CLINICAL AND VACCINE IMMUNOLOGY, Sept. 2006, p. 981–990 Vol. 13, No. 9
1556-6811/06/$08.00+0 doi:10.1128/CVI.00156-06
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Distinct Contributions of Vaccine-Induced Immunoglobulin G1 (IgG1) and IgG2a Antibodies to Protective Immunity against Influenza

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Received 26 April 2006/Returned for modification 20 June 2006/Accepted 5 July 2006

Vaccination represents the most effective form of protection against influenza infection. While neutralizing antibodies are typically measured as a correlate of vaccine-induced protective immunity against influenza, nonneutralizing antibodies may contribute to protection or amelioration of disease. The goal of this study was to dissect the individual contributions of the immunoglobulin G1 (IgG1) and IgG2a antibody isotypes to vaccine-induced immunity against influenza virus. To accomplish this, we utilized an influenza vaccine regimen that selectively enhanced IgG1 or IgG2a antibodies by using either DNA or viral replicon particle (VRP) vectors expressing influenza virus hemagglutinin (HA) (HA-DNA or HA-VRP, respectively). After HA-DNA vaccination, neutralizing antibodies were detected by both in vitro (microneutralization) and in vivo (lung viral titer) methods and were associated with increased IgG1 expression by enzyme-linked immunosorbent assay (ELISA). Vaccination with HA-VRP did not strongly stimulate either neutralizing or IgG1 antibodies but did induce IgG2a antibodies. Expression of IgG2a antibodies in this context correlated with clearance of virus and increased protection against lethal influenza challenge. Increased induction of both antibody isotypes as measured by ELISA was a better correlate for vaccine efficacy than neutralization alone. This study details separate but important roles for both IgG1 and IgG2a expression in vaccination against influenza and argues for the development of vaccine regimens that stimulate and measure expression of both antibody isotypes.

Despite the availability of an effective vaccine, the World Health Organization estimates that annual influenza epidemics exact a toll of 3 to 5 million severe illnesses and 250,000 to 500,000 deaths in the industrialized world (63). Part of this failure is due to limited distribution of the vaccine, but part can be attributed to reduced efficacy in groups at high risk for complications. The incipient pandemic developing in Southeast Asia is a warning that we need more-effective influenza vaccines (3, 70). Particularly troubling is the difficulty in generating a robust immune response against highly pathogenic avian influenza viruses of the H5N1 subtype by use of traditional vaccine approaches (67, 72). Refinement of the methodologies used to prevent this important disease and to evaluate the immune response to influenza vaccines is needed.

Typical assays used to measure vaccine responses against influenza antigens include hemagglutination inhibition (HI) and microneutralization assays. These standardized tests are easy to perform and provide a quantitative measure of antibodies based on their ability to neutralize viral particles (57). Use of these assays has shown that high levels of antibody are required to see effective neutralization in vivo (54). While neutralizing titers immediately following vaccination may be high enough to meet this threshold, antibody titers wane over time. In many cases, it may be difficult for the host to maintain a neutralizing antibody titer sufficient to prevent infection during an entire influenza season and into subsequent seasons.

In addition to their neutralizing properties, antibodies can mediate host effector functions and facilitate the removal of a pathogen from a host. Specifically, the Fc portion of immunoglobulin G2a (IgG2a) antibodies interacts with complement components (51) and activatory Fc receptors (21, 25, 69) with a high affinity. This interaction can efficiently activate Fc receptor-mediated effector functions, which include the stimulation of antibody-dependent cell-mediated cytotoxicity (36) and opsonophagocytosis by macrophages (64), the latter of which has been shown to contribute to the clearance of influenza virus from infected hosts (31). The Fc portion of IgG1 antibodies mediates a lower-affinity interaction with activatory Fc receptors and does not stimulate Fc receptor-mediated immune responses as effectively (52, 53). Interestingly, protective anti-influenza immunity in the absence of measurable neutralizing antibodies has been described to occur in influenza vaccine trials with both animals (38, 39) and humans (4, 9), but the explanation for this observation has yet to be determined.

BALB/c mice typically respond to inactivated influenza vaccines and subunit vaccines with a Th2-type immune response (2, 5, 27, 48), which is associated with the stimulation of IgG1 antibodies (60). However, the major antibody isotype present in the sera of mice that survive viral infections is IgG2a (10, 11), which is stimulated during Th1-type immune responses (60). Stimulation of IgG2a antibodies has been associated with increased efficacy of influenza vaccination (1, 30, 31, 48). Ad-

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ditionally, monoclonal antibodies of the IgG2a isotype are more efficient at clearing influenza (20, 50), Ebola (71), and yellow fever (58) virus infections than monoclonal antibodies of the IgG1 isotype displaying similar antigenic specificities.

In the present study, we specifically stimulated immunity against the hemagglutinin (HA) surface glycoprotein of influenza virus without complementary immunity from other external (neuraminidase) and internal (nucleoprotein [NP] and acid polymerase [PA]) components of the virus that are known to play a role in immunity against influenza viruses (13, 33–35). While focusing on the HA alone does not induce optimal protection against influenza challenge, it allowed us to dissect the distinct contributions of different elements of the immune response. We delivered influenza HA expressed in plasmid DNA via the gene gun, a route of vaccination that is known to induce a predominantly IgG1 response in BALB/c mice (16, 41, 76). We then vaccinated mice with replication-deficient viral replicon particles (VRP) from Venezuelan equine encephalitis (VEE) virus, which express the influenza HA in a manner known to enhance IgG2a antibody levels in mice (23, 75, 76). Our results support a role for IgG1 antibodies in the neutralization of viral particles both in vitro and in vivo. In contrast, the specific induction of IgG2a antibodies was not associated with neutralization of influenza virus but appears to assist in the clearance of influenza virus from the infected host. The data are discussed with emphasis on the different roles of antibody isotypes in antiviral immunity.

MATERIALS AND METHODS

Mice. Adult (6- to 8-week-old) female BALB/cJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of four to six in high-temperature 31.2- by 23.5- by 15.2-cm polycarbonate cages with isolator lids. Rooms used for housing mice were maintained on a 12-h:12-h light:dark cycle at 22 ± 2°C with a humidity of 50% in the biosafety level 2 facility at St. Jude Children's Research Hospital (Memphis, TN). Prior to inclusion in experiments, mice were allowed at least 7 days to acclimate to the animal facility. Laboratory autoclavable rodent diet (PMI Nutrition International, St. Louis, MO) and autoclaved water were available ad libitum. All experiments were performed in accordance with the guidelines set forth by the Animal Care and Use Committee at St. Jude Children's Research Hospital.

Coupling plasmid DNA to gold particles. HA from the A/Hong Kong/1/68 (H3N2) (HK68) (GenBank accession no. AF348176) strain of influenza virus was cloned into pHW2000 plasmid DNA as described previously (29). The HA cloned in these studies differed from the GenBank sequence at N153I (A458T).

Spermidine (0.1 M) (Sigma, St. Louis, MO), 2.5 M $CaCl_2$ (Fisher, Fair Lawn, NJ), and 2.5 μg plasmid DNA per 1 mg gold (1-μm particle size) (Bio-Rad Laboratories, Hercules, CA) were incubated at room temperature. Ethanol-washed gold beads were suspended in ethanol containing 0.2 mg ml^{-1} polyvinylpyrrolidone (molecular weight, 360,000) and dried onto Tefzel tubing (Saint-Gobain Performance Plastics, Mickleton, NJ).

VRP creation. VEE VRP expressing an identical HA sequence were produced as described previously (46, 55) using constructs provided by Alphavax (Alphavax, Inc., Research Triangle Park, NC). Briefly, RNA from a single construct expressing both VEE nonstructural proteins and HK68 HA in place of VEE structural proteins was transfected into baby hamster kidney (BHK) cells by electroporation. Concurrently, RNA from two helper constructs that expressed VEE structural proteins but lacked packaging signals was transfected into BHK cells. Coelectroporation of these three RNA constructs results in the production of VRP that express the nonstructural proteins of VEE and the influenza HA. Supernatants from transfected BHK cells containing VRP were purified and concentrated prior to inoculation. VRP encoding green fluorescent protein (GFP) in place of influenza HA were used as a heterologous antigen control (46).

HK/Syd reassortant influenza virus. Individual influenza genes were cloned into pHW2000 plasmid vectors as described previously (29). The HA component of the virus was derived from the HK68 HA plasmid described above. The neuraminidase component used for creation of these viruses was from the A/Sydney/5/97 (H3N2) strain of influenza virus. The remaining genes used to create influenza virus were from A/Puerto Rico/8/34 (Erich Hoffmann, St. Jude Children's Research Hospital). Influenza virus (HK/Syd) was created using the reverse genetics technique described previously (28), and the rescued virus was propagated in 10-day-old embryonated chicken eggs for 72 h at 37°C.

HK/Syd virus created using reverse genetics had an egg 50% infective dose (ID_{50}) of $10^{7.50}$ and a Madin-Darby canine kidney (MDCK) 50% tissue culture infective dose ($TCID_{50}$) of $10^{7.375}$, measured using techniques described previously (74). With BALB/c mice (Jackson Laboratories, Bar Harbor, ME), the ID_{50} was 1 $TCID_{50}$, while the 50% lethal dose was $10^{5.5}$ $TCID_{50}$. Following administration of lethal doses of influenza (either 3 or 10 50% minimum lethal doses [MLD_{50}]), mice were monitored for signs of morbidity (weight loss) and mortality (survival). Mice that lost more than 33% of their initial body weight were euthanized and recorded as dying on the following day. Either 3 or 6 days after sublethal challenge (100 50% median infective doses [MID_{50}]), mice were euthanized, lungs were removed, rinsed in sterile phosphate-buffered saline (PBS), and homogenized, and $TCID_{50}$ values for dilutions of these homogenates were determined. ID_{50} and 50% lethal dose values were calculated using the method of Reed and Muench (56).

Vaccination. For DNA vaccination, 2.5 μg of either HA- or vector control DNA-coated gold particles (1 mg) was delivered at two nonoverlapping sites on the abdomen, using a Helios (Bio-Rad) gene gun, at 21-day intervals. Mice that were boosted with VRP received 1×10^6 infectious units expressing either HA or GFP (vector control) delivered subcutaneously in a 10-μl volume in the right rear footpad (46) at 28-day intervals. For an additional control group, mice were inoculated in the right rear footpad with PBS. When DNA was delivered without subsequent VRP administration, mice received three DNA inoculations (primary, secondary, and tertiary exposures). For groups that received DNA and VRP, mice were inoculated with two doses of DNA (primary and secondary) followed by two vaccinations with either VRP or PBS in the footpad (tertiary and quaternary). For all experiments, when DNA was administered, serum was collected 14 days after each vaccination, and when VRP or PBS was inoculated into the footpad, serum was collected at day 21 postinoculation. Serum was obtained from blood collected via the orbital plexus of isoflurane-anesthetized mice. Vaccination and serum collection time points were optimized through prior preparatory experiments conducted in our lab with these immunogens.

ELISA. Egg-grown HK/Syd virus was concentrated, purified over a sucrose gradient as described previously (42), and inactivated with 0.025% formalin treatment for 3 days at 4°C (68). HA content of this virus preparation was quantitated after resolution through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad) using bovine serum albumin (BSA) (Pierce, Rockford, IL) as a standard. Enzyme-linked immunosorbent assay (ELISA) plates (Becton Dickinson and Company, Franklin Lakes, NJ) were coated with 1 μg HA ml^{-1} in PBS. Plates were washed with PBS containing 0.05% (vol/vol) Tween 20 (Sigma) (PBST) and blocked with 10% fetal bovine serum (HyClone, Logan, UT) in PBST (FBS-PBST). Receptor-destroying-enzyme-treated (Accurate Chemical & Scientific Corp., Westbury, NY), heat-inactivated sera were diluted in FBS-PBST. Alkaline phosphatase-conjugated goat anti-mouse IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, or IgG (γ heavy chain specific) (Southern Biotechnology Associates, Birmingham, AL), diluted 1:1,000 in FBS-PBST, was used as the detection antibody, with *p*-nitrophenylphosphate as a substrate (Sigma). The optical density (OD) was read at 405 nm using a Multiskan Ascent plate reader (Labsystems, Helsinki, Finland) 1 h after substrate addition. Reciprocal serum antibody titers were calculated at 50% maximal binding on the titration curve. Samples with OD values of less than 0.5 at the starting dilution (1:50) were reported as having a titer of less than 50.

Microneutralization. HK/Syd (2×10^3 $TCID_{50}$ ml^{-1}) was incubated for 2 h with serum diluted in infection media as described previously (57). Virus-serum mixtures were then added to PBS-washed MDCK cell monolayers in 96-well Falcon plates (Becton Dickinson) and incubated for 2 h. Inoculum was removed, and cells were incubated with media containing 2 μg ml^{-1} TPCK-trypsin for 18 to 22 h. Influenza virus was detected using mouse monoclonal antibody specific for influenza A virus nucleoprotein (kindly provided by Robert G. Webster, St. Jude Children's Research Hospital) diluted 1:2,000 in 1% BSA (Invitrogen, Grand Island, NY) in PBS containing 0.1% (vol/vol) Tween 20 (Sigma), as described previously (57). Horseradish peroxidase-conjugated goat anti-mouse IgG (Fc specific) (Sigma), diluted 1:2,000 in 1% BSA in PBS containing 0.1% (vol/vol) Tween 20, was used as the detection antibody, with *o*-phenylenediamine dihydrochloride as the substrate (Sigma). Upon addition of 1 N H_2SO_4, the OD at 492 nm was measured using a Multiskan Ascent plate reader. The microneutralization titer is reported as the reciprocal of the final serum dilution that exhibits an OD value less than one-half of that measured in virus control wells.

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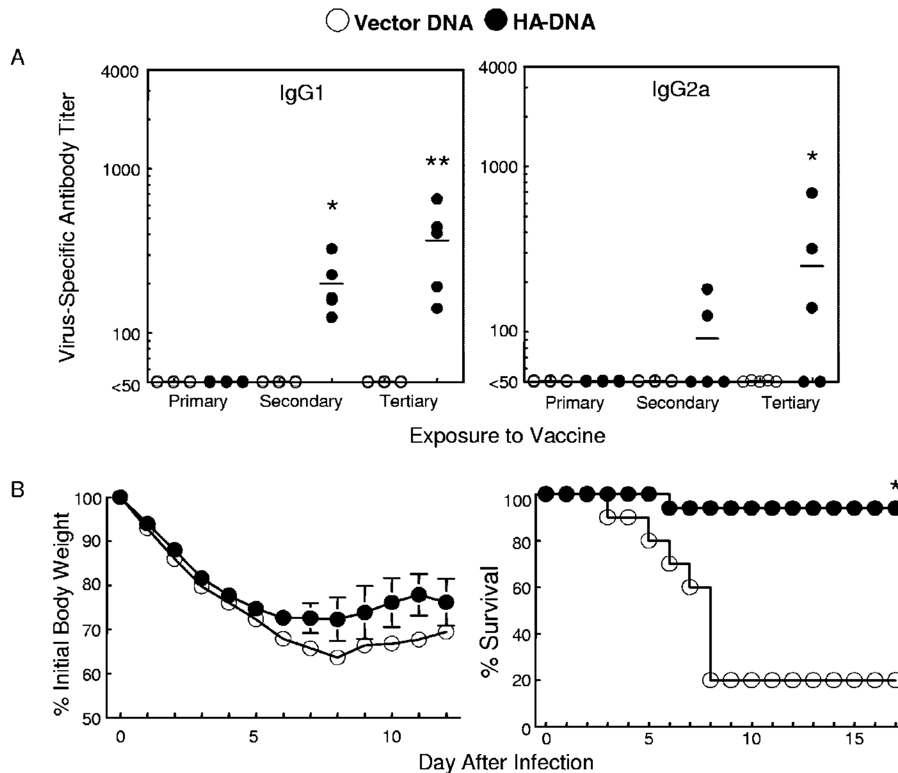

FIG. 1. Response of BALB/c mice to HA-DNA gene gun vaccination and influenza challenge. (A) IgG1 and IgG2a antibody levels were measured by ELISA 14 days after primary, secondary, and tertiary exposures to vector control or influenza HA delivered by DNA vaccine at 3-week intervals. Data are reported for six vector control-inoculated mice, with the exception of IgG2a readings at day 14 of the tertiary response (five mice). Data are shown for six HA-inoculated mice for the primary response and five HA-inoculated mice for the secondary and tertiary responses for both isotypes. An asterisk indicates a significant difference in titer compared to that for mice inoculated with vector DNA ($P < 0.01$ by ANOVA). A double asterisk indicates a significant difference in titer compared to those for all other groups ($P < 0.01$ by ANOVA). (B) Mice were challenged with 3 MLD_{50} HK/Syd on day 21 of the tertiary response to the vaccine. Mean levels of weight loss ± standard deviations are pictured for seven randomly selected mice per group. Survival data are reported for 10 vector control mice and 17 HA-vaccinated mice. An asterisk indicates a significant difference in survival compared to that of controls ($P < 0.01$ by log rank test of the Kaplan-Meier survival data).

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Flow cytometry. Mice were euthanized, the axillary artery was cut, and cells were recovered from the bronchoalveolar lavage (BAL) fluid by three 1-ml washes with Hanks balanced salt solution. The inferior vena cava was cut, and the liver was perfused via the hepatic portal vein. The gallbladder was excised during the liver's removal. Livers were minced and plunged through fine-mesh filters. The cell suspension was washed twice in cold PBS with 2% fetal bovine serum and 0.02% sodium azide and spun through 33.8% Percoll (Amersham Biosciences, Sweden) for 12 min at 693 × *g* to isolate lymphocytes as previously described (24). Isolated cells were incubated for 20 min on ice with phycoerythrin-conjugated anti-CD8 (BD Pharmingen) and an unconjugated rat anti-mouse CD16/32 antibody (BD Pharmingen) to block nonspecific Fc receptor-mediated binding. Cell populations were analyzed using a FACSCalibur system and CellQuest software (BD Biosciences, San Jose, CA).

Statistical analysis. Comparison of survival between groups of mice was done with a log rank chi-square test of the Kaplan-Meier survival data. Comparison between antibody titers was done using repeated-measures analysis of variance (ANOVA) by the Holm-Sidak method. SigmaStat for Windows (v3.11; SysStat Software, Inc.) was utilized for all statistical analyses. A P value of <0.05 was considered significant for these comparisons. Because of small group sizes, the analysis lacked the power to distinguish statistically between lung titer values.

RESULTS

HA-DNA vaccination induces protective immunity in mice. To test the efficacy of our vaccine using the plasmid DNA vector alone, we initially delivered plasmid DNA (pHW2000) expressing influenza HA (HA-DNA) from the A/Hong Kong/1/68 strain of influenza three times via gene gun. After the first exposure to the DNA, neither IgG1 nor IgG2a antibody levels were detectable by ELISA (Fig. 1A). After two doses of the DNA vaccine (secondary response), influenza-specific IgG1 antibodies had increased significantly ($P < 0.01$ by ANOVA) in the group inoculated with HA-DNA compared to levels in the group receiving vector DNA. After a third dose of the vaccine (tertiary response), a significant increase ($P < 0.01$ by ANOVA) in IgG1 antibody expression was seen in the HA-DNA-vaccinated group compared to levels in all other groups, including in comparison to the IgG1 titers seen after the secondary exposure to HA-DNA. Two of the five HA-DNA-vaccinated mice had measurable levels of IgG2a at day 14 after the secondary exposure to DNA. After the third exposure to HA-DNA, three of the five mice expressed IgG2a to a level that resulted in a significant difference ($P < 0.01$ by ANOVA) in IgG2a antibody titers upon comparison to those of vector DNA-inoculated mice. Upon challenge with a lethal dose (3 MLD_{50}) of influenza virus (Fig. 1B), all of the mice were infected, as evidenced by a drop in body weight. Mice that received three doses of HA-DNA were significantly protected ($P < 0.01$ by log rank test of the Kaplan-Meier survival data) from the challenge (94% survival), while mice that received vector DNA were not (20% survival).

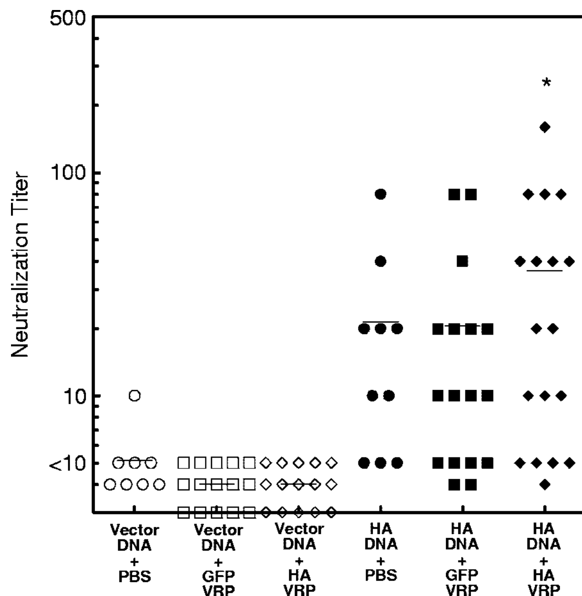

FIG. 2. Serum neutralizing antibody response of BALB/c mice to DNA prime followed by VRP boost. Microneutralization titers against HK/Syd (2,000 $TCID_{50}$ ml^{-1}) at day 21 of the quaternary response to the vaccine are reported. Data are reported for the following numbers of mice in the various groups: for vector DNA plus PBS, $n = 8$; for vector DNA plus GFP-VRP, $n = 15$; for vector DNA plus HA-VRP, $n = 15$; for HA-DNA plus PBS, $n = 10$; for HA-DNA plus GFP-VRP, $n = 17$; and for HA-DNA plus HA-VRP, $n = 18$. An asterisk indicates a significant difference in titer compared to those for groups inoculated with vector DNA ($P < 0.01$ by ANOVA).

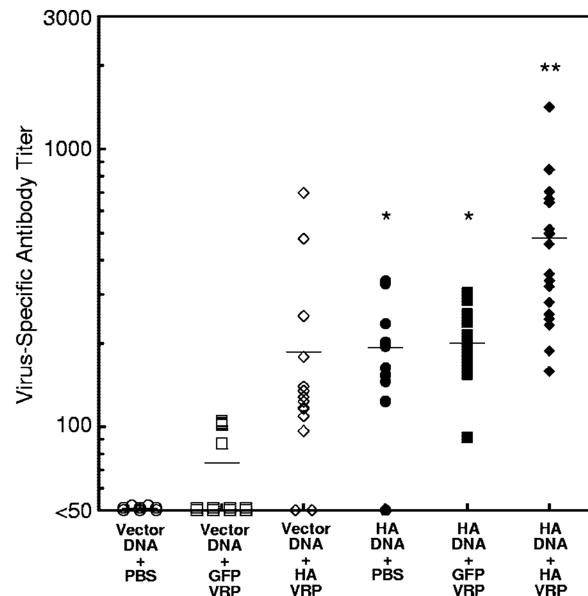

FIG. 3. Virus-specific serum antibody response of BALB/c mice to DNA prime followed by VRP boost. IgG antibody titers against HK/Syd (1 μg HA ml^{-1}) were measured by ELISA and are shown at day 21 of the quaternary response to the vaccine. Data are reported for the following numbers of mice in the various groups: for vector DNA plus PBS, $n = 8$; for vector DNA plus GFP-VRP, $n = 15$; for vector DNA plus HA-VRP, $n = 15$; for HA-DNA plus PBS, $n = 10$; for HA-DNA plus GFP-VRP, $n = 17$; and for HA-DNA plus HA-VRP, $n = 18$. An asterisk indicates a significant difference in titer for mice vaccinated with HA-DNA plus PBS and mice vaccinated with HA-DNA plus GFP-VRP compared to those for mice in groups inoculated with vector DNA plus PBS and vector DNA plus GFP-VRP ($P < 0.01$ by ANOVA). A double asterisk indicates a significant difference in titer for mice vaccinated with HA-DNA plus HA-VRP compared to those for all three groups of vector DNA-vaccinated mice ($P < 0.01$ by ANOVA).

Influenza virus-neutralizing antibodies are induced following administration of HA-DNA but not HA-VRP. Having established that this DNA vaccine regimen induced the expected result of consistent IgG1 expression and protected mice from a lethal challenge with influenza, we next used a VEE VRP vector expressing the same influenza HA in an attempt to understand the relevant role of vaccine-induced IgG2a responses within these animals. Our initial assessment of vaccine-induced immunity involved measuring neutralizing antibodies induced using these different HA-expressing vectors. Groups initially received two exposures to either vector or HA-DNA, followed by two inoculations with VRP expressing either HA (HA-VRP) or GFP (GFP-VRP) as a control. As a further control for the potential effects of the VRP alone, additional groups were boosted with PBS instead of VRP. The serum samples analyzed here were obtained 21 days after the quaternary exposure to the immunogen (two exposures to DNA followed by two exposures to either VRP or PBS). Mice primed with vector DNA did not produce specific neutralizing antibodies as measured by microneutralization (Fig. 2), even after two subsequent inoculations with HA-VRP. Groups primed with HA-DNA, however, expressed detectable levels of neutralizing antibody in most animals. The group that received HA-DNA plus HA-VRP was the only group that exhibited a statistically significant increase in neutralizing antibody titers ($P < 0.01$ compared to all three groups inoculated with vector DNA). While boosting HA-DNA-vaccinated animals with HA-VRP modestly enhanced this response, it is clear that induction of neutralizing antibodies in this model was dependent on the HA-DNA prime. Analysis of sera using the traditional HI assay showed a similar pattern of neutralizing antibody induction (data not shown).

Expression of HA-specific IgG antibody isotypes depends on the vector used for antigen delivery. We next employed an ELISA technique to measure serum antibody expression 21 days after the quaternary exposure to the immunogen (two exposures to DNA followed by two inoculations with either VRP or PBS). This analysis revealed that IgG antibodies were induced to similar levels in animals vaccinated with either HA-DNA or HA-VRP (Fig. 3). This is of interest because influenza virus-specific antibodies present within the sera of mice vaccinated with HA-VRP were not detected by microneutralization (Fig. 2) but were detectable by ELISA. The groups inoculated with HA-DNA followed by a boost with either PBS or GFP-VRP expressed influenza virus-specific IgG antibody levels that were significantly higher ($P < 0.01$ by ANOVA) than those detected in groups that received vector DNA followed by either PBS or GFP-VRP. As expected, combined vaccination with HA-DNA and HA-VRP yielded maximal IgG expression, resulting in a significant increase in IgG antibody titers ($P < 0.01$ by ANOVA) compared to those of mice in all three groups that received vector DNA. Based upon

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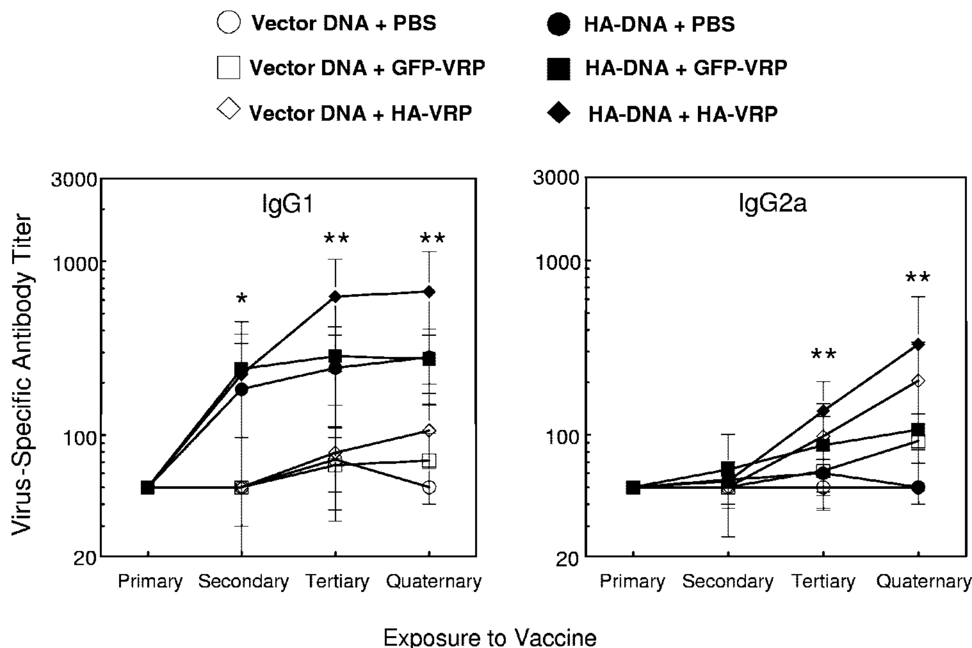

FIG. 4. Virus-specific serum antibody response of BALB/c mice to DNA prime followed by VRP boost. IgG1 and IgG2a antibody isotype titers against HK/Syd (1 μg HA ml^{-1}) were measured by ELISA and are shown after primary (day 14), secondary (day 14), tertiary (day 21), and quaternary (day 21) responses to the vaccine. For all days and isotypes measured, the following numbers of mice were included in the various groups: for vector DNA plus PBS, $n = 8$; for vector DNA plus GFP-VRP, $n = 15$; for vector DNA plus HA-VRP, $n = 15$; for HA-DNA plus PBS, $n = 10$; for HA-DNA plus GFP-VRP, $n = 17$; and for HA-DNA plus HA-VRP, $n = 18$. An asterisk indicates a significant difference in titer for HA-DNA-vaccinated mice compared to that for vector DNA-vaccinated mice ($P < 0.01$ by ANOVA). A double asterisk indicates a significant difference in titer for mice vaccinated with HA-DNA plus HA-VRP compared to those for all other groups ($P < 0.01$ by ANOVA).

this finding, we investigated the contributions of the different IgG isotypes to this total IgG response.

The IgG isotype response was dominated by IgG1 and IgG2a antibodies, which are reported for the six groups after each individual administration of immunogen (Fig. 4). IgG1 antibodies were not detected 14 days after a primary inoculation with HA-DNA. A second vaccination with HA-DNA induced a significant ($P < 0.01$ by ANOVA) and consistent IgG1 antibody response by all three groups inoculated with HA-DNA, as shown at 14 days after this secondary inoculation. The IgG1 response to HA-DNA in this experiment was similar to that reported in the first study (Fig. 1). Inoculation of mice with HA-VRP alone (tertiary and quaternary inoculations) did not yield significant IgG1 expression, but the group inoculated twice with HA-DNA and boosted with HA-VRP had significantly higher IgG1 titers ($P < 0.01$ by ANOVA) than all groups at 21 days after both tertiary and quaternary exposures to the HA antigen.

As with IgG1 expression, and consistent with what we saw in the initial experiment (Fig. 1), IgG2a levels were below the detectable limit 14 days after primary exposure to the antigen via the gene gun (Fig. 4). In fact, delivery of HA-DNA alone did not significantly induce an IgG2a response to this antigen even at day 14 of the secondary response to HA-DNA. However, 21 days after exposure to HA-VRP (tertiary response), IgG2a levels were increased, regardless of the DNA prime. Influenza-specific IgG2a expression by HA-VRP-vaccinated mice reached a significant difference ($P < 0.01$ by ANOVA) for the group that received two inoculations with HA-DNA, compared to all other groups, at 21 days after both the tertiary and the quaternary exposure to the antigen. Thus, HA-DNA vaccination induced primarily IgG1 antibodies, while HA-VRP inoculation consistently stimulated IgG2a antibodies. When HA was administered by both DNA and VRP vectors, the highest titers for influenza-specific IgG1 and IgG2a antibodies were achieved. On day 21 of the quaternary response to influenza HA, IgA, IgG2b, and IgG3 antibody titers were measured. Influenza-specific serum antibodies of the IgA and IgG3 isotypes did not achieve a measurable titer in this study, and only two mice in the vector DNA-plus-HA-VRP group (titer values of 113 and 119) and three mice in the HA-DNA-plus-HA-VRP group (titer values of 128, 153, and 290) expressed measurable levels of IgG2b antibodies. Influenza-specific antibodies of the IgM isotype were detected but levels did not differ between the groups (data not shown).

In vivo neutralization and protective immunity against influenza are optimized when both HA-DNA and HA-VRP are delivered. To assess the role of vaccine-induced antibodies in protection against influenza, mice were first challenged with a sublethal dose (100 MID_{50}) of influenza virus. At both 3 and 6 days after challenge, mice that were primed with HA-DNA exhibited lower viral titers than those that were primed with vector DNA (Fig. 5). The most effective in vivo neutralization was seen with mice that received a prime with HA-DNA and a boost with HA-VRP. On day 6 after viral challenge, two mice in the group inoculated with HA-DNA plus HA-VRP had undetectable levels of virus in their lungs, and the third mouse in that group had a lung viral titer of 2×10^3 $TCID_{50}$ ml^{-1}.

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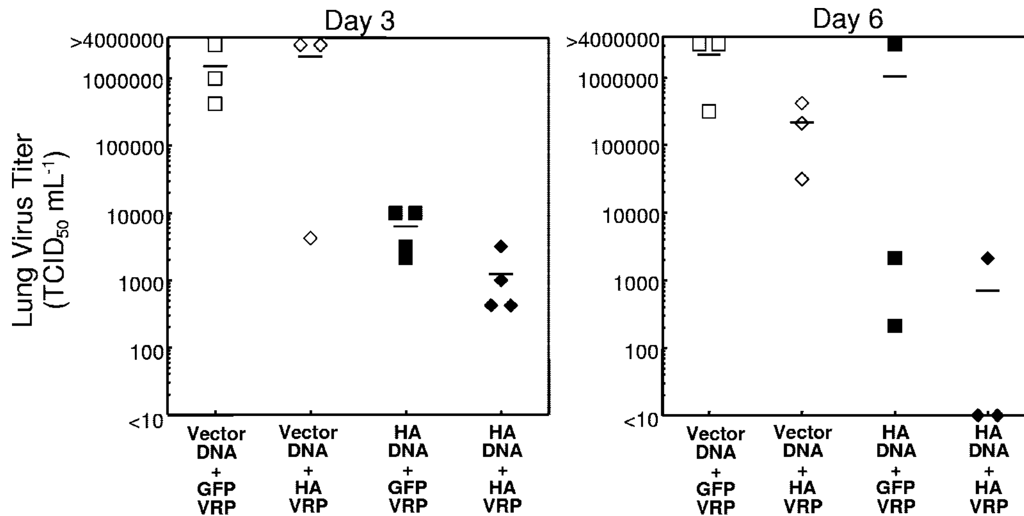

FIG. 5. Lung viral titers of BALB/c mice after HK/Syd challenge. After the quaternary exposure to the vaccine, mice were infected with 100 MID_{50} HK/Syd, and lung viral titers at days 3 and 6 after inoculation were determined. Data are reported for the following numbers of mice in the various groups: for vector DNA plus GFP-VRP, $n = 3$; for vector DNA plus HA-VRP, $n = 3$; for HA-DNA plus GFP-VRP, $n = 4$; and for HA-DNA plus HA-VRP, $n = 4$. On day 6 after viral challenge, all four groups contained three mice each.

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Next, we utilized an influenza virus challenge study to assess the impact of vaccine-induced IgG1 and IgG2a expression on survival. In our previous experiment, HA-DNA was effective at preventing death in 94% of BALB/c mice challenged with 3 MLD_{50} of influenza virus. To test the contributions of HA-DNA and HA-VRP delivery to protection against lethal influenza challenge, we exposed these mice to a much higher dose (10 MLD_{50}) of HK68 HA-expressing influenza virus as a more stringent measure of immunity (Fig. 6). After exposure to this high viral load, all mice were infected and lost weight (Fig. 6, top). Mice in the vector DNA-vaccinated groups that were boosted with either PBS or GFP-VRP were not protected from the challenge (0% survival for both groups) (Fig. 6, bottom). Mice that were vaccinated with HA-DNA alone showed survival rates of 30% (HA-DNA plus PBS) ($P < 0.05$ compared to groups vaccinated with vector DNA plus PBS and vector DNA plus GFP-VRP by log rank test) and 20% (HA-DNA plus GFP-VRP) by use of this higher challenge dose. Interestingly, the two groups that received HA-VRP showed similar, significant levels of survival ($P < 0.05$ compared to groups vaccinated with vector DNA plus PBS and vector DNA plus GFP-VRP by log rank test), regardless of whether they were initially inoculated with vector DNA (44% survival) or HA-DNA (45% survival). Challenge with 10 MLD_{50} influenza virus allowed for an assessment of the increased immunity observed with HA-VRP inoculation that could not have been determined using 3 MLD_{50}.

Vaccination against influenza HA by use of this regimen does not enhance CD8 T-cell immunity. $CD8^+$ T cells contribute significantly to protection against influenza, primarily through presentation of peptides from internal viral proteins (NP or PA) (66). We used a vaccine regimen directed specifically against the HA component of influenza virus to avoid confounding immunity from T cells. However, in the absence of NP and PA proteins, it is possible that T-cell immunity may be stimulated through a compensatory mechanism (7). Thus,

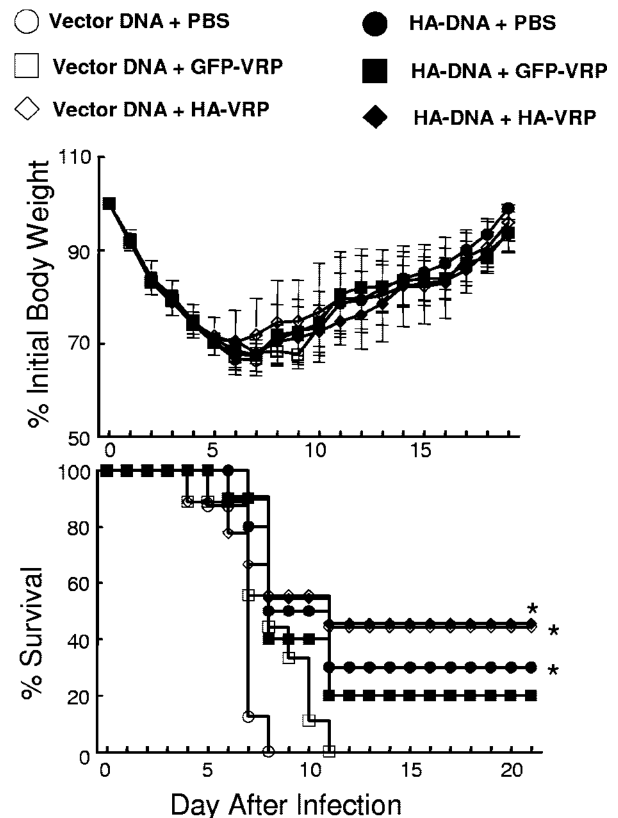

FIG. 6. Survival of BALB/c mice after HK/Syd challenge. After the quaternary exposure to the vaccine, mice were infected with 10 MLD_{50} HK/Syd and monitored for morbidity (percent weight loss) (top) and mortality (percent survival) (bottom). Data are reported for the following numbers of mice in the various groups: for vector DNA plus PBS, $n = 8$; for vector DNA plus GFP-VRP, $n = 9$; for vector DNA plus HA-VRP, $n = 9$; for HA-DNA plus PBS, $n = 10$; for HA-DNA plus GFP-VRP, $n = 10$; and for HA-DNA plus HA-VRP, $n = 11$. An asterisk indicates a significant difference in results compared to those for vector DNA-plus-PBS and vector DNA-plus-GFP-VRP groups ($P < 0.05$ by log rank test of the Kaplan-Meier survival data).

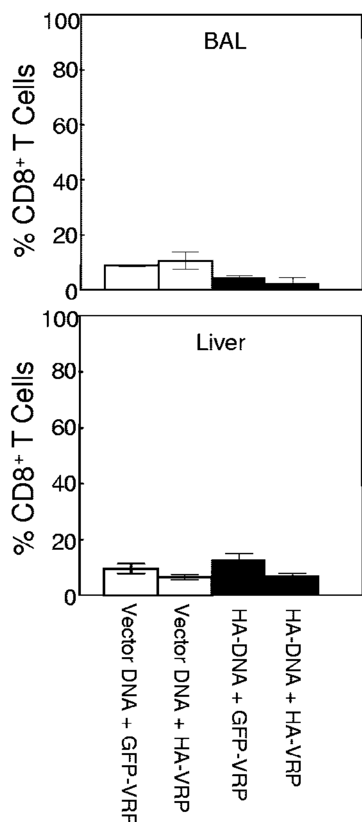

FIG. 7. Effector $CD8^+$ T-cell populations in the BAL fluid and livers of BALB/c mice after HK/Syd challenge. After the quaternary response to the vaccine, mice were infected with 100 MID_{50} HK/Syd. BAL fluid and liver cells were isolated and analyzed for $CD8^+$ T-cell populations on day 6 after challenge. All groups consisted of three mice.

we tested the ability of this vaccine regimen to enrich CD8 T cells within the cell populations of the BAL fluid and livers of these mice. The BAL fluid was measured for the presence of CD8 T cells within the local infection environment, whereas the CD8 populations within the liver were analyzed based on the role of this organ as a reservoir for memory effector T cells (12, 32). On day 6 after sublethal (100 MID_{50}) viral challenge, there were suboptimal levels of effector T cells in both the lungs and the livers of these mice (Fig. 7). The low-level expression of these cells indicates that this vaccine regimen is not effective at enriching T cells, although a role for T cells in the resolution of influenza virus past day 6 cannot be excluded.

DISCUSSION

In this study, we attempted to parse the contribution of vaccine-stimulated IgG antibody subclasses to protection against influenza infection. We utilized two routes of vaccination known to differentially stimulate immune responses and focused only on the HA as an antigen so our results would not be confounded by a contribution of cellular immunity. Upon HA-DNA vaccination via gene gun, antibody stimulation could be measured using a traditional microneutralization assay. These antibodies could also be detected by ELISA and were shown to be of the IgG1 isotype. Induction of these antibodies plays a role in protection against influenza, as HA-DNA-vaccinated mice exhibited reduced lung viral titers upon challenge with a low dose (100 MID_{50}) of influenza virus and protection against challenge with a modest dose (3 MLD_{50}) of influenza virus. However, the groups that received HA-DNA exhibited suboptimal survival upon challenge with a significantly higher dose of influenza virus (10 MLD_{50}), even in the presence of high IgG1 expression and viral neutralization.

When mice were exposed to both HA-DNA and HA-VRP, antibody levels reached their highest as measured by both microneutralization and ELISA. These elevated antibody levels correlated with protection against a more stringent challenge dose of influenza virus (10 MLD_{50}). While it was expected that mice inoculated with both HA-DNA and HA-VRP would achieve optimal survival in this experiment, it was surprising that the mice that received HA-VRP alone, a group which had low levels of viral neutralization and IgG1 expression, achieved equivalent survival rates. Analysis of antibody expression by ELISA revealed IgG antibody levels that were comparable to those seen with HA-DNA-vaccinated mice, but the major IgG isotype expressed within these animals was IgG2a, not IgG1. The inability of the observed protection to be explained by vaccine-induced memory effector T cells within these mice implicates influenza-specific antibodies in both the neutralization and the clearance of this virus. It has been reported that antigen delivery using a VRP vehicle can result in enhanced antigen-specific serum IgA expression (76), but we were unable to detect this isotype after HA-DNA and HA-VRP vaccination. The low levels of IgG2b and IgG3 observed after gene gun and VRP vaccination are similar to what has been reported previously (16, 23). Taken together, the analyses of different serum antibody isotypes after vaccination with this regimen strengthen the argument that vaccine-induced IgG1 and IgG2a antibodies contribute to the protective responses observed.

Of specific interest is the observation with this model that mice were protected from influenza infection even when serum neutralizing activity was not detectable by standard assays. Protective immunity in the absence of strong neutralizing antibody titers has been observed previously with influenza vaccine studies (4, 38, 39, 44). Here we show that antibody levels are measured more efficiently by an ELISA method, allowing for the quantitation of vaccine-induced antibodies that remain undetected when traditional neutralization assays are employed. Furthermore, by using the ELISA technique, not only were we able to detect vaccine-induced antibodies within these mice but we were able to analyze the individual isotypes stimulated and the differential contributions of these isotypes to immunity against influenza. Specifically, we describe contributions of both neutralizing and host effector response-activating antibody isotypes that together result in strong immunity against influenza. Since the induction of these isotypes could be detected and differentiated only by ELISA, our findings argue for the incorporation of this technique in studies designed to assess correlates of immunity after influenza vaccination, in particular, when neutralizing antibody levels are either low or undetectable.

In support of the hypothesis that antibody isotypes play different roles in antiviral immunity, experiments conducted with monoclonal antibodies against Ebola envelope antigens

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showed that IgG2a antibodies were more effective at clearing infections than antibodies of the IgG1 isotype, even when each was specific for the same epitope (71). Protection against Ebola was achieved with lower concentrations of IgG2a antibodies, making it possible that, in addition to neutralizing viral particles, antibodies of the IgG2a isotype can stimulate host effector mechanisms that aid in the clearance of viral infections (71). Host effector mechanisms that are stimulated by IgG2a antibodies include complement fixation (51) and Fc receptor activation (52, 53). Since high levels of antibody (approximately 70 antibody molecules) are required to neutralize a single influenza virion (65), the presence of antibodies that are more active inducers of host clearance mechanisms and that are effective at lower concentrations (71) may aid in the resolution of the infection when levels of neutralizing antibodies begin to wane.

A contribution of the complement system in stimulating anti-influenza T-cell immunity has been described previously (40), but its role in the clearance of influenza virus is still unclear (17, 50). Alternatively, a role for Fc receptor-mediated clearance of influenza from vaccinated hosts has been described previously (31) through the use of mice deficient in expression of activating Fc receptors that signal through the common γ chain (64), a group that now includes the recently described FcγRIV (52). Furthermore, antibodies of the IgG2a isotype exhibit the best Fc receptor activatory-to-inhibitory ratio of all IgG isotypes (53), making it the isotype predicted to have the greatest ability to activate Fc receptor-mediated host effector responses.

Vaccination against the HA component of influenza by use of either HA-DNA or HA-VRP does not appear to induce strong T-cell-mediated immunity, as enriched memory populations of CD8 T cells were not detected in the local (BAL fluid) or systemic (liver) populations after challenge of these mice with influenza virus. While the absence of these cells implies that antibodies are the main contributors to the protection seen within this model, the important role of T cells in the eventual resolution of the virus infection cannot be excluded. T cells are important in viral clearance due to their ability to lyse infected cells (45, 73) and stimulate cytokine expression, which activates cells, like macrophages, that are involved in the clearance of pathogens (18, 37, 49). Since the vaccine itself does not specifically enrich T cells but the antibodies present can significantly reduce the amount of virus to which the host is exposed, it is possible that the stimulation of T cells is delayed past day 6 after challenge and that these cells do not reach their maximal capacity until later in the infection process, during the resolution phase. For this study, we deliberately used a vaccine strategy that would not be predicted to induce T-cell immunity. A vaccine of similar design, utilizing a protein that stimulates cellular immunity, such as NP, would be predicted to be more efficacious in terms of improved overall survival.

The vaccine design described in this study stimulates IgG1 and IgG2a antibodies in a way that makes it possible to study the contribution of these isotypes both individually and in concert. This study reveals that even in the absence of neutralizing antibodies a vaccinated host may have protective immunity against influenza. These findings have implications in the development of vaccines for pandemic preparedness, as subunit and recombinant vaccines against H5 antigens have shown low immunogenicity in humans, as measured by HI titers, microneutralization, single radial hemolysis, and ELISA for total IgG (61, 62). In order to increase the immunogenicity of a vaccine against the H5N3 virus A/Duck/Singapore/97, the adjuvant MF59, which has been licensed for use in Europe (59), was included in the vaccine (62). MF59 increases influenza-specific antibody responses in humans (14, 47), with evidence of modest IgG2a antibody induction after vaccination of BALB/c mice (26).

Additionally, H5 HA-expressing DNA delivered via the gene gun to either mice or chickens induces protection against homologous and heterologous virus challenge, even in the absence of high HI titers (38, 39). Our results suggest that reduced expression of neutralizing antibodies in response to H5 antigens does not necessarily indicate an ineffective vaccine and that characterization of the immune response induced using more-sensitive, isotype-specific assays may better predict vaccine efficacy. Pursuit of a vaccine strategy to induce complement-fixing and Fc receptor-activating antibodies in addition to neutralizing antibodies might improve vaccine efficacy.

DNA vaccines provide an advantage over conventional influenza vaccines in their ability to be mass-produced safely in a short period of time and their ability to be quickly altered to deal with the rapidly changing antigens of influenza viruses that circulate within the human population (19). Unfortunately, the applicability of DNA vaccines to humans has been limited by low efficacy (15, 19). One proposed way to increase the immunogenicity of DNA vaccines is to deliver them in a prime-boost manner using viral vectors as the boosting vehicle (6, 15, 22). This vaccination regimen has been shown by other groups to be beneficial over repeated exposures to the antigen delivered by either the DNA or the viral vector alone (8, 43). Our data support this concept and demonstrate that the increased immunity seen after delivery of both the DNA and the viral vector is not simply due to increases in total antibody responses. Instead, the prime-boost regimens may be more effective due to their ability to stimulate both neutralizing and host effector response-activating antibodies, thus better equipping the host to deal with infection.

ACKNOWLEDGMENTS

We thank Robert G. Webster for providing us with the mouse monoclonal antibodies against the nucleoprotein of influenza A virus. We also thank Erich Hoffmann for providing us with plasmids for the creation of influenza viruses by reverse genetics and Alphavax, Inc., for providing constructs used for VEE VRP creation.

We have no conflicting financial interests.

This work was supported by the American Lebanese Syrian Associated Charities (ALSAC).

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