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Cross-Protection among Lethal H5N2 Influenza Viruses Induced by DNA Vaccine to the Hemagglutinin

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Inoculation of mice with hemagglutinin (HA)-expressing DNA affords reliable protection against lethal influenza virus infection, while in chickens the same strategy has yielded variable results. Here we show that gene gun delivery of DNA encoding an H5 HA protein confers complete immune protection to chickens challenged with lethal H5 viruses. In tests of the influence of promoter selection on vaccine efficacy, close correlations were obtained between immune responses and the dose of DNA administered, whether a cytomegalovirus (CMV) immediate-early promoter or a chicken β-actin promoter was used. Perhaps most important, the HA-DNA vaccine conferred 95% cross-protection against challenge with lethal antigenic variants that differed from the primary antigen by 11 to 13% (HA1 amino acid sequence homology). Overall, the high levels of protection seen with gene gun delivery of HA-DNA were as good as, if not better than, those achieved with a conventional whole-virus vaccine, with fewer instances of morbidity and death. The absence of detectable antibody titers after primary immunization, together with the rapid appearance of high titers immediately after challenge, implicates efficient B-cell priming as the principal mechanism of DNA-mediated immune protection. Our results suggest that the efficacy of HA-DNA influenza virus vaccine in mice extends to chickens and probably to other avian species as well. Indeed, the H5 preparation we describe offers an attractive means to protect the domestic poultry industry in the United States from lethal H5N2 viruses, which continue to circulate in Mexico.

The ability of directly injected DNA expression vector for viral protein to elicit protective immune responses has been demonstrated in numerous experimental systems (2, 3, 5, 22, 31, 33, 35, 40). Studies to assess this strategy for protection against influenza virus have used both envelope and internal viral proteins (9, 10, 29, 36). These DNA vaccinations have produced both cell-mediated and humoral responses, similar to results obtained with live viruses (24, 25, 27, 33, 35, 40). Studies with ferrets indicate that DNA vaccines against conserved internal viral proteins of influenza virus, together with surface glycoproteins, are more effective against antigenic variants of influenza virus than are either inactivated or subvirion vaccines (6). Indeed, reproducible and long-lasting (12-month) immune responses to DNA encoding nucleoprotein have been reported to occur in mice (41).

The possibility of species-specific differences in the responsiveness to DNA-based vaccines was first raised in studies by Robinson et al. in 1993, showing that direct inoculation of a defective retroviral vector expressing H7 HA could protect chickens against lethal H7 influenza virus challenge (29). These investigators reported a range of protection rates, ranging from a high of 100% to a low of 28% (10, 29), in contrast to the generally high responsiveness seen with mice (10).

The variable results reported by Robinson et al. (29) could reflect several factors that influence the efficiency of expression of antigen genes and the immunogenicity of DNA vaccines, including the reproducibility of inoculation and the choice of the promoter used to drive antigen gene expression. Depending on their origin, promoters differ in tissue specificity and efficiency in initiating mRNA synthesis (1, 39). To date, most DNA vaccines in mammalian systems have relied upon viral promoters derived from cytomegalovirus (CMV). These have had good efficiency in both muscle and skin inoculation in a number of mammalian species but may not be as effective in chickens. Another factor known to affect the immune response elicited by DNA immunization is the method of DNA delivery; parental routes can yield low rates of gene transfer and produce considerable variability of gene expression (22). High-velocity inoculation of plasmids into the epidermis with a gene gun enhanced the immune responses of mice (8, 10), presumably because of a greater efficiency of DNA transfection and more effective antigen presentation by dendritic cells. Finally, the variability among chicken experiments might well reflect the use of outbred birds.

We were interested in whether DNA vaccination via a gene gun could reliably produce protective immunity in chickens against lethal antigenic variants of H5 influenza virus, by comparison to the contemporary inactivated virus vaccine. The emergence of A/Chick/Queretaro/95 (H5N2) provided the opportunity to test the efficiency of a hemagglutinin (HA) DNA vaccine against antigenic variants with up to 13% variability in amino acid sequence homology in the antigenic region of the HA1. The experiments described here were also designed to test the role of the promoter element in a DNA vaccine that expressed the HA of influenza virus A/Turkey/Ireland/83 (H5N2) in chickens. Our findings demonstrate reproducible induction of immune protection against a lethal H5 virus and its antigenic variants, at rates comparable to those achieved with a conventional whole-virus vaccine.

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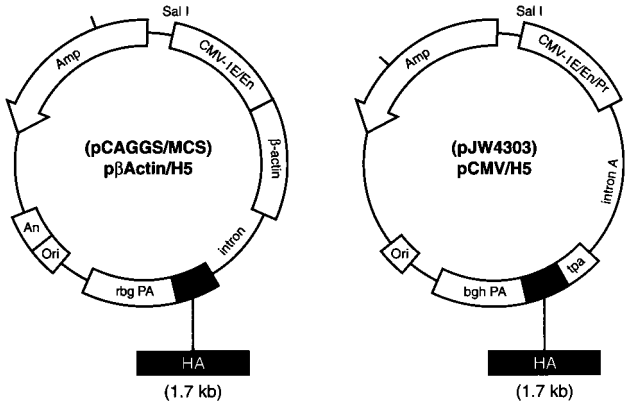

FIG. 1. Schematics of pJW4303/H5 and pCAGGS/MCS/H5 DNAs used for immunizations. Ori, simian virus 40 origin of replication; CMV-IE/En/Pr CMV immediate-early enhancer/promoter; CMV-IE/En CMV enhancer; H5, hemagglutinin cDNA from Ty/Ir/83 (H5N8) influenza virus (20); β-actin, chicken β-actin promoter; bgh PA, bovine polyadenylation site; rbg PA, rabbit β-globulin polyadenylation site; An, simian virus 40 antigen.

MATERIALS AND METHODS

Viruses and cells. A/Chick/Queretaro/19/95 (H5N2) (Ck/Quert/95), A/Turkey/Ireland/83 (H5N8) (Ty/Ire/83), and A/Chick/Pennsylvania/1370/83 (H5N2) (Ck/Penn/83) were from the influenza virus repository at St. Jude Children's Research Hospital, Memphis, Tenn. These viruses were cultivated in the allantoic cavities of embryonated eggs (37) and handled in the hospital's U.S. Department of Agriculture (USDA)-approved BL3 containment facility. Cos-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum. Chicken embryo fibroblasts were prepared from 10-day-old chicken embryos, as described previously (32).

Preparation and standardization of inactivated vaccine. Influenza Ty/Ire/83 virus was purified by equilibrium density centrifugation through 25 to 75% sucrose gradients (20). Purified virus at 40,000 HA units/ml was inactivated with 0.025% formalin at 4°C, resulting in complete loss of infectivity (15). The amount of HA per total virus protein was determined by polyacrylamide gel electrophoresis. The relative HA content of viruses was evaluated by determining the ratio of HA activity to total viral protein content following the staining of gels with Coomassie brilliant blue and scanning with a Sparc station 2 densitometer (Sun Systems, Foster City, Calif.) and Bioimaging Visage 110 software (Millipore, Bedford, Mass.). The relative HA content of influenza Ty/Ire/83 virus was 35% of total virus protein. The protein concentration was estimated by a modification of the Bradford method (Repligen, Cambridge, Mass.).

Serologic testing. HA and HA inhibition (HI) assays were performed with 0.5% chicken erythrocytes (RBCs) as previously described (38). Sera from chickens were tested individually after treatment with receptor-destroying enzyme (37). HI titers were determined as the reciprocal of the highest serum dilution giving complete HI.

Antigenicity and sequence analysis of HA. To investigate the relationship between antigenicity and immunogenicity, we performed HI tests with a panel of monoclonal antibodies prepared against the HA of Ck/Penn/83 (17). The amino acid sequence homology among Ck/Penn/83, Ck/Quert/95, and Ty/Ire/83 was analyzed by sequence comparison (GCG Bestfit software).

Influenza virus genes and expression vectors. A full-length cDNA copy of the HA gene of Ty/Ire/83, designated pTH29, has been cloned (16). The full-length HA gene from this clone was obtained by digestion with *Hin*dIII-*Bam*HI, and the resulting fragments were ligated into a pJW4303 vector (generously provided by B. R. Cullen, Duke University, N.C.) (4) under the control of a CMV immediate-early promoter [pJW4303/H5(Ty/Ir/83)] (Fig. 1). A plasmid expression vector, pCAGGS (23), was modified by cloning oligonucleotides containing recognition sites of *Eco*RI, *Sac*I, *Cla*I, *Nsi*I, *Asp*718, *Sma*I, *Sph*I, *Xho*I, *Nhe*I, and *Bgl*II between the *Eco*RI and *Bgl*II sites (pCAGGS/MCS). The full-length HA gene from the pTH29 clone was also obtained by digestion with *Cla*I-*Nhe*I and ligated into the *Cla*I-*Nhe*I sites of the pCAGGS/MCS vector (generously provided by Y. Kawaoka, St. Jude Children's Research Hospital, Memphis, Tenn.) under the control of a chicken β-actin promoter [pCAGGS/MCS/H5(Ty/Ir/83)] (Fig. 1). DNAs were grown in *Escherichia coli* HB101 and purified on Qiagen plasmid purification columns. DNA concentrations were determined by optical density measurements at 260 nm (10).

Transfection. Cos-1 cells were transfected with either pJW4303/H5(Ty/Ir/83) (pCMV/H5) or pCAGGS/MCS/H5(Ty/Ir/83) (pβactin/H5), as described previously (13). Six-well tissue culture plates were seeded with Cos-1 cells (10^6/well), and the cells were grown until they were about 70% confluent and then washed with 2 ml of reduced-serum medium (OPTI-MEM; Life Technologies, Inc., Gaithersburg, Md.) per well. For each transfection (or well), 2 μg of plasmid DNA in 100 μl of OPTI-MEM was mixed with 6 μl of Lipofectamine in 100 μl of OPTI-MEM. This mixture (0.2 ml) was then incubated at room temperature for at least 30 min before it was added to the cells. After incubation for 3 h at 37°C in a humidified 5% CO_2 incubator, 1 ml of medium–10% fetal calf serum per well was added to the cells.

Hemadsorption. The expression and biological activity of the influenza virus HA were examined by hemadsorption with chicken RBCs (18). At 48 h post-transfection, the cells were washed and treated with bacterial neuraminidase for 1 h at 37°C to remove host cell sialic acid. After 1 h of incubation followed by washing to remove neuraminidase, the cells were overlaid at 4°C with 1% chicken RBCs in isotonic phosphate-buffered saline (PBS). Unbound RBCs were removed 30 min later by washing with PBS, after which the cells were fixed with 10% buffered formalin phosphate. Bound RBCs were visualized by staining with Giemsa. Primary chicken embryo fibroblasts were prepared as described previously (32), with transfection and hemadsorption performed by standard methods (18).

Indirect immunofluorescence. A total of 2×10^6 cells were incubated at 4°C for 45 min with 50 μl of a 1:100 dilution of antibody-containing ascitic fluid (a pool of monoclonal antibodies CP34, CP46, and CP59) prepared against the HA of Ck/Penn/83. The cells were washed twice with PBS and incubated for 45 min at 4°C with 100 μl of a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. The cells were washed twice with PBS and analyzed in a fluorescence-activated cell sorter (39).

Gene gun delivery of DNA. Plasmid DNAs were accelerated into a defeathered area of breast in 3- to 4-week-old chickens with the use of an helium pulse gene gun (Accell; Auragen, Inc., Middleton, Wis.) as previously described (25). Plasmid DNA was affixed to gold particles by adding 10 mg of 0.95-μm-diameter gold powder (Degussa, South Plainfield, N.J.) and an appropriate amount of plasmid DNA to a 1.5-ml centrifuge tube containing 50 μl of 0.1 M spermidine. Plasmid DNA and gold were coprecipitated by adding 100 μl of 2.5 M $CaCl_2$ during vortex mixing, after which the precipitate was allowed to settle, washed with absolute ethanol, and resuspended in 2.0 ml of ethanol. This suspension was transferred to Mylar sheets and allowed to settle for several minutes; then the excess ethanol was removed by aspiration. The gold-DNA-coated sheets were dried and stored under vacuum. DNA-coated gold particles were delivered into the skin with the Accell instrument at a helium pressure setting of 400 lb/in^2.

Dose-response relationship of DNA vaccine. Dose-response trials with chickens were conducted in a USDA-approved BL3 facility. The bead-loading rate used for coating was 0.5 mg of 0.95-μm gold particles/shot. The DNA loading concentrations used for coating were 0.5, 1.0, 2.0, and 10 μg, which deliver 0.25, 0.5, 1.0, and 5 μg of DNA per shot, respectively. Thus, gene gun inoculations delivered 0.5 to 1.0 mg of 0.95-μm gold beads depending on the number of shots (single or double) used. Five groups of 10 chickens each were inoculated via a gene gun with amounts of plasmid DNA ranging from 0.25 to 10 μg, in either the pCMV/H5 or the pβactin/H5. The groups which received 0.25 to 5 μg of DNA were inoculated with a single shot of beads containing the appropriate amount of DNA, whereas the group which received the 10-μg dose of DNA vaccine was given two shots containing 5.0 μg of DNA per shot. The energy level for the gene gun was a helium pressure setting of 400 lb/in^2. At 3 weeks after immunization, the chickens were challenged intranasally with 100 50% lethal doses of (LD_{50}) of Ty/Ire/83 virus.

Immunization and challenge infection. Vaccine trials in chickens were conducted in a USDA-approved BL3 facility. We studied two groups of chickens. In one (n = 30), the vaccine consisted of formalin-inactivated whole-virus vaccine. These chickens were primed subcutaneously at the base of the neck on day 0 with 100 μl of inactivated virus containing 10 μg of HA in Freund's incomplete adjuvant and boosted 4 weeks later with the same dose of vaccine lacking the adjuvant. In the DNA vaccine group, the chickens were inoculated with 10 μg of DNA encoding HA via a gene gun on day 0 and boosted with the same dose 4 weeks later. Thirty control chickens either received DNA that lacked the viral HA gene or were left untreated. At 10 days after the boost, chickens in each vaccine group and the controls were divided into three subgroups of 10 birds each and challenged with 100 LD_{50} of either Ck/Penn/83, Ck/Quert/95, or Ty/Ire/83.

RESULTS

Expression of HA protein in vitro. The expression and biological activity of the cloned HA gene were assessed by a hemadsorption assay. Each of the promoters was able to drive cell membrane expression of the HA protein. To determine the influence of the promoter on the level of HA expression in vitro, we transfected Cos-1 and chicken embryo fibroblasts with equal amounts of either pCMV/H5 or pβactin/H5. At 2 days later, the cells were stained by indirect immunofluorescence with a pool of monoclonal antibodies directed to the H5 HA protein and then analyzed by fluorescence-activated cell sorting. The percentages of transfected cells were comparable

TABLE 1. Antigenic and genetic relatedness of H5 influenza viruses by HI analysis with monoclonal antibodies to influenza A/Chicken/Pennsylvania/83 (H5N2) virus

Monoclonal antibody	HI titer for:		
	Ck/Penn/83	Ck/Quert/95	Ty/Ire/83
CP22	12,800	1,600	160
CP25	12,800	25,600	160
CP30	6,400	<100	<100
CP34	25,600	102,400	320
CP38	12,800	<100	<100
CP55	12,800	1,600	<100
CP59	25,600	1,600	640
CP46	25,600	1,600	320
CP45	25,600	<100	<100
% Difference in amino acid sequence of HA1[a]	11	13	0

[a] Based on amino acid sequence homology of the HA1 region.

upon transfection with either vector (i.e., 25% for pCMV/H5 or pβactin/H5 in Cos-1 cells and 15 to 20% for pCMV/H5 and pβactin/H5, respectively, in chicken embryo fibroblasts).

Antigenic and genetic relationships among H5 viruses. Memory responses developed against one antigen affect subsequent immune responses to antigens that are antigenically and structurally related to the primary antigen. To test the antigenic relationship between Ty/Ire/83 and two H5 subtype variants, we assessed the reactivity of the viruses with a panel of monoclonal antibodies to the HA region. The CP25 and CP34 monoclonal antibodies revealed common epitopes between Ck/Quert/95 and Ck/Penn/83, but Ck/Quert/95 was less reactive with the other seven monoclonal antibodies tested. Ty/Ire/83 reacted poorly (10- to 40-fold lower HI titer) than Ck/Penn with each of the nine test antibodies (Table 1).

Dose-response curve for an H5 gene vaccine with different promoters. To investigate the relationship between the amount of plasmid DNA administered via a gene gun and the magnitude of the immune response, we vaccinated groups of 10 chickens each with various doses of pCMV/H5 and pβactin/H5 plasmid and measured the protective efficacy against lethal homologous challenge.

Although at 0.5 and 1 μg, the pβactin/H5 construct appeared to offer better protection, statistical analysis (Fisher's exact tests) indicated no significant difference in the protection achieved by these two vectors at each dose level (Fig. 2). These studies demonstrate that both pβactin/H5 and pCMV/H5 were effective in chickens.

Protection and cross-protection induced by the pCMV/H5 vector. The vaccine efficacy for influenza is ultimately determined not only by efficacy for homologous virus but also by efficacy for antigenic drift variants. We therefore sought to determine the extent of protection conferred by gene gun-inoculated DNA against challenge with homologous H5 virus or an antigenic variant.

Gene gun immunization of birds with 10 μg of pCMV/H5 DNA provided complete protection (9 of 9 birds) after challenge with homologous virus, with no evidence of virus shedding in either the trachea or cloaca (Table 2). Immunization with 10 μg of H5-DNA administered via gene gun also provided complete cross-protection against challenge with the Ck/Penn/83 variant and 90% protection against Ck/Quert/95, again in the absence of virus shedding. Similar cross-protection was obtained with 10 μg of HA-inactivated Ty/Ir/83 whole-virus vaccine, except that one chicken challenged with Ty/Ire/83 continued to shed virus in the trachea (Table 2). None of the control chickens (given 10 μg of pCMV control DNA) survived their infections. It is interesting that Ty/Ir/83 virus, which killed 100% of birds, was shed in the cloaca of only 20% of the birds. Of the 30 chickens vaccinated with H5-DNA, only 2 became sick (1 of them died), compared with 4 of the 30 given the whole-virus vaccine (2 of which died). Thus, the DNA vaccine was at least as efficacious as the conventional vaccine, affording high levels of protection against homologous H5 virus and its antigenic variants.

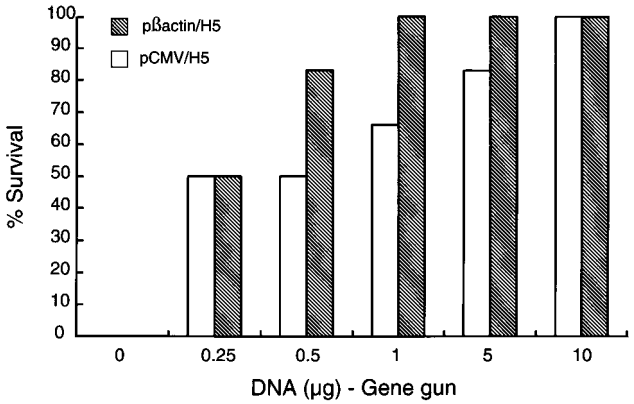

FIG. 2. Dose response of chickens to DNA-HA vaccine with different promoters. Six groups of 10 chickens each were immunized on day 0 with the indicated amount of either pCMV or pβactin HA DNA via particle-mediated delivery (gene gun) to the abdominal epidermis. On day 28, all the chickens were challenged intranasally with 100 LD_{50} of Ty/Ire/83. Protection was measured on the basis of survival of the lethal infection. None of the survivors showed signs of influenza. All controls died of lethal infection within 5 to 7 days of challenge.

Antibody responses of chickens immunized with DNA and conventional vaccine. The relative abilities of formalin-inactivated and DNA vaccines to induce serum antibodies were also examined. Within the first 3 weeks postimmunization, no detectable antibodies were found in any of the vaccine groups. However, in chickens given formalin-inactivated vaccine, vaccination with Ty/Ire/83 (on day 31) generated low levels of antibodies (geometric mean titer [GMT], 42) specific to the immunizing antigens. Antibodies to the Ck/Penn/83 or Ck/Quert/93 antigenic variant were not detected, and no antibodies to any of the three H5 virus antigens were found following booster immunization of the DNA vaccine group.

The profile of serum antibodies induced by homologous challenge was similar in both the DNA and whole-virus vaccine groups (Fig. 3) and was characterized by high levels of HI antibodies specific to Ty/Ire/83 antigens. Antibody levels in serum against the two antigenic variants, Ck/Penn/83 and Ck/Quert/95, did not differ substantially whether chickens were inoculated with HA-DNA or given the whole-virus vaccine. These levels were uniformly threefold lower than antibody levels against homologous Ty/Ire/83 antigen.

The profiles of serum antibodies induced by challenge with the antigenic variants Ck/Penn/83 and Ck/Quert/95 were similar in both the DNA and whole-virus vaccine groups and were again characterized by high levels of HI antibodies specific to Ty/Ire/83 antigen.

DISCUSSION

In these studies, gene gun delivery of DNA encoding the H5 HA was an effective method of immunization. Despite the lack of detectable HI antibodies against H5 influenza virus after

TABLE 2. Protection of chickens against challenge with antigenic variants of lethal H5 influenza virus by either pCMV/H5 or inactivated whole-virus vaccine

Vaccine (dose)	Challenge virus	No. of survivors/ no. tested[a]	Virus shedding at 3 days postinfection in:			
			Trachea		Cloaca	
			No. of chickens shedding/total no.	Mean virus titer[b]	No. of chickens shedding/total no.	Mean virus titer[b]
DNA (pCMV-HA) (10 μg)	Ty/Ire/83	9/9	0/9	<1	0/9	<1
	Ck/Penn/83	10/10	0/10	<1	0/10	<1
	Ck/Quert/95	9/10[c]	0/9	<1	0/9	<1
DNA controls (pCMV) (10 μg)	Ty/Ire/83	0/10	8/10	3.6	2/10	2.8
	Ck/Penn/83	0/10	10/10	3.1	8/10	2.5
	Ck/Quert/95	0/10	10/10	3.3	9/10	3.1
Inactivated whole virus (Ty/Ire/83) (10 μg of HA)	Ty/Ire/83	8/10[c] (1)	1/9	2.8	0/9	<1
	Ck/Penn/83	9/10[c]	0/9	<1	0/9	<1
	Ck/Quert/95	10/10	0/10	<1	0/10	<1

[a] Numbers in parentheses denote surviving chickens that became sick but recovered.
[b] The swabs were suspended in 1 ml of isolation medium, and the mean virus titer is expressed as $\log_{10}$ 50% egg infective doses per milliliter.
[c] Chickens died 2 days postinfection, and samples were not collected from these birds.

two DNA injections, 100% of the DNA-vaccinated chickens were protected against homologous challenge and 95% of the DNA-vaccinated chickens were protected against two H5 variants that otherwise cause lethal infection in chickens. This result was at least as good as that obtained with a standard whole-virus vaccine. The cross-protection induced against challenge with antigenic variants that differed from the primary antigen by 11 to 13% is significant considering that a 20 to 25% difference in amino acid sequence homology between influenza viruses constitutes a new subtype. The extent of protection correlated well with the amount of HA DNA administered, whether a pCMV or chicken pβactin promoter construct was used.

How does DNA vaccination confer protection against influenza virus? Neutralization of influenza virus to prevent infection requires antibodies to the HA molecule (9, 29, 36). In both our current and previous studies (10, 29), there were no detectable prechallenge antibodies to influenza virus antigens in DNA-immunized chickens. The inactivated vaccine (10 μg of HA protein) also induced very low levels of HI antibodies (GMT, <42), suggesting that generation of primary antibodies requires high concentrations of antigen. Nevertheless, at 10 days postchallenge, we noted very high antibody titers (GMT, 600 to 800) that were associated with complete protection from lethal virus challenge. This observation indicates a large protective contribution from the B-cell memory response, in keeping with the recognized role of B cells in mediating the immune defenses against influenza virus infection (11).

Since the HA protein is not secreted by cells, how might HA DNA elicit a strong B-cell memory response? In the particle bombardment method, cells of the dermis and epidermis are transfected by direct penetration of the DNA-coated gold beads. The dendritic cells in these layers of skin cells could incorporate the DNA, synthesize HA protein, and initiate an immune response by transporting the antigen intact to B cells as part of their antigen-presenting function. In this manner, a small number of B cells primed with antigen could become memory cells and generate robust antibody levels after challenge. In contrast, intramuscular injection results in the uptake of DNA and antigen expression by muscle cells and the induction of a protective immune response possibly mediated by transfer of antigen from muscle cells to antigen-presenting cells (34).

In DNA-vaccinated mice, memory responses develop at the site of virus infection, with lung-associated lymphoid tissue containing more than 90% of all HA-specific antibody-producing cells during the early stages of infection (14). The immune response increased for 2 to 3 months in mice inoculated with a gene gun, indicating that responses to this type of inoculation

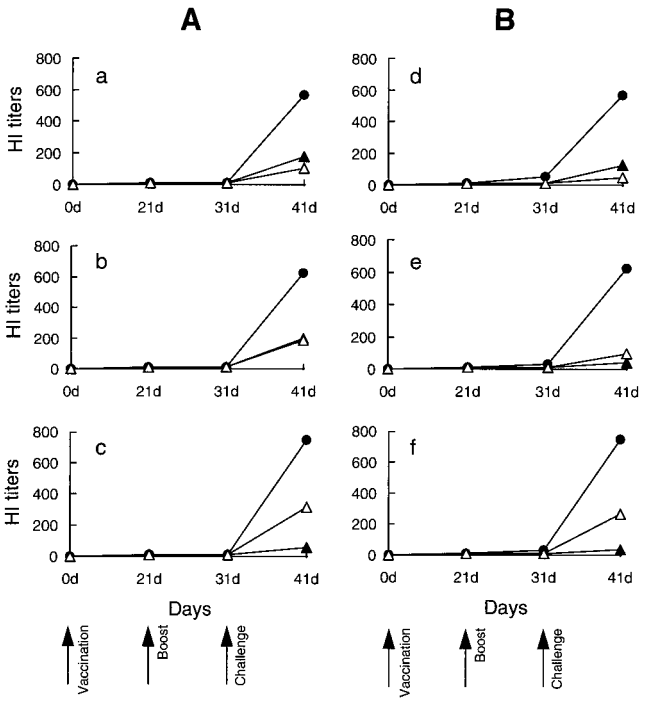

FIG. 3. Antibody titers in chickens immunized with DNA-HA or inactivated whole-virus vaccine and challenged with antigenic variants. The data are the geometric means of the reciprocal of the final dilutions of pooled sera positive for inhibition. Serum samples were collected on day 21 (3 weeks after primary immunization), day 31 (10 days after boosting), and day 41 (10 days postchallenge). (A) DNA vaccines; (B) inactivated whole-virus vaccine; (a and d) Ty/Ir/83 challenge; (b and e) Ck/Penn/83 challenge; (c and f) Ck/Quert/95 challenge. Solid circles, HI titers to Ty/Ire/83 antigen; solid triangles, HI titers to Ck/Penn/83 antigen; open triangles, HI titers to Ck/Quert/95 antigen. Details of the vaccination trials and challenge are given in Materials and Methods.

can come up slowly and then persist for a long time (28). The site of memory B-cell localization in chickens is not clearly understood. In contrast to its confinement to the respiratory tract in mice, highly pathogenic H5- and H7-mediated influenza is a systemic disease in chickens, suggesting that memory B cells may be localized in all lymphoid tissues of this species.

Antigenic determinants of the H5 variants. High levels of postchallenge antibody response to the priming antigen were observed in both the inactivated and DNA-immunized chickens, irrespective of the challenge antigen. This recall memory response to the priming antigen, in which the response to challenge depends not only on the nature of the challenge antigen but also on its antigenic relatedness to priming antigen, has been termed "original antigenic sin." Our previous studies (15, 19) indirectly addressed this issue and showed that close antigenic relatedness between the priming and challenging antigen is responsible for the greater immunogenicity of challenge antigen if it is very closely related to the primary antigen (19). Whatever the explanation, vaccination with Ty/Ire/83 DNA is sufficient to induce protective cross-reacting antibodies against antigenic variants.

Immunity induced by DNA vaccines is at least as good as that afforded by inactivated vaccines. Few published studies have addressed the range of protection induced by DNA vaccines compared with conventional inactivated virus vaccines (6, 14). Donnelly et al. (6) demonstrated in ferrets that DNA vaccines encoding the HA and internal proteins are more effective against antigenic variants than is either inactivated or subunit vaccine. Other investigators, using a murine model to analyze the effects of vaccine or antibody-producing B cells, found fewer antigen-specific B cells in mice vaccinated with subunit vaccine (purified HA) than in those vaccinated with live virus or DNA (14). In our study, the induction of cross-protection against antigenic variants was not unique to the DNA vaccine; rather, the kinetics of protection against homologous and antigenic variants were similar whether DNA or conventional vaccine was used. However, based on analysis of postchallenge antibody responses (41 days), the DNA vaccine induced slightly higher levels of cross-reacting antibodies against antigenic variants than did the formalin-inactivated vaccine, suggesting a difference in the priming of cross-reacting T and B cells by these two preparations. The differences in priming of B or T lymphocytes by these two preparations could be due to (i) in vivo antigen synthesis and better antigen presentation with DNA vaccines or (ii) loss of immunogenicity due to denaturation resulting deformation of epitopes on live virus by formalin treatment.

Relation of the promoter to DNA vaccine efficacy. Studies have shown that proper selection of a promoter/enhancer element to drive the expression of transferred genes is critical to successful gene delivery. This choice depends upon both the target cell type and the functional design of the vector construct (13). We therefore tested two different vector constructs to reduce the variability in the magnitude of immune responses previously seen with the use of DNA vaccines in chickens (10, 29). Chicken β-actin is a major component of the cytoskeleton and one of the most abundant proteins in many cell types (26). Since the rate of transcription of an abundantly expressed gene is very high (21), the β-actin gene promoter is probably a potent transcriptional initiator in chicken cells and could be expected to enhance the expression of the HA gene. This prediction was not substantiated by results of the present study. There was no statistically significant difference in the protection obtained by these two vectors at each dose level, indicating that at least with regard to the influenza virus HA protein, these promoters are equably suitable for a DNA vaccine. However, this does not necessarily indicate that these two vectors induce similar levels of protein expression in vivo. Higher levels of antigen expression may not necessarily mean stronger immune responses, because the induction of immune responses also depends on the induction of cytokines like alpha/beta interferon and interleukin-12 due to immunostimulatory sequences in the DNA of plasmid (30).

Reproducibility of the immune response induced by HA gene immunization. In our previous studies, the pCMV vector encoding the HA of an H7 influenza virus yielded variable protection in chickens (9, 29). A possible reason for this result may be irreproducibility of saline inoculations. In the present study, gene gun inoculation of low doses of DNA encoding an H5 HA reproducibly conferred complete protection against an otherwise lethal challenge.

Need for protection against H5N2 infection in chickens. Pathogenic avian influenza viruses of the H5 and H7 subtypes emerge at irregular intervals and can decimate concentrations of poultry (7). The feasibility of using a vaccine to prevent virus spread will depend on the availability of preparations that induce durable protective immunity. Although available for restricted use, inactivated vaccines are not standardized in terms of HA content (38), and they generate antibodies to cross-reactive antigens such as nucleoprotein, precluding the detection of virus in the flock. Destruction of entire flocks, as practiced in the past in the United States (12), will effectively control avian influenza virus infection; however, this strategy is not considered tenable under conditions of widespread infection as in Mexico. Since the avian H5N2 virus is still active in Mexico in 1997 and could spread rapidly to the United States, a vaccine that would protect chickens from lethal infection and prevent the spread of virus is urgently needed.

Vaccination of chickens with DNA would offer a number of advantages over immunization with whole inactivated virus. DNA vaccines are easier to manufacture than inactivated vaccines and prevent the spread of infection more effectively. In our study, 13% of chickens vaccinated with inactivated whole-virus vaccine shed virus after challenge, compared with only 6% of DNA-vaccinated chickens. Furthermore, plasmid DNA is not subject to the same selective pressures that cause antigen changes among in vitro-propagated viruses and would therefore be expected to yield highly reproducible results. Most important are the simplicity and relative ease with which one can manipulate the delivery system; thus, it may be possible to improve future generations of vaccines by incorporating additional genes against multiple subtypes (e.g., H4, H5, and H7) into the plasmid.

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