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Protection of Mice and Poultry from Lethal H5N1 Avian Influenza Virus through Adenovirus-Based Immunization

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The recent emergence of highly pathogenic avian influenza virus (HPAI) strains in poultry and their subsequent transmission to humans in Southeast Asia have raised concerns about the potential pandemic spread of lethal disease. In this paper we describe the development and testing of an adenovirus-based influenza A virus vaccine directed against the hemagglutinin (HA) protein of the A/Vietnam/1203/2004 (H5N1) (VN/1203/04) strain isolated during the lethal human outbreak in Vietnam from 2003 to 2005. We expressed different portions of HA from a recombinant replication-incompetent adenoviral vector, achieving vaccine production within 36 days of acquiring the virus sequence. BALB/c mice were immunized with a prime-boost vaccine and exposed to a lethal intranasal dose of VN/1203/04 H5N1 virus 70 days later. Vaccination induced both HA-specific antibodies and cellular immunity likely to provide heterotypic immunity. Mice vaccinated with full-length HA were fully protected from challenge with VN/1203/04. We next evaluated the efficacy of adenovirus-based vaccination in domestic chickens, given the critical role of fowl species in the spread of HPAI worldwide. A single subcutaneous immunization completely protected chickens from an intranasal challenge 21 days later with VN/1203/04, which proved lethal to all control-vaccinated chickens within 2 days. These data indicate that the rapid production and subsequent administration of recombinant adenovirus-based vaccines to both birds and high-risk individuals in the face of an outbreak may serve to control the pandemic spread of lethal avian influenza.

Wild waterfowl, the natural hosts of all known influenza A viruses, are the source of viruses that cause sporadic outbreaks of highly fatal disease in domestic poultry. The highly pathogenic avian influenza virus (HPAI) H5N1 strain, which recently reached Europe, has likely become endemic in Southeast Asia, with frequent outbreaks in poultry leading to the destruction of hundreds of millions of animals and raising concerns about the potential pandemic spread of lethal disease (22). In 1997, H5N1 was transmitted from poultry to humans in Hong Kong, resulting in 18 infected people and six deaths (40), and reemerged in 2003, causing two similar cases with one fatality (27). In the period from 2003 to 2005, extensive outbreaks of H5N1 occurred in nine Asian countries, resulting in 19 human cases in Thailand, 91 in Vietnam, 7 in Indonesia, and 4 in Cambodia, with a total of 62 reported deaths. H5N1 infections in family clusters have raised the possibility of human-to-human transmission of H5N1 virus. As human exposure to and infection with H5N1 viruses continue to increase, so too does the likelihood of the generation of an avian-human reassortant virus that may be transmitted efficiently within the global human population, which currently lacks H5N1-specific immunity. Such reassortment events between avian-human and swine-human influenza A viruses have been associated with the 1957 and 1968 influenza pandemics; the 1918 pandemic events remain unclear.

Concerns about the potential for the generation of a pandemic H5 strain and its concomitant morbidity and mortality are spurring the search for an effective vaccine. An inactivated H5N1 influenza virus candidate vaccine has been rapidly produced by use of a reverse genetics technique (23, 38) and is being evaluated in clinical trials; however, the limited capability of producing conventional inactivated influenza virus vaccines could severely hinder the ability to control the pandemic spread of avian influenza through vaccination (7).

Recombinant DNA vaccines are highly effective inducers of both humoral and cellular immunity and show promise in the prevention of human disease in nonhuman primate models (3, 4, 9, 31). Here, we generated E1/E3-deleted adenovirus serotype 5-based vectors that express the codon-optimized hemagglutinin (HA) gene from A/Vietnam/1203/2004 influenza virus (VN/1203/04). We also tested their abilities to protect mice and chickens from challenge with wild-type influenza virus H5N1.

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MATERIALS AND METHODS

Influenza viruses. Influenza viruses used in this study were (H5N1) VN/1203/04 and A/Hong Kong/156/97 (H5N1) (HK/156/97). Virus stocks were propagated at 37°C in the allantoic cavity of 10-day-old embryonated hens' eggs for 26 h and aliquoted and stored at −70°C until use.

Gene synthesis and adenoviral vector construction. The HA, HA1, and HA2 genes from VN/1203/04 and the HA1 gene from HK/156/97 were codon optimized using the UpGene algorithm (www.vectorcore.pitt.edu/upgene.html) by overlapping oligonucleotides as previously described (15). E1/E3-deleted adenoviral vectors expressing the codon-optimized genes were constructed using Cre-*lox* recombination into the adenovirus-packaging cell line CRE8. The recombinant adenoviruses were propagated in CRE8 cells, purified by cesium chloride density gradient centrifugation and dialysis, and stored at −70°C (16). Adenovirus particle concentration was determined by spectrophotometric analysis using a validated assay based on Adenovirus Reference Material obtained from the ATCC.

Animal experiments. Six-week-old BALB/c mice were used in murine experiments. Eight groups of 10 mice each were immunized with an intramuscular injection of 5×10^{10} virus particles of Ad.VNHA, Ad.VNHA1, Ad.HKHA1, Ad.VNHA2, and Ad.ψ5 at day 0 and day 14. At day 70, mice were lightly anesthetized with CO_2 and inoculated intranasally with 50 μl of 100 50% lethal doses (LD_{50}s) of VN/1203/04 virus diluted in phosphate-buffered saline. Mouse LD_{50} titers were determined as previously described (24). To evaluate the degree of protection from challenge, eight vaccinated mice in each group were infected intranasally with 100 LD_{50}s of VN/1203/04 virus. Five mice per group were observed for illness, weight loss, and death each day for 14 days after infection, and three mice per group were sacrificed for virus isolation on day 3 or day 6 postinfection, depending on the experiment. For avian studies, 3-week-old specific-pathogen-free single comb white leghorn chickens from an in-house flock (SEPRL, USDA) were used. Four groups of 10 chickens each were immunized with an intranasal or subcutaneous administration of 5×10^{10} virus particles of Ad.VNHA or Ad.ψ5. At 6 weeks of age, the chickens were challenged with 10^6 50% embryo infectious doses (EID_{50}s) of VN/1203/04 virus intranasally through the choanal slit to determine protection. The chickens were observed for illness, weight loss, and death each day for 14 days after infection. Serum samples were obtained at 3, 6, and 8 weeks of age for detection of hemagglutination inhibition (HI) antibodies.

HI assay and ELISA. Immune sera from mice were collected by bleeding from the saphenous vein and treated with receptor-destroying enzyme from *Vibrio cholerae* (Denka-Seiken, San Francisco, CA) before being tested for the presence of H5-specific antibodies as described previously (24). The HI assay was performed using four HA units of virus and 1% horse red blood cells as described previously (32). H5N1 influenza virus-specific immunoglobulin G (IgG) antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as previously described (20), except that 1 μg/ml of purified baculovirus-expressed recombinant H5 HA protein from VN/1203/04 virus (Protein Sciences Corporation, Meriden, CT) was used to coat plates. ELISA end point titers were expressed as the highest dilution that yielded an optical density greater than twice the mean plus one standard deviation of that of similarly diluted negative control samples.

Enzyme-linked immunospot (ELISPOT) assay for IFN-γ. Ninety-six-well membrane-coated plates (Millipore, Bedford, MA) were incubated with 10 μg/ml monoclonal antibody to mouse gamma interferon (IFN-γ) (AN-18; Mabtech AB, Mariemont, OH) in 0.1 M carbonate buffer overnight. Previously frozen splenocytes were thawed and plated at 1×10^5 to 2×10^5 cells per well in medium supplemented with 10% fetal bovine serum. Individual 15-mer peptides overlapping by 11 amino acids and representing the entire HA sequences from H5N1 influenza virus strains VN/1203/04 and A/HK/156/97 (Sigma Genosys, The Woodlands, TX) were dissolved in dimethyl sulfoxide at 10 mg/ml and used in pools of 19 to 30 peptides (final concentration, 3.33 to 5.26 μg/ml) or 9 to 10 peptides (5.0 to 5.5 μg/ml) or used individually at 5.0 μg/ml as previously described (6).

RESULTS

Rapid generation of adenoviral vectors encoding H5N1 hemagglutinin. In this study, we generated E1/E3-deleted adenovirus serotype 5-based vectors that express the codon-optimized (15) HA gene either as the full-length protein or as the HA1 or HA2 subunits from the VN/1203/04 virus (Ad.VNHA, Ad.VNHA1, Ad.VNHA2). Additionally, we constructed a vector containing the HA1 portion of the (H5N1) HK/156/97 viral isolate (Ad.HKHA1). The generation of the recombinant adenoviral vectors was completed 36 days after the acquisition of the VN/1203/04 HA sequence, illustrating the rapid construction and ease of manipulation necessary for adenovirus-based vaccine development.

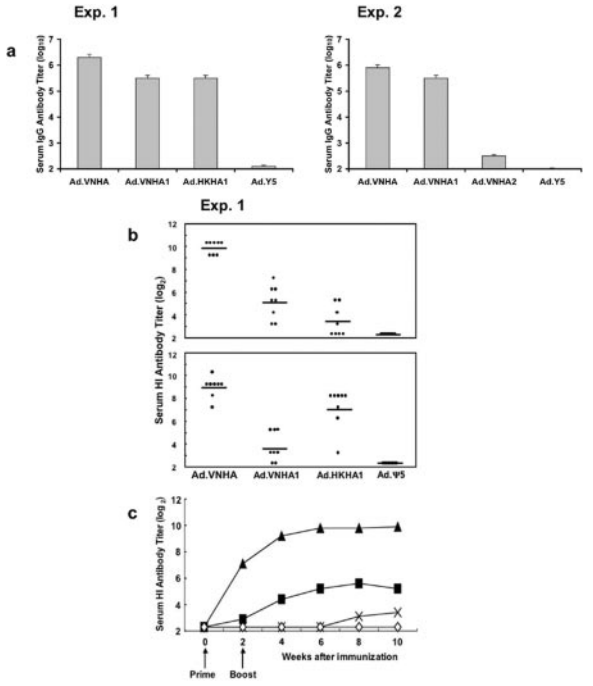

FIG. 1. Humoral immune responses in vaccinated mice. (a) Anti-H5N1 HA IgG antibody responses. Sera from eight mice per group were collected 8 weeks after the second immunization and tested by ELISA for the presence of H5N1 subtype-specific IgG antibodies by using purified VN1203 HA recombinant protein. Antibody titers are expressed as the $\log_{10}$ values of reciprocal end point titers. (b) Serum HI antibody responses. Sera were collected 8 weeks after the second vaccination and tested individually for HI antibody against VN/1203/04 (top) or HK/156/97 (bottom) virus. HI antibody titers for individual mice are expressed as a $\log_2$ value of the reciprocal of the highest dilution of serum inhibiting agglutination of 1% horse erythrocytes by four HA units of virus. Horizontal lines represent the geometric mean of each group. (c) Kinetics of serum anti-VN/1203/04 HI antibody production. Symbols: Ad.VNHA (▲), Ad.VNHA1 (■), Ad.HKHA1 (×), empty vector Ad.ψ5 (◇). Exp., experiment.

Broad immunity and protection from challenge in vaccinated mice. Eight groups of susceptible BALB/c mice (10 mice/group) (24) were immunized intramuscularly with 5×10^{10} viral particles of Ad.VNHA, Ad.VNHA1, Ad.HKHA1, or empty vector Ad.ψ5 and boosted 14 days later (experiment 1). Additional groups of mice were similarly vaccinated and boosted with Ad.VNHA, Ad.VNHA1, Ad.VNHA2, or empty vector Ad.ψ5 (experiment 2). Serum samples were obtained from all mice to screen for antibody responses as a marker of immunogenicity (18). On week 10, 8 weeks after the booster immunization, high titers of H5-specific antibodies were detected in all vaccinated animals except for those in the Ad.VNHA2 group, which had titers more than 3 orders of magnitude lower than those of all other vaccinated groups (Fig. 1a). We then determined the degree to which antibody responses could neutralize homologous VN/1203/04 and het-

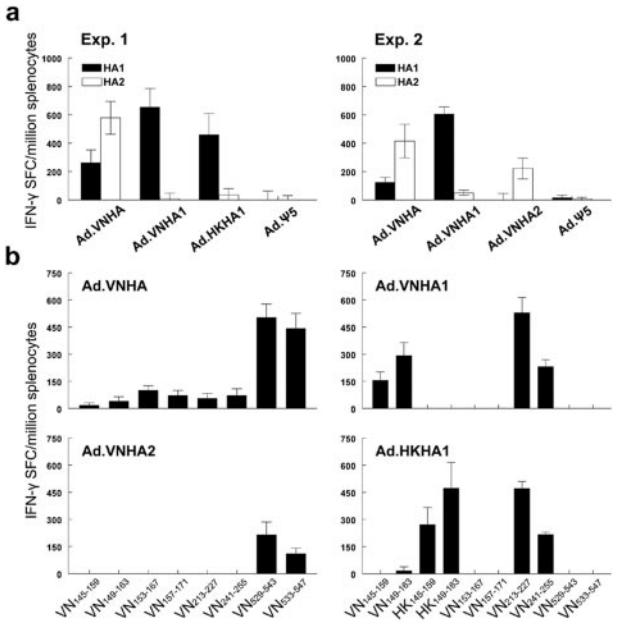

FIG. 2. Cellular immune responses in vaccinated mice. (a) HA1- and HA2-specific responses of splenocytes taken 3 to 5 days after a second boost as determined by IFN-γ ELISPOT assay using pools of 15-mer peptides. (b) Identification of individual epitope specific-responses as determined by IFN-γ ELISPOT assay using individual 15-mer peptides as shown. Data represent means ± standard errors of the mean of triplicate determinations for a minimum of two mice per group. SFC, spot-forming cells; Exp., experiment.

erosubtypic HK/156/97 influenza virus strains by using the horse red blood cell HI assay (32). Vaccination with full-length HA induced homologous and heterotypic antibody responses, whereas vaccination with Ad.VNHA1 or Ad.HKHA1 induced primarily antibody responses specific to the vaccinating strain (Fig. 1b). The kinetics of serum HI responses suggest that a single immunization may be sufficient to achieve a high-level anti-HA antibody response (Fig. 1c).

Given that vaccination induced various degrees of humoral immunity, with the Ad.VNHA2-immunized group having a markedly reduced H5-specific antibody response, we next analyzed the cellular immune response to vaccination after an additional boost immunization using the IFN-γ ELISPOT assay for two mice per group. Overlapping 15-mer peptides representing the entire VN/1203/04 HA protein and nonconserved sequences of HK/156/97 were pooled to evaluate the strength and breadth of immunity. Individual epitope-containing peptides were then identified through analysis of matrices in which each peptide was represented by two pools (6). All animals immunized with full-length HA or the HA1 or HA2 subunits developed strong cellular responses to HA peptides, reaching an average peak intensity of one HA-specific T cell per 1,200 freshly isolated splenocytes in the Ad.VNHA group (Fig. 2a). Cumulative cellular immune responses were HA region specific, with only the animals immunized with full-length HA developing T-cell responses spanning both HA1 and HA2 (Fig. 2a). Detailed characterization of vaccine-induced immune responses identified both conserved and unique peptide targets (Fig. 2b). As expected, cellular responses against the conserved HA1 regions $VN_{213-227}$ and $VN_{241-255}$ were elicited regardless of HA1 immunization strain, whereas responses to peptides spanning the regions from amino acid 145 to amino acid 163, which differed between VN/1203/04 and HK/156/97, were limited to animals immunized with the respective subtype (Fig. 2b). Ad.VNHA2 immunization revealed the presence of an immunodominant epitope within HA2 represented by $VN_{529-543}$/$VN_{533-547}$ peptides. Immunization with Ad.VNHA induced a subdominant response to the previously identified SFFRNVV WLIKK epitope (17, 19) contained within the HA1 peptides $VN_{153-167}$/$VN_{157-171}$. Immunization with Ad.VNHA altered the nature of HA1-specific immune responses seen when Ad.HA1 was the sole immunogen, generating more-modest responses to $VN_{145-159}$/$VN_{149-163}$, $VN_{213-227}$, and $VN_{241-255}$ that were subdominant to the responses to $VN_{529-543}$/$VN_{533-547}$ (Fig. 2b). These data demonstrate that adenovirus-based vaccination generates robust cellular immune responses to HA, which in the case of HA2 vaccination appear to be dominant to the humoral immune response.

Eight weeks after the second immunization, all mice were challenged by intranasal inoculation with 100 LD_{50}s of VN/1203/04. All animals immunized with control Ad.ψ5 vector experienced substantial weight loss beginning at day 3 postchallenge and death by 6 to 9 days postchallenge. In contrast, all animals immunized with Ad.VNHA, Ad.VNHA1, or Ad.HKHA1 showed only mild and transient loss of body weight and survived the lethal challenge (Fig. 3a and b). All animals immunized with Ad.VNHA2 experienced substantial weight loss, but three out of five animals in this group regained weight after day 8 and recovered fully (Fig. 3a and b). This recovery is notable given that vaccination with HA2 induced primarily cellular immune responses, which previously have been associated only with enhanced viral clearance and recovery from influenza infection (26). At day 3 or day 6 postchallenge, three animals per group were sacrificed for virus isolation. Infectious virus was isolated from multiple organs in the control-vaccinated group and to various degrees from animals vaccinated with HA1 or HA2 subunits. In contrast, virus was isolated at extremely low levels on day 3 postinfection ($\log_{10}$ mean virus titer, 0.5; experiment 1) and not at all on day 6 postinfection ($\log_{10}$ mean virus titer, $\leq$0.5; experiment 2) from organs from mice vaccinated with full-length HA (Fig. 3c).

Efficacy of vaccination in chickens. Following the encouraging responses to vaccination and challenge in the murine model, we evaluated the efficacy of adenovirus-based vaccination in domestic chickens, given the critical role that fowl species play in the spread of HPAI in Southeast Asia (8). The severity of H5N1 infection in chickens differs from that in mice, as chickens rarely survive past the second day after challenge, whereas the median survival period in naive mice is 8 days. We restricted our experiment to vaccination using full-length HA, given the superior protection noted in mice immunized with this vaccine. Four groups of 10 3-week old chickens received one immunization intranasally or subcutaneously with 5×10^{10} viral particles of Ad.VNHA or empty vector Ad.ψ5 and were challenged with an intranasal inoculation of 10^6 EID_{50}s of VN/1203/04 21 days later (at 6 weeks of age). This virus dose was 10,000-fold higher than that given to mice and likely represented a challenge significantly greater than that experienced by chickens in a natural outbreak. The vaccination induced HI

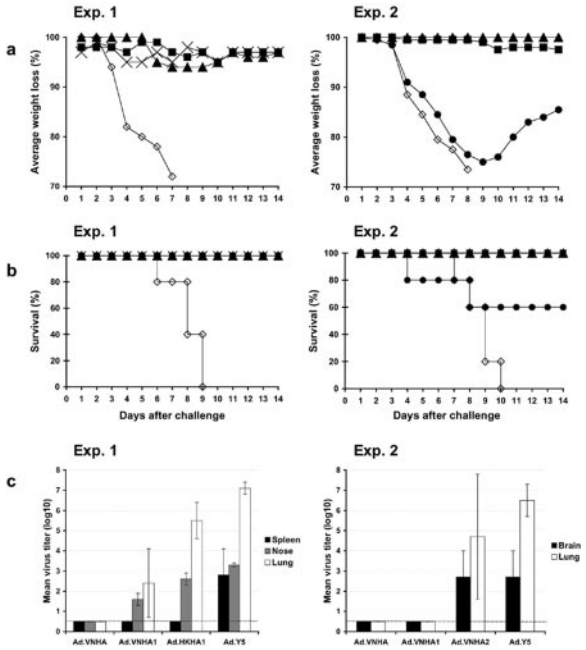

FIG. 3. Outcomes in vaccinated mice following lethal intranasal challenge with VN/1203/04. (a) Weight loss and (b) survival in mice challenged by intranasal inoculation with 100 LD_{50}s of VN/1203/04 influenza virus 8 weeks after the second immunization. Mean weight loss is expressed as a percentage of original weight. Symbols: Ad.VNHA (▲), Ad.VNHA1 (■), Ad.HKHA1 (×), Ad. VNHA2 (●), empty vector Ad.ψ5 (◇). Data shown are the means for five mice per group. (c) Virus titers in different tissues determined 3 and 6 days after challenge for experiments (Exp.) 1 and 2, respectively. Data represent means ± standard deviations of $\log_{10}$ EID_{50}s. Dotted lines indicate the thresholds of viral detection.

antibodies to VN/1203/04 in all chickens belonging to the subcutaneous immunization group whose members were boosted upon virus challenge. All animals in this group survived challenge with no detectable clinical signs of disease (Table 1). In contrast, all control-immunized chickens died (median survival period of 1.8 days). Only one of the chickens immunized with Ad.VNHA intranasally had HI antibodies, while, as a group, the chickens experienced 50% morbidity and 50% mortality following challenge (Table 1). The poorer protection afforded by intranasal immunization may reflect an infection by adenovirus serotype 5 via this route that is limited in comparison to that via the subcutaneous route. Oral and cloacal measurements of virus titers showed that subcutaneously administered vaccine greatly reduced replication of the challenge virus such that virus could not be detected in the gastrointestinal tract and levels were reduced by 3 orders of magnitude in the respiratory tract (Table 1).

DISCUSSION

It is widely accepted that novel influenza virus vaccination strategies are urgently needed both to control the spread of HPAI within fowl species and to prevent the pandemic spread of HPAI in humans should the capacity for human-to-human transmission emerge. In this study, we have tested the ability of adenovirus-based immunization to induce both broad and potent HA-specific humoral and cellular immune responses able to confer protection against lethal intranasal challenge, given its promise in other vaccine applications (31, 34) and recent promising results of immunization of humans with an influenza virus HA vaccine based on an adenoviral platform (37). A broadly cross-protective vaccination could be useful for the treatment of domestic animals as well as humans, and adenoviral vectors may be a practical alternative to propagating vaccines with conventional methods in embryonated chicken eggs (2).

Previous studies using inactivated whole H5N1 influenza virus vaccines in mice have indicated that strain-specific neutralizing antibodies provide long-lasting protection against homologous influenza virus challenge (33), but protection is limited against antigenically variant strains Two recent studies have demonstrated the efficacy and immunogenicity of adenovirus-vectored influenza virus HA (H3N2) vaccines in swine and mice and have revealed that cross-protection from heterotypic challenge can occur in the absence of neutralizing humoral immunity (37, 39). Our study builds upon these findings, going one step further in trying to understand the role of the T-cell response to an adenovirus-based influenza virus HA vaccine. The presence of heterotypic H5N1 protection in the absence of a strong humoral neutralizing response in our initial studies, further reinforced by the ability of adenovirus-based HA2 vaccine to partially protect the immunized animal (the HA2 region is known not to induce neutralizing humoral immunity), strongly suggests a complementary role for the cellular response to its humoral counterpart. This idea is supported by recent findings showing that DNA-prime adenoviral boosting using the anti-H1N1 nucleoprotein (NP) influenza virus vaccine induced a T-cell response that was able to protect from heterosubtypic challenge (11). Together, these data suggest that humoral immunity in the context of an influenza virus

TABLE 1. Efficacy of VN/1203/04 vaccination in chickens[a]

Group	Route	Morbidity	Mortality (MDT)	Virus isolation[b]		Serum HI antibody titer (GMT)		
				Oral	Cloacal	d0 PV	d21 PV	d14 PC
Ad.ψ5	IN	10/10	10/10 (1.8)	NA	NA	0/10	0/10	NA
Ad.ψ5	SQ	10/10	10/10 (1.8)	6.96 (0.81)	6.26 (0.50)	0/10	0/10	NA
Ad.VNHA	IN	5/10	5/10 (6.0)	NA	NA	0/10	1/10 (4)	5/5 (97)
Ad.VNHA	SQ	0/10	0/10	3.84 (1.22)	≤0.9 (0.00)	0/10	10/10 (13)	10/10 (315)

[a] Data are shown as the ratio of the number of animals affected to the total number of animals per group. GMT, geometric mean reciprocal end point titer; MDT, median time to death in days; PV, postvaccination; PC, postchallenge; IN, intranasal; SQ, subcutaneous; d0, day 0; NA, not available.

[b] VN/1203/04 (H5N1) virus was isolated 2 days after challenge; mean virus titers are represented as $\log_{10}$ EID_{50}/ml (± standard deviation). Sensitivity threshold, 0.9 $\log_{10}$ EID_{50}/ml.

vaccine is a ready response that, when strong, fully protects an animal from homologous challenge. In contrast, a T-cell recall response is delayed in its action (7 to 8 days in mice) but has the advantage of giving broader protection against different influenza virus subtypes. The ability of DNA-based influenza virus vaccination using adenoviral delivery or other methods (1, 5, 10, 13, 21, 35, 36) to induce a T-cell response able to cross-protect at least partially against heterotypic infection could represent an added value, rendering further investigation in the influenza virus recombinant vaccine development field worthwhile. Adenovirus might have some advantages over other DNA-based technologies based on its relatively safe profile, which has been proven through the vaccination of volunteers in more than 100 phase I, II, and III clinical trials. Moreover, the adenovirus production yield, particularly the ability to scale up to large quantities, make this technology one of the most promising DNA-based platforms for vaccination.

Natural vector-specific immunity of some human populations against adenovirus serotype 5 (28) could potentially reduce vaccine efficacy in the event that global vaccination against HPAI is implemented. In a recent report, however, adenovirus serotype 5-based vaccines against influenza virus A/PR/8 (H1N1) were tested successfully in humans in a phase I clinical trial and shown to be safe (37). Importantly, vaccination was highly effective in inducing anti-influenza virus-neutralizing antibodies despite the presence of preexisting antiadenoviral antibodies, suggesting that vector-specific immunity may be overcome (37). Alternatively, a wide range of different human and simian adenovirus serotypes are being developed as alternative vectors, which will likely negate the issue of preexisting serotype 5-specific immunity (12, 14, 25, 29, 30).

In conclusion, our findings as well as those from other adenovirus-based vaccine studies support the development of replication-defective adenovirus-based vaccines as a rapid response in the event of the pandemic spread of HPAI. Given the induction of protective immunity in chickens, widespread immunization of susceptible poultry would likely provide a significant barrier to the spread of HPAI and also be economically advantageous. Finally, in the worst-case scenario of the pandemic spread of lethal human disease, an adenovirus-based vaccine could be utilized to complement traditional inactivated influenza virus vaccine technology, which is still the primary choice but at the same time may become hampered by the limits to production capability in fertilized eggs.

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W. Gao, A. Montecalvo, and P. D. Robbins generated the codon-optimized HA recombinant adenoviral vectors and performed the Western and dot blot analyses and mice immunizations. J. M. Katz, X. Lu, and D. C. Nguyen performed the microneutralization assay and the in vivo viral challenge. S. M. Barratt-Boyes and A. C. Soloff were responsible for the IFN-γ ELISPOT T-cell assay. Y. Matsuoka and R. O. Donis were responsible for the A/Vietnam/1203/2004 (H5N1) strain sequencing and codon-optimized expression level studies. D. E. Swayne was responsible for the chicken studies. A. Gambotto was the principal investigator; he conceived and coordinated the study.

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