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Vesicular stomatitis virus vectors expressing avian influenza H5 HA induce cross-neutralizing antibodies and long-term protection

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Abstract

Given the lethality of H5N1 avian influenza viruses (AIV) and the recurring spread from poultry to humans, an effective vaccine against H5N1 viruses may be needed to prevent a pandemic. We generated experimental vaccine vectors based on recombinant vesicular stomatitis virus (VSV) expressing the H5 hemagglutinin (HA) from an H5N1 virus isolated in 1997. The *HA* gene was expressed either from an attenuated wild-type VSV vector or from a single-cycle vector containing a deletion of the VSV *G* gene. We found that all of the vectors induced potent neutralizing antibody titers against the homologous and antigenically heterologous H5N1 viruses isolated in 2004 and 2005. Vaccination of mice with any combination of prime or prime/boost vectors provided long-lasting protection (>7 months) against challenge with AIV, even in animals receiving a single dose of single-cycle vaccine. Our data indicate that these recombinants are promising vaccine candidates for pandemic influenza.

Keywords: VSV; AIV; Vaccine; H5N1; Cross neutralizing antibody

Introduction

The influenza pandemics that occurred in 1918, 1957, and 1968 killed over 40 million people worldwide. Pandemics arise when novel influenza A subtypes emerge and spread to humans, generally from avian reservoirs (Wright and Webster, 2001). Viruses in the influenza A genus are subdivided on the basis of the antigenicity of the envelope glycoproteins, hemagglutinin (HA), and neuraminidase (NA). Currently, there are 16 known types of HA (H1–16) and 9 of NA (N1–9) (Fouchier et al., 2005; Wright and Webster, 2001).

In 1997, outbreaks of the H5N1 subtype of avian influenza virus (AIV) occurred in Hong Kong, in both poultry and humans. This was the first time that a H5 subtype was known to cause disease in humans. H5N1 viruses emerged again in 2003 and continue to cause disease in poultry and humans to the present day (Gillim-Ross and Subbarao, 2006). Since 2003, at least 261 people have been infected by H5N1 viruses with an associated mortality rate of over 60% (http://www.who.int/csr/disease/avian_influenza). Despite the unusually high mortality rate, the human outbreaks probably have been contained because the current H5N1 strains do not spread efficiently from person to person (Katz et al., 1999; Ungchusak et al., 2005). This high mortality rate and continued spread triggered the World Health Organization and the U.S. Centers for Disease Control and Prevention to issue warnings about a possible H5N1 pandemic. The H5N1 strains that have emerged since 1997 have already split into numerous sublineages or clades (2005; Chen et al., 2006), illustrating the rapid evolution of this

Abbreviations: AIV, avian influenza virus; HA, hemagglutinin; HK/156, AIV strain A/Hong Kong/156/1997; HK/483, AIV strain A/Hong Kong/483/1997; HPAI, highly pathogenic influenza virus; i.m., intramuscular; i.n., intranasal; INA/5, AIV strain A/Indonesia/5/2005; NA, neuraminidase; VSV, vesicular stomatitis virus; rVSV, recombinant VSV.

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virus. The ability of H5N1 subtypes of AIVs to infect humans has emphasized the need for vaccines protecting against AIV infection.

Compared to human influenza vaccines targeting epidemic influenza, development of AIV vaccines is more problematic. Traditional influenza vaccines are prepared by growing attenuated reassortant viruses in embryonated chicken eggs. These attenuated viruses encode the HA and NA proteins predicted to be present in the upcoming seasonal epidemic viruses. However, the AIVs with the greatest potential public health threat are highly pathogenic avian influenza (HPAI) viruses that can be too pathogenic to grow efficiently in eggs and require costly levels of biocontainment. Because a newly emerged H5N1 virus might spread rapidly in the population, the long lead-time required for traditional vaccine production is also a major concern.

In an effort to create an H5N1 AIV vaccine, many groups are exploring new approaches to generate a vaccine that will protect against H5N1 strains (reviewed in Gillim-Ross and Subbarao, 2006; Horimoto and Kawaoka, 2006). Both inactivated and live attenuated AIV vaccines are under development. Other strategies such as inactivated subvirion, recombinant subunit, and DNA vaccines are being investigated. Virus vectors, such as adenovirus and Newcastle disease virus vectors, encoding influenza antigens are also being explored as potential AIV vaccines for both avian and human use (Ge et al., 2007; Horimoto and Kawaoka, 2006; Park et al., 2006; Veits et al., 2006).

Experimental virus vaccine vectors based on attenuated vesicular stomatitis viruses (VSV) that express foreign viral antigens have been developed (Brandsma et al., 2007; Daddario-DiCaprio et al., 2006; Egan et al., 2004; Garbutt et al., 2004; Jones et al., 2005; Kahn et al., 2001; Kapadia et al., 2005; Publicover et al., 2005; Ramsburg et al., 2004; Reuter et al., 2002; Roberts et al., 1998, 1999, 2004; Schlereth et al., 2000; Schnell et al., 1997). These vectors protect animals against numerous viral challenges. The initial studies on VSV as a vaccine vector showed that a VSV expressing the HA protein of the WSN strain of influenza elicited high neutralizing antibody titers in mice and protected mice against challenge with a lethal dose of the WSN virus (Roberts et al., 1998, 1999).

VSV is a versatile vaccine vector for many reasons including the fact that exposure and seropositivity to VSV is negligible in the human population. Additionally, it replicates solely in the cytoplasm, does not undergo recombination, and generates no DNA intermediates that might integrate into host genomes (Rose and Whitt, 2001). VSV's relatively simple genome can accommodate at least 4.5 kb of foreign gene(s) that are highly expressed in infected cells. Moreover, VSV is effective when administered intranasally and is easily manufactured on a large scale in cell lines already approved for vaccine production. There are also highly attenuated mutants of VSV as well as replication defective, single-cycle VSV vectors that still generate potent immune responses (Publicover et al., 2004, 2005; Roberts et al., 1999).

In the present study, we have generated replicating and single-cycle VSV vectors that express the H5 *HA* gene derived from a prototypic H5N1 AIV, A/Hong Kong/156/97 (HK/156), originally isolated from a lethal human infection (Subbarao et al., 1998). To assess the efficacy of these vectors, we examined the ability of the VSVs expressing H5 HA to induce a neutralizing antibody response against the homologous virus. Additionally, we determined if these vaccines could elicit cross-neutralizing antibody titers against distantly related H5N1 viruses. All of our vectors, with and without boosting, were able to induce a neutralizing antibody response against all H5N1 viruses tested. Furthermore, the response was 100% protective in a mouse model of AIV challenge. This protection was achieved with a single dose of vaccine and was long-lasting.

Results

Construction of replication competent and single-cycle VSV vectors expressing an avian influenza H5 HA protein

In order to generate VSV-based vaccine vectors for H5N1 avian influenza, we incorporated the *HA* gene from the H5N1 AIV strain, HK/156, into the three different recombinant VSV (rVSV) vectors shown in Fig. 1A. The two replication competent vectors had the *HA* gene inserted into the fifth genome position downstream of the VSV *G* gene. One of these vectors (VSV-H5 HA, Fig. 1A) contained the VSV *G* from the Indiana serotype. To allow for effective boosting after priming with the vector containing the Indiana G protein, the boosting vector substituted this gene with the VSV *G* gene from the New Jersey serotype (VSV-NJG-H5 HA, Fig. 1A). Priming with VSV vectors precludes effective boosting with the same vector because of the high level of neutralizing antibody generated against the VSV G protein (Rose et al., 2000). Additionally, we generated a single-cycle vector where the *G* gene was deleted (VSVΔG-H5 HA, Fig. 1A). This vector was propagated in a complementing cell line expressing VSV G (Schnell et al., 1997). However, it cannot spread in animals beyond initially infected cells because it does not encode the VSV G protein. Such single-cycle vectors eliminate concerns about pathogenesis of VSV recombinants.

Expression of the HA gene from the recombinant VSV vectors

To determine if the H5 *HA* gene was expressed from these recombinant vectors, western blot analysis was performed on whole cell extracts of infected cells. Proteins with the mobilities expected of the H5 HA protein (HA_0) and its cleaved forms (HA_1 and HA_2) accumulated in cells infected with all three vectors, but not in cells infected with the parent wild-type (WT) virus (Fig. 1B, left panel). When the same blot was stripped and re-probed with anti-VSV (Indiana) antibodies, VSV proteins were detected in the infected cells, except for the G protein in cells infected with the single-cycle vector, VSVΔG-H5 HA, which does not encode VSV *G*, or in cells infected with VSV-NJG-H5 HA because the NJ G protein is not detected well by the anti-VSV (Indiana) serum (Fig. 1C, left panel). A trace of cross reacti-

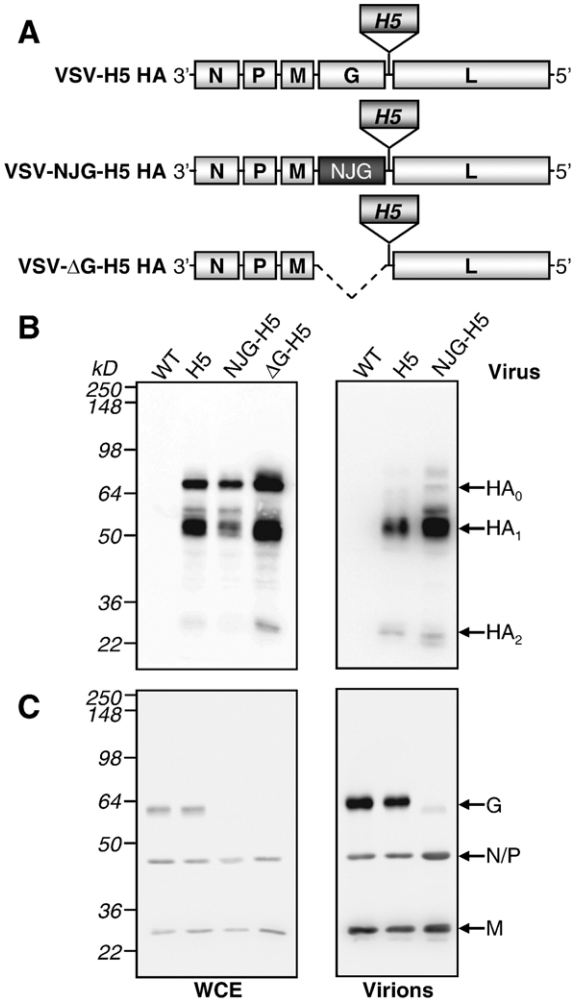

Fig. 1. Recombinant VSV vectors expressing the H5 *HA* from the A/HK/156/97. (A) Diagram of the recombinant VSV vector genomes showing the insertion site of the H5 *HA* gene, the replacement of the *G* gene (Indiana serotype) with that from the New Jersey (NJ) serotype in the VSV-NJG-H5 HA vector, and the deletion of the *G* gene in the VSV-ΔG-H5 HA vector. (B, C) Whole cell extracts (WCE; left panels) prepared from BHK-21 cells infected with the indicated viruses or virions purified from infected cell supernatants (right panels) were subjected to analysis by SDS-PAGE. Western blot analyses were performed using antibodies specific for (B) H5 HA or (C) VSV. The anti-VSV blot (C) is the same blot from (B) that was stripped and re-probed with anti-VSV (Indiana) antibody. The full-length (HA_0) and cleaved isoforms (HA_1, HA_2) of H5 HA as well as the VSV proteins (G, N, P and M) are indicated by the arrows.

vity to the NJ G protein is detected in the blot of virion proteins (Fig. 1C, right panel).

H5 HA is incorporated into the recombinant VSV virions

To determine if the H5 HA protein was incorporated into recombinant VSV virions, we purified virions by ultracentrifugation and then performed a western blot analysis of the virion proteins with anti-HA antibodies. Fig. 1B (right panel) shows that the cleaved forms, HA_1 and HA_2, were incorporated into virions of rVSVs expressing HA, but not into WT virions. The presence of little or no HA_0 in the recombinant virions is expected because HA_0 should be cleaved before reaching the cell surface (Wright and Webster, 2001) where VSV budding occurs (Rose and Whitt, 2001). Re-probing of the same blot with anti-VSV serum showed that the expected VSV proteins were also present in the virions (Fig. 1C, right panel).

Immunization with the VSV recombinants elicits a potent neutralizing antibody response to H5N1 AIV

To evaluate the ability of the rVSV vaccine vectors to generate neutralizing antibodies against avian influenza, mice were primed intramuscularly (i.m.) or intranasally (i.n.) with VSV-H5 HA, or i.m only with VSVΔG-H5 HA. Half of the mice in each group were boosted 1 month later by the respective route with the G serotype switch vector, VSV-NJG-H5 HA. An rVSV vector that expresses an unrelated protein was used as a negative control vector.

Serum antibody responses against the homologous AIV, HK/156, were analyzed in individual mice at various times post-prime (Fig. 2) using an AIV microneutralization assay that measures 100% neutralization of infectivity (Suguitan et al., 2006). A single dose of either VSV-H5 HA or the single-cycle vector, VSVΔG-H5 HA, elicited neutralizing antibody titers against the homologous virus as early as 1 and 2 months post-prime (data not shown), but peaked at 3 months post-prime (Fig. 2, gray bars). These antibodies persisted at 5.5 months post-prime (Fig. 2, black bars). Neutralizing titers against HK/156 were enhanced substantially after boosting in the animals vaccinated i.m. After the prime, the animals vaccinated i.n. had higher titers than the animals vaccinated i.m. (Fig. 2). Boosting of the i.n. vaccinees was less effective (Fig. 2) perhaps because of the presence of a higher initial level of neutralizing antibodies directed against H5 HA after the prime.

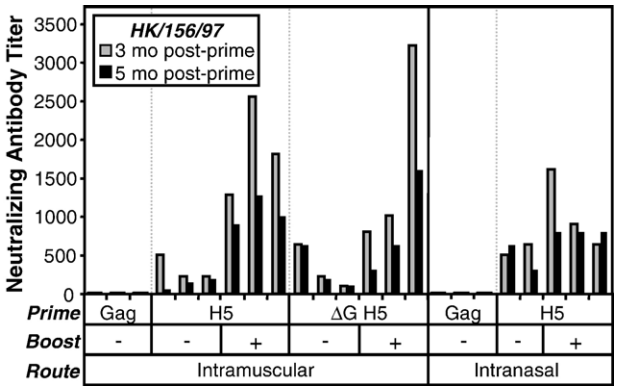

Fig. 2. Neutralization of the homologous AIV strain by sera from VSV vaccinated mice. Mice were vaccinated i.m. or i.n. with VSV-H5 HA (H5; $n=6$), VSV-ΔG-H5 HA (ΔG H5; $n=6$) or the negative control vector, VSV-Gag (Gag; $n=3$). At 1 month post-prime, animals were boosted with VSV-NJG-H5 HA ($n=3$) where indicated. At various times post-prime, sera from individual mice were analyzed for a neutralizing antibody response against the homologous HK/156 AIV strain (3 or 5.5 months post-prime, gray and black bars, respectively). Each bar represents the neutralizing antibody titer from an individual mouse as determined by 100% inhibition of CPE in a microneutralization assay.

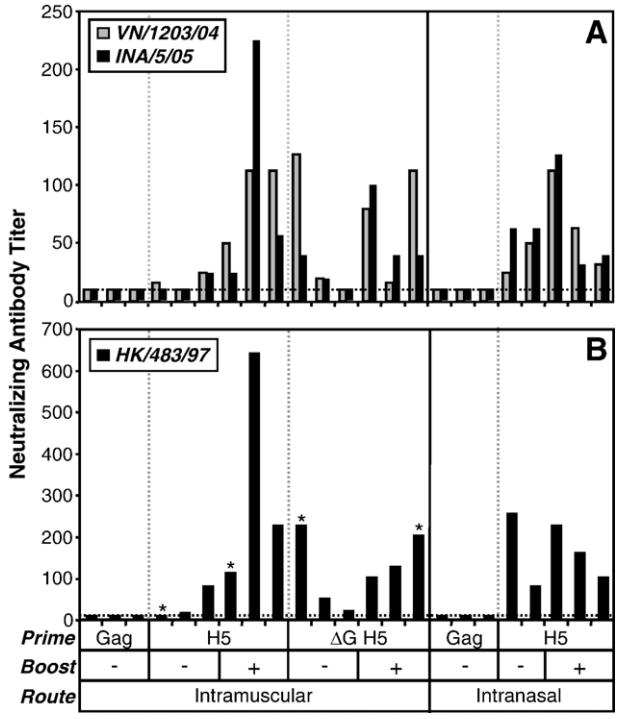

Fig. 3. Cross-neutralization of heterologous AIV strains by sera from VSV vaccinated mice. Mice were vaccinated i.m. or i.n. with VSV-H5 HA (H5; $n=6$), VSV-ΔG-H5 HA (ΔG H5; $n=6$), or the negative control vector, VSV-Gag (Gag; $n=3$). At 1 month post-prime, animals were boosted with VSV-NJG-H5 HA ($n=3$) where indicated. At various times post-prime, sera from individual mice were analyzed for a neutralizing antibody response against the heterologous strains (A) VN/1203 (gray bars), INA/5 (black bars) (3 months post-prime), or (B) HK/483 (5.5 months post-prime). Each bar represents the neutralizing antibody titer from an individual mouse as determined by 100% inhibition of CPE in a microneutralization assay. The dotted horizontal line at a value of 10 represents the limit of detect for this assay. A value of 10 is considered negative. Asterisks (*) indicate mice that were not challenged with HK/483 (Fig. 4) because they died of unrelated causes (e.g. during anesthesia) prior to challenge.

Vaccination elicits a broad cross-neutralizing antibody response against heterologous AIV strains

Because of the frequency of antigenic change (drift) in influenza viruses, it is important to determine if vaccine candidates can generate neutralizing antibodies against AIVs from other clades within the same subtype. We therefore examined the ability of our rVSVs to generate a cross-neutralizing antibody response against the heterologous AIVs, A/Vietnam/1203/04 (VN/1203) and A/Indonesia/5/05 (INA/5). These are viruses from other clades that emerged 7–8 years after isolation of the HK/156 strain and were isolated in geographically distinct areas. We found that a single i.m. or i.n. dose of either VSV-H5 HA or VSVΔG-H5 HA elicited detectable cross-neutralizing antibodies against the clade 1 virus, VN/1203 (Fig. 3A, gray bars), and the clade 2 virus, INA/5 (Fig. 3A, black bars) in some of the animals. In general, boosting of the animals substantially increased the cross-neutralizing antibody titers against both AIVs (Fig. 3A).

We also examined whether vaccination with these vectors could induce a cross-neutralizing antibody response against another more closely related clade 3 virus, A/Hong Kong/483/97 (HK/483), a HPAI virus. HK/483 is much more pathogenic in mice than the homologous HK/156 strain (Lu et al., 1999), and was therefore more suitable for use in the subsequent challenge studies. A single vaccination elicited substantial cross-neutralizing antibody titers against HK/483 in individual mice that were enhanced by boosting (Fig. 3B, Table 1). The response generated against HK/483 (Fig. 3B) was greater than that seen against VN/1203 and INA/5 (Fig. 3A), but was lower than that raised against the homologous clade 3 strain, HK/156 (Fig. 2).

Vaccination confers long-term protection against AIV challenge

While several experimental vaccine vectors expressing the H5 HA show promise, to our knowledge no study has examined the long-term efficacy of vaccination in a mammalian model. Mice vaccinated with the rVSVs expressing the HK/156 H5 HA were challenged i.n. with the HPAI HK/483 at 7.5 months post-prime or 6.5 months post-boost in those animals that were boosted. The mice that were challenged were the same mice for which the immunogenicity of the vaccine vectors was determined (Figs. 2 and 3) except for 5 mice that died prior to challenge. Mice vaccinated with the negative control virus, VSV-Gag, lost approximately 30% of their body weight within the first week post-challenge (Fig. 4) and these mice died by 8–

Table 1
Serologic response to and protection from challenge with HK/483

Antigen[a]	nAb titer[b]	% wt loss[c]	% survival[d]	*P*-value[e]	TTD (days)[f]
H5[g]	104 (16–640)	2.9 (1.5–6.6)	100 (13/13)	<0.0001	NA
H5 (Prime only)	54 (16–254)	2.4 (1.6–4.8)	100 (6/6)	0.0005	NA
H5 (Prime+Boost)	185 (101–640)	3.4 (1.5–6.6)	100 (7/7)	0.0002	NA
Gag	10 (10)	31.6 (25–34.3)	0 (0/6)	NA	8–9
Naive	ND	32.4 (30.5–34.5)	0 (0/2)	NA	8

[a] Each row represents the combined data for both i.n. and i.m. routes and for all vaccine vectors expressing the indicated antigen.
[b] Summary of individual mouse geometric mean neutralizing antibody (nAb) titers from Fig. 3B, 5.5 months post-prime; range of titers in parentheses.
[c] Summary of Fig. 4 data as the geometric mean of maximum percent weight (wt) loss; percent weight loss range in parentheses.
[d] In parentheses, number of survivors over total number of animals per group.
[e] When compared to the Gag control group (Logrank test).
[f] TTD, time to death.
[g] Sum of rows 2 [H5 (Prime only)] and 3 [H5 (Prime+Boost)].

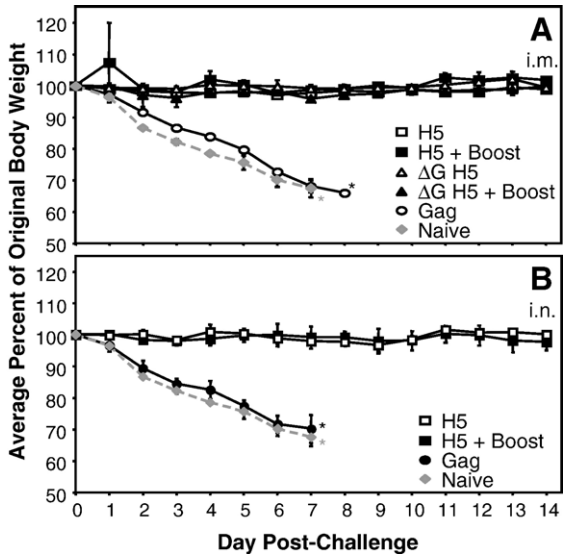

Fig. 4. Vaccination with VSV vectors expressing a H5 HA protects mice against a HPAI virus. Balb/c mice were vaccinated (A) i.m. or (B) i.n. with VSV-H5 HA (squares; $n=4$ and $n=5$, respectively), VSVΔG-H5 HA (triangles; $n=4$), or VSV-Gag (circles; $n=3$). At 1 month post-prime, some mice were boosted with VSV-NJG-H5 HA [solid squares (A, $n=2$; B, $n=3$) and solid triangles (A, $n=2$; B, $n=3$)]. At 7.5 months post-prime, mice were challenged i.n. with 100 LD_{50} of HK/483. Naive mice ($n=2$) were also challenged (gray diamonds, dashed line). For comparison the same group of naive mice is depicted in both panels. Body weights and survival were assessed daily. *Termination of analysis due to death of animals.

9 days post-challenge as did the naive control mice (Fig. 4, Table 1). Mice that were vaccinated with any combination of the prime and boost vectors, including all mice receiving a single vaccine dose of either the replicating VSV-H5 HA or the single-cycle vector, VSVΔG-H5 HA, remained healthy throughout the duration of the experiment and did not exhibit any weight loss or other signs of disease (Fig. 4, Table 1). Survival was highly significant when vaccinated mice were compared to the VSV-Gag vaccinated control mice with *P*-values ranging from <0.0001 to 0.0005 (Table 1). The mean geometric titer against the challenge virus in these vaccinated animals was 104, ranging from 16 to 640 (Table 1). Even the animals with the lowest titers at 5.5 months post-prime (Fig. 3B, Table 1) were completely protected from challenge at 7.5 months post-prime (Fig. 4, Table 1). Complete protection was achieved by both i.m. (Fig. 4A) and i.n. vaccination (Fig. 4B).

Discussion

The highly pathogenic nature of recently emerged H5N1 viruses, the continued spread through poultry populations, and the high mortality they cause in humans have generated a major concern about a possible AIV pandemic. This concern has led to new efforts to develop AIV vaccines including development of non-traditional methods. VSV vaccine vectors expressing foreign antigens are capable of providing protection against a number of respiratory viral diseases, such as SARS (Kapadia et al., 2005), influenza (Roberts et al., 1998, 1999), and RSV (Kahn et al., 2001). VSV vectors have shown promise as AIDS vaccines in a monkey model (Egan et al., 2004; Ramsburg et al., 2004; Rose et al., 2001) and highly attenuated VSVs expressing HIV antigens will soon be entering clinical trials as experimental AIDS vaccines.

We generated experimental VSV-based H5N1 AIV vaccines by inserting the H5 *HA* gene from the HK/156 strain into recombinant WT and single-cycle VSV vectors. We chose the H5 HA protein because the only major correlate of protection against influenza is antibody to its surface glycoproteins, and primarily to HA (Wright and Webster, 2001). Recovered rVSVs expressed full-length and cleaved HA in infected cells and the cleaved HA_1 and HA_2 were incorporated into the rVSV virions (Fig. 1). We assessed the serological response to vaccination in mice after i.n. and i.m. inoculation. Some mice received a single dose of vaccine while others received a boost with a serotype switch vector. We found that high neutralizing antibody titers were present in all mice receiving any combination of the H5-expressing rVSV vectors by either route. The titers peaked at about 3 months post-prime, but were still high after 5.5 months post-prime (Fig. 2).

Because influenza viruses undergo frequent mutation, it is important that any vaccine intended for pandemic influenza be able to cross-neutralize antigenically distinguishable AIVs of the same subtype. We found that our rVSV vectors elicited cross-neutralizing antibodies against three distinct H5N1 viruses, VN/1203, INA/5 and HK/483 (Fig. 3, Table 1) at levels suggestive of protection against these heterologous strains. The vaccinated mice were challenged at 7.5 months post-prime with a lethal dose of the HPAI HK/483. All vaccinees, including those that received a single dose of the single-cycle vector, survived challenge and were protected from AIV-associated disease (Fig. 4, Table 1), while all control mice died within 8–9 days post-challenge.

Our serologic data correlate with complete protection from pulmonary replication as seen in a previous study after administration of two doses of a cold-adapted (ca) H5N1 vaccine (Suguitan et al., 2006). The titers induced by the rVSV vaccines are higher than were achieved after two doses of H5N1 ca vaccine (Suguitan et al., 2006). Moreover, vaccination with rVSV expressing HA from the WSN strain shows that rVSV vaccination against influenza can completely prevent influenza replication in the lungs following challenge (Roberts et al., 1999). The prevention of AIV-associated weight loss and the high neutralizing antibody titers after rVSV vaccination in this study is suggestive of protection against replication. Direct assay of virus replication was not possible in the BSL-4 facility where the experiments were carried out and regulations prevented removal of samples for assay outside of the facility. Replication will therefore be addressed in future studies.

VSV-based AIV vaccine candidates have potential advantages over other vaccine candidates including ease of delivery by the intranasal route, negligible VSV seropositivity in humans, and production in cell lines already approved for manufacture of human vaccines rather than in eggs. A VSV-based vaccine also eliminates safety concerns about growing AIV-based vaccines, such as risk of exposure or accidental spread during production. Furthermore, VSV vectors are effec-

tive at relatively low doses and are typically effective, as seen here, after a single vaccination not requiring the use of an adjuvant.

Although WT VSV does not cause neurological disease in natural host animals, it can be neuroinvasive following intranasal inoculation of mice (Sabin and Olitsky, 1937). Safety concerns about use of replication competent VSV vectors have therefore been addressed. The prototype attenuated VSV vector (Clarke et al., 2006) is not neuroinvasive in non-human primates after intranasal administration (Johnson et al., 2006). Furthermore, more highly attenuated live VSV vectors have been generated that lack neurovirulence in non-human primates following direct intracranial (thalamic) inoculation (Johnson et al., 2006). Also, the vector we described here lacking the VSV *G* gene eliminates concerns about neurovirulence because it replicates for only a single cycle.

Another potential safety concern arises from the fact that the H5 HA protein is incorporated into VSV virions. However, we have been unable to demonstrate any infectivity of the viruses due to the H5 HA protein in the vectors expressing HA. Because influenza virus requires the receptor-destroying activity of NA for propagation (Lamb and Krug, 2001), the absence of the influenza *NA* gene in our constructs also predicts lack of pathogenicity due to HA incorporation into virions. We are currently generating VSV recombinants expressing soluble, secreted forms of H5 HA that could not be incorporated into virion membranes. Such recombinants would provide an additional safety factor, and could potentially be just as effective at generating neutralizing antibody to influenza. VSV-based vectors have excellent potential as vaccines protecting against pandemic AIV and warrant further development.

Materials and methods

Cells and viruses

BHK-21 and BHK-G (Schnell et al., 1997) cells were grown in Dulbecco's Modified Essential Medium (DMEM) containing 5% fetal bovine serum (FBS) and 1× Pen-Strep. The BHK-G cell maintenance media were supplemented with 75 μg/ml G418 and 5 ng/ml tetracycline (Tet). MDCK cells were propagated in MEM/BSA [minimal essential medium (MEM) supplemented with 0.3% bovine serum albumin (BSA) and 1× Pen-Strep].

Recombinant VSVs were grown and titrated by plaque assay on BHK-21 or BHK-G cells as previously described (Lawson et al., 1995; Schnell et al., 1997). The VSVΔG-H5 HA stock used for vaccination was resuspended in PBS after centrifugation of the infected cell media at 25,000 rpm in a Beckman SW28 rotor (112,398×*g*) for 1 h at 4 °C. The negative control rVSV vector, VSV-Gag, containing the SIV *Gag* gene in the fifth VSV genome position has been described previously (Rose et al., 2001).

The AIVs, A/Hong Kong/156/1997 (HK/156), A/Hong Kong/483/1997 (HK/483), A/Vietnam/1203/2004 (VN/1203), and A/Indonesia/5/2005 (INA/5), were kindly provided by Drs. Nancy Cox and Alexander Klimov, Influenza Branch, Centers for Disease Control and Prevention, Atlanta, GA, and were propagated in the allantoic cavity of specific pathogen-free eggs as described previously (Suguitan et al., 2006). HK/483 used for mouse challenge experiments was grown and titrated by plaque assay on MDCK cells in media supplemented with 1.0 μg/ml TPCK-treated trypsin.

Construction and recovery of recombinant VSV vectors

The H5 *HA* gene (Accession #AF046088; kindly provided by Y. Kawaoka, University of Wisconsin, Madison, WI) was amplified by PCR with the primers 5′-GGACCCGGG-AAAATGGAGAAAACAGTGCTTCTTC-3′ and 5′-GGA-CTCGAGATCGATCTCTGTTAGTTTTTTCATACCT-TAAATGCAAATTCTGCATTG-3′ that introduce 5′ *Xma*I and 3′ *Xho*I sites (underlined), respectively, and then cloned into *Xma*I–*Xho*I digested pBlueScript II SK to generate pBS-H5. The H5 *HA* gene was amplified by PCR from pBS-H5 using the primers, 5′-GATCGATCCTCGAGATCATGGAGAAAACA-GTGCTTCTTCTTGC-3′ and 5′-GAAATACTACGCTAGC-TTAAATGCAAATTCTGCATTGTAAAC-3′ that introduce 5′ *Xho*I and 3′ *Nhe*I sites (underlined), respectively. The *Xho*I–*Nhe*I digested PCR product was cloned into the *Xho*I and *Nhe*I sites of the pVSV-XN2 (Schnell et al., 1996) and pVSV-NJG-XN (Rose et al., 2000) vectors to generate pVSV-H5 HA and pVSV-NJG-H5 HA, respectively. The pVSVΔG-H5 vector was generated by replacing the *HA* gene (A/WSN strain) from pVSVΔG-HA (Roberts et al., 1999) with the H5 *HA* PCR product.

The recombinant VSVs, VSV-H5 HA, and VSV-NJG-H5 HA, were recovered using the above plasmids as previously described (Lawson et al., 1995). Briefly, BHK-21 cells were infected with the T7 polymerase expressing vaccinia virus, vTF7-3 (Fuerst et al., 1986), at a MOI of 10. At 1 h post-infection (hpi), the cells were transfected with the appropriate H5 HA plasmids plus the support plasmids, pBS-N, pBS-P, pBS-G, and pBS-L. The cell culture media was collected, filtered through a 0.2 μm filter, and passaged onto BHK-21 cells 48 h post-transfection. Once CPE was observed the culture media was filtered through a 0.1 μm filter, plaque purified, and used to grow up virus stocks. Growth and recovery of VSVΔG-H5 HA were achieved by complementation from BHK-G cells expressing VSV G (Schnell et al., 1997). In order to optimize titers, either BHK-G cells or BHK-21 cells transiently expressing G from pCAGGS-G (Publicover et al., 2005) were used.

Western blot analysis

Cell extracts

BHK-21 cells were infected with the indicated virus. At 4.5 hpi, cells were washed twice with ice-cold PBS and incubated with lysis buffer (1% NP-40, 0.4% deoxycholate, 62.5 mM EDTA, 50 mM Tris–HCl pH 8) for 5 min on ice.

VSV virions

The culture media of infected cells exhibiting 100% CPE were clarified by low speed centrifugation, layered on top of

20% sucrose in TE (pH 7.4), and subjected to ultracentrifugation in a Beckman SW50.1 rotor at 38,000 rpm (173,069×*g*) for 1 h at 4 °C. The pellet was then resuspended in lysis buffer.

SDS-PAGE/Western blot

Infected cell or virion lysates were combined with SDS-sample buffer [250 mM Tris–HCl pH 6.8, 30% glycerol (v/v), 8% SDS, 0.02% bromophenol blue, 10% 2-mercaptoethanol (v/v)] and subjected to SDS-PAGE on a 10% acrylamide gel. The proteins were transferred to nitrocellulose with a semi-dry transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions. The blots were incubated overnight at 4 °C in PBST-milk [PBS containing 0.5% Tween-20 (PBST) and 4% non-fat milk] and then in a 1:1000 dilution of NR-665 polyclonal anti-influenza virus H5 HA A/Hong Kong/156(483)/97 sheep antiserum in PBST-milk for 1 h at room temperature. The NR-665 antibody was obtained through the NIH Biodefense and Emerging Infections Research Repository, NIAID, NIH. After washing 3 times with PBST, the blots were incubated with donkey anti-sheep IgG conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:10,000 dilution in PBST-milk for 1 h. The blots were washed as before and incubated with a 1:1 mixture of ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ). Chemiluminescence was detected using a LAS-3000 imaging system (Fujifilm). The blots were then incubated in stripping buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) for 30 min at 50 °C, washed several times with PBS, and probed for VSV proteins as described above using a rabbit polyclonal anti-VSV antibody at a 1:1000 dilution and mouse anti-rabbit IgG conjugated to HRP (Pierce Biotechnology, Rockford, IL) at a 1:10,000 dilution.

Vaccinations

The Yale University Institutional Animal Care and Use Committee approved all vaccinations. Six to eight week old Balb/c female mice (Charles River Laboratories, Wilmington, MA) were primed i.m. with 50 μl or i.n. with 25 μl containing 10^6 plaque forming units (pfu) of VSV-H5 HA in DMEM or approximately 10^6 pfu of VSVΔG-H5 HA in PBS. Mice vaccinated i.n. were lightly anesthetized with 20% isoflurane (v/v; Baxter, Deerfield, IL) in propylene glycol prior to vaccination. At 1 month post-prime, half of the mice were boosted i.m. or i.n. with 10^6 pfu of VSV-NJG-H5 HA in DMEM as described above.

Microneutralization assay

Anti-H5 HA neutralizing antibody titers were determined as previously described (Suguitan et al., 2006). Briefly, serum obtained from vaccinated mice was heat inactivated at 56 °C for 30–60 min and subjected to serial 2-fold dilutions starting at a 1:10 dilution. Equal volumes of 100 $TCID_{50}$ of the indicated virus and diluted serum were combined, incubated at room temperature for 1 h, and added to MDCK cells in quadruplicate. Neutralizing titers are represented as the reciprocal dilution at 100% neutralization as defined by the absence of CPE at 4 days. Samples that did not display any neutralizing activity were arbitrarily given a value of 10. These assays were conducted using enhanced BSL-3 containment procedures in laboratories approved for use by the U.S. Department of Agriculture and Centers for Disease Control and Prevention.

H5N1 challenge

At 7.5 months post-prime, vaccinated mice were i.n. challenged with 100 LD_{50} (4.5 pfu) of HK/483 in 50 μl MEM/BSA. The LD_{50} was determined by the Reed and Muench method (Reed and Muench, 1938). Mice were weighed daily and analyzed for disease for 14 days. Mice exhibiting severe morbidity were humanely euthanized. Data are shown as the average percent of original pre-challenge weight. The challenge was performed using BSL-4 containment procedures under an approved animal use protocol and according to the guidelines of the Canadian Council on Animal Care.

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