Plasmid DNA–Based Vaccines Protect Mice and Ferrets against Lethal Challenge with A/Vietnam/1203/04 (H5N1) Influenza Virus

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Plasmid DNA (pDNA) vaccines represent an alternative to conventional inactivated influenza vaccines that are likely to experience supply constraints during a pandemic. Several Vaxfectin-formulated pDNA vaccines were tested in mice and ferrets for efficacy against a lethal challenge with the highly pathogenic A/Vietnam/1203/04 (H5N1) influenza virus strain; the vaccines encoded influenza A virus hemagglutinin (HA), and/or nucleoprotein (NP), and M2 protein. Complete protection from death and disease was achieved in mice and ferrets with 2 doses of a Vaxfectin-formulated vaccine containing H5 HA, NP, and M2 plasmids and in ferrets with only 1 dose. A Vaxfectin-formulated vaccine containing NP and M2 pDNA provided significant protection against death in mice and provided some benefit in ferrets (i.e., 17% survival, delayed time to illness and death, and significant reduction in viral load compared with that in negative control animals). These experiments support the clinical testing of pDNA vaccine candidates that may ultimately increase global vaccine supply options during pandemics.

Influenza pandemics have occurred at unpredictable intervals over many centuries, with the 3 most recent occurring in 1918, 1957, and 1968 [1, 2]. An increasing number of pandemic alerts have occurred in recent years [3, 4]. Of particular concern since 1997 are numerous instances of human infections by highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype. Since 2003, HPAI viruses have become endemic in wild aquatic birds and poultry and to date have been isolated in 54 countries in Asia, Europe, and Africa [3]. These viruses have infected at least 322 humans in 12 countries and caused 195 deaths as of 23 August 2007 [5]. Because of the unprecedented geographic range of H5N1 viruses, the continued zoonotic infections and high fatality rates (60%) in infected humans, and the occasional reports of human-to-human transmission among blood relatives [6], many governments have developed pandemic preparedness plans that include stockpiling antivirals and vaccines.

Vaccination represents a critical control measure against yearly seasonal influenza viruses and is an essential component of pandemic preparedness plans [7]. To date, vaccine approaches tested in humans include inactivated subunit vaccines derived from low-pathogenicity H5N3 viruses with or without adjuvants [8–10], a recombinant H5 hemagglutinin (HA) protein vaccine [11], inactivated split H5N1-derived vaccines with or without adjuvant [12, 13], and inactivated whole-virus vaccines with adjuvant [14]. In approximately half of vaccinees, nonadjuvanted H5 vaccines require high doses given twice to reach putative seroprotective levels of hemagglutination inhibition (HI) antibody titers (∼40) [12], sug-
gesting that adjuvants may be required to increase conventional vaccine potency and supplies. Adjuvants such as MF-59, aluminum hydroxide, and AS03 (GlaxoSmithKline) have been shown to increase HI titers, afford dose sparing, and broaden antigenic reactivity [8–10, 13]. Despite this, there are several limitations in worldwide influenza vaccine production that may affect H5 vaccine supplies, including limited manufacturing capacity (~300 million doses of trivalent vaccine per year), long manufacturing cycles (6–9 months for product release), dependence on eggs or cell culture, the need for biosafety level 3 facilities, and low production yields of bulk H5N1 viruses [15, 16]. Therefore, there is an urgent need to develop alternative vaccine approaches that may overcome many of these limitations.

Vaccination with plasmid DNA (pDNA)-based vaccines encoding at least 1 influenza virus protein is a promising alternative vaccine strategy that has demonstrated efficacy in multiple animal challenge models, including lethal influenza challenge models [17–25]. DNA vaccines have advanced from proof-of-concept studies in animal models to licensure of 3 animal health products: vaccines for the prevention of infectious hematopoietic necrosis virus in salmon and West Nile virus in horses and a treatment for canine oral melanoma. In humans, pDNA vaccines encoding antigens from diverse pathogens—for example, malarial parasites, HIV-1, Ebola virus, and influenza virus—have been tested in hundreds of subjects and have been shown to be generally safe, well tolerated, and immunogenic [26–29]. Key advantages of pDNA vaccines for pandemic influenza include expeditious vaccine production, independence from substrates and conventional manufacturing processes, the antigen-independent universal pDNA manufacturing process, the potential to include additional influenza genes encoding conserved antigens to possibly broaden antiviral immune responses, and amenability to long-term stockpiling of stable formulations.

We recently reported that vaccination with pDNA encoding consensus sequences of influenza A nucleoprotein (NP) and M2 cross-protects mice against lethal challenge with H3N2 and H1N1 viruses at low pDNA doses when formulated with Vaxfectin [30]. Vaxfectin is a cationic lipid delivery system that, compared with pDNA vaccines administered in PBS, increases immune responses to a variety of immunogens and provides significant protection against lethal influenza virus infection [30–32]. In the present study, we tested several Vaxfectin-formulated pDNA vaccines for protective efficacy in mice and ferrets against the highly pathogenic A/Vietnam/1203/04 (H5N1) virus. Because pandemics may arise from avian influenza virus reservoirs by either reassortment or direct transmission [33, 34], we compared vaccines containing pDNA encoding the conserved antigens NP and M2 derived from consensus sequences of H1 and H3 viruses with vaccines encoding NP and M2 antigens derived from A/Vietnam/1203/04 sequences. The inclusion of NP and M2 plasmids with an H5 HA plasmid in a pandemic vaccine was intended to increase the breadth of the antiviral response in the likely event of a suboptimal antigenic match between the HA of a stockpiled vaccine and the HA of the circulating pandemic human virus.

**MATERIALS AND METHODS**

**Virus.** The human H5N1 virus A/Vietnam/1203/04 was obtained from the World Health Organization influenza collaborating laboratories. Stock virus was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 36 h, harvested, and stored at −70°C. All work with this virus was performed in a biosafety level 3 containment facility approved for use by the US Department of Agriculture and the Centers for Disease Control and Prevention (CDC).

**pDNA vaccines.** Five plasmid constructs were created using the backbone expression plasmid VR10551 [35]. The plasmids encoding NP and M2 consensus sequences were created as described elsewhere [30]. The H5 HA, NPA, and M2A protein sequences derived from the A/Vietnam/1203/04 (H5N1) virus were obtained from the Influenza Sequence Database (accession numbers ISDN38687, AY651499, and AY818144, respectively). All 5 influenza virus gene sequences were human codon optimized and synthesized by GENEART. All of the plasmids were manufactured according to current good manufacturing practice regulations. All vaccines were formulated with Vaxfectin [31], composed of a 1:1 molar ratio of (+)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(cis-9-tetradecenyl-oxy)-1-propanaminium bromide (GAP-DMORIE) and 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPyPE) at a final pDNA (phosphate) to cationic lipid molar ratio of 4:1, as described elsewhere [30].

**Animal experiments.** All animal experiments were performed in compliance with the guidelines given in the Guide for the Use and Care of Laboratory Animals [36] and in accordance with Institutional Animal Care and Use Committee–approved protocols. Viral challenges were conducted in a CDC-approved biosafety level 3 containment facility.

**Mouse experiments.** BALB/c mice (Jackson Laboratory), 6–8 weeks old, received bilateral, intramuscular injections (50 μL/quadriceps) of Vaxfectin-formulated pDNA vaccines (table 1) on days 0 and 21. A negative control group received pDNA backbone (pDNA with no gene insert). A positive control group received a nonadjuvanted, β-propiolactone–inactivated whole-virus vaccine produced from A/Vietnam/1203/04, as described elsewhere [37]. On day 42, mice were sedated (Avertin; 300 mg/kg intraperitoneally), infected intranasally (50 μL) with 100 times the LD₅₀ of A/Vietnam/1203/04 (H5N1) virus (~160 times the median EID₅₀), and monitored closely for ~21 days for weight loss and survival. Animals found to be in moribund condition during the course of the study were euthanized.

**Ferret experiments.** Ferrets were obtained from either Triple F Farms or the breeding program at St. Jude Children’s Re-
search Hospital. In the first experiment, 12-week-old Fitch ferrets received bilateral, intramuscular injections of Vaxfectin-formulated pDNA vaccines (0.5 mL/gastrocnemius) on days 0 and 21 (table 1). In the second experiment, groups of 6 ferrets each received either 1 or 2 doses of Vaxfectin-formulated pDNA vaccines. Control ferrets received the DNA backbone plasmid. For both experiments, at 3 weeks after the last vaccination, ferrets were sedated using isoflurane, infected intranasally (50 H9262) on day 42 with 100 times the LD₅₀ of A/Vietnam/1203/04 (H5N1) virus, and monitored closely for weight loss, body temperature, and survival. Moribund animals were euthanized.

Serological assays. For all studies, serum was collected on days 0 and 35 from all animals and on day 63 (3 weeks after infection) from surviving animals. For the second ferret experiment, serum was also collected on day 14. HI assays were performed using serum samples and the S223N mutant A/Vietnam/1203/04 virus [38]. Antibodies against the M2 protein ectodomain (M2e) were measured by ELISA [30] using an M2e peptide derived from the consensus M2e sequence or from the A/Vietnam/1203/04 M2e sequence.

Nasal wash viral titers. Nasal wash samples were collected from ferrets in the second experiment only on days 3, 5, and 7 after infection. Ferrets were anesthetized with ketamine (30 mg/kg intramuscularly), and 0.5 mL of PBS containing antibiotics was introduced into each nostril. Virus was titrated in eggs, and the results were expressed as the EID₅₀/mL for each nasal wash sample, calculated as described elsewhere [39].

Statistical analyses. All statistical calculations were done using SAS software (version 9.1; SAS Institute). Data were analyzed using the nonparametric Wilcoxon rank-sum (intergroup comparisons) or signed-rank (intragroup comparisons) test. Survival analyses were applied for time to death in comparisons between groups. Kaplan-Meier survival curves were plotted for each group by means of GraphPad Prism (version 4.03), and the log-rank test was employed to test for significant differences in overall survival between groups. In the analysis of time to death, animals were censored at the last date they were alive. Antibody titer and viral titer data were log transformed. Between-group differences were considered statistically significant if P < .05. No adjustments to α errors were made for multiple pairwise comparisons between groups.

RESULTS

Protection of mice against A/Vietnam/1203/04. The results are shown in figure 1. All negative control mice died within 9 days of infection, but 100% of mice in the positive control group

<table>
<thead>
<tr>
<th>Species, group</th>
<th>Vaccine*</th>
<th>Total dose, µg</th>
<th>Sex of animals, no. male/female</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Inactivated H5 HA vaccine</td>
<td>15.0</td>
<td>0/16</td>
</tr>
<tr>
<td>B</td>
<td>pDNA backbone</td>
<td>100.0</td>
<td>0/16</td>
</tr>
<tr>
<td>C</td>
<td>H5 pDNA</td>
<td>33.3</td>
<td>0/16</td>
</tr>
<tr>
<td>D</td>
<td>NPA + M2A pDNA</td>
<td>66.6</td>
<td>0/16</td>
</tr>
<tr>
<td>E</td>
<td>H5 + NPA + M2A pDNA</td>
<td>100.0</td>
<td>0/16</td>
</tr>
<tr>
<td>F</td>
<td>NP + M2 pDNA</td>
<td>66.6</td>
<td>0/16</td>
</tr>
<tr>
<td>G</td>
<td>H5 + NP + M2 pDNA</td>
<td>100.0</td>
<td>0/16</td>
</tr>
<tr>
<td>Fitch ferrets</td>
<td></td>
<td></td>
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<tr>
<td>Experiment 1c</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>pDNA backbone</td>
<td>1000</td>
<td>2/4</td>
</tr>
<tr>
<td>B</td>
<td>NPA + M2A pDNA</td>
<td>1000</td>
<td>2/4</td>
</tr>
<tr>
<td>Experiment 2c,d</td>
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<tr>
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<td>pDNA backbone</td>
<td>1000</td>
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<tr>
<td>B</td>
<td>NP + M2 pDNA</td>
<td>666</td>
<td>3/3</td>
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<tr>
<td>C</td>
<td>H5 + NP + M2 pDNA</td>
<td>1000</td>
<td>3/3</td>
</tr>
<tr>
<td>D</td>
<td>H5 + NP + M2 pDNA (×1)</td>
<td>1000</td>
<td>3/3</td>
</tr>
</tbody>
</table>

NOTE. NP, nucleoprotein.
* All plasmid DNA (pDNA)-based vaccines were formulated with Vaxfectin; the inactivated H5 hemagglutinin (HA) vaccine was formulated with PBS.
** 15 µg of HA.
*** All ferrets were seronegative for A/Vietnam/1203/04 before vaccination.
**** Ferrets used for experiment 2 were seropositive for A/New York/55/04 (H3N2) (hemagglutination inhibition titers of 40–80). Animals were allocated to groups such that weights and titers were similar in each group. Ferrets in group D received a single vaccine dose on day 21 (denoted ×1).
survived ($P < .001$, log-rank test). All animals vaccinated with the H5, H5+NP+M2, or H5+NP+M2A pDNA vaccines survived ($P < .001$, compared with negative control mice). In each group vaccinated with the NP+M2 or NP+M2A vaccine, 14 (88%) of 16 mice survived ($P < .001$, compared with the negative control mice) (figure 1A).

When compared with positive control mice, no significant difference in body weight change after infection was observed among groups vaccinated with the H5+NP+M2 or H5+NP+M2A pDNA vaccine ($P > .05$) (figure 1B), but a significant loss in body weight was observed in the negative control mice ($P < .001$) (figure 1B). A significant but transient loss in body weight was observed in mice vaccinated with the NP+M2 or NP+M2A pDNA vaccine ($P < .001$). Body weight declined in these groups by 14% and 21% of baseline values, respectively, within 7–9 days after infection, followed by weight gain to baseline levels by 21 days after infection (figure 1B).

All mice were seronegative for A/Vietnam/1203/04 H5N1 influenza virus before vaccination (HI geometric mean titer [GMT] of <10). Mice that received any of the vaccines containing H5 pDNA seroconverted by day 35, with HI GMTs ranging from 118 to 334 (figure 2). Prechallenge HI titers were significantly lower ($P < .05$) for mice immunized with the H5+NP+M2 pDNA vaccine than for mice immunized with the H5+NP+M2A pDNA vaccine (figure 2). Despite this differ-

Figure 1. Survival (A) and body weight changes (B) in vaccinated and control mice after infection with A/Vietnam/1203/04. Groups of 16 BALB/c mice were vaccinated on days 0 and 21 with the indicated vaccines, infected on day 42 with 100 times the LD$_{50}$ of virus (target dose of ~160 times the median EID$_{50}$), and monitored for 21 days. NP, nucleoprotein; WV, whole virus.

Figure 2. Hemagglutination inhibition (HI) serum antibody titers in vaccinated mice before vaccination (day 0), 2 weeks after the second vaccine dose (day 35), and 3 weeks after infection (day 63). The HI titers are the reciprocal of the highest dilution that inhibited the hemagglutination of 4 hemagglutinin units of A/Vietnam/1203/04 containing the S22N mutation [38] and are expressed for each group as geometric mean titers (GMTs). NP, nucleoprotein; WV, whole virus.
ence, survival rates were comparable between the 2 groups. The day 63 HI GMT increased in all groups and ranged from 190 to 1560.

Protection of ferrets against A/Vietnam/1203/04. In the first of 2 experiments in ferrets, all negative control animals demonstrated clinical signs of illness and died within 6 days after infection. All animals that received the Vaxfectin-formulated H5/H11001 NPA/H11001 M2A pDNA vaccine survived with only a slight, transient decrease in activity and no significant change in temperature (data not shown) or body weight (figure 3) relative to baseline values, except for a decrease on day 2 only (P = .03). The HI GMTs in this group at days 35 and 63 were 640 and 1810, respectively.

In the second ferret experiment, all negative control animals died between 5 and 7 days after infection (figure 4A). All ferrets that received the Vaxfectin-formulated H5+NPA+M2A pDNA vaccine survived with only a slight, transient decrease in activity and no significant change in temperature (data not shown) or body weight (figure 3) relative to baseline values, except for a decrease on day 2 only (P = .03). The HI GMTs in this group at days 35 and 63 were 640 and 1810, respectively.

In the second ferret experiment, all negative control animals died between 5 and 7 days after infection (figure 4A). All ferrets that received either 1 or 2 vaccinations with the Vaxfectin-formulated H5+NP+M2 pDNA vaccine survived (P < .001, compared with negative control ferrets), with no clinical signs of illness throughout the study period except for a transient loss of appetite (seen on day 4 only) in 2 animals vaccinated twice and in 1 animal vaccinated once. Of ferrets receiving the Vaxfectin-formulated NP+M2 pDNA vaccine, 1 (17%) of 6 survived (P = .14 compared with negative control ferrets), with deaths occurring 6–7 days after infection. Clinical signs of illness were delayed by several days, compared with that in negative control ferrets. Within 3–4 days, symptoms included a slight to severe decrease in activity, loss of appetite and/or cessation of fluid intake, diarrhea, nasal discharge, neurological distress, paralysis, and seizures.

The decrease from baseline body weight in the negative control ferrets, reaching 15% by day 5 after infection (figure 4B), was significantly greater than the decrease in ferrets that received the NP+M2 pDNA vaccine (~8%) (P = .01). No difference in mean body weight change on day 5 was evident between groups that received either 1 or 2 vaccinations with the H5+NP+M2 pDNA vaccine. A similar overall mean body weight gain during the 21-day period (6%–8% increase, compared with mean baseline values) was observed in ferrets that received either 1 or 2 vaccinations with the H5+NP+M2 pDNA vaccine (figure 4B).

Compared with mean baseline values, body temperature did not change significantly in the negative control group through day 5 after infection, when the last ferret in that group died. Ferrets in the group that received the NP+M2 pDNA vaccine had a significantly higher mean body temperature on days 3

Figure 3. Survival (A) and body weight changes (B) in vaccinated and control ferrets after infection with A/Vietnam/1203/04. Six ferrets per group were vaccinated on days 0 and 21 with either a plasmid DNA (pDNA) backbone (plasmid without insert) or a pDNA vaccine encoding H5 hemagglutinin, NPA, and M2A; infected on day 42 with 100 times the LD_{50} of virus (target dose of ~5000 times the median EID_{50}); and monitored for 21 days. Clinical signs of illness (with onset on day 3) included decreased activity, loss of appetite and/or cessation of fluid intake, and rapid weight loss.
(40.6°C) and 5 (40.5°C) after infection, compared with the baseline value (38.7°C) (P = .031, Wilcoxon signed-rank test). In addition, the day 5 mean temperature value for this group was significantly higher than the mean value for the negative control group (38.4°C) (P = .01, Wilcoxon rank-sum test). No significant change in mean body temperature was observed over time in groups that received either 1 or 2 vaccinations with the H5/HA + NP + M2 pDNA vaccine during the 21-day period after infection.

**Serum HI titers and nasal wash virus titers.** All ferrets that had received 1 or 2 doses of the H5+NP+M2 pDNA vaccine seroconverted, with HI GMTs of 10 and 226, respectively, on day 35 (figure 5A). On day 63, the GMTs in those groups were 143 and 113, respectively. Nasal wash samples were collected on days 3, 5, and 7 to determine the level of viral replication in the upper respiratory tracts of ferrets in each vaccine group. Compared with the negative control group, animals that received 2 doses of the H5+NP+M2 pDNA vaccine had significantly lower viral titers on days 3 and 5 after infection (P = .041 and P = .004, respectively; Wilcoxon rank-sum test) and undetectable viral titers by day 7 (figure 5B). Again compared with the negative control group, animals that received a single dose of the H5+NP+M2 pDNA vaccine on day 21 had significantly lower nasal wash viral titers on day 5 after infection (P = .021) and undetectable viral titers by day 7. The group that received the NP+M2 pDNA vaccine on days 0 and 21 also had significantly lower nasal wash viral titers on day 5 after infection (P = .044) (figure 5B), and in the 1 survivor the virus titer was markedly reduced by day 7.

**Serum anti-M2e antibody titers.** By day 35, all ferrets that received 2 doses of the NP+M2 pDNA vaccine seroconverted (≥4-fold rise from baseline titers; either peptide), compared
with 4 of 6 ferrets receiving 2 doses of H5+NP+M2 and 0 of 6 ferrets receiving a single dose of H5+NP+M2 (figure 6). An increase in titer was observed in some animals 3 weeks after challenge (day 63), including the NP+M2–vaccinated survivor (figure 6).

DISCUSSION

The findings reported here demonstrate that 2 immunizations with Vaxfectin-formulated vaccines containing pDNA encoding an H5 HA component provided the highest levels of protection from disease and death caused by a highly pathogenic A/Vietnam/1203/04 (H5N1) virus strain in both mice and ferrets (figures 1, 3, and 4) and reduced viral shedding in ferrets. A single dose of the Vaxfectin-formulated H5+NP+M2 pDNA vaccine provided the same degree of protection in ferrets as did 2 doses. A single dose of vaccine significantly reduced viral titers in nasal wash samples from ferrets within 5 days after infection, but 2 doses also provided a significant reduction that was evident earlier (by day 3) (figure 5B). Therefore, 2 doses provided the greatest benefit, with protection from clinical symptoms and death and with reduced upper respiratory tract viral shedding.

Figure 5.  A, Hemagglutination inhibition (HI) titers in vaccinated ferrets before vaccination (day 0), 2 weeks after the first and second vaccine doses (days 14 and 35), and 3 weeks after infection (day 63), for each vaccine group. All animals were vaccinated twice, except for those in one group, which were vaccinated once on day 21 (denoted x). All animals were infected with A/Vietnam/1203/04 virus 21 days after the last vaccination, including animals vaccinated only once. The day 63 HI titer for the sole survivor in the NP+M2 group reached 640 (not shown). B, Virus titers in the upper respiratory tract of vaccinated and control ferrets. Titers were measured in nasal wash samples collected on days 3, 5, and 7 after infection with A/Vietnam/1203/04. GMT, geometric mean titer; NP, nucleoprotein.
Although survival outcomes after lethal challenge served H-2d T cell epitopes defined for NP (http://www.flu.lanl.gov). Although survival outcomes after lethal challenge served H-2d T cell epitopes defined for NP (http://www.flu.lanl.gov) were indistinguishable between groups receiving H5+NP+M2 or H5+NP+M2A and in vitro expression was comparable between these vaccines for same-type plasmids (data not shown), the former group receiving H5+NP+M2 had significantly lower HI titers than did the group receiving H5+NP+M2A on days 35 and 63 (figures 1 and 2). This effect may be related to differences in immunodominant epitopes contained within the NP and/or M2 consensus proteins, which may result in more effective T cell responses that rapidly clear virus after infection, resulting in a lower boost from live virus.

The NP and M2 consensus sequence constructs were also tested for efficacy in ferrets, which are highly susceptible to infection with A/Vietnam/1203/04 and other H5N1 viruses [40]. Only 1 of 6 ferrets vaccinated with plasmids encoding the NP and M2 consensus sequences was protected from death, although nasal wash viral titers were significantly reduced on day 5, compared with titers for negative control ferrets (figure 5B). The different survival outcomes for mice and ferrets immunized with vaccines containing NP and M2 may relate to differences in the 2 animal models, in particular, the rapid onset and disease progression in ferrets that resulted in death in \( \sim \)5 days, compared with \( \sim \)9 days in mice.

Although assays to measure T cell responses to NP and M2 in ferrets are not available, serum antibody responses to M2e were evaluated. Previous studies in mice have shown that serum anti-M2e antibodies can reduce virus replication and death but cannot prevent infection or disease [24, 41–43], whereas one study in ferrets vaccinated three times with QS21–adjuvanted M2e-conjugate vaccines revealed reductions in viral recovery but not viral lung titers after A/Puerto Rico/8/34 (H1N1) virus infection [44]. However, the anti-M2e antibody titer in the surviving ferret (1600 before challenge) was not predictive of survival, given the 800–3200 prechallenge titers in nonsurvivors. Furthermore, ferrets vaccinated once with H5+NP+M2 did not seroconvert to M2e but were primed, as indicated by postchallenge increases in titers, suggesting a minimal contribution of anti-M2e antibodies to protection.

In humans, HI titers \( \geq 40 \) are currently recognized as a surrogate end point likely to predict benefit for a pandemic influenza vaccine, including pDNA-based vaccines [45]. The pDNA vaccines containing an H5 component generated HI titers \( >40 \) to A/Vietnam/1203/04 S223N [38] in both mice and ferrets after 2 doses (figures 2 and 5A). In mice, comparable HI titers were induced by an inactivated whole-virus vaccine and the H5-containing pDNA vaccines (GMT between 118 and 320) (figure 2). In ferrets, 2 vaccine doses elicited titers well over 40, whereas a single vaccine dose elicited low HI titers (GMT of 10) at 1 week before infection (day 35) (figure 5A). Despite the low titers, these ferrets were fully protected from disease and death (figure 4). This result is not unexpected, because the correlates of protection in the ferret model have not been clearly defined and may be a combination of serological and cellular mediators. Several re-

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**Figure 6.** Serum antibody titers to the extracellular domain of M2 (M2e) in vaccinated ferrets on day 0 (D0), day 35 (D35), and day 63 (D63), for each group. Vaccine groups received 2 doses of NP+M2 (days 0 and 21), 2 doses of H5+NP+M2 (days 0 and 21), and 1 dose of H5+NP+M2 (day 21) (designated groups B, C, and D, respectively). Antibodies were measured by ELISA using a peptide corresponding to the first 23 aa of either the M2 consensus sequence (MSLLTEVETPIRNEWGCRCNDSS) (A) or the M2 sequence from the A/Vietnam1203/04 virus (MSLLTEVETPIRNEWGCRCNDSS, which differs by 3 aa [underscored]) (B). A peroxidase-conjugated goat anti-ferret secondary IgG diluted 1:1600 was used. Absorbance was measured at 405 nm within 30 min after the reaction was stopped. End point antibody titers were the reciprocal of the serum dilution resulting in an absorbance value twice that of negative control serum. Each curve represents the longitudinal titers of an individual ferret. NP, nucleoprotein.
cent heterologous challenge studies testing inactivated whole-virus vaccines have shown that neither HI nor neutralization titer appears to provide a correlate of protection in the ferret model [37, 46].

The data presented here support clinical testing of Vaxfectin-formulated H5-containing vaccines, which may ultimately lead to the diversification of pandemic vaccine stockpiling options. Although H5N1 subtypes of HPAI viruses are a looming pandemic threat, H2, H7, H9, and H10 subtypes can also infect humans [4]. Inclusion of conserved NP and M2 influenza antigens in a pandemic vaccine may provide increased protection against death (and perhaps against severe disease) and may decrease transmission before the actual pandemic strain is isolated and a matched vaccine distributed, particularly if an unexpected subtype emerges. Because ferrets vaccinated only once had low HI titers but were fully protected, a low HI titer alone may be sufficient to protect ferrets, given that the anti-M2e antibodies did not appear to play an important role in protecting them from viral challenge. However, because T cell responses may be important in lessening the severity of influenza virus infections [47, 48], induction or augmentation of T cell responses against NP and/or M2 may improve the efficacy of this vaccine, provided that seroprotective HI titers can also be elicited for the HA component included in the vaccine.

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