A Broadly Protective Vaccine against Globally Dispersed Clade 1 and Clade 2 H5N1 Influenza Viruses

Mary A. Hoelscher,1,4 Neetu Singh,2,4 Sanjay Garg,1 Lakshmi Jayashankar,1 Vic Veguilla,1 Aseem Pandey,1 Yumi Matsuoka,1 Jacqueline M. Katz,1 Ruben Donis,1 Suresh K. Mittal,2 and Suryaprakash Sambhara1

1Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; and 2Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

Development of effective and immunogenic vaccines against highly pathogenic avian influenza H5N1 viruses with the potential to cause a pandemic is a public health priority. The global demand for a vaccine cannot be met in the event of an influenza pandemic because of the limited capacity to manufacture egg-derived vaccines as well as potential problems with the availability of embryonated eggs. Thus, there is an urgent need to develop alternative, egg-independent vaccines. We developed an adenoviral vector–based vaccine that contains hemagglutinin protein from clade 1 and clade 2 viruses, as well as conserved nucleoprotein, to broaden the vaccine coverage against H5N1 viruses.

Highly pathogenic avian influenza H5N1 viruses are currently endemic in domestic poultry in southeast Asia and have spread to >60 countries on 3 continents. Furthermore, they have crossed the species barrier to infect humans, resulting in >300 cases of infection, with a 60% mortality rate [1, 2]. Although limited human-to-human transmission has been reported, H5N1 spread to other species has occurred primarily through direct contact with infected poultry. In addition, H5N1 viruses have diverged into genetically and antigenically distinct clades and subclades, thereby complicating the development of a “perfect match” vaccine [3]. Traditional influenza vaccine approaches rely on embryonated eggs for vaccine production. Egg-derived vaccines against H5N1 viruses are not very immunogenic and require large doses of vaccine to elicit a protective immune response, and the seroconversion rates and the magnitude of immune response are suboptimum [4–6]. However, with novel adjuvants, significant dose sparing has been achieved, and the humoral immune responses induced by a vaccine with a clade 1 (C1) viral adjuvant showed considerable cross-reactivity with a clade 2 (C2) virus [7]. However, the capacity to manufacture egg-derived vaccines is severely limited and unlikely to meet global needs, particularly if a novel influenza virus strain arises that is lethal to chickens, the sources of the embryonated eggs needed to manufacture the vaccine. Hence, we and others tested the feasibility of an adenoviral vector–based vaccine as an alternative to the conventional egg-derived vaccine against pandemic influenza [8, 9]. We demonstrated that a replication-defective human adenoviral 5 vector (HAd) expressing the gene encoding hemagglutinin (HA) in A/Hong Kong/156/97 (HAd-H5HA) H5N1 viruses conferred long-lasting immunity and cross-protection in mice against challenge with more-recent strains of H5N1 virus [10]. Thus, it may be worthwhile to stockpile this influenza vaccine as a means to control pandemic influenza.

Materials and methods. To broaden vaccine-induced immunity, we developed adenoaviral vaccine candidates that express the HA gene in currently circulating and divergent C1 (A/Vietnam/1203/04) and C2 (A/Indonesia/05/05) viruses, as well as the gene encoding nucleoprotein (NP) in A/Vietnam/1203/04 viruses, and assessed humoral and cell-mediated immune responses in a mouse model. The NP gene is highly conserved across influenza A virus subtypes. A Cre-recombinase–mediated site-specific recombination technique was used to insert the full-length coding region of the H5 gene (with a modified polybasic site) of A/Vietnam/1203/04 and A/Indonesia/05/05 viruses into the early region (E1) of the HAd genome, which is under the control of the immediate early promoter of cytomegalovirus (CMV) and bovine growth hormone (BGH) polyadenylation (polyA) signaling, to create HAd-1203HA and HAd-05HA vaccine constructs.
respectively [11]. Similarly, the HAd-NP vector, containing the NP gene of A/Vietnam/1203/04 under the CMV promoter and BGH polyA signaling, was created. We also created the HAd-05H5-NP vaccine construct, which contained the full-length coding region of the H5 HA gene (with a modified basic amino acid cleavage site) of A/Indonesia/05/05 virus under control of the CMV promoter and BGH polyA signaling and the full-length coding region of the NP gene of A/Vietnam/1203/04 under the CMV promoter and simian virus 40 polyA signaling. Both genes in HAd-05H5-NP were in the E1-parallel orientation. Western blot analyses with polyclonal serum specimens against H5HA and NP confirmed the expression of HA and NP proteins. An empty vector construct (HAd-ΔE1E3) was used as a negative control in all experiments. C1 and C2 H5N1 virus vaccine strains containing the H5 HA gene (with a modified basic amino acid cleavage site) and the N1 neuraminidase (NA) gene on a A/PR/8/34 background were generated by reverse genetics (RG) technology. These C1RG and C2RG vaccine strains served as positive controls and were also used as challenge viruses.

To assess the breadth of immune response, we vaccinated 10-week-old BALB/c mice obtained from Taconic Laboratories (20 animals per group) intramuscularly with $$1 \times 10^8$$ pfu of vaccine constructs HAd-1203HA, HAd-05HA, HAd-05HANP, HAd-NP, and HAd-ΔE1E3. To keep the total dose of vaccine constant at $$1 \times 10^8$$ pfu, the HAd-1203HA plus HAd-05HA group was vaccinated with $$5 \times 10^7$$ pfu of each vector. Two additional groups of mice were vaccinated interperitonally with 200 hemagglutination units of C1RG (A/Vietnam/1203/04) or C2RG (A/Indonesia/05/05) vaccine strain viruses. Four weeks later, mice received a booster containing the same vaccine construct, and blood was obtained 3 weeks later for detection of neutralizing antibodies by a virus microneutralization assay (lower limit of detection, $$\geq 10$$) and detection of hemagglutination-inhibiting antibodies by a horse red blood cell test in pooled serum specimens [12, 13]. Four weeks after receipt of the booster, 5 mice from each group were challenged in each nostril with a 25-$$\mu$$L 100x 50% mouse infectious dose of C1RG virus or C2RG virus. Because C1RG and C2RG viruses are not lethal and do not produce clinical disease and weight loss in mice, protection was monitored by clearance of virus from lungs. Mice were sacrificed under anesthesia on day 3 after challenge, and lungs were collected and processed in embryonated eggs to determine viral titers. The 10 remaining mice were euthanized to collect spleen cells for evaluation of cellular responses by means of epitope-specific T cell ELISpot and pentamer staining, as described previously [9].

**Results.** Mice vaccinated with either HAd-1203HA or HAd-05HA produced hemagglutination-inhibiting antibodies (data not shown) and neutralizing antibody titers of 320 against C1 or C2 wild-type viruses, but no cross-reactivity was detected (table 1). However, mice vaccinated with both HAd-1203HA and HAd-05HA elicited protective neutralizing antibody titers of 160 against viruses from both clades. The 2-fold reduction in the neutralization antibody titer after vaccination with both constructs is probably associated with the vaccine dose, because the animals received 5 $$\times$$ 107 pfu of each vector and because a 2-fold reduction is not statistically significant. The addition of the NP gene to the HAd-05HA vaccine construct (HAd-05HANP) did not significantly increase the neutralizing antibody response to A/Indonesia/05/05, because NP is an internal protein, and antibodies against NP will not neutralize the virus. Similarly, the HAd-NP construct failed to induce any neutralizing antibodies either C1 or C2 viruses. C1RG and C2RG viruses also express
other proteins and may induce NA-specific and M2-specific antibodies that may be playing a role, whereas Ad-vector constructs just express HA alone or HA and NP. On the basis of Western blot findings, it is clear that polyclonal serum specimens raised against H5 HA or monoclonal antibodies raised against NP recognized Ad-vector–expressed H5HA and NP, indicating that these proteins were folded properly (data not shown). However, we cannot rule out subtle changes in the conformation of Ad-vector–expressed HA. These proteins were functional, as the immune responses induced by them conferred protection against viral challenge. The C1RG and C2RG vaccine constructs induced neutralizing antibody titers of 320 and 640, respectively, against their specific wild-type viruses. However, the C1RG virus also induced a substantial neutralizing antibody titer of 80 against the C2 virus. In contrast, the neutralizing antibody response induced by the C2RG virus was not cross-reactive against the C1 virus. It is not clear why there was a lack of reciprocal neutralization, and detailed structure-function studies of HA and NA from C1RG and C2RG viruses are required to understand this finding.

When challenged with C1RG or C2RG viruses, all vaccinated mice had lung viral titers at or below the level of detection (defined as \(1.5 \log_{10}\) 50% egg infective dose per mL), with the exception of the empty vector and the HAd-NP groups (table 1). The HAd-NP group had lung viral titers substantially lower than those in the HAdAE1E3 vector group after challenge with either C1RG or C2RG viruses; however, the addition of NP to the HAd-HA vector further enhanced the vaccine’s efficacy. This is quite evident because HAd-05HA–NP vaccine provided complete protection against both clades. It is known that NP is a CD8+ T cell target, and T cell responses against NP will not prevent infection but will aid in viral clearance.

Despite the lack of the neutralizing antibody titers against the heterologous clade virus, mice vaccinated with either HAd-1203HA or HAd-05HA had little or no detectable virus in the lungs after challenge with antigenically distinct (i.e., C1RG or C2RG) virus, indicating that mechanisms other than the virus neutralizing antibody response, such as cell-mediated immune responses and/or nonneutralizing antibody–mediated or antibody-dependent cell-mediated cytotoxicity [14, 15], may contribute to viral clearance. Indeed, groups vaccinated with the HAd-1203HA and/or HAd-05HA vaccine construct(s), C1RG virus, or C2RG virus had significantly higher levels of HA518 epitope–specific CD8+ T cells detected by pentamer staining (table 1) and by interferon-γ ELISPOT analysis (data not shown), compared with groups that received HAd-NP or HAd-AE1E3 (\(P < .001\)). The groups that received the vaccine construct with the NP gene alone (i.e., HAd-NP), C1RG virus, or C2RG virus had significantly higher levels of NP147 epitope–specific CD8+ T cells than did other groups (\(P < .001\)), and the group that received HAd-05HANP had significantly higher levels of both HA518–epitope–specific and NP147–epitope–specific CD8+ T cells (\(P < .001\)).

Discussion. C1 H5N1 adjuvant vaccines demonstrated enhanced immunogenicity at low doses and significant cross-reactive antibody responses against C2 viruses, compared with vaccines without an adjuvant [7]. However, the cross-reactive responses were significantly lower than those against C1 viruses. At present, the immunogenicity of C2 adjuvant viral vaccines and their cross-reactivity against C1 viruses are not known. It is not clear whether these adjuvanted vaccines stimulate CD8+ T cell responses, which are crucial in conferring cross-protection against antigenically distinct, novel influenza viruses if the antibodies induced by C1 viral vaccine fail to neutralize the virus. We demonstrated that the adeno-viral vector–based strategy elicited cell-mediated CD8+ T cell immune responses as well as neutralizing antibodies against C1 and C2 strains, thereby broadening the vaccine’s coverage. It is known that NP and HA proteins produced by influenza virus also induce T cell responses in humans. Thus, this approach may prevent severe illness and death or shorten the course of future infection with H5N1 virus strains that are antigenically distinct from currently circulating strains, and it may offer stockpiling advantages that overcome the limitations associated with storage of egg-derived vaccines.

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References

7. Leroux-Roels I, Borkowski A, Vanvolleghem T, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pan-