; Fig. 1*B*) and at the common ancestor for all NA genes with a 20-aa deletion in the stalk region (d; Fig. 1*B*). Both NA ancestral genes differed not only by presence or absence of 20 aa in the stalk region but also by an additional 8 aa mutations. Sequences of the four HA and two NA ancestral constructs are presented in *Appendix S1*. Each of the four HA and two NA genes were synthesized and cloned into pHW2000 (15).

Virus Rescue. Currently licensed H5N1 influenza vaccines are based on the growth of vaccine seed strains in embryonated chickens eggs or cultured cells. The seed viruses contain six gene segments (PB2, PB1, PA, NP, M, NS) from A/Puerto Rico/8/34 and the HA and NA of the target virus (so-called 6+2 viruses). As we wished to remain within the framework of currently licensed influenza vaccine platforms, we next attempted to create replicating influenza viruses containing combinations of the computed ancestral HA and NA genes by using plasmid-based reverse genetics (rg). rg 6+2 viruses were successfully generated for the different H5N1 clade representatives and for the eight ancestral H5N1 vaccine candidates—A+d, A+r, B+d, B+r, C+d, C+r, D+d, and D+r—showing that the computationally derived sequences coded for functional proteins and could be used to create a replicating virus. Moreover, the eight rescued ancestral viruses grew to similar titers in Madin–Darby canine kidney cells as similar strains derived from contemporary H5N1 viruses (as shown by the end point of 50% tissue culture infectious dose; Table S1). The four viruses containing the r N1 ancestral gene grew to a higher titer in eggs than the viruses containing the d N1, with a difference of as much as 3 $\log_{10}$ (Table S1). A+r, B+r, C+r, and D+r were therefore selected for the work presented in this study, and are referred to as "A", "B", "C", and "D" from now on.

Antigenic Properties of Ancestral Viruses. To assess the potential of the four ancestral viruses A, B, C, and D, and the reference H5N1 isolates A/Vietnam/1203/04 (VN1203, clade 1), A/Hong Kong/213/03 (HK213, clade 1), A/duck/Hunan/795/02 (DKHUN795, clade 2.1), A/whooper swan/Mongolia/244/05 (WSM244, clade 2.2), A/turkey/Egypt/7/07 (TYEGY7, clade 2.2), A/Japanese white-eye/Hong Kong/1038/06 (JWEHK1038, clade 2.3.4), A/duck/Laos/3295/06 (DKLAO3295, clade 2.3.4), and A/goose/Guiyang/337/06 (GG337, clade 4) as vaccine antigens, the cross-reactivity of their homologous antisera toward various H5N1 viruses was studied by ELISA, hemagglutination inhibition (HI) assay, and microneutralization (MN) assay. Table 1 summarizes the results and shows the considerable diversity in the degree of reactivity to the various reference viruses. Of the reference antisera, only those from DKHUN795 and GG337 had ELISA titers to the clade 1 VN1203 antigen that were within twofold of the homologous serum values (i.e., VN1203 antiserum); three of the four ancestral virus antisera (A, C, and D) fell within this range. In comparison, reactivities to the clade 2.2 (A/Egypt/2321-NAMRU3/06), 2.3.4 (JWEHK1038), and 4 (GG337) antigens were somewhat reduced, with only the VN1203, DKHUN795, A, and D-specific serum (and JWEHK1038 serum for GG337 antigen) less than twofold lower than the homologous antiserum values. With JWEHK1038, titers from VN1203, DKHUN795, and D-specific serum were actually higher than that of the homologous. Of note, the clade 2.1 antigen, A/Indonesia/5/05, was well recognized by all the sera tested (ELISA normalized titers >0.5; Table 1). HI and MN assays both gave overall low values, suggesting a modest cross-neutralization for ancestral as well as reference H5N1 strains. A and D showed a slightly higher cross-reactivity versus their ancestral counterparts in HI and MN assays, respectively (Table 1). Overall, the postinfection ferret antiserum raised to VN1203, DKHUN795, and ancestors A and D were consistently the most cross-reactive, a trend also seen in HI and MN assays and one confirming the ability of the synthetic proteins to induce antibodies able to recognize a range of H5 viruses.

Ancestral Viruses as Vaccine Antigens. To determine the ability of the computationally derived ancestral HA and NA proteins to act as vaccine antigens, groups of ferrets were immunized twice with inactivated whole virus vaccines made from A and D and then challenged with H5N1 viruses representative of currently circulating clades: clade 1 (VN1203), clade 2.1 (DKHUN795), and clade 2.2 (TYEGY7). As a positive control, whole virus vaccines to the most broadly in vitro reactive reference viruses, VN1203 and DKHUN795, were also included. Because of the relatively mild virulence of DKHUN795 in ferrets, a reassortant virus was used as the challenge virus in these experiments (HA and NA of DKHUN795 and internal genes from VN1203). Table S2 shows the differences between the HA proteins of the vaccine antigens DKHUN795, VN1203, A, and D. DKHUN795 and D were the most similar strains with only 3 aa differences. A and D, A and DKHUN795, D and VN1203, A and VN1203, and VN1203 and DKHUN795 had 9, 10, 12, 13, and 13 aa differences, respectively. Whereas mutations at position 8 (signal peptide) and positions 494 to 525 (transmembrane domain) likely have little effect on the immunogenicity of the vaccine antigens, the 12 mutations in the globular head of the HA (between positions 52 and 336; Table S2) likely play a more important role. Most notably, positions 145 and 172 (129 and 156 H5 numbering) were located within antigenic sites 3 and 2, respectively. Mutations at these sites distinguish VN1203 and A, and DKHUN795 and D.

Before challenge, ferrets were bled, and the development of antibodies against the immunizing antigen was investigated by HI assays (Table S3). MN titers were also determined, and they correlated with HI titers. "Protective" serum HI titers of 40 against their respective homologous strains were reached in 33% to 83% of the vaccinated ferrets. The most immunogenic antigens (as measured in homologous HI) were those derived from D and DKHUN795, for which 67% and 83% of ferrets, respectively, had HI titers of at least 40 (whereas it was true for only one third of the A- and VN1203-vaccinated ferrets). The arithmetic mean titers of the D- and DKHUN795-vaccinated animals were 95 and 87, respectively: significantly higher than those of the A- and VN1203-vaccinated ferrets (37 and 26, respectively; $P = 0.011$, ANOVA). The titers against the challenge strains ranged from undetectable to equivalent to those of the homologous strains. Mock-vaccinated ferrets did not have any detectable HI titers against the different H5N1 strains (Table S3).

After challenge with H5N1 strains VN1203, DKHUN795, and TYEGY7, all vaccinated ferrets survived irrespective of their vaccine regimen (Fig. 2), and they had no significant weight loss (Fig. S1). Mock-vaccinated animals all died or were euthanized by day 5 or by day 8 when infected with VN1203 or DKHUN795, respectively. Although mock-vaccinated animals challenged with TYEGY7 survived, they lost weight (Fig. S1), were lethargic, and showed neurological signs.

There were significant differences between vaccinated and control groups in the amount of virus detected in the nasal washes of DKHUN795- and TYEGY7-infected animals 3, 5, and

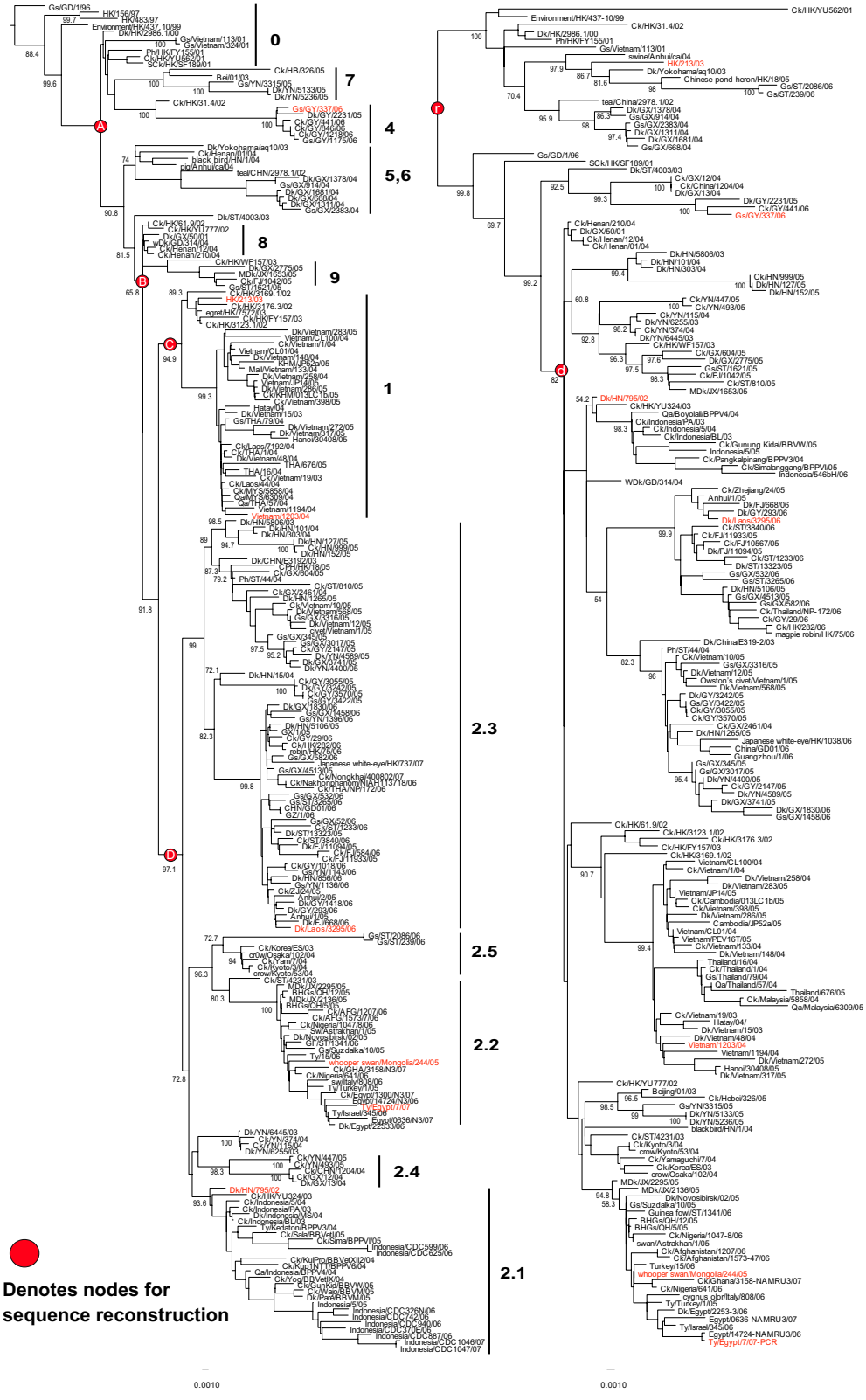

Fig. 1. Phylogenetic relationship of influenza A viruses used to calculate putative ancestral sequences. Four nodes on the HA gene (a) were chosen to represent (*A*) the midpoint root of the tree; (*B*) the ancestral node common to clades 1, 2.1, 2.2, 2.3, 2.4, 2.5, and 8; (*C*) the ancestral node to clade 1 viruses; and (*D*) the ancestral node for all clade 2 viruses. For the NA gene (b), two nodes were predicted, one at the root of the phylogeny (r) and another at the common ancestor for all NA genes with a 20-aa deletion in the stalk region (d). Numbers at branch nodes indicate neighbor-joining bootstrap values of at least 70% for major clades. Analyses were based on nucleotides 1 to 1,707 and 1 to 1,407 of the HA and NA genes, respectively. The HA tree was rooted to A/goose/Guangdong/1/96 and the NA tree was midpoint rooted. Numbers to the right of the figure refer to WHO H5N1 clade designations (5). (Scale bar, 0.001 substitutions per site.) Viruses used in the present study are in red font.

Table 1. ELISA, HI and MN titers in infected ferret serum

Antiserum/ titer	Antigen				
	Clade 1 (VN1203)	Clade 2.1 (A/Indonesia/5/05 or DKHUN795)	Clade 2.2 (A/Egypt/2321-NAMRU3/06 or WSM244)	Clade 2.3.4 (JWEHK1038)	Clade 4 (GG337)
VN1203					
ELISA	*1 (135)*	0.75	0.95	2.1	3.6
HI	*1 (26)*	0.33	0.29	0.09	0.79
MN	*1 (113)*	0.13	0.50	0.02	0.63
DKHUN795					
ELISA	0.71	*1 (80)*	1.76	1.9	1.1
HI	0.16	*1 (160)*	0.88	0.08	0.06
MN	0.16	*1 (842)*	0.90	0.03	0.10
WSM244					
ELISA	0.27	0.63	*1 (113)*	0.27	0.33
HI	0	0.24	*1 (74)*	0.02	0.05
MN	0.53	0.94	*1 (125)*	0.17	0.43
JWEHK1038					
ELISA	0.25	0.56	0.40	*1 (57)*	1.1
HI	0	0.13	0.06	*1 (37)*	0
MN	0.10	0.12	0.29	*1 (258)*	0.26
GG337					
ELISA	0.89	1.23	0.18	0.47	*1 (40)*
HI	0.12	0	0	0	*1 (226)*
MN	0.13	0.02	0.11	0.01	*1 (1,417)*
A					
ELISA	0.79	0.75	0.60	1	0.55
HI	0.28	0.03	0.53	1.28	0.31
MN	0.16	0.12	0.21	0.12	0.19
B					
ELISA	0.32	0.56	0.12	0.4	0.33
HI	0.02	0.09	0.11	0.70	0.05
MN	0.10	0.11	0.19	0.08	0.11
C					
ELISA	0.57	0.69	0.02	0.23	0.22
HI	0.25	0.22	0.23	0.34	0.04
MN	0.11	0.08	0.11	0.05	0.08
D					
ELISA	0.79	0.88	0.88	3.9	4.9
HI	0.02	0.21	0.35	0.63	0
MN	0.13	0.21	1.18	0.17	0.17

Normalized titer: ratio of heterologous titer to homologous titer, ratio calculated animal per animal. Clade-homologous titers have both normalized and geometric mean titers (e.g., "1 (135)" for VN1203 ELISA titer) and are indicated in italics. Four ferret sera were tested against each antigen. Geometric mean titers are indicated in parentheses.

7 d after infection ($P < 0.01$; Fig. 3). Despite a trend for lower viral titers in vaccinated versus control animals 3 d after infection in the VN1203 challenge, the differences were not significant. All VN1203 control animals died by 5 d after infection. No statistically significant differences were observed among the different vaccine groups for any of the three challenge viruses, indicating that the ancestral vaccine antigens were able to induce antibodies that were indeed protective and generally similar in nature to those induced by reference viruses.

Discussion

The challenge in influenza vaccination is that the most potent form of immunity, neutralizing antibodies, targets the most variable viral protein, HA. This dilemma has proven to be especially challenging in the context of developing H5N1 stockpile vaccines, because the virus has evolved into several antigenically and genetically distinct clades in the 14 y it is known to have circulated (16). The current solution of the WHO to this diversification is the creation of additional vaccine seed strains to cover this diversity; the current tally of such seed stocks is 13, with six more in various stages of development (17). Newer approaches must be evaluated, as not only is it economically restrictive to produce vaccines from each seed virus, but it is also becoming less clear which are the most useful seed strains for a given region. Our hypothesis that viable viruses derived from computationally generated ancestral HA and NA proteins could be generated was validated.

We found that each of the four computationally derived HA proteins and the two NA proteins were functional and able to be incorporated into replicating influenza viruses through the use of the eight-plasmid reverse genetics system (15). We did find, however, that viruses containing the NA protein with a stalk deletion were less able to replicate in chicken eggs. Castrucci and Kawaoka demonstrated in 1993 that mutated A/WSN/33 (H1N1) strains with a shortened stalk replicated poorly in eggs and did not cause systemic disease (18). In 2009 Matsuoka et al. (19) and Zhou et al. (20) both showed higher virulence of H5N1 viruses with longer NA stalks in chickens, but their studies reported varying effects in mice (19, 20). Although isolates with the NA stalk deletion did grow, we did not proceed with them because of the importance of egg growth for current manufacturing of influenza vaccines. In addition, the immunogenicity of the varying

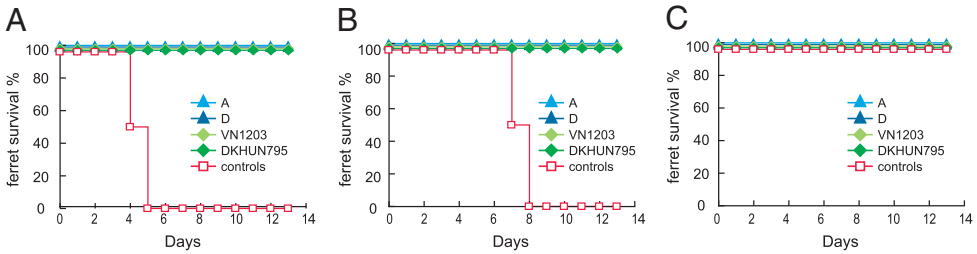

Fig. 2. Ferrets' survival rates after infection with VN1203 (*A*), DKHUN795 (*B*), and TYEGY7 (*C*). Control (mock-vaccinated) ferrets are represented by red open squares. Ferrets vaccinated with ancestral strains are represented by closed blue triangles: A in light blue and D in dark blue. Ferrets vaccinated with positive control isolate-based strains are represented by green closed diamonds: VN1203 in light green and DKHUN795 in dark green.

ancestral NA proteins was not tested here because of the greater importance of HA-specific antibodies in the protective response. Although we found a growth advantage for viruses containing the ancestral NAs without deletions, it is interesting that this was not necessarily seen with reassortant viruses containing WT NAs; good egg growth properties were seen with reassortant viruses containing WT NAs with stalk deletions. The reasons underlying egg growth phenotypes remain unclear, but it is clear that factors other than the length of the NA stalk can also play important roles.

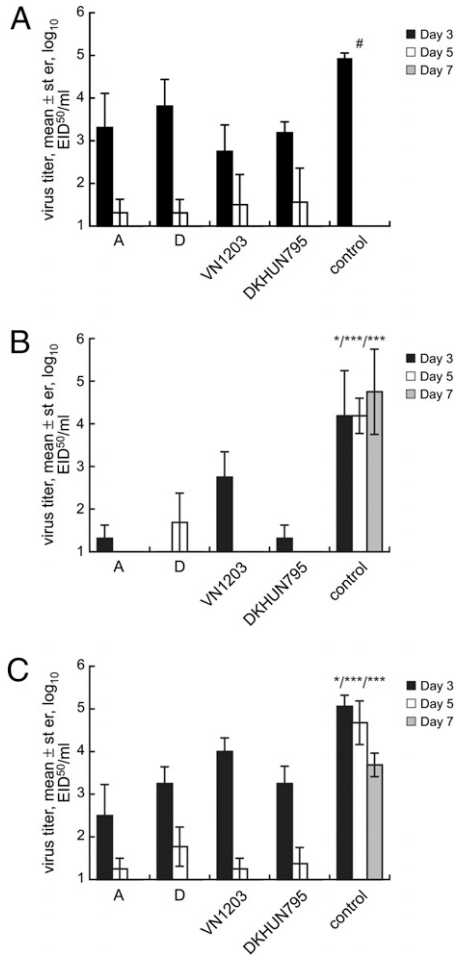

Fig. 3. Ferrets' nasal wash titers after infection with VN1203 (*A*), DKHUN795 (*B*), and TYEGY7 (*C*). The mean virus titers of nasal washes for the four animals per group (same vaccine and same challenge virus) are expressed in $\log_{10}$ EID_{50}/mL ± SEM (st er). Titers are grouped by vaccination regimen on the *x* axis. Black, white, and gray filled bars represent titers 3, 5, and 7 d after infection, respectively. (*$P \leq 0.05$ and ***$P \leq 0.001$ by ANOVA comparing the five vaccine regimen groups virus titers.) Slashes separate ANOVA *P* values at days 3, 5, and 7. [#All mock-vaccinated (control) VN1203-infected ferrets were dead before nasal washes could be collected at days 5 and 7 after infection.]

The premise of the ancestral virus approach is that such viruses may be broadly representative of the population of influenza viruses circulating. Even though this study was primarily designed as a proof of principle of the approach, we did evaluate the ability of the ancestral proteins to induce antibodies able to bind to H5N1 influenza strains. Despite some heterogeneity, antiserum specific to the A and D antigens was able to recognize each of the reference antigens with less than a 50% ELISA titer decrease compared with the values for the homologous antigens. Interestingly, antigen A induced cross-reactive antiserum as this virus contains the putative ancestor HA antigen of all circulating clades. Given the basal position of virus A, the broad cross-reactivity of its antiserum appears to confirm the rationale for these experiments.

Although the approach used in this study incorporates the evolutionary history of the virus population, the method is dependent on sampled virus sequences, a large portion of which have been collected from disease outbreaks and not systematic surveillance. As a result, we often see long branches in the phylogenetic trees that indicate extended periods of unsampled diversity. This sampling bias may potentially affect ancestral sequence reconstruction to, in some instances, produce a suboptimal antigen. However, advanced methods that incorporate the phylogenetic uncertainty along with antigenic cartography into the vaccine design could overcome these limitations. The biological factors underlying the antigenic differences of the antigens tested in this study are unknown but likely related to differences in immunodominant antigenic site residues or glycosylation patterns, which should be more deeply evaluated. A, B, C, and D all contain the same number of potential glycosylation sites ($n = 6$), although differences were observed in antigenic site 1 residues 140 and 145 (H5 numbering in ref. 21).

In addition to the ability of computationally derived HAs to induce antibodies that react with H5N1 viruses, we were also able to show that these antibodies were indeed protective. Our ferret challenge experiments were not powered to detect differences between any of the tested antigens but rather to provide support for their immunogenic nature. Whole-virus vaccines based on A and D viruses conferred protection to ferrets against morbidity and mortality when challenged with clade 1 (VN1203), clade 2.1 (DKHUN795), and clade 2.2 (TYEGY7) viruses that was of at least equal efficacy to that induced by the most cross-reactive reference viruses. Although it is possible that all four antigens are equally effective, further analysis with split vaccines (whole-virus preparations are inherently more immunogenic but also rarely used in humans in view of associated febrile responses), titrations of antigen dose, and more divergent challenge viruses are warranted and required to test the hypotheses that the ancestral antigens are more cross-reactive than some or all naturally occurring counterparts. Nevertheless, the ability of

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single antigens to protect across a range of challenge viruses is encouraging from the standpoint of vaccine stockpiling, as has been suggested by other ferret (22, 23, 6, 24) and human studies (25). Although we observed no mortality or morbidity in H5N1 cross-clade ferret challenges, Govorkova et al. (22) challenged HK213 (clade 1)-vaccinated animals with A/Hong Kong/156/97 (clade 0, 96% HA aa identity with the vaccine strain, excluding the cleavage peptide) and reported morbidity but no mortality. Baras et al. (23) used an adjuvanted VN1203 (clade 1)-based split vaccine and were able to show that it protected 96% of ferrets against mortality when challenged with homologous or clade 2.1 (A/Indonesia/5/05, 97% HA aa identity with the vaccine) strains. Finally, Forrest et al. (6) showed ferrets immunized with inactivated whole JWEHK1038 (clade 2.3) were partially protected from VN1203 (clade 1, 96% HA aa identity with the vaccine) challenge. One of the difficulties of evaluating and comparing vaccine studies using different H5N1 challenge viruses is the substantial differences in virus lethality. In our study, this was overcome by generating a reassortant virus carrying the HA and NA of DKHUN795 on the backbone of VN1203. The parental DKHUN795 virus is not lethal in ferrets, whereas this reassortant virus was. Similarly, it has been previously shown that substitution of the polymerase genes (PB2, PB1, and PA) of A/chicken/Vietnam/C58/04 for those of VN1203 was able to convert this virus from sublethal to lethal in ferrets (26).

In summary, we were able to provide proof of concept that a most recent common ancestor computational method can be used to design H5N1 vaccine by using methodologies consistent with currently licensed vaccine platforms. We obtained replicating viruses able to induce antibodies consistent with that of reference strains that should be further examined as a means to increase the breadth of such responses.

Materials and Methods

Generation of Ancestral Sequences. Ancestral amino acid sequences were calculated from nucleotide alignments used to generate the HA and NA phylogenetic trees. A marginal reconstruction method as implemented in ANCESCON (26) was used with default settings except that a maximum likelihood rate factor was used. Marginal reconstruction compares the probabilities of different amino acids at an internal node at a site, and the amino acid that yields the maximum likelihood for the tree at that site is then selected (26). This method also takes into account the variability of substitution rates among sites. By using this method, ancestral protein sequences were determined at four nodes of the HA phylogenetic tree and two nodes of the NA phylogenetic tree. To generate HA and NA nucleotide sequences that expressed the putative ancestral proteins, the proteins were compared with sequences available in GenBank using BLAST and then the minimum mutations to code for phenotypic changes were made. The connecting peptide was modified to match that of low-pathogenicity viruses so that the reverse genetic strains could be used in biosafety level (BSL)-2+ laboratories.

Ferrets Immunization and Challenge. Vaccines in two doses given at a 3-wk interval were injected intramuscularly into 3- to 4-mo-old ferrets. Each dose of vaccine contained 7.5 μg of rg VN1203, DKHUN795, A, or D HA (15 μg of HA total per ferret). Twelve ferrets were vaccinated with each vaccine: rg 6+2 A, D, VN1203, and DKHUN795. Twelve control ferrets were given PBS solution only. Three weeks after the second (i.e., boost) dose of vaccine, ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 50% egg infectious doses (EID_{50}s) of a HPAI H5N1 challenge virus (VN1203, DKHUN795, and TYEG7, representative of different clades) in a BSL-3+ laboratory. Ferrets were then monitored daily for 14 d for weight change, temperature, and clinical disease signs. Body temperature was measured via transponders s.c. implanted between the shoulder blades (BioMedic Data Systems).

A more complete description of the materials and methods used in the present study is available in *SI Materials and Methods*.

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