Protection of Chickens from Infectious Bronchitis by In Ovo and Intramuscular Vaccination with a DNA Vaccine Expressing the S1 Glycoprotein

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Protection of Chickens from Infectious Bronchitis by In Ovo and Intramuscular Vaccination with a DNA Vaccine Expressing the S1 Glycoprotein

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SUMMARY. We have constructed a DNA vaccine (pDKArkS1-DPI) expressing the S1 glycoprotein (Arkansas DPI) of infectious bronchitis virus (IBV) to examine protective immunity after *in ovo* and intramuscular DNA immunization. Birds receiving *in ovo* DNA followed by live virus vaccination at 2 wk of age were 100% protected from clinical disease. Birds receiving only live virus vaccine or only *in ovo* DNA vaccination were ≤80% protected. IBV was detected up to 10 days postchallenge in unvaccinated control groups, whereas birds receiving *in ovo* DNA and live virus vaccination cleared IBV from tracheal samples before day 5 postchallenge. Transcription of the S1 gene was confirmed in lung tissue after *in ovo* vaccination by an antisense riboprobe, and the S1 protein was detected by immunohistology in the heart and bursa. In a separate experiment, birds were injected intramuscularly with either 50, 100, or 150 μg of the DNA vaccine at 1 day of age and then again with either 100, 200, or 300 μg of the DNA vaccine, respectively, at 14 days of age. At 10 days postchallenge, no clinical signs were observed and no challenge virus was reisolated from the birds vaccinated with 150 μg and 300 μg of DNA. Between DNA-vaccinated birds and nonvaccinated control birds, no statistical differences were observed for IBV-specific serum antibodies as detected by enzyme-linked immunosorbent assay or the virus neutralization test. These data indicate that DNA vaccination with the S1 gene either *in ovo* or intramuscularly can provide birds with some protection against clinical disease after homologous IBV challenge.

RESUMEN. Protección contra la bronquitis infecciosa mediante la vacunación in ovo o por vía intramuscular con una vacuna de ADN expresando la glicoproteína S1.

Se construyó una vacuna de ADN (pDKArkS1-DPI) expresando la glicoproteína S1 de la cepa Arkansas DPI del virus de bronquitis infecciosa con el fin de examinar su inmunidad protectora después de la inoculación *in ovo* y por vía intramuscular. Se observó una protección del 100% contra la enfermedad clínica en las aves que recibieron la vacuna de ADN *in ovo* seguida por una vacunación a virus vivo a las dos semanas de edad. Se observó una protección menor o igual al 80% en las aves que recibieron únicamente la vacuna a virus vivo ó la vacuna de ADN *in ovo.* En las aves no vacunadas de los grupos control se detectó el virus de bronquitis infecciosa hasta los 10 días posteriores al desafío, mientras que las aves que recibieron la vacuna de ADN *in ovo* y la vacuna a virus vivo eliminaron el virus de bronquitis infecciosa de las muestras de tráquea antes de los 5 días posteriores al desafío. Se confirmó la transcripción del gen S1 en el tejido pulmonar después de la vacunación *in ovo* mediante una ribosonda complementaria y se detectó la proteína S1 en el corazón y la bolsa de Fabricio mediante inmunohistología. En un experimento diferente se inocularon aves con 50, 100 ó 150 μg de vacuna de ADN al día de edad y nuevamente a los 14 días con 100, 200 y 300 μg, respectivamente. No se observaron signos clínicos ni se aisló el virus de desafío a partir

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de las aves vacunadas con 150 y 300 μg de ADN a los 10 días posteriores al desafío. No se observaron diferencias estadísticamente significantes entre los anticuerpos específicos para el virus de bronquitis infecciosa, detectados mediante las pruebas ELISA o virus neutralización, entre las aves que recibieron la vacuna de ADN y las aves control no vacunadas. Estos datos indican que la vacuna de ADN que contiene el gen S1 administrada *in ovo* o por vía intramuscular puede suministrar a las aves alguna protección contra la enfermedad clínica ocasionada por el desafío con cepas homólogas del virus de bronquitis infecciosa.

Key words: infectious bronchitis virus, DNA vaccine, S1 gene, *in ovo* vaccination

Abbreviations: CTL = cytotoxic T lymphocytes; DIG = digoxigenin; EID_{50} = 50% embryo infectious dose; ELISA = enzyme-linked immunosorbent assay; HPI = hours postinoculation; IBV = infectious bronchitis virus; IgG = immunoglobulin G; IIF = indirect immunofluorescence; PBS = phosphate-buffered saline; PC = postchallenge; PCR = polymerase chain reaction; RT = reverse transcriptase; S = spike; SDS = sodium dodecyl sulfate; SPF = specific-pathogen free; SSC = standard sodium citrate; TE buffer = 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.6; VN = virus neutralization

Avian infectious bronchitis virus (IBV) is the etiologic agent of infectious bronchitis, which causes an acute, highly contagious upper respiratory tract disease in chickens (6). In addition to respiratory disease, some strains of IBV cause nephritis and urolithiasis. IBV is distributed worldwide and is of considerable economic importance to the poultry industry (6).

IBV is the prototype virus of the genus *Coronavirus* of the family *Coronaviridae*. The genome of IBV is comprised of a single-stranded RNA molecule of positive polarity (14). IBV contains four structural proteins: spike (S), membrane, small membrane, and nucleocapsid proteins. The spikes of IBV are composed of two glycopeptides, S1 and S2. The S1 glycopeptide is found at the distal end of the spike protein and contains both virus-neutralizing and hemagglutination-inhibiting antibody epitopes (4,5,16). Attachment to host cells is also associated with S1.

Vaccination with either attenuated live viruses or killed vaccines is the only means to control the disease. However, many different serotypes of IBV are present in the field, and for the most part, different serotypes do not cross protect. In addition, the attenuated vaccines can cause severe upper respiratory tract reactions especially in 1-day-old birds. These vaccines also can revert to pathogenicity, and there is a potential for the formation of variant viruses by recombination with field strains (1,6,9,17).

The purpose of this study was to determine the potential of a DNA vaccine to protect chickens against IBV. DNA vaccines represent a novel approach to controlling infectious diseases, and numerous studies have indicated the effectiveness of these vaccines (7,8,18,19,27, 29). Typically, DNA vaccines are bacterial plasmids that contain the genetic sequence of an immunogenic protein. They are not infectious and do not replicate in the bird. After uptake of the DNA vaccine, the protein is expressed by host cells and presented to the immune system for processing. An advantageous feature of DNA vaccines is their ability to elicit both humoral and cell-mediated immune responses (2, 3,7,8,15,19,27,29). In this study, a DNA vaccine containing the S1 glycoprotein gene of the Arkansas serotype of IBV was administered to chickens either *in ovo* or intramuscularly, and protection against the homologous serotype was examined.

MATERIALS AND METHODS

Virus and cells. The pathogenic Arkansas (Ark) DPI strain of IBV was used in this study and was propagated by inoculating the allantoic sac of 9-to-11-day-old chicken embryos (10). Allantoic fluid was harvested 48 hr postinfection and was used for virus challenge and virus neutralization tests. African green monkey kidney cells (COS-1) obtained from American Type Culture Collection (ATCC CRL1650; Rockville, MD) were used for *in vitro* transfection studies and were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD).

Chickens and embryonated eggs. Fertile specific-pathogen-free (SPF) eggs and SPF white leghorn chickens were obtained from Merial Laboratories (Gainesville, GA). Chicks were maintained in positive-pressure Horsfall–Bauer-type isolation units lo-

cated at the Poultry Diagnostic Research Center, University of Georgia (Athens, GA). Chickens were given feed and water *ad libitum.*

Cloning and sequencing of the S1 glycoprotein gene. The reverse transcriptase (RT)–polymerase chain reaction (PCR) was used to amplify the S1 glycoprotein gene from viral genomic RNA (23). The following PCR primers were used: IBVNPVS15′ (5′-TTAGATCT-ATGTTGGTAACACCTCTTTTACTA-3′) containing the ATG start codon for the S1 gene (underlined) and IBVNPVS13′ (5′-TTGGTACCTCATGTTCCATTA-GTGATTTTGATGTA-3′), which contains a UGA stop codon (underlined). The UGA stop codon is located at the S1/S2 cleavage site. PCR product was cloned with the TA cloning® kit (Invitrogen, San Diego, CA) according to the manufacturer's recommendations. One ampicillin-resistant clone designated pMJTAS1Ark was selected and subjected to sequence analysis with the Ready Reaction Dye Terminator Ampli-Taq kit (Perkin Elmer, Foster City, CA).

Construction of the plasmid pDKArkS1-DPI. The S1 gene from pMJTAS1Ark was obtained by digestion with *Hin*dIII-*Eco*RV and ligated into the *Hin*dIII-*Eco*RV sites of the pcDNA 3.1(+) vector (Invitrogen) under control of the CMV immediate early promoter to create plasmid pDKArkS1-DPI (Fig. 1). The integrity of the cloned S1 gene was determined by restriction enzyme analysis and automated nucleic acid sequencing as described above. Plasmid DNA was grown in *Escherichia coli* INVaF′ and purified on Qiagen, Inc. (Chatsworth, CA) plasmid purification columns. DNA concentrations were determined by spectrophotometry at 260 nm (23).

S1 glycoprotein gene expression. Expression of the S1 glycoprotein from plasmid pDKArkS1-DPI was demonstrated in COS-1 cells. Briefly, COS-1 cells were grown on 18-mm-round glass coverslips (Hampton Research, Laguna Hills, CA) until approximately 70% confluent, then transfected with 5 μg of pDKArkS1-DPI in 30 μl of SuperFect Transfection Reagent (Qiagen, Inc.) according to the manufacturer's recommendations. After incubation for 24–48 hr, cells were fixed in ice cold acetone, allowed to air dry, and reacted with polyclonal antisera produced against IBV Arkansas DPI diluted (1:250) in phosphate-buffered saline (PBS). The cells were washed with PBS and reacted with fluorescein-labeled rabbit anti-chicken immunoglobulin G (IgG) (Sigma Chemical Co., St. Louis, MO) diluted 1:1000. Cells were washed again as above, air dried, and mounted on microscope slides with 50% glycerol in sterile distilled water.

Construction of digoxigenin (DIG)-labeled riboprobe. The S1 gene from the Arkansas DPI strain of IBV was RT-PCR amplified as previously described (11) and cloned into the pcDNA 3.1(−) vector (Invitrogen) in reverse orientation to construct the plasmid pDKArkS1-rev (13). A 1700 nucleotide antisense S1 DIG-labeled riboprobe was prepared by digesting pDKArkS1-rev with *Hin*dIII followed by phenol-chloroform-ethanol extraction and *in vitro* transcription with the T7 RNA polymerase DIG-labeling kit (Roche, Indianapolis, IN). The riboprobe concentration was determined by dot blot comparison with a known standard DIG-labeled RNA (Roche).

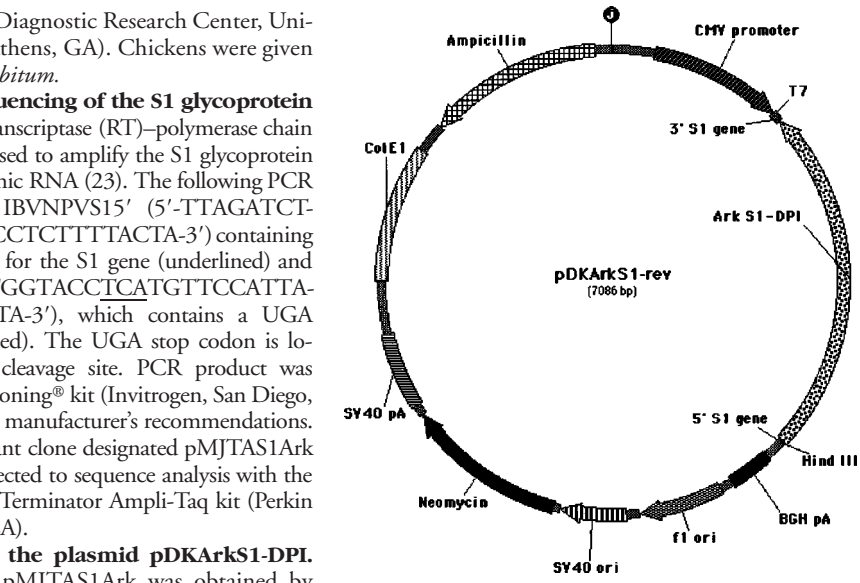

Fig. 1. A gene map of pDKArkS1-DPI used in this study. CMV promoter: human cytomegalovirus immediate-early promoter; Ark S1-DPI: IBV Arkansas DPI S1 gene; BGH pA: bovine growth hormone transcription and processing signals; SV40 ori: Simian virus 40 promoter and origin; neomycin: neomycin resistance gene; SV40 pA: simian virus 40 polyadenylation signals; ColE1: *E. coli* origin of replication; ampicillin: ampicillin resistence gene.

***In ovo* DNA immunization and challenge.** Embryonating eggs were inoculated on the 18th day of embryonation with 0.2 ml of pDKArkS1-DPI (see below for DNA concentrations) or sterile TE buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.6). Embryo vaccination was performed as described previously (28). Briefly, the large end of the egg was cleaned with 70% ethanol, a small hole was made with a 16-gauge needle, through which the vaccine was delivered into the allantoic sac with a 1.5-inch 26-gauge needle.

One hundred eighty embryonating eggs were randomly separated into nine equal groups and vaccinated as described in Table 1. Embryos in groups 7, 8, and 9 received 300 μg of DNA mixed with 1.5 mg/ml of an activated dendrimer molecule (SuperFect™; Qiagen, Inc.) that promotes uptake of DNA by eukaryotic cells. At 2 wk of age, birds in groups

Table 1. Experimental procedure for *in ovo* vaccination and challenge with homologous Arkansas IBV.

Group	*In ovo* vaccine[A]	Secondary vaccine[B]	Challenge[C]
1	TE buffer	None	No
2	TE buffer	None	Yes
3	TE buffer	Live virus	Yes
4	DNA[D]	None	No
5	DNA[D]	None	Yes
6	DNA[D]	Live virus	Yes
7	DNA + SuperFect™[E]	None	No
8	DNA + SuperFect™[E]	None	Yes
9	DNA + SuperFect™[E]	Live virus	Yes

[A]*In ovo* vaccination at embryonation day 18.

[B]Live attenuated Arkansas DPI IBV vaccine (Merial Laboratories, Gainesville, GA) administered at 2 wk of age.

[C]Intratracheal challenge with 5.5×10^5 EID_{50}/bird pathogenic Arkansas DPI IBV 2 wk after secondary vaccination.

[D]300 μg pDKArkS1-DPI per embryo.

[E]300 μg pDKArkS1-DPI in 1.5 mg/ml SuperFect™ per embryo.

3, 6, and 9 were given a secondary vaccination with live attenuated Arkansas DPI vaccine (Merial Laboratories). Then, birds in groups 2, 3, 5, 6, 8, and 9 were challenged intratracheally with 5.5×10^5 50% embryo infectious dose (EID_{50})/bird with pathogenic IBV Arkansas DPI at age 28 days as indicated in Table 1. Birds were necropsied 5, 10, and 21 days after virus challenge. At necropsy, the birds were weighted, clinical signs (tracheal rales or airsacculitis) were recorded, tracheal swabs were taken for virus isolation, and serum was collected. In addition, the middle third of the trachea, below the area swabbed, was fixed in 10% neutral buffered formalin for histopathology.

Detection of S1 gene expression. The following organs were harvested from embryos at 24 and 48 hr postvaccination and fixed in 10% buffered formalin: lung, heart, thymus, spleen, and bursa. After 24 hr in formalin, tissues were placed in 50% ethanol in water and stored at 4 C until embedded in paraffin. Formalin-fixed, paraffin-embedded tissues were sectioned (4–6 μm) and placed on either silicon-charged slides for *in situ* hybridization or untreated slides for immunohistology (13). Positive control tissue was obtained by inoculating embryos with 100 EID_{50} of pathogenic Arkansas DPI IBV via the chorioallantoic sac and processing as above.

In situ hybridization. Paraffin sections were deparaffinized in Hemo-De (Fisher Scientific, Pittsburgh, PA), rehydrated in 0.2 M Tris, pH 7.6, with 0.1 M glycine, and digested with 30 μg/ml proteinase K for 15 min at 37 C. Tissues were prehybridized at 37 C for 1 hr in 50% formamide, 5× standard sodium citrate (SSC), 5% blocking reagent (Boehringer-Mannheim), 1% N laurylsarcosine, and 0.02% sodium dodecyl sulfate (SDS). After prehybridization, approximately 15 ng of riboprobe in 100 μl prehybridization buffer was added to each slide, which was then covered with a siliconized coverslip and sealed with nail hardener. Hybridization was carried out overnight at 42 C. All incubations were carried out in a humidified chamber unless otherwise noted. After hybridization, samples were washed at 50 C and room temperature with decreasing concentrations of SSC and SDS. Unbound protein binding sites were blocked with 5% blocking reagent (Boehringer-Mannheim, Mannheim, Germany) and incubated with a 1:300 dilution of alkaline phosphatase–labeled goat anti-DIG antibody in 1% sheep serum for 2 hr at 37 C. Sections were then developed with substrate (5-bromo-4-chloro-3-indoylphosphate) and chromogen (nitroblue tetrazolium) (Boehringer-Mannheim) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ with 5 mM Levamisol (ICN, Irvine, CA) for 90 min at room temperature. The slides were counterstained with hematoxylin and coverslipped in Permount (Fisher Scientific) for permanent record.

Immunohistology. Samples for immunohistology were deparaffinized and rehydrated as described above. Unbound protein binding sites were blocked by incubation for 2 hr with 3% bovine serum albumin in PBS in a humid chamber at 37 C. Hyperimmune polyclonal antisera produced by our laboratory in chickens against the Arkansas DPI strain of IBV was diluted (1:250) in blocking buffer and incubated with sections for 1.5 hr at 37 C. After washing as above, tissues were incubated with a 1:500 dilution of alkaline phosphatase–labeled goat anti-chicken IgG antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in 1% sheep serum for 2 hr at 37 C. Substrate and chromogen (as described above) were added, and development progressed for 90 min at room temperature.

DNA vaccination of 1-day-old chickens and challenge. One-day-old SPF leghorn chicks were randomly separated into six groups of 40 birds each. All vaccinations were given as intramuscular injections into the thigh region of the bird except in group 3, which was vaccinated as described below. Birds in group 1 received injection with sterile TE buffer at 1 and 14 days of age. Birds in group 2 received injections containing 150 μg and 300 μg of the vector DNA without the S1 gene on day 1 and day 14, respectively. The birds in group 3 were vaccinated with a live commercial IBV vaccine (Arkansas DPI serotype) as per the manufacturer's recommendations (Merial Laboratories). Groups 4, 5, and 6 were injected with either 50 μg, 100 μg, or 150 μg of pDKArkS1-DPI at 1 day of age and then again at 14 days of age with 100 μg, 200 μg, or 300 μg of pDKArkS1-DPI, respectively. Twenty of the birds in each group were challenged intratracheally with 5.5×10^5 EID_{50}/bird with pathogenic IBV Ark DPI at 28 days of age, whereas the other 20 were held as nonchallenged groups.

Five birds from virus-challenged and nonchallenged groups were necropsied at 33, 38, 42, and 49 days of age, which corresponded to 5, 10, 14, and 21 days after virus challenge. At necropsy, the birds were weighed, clinical signs (tracheal rales or airsacculitis) were recorded, tracheal swabs were taken for virus isolation, and serum was collected. In addition, the middle third of the trachea, below the area swabbed, was fixed in 10% neutral buffered formalin for histopathology.

Virus isolation. Tracheal swabs were tested for IBV by the RT-PCR–based DNA diagnostic test as described (11). Briefly, tracheal swabs taken from each bird in each group on each necropsy day were pooled, filter sterilized, and inoculated into embryonating eggs at 9–11 days of incubation. Allantoic fluid from inoculated eggs was tested for the presence of IBV by RT-PCR. Samples were considered negative for IBV if two passages in 9-to-11-day-old embryonating eggs did not yield a positive result.

Serology. Serum was collected from all birds on each necropsy day and tested individually for antibodies to IBV by enzyme-linked immunosorbent assay (ELISA) (IDEXX, Portland, ME). Statistical analysis of the mean ELISA titers was performed by Tukey–Kramer HSD comparisons with the JMP® (SAS Institute, Inc., Cary, NC) statistics software. Measurement of virus-neutralizing antibodies was performed as previously described with slight modifications (10). For *in ovo* vaccination, sera from each group were equally pooled, serially diluted, and mixed 1:1 with Arkansas DPI IBV (2×10^2 EID_{50}) in a microfuge tube. Sera from birds vaccinated at 1 day of age were individually tested. Serum and virus were allowed to incubate together for 1 hr at room temperature before inoculation into four 9-to-11-day-old SPF embryonating eggs via the chorioallantoic sac. Embryos were incubated at 37 C for 7 days and examined for typical IBV lesions (curling, stunting, or death). Virus-neutralization titers were determined by the method of Reed and Muench (22).

Histopathology. Tracheal tissue samples fixed in 10% formalin were routinely processed to paraffin cut into 5-μm sections and stained with hematoxylin and eosin for histopathologic examination. The following tracheal lesions, epithelial hyperplasia, lymphocyte infiltration, and severity of epithelial deciliation were scored from 1 to 4 with 1 being normal; 2, focal (1%–29%); 3, multifocal (30%–60%); and 4, diffuse (>60%). Statistical analysis of the data by Tukey–Kramer HSD comparisons was performed with the JMP® (SAS Institute, Inc.) statistics software.

RESULTS

S1 gene expression in COS cells. After transfection of COS-1 cells with the pDKArkS1-DPI DNA vaccine, the S1 glycoprotein was visualized by indirect immunofluorescence (IIF) with hyperimmune polyclonal antisera produced against the Ark DPI serotype of IBV (Fig. 2). COS-1 cells transfected with vector DNA without the S1 gene and cells alone tested negative by IIF.

Protective immunity conferred after *in ovo* DNA vaccination. Chicken embryos were vaccinated with a DNA vaccine containing the S1 gene of Arkansas DPI IBV or TE buffer, followed at 14 days of age by a live Arkansas vaccine or TE buffer. After homologous challenge with pathogenic Arkansas DPI virus, birds were examined for clinical signs of IBV infection 5, 10, and 21 days postchallenge (PC). As expected, all nonchallenged groups were negative for clinical signs of IBV infection (groups 1, 4, and 7). Birds receiving only TE vaccinations (group 2) were 50% and 60% protected 5 and 10 days PC, respectively (Table 2). However, when a live vaccine was administered at 14 days of age after *in ovo* vaccination with buffer (group 3), birds were 80% and 100% protected 5 and 10 days PC, respectively. All birds receiving pDKArkS1-DPI *in ovo* followed by live virus vaccine at 14 days of age (groups 6 and 9) were 100% protected from clinical disease. Birds receiving only *in ovo* DNA vaccination without a secondary vaccination at 14 days of age (groups 5 and 8) were ≤40% protected on day 5 and between 75% and 83%

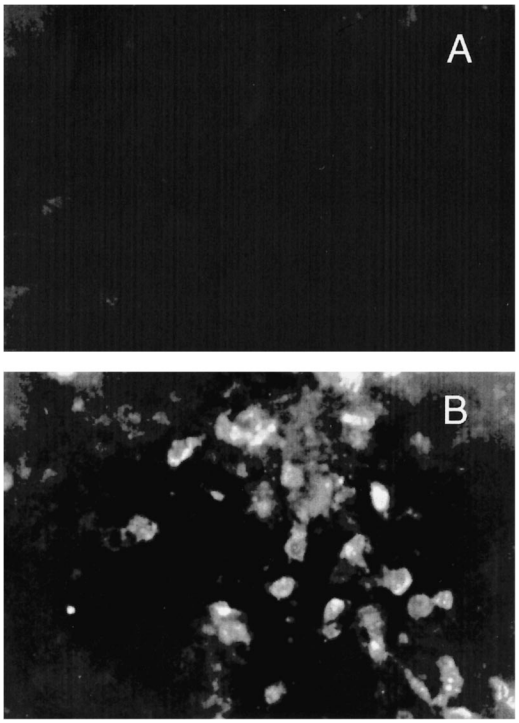

Fig. 2. Immunofluorescent antibody staining of COS-1 cells transfected with plasmid DNA. Cells were either transfected with (A) 5 μg of vector DNA without S1 insert or (B) 5 μg PDKArkS1-DPI. See Materials and Methods for details.

protected 10 days PC. No clinical signs were observed in any group after 10 days PC.

Virus isolation. All nonchallenged groups (1, 4, and 7) were negative for IBV by RT-PCR (Table 2). All virus-challenged groups protected 100% from clinical disease (groups 6 and 9) were negative for IBV on all days tested. Birds that received only TE buffer (group 2) were positive for IBV 5 and 10 days PC. At 5 days PC, birds receiving buffer *in ovo* and live virus at 14 days of age (group 3) were positive for IBV. Likewise, birds receiving only *in ovo* DNA vaccination (groups 5 and 8) were positive for IBV 5 days PC but were negative on day 10.

Humoral immune response. In addition to protective immunity, the humoral immune response after *in ovo* DNA vaccination was measured by ELISA and virus-neutralization tests. The mean titers of each group were compared by Tukey–Kramer HSD procedure. As expected, all unchallenged *in ovo*–vaccinated groups (1, 4, and 7) had low ELISA titers on all days tested (Table 3). No statistical difference in ELISA titer was observed between any groups 5 days PC. However, birds receiving both *in ovo* DNA and live virus vaccinations (groups 6 and 9) had the highest titers on that day. Ten days PC, birds in groups 2 and 8 displayed the highest ELISA titers, which were significantly different from groups 1, 3, 4, 6, and 7 ($P \leq$

Table 2. Protective immunity against IBV after *in ovo* vaccination.

Group[A]	*In ovo* vaccine	Secondary vaccine	Clinical signs[B] protected/total (%) 5 days PC	Clinical signs[B] protected/total (%) 10 days PC	Virus detection by RT-PCR[C] 5 days PC	Virus detection by RT-PCR[C] 10 days PC
1	TE buffer	None	5/5 (100)	5/5 (100)	−	−
2	TE buffer	None	2/4 (50)	3/5 (60)	+	+
3	TE buffer	Live virus	8/10 (80)	9/9 (100)	+	−
4	DNA	None	5/5 (100)	5/5 (100)	−	−
5	DNA	None	0/3 (0)	3/5 (60)	+	−
6	DNA	Live virus	8/8 (100)	8/8 (100)	−	−
7	DNA[D]	None	5/5 (100)	5/5 (100)	−	−
8	DNA[D]	None	2/5 (40)	5/6 (83)	+	−
9	DNA[D]	Live virus	9/9 (100)	9/9 (100)	−	−

[A]Groups 1, 4, and 7 did not receive IBV challenge.

[B]Challenge by intratracheal inoculation with 5.5×10^5 EID_{50}/bird pathogenic IBV 2 wk after secondary vaccination. No clinical signs were observed in any group after day 10 PC. Values are number protected/total (percentage in parentheses).

[C]Tracheal swabs were pooled from all birds within a group and inoculated in 9-to-11-day-old embryos. After two blind passages, RNA from allantoic fluid was isolated and used for RT-PCR.

[D]DNA plus SuperFect™.

Table 3. Humoral immune response after *in ovo* DNA vaccination determined by ELISA and virus neutralization (VN).

	5 days PC		10 days PC		21 days PC	
Group	ELISA[A]	VN	ELISA[B]	VN	ELISA[A]	VN
1	40	5	22*	<8	23	13
2	10	10	1611	10	514	21
3	328	17	461*	24	653	22
4	1	5	10*	14	16	<8
5	334	13	1270	20	2014	22
6	386	9	261*	<8	391	10
7	1	9	13*	<8	14	<8
8	18	12	1557	8	1141	11
9	373	9	888	18	1125	13

[A]No significant difference between any groups ($P \leq 0.05$).
[B]Groups with asterisk were significantly different from groups 2 and 8 on that day ($P \leq 0.05$).

0.05). Birds in group 5 had the highest ELISA titers 21 days PC. However, no statistical difference was observed between any groups on this day regardless of vaccination route or dose.

Virus-neutralization experiments were performed on sera from each group that had been equally pooled prior to testing. For virus-challenged groups (2, 3, 5, 6, 8, and 9), serum virus-neutralization titers were in general comparable between the groups regardless of vaccine strategy. Chickens in group 3 had the highest serum virus-neutralization titers 5, 10, and 21 days PC (Table 3).

Histopathology. To examine local immunity against IBV, we collected tracheas from birds in each group to compare cilia loss, hyperplasia, and lymphocyte infiltration by histopathology. As expected, all nonchallenged groups had mean tracheal lesion scores of approximately 1.0 on each day tested (Fig. 3). All groups of birds that did not receive secondary vaccination with live virus (groups 2, 5, and 8) had mean tracheal lesion scores ≥2.3, which was significantly higher than all other groups examined both 5 and 10 days after virus challenge ($P \leq 0.05$). There was no significant difference in lesion score between any other groups on those days. Twenty-one days after virus challenge, birds in groups 2 and 5 had mean lesion scores that were significantly higher than lesion scores of groups 1, 3, 7, and 9 ($P \leq 0.05$).

In situ hybridization and immunohistology. In an effort to determine sites of S1 gene transcription and translation after *in ovo* DNA vaccination, we probed spleen, heart, thymus, lung, and bursal tissue with a DIG-labeled S1 antisense riboprobe and anti-Arkansas DPI antibodies, respectively. As expected, no positive staining was observed in any tissue from birds receiving TE buffer *in ovo* (Figs. 4A, 5A,C). With lung tissue from embryos inoculated with IBV, the S1 riboprobe detected virus-infected cells in the epithelium of the primary bronchus of the lung at 24 hr postinoculation (HPI) (Fig. 4B). Staining was localized to the cytoplasm of respiratory epithelial cells. Virus-infected cells were also detected by the S1 riboprobe at 48 HPI in the lungs and bursa (data not shown). After *in ovo* DNA vaccination, *in situ* hybridization detected S1 gene expression in lung tissue 24 HPI (Fig. 4C). Expression was also detected in the lungs at 48 HPI (data not shown). No other tissue tested reacted positively for S1 gene expression. By immunohistology for S1 protein expression, positive stained regions within the bursa and heart of DNA-vaccinated embryos was detected at 48 and 24 HPI, respectively (Fig. 5B,D, respectively).

DNA vaccination of 1-day-old chickens and challenge. Chickens were immunized with a DNA vaccine containing the S1 gene via a dose titration by intramuscular injections at 1 and 14 days of age. After homologous challenge with pathogenic Ark DPI virus, all of the birds in group 6 receiving 150 μg and 300 μg of DNA were protected from the disease on the basis of clinical signs (Table 4). Birds in groups 4 (50 μg and 100 μg DNA) and 5 (100 μg and 200 μg DNA) were 80% protected at 5 days PC (day 33), and no bird in groups 4 and 5 had clinical signs at 10 days PC. Birds vaccinated with a commercial live IBV vaccine

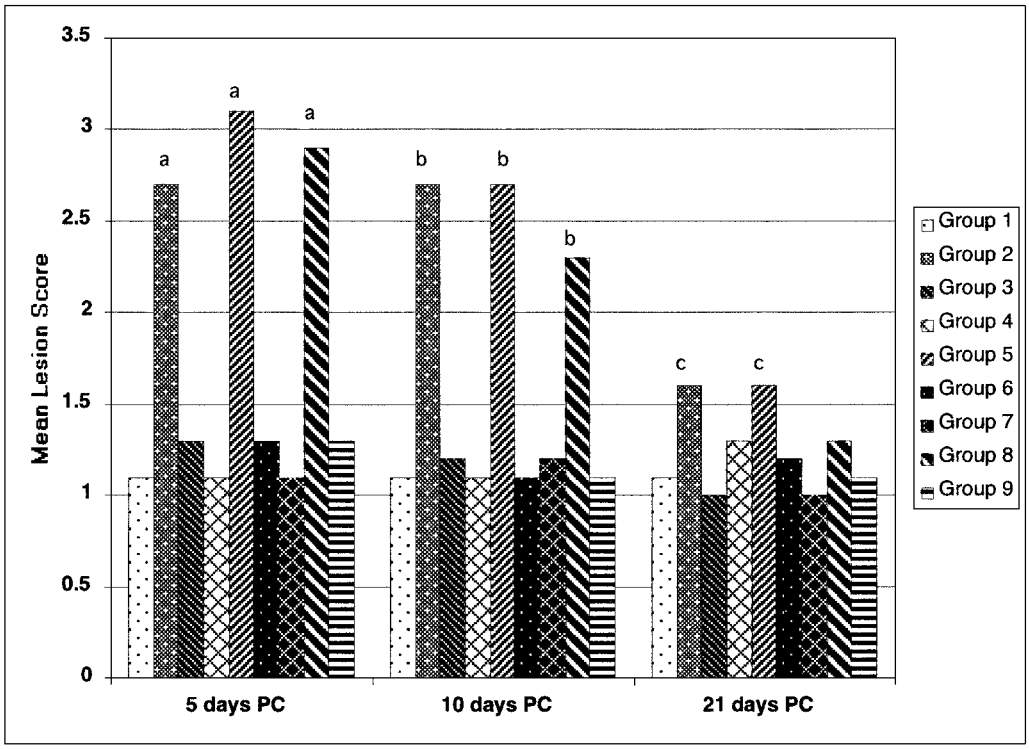

Fig. 3. Histopathologic tracheal lesion scores determined by cilia loss, hyperplasia, and lymphocyte infiltration. Birds received either buffer (groups 1–3) or DNA vaccination (groups 4–9) *in ovo* at day 18 of embryonation, followed by a standard live IBV vaccine at day 14 (groups 3, 6, 9). Birds were challenged with 5.5×10^5 EID_{50}/bird (groups 2, 3, 5, 6, 8, 9). Tracheas were taken for histopathology at 5, 10, and 21 days postchallenge (PC). Mean lesion scores are presented.

(group 3) were protected at similar levels as groups 4 and 5 by the same criteria. None of the birds receiving DNA without S1 (group 2) were protected from clinical signs 5 days PC, and only 20% of the birds were protected after 10 days PC by pooled results. Birds receiving buffer vaccination (group 1) were 40% protected from clinical disease 5 and 10 days PC. No clinical signs were seen in any group after day 38. No difference in body weight was observed between any groups on each day tested (data not shown).

Virus isolation. All groups receiving virus challenge were positive for IBV at day 33 (Fig. 6A). At day 38, only the groups receiving either buffer (group 1) or vector (group 2) were positive for IBV (Fig. 6B). None of the virus-challenged DNA-vaccinated birds (groups 4, 5, and 6) were positive for IBV after day 33. On day 42, two of the groups not receiving virus challenge became positive for IBV (Fig. 6C). None of the groups was positive for IBV at day 49.

Humoral immune response. To determine if DNA vaccination with the S1 gene could induce virus-specific antibodies, the humoral immune response to IBV was measured during the course of the experiment. The geometric mean serum ELISA antibody titers of IBV-challenged groups are given in Table 5. Birds receiving the standard live vaccine (group 3) had the highest antibody titers of any group at any day tested. At 33 and 42 days of age, ELISA titers from birds in this group were statistically higher than any other group ($P \leq 0.05$) on that day (Table 5). Antibody titers were also significantly higher in the standard live vaccine group 3 than any DNA-vaccinated group at days 38 and 49 ($P \leq 0.05$). No statistical difference was observed between groups receiving the standard live vaccine and either buffer (group 1) or vector

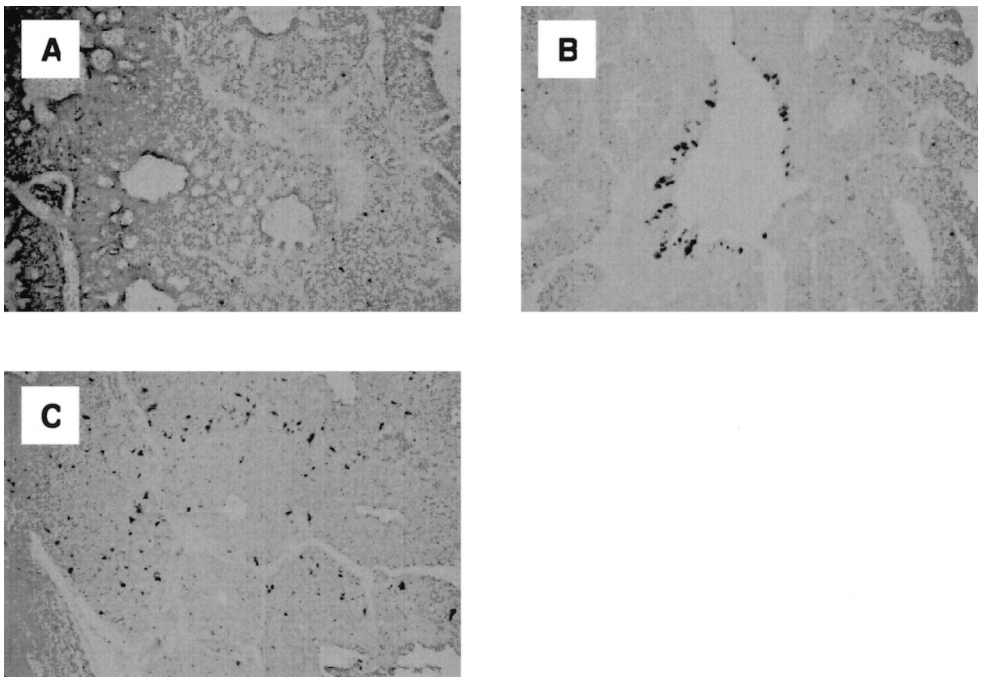

Fig. 4. *In situ* hybridization with a DIG-labeled S1 antisense riboprobe of lung tissue (10×) after either IBV inoculation or *in ovo* DNA vaccination with pDKArkS1-DPI. (A) Control embryo, 24 HPI. (B) IBV-infected embryo, 24 HPI. (C) pDKArkS1-DPI–vaccinated embryo, 24 HPI.

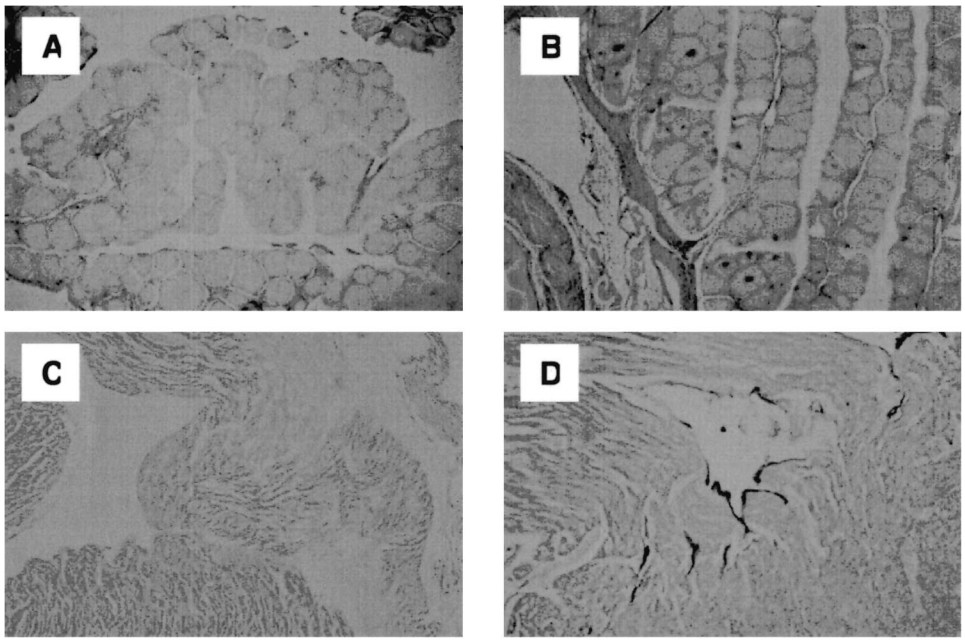

Fig. 5. Immunohistology of bursa and heart after *in ovo* DNA vaccination with pDKArkS1-DPI. (A) Control embryo bursa (10×), 48 HPI. (B) pDKArkS1-DPI–vaccinated embryo bursa (10×), 48 HPI. (C) Control embryo heart (4×), 24 HPI. (D) pDKArkS1-DPI–vaccinated embryo heart (10×), 24 HPI.

Table 4. DNA vaccination and protection of chickens against Arkansas DPI IBV after intramuscular immunization.[A]

Group	Vaccination		Clinical signs[B]	
	1 day of age	14 days of age	Day 33 PC (5)[C]	Day 38 PC (10)
1	TE buffer	TE buffer	3/5	3/5
2	150 μg pcDNA 3.1 (+)	300 μg pcDNA 3.1 (+)	5/5	3/5
3	Live Arkansas vaccine	Live Arkansas vaccine	0/5	1/5
4	50 μg pDKArkS1-DPI	100 μg pDKArkS1-DPI	1/5	0/5
5	100 μg pDKArkS1-DPI	200 μg pDKArkS1-DPI	1/5	0/5
6	150 μg pDKArkS1-DPI	300 μg pDKArkS1-DPI	0/5	0/5

[A]See text for details; data for nonchallenged birds are not presented.
[B]Values are number of birds with tracheal rales/number of birds examined; no clinical signs were observed after day 38.
[C]Number of days PC in parentheses.

(group 2) at 10 and 21 days PC. No significant difference in antibody titer was observed between birds given either buffer or vector (groups 1 and 2) and any DNA-vaccinated birds (groups 4, 5, and 6) at any day tested. Despite the different amounts of pDKArkS1-DPI given DNA-vaccinated groups, no difference was observed in antibody titers between these groups on each day tested.

Serum samples from each group on each necropsy day were equally pooled and tested for virus-neutralizing antibodies in 9-to-11-day-old embryos. Because a wide range of ELISA titers was observed between all groups during this study, we varied the serum dilutions used on days 33 (1:4–1:32), 38 (1:8–1:64), and 42 and 49 (1:16–1:128) in response to the corresponding increase in ELISA titers. Birds receiving the standard live vaccine (group 3) had virus-neutralization titers greater than the highest dilution of serum tested for each day and were equal to or higher than any group tested on that day (Table 5). DNA-vaccinated groups had higher virus-neutralization titers on days 33 and 38 than birds given buffer or vector (groups 1 and 2) despite having lower ELISA titers. By 21 days PC, all groups of birds had become seropositive for IBV.

Histopathology. To determine protection against local infection, we collected tracheas from each bird in each group and examined and scored for cilia loss, hyperplasia, and lymphocyte infiltration by histopathology. Mean lesion scores for all nonchallenged groups were ≤1.5 on each day tested (data not shown). For virus-challenged groups, birds receiving the standard live vaccine (group 3) had mean lesion scores of 2.0 at day 33 and were statistically lower than all other groups on that day ($P \leq 0.05$; Fig. 7). On day 38, statistical differences in lesion scores were observed between groups receiving the standard live vaccine (group 3) or buffer (group 1) and birds given DNA in groups 5 and 6. Birds in group 4 were also significantly different from birds in group 5 on day 38. On days 42 and 49, all virus-challenged groups had mean lesion scores similar to nonchallenged groups.

DISCUSSION

Previous research has indicated *in ovo* vaccination of chickens can lead to protective immunity against a number of avian viral pathogens (12,25,26). However, a majority of these *in ovo* vaccines are attenuated infectious agents by nature with the capability to revert to a virulent state. The results of the present study demonstrate that *in ovo* DNA vaccination can increase protective immunity against IBV. To our knowledge this is the first known report of protective immunity induced by *in ovo* DNA vaccination against IBV. Immunization of 18-day-old embryonating chicken eggs with a DNA vaccine containing the S1 glycoprotein gene from IBV followed by live virus vaccination completely protected birds from virus challenge as determined by clinical signs and virus detection in tracheal samples. A single *in ovo* application of the DNA vaccine was not sufficient for protection against IBV.

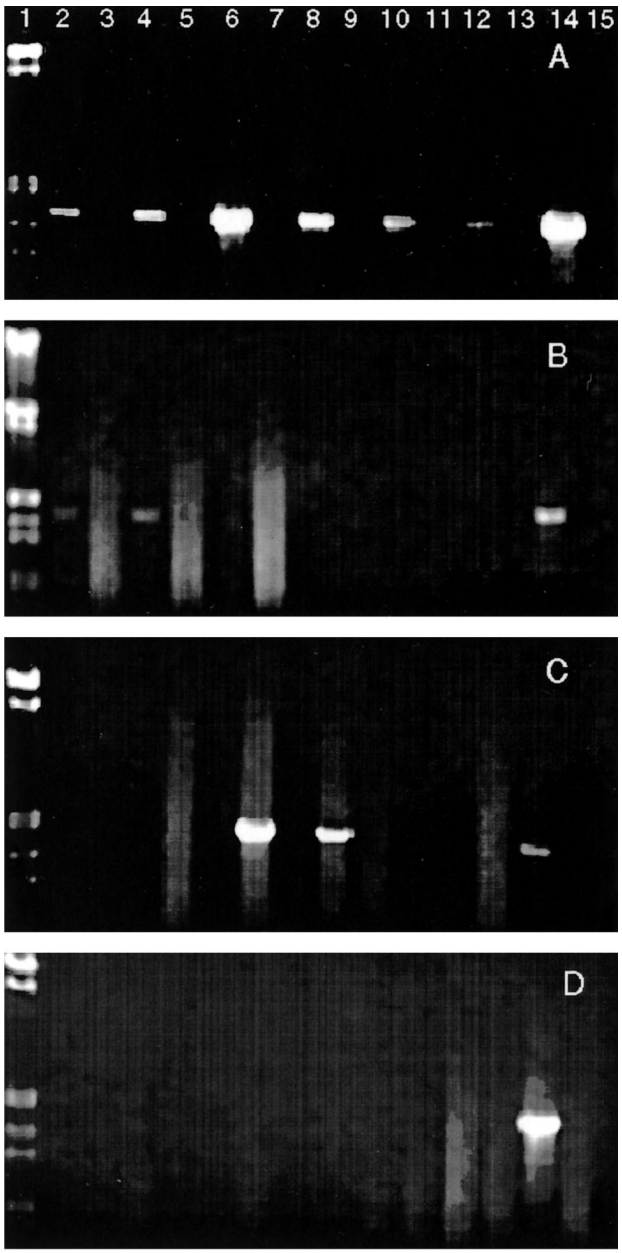

Fig. 6. Results of RT-PCR from tracheal swabs with primers specific for the S1 gene. Days tested: (A) day 33, (B) day 38, (C) day 42, (D) day 49. Lane 1, DNA digested with *Eco*RI/*Hin*dIII; lane 2, buffer virus challenged; lane 3, buffer nonchallenged; lane 4, vector virus challenged; lane 5, vector nonchallenged; lane 6, standard live virus challenged; lane 7, standard live nonchallenged; lane 8, DNA 1 virus challenged; lane 9, DNA 1 nonchallenged; lane 10, DNA 2 virus challenged; lane 11, DNA 2 nonchallenged; lane 12, DNA 3 virus challenged; lane 13, DNA 3 nonchallenged; lane 14, positive virus control; lane 15, negative virus control.

Table 5. Humoral immune response after DNA vaccination determined by IBV ELISA and virus neutralization (VN).[A]

	Day 33		Day 38		Day 42		Day 49	
Group	ELISA[B]	VN	ELISA	VN	ELISA	VN	ELISA	VN
1	84	<4	3289	32	3268	25	2605	>128
2	127	<4	4302	45	1093	<16	2809	>128
3	5487[C]	>32	7642	<64	9595[C]	>128	9407	>128
4	58	18	492[D]	32	3068	>128	2127[D]	>128
5	2	>32	864[D]	40	286	45	853[D]	>128
6	10	23	1504[D]	64	1387	90	4382[D]	>128

[A]See text for details; data for nonchallenged birds are not presented.
[B]Geometric mean titer.
[C]Significantly different from any other group on that day ($P \leq 0.05$).
[D]Significantly different from standard live on that day ($P \leq 0.05$).

Antibody responses after *in ovo* DNA vaccination differed considerably. Without virus challenge, antibody levels from both of the *in ovo* DNA-vaccinated groups were similar to the unchallenged buffer-vaccinated birds. Failure to show an antibody response in DNA-vaccinated birds has been reported previously (18). However, in that study (18), an immune response was induced in those vaccinated birds because they were protected at various levels. Those observations are similar to our results, but still unclear is why antibodies were not detected in DNA-vaccinated birds because, in our study, we detected S1 protein in the bursa at 48 hr post *in ovo* DNA vaccination. Vaccination of chickens with a DNA vaccine containing IBV-specific cytotoxic T lymphocyte (CTL) epitopes has been shown previously to stimulate cross-reactive splenic T cells that could lyse target cells from birds infected with different serotypes of the virus (24). Although not examined in the present study, induction of CTL activity against

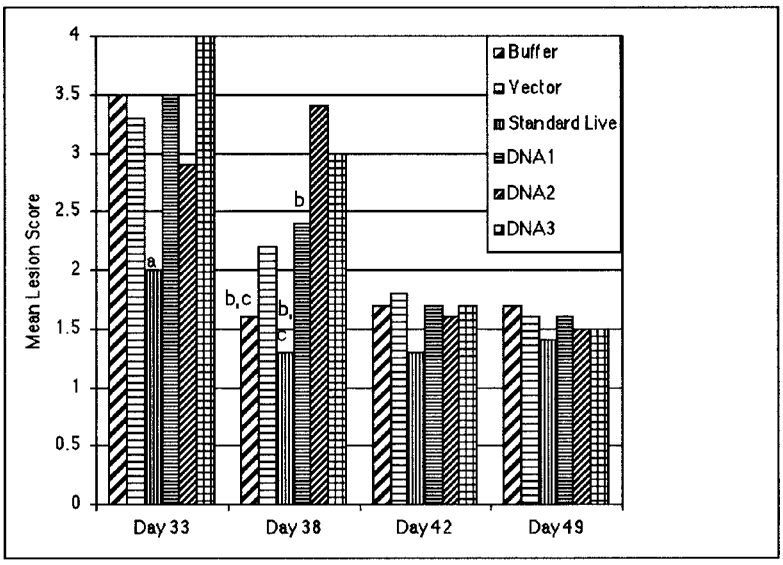

Fig. 7. Mean histopathologic tracheal lesion scores determined by cilia loss, hyperplasia, and lymphocyte infiltration of virus-challenged groups. See text for details. All nonchallenged groups had mean scores <1.5 on each day tested. a = Significantly different from all other groups tested on that day ($P \leq 0.05$). b = Significantly different from DNA 2 on that day ($P \leq 0.05$). c = Significantly different from DNA 3 on that day ($P \leq 0.05$).

IBV by *in ovo* DNA vaccination may have contributed to the protective immunity observed.

Intramuscular injection of a DNA vaccine containing the S1 gene at 1 and 14 days of age induced protection against homologous IBV challenge as determined by clinical signs and virus recovery from the trachea. In addition, birds that received 150 μg at 1 day of age followed by 300 μg at 14 days of age were protected as well as birds receiving the commercial attenuated live vaccine on the same days. Similar to *in ovo* DNA vaccination, why the intramuscular DNA-vaccinated birds had little or no detectable antibody response is unclear. However, we did observe that virus-neutralization titers were generally higher (although not statistically significant) in intramuscular DNA-vaccinated groups when compared with buffer- or vector-vaccinated birds.

The results of the histopathologic studies indicate that *in ovo* or intramuscular DNA vaccination cannot provide local immunity against IBV. To induce a local immune response, it may be necessary to target the vaccine to tracheal tissue. This could be accomplished by incorporating the DNA vaccine into a delivery vehicle that targets mucosal surfaces (i.e., virosomes). Ramani *et al.* (20,21) reported that F-virosomes produced from Sendai virus envelopes containing reporter gene constructs could target liver cells of BALB/c mice *in vivo.* Targeted delivery vehicles could provide precise delivery of DNA vaccines to tissues naturally infected by the disease agent and possibly induce a protective local immune response.

On the basis of our results, it appears that DNA vaccination, when given *in ovo* or intramuscularly, was able to induce an immune response against IBV. When given *in ovo* and used in combination with a standard live virus or when two intramuscular injections were given, protection from clinical disease was observed.

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