

# A DNA Vaccine Prime Followed by a Liposome-Encapsulated Protein Boost Confers Enhanced Mucosal Immune Responses and Protection<sup>1</sup>

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A variety of DNA vaccine prime and recombinant viral boost immunization strategies have been developed to enhance immune responses in humans, but inherent limitations to these strategies exist. There is still an overwhelming need to develop safe and effective approaches that raise broad humoral and T cell-mediated immune responses systemically and on mucosal surfaces. We have developed a novel mucosal immunization regimen that precludes the use of viral vectors yet induces potent T cell responses. Using hepatitis B surface Ag (HBsAg), we observed that vaccination of BALB/c mice with an i.m. HBsAg-DNA vaccine prime followed by an intranasal boost with HBsAg protein encapsulated in biologically inert liposomes enhanced humoral and T cell immune responses, particularly on mucosal surfaces. Intranasal live virus challenge with a recombinant vaccinia virus expressing HBsAg revealed a correlation between T cell immune responses and protection of immunized mice. A shortened immunization protocol was developed that was successful in both adult and neonatal mice. These results support the conclusion that this new approach is capable of generating a Th-type-1-biased, broad spectrum immune response, specifically at mucosal surfaces. The success of this design may provide a safe and effective vaccination alternative for human use. *The Journal of Immunology*, 2008, 180: 6159–6167.

The development of various vaccines has significantly improved the quality of human life resulting in drastic decreases in morbidity and mortality (1, 2). Although the strategies used to develop these vaccines have proven greatly effective, they have not been sufficient to combat pathogens that demonstrate unique pathogenesis or which require multiple components of the immune system for their elimination (3–6). The vast majority of data indicate that new, innovative vaccine strategies are required to combat the array of pathogens for which preventative and therapeutic avenues are lacking. Development of robust, practical vaccines for sexually transmitted diseases (e.g., HIV, HSV, *Chlamydia trachomatis*), new and re-emerging pathogens (e.g., avian influenza virus, antibiotic-resistant tuberculosis), and bioterrorism threats (e.g., anthrax, smallpox) remains a challenge (6–9).

Currently, only limited numbers of vaccines are safe and effective in raising protective immunity at mucosal surfaces, the most common entry portal of pathogens (10). Vaccines are also limited in their ability to raise robust cell-mediated immune responses that are usually required to eliminate intracellular infections (11, 12). One new approach is a heterologous vaccine regimen involving a

DNA prime followed by an attenuated live or nonreplicating virus boost. This prime and boost vaccination strategy has been used to overcome the disappointingly ineffective induction of immune responses displayed by DNA immunization alone in nonhuman primates and humans (4, 13–18). The advantages of this approach include a synergistic effect on the induction of immune responses and the generation of a robust T cell-mediated immune response (3, 4, 12–16, 19–25).

Limitations to the use of viral vectors are apparent. Viral vectors are usually not administered intranasally (i.n.)<sup>3</sup> to raise mucosal immune responses due to the concern that they could result in localization to the CNS (26). Instead, generation of mucosal immune responses usually requires the inclusion of potentially toxic adjuvants delivered with a protein component of the vaccine (27–29). Development of immune responses to the viral vector raises concerns over widespread and repeated use of viral vectors (4, 30). Furthermore, recent results using a nonreplicating adenoviral-based HIV vaccine led to halting of two clinical trials (31–36). Not only had the vaccine failed to prevent infection or reduce viral load, but further analysis suggested that the vaccine might have actually increased susceptibility to infection (32, 35). The failure of the vaccine raises the question as to whether vaccines that only induce cell-mediated responses will prove effective without increasing risk to the vaccinated individual (32).

To address these issues, we have developed a new heterologous immunization strategy that relies on a DNA vaccine prime followed by a liposome-encapsulated protein boost. Both of these components are safe, easily manufactured, and have demonstrated capability in the development of vaccines (37–39).

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<sup>3</sup>Abbreviations used in this paper: i.n., intranasal; HBsAg, hepatitis B virus surface Ag; vHBs4, a recombinant vaccinia virus expressing HBsAg.

Using the model Ag, hepatitis B virus surface Ag (HBsAg), that is well-characterized and commercially available, we demonstrated that this protocol was highly effective in inducing systemic B and T cell-mediated responses and robust mucosal immune responses when the boost was delivered intranasally. The advantage of this protocol was that it not only induced a broad spectrum of systemic and mucosal immune responses, but also induced protective immune responses that were associated with elevated levels of Ag-specific CD8<sup>+</sup> T cells in lungs of vaccinated mice. We also demonstrated a marked ability of the protocol to induce responses in HBsAg-nonresponder and neonatal mice, and the ability to induce potent responses after delivery of the components in a shortened time span. This newly developed immunization protocol demonstrates all of the hallmarks of a successful vaccination regimen and may be an ideal candidate for the formulation of new preventative and therapeutic vaccines.

## Materials and Methods

### Immunogens

HBsAg vaccine (HBsAg adw subtype, Engerix-B; GSK Biologicals), HBsAg-DNA vaccine pRc/CMV-HBs(S) ayw subtype (40) (Alvecon), purified recombinant HBsAg protein (ayw subtype; Advanced Immunotechnical), and the L<sup>d</sup>-restricted peptide of HBsAg (amino acids 28–39, i.e., S28–39, IPQSLDSWWTSL-OH; Biopeptide) were purchased from commercial sources.

Large multilamellar liposomes were prepared at the following lipid concentrations: Phosphatidylcholine/cholesterol/dicetyl phosphate 7/3/0.5 mole %. Dicetyl phosphate was dissolved in chloroform/5% ethanol and sonicated before the addition of phosphatidylcholine and cholesterol. Lipids were dried in a Labconco rotary evaporator for 1 h and traces of chloroform were removed by freeze-drying with a Freezone 4.5 Freeze Dry System overnight. The lipid film was hydrated with Ag at a concentration of 125–300 µg/ml in 10 mM HEPES-buffer, 150 mM NaCl (pH 7.4) (HBS), and filtered through a 0.2-µm nylon filter. The mixture was vortexed thoroughly and allowed to sit for 1 h and then vortexed again to ensure the formation of multilamellar vesicles. The resultant liposomes were then subjected to three freeze-thaw cycles (1 cycle = freezing for 1 h and thawing for 1 h at room temperature). The size of the liposomes was measured with a N4 MD Submicron Particle Size Analyzer (Coulter Electronics). The ζ potential was measured using the ζ-Puls ζ-potential analyzer (Brookhaven Instruments) in 5 mM HEPES buffer, 1.0 mM NaCl (pH 7.4).

### Mice and immunizations

Pathogen-free, barrier-maintained female BALB/c mice (H-2<sup>d</sup>) and female C57BL/6 (H-2<sup>b</sup>) mice 6–7 wk of age were obtained from Harlan Breeders. All mice were maintained under specific pathogen-free conditions. After 1 wk of acclimation, mice were anesthetized with a ketamine/xylazine mixture, and administered the appropriate amount of DNA vaccine i.m. or HBsAg liposomes or empty liposomes (10–50 µl total dose/mouse/time point) in both nostrils. All animals were housed in sterile microisolator cages and had no evidence of spontaneous infection. Animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committees of both the University of Massachusetts Medical School and Biomedical Research Models, and in accordance with the "Guide for the Care and Use of Laboratory Animals."

### Sample collection

Blood samples were collected by snipping the end of the tail and collecting blood by capillary action into clot-activating Microvette microtubes (Sarstedt). Vaginal washes were performed by instilling 2 × 40 µl of sterile saline intravaginally to anesthetized mice, flushing the cavity, and collecting the wash with a pipette tip. This procedure was repeated twice. Vaginal washes were preserved with a 20% solution (v:v) of 2.2% protease inhibitor mixture (Sigma-Aldrich) in PBS.

### Measurement of Ab responses

HBsAg-specific Ab responses were measured using ELISA. Assays included measurements for total Ig, IgG, IgG1, and IgG2a (three layers consisting of HBsAg, sample, and anti-Ig-HRP-detecting Abs), and mucosal IgG and IgA (five layers consisting of anti-mouse IgG or IgA capture, sample, HBsAg, rabbit anti-HBsAg, and goat anti-rabbit Ig-HRP detecting Ab). Abs and mouse Ig isotype standards were purchased from BD Bio-

sciences, Novus Biologicals, and Southern Biotechnology Associates. High-binding ELISA plates were purchased from Costar. Assays were developed and optimized based on the conditions recommended by BD Biosciences using 10 µg/ml (50 µl/well) of rHBsAg in binding buffer to coat the plates for direct assays, and 2–5 µg/ml (100 µl/well) for the Ab-sandwich layers. Checkerboard titrations were performed to optimize the signal:noise ratio for each assay. ELISAs were developed using Sure-Blue TMB microwell peroxidase substrate (KPL) and 2 N sulfuric acid to stop the reaction. Plates were read using a Multiskan Ascent plate reader (Thermo Electron). Results are expressed either as OD at 450 nm (total IgG and IgA assays) or as micrograms per milliliter of IgG (quantitative assays for IgG, IgG1, and IgG2a).

### Virus and viral challenge

A recombinant vaccinia virus expressing HBsAg (vHBs4) was provided by Dr. B. Moss (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (41). Virus was grown in NIH L929 cells and titrated in Vero cells. Anesthetized mice received 2–5 × 10<sup>5</sup> PFU of vHBs4 i.n. in a volume of 50 µl.

### Virus titration

The number of PFU of vaccinia virus was determined by plaque assay using 10-fold serial dilutions of a 10% homogenate of lungs taken from individual mice. Results were expressed as the geometric mean titers, i.e., the arithmetic averages of the logs for five separate animals titrated for virus individually plus or minus the SEM. Titers reported are log<sub>10</sub> PFU (42).

### Intracellular cytokine staining assay

Intracellular staining for IFN-γ was performed on cells using commercial reagents and protocols (Cytotfix/Cytoperm kit including GolgiPlug; BD Pharmingen). Briefly, mononuclear cells were first stimulated *in vitro* for 5 h at 37°C in complete medium with the L<sup>d</sup>-restricted HBsAg T cell epitope S28-39 or with no stimulus in the presence of GolgiPlug. Cells were then harvested and incubated with Fc block (anti-mouse CD16/CD32 unlabeled) followed by anti-CD8α-PerCP and anti-CD4-FITC. Cells were then permeabilized with Cytotfix/Cytoperm and incubated with anti-mouse IFN-γ-PE in the presence of PermWash buffer. Appropriate isotype control Abs were also included to determine background staining levels. All staining procedures were performed in 96-well U-bottom plates using standard immunofluorescence staining protocols. Freshly stained samples were analyzed on a BD Biosciences LSR II using FlowJo software. At least 200,000 events were typically analyzed.

### ELISPOT assays

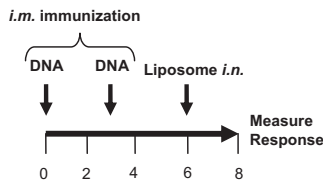
ELISPOT assays for IFN-γ and IL-4 release were performed using Ab pairs and reagents from BD Pharmingen, and 96-well ELISPOT plates from Millipore. Samples (1 × 10<sup>6</sup> and 5 × 10<sup>5</sup> cells/well) were incubated with either rHBsAg protein (starting at a 10 mM concentration), Con A (2.5 µg/ml), or anti-mouse CD3α (10 µg/ml) for positive controls or were left untreated or incubated with irrelevant Ag (negative controls) for 1.5 days in a 37°C incubator (5% CO<sub>2</sub>). All samples were tested in duplicate. ELISPOT assays were developed according to manufacturer's directions. Plates were scanned and digitally quantified to determine the number of spots per well and size distribution of the spots (CTL).

### Plaque reduction neutralization assay

Two serum pools were used for these assays: a pool obtained from mice immunized with the heterologous immunization protocol, and hyperimmune anti-vaccinia virus-specific reference serum (positive control). Serial 2-fold dilutions of serum were combined with 300 PFU of either recombinant vHBs4 or wild-type vaccinia virus (WR strain) as a control. After incubation for 1 h at 37°C, the virus was added to 6-well plates containing 80% confluent Vero cells. Cells were incubated for 2 days at 37°C, overlaid with 1% Seakem agarose containing normal cell growth medium and neutral red stain, incubated for an additional day and analyzed for the number of plaques per well to determine the plaque inhibition efficiency of sample sera.

### Statistical analysis

Student's *t* tests were performed to compare statistical differences between groups. Probability (*p*) values <0.05 were considered to be significant, and *p* values <0.01 were considered to be highly significant.



**FIGURE 1.** Prime boost schedule for the heterologous vaccine protocol. Naive mice are immunized with two doses of HBsAg-DNA delivered i.m. (10–100  $\mu\text{g}/\text{dose}$ ) followed by one dose of HBsAg liposomes delivered i.n. (3 or 15  $\mu\text{g}/\text{dose}$ ). DNA immunizations may be delivered in 3 wk (long protocol) or 3 days (short protocol). Liposomes are delivered 2–4 wk after the last DNA immunization. Maximal immune responses are detected beginning 2 wk following the final immunization.

## Results

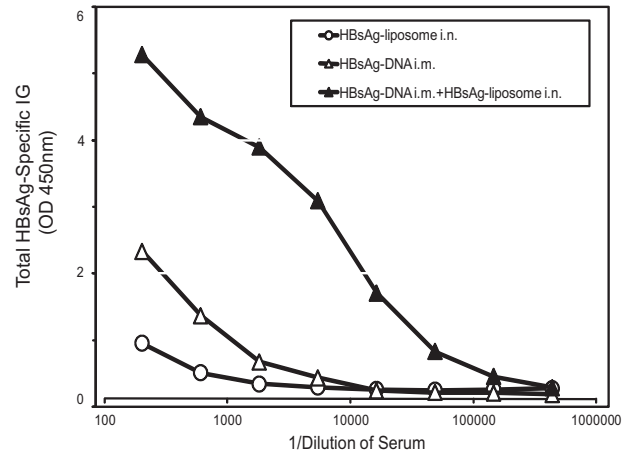
### Development of a DNA- and liposome-based heterologous immunization protocol

We first performed a series of experiments to optimize the conditions for immunization. Dose, route of administration (i.m. vs i.n.), sequence of delivery (DNA and liposome components), the composition, electric charge, and the size (0.2–4  $\mu\text{m}$ ) of the liposomes were all evaluated (data not shown). A negatively charged liposome of 1- to 4- $\mu\text{m}$  size was determined to be the chosen vehicle for encapsulation of HBsAg to raise optimal immune responses. Based on these data, we devised the heterologous immunization protocol shown in Fig. 1. Critical features of this protocol include i.m. injection of DNA, i.n. administration of liposome-encapsulated Ag, and order of administration of the components. Two rounds of DNA immunization were chosen to ensure successful inoculation into the quadriceps. The time interval between immunizations is described below. Measurement of immune responses occurred between 2 and 8 wk after the last immunization.

### Heterologous immunization induces synergistic HBsAg-specific IgG responses

We determined whether i.m. immunization with HBsAg-DNA (prime) followed by i.n. delivery of HBsAg liposome (boost) would induce greater Ab responses than the individual components alone. Mice were either immunized with the individual components of the heterologous vaccine (two times i.m. with HBsAg-DNA or one time i.n. with HBsAg liposome), or were immunized first with 2 $\times$  i.m. HBsAg-DNA on weeks 0 and 6 followed by i.n. immunization with HBsAg liposome on week 10. As shown in Fig. 2, the individual components induced weak HBsAg-specific total serum Ab responses. However, DNA immunization followed by i.n. boosting with HBsAg liposomes markedly increased the total HBsAg-specific Ab responses. Quantitative ELISA were used to determine the amount of specific IgG in the sera. HBsAg-specific IgG levels after two rounds of HBsAg-DNA immunization or one round of administration of HBsAg liposomes averaged 43.2  $\mu\text{g}/\text{ml}$  and 1  $\mu\text{g}/\text{ml}$  Ab, respectively. In contrast, HBsAg-specific serum IgG levels were 248  $\mu\text{g}/\text{ml}$  after the combined heterologous immunization protocol, demonstrating a clear synergistic effect ( $p < 0.01$ ).

A similar pattern was observed when the HBsAg-liposome boost was delivered intramuscularly. HBsAg-specific serum IgG levels after two rounds of HBsAg-DNA immunization or one round of administration of HBsAg liposomes averaged 27  $\mu\text{g}/\text{ml}$  and  $<1$   $\mu\text{g}/\text{ml}$  Ab, respectively. HBsAg-specific serum IgG levels were 424  $\mu\text{g}/\text{ml}$  after the combined heterologous immunization protocol. This synergy was only observed when DNA immunization preceded HBsAg-liposome immunization ( $p < 0.01$ ). Revers-



**FIGURE 2.** Heterologous immunization generates synergistic serum HBsAg-specific Ig responses. Groups of female BALB/c mice were vaccinated with either HBsAg-DNA i.m. (2  $\times$  100  $\mu\text{g}/\text{dose}$ ) alone on weeks 0 and 6, HBsAg liposome delivered i.n. once (15  $\mu\text{g}/50$   $\mu\text{l}$ ) on week 10 or HBsAg-DNA (delivered i.m. on weeks 0 and 6) followed by HBsAg liposomes (3  $\mu\text{g}/\text{dose}$ ; i.n.) on week 10. Total HBsAg-specific Ig responses were measured using Ag-specific ELISA 4 wk after the last immunization. The plotted data represent the mean responses in pooled sera from eight mice per group.

ing the order of the immunizations revealed no synergy in the Ab responses (data not shown).

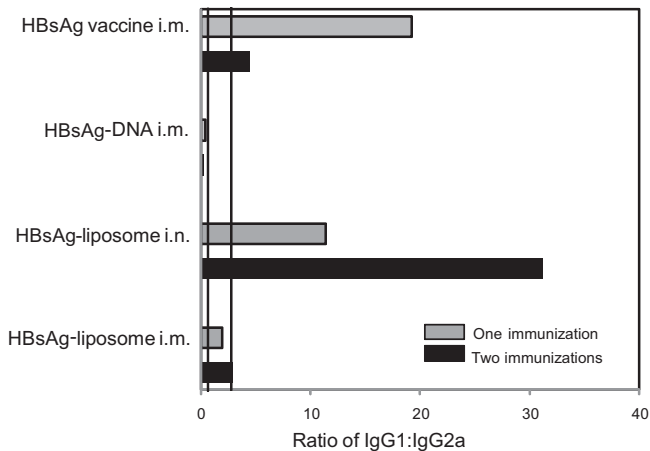
Intranasal inoculation volume was also evaluated for its role in Ab responses. We observed no statistical difference among the Ab responses of different groups of mice receiving equivalent amounts of HBsAg liposomes delivered in 20-, 30-, 40-, or 50- $\mu\text{l}$  volumes ( $p > 0.5$ ). Only mice receiving a 10- $\mu\text{l}$  inoculation volume showed a significantly lower Ab response ( $p < 0.05$ ).

### The heterologous immunization regimen induces robust responses in nonresponder C57BL/6 mice

It has been previously shown that C57BL/6 mice are nonresponsive to the HBsAg protein vaccine; however, DNA vaccination can prime Ag-specific CD8<sup>+</sup> T cells successfully (43). To determine whether the heterologous immunization protocol could overcome this unresponsiveness, the following experiment was performed. One group of C57BL/6 mice was immunized with two rounds of HBsAg liposome delivered i.n. (group A,  $n = 5$ ). As expected, these mice failed to generate a detectable level of anti-HBsAg serum IgG. These mice and a second group of naive mice (group B,  $n = 8$ ) were then immunized with two rounds of HBsAg-DNA delivered intramuscularly. Serum IgG levels in both groups of mice in response to DNA vaccination were comparable (12.8 and 8.0  $\mu\text{g}/\text{ml}$ , for groups A and B, respectively). Each group was then further boosted with HBsAg liposomes delivered intranasally. Surprisingly, both groups of C57BL/6 mice demonstrated equivalent high levels of anti-HBsAg serum IgG (group A, 238  $\mu\text{g}/\text{ml}$ ; group B, 310  $\mu\text{g}/\text{ml}$ ). The results from this experiment illustrate three points: 1) heterologous immunization can stimulate high Ab responses in a historically nonresponsive mouse strain; 2) the order of immunization is critical for the induction of serum IgG responses; 3) synergistic Ab responses are observed after heterologous immunization.

### Heterologous immunization generates a Th1-biased immune response

A Th1-biased response is the objective for vaccines that are effective at controlling intracellular pathogens such as viruses. The ratio



**FIGURE 3.** Heterologous immunization induces IgG1: IgG2a ratios indicative of a Th1-biased immune response. Groups of five female BALB/c mice were vaccinated with a human HBV vaccine, the indicated individual components of the immunization regimen. All immunizations were given at 3-wk intervals. IgG1:IgG2a ratios were determined from quantitative measurements of HBsAg-specific serum IgG1 and IgG2a measured 2 wk after the last immunization on pools of sera from five mice. □, IgG1:IgG2a ratios from mice that received one immunization. ■, Ratios from mice that received two immunizations. Two vertical lines at 0.5 and at 2.0 demarcate three different patterns of Ab responses. A ratio of 0.5 or less indicates a Th1-polarized response. A ratio of 2.0 or more indicates a Th2-polarized response. Ratios between 0.5 and 2.0 indicate a mixed or balanced response.

of Ag-specific IgG1 and IgG2a Abs and cytokine profiling can be used to characterize the Th bias of an immune response. IgG1: IgG2a ratios  $\leq 0.5$  indicate a Th1-biased immune response, while a ratio of  $\geq 2.0$  indicates a Th2-biased immune response. Ratios between 0.5 and 2.0 indicate a mixed response (44, 45). To evaluate the contribution from each vaccine component, the IgG1: IgG2a ratio obtained after administration of individual components of the heterologous protocol was determined. Fig. 3 shows the ratios of HBsAg-specific IgG1:IgG2a following i.m. immunization with HBsAg-DNA, or with HBsAg liposomes delivered either intranasally or intramuscularly. The ratios following immunization with a commercial human HBsAg vaccine, which is known to result in Th2-biased responses in humans, is also shown for comparison. The ratios following one or two homologous immunizations are also shown. As predicted from published data, the human vaccine and the HBsAg-DNA resulted in Th2 and Th1-biased immune responses respectively when administered intramuscularly. HBsAg liposomes gave a strong Th2-biased response when administered i.n. and a mixed response when administered intramuscularly.

The IgG1:IgG2a ratio was obtained after heterologous immunization with DNA followed by liposome-encapsulated Ag. As demonstrated in Fig. 3, a Th1-biased response was observed following immunization with HBsAg-DNA (preboost, □). Notably, a Th1 response was still observed after a boost with HBsAg liposomes administered either i.n. or i.m. 2 wk after immunization (IgG1: IgG2a ratio is  $< 0.3$ ). These results demonstrate that the Th1-biased response that is established by the DNA immunization is not diverted to either a mixed or a Th2-biased response following the liposome boost.

To confirm Th type of the immune responses, we performed ELISPOT assays to compare cytokine responses from naive mice, mice immunized with two doses of Ag encapsulated in liposome (homologous) or mice that received a heterologous immunization.

Spleen cells isolated from mice immunized with the heterologous protocol predominantly produced IFN- $\gamma$  (Th1-biased), while those isolated from mice immunized with the homologous protocol predominantly expressed IL-4 (Th2 biased; data not shown). An automatic Lincoplex assay for testing cytokine profiles not only confirmed high levels of IFN- $\gamma$  in the serum of mice immunized with the heterologous protocol but also detected elevated IL-12 levels in these same samples (data not shown). Interestingly, IL-12 has been shown to be one of the crucial cytokines for induction of systemic and mucosal T cell responses (46–49). Taken together, these results suggest that the heterologous immunization protocol induces a Th1-biased response that should be associated with high, Ag-specific CD8 T cell activity for clearance of virally infected cells.

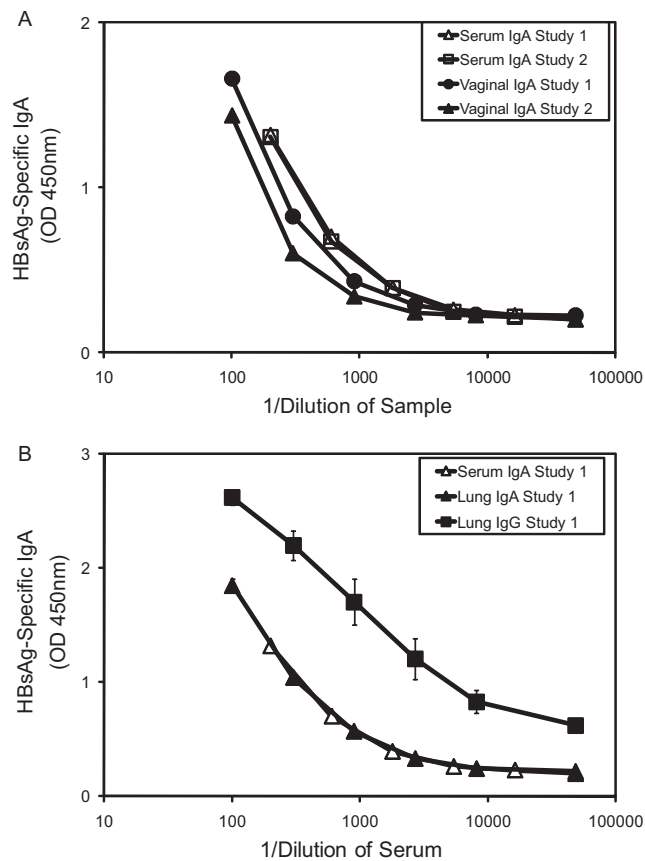
#### *Heterologous immunization induces mucosal IgA and IgG responses*

The levels of IgA and IgG following heterologous immunization with an i.n. boost with HBsAg liposomes were determined in the serum, and in vaginal and lung washes. Fig. 4 shows compiled results from two separate experiments. Circulating serum IgA was detected at comparable levels in both experiments. Equivalent or nearly equivalent levels of secretory IgA were also detected in vaginal washes and lung washes. In addition, substantial levels of lung IgG were also observed. IgA was also observed in a more limited number of samples from nasal washes and from saliva collected  $\geq 4$  wk following the i.n. HBsAg-liposome boost (data not shown). However, only low or marginal levels of mucosal Abs were detected when the liposome HBsAg vaccine was delivered by the i.m. route (data not shown). The heterologous immunization protocol is therefore effective at inducing Ab responses at both local (lung) and distant (vagina) mucosal sites.

#### *Both long and abbreviated DNA immunization regimens are equally effective at priming for the HBsAg-liposome boost to raise HBsAg-specific IgG responses*

In our initial experiments (i.e., Fig. 1), immunizations were performed at intervals of 4–6 wk. We later observed comparable results when those intervals were reduced to 3 wk (data not shown). We therefore tested whether the DNA priming portion of the heterologous immunization could be further reduced from weeks to days without compromising the immune responses generated. Fig. 5 shows a comparison between heterologous immunizations in which the DNA was given at an interval of 2 days (short protocol) vs an interval of 3 wk (long protocol). Two rounds of DNA injection were used to ensure immunization in the small leg muscle mass of the mouse. The short interval between DNA injections precludes a prime-boost induction of immune responses. After i.n. boosting with HBsAg liposomes, both protocols were shown to be equally effective in eliciting HBsAg-specific serum IgG responses, as well as comparable mucosal IgA responses (data not shown).

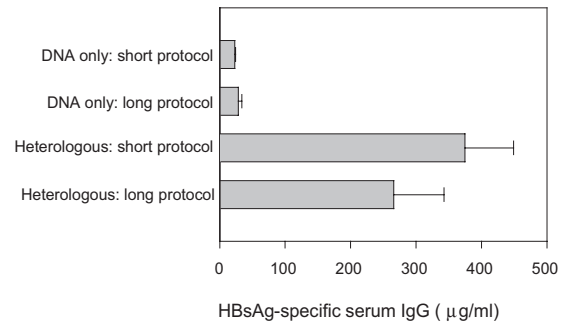
In addition, we tested whether the shortened protocol could be used to induce immune responses in neonatal mice. Neonates were injected i.m. with 50  $\mu$ g of DNA at 2 days of age followed by i.n. administration of 20  $\mu$ l of HBsAg liposomes 3 wk later. Total IgG Ab responses were measured 2 wk after the last immunization. Similar to those results obtained in adult mice, we observed a synergistic robust response after DNA and liposome administration (331.9  $\mu$ g of IgG/ml) and a minimal response after DNA immunization alone (0.7  $\mu$ g/ml IgG). These differences were highly significant ( $p = 0.002$ ;  $n = 6$ ).



**FIGURE 4.** Heterologous immunization induces mucosal IgA and IgG responses. Groups of six female BALB/c mice were immunized i.m. on weeks 0 and 4 with 100  $\mu$ g of HBsAg-DNA and i.n. on week 8 with 15  $\mu$ g of HBsAg liposomes. Serum and vaginal wash samples were collected on week 12, and lung washes were collected on week 14. *A*, Serum and vaginal IgA responses from two independent experiments. Results show the HBsAg-specific IgA levels determined using a five-layer sandwich ELISA. The results are from sample pools using equal volumes of serum or vaginal washes from six mice per group. Negligible ( $<2$  SD from levels in naive mice) HBsAg-specific IgA was detected from the following treatment groups: 1) mice that received DNA immunization alone, 2) mice that received HBsAg liposome alone, and 3) mice that received DNA immunization followed by HBsAg liposome delivered i.m. (data not shown). *B*, HBsAg-specific IgA and IgG from the lungs. Results show the HBsAg-specific IgA and IgG levels determined using a five-layer sandwich ELISA. Results shown are the mean  $\pm$  SEM from six mice per group. The serum IgA levels are shown again in this panel for reference.

#### *The adjuvant role of liposomes in the heterologous immunization protocol*

HBsAg is known to be a moderately strong immunogen containing both T and B cell epitopes (50–54). Liposomal protein vaccines alone have demonstrated only weak to moderate immune stimulating effect (55, 56). The contribution of the liposome element of the booster immunization to immunogenicity in the heterologous immunization protocol was evaluated in two independent experiments (Table I). Pairwise comparisons of HBsAg protein vs HBsAg liposomes showed that HBsAg protein alone contributed a significant boosting effect when it was given after priming with HBsAg-DNA ( $p < 0.001$ ). Further enhancement was observed upon encapsulation of the HBsAg protein within liposomes. Encapsulated protein induced greater levels of HBsAg-specific serum IgG when compared with DNA priming alone ( $p < 0.001$ ) and DNA priming followed by a nonencapsulated protein boost ( $p < 0.01$ ).



**FIGURE 5.** Short (3 days) and long (3 wk) DNA priming regimens are both effective at priming for Ab responses. Groups of female BALB/c mice were immunized with 50  $\mu$ g of HBsAg-DNA i.m. on days 0 and 2 (short protocol) or 100  $\mu$ g of HBsAg-DNA i.m. on day 0 and week 3 (long protocol) followed by 15  $\mu$ g of HBsAg liposomes i.n. 3 wk after the last DNA immunization. Ab responses were measured 2 wk after the liposome immunization. Average HBsAg-specific IgG responses (micrograms per milliliter)  $\pm$  SEM are shown. From top to bottom,  $n = 8, 6, 8,$  and 12/group.

#### *Heterologous immunization provides protection against i.n.-delivered, live recombinant vaccinia virus expressing HBsAg without causing immunopathology*

An important objective of our vaccine development research is to increase the levels of Ag-specific T cell responses that are important in the control of intracellular pathogens. We evaluated the protective immunity conferred by the heterologous immunization protocol. Because BALB/c mice are not susceptible to infection with human hepatitis B virus, we used a recombinant vaccinia virus expressing HBsAg (vHBs4) to evaluate protective immunity in BALB/c mice.

The first challenge experiment applied the long protocol (1, 3, and 6 wk as immunization intervals), high DNA vaccine priming (100  $\mu$ g), and lower vHBs4 dose for challenge ( $2 \times 10^5$  PFU). Three different groups were evaluated: naive mice (group A), mice that received two i.m. immunizations with HBsAg-DNA followed by i.n. immunization with empty liposomes (group B), and mice that received two i.m. immunizations with HBsAg-DNA followed by one i.n. immunization with HBsAg liposomes (group C). Because vHBs4 is an attenuated recombinant vaccinia virus with a thymidine kinase (TK) gene disruption, it usually does not cause death in mice. A dose of  $2 \times 10^5$  PFU of recombinant vHBs4 was administered i.n. 2 wk after the HBsAg liposome boost. Viral titers in the lung and the frequency of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells detected after ex vivo stimulation with an immunodominant HBsAg CTL peptide were measured 5 days postinfection. The results are shown in Fig. 6.

Viral titers in the lungs of mice that received the HBsAg-DNA immunization and empty liposomes were significantly reduced compared with naive mice (Fig. 6A, group A vs group B). All naive mice had detectable virus in the lungs ( $3.72 \pm 0.4$  log PFU) while three of four mice immunized with DNA and empty liposomes had detectable but significantly lower viral titers ( $1.86 \pm 0.4$  log PFU;  $p = 0.01$ ). In comparison, no detectable virus could be recovered from the lungs of mice that were immunized with both HBsAg-DNA and HBsAg liposomes (Fig. 6A, group C), indicating complete clearance of virus.

High percentages of CD8<sup>+</sup> T cells were observed in the lungs of mice immunized with both DNA and HBsAg liposomes ( $\sim 28\%$  of viable lymphocytes; Fig. 6B, group C). In comparison, naive mice or mice immunized solely with DNA demonstrated CD8<sup>+</sup> T cell frequencies of  $\sim 3$  and  $\sim 12\%$ , respectively, in the lung (Fig. 6B,

Table I. HBsAg-specific Ab responses are enhanced by liposomes (long protocol)

Group <sup>a</sup>	Prime	Boost	Ag-Specific IgG ( $\mu\text{g/ml}$ ) <sup>b</sup>	Comparison	p Value
A1	HBsAg-DNA	None	23.9 $\pm$ 8.8	A1 vs A2	<0.001
A2	HBsAg-DNA	HBsAg	109.5 $\pm$ 17.6	A2 vs A3	<0.01
A3	HBsAg-DNA	HBsAg liposome	374.8 $\pm$ 64.4	A3 vs A1	<0.001
B1	HBsAg-DNA	None	28.6 $\pm$ 5.1	B1 vs B2	<0.001
B2	HBsAg-DNA	HBsAg	203.4 $\pm$ 43.9	B2 vs B3	<0.01
B3	HBsAg-DNA	HBsAg liposome	618.0 $\pm$ 99.6	B3 vs B1	<0.001

<sup>a</sup> Two independent experiments A and B were performed with three subgroup comparisons in each experiment (1, 2, and 3).

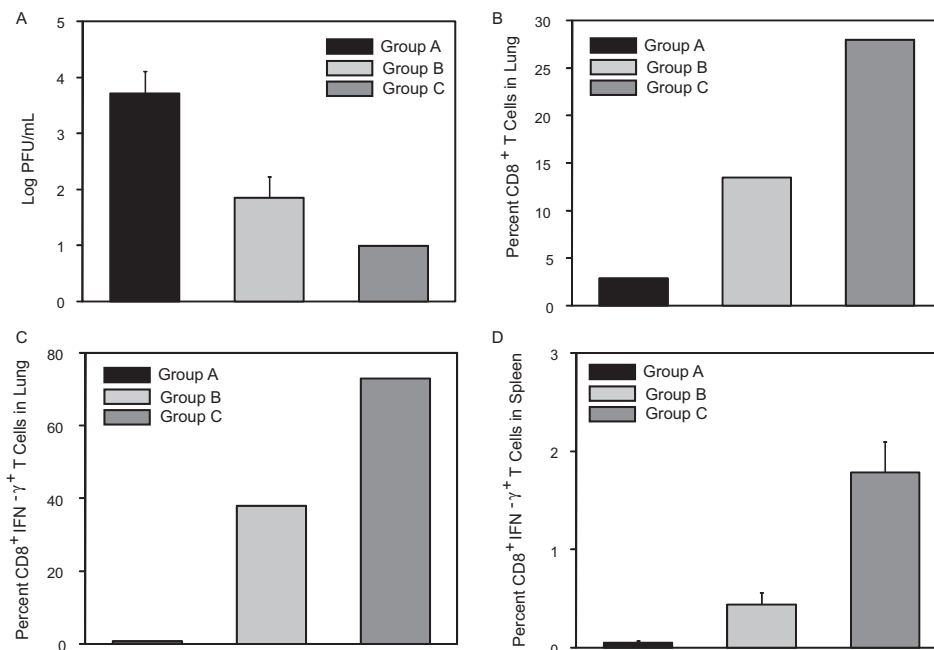
<sup>b</sup> HBsAg-specific serum IgG was evaluated using a quantitative ELISA on individual mouse serum samples. Experiments A and B contained eight ( $n = 8$ ) and six ( $n = 6$ ) mice per group, respectively.

groups A and B). To determine whether the CD8<sup>+</sup> T cells were Ag specific, we performed an intracellular cytokine staining assay to determine the frequency of CD8<sup>+</sup> T cells that expressed IFN- $\gamma$ . The challenge with vHBs4 revealed highly significant differences between groups. Mice immunized with the DNA prime and liposome-HBsAg boost protocol demonstrated high frequencies of Ag-specific CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells in the lung (>70% of CD8 T cells; Fig. 6C) and also systemically in the spleen, albeit on a much lower scale (1.79  $\pm$  0.31%, Fig. 6D). The DNA portion of the heterologous protocol contributed to induction of these responses as immunization with DNA alone only induced Ag-specific CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells in the lung (38% of cells) and in the spleen (0.44% of the cells). In contrast, naive mice exhibited few Ag-specific CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells in the lung and spleen.

Reduced viral titers at 5 days post-viral challenge was also observed in experiments using the shortened immunization protocol (2 $\times$  DNA priming within 3 days), a lower DNA vaccine dose (10  $\mu\text{g}$ ), and higher vHBs4 challenge dose (5  $\times 10^5$  PFU). Mice in one experiment were either left untreated (naive), or were immunized

with HBsAg-DNA plus empty liposomes or HBsAg-DNA plus HBsAg liposomes (15  $\mu\text{g}$  HBsAg). Although immunization of mice with HBsAg-DNA plus empty liposomes significantly reduced viral titers in the lung (3.06  $\pm$  0.08 log), heterologous immunization with HBsAg-DNA plus HBsAg liposomes reduced viral titers even further (1.7  $\pm$  0.26 log), in comparison to the naive group (4.95  $\pm$  0.08 log). This demonstrates that significantly better protection was achieved in mice immunized with both the DNA and liposome components of the vaccine as compared with mice immunized with HBsAg DNA plus empty liposome vector ( $p < 0.01$ ,  $n = 5$ ).

We also analyzed Ag-specific T cell responses in immunized mice before viral infection. BALB/c mice were immunized with either the DNA portion or HBsAg-liposome portion of the vaccine, or were immunized with both HBsAg-DNA and HBsAg liposomes. Pooled lung samples from all mice were tested for Ag-specific CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells 2 wk after the final immunization. Significantly higher frequencies of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (5.7%,  $n = 5$ ) were observed in the lungs of mice



**FIGURE 6.** Heterologous immunization induces protective immune responses and high frequencies of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells. Groups of five female BALB/c mice were either left untreated (group A), were immunized with HBsAg-DNA + empty liposomes (group B) or were immunized with HBsAg-DNA + HBsAg liposomes (group C) using the long immunization protocol. Two weeks after the last immunization, mice were infected i.n. with  $2 \times 10^5$  PFU of vHBs4. Five days after infection, lungs from four mice per group were harvested for determination of viral titers (A), whereas the final lung was analyzed for total frequency of CD8<sup>+</sup> T cells in the mononuclear population (B) and frequency of Ag-specific CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (C). Individual spleens (four per group  $\pm$  SEM) were also analyzed for Ag-specific CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (D, A vs B:  $p = 0.01$ ; A vs C:  $p = 0.0005$ ; B vs C:  $p < 0.004$ ). All analyses were performed as described in *Materials and Methods*. For C and D, cells incubated without peptide showed <0.1% positive staining for IFN- $\gamma$  within the CD8 population (data not shown).

primed with DNA and boosted with liposome vaccine, but not in the lungs of mice immunized solely with DNA or liposome vaccine (0.5%, 0.4%,  $n = 5$ ). No significant differences in CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells were observed in any of the groups. These data suggest that whereas no Ag-specific T cells were detected in the lungs of mice immunized by DNA or liposome vaccine alone, Ag-specific T cells were induced in the lung at elevated levels in mice immunized using the heterologous immunization protocol.

Importantly, no immunopathology was observed in the lungs of mice immunized with this strategy and challenged with the  $5 \times 10^5$  PFU dose of vHBs4, highlighting the safety of this approach (data not shown).

#### *Neutralizing Abs do not account for clearance of virus*

Current commercial HBsAg vaccines usually raise a Th-2 type Ab response and the mechanism of protection is primarily through neutralizing Abs. Due to the lack of mouse models for HBV infection to help dissect the protective mechanism, a pulmonary infection with recombinant vaccinia virus expressing HBsAg Ag (vHBs4) was designed to evaluate the role of T cell-mediated responses in the mice immunized with our protocol. Because the expressed HBsAg is foreign protein that is neither integral to nor incorporated into the recombinant vaccinia viral particle, it is unlikely that neutralizing Abs are responsible for immune protection against the vHBs4 virus. To demonstrate that neutralizing Abs were not responsible for clearance of virus in this model, we performed an *in vitro* plaque reduction neutralization assay. Sera from mice that received the heterologous immunization protocol did not neutralize either recombinant vHBs4 or wild-type vaccinia virus even at the highest concentrations of serum used (dilution 1/25 of whole serum). In contrast, a serum pool composed of known anti-vaccinia Abs neutralized both viruses. A 96% reduction in plaque formation was observed when this serum was diluted 1/200. This *in vitro* data strongly supports the conclusion that neutralization activity is not responsible for the observed protection in this experimental system, but T cell-mediated immunity plays a protective role against virus infection in the lung.

## Discussion

We have developed a heterologous immunization protocol consisting of immunization with DNA vaccine encoding Ag followed by administration of Ag encapsulated in liposomes. Our approach combines liposome encapsulation, specific delivery to mucosal sites, and the use of a heterologous immunization regimen. To the best of our knowledge, this is the first report of a heterologous immunization protocol combining systemic priming with DNA and a mucosal boost with liposome-encapsulated protein. The enhanced boosting effect seen after use of protein encapsulated in liposomes rather than a simple (not encapsulated) protein boost suggests that the liposome acts as both a delivery vehicle for the protein Ag and as a potent vaccine adjuvant. Unlike other heterologous immunization protocols, this regimen was specifically designed for enhanced safety by excluding the use of live viral vectors and/or potentially toxic adjuvants. The unique benefits of this protocol include induction of Th1-biased, humoral, and cellular immune responses on mucosal surfaces and flexibility in delivery of individual components and induction of responses in "non-responder" animals and neonates.

Although heterologous immunization using the *i.n.* boost induced strong systemic Ab responses, we also observed induction of Ag-specific IgA and IgG on multiple mucosal surfaces such as respiratory tracts and the remote vaginal cavity. However, we were unsuccessful in enhancing immune responses after delivery of liposome HBsAg by the vaginal route (data not shown) supporting

the general belief that although immune responses can be detected in the vagina they are not directly generated in the vaginal mucosa (57). In contrast, heterologous immunization with an *i.m.*-delivered liposome boost induced strong systemic responses, but only induced marginal mucosal immunity. Robust Ag-specific CD8<sup>+</sup> T cell responses were also induced after heterologous immunization as revealed by intracellular cytokine-staining assays before and after viral challenge. These CD8<sup>+</sup> T cell responses provided enhanced protection from a mucosal virus challenge. To our best knowledge, this is the first time that a liposome-based vaccine has been shown to enhance protective CD8<sup>+</sup> T cell responses in mucosal sites of mice primed with DNA vaccines.

It is well-established that homologous immunization with an *i.m.* DNA vaccine induces a Th-type 1 (Th1) biased immune response which may favor T cell-mediated immune responses (44, 45). We observed similar results confirmed by IgG subclass profiles and enhanced IFN- $\gamma$  production by CD8<sup>+</sup> HBsAg-specific T cells. In contrast, homologous immunization with *i.n.*-delivered liposomal HBsAg induced greater levels of IgG1 than IgG2a Abs, suggesting a Th-type 2 (Th2) biased response. The combination of *i.m.* DNA priming followed by an *i.n.* liposomal HBsAg boost maintained Th1-type IgG subclass and cytokine profiles, suggesting that initial priming with DNA vaccine determines the ultimate Th type, in agreement with others' findings (4, 16, 19, 21). The maintenance of Th1-biased responses suggests that this protocol will be useful for any situation where induction of robust CD8<sup>+</sup> T cell responses is desired.

Other important experiments highlight the strengths of this vaccination protocol. C57BL/6 mice, typically described as nonresponders to HBsAg, can be primed by DNA vaccine against HBsAg (43). C57BL/6 mice were vaccinated using the heterologous immunization protocol with an *i.n.* liposomal-HBsAg boost. Surprisingly, we also observed synergistic induction of robust systemic Ab responses. It will be interesting to determine whether this vaccine approach may also induce responses in the ~5–10% of healthy vaccine recipients who fail to respond to the currently available HBV vaccine (40, 58).

We also compared long and short DNA priming protocols. We observed no significant differences in Ab titers after either protocol, suggesting there is flexibility in timing of delivery of the vaccine components and that a single DNA vaccine prime may be sufficient.

The long protocol consists of a prime and a boost with the same DNA vaccine at an interval of 3 wk. In the short protocol, we used *i.m.* injection of DNA on days 0 and 2 for technical reasons to ensure successful inoculation of DNA into the small target area (quadriceps muscle). However, this short interval between injections virtually precludes the development of a prime and boost scenario. Because *i.m.* administration of vaccines in humans is more efficient simply due to the abundance of muscle tissue and ease of administration, the success of the short protocol (one prime) in comparison with the long protocol (two primes) in mice suggests the feasibility of applying only one prime with DNA vaccine and one boost with liposome vaccine in clinical trials. This short protocol may prove ideal for emerging or pandemic threats with the potential to rapidly raise protective immunity against mucosally transmitted pathogens. It will be very interesting and significant to further investigate whether the 3-wk interval between the prime and boost used in the shortened protocol can be further decreased to <3 wk without compromising immune responses and protection. Finally, the demonstrated success of the shortened protocol in both adult and neonatal mice can greatly broaden the application potential of this heterologous immunization regimen by which rapid protective immunity can be generated.

Our data demonstrate the feasibility of eliciting mucosal T cell responses that are primarily responsible for protective immunity using a heterologous immunization protocol. Protective immunity was demonstrated by the complete clearance of virus from the lung, enhanced frequencies of CD8<sup>+</sup> IFN- $\gamma$  cells before viral challenge, and the exclusion of neutralizing Abs as a mechanism of protection for this specially designed challenge model.

Additional success in raising broad and effective protective immunity was also seen in HSV-2 and influenza virus A (H1N1) models by applying this prime and boost immunization protocols (data not shown).

This novel heterologous immunization approach using a DNA and liposome-based protein vaccine is safe and effective in raising enhanced immune responses and immune protection especially at mucosal surfaces. Recent studies show that humoral and cellular immune responses at the systemic immune level can be augmented in mice by priming with a hepatitis B DNA vaccine and boosting s.c. with a complex of HBsAg protein and alum hydroxide (59, 60). Delivery of liposomal HBsAg vaccine i.n. using our strategy raised both systemic and mucosal immune responses. Although HBsAg was used as a model Ag for development of the vaccine, the regimen was designed for application to a variety of pathogens. The robust immune responses observed in mice encourage further investigation in larger animal models including nonhuman primates. In addition, studies investigating the relationship between enhanced mucosal Ab responses and immune protection from other pathogens are underway.

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## Disclosures

The authors have no financial conflict of interest.

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