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SUMMARY. Infectious bronchitis virus (IBV) is a highly contagious coronavirus prevalent in all countries with an extensive poultry industry and continues to cause economic losses. IBV strains of the Ark serotype are highly prevalent in the Southeastern United States despite extensive vaccination. One explanation for this observation is the high genetic variability of IBV. In addition, IBV Ark-type vaccines may induce suboptimal mucosal immune responses, contributing to the prevalence and persistence of the Ark serotype. To test this hypothesis, chickens were ocularly vaccinated with a commercially available live attenuated IBV Ark–Delmarva Poultry Industry vaccine strain and both mucosal and systemic antibody responses were measured. The highest immunoglobulin A (IgA) spot-forming cell (SFC) response was observed in the Harderian glands (HG) and to a lesser extent in the spleen and conjunctiva-associated lymphoid tissues, while a limited IgG SFC response was observed in either the mucosal or systemic immune compartment. Interestingly, the peak IgA SFC response occurred 2 days earlier in spleen than in the head-associated lymphoid tissues despite ocular vaccination. Furthermore, IgA IBV-specific antibody levels significantly increased over controls 3 days earlier in tears and 4 days earlier in plasma than did IgG antibodies. IgA antibody levels were higher than IgG antibody levels throughout the primary response in tears and were similar in magnitude in plasma. In addition, a very early increase in IgA antibodies on day 3 postvaccination was observed in tears; such a response was not observed in plasma. This early increase is consistent with a mucosal T-independent IgA response to IBV. In the secondary response the IBV antibody levels significantly increased over controls starting on day 1 after boosting, and the IgG antibody levels were higher than the IgA antibody levels in both tears and plasma. In summary, ocular vaccination induced higher IgA antibodies in the primary IBV response, while the memory response is dominated by IgG antibodies. Thus, lower mucosal IgA antibody levels are observed upon secondary exposure to IBV, which may contribute to vulnerability of host epithelial cells to infection by IBV and persistence of the Ark serotype.

RESUMEN. La inmunoglobulina A como una respuesta humoral temprana después de la vacunación en las mucosas con coronavirus aviar.

El virus de la bronquitis infecciosa (IBV) es un coronavirus muy contagioso prevalente en todos los países con una industria avícola extensa y sigue causando pérdidas económicas. Las cepas del virus de bronquitis del serotipo Arkansas son altamente prevalentes en el sureste de los Estados Unidos a pesar de la vacunación extensiva. Una explicación de esta observación es la alta variabilidad genética del virus de bronquitis. Además, las vacunas del tipo Arkansas pueden inducir respuestas inmunitarias subóptimas en las mucosas, que contribuyen a la prevalencia y persistencia del serotipo Arkansas. Para comprobar esta hipótesis, los pollos fueron vacunados ocularmente con una cepa vacunal viva atenuada disponible comercialmente del serotipo Arkansas DPI (Delmarva Poultry Industry) y las respuestas inmunitarias de las mucosas y de anticuerpos sistémicos fueron medidas. El sitio de células formadoras de inmunoglobulina A con mayor producción se observó en la glándula de Harder y en menor medida en el bazo y en los tejidos linfoides asociados a la conjuntiva, mientras que se observó una limitada respuesta de los sitios de células formadoras de IgG, ya sea en la mucosa o en el compartimento inmune sistémico. De manera interesante, el pico de respuesta de los sitios celulares formadores de IgA se produjo dos días antes en el bazo que en los tejidos linfoides asociados con la cabeza a pesar de que la vacunación fue por vía ocular. Por otra parte, los niveles de anticuerpos IgA específicos contra el virus de bronquitis aumentaron significativamente en los controles tres días antes en lágrimas y cuatro días antes en el plasma en comparación con los anticuerpos IgG. Los niveles de anticuerpos IgA fueron más altos que los niveles de anticuerpos de IgG a lo largo de la respuesta primaria en las lágrimas y fueron similares en magnitud en el plasma. Además, un aumento muy temprano en la IgA en el día tres después de la vacunación se observó en las lágrimas; dicha respuesta no fue observada en el plasma. Este aumento precoz es consistente con una respuesta de IgA T-independiente en la mucosa contra el virus de bronquitis. En la respuesta secundaria, los niveles de anticuerpos contra el virus de la bronquitis aumentaron significativamente en los controles a partir del día uno después de la vacunación de refuerzo y los niveles de anticuerpos IgG fueron más altos que los niveles de anticuerpos IgA tanto en lágrimas como en el plasma. En resumen, la vacunación ocular induce niveles de anticuerpos IgA más altos en la respuesta primaria contra el virus de bronquitis, mientras que la respuesta de memoria está dominada por anticuerpos IgG. De esta manera, niveles bajos de IgA en la mucosa se observan después de la exposición secundaria al virus de la bronquitis infecciosa, lo que puede contribuir a la vulnerabilidad de las células epiteliales del huésped a la infección por dicho virus y a la persistencia del serotipo Arkansas.

Key words: infectious bronchitis virus, IBV-specific antibody kinetics, mucosal immunity, T-independent IgA response, head-associated lymphoid tissue, avian coronavirus

Abbreviations: APCs = antigen-presenting cells; Ark DPI = Arkansas Delmarva Poultry Industry; CALT = conjunctiva-associated lymphoid tissue; E = envelope protein; ELISPOT = enzyme-linked immunospot; HALT = head-associated lymphoid tissues; HG = Harderian gland; HRP = horseradish peroxidase; IBV = infectious bronchitis virus; Ig = immunoglobulin; IL = interleukin; MDA5 = melanoma differentiation-associated protein antigen 5; PBS = phosphate-buffered saline; pIgR = polymeric immunoglobulin receptor; S = spike glycoprotein; SFC = spot-forming cell; SPF = specific pathogen free; TLR = Toll-like receptor; TMB = 3,3′,5,5-tetramethylbenzidine; TNF = tumor necrosis factor

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Infectious bronchitis virus (IBV) is a highly contagious group 3 coronavirus prevalent in all countries with extensive poultry industry. The IBV virion contains four structural proteins (i.e., the nucleoprotein [N], the membrane protein [M], the envelope protein [E], and the spike glycoprotein [S]). The N protein associates with the 27.6-kb single-stranded positive sense RNA genome. The remaining structural proteins are associated with the lipid envelope. The coronavirus spike protein, a large surface protein associated with the viral envelope (7), is cleaved into two subunits, the amino-terminal S1 subunit responsible for attachment to host cells and the carboxy-terminal S2 subunit, which mediates membrane fusion (3,23,38). S genes can have a very high mutation rate. The S1 protein's amino acid sequences from different serotypes differ normally between 20% and 25% from each other and by as much as approximately 50% in some serotypes, while most other IBV proteins don't exceed 15% change in amino acid sequence (5,6).

IBV initially infects the host mucosal surfaces of the respiratory tract, including the conjunctiva, the Harderian gland (HG), the nostrils, and the trachea, followed by a short period of viremia, which leads to infection of distant sites such as the kidneys and the urogenital and gastrointestinal tracts. IBV has an incubation period of 24 to 48 hr, with coughing, sneezing, and tracheal rales becoming evident within only a few days after initial infection of the flock (8). Symptoms include, but are not limited to, wet eyes, swollen face, tracheal and kidney lesions, respiratory disease, reduced weight gain in broilers, and decreasing and poor egg quality in layers (8,18). The severity of the symptoms is predicated by the virulence of the IBV strain present as well as the age and immune status of the flock. The existence of various IBV serotypes as well as antigenic variants (18) complicates vaccination programs. Immunity induced by vaccination against a single serotype generally provides insufficient protection against other serotypes (5,7).

As is the case with most viral infections, the immune response to IBV consists of an innate and an adaptive component. The innate immune system provides nonspecific protection through physical and chemicals barriers, including skin, mucosal epithelium, blood proteins, and phagocytic cells, to control infection (30). Many factors of the innate immune response are essential in controlling IBV. The innate immune mechanisms that detect RNA virus entry into the host cells include Toll-like receptors (TLRs), such as TLR-3, TLR-7, and TLR-21, which are located in the endosomal compartment. These TLRs play a role in the immune response to avian influenza (37), as does the melanoma differentiation-associated protein 5 (MDA5) located in the cytoplasm (20). Interestingly, the retinoic acid–inducible gene 1 receptor, which reacts with short blunt 5′-ppp dsRNA found in RNA viruses such as IBV (27,28), is absent in chickens (2). Whether IBV can trigger MDA5, as has been reported for negative stranded RNA viruses such as avian influenza (40), is not known at this point. Activation of these pathogen-associated molecular pattern recognition receptors result in type 1 interferon production, inflammatory cytokine production, such as interleukin (IL)-1 and IL-6, and activation of antigen presenting cells, which activate lymphocytes to generate an IBV-specific Th1 response and B-cell proliferation (4,30).

Since IBV enters through mucosal surfaces and initially replicates in the HG and trachea it seems logical that antibodies at mucosal surfaces could have an impact on the severity of an IBV infection. The presence of IBV-specific immunoglobulin A (IgA) and IgG in tracheal washes suggests that both immunoglobulin classes may contribute in controlling IBV at mucosal surfaces (17). It is thought that IgA is locally synthesized within the respiratory tract, while IgG is both locally synthesized and transduced to the respiratory tract from the systemic compartment (33). It has been demonstrated that IBV-specific IgG responses were less associated with protection against IBV than were IBV-specific IgA antibodies found in tears (31). Others have confirmed that IBV-specific IgA levels in lachrymal fluid correlated with the resistance to IBV reinfection (22). Furthermore, Cook *et al.* (10) demonstrated that resistance and/or susceptibility to IBV in inbred chicken lines was correlated with higher IgA levels in tears and saliva. Measuring weekly antibody titers in tears and serum after eyedrop vaccination on day 1 or day 14 of age with the Massachusetts Connaught strain of IBV demonstrated an increase of lachrymal antibodies prior to that in serum (14). These observations suggested that IgA is important in neutralizing IBV at mucosal surfaces (10). Dimeric IgA is transported across mucosal surfaces in chickens based on the expression of the polymeric immunoglobulin receptor (pIgR) on mucosal surfaces including HG and conjunctiva-associated lymphoid tissue (CALT) (36,39) and the prevalence of dimeric IgA in tears (36). Although mucosal IgA antibodies are thought to play a role in the control of IBV (8), the ability of live-attenuated Arkansas Delmarva Poultry Industry (Ark IBV) vaccines to induce IgA and IgG IBV-specific antibodies in the mucosal and systemic immune compartment is poorly understood.

The aim of this study is to provide a better understanding of vaccine-induced mucosal and systemic humoral immune responses to live attenuated Ark DPI IBV vaccines. Specifically, the production of antibodies in head-associated lymphoid tissue (HALT) and spleen as well as IgA and IgG antibody levels in the systemic and mucosal immune compartment were analyzed.

MATERIALS AND METHODS

Chickens. Specific-pathogen-free (SPF) white leghorn eggs were obtained from Sunrise Farms, Inc. (Catskill, NY), hatched, and used in all experiments. Chickens were housed in Horsfall-type isolation units in Biosafety Level 2 facilities throughout the duration of the experiment. Food and water were provided *ad libitum*. All experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. The Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)–accredited institution.

IBV vaccination. Four-week-old SPF chickens were ocularly vaccinated with 3×10^5 median embryo infectious dose (EID_{50}) per bird of live attenuated IBV Ark DPI vaccine (Zoetis, New York, NY) in 50 μl phosphate-buffered saline (PBS). Four weeks later they were boosted ocularly with the same vaccine dose.

Sample collection. Tears were collected as previously described (33). Approximately 250 μl of tears was collected from each chicken and placed into 1.5-ml microcentrifuge tubes. The tubes were kept on ice, followed by centrifugation at 16,100 × *g* for 10 min and stored at −80 C until used. Tears were collected prior to vaccination and every day postvaccination. Tear collections were rotated between three groups of vaccinated chickens to allow daily collection. Chickens were revaccinated 28 days after primary vaccination. Collection of samples continued until the 17th day of the primary response and the 14th day of the secondary immune response.

Blood samples were obtained by puncturing the brachial vein with a sterile 20-gauge needle. Approximately 1 ml of blood was collected into Kendall monoject blood collection tubes (Tyco Healthcare Group LP, Mansfield, MA) and incubated on ice. Blood samples were centrifuged at 500 × *g* for 30 min. Plasma was collected and stored at −80 C until tested for antibodies. Blood samples were collected from all chickens prior to primary vaccination and every day postvaccination, as described above for tears.

IBV propagation and purification for ELISA and enzyme-linked immunospot (ELISPOT). IBV was propagated in SPF white leghorn embryonated chicken eggs (Sunrise Farms, Inc.) by inoculation on day 10 of embryonation. Five thousand vaccine doses of IBV Ark vaccine were reconstituted in 18 ml tryptose broth, and 200 µl of this suspension was injected into each of the eggs' allantoic sac. Eggs were incubated for 2–3 days, and the allantoic fluid was collected after the eggs were refrigerated for ~4 hr. Cloudy allantoic fluid was discarded. Fluid was further clarified by centrifugation for 30 min at 3000 × *g*. Supernatants were titrated for the IBV virus using the Reed and Muench method (25).

IBV was heat inactivated at 56 C for 15 min, and inactivation of the virus was confirmed by injection into embryonated eggs. The inactivated IBV was purified based on a previously published protocol (28). In brief, allantoic fluids were placed in an ultracentrifugation tube and underlaid with 2 ml 30% sucrose in PBS and centrifuged at 90,000 × *g* for 6 hr at 4 C. The resulting IBV suspension was subsequently underlaid with 55% and 30% sucrose layers in PBS and centrifuged. Virus was collected at the 30%–55% sucrose interface and diluted in PBS to approximately 60% of the tube volume. The suspension was then underlaid with 30% sucrose and centrifuged. The resulting pellet was resuspended in PBS. IBV protein concentration was measured using the Pierce Micro BCA protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL). The virus was then stored at −80 C until used.

IgA and IgG ELISPOT assays. IgA and IgG ELISPOT assays were performed as previously reported (35). In brief, lymphocytes from HGs, CALT, and spleen were isolated and plated on IBV-coated (5 µg/ml) ELISPOT plates, which were blocked with complete RPMI medium containing 10% fetal calf serum. The plates were incubated overnight at 41 C in a humidified CO_2 incubator. After washing, the plates were incubated with goat–anti-chicken IgG or goat–anti-chicken IgA conjugated to horseradish peroxidase (HRP; Gallus Immunotechnology, Inc., Cary, NC) and the spots were developed with HRP substrate (Moss, Inc., Pasadena, MD) for 15–30 min prior to stopping the reaction by washing the plates with water.

IBV-specific ELISA. In order to measure IgG and IgA antibody levels in plasma and tears, an IBV-specific ELISA was developed. ELISA plates (Nunc MaxiSorp Immuno Plates; Thermo Scientific) were coated at 4 C overnight with 100 µl of 5 µg/ml purified, heat-killed IBV in carbonate buffer (50 mM $NaCHO_3$ and Na_2HCO_3, pH 9.4). Plates were then drained and blocked with 200 µl of ELISA assay buffer (1% bovine serum albumin and 0.05% Tween 20 in PBS) for 1 hr at room temperature. Plates were drained, and serial twofold dilutions of plasma or tear samples were loaded onto the plates. Plates were incubated at 4 C overnight. After washing the plates with PBS-Tween 20 (PBS pH 7.4, 0.05% Tween 20), 100 µl of biotinylated monoclonal mouse–anti chicken IgG (clone G-1) or anti-chicken IgA (clone A-1; 0.5 µg/ml; Southern Biotechnology Associates, Inc., Birmingham, AL) was loaded in each well and incubated at room temperature for 2 hr. After washing, 100 µl streptavidin-conjugated HRP at 1 µg/ml (Southern Biotech Associates, Inc., Birmingham AL) was incubated for 1 hr at room temperature. Plates were developed with 100 µl of 3,3′,5,5-tetramethylbenzidine (TMB; Invitrogen Corp., Frederick, MD) HRP substrate. TMB reaction was stopped with 100 µl of 1 N HCl solution after 30 min. Absorbance at 450 nm was measured with a Powerwave XS (BioTek Instruments, Inc., Winooski, VT). The highest sample dilution with an optical density of at least 450 nm of 0.100 above background level was defined as the endpoint titer.

Statistical analysis. ELISPOT data were analyzed using a Mann-Whitney *U*-test, and ELISA data were analyzed using a one-way ANOVA test with Dunnett posttest with exception of data pertaining to analysis of T-independent IgA, which was analyzed by Student *t*-test using GraphPad Prism5 software. Groups were considered significantly different at $P < 0.05$.

RESULTS

IBV-specific IgA ELISPOT. To measure the humoral response in lymphoid tissues the anti-IBV IgA and IgG spot-forming cell (SFC) response after ocular vaccination with IBV Ark serotype is measured in HG, CALT, and spleen by the ELISPOT assay. The HG displayed increased numbers of IBV-specific IgA SFC over controls starting on day 7 and remained elevated through day 13 (Fig. 1A). The peak IgA SFC response on day 10 of 60.9 ± 26.6 SFC/10^6 lymphocytes was significantly ($P < 0.05$) higher than in control birds. The CALT displayed a lower and more restricted IgA SFC response to IBV than did the HG, consisting almost exclusively of a significant elevated IgA SFC peak response on day 10, 16.7 ± 8.4 SFC/10^6 lymphocytes ($P < 0.05$), after which no more IgA SFC were detected. The spleen displayed an increase in IBV-specific IgA SFC starting on day 7 and achieved the highest response on day 8, 26.0 ± 4.7 SFC/10^6 lymphocytes ($P < 0.05$), followed by another significant increase on day 10, 10.0 ± 1.2 SFC/10^6 lymphocytes ($P < 0.05$) . The highest peak IBV-specific IgA SFC response was observed in the HG followed by the spleen and the CALT, respectively. The CALT was the slowest to respond, with an increase in IgA SFC after ocular immunization of the lymphoid organs tested.

The IgG SFC response was low in all three lymphoid organs tested. The HG displayed the earliest increase, followed by the spleen. As a result of the variability in the number of SFC in the HG the values were not significantly higher than those associated with controls. The spleen exhibited a significantly elevated number of IBV-specific IgG SFC on days 8–13 of the response. No IgG response was observed in CALT, with the exception of 1 IgG SFC on day 8.

Primary IBV-specific IgA and IgG response in plasma. Plasma samples were evaluated over the course of a 17-day period after primary vaccination and compared to unvaccinated controls. IBV-specific IgA plasma titers showed a significant increase over controls ($P < 0.05$) starting 6 days after IBV vaccination (Fig. 2A) and remained significantly elevated over the period during which the chickens were monitored. The IgA titers gradually increased until day 9. Thereafter, the values slightly fluctuated but gradually declined until day 15, after which they again increased to an endpoint titer of 10.0 ± 0.4 on day 17. The IgG plasma IBV-specific antibody titers showed a significant increase starting on day 10 after IBV vaccination (Fig. 2B). A peak response was observed on day 13 postvaccination, after which the IgG antibody titers decreased and plateaued at a $\log_2$ endpoint titer of approximately 9.0, remaining significantly elevated over control values. Therefore, the plasma IgA antibody levels significantly increased 4 days earlier than the IgG antibody levels compared to controls after ocular IBV vaccination, and both IgA and IgG antibody levels fluctuated around an endpoint titer of 8.0–9.0.

Primary IBV-specific IgA and IgG antibody response in tears. The IgA and IgG IBV-specific antibody levels in tears were measured over a 17-day period after ocular IBV vaccination. IgA titers in tears showed a significant increase in IBV-specific antibody titers compared to controls ($P < 0.05$) starting 6 days after IBV vaccination. IgA titers remained significantly elevated over controls during the period during which tears were collected, with the exception of the last day of monitoring (Fig. 3A). The highest IgA response was observed on day 9, after which the IgA titers in tears gradually declined and were no longer significantly elevated over control values by day 17 of the immune response. No IgG antibody responses were detected during the first 6 days after ocular IBV vaccination. The IgG antibody titers in tears showed a significant ($P < 0.05$) increase in IBV-specific antibody levels starting on day 9 and remained significantly elevated over controls through day 17 (Fig. 3B). A peak IgG response was observed on day 10. The IgA antibody titers in tears increased over controls 3 days earlier than did

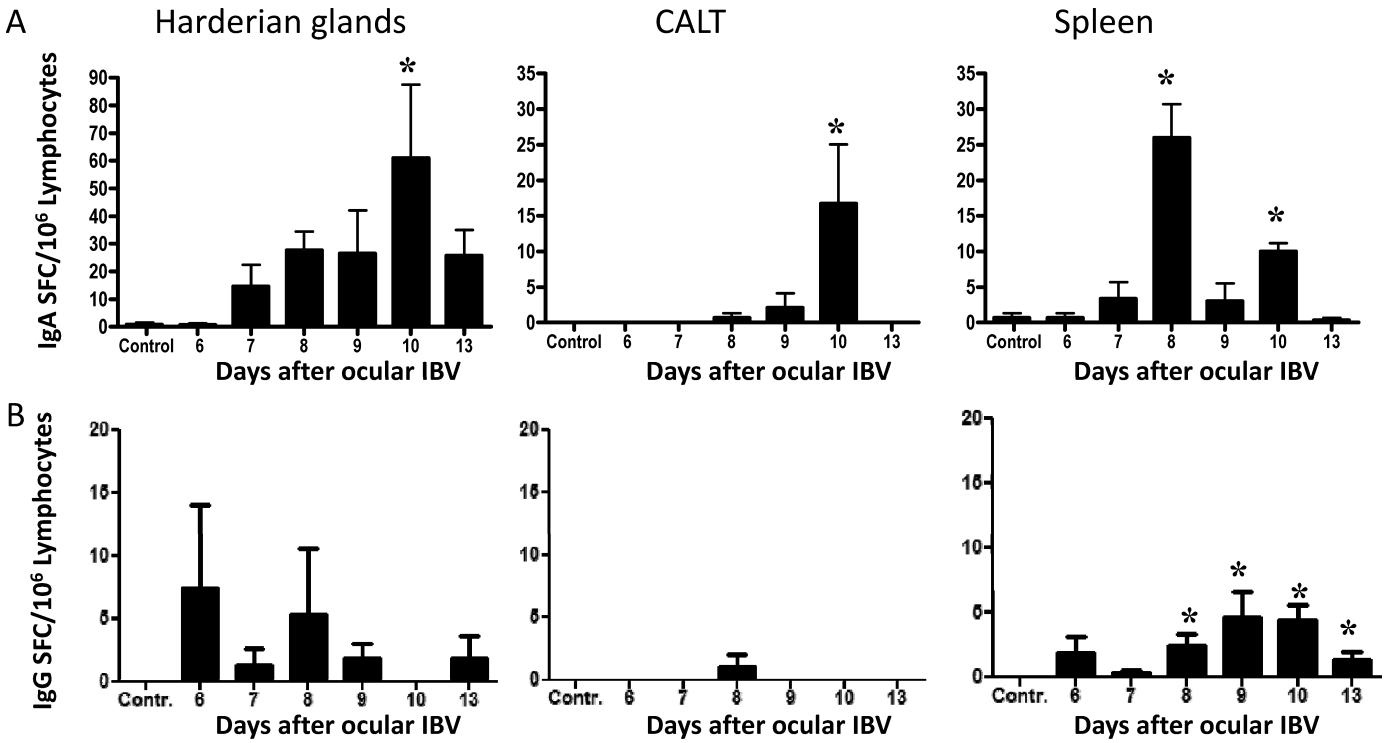

Fig. 1. IBV-specific antibody SFC in HG, CALT, and spleen after ocular IBV vaccination. Lymphocytes from Harderian glands, CALT, and spleen were isolated on days 6, 7, 8, 9, 10, and 13 after ocular IBV vaccination. Lymphocytes were plated on IBV-coated ELISPOT plates, and the IBV-specific IgA (A) and IgG (B) antibody–secreting B cells generating SFC were counted. Indicated are the mean SFC/10^6 lymphocytes and one standard error (IBV vaccinated, $n = 3$–4; controls, $n = 5$–6). The data were analyzed using the one-way ANOVA with the Dunnett posttest. The * indicates significant differences ($P < 0.05$).

the IgG antibody titers in tears. The IgA response also peaked 1 day earlier than the IgG response.

The primary IgA response to IBV in tears was more robust than the IgG response based on the higher IgA titers after the peak response, which reached a plateau around an endpoint titer of 9.0, while the IgG antibody titers plateaued around 6.0. Thus, IgA antibodies prevailed over IgG antibodies in tears during the primary IBV response.

Early mucosal IgA antibody response. To determine whether the increase in IgA 3 days after IBV vaccination (Fig. 3A) represents a true increase in IBV-specific IgA antibodies, an additional 26 birds were analyzed. As depicted in Fig. 4A, no change in IgA plasma titer was observed on day 3 after vaccination, while a significant ($P = 0.009$) increase in IBV-specific IgA antibodies was measured in tears. Furthermore, no significant change was observed in IBV-specific IgG antibodies in either plasma or tears (Fig. 4B). These data are consistent with an early T-independent mucosal IgA response.

Secondary IBV-specific IgA and IgG response in plasma. Chickens ocularly boosted 28 days after initial vaccination with IBV were analyzed for IgA and IgG plasma titers throughout a 14-day period. IBV-specific IgA and IgG plasma titers significantly increased starting 1 day after the IBV boost compared to those observed in control chickens (Fig. 5). After day 1, IgA and IgG titers dropped slightly, but both titers remained significantly elevated through the 14-day monitoring period compared to controls. The IgA plasma levels in the secondary IBV response plateaued around an endpoint titer of 8–9, while the IgG levels plateaued around 9–10. Unlike the primary IBV response, in which IgG and IgA antibodies were present at similar levels, the secondary antibody response in plasma showed higher IgG antibody levels than levels of IgA in plasma.

Secondary IBV-specific IgA and IgG response in tears. Chickens ocularly boosted with IBV 28 days after initial vaccination were examined for IgA and IgG antibody titers in tears throughout a 14-day period after the boost. IBV-specific IgA antibody titers in tears showed no significant increase when compared to controls, with exception of day 1 postboost (Fig. 6A). IBV-specific IgG antibody titers significantly increased in tears 1 day after the ocular IBV boost, compared to controls, and the levels gradually decreased over time from an endpoint titer of 10 on day 1 to an endpoint titer of 8.0 on day 14, but they remained significantly elevated over controls until the last collection day (i.e., day 14; Fig. 6B). Compared to the primary antibody response, plasma levels of IBV-specific IgA in tears decreased, while the IgG levels increased after boosting.

DISCUSSION

IBV enters the host through mucosal surfaces by infecting epithelial cells. Therefore, mucosal immunity should play an important role in controlling IBV. Consistent with this notion are previous observations that resistance and or susceptibility to IBV in inbred chicken lines are correlated with mucosal protection (i.e., IgA levels in lacrimal fluid and saliva) (10). Resistance to IBV was also correlated with IgA in tears in non-inbred chickens (31). To better understand mucosal and systemic humoral responses after IBV vaccination, the ability of an Ark DPI–derived live attenuated IBV vaccine to induce systemic and mucosal humoral immune responses was examined after ocular application.

IBV-specific IgA antibody levels in both tears and plasma showed a significant increase over controls 6 days after vaccination, while

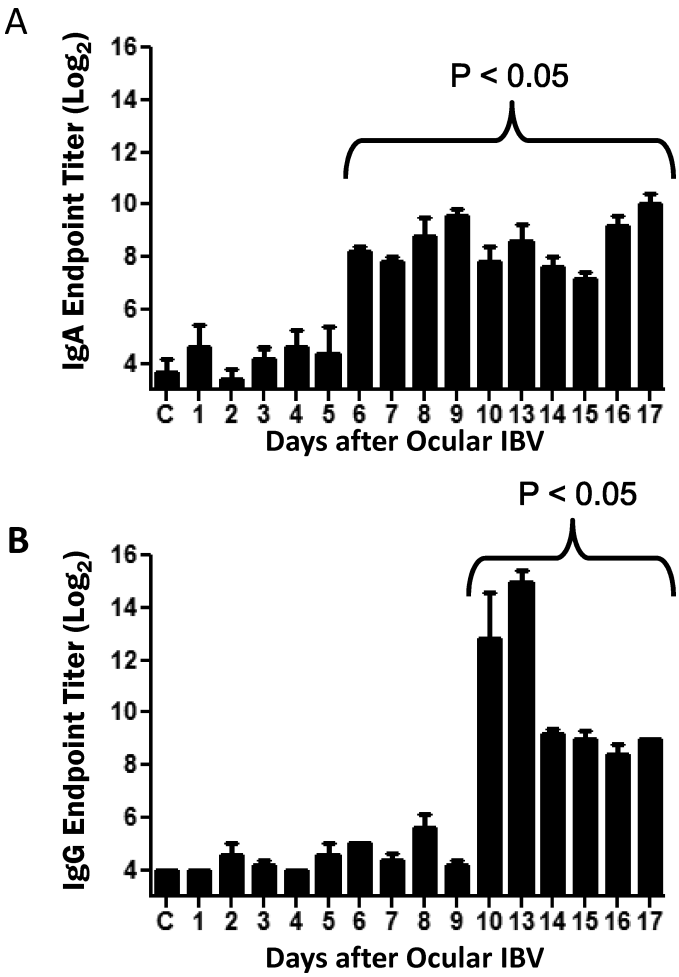

Fig. 2. Primary IBV-specific IgA and IgG antibody responses in plasma after ocular IBV vaccination. Chickens were ocularly vaccinated with live attenuated IBV vaccine. Plasma was collected at various days postvaccination and analyzed by an IBV-specific ELISA. Five samples were analyzed for each time point, and 15 pre-bleeds were used as controls. The mean (±1) standard error values of the IgA (A) and IgG (B) endpoint titers in tears are depicted. The one-way ANOVA test with the Dunnett posttest was used for statistical analyses. A significant increase over control values is indicated ($P < 0.05$).

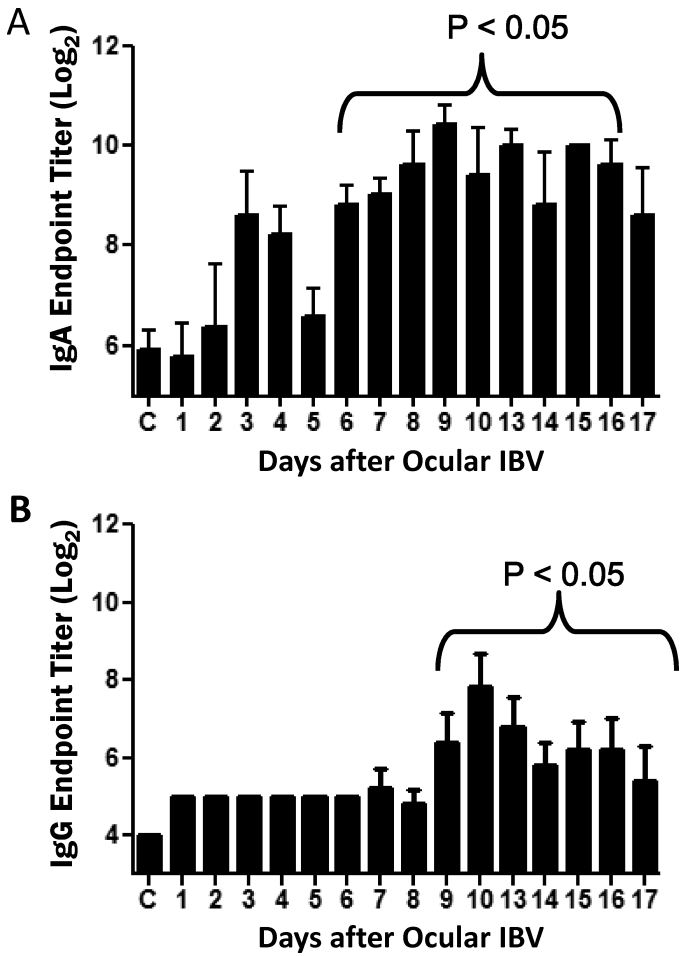

Fig. 3. Primary IBV-specific IgA and IgG antibody responses in tears after ocular IBV vaccination. The chickens were ocularly vaccinated with live attenuated IBV vaccine. Tear samples were collected at various days postvaccination and analyzed by an IBV-specific ELISA. Five samples were analyzed for each time point, and 15 pre-bleeds were used as controls. The mean (±1 standard error) values of the IgA (A) and IgG (B) endpoint titers in tears are depicted. The one-way ANOVA test with the Dunnett posttest was used for statistical analyses. A significant increase over control values is indicated ($P < 0.05$).

IgG levels did not increase significantly until 10 days postvaccination in plasma and 9 days postvaccination in tears. Both mucosal IgA in tears and systemic IgA in plasma are early responders in the primary humoral response to IBV and appeared 3 and 4 days earlier than IgG antibodies, respectively. Previous studies on IBV-specific IgM in sera demonstrated that the induction of IgM antibodies occurred around 5 days postvaccination (13), reaching peak levels around 8 days and waning to marginal levels by 24 days (21). Thus, the IgM response to IBV occurs 1 or 2 days earlier than do the IgA antibodies observed in Fig. 3, and, like the mucosal IgA in tears, the response is also transient. The notion of IgA serving as an early responder has also been demonstrated in humoral responses to *Mycoplasma pneumonia* in humans, in which IgA antibodies developed much earlier than IgG and even earlier than IgM antibodies (15). Furthermore, the presence of only IgA antibodies in human sera to *Chlamydia trachomatis* was correlated with early infection (19). Mucosal IgA antibodies specific for IBV are transient, as has been reported for IgM antibodies (13), based on the observation that IgA titers in tears were not significantly elevated over controls starting on day 17 of the primary response (Fig. 3A). In contrast, the IgA antibody levels in plasma 17 days after vaccination were the highest levels recorded.

The IgA and IgG SFC responses to IBV in HALT and spleen (Fig. 1) are consistent with a prominent role for IgA in the primary IBV response. The HG contained by far the highest number of IgA SFC. This finding was consistent with a lack of IgA following IBV vaccination in tears after HG removal (12) and with the notion that HG is the main contributors of IgA to lacrimal fluid (34). Our data as well as those of other investigators are consistent with a prominent role for the HG in mucosal humoral immunity. Specifically, HG B cells express the J chain, which is needed for generating polymeric IgA and IgM (29). Polymeric IgA binds to the pIgR, which has been cloned for chickens (35) and is expressed in the HG (36). Furthermore, chicken tears contain almost exclusively polymeric IgA, while plasma contains predominantly monomeric IgA (36). All of these observations are consistent with a role of the HG in generating mucosal humoral immunity to protect against pathogens such as IBV. After initially replicating in the upper respiratory tract, IBV disseminates via viremia to other tissues over the next few days (32). IBV's early replication in the HG, followed by a progression

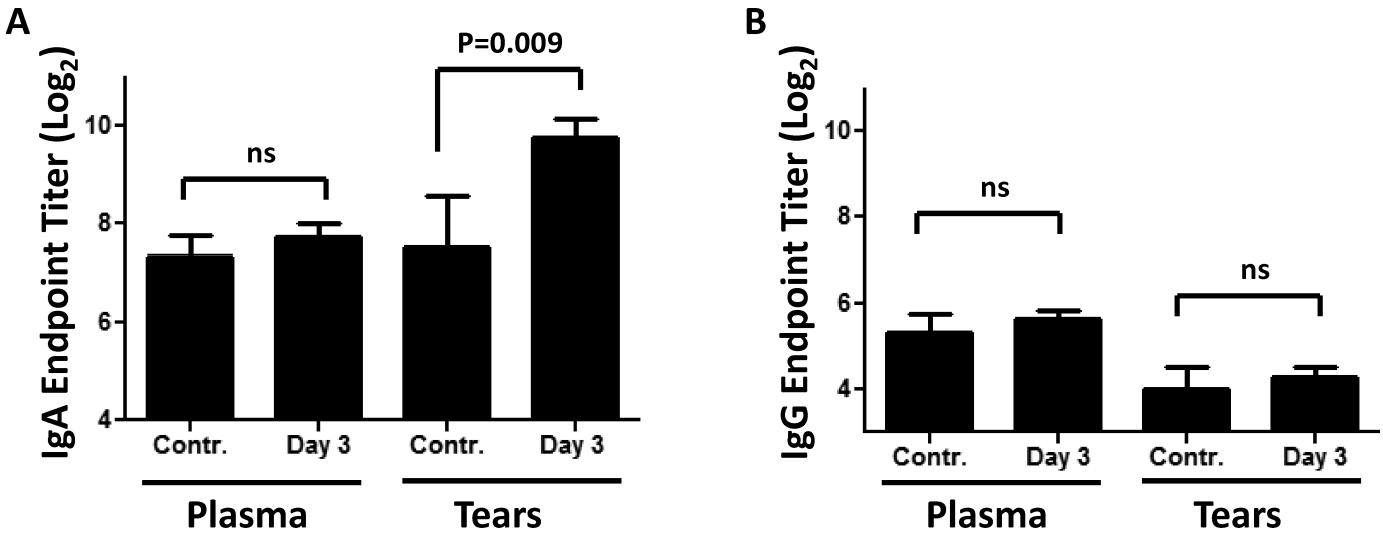

Fig. 4. IBV-specific antibody response 3 days after ocular IBV vaccination. Three days after primary IBV vaccination tears and plasma were collected and analyzed for IBV-specific IgA and IgG antibodies. The mean (± 1 standard error) values of the IgA (A) and IgG (B) endpoint titers in plasma are depicted. Data from six controls and 26 vaccinated birds were analyzed using the Student *t*-test. The absence (ns) or presence ($P < 0.05$) of a significant increase over controls is indicated.

into the systemic immune compartment, is consistent with IgA prevailing in the primary IBV response over IgG.

Polymeric secretory IgA plays an important role in the protection of epithelia at mucosal surfaces. Secretory IgA provides a barrier at mucosal surfaces by eliminating pathogens within epithelial cells and polymeric-IgA containing immune complexes in the lamina propria and by preventing entry of microorganisms into the host (11). Furthermore, passive administration of IgA antibodies specific for influenza virus to mice demonstrated that polymeric IgA protected against subsequent challenge with this respiratory pathogen, while monomeric IgA did not (26). The lower mucosal IgA antibody levels observed upon secondary exposure to IBV may therefore contribute to vulnerability of host epithelial cells to infection by IBV and persistence of the Ark serotype in the host.

The observation that IgA SFC response in the spleen peaked on day 8 after vaccination preceding the peak SFC response in HG and CALT on day 10 is puzzling. The mucosal head-associated tissues encounter the virus prior to the spleen after ocular vaccination, which theoretically supports the notion that mucosal lymphoid tissues should peak earlier in IgA SFC than does the spleen. One possible scenario that could explain this observation would be homing of HG lymphocytes/antigen-presenting cells (APCs) to the spleen very early during the IBV infection, after which the spleen is able to generate a humoral response faster than HALT (i.e., HG and CALT). Surface IgA-positive B cells from the HG migrate to cecal tonsils (1) and possibly other mucosal sites. However, no data are available with regard to whether APCs from the HG can migrate to other lymphoid tissues, such as the spleen. If an APC migration would occur early during ocular IBV exposure one could imagine that the spleen may be well equipped to generate an antibody response of limited duration early in the immune response, as was observed during our experiments. Alternatively, IgA plasma cells migrate directly to the spleen to induce this immune response. This is a less likely scenario based on the observation that the HG does not peak until day 10 of the immune response, while the peak response in spleen is observed on day 8. The latter explanation represents the more likely scenario for the low response observed in the spleen on day 10.

The IgA response in the spleen seems biphasic in nature based on the observation that the peak IgA SFC response was observed on day 8, followed by a decline on day 9 and another increase on day 10. The peak IgA SFC response for mucosal lymphoid tissues was also on day 10 post–IBV vaccination, indicating a potential link to this second peak in the spleen and the response in the HG. The IgG SFC response was low compared to the IgA SFC response and was most prevalent in the spleen, with peaks around 9–10 days after vaccination. In the HG the IgG SFC response was low and erratic, and in CALT it was almost absent. Based on expression of granzyme A, perforin, and interferon gamma, we demonstrated that the CALT expressed the highest levels of these cell-mediated immunity-associated mRNAs after IBV vaccination when compared to HG and spleen (16). This indicates that there may be a divergence in function between the CALT and HG pertaining to IBV-specific immunity.

The early increase in IBV-specific IgA levels on day 3 post–primary vaccination strongly indicates the existence of a T-independent IgA response at mucosal surfaces after IBV vaccination, since this early increase was only observed in tears and not in plasma. The day 3 IgA peak in tears precedes the peak in IBV viral load in the HG after vaccination, which occurs around day 7 after vaccination (24), indicating that this increase in IgA may potentially have a considerable impact on resistance to IBV. In mammals, the T-independent IgA class switch has been well documented and seems to occur in the nonorganized lymphoid tissue of the lamina propria and is amplified by the production of APRIL (a proliferation-inducing ligand) and BAFF (B-cell activating factor belonging to the tumor necrosis factor [TNF] family), TNF-family ligands, by dendritic cells (9). To our knowledge no T-independent IgA production has been previously reported in chickens, and this study represents the first evidence of a T-independent mucosal IgA response in a nonmammalian species. This T-independent mucosal IgA response is observed prior to the reported IgM response (13). This early IgA response may also explain the importance of IgA in the resistance to IBV.

The secondary humoral immune response to IBV is observed as early as 1 day after vaccination for IgA and IgG in plasma and tears

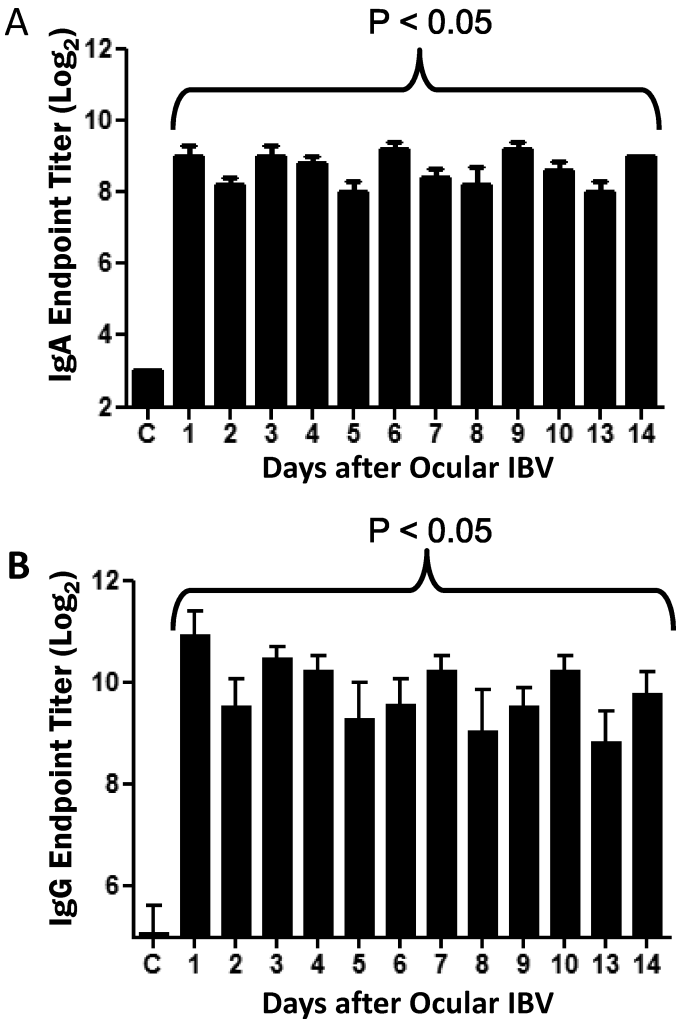

Fig. 5. Secondary IBV-specific IgA and IgG antibody responses in plasma after ocular IBV boost. Chicken were ocularly boosted 4 wk after primary IBV vaccination. Plasma samples were collected to measure IBV-specific IgA (A) and IgG (B) antibody responses by ELISA. Each data point contains five samples including the controls. Depicted is the mean (±1 standard error). The one-way ANOVA test with the Dunnett posttest was used for statistical analyses. A significant increase of antibody titers over control values is indicated ($P < 0.05$).

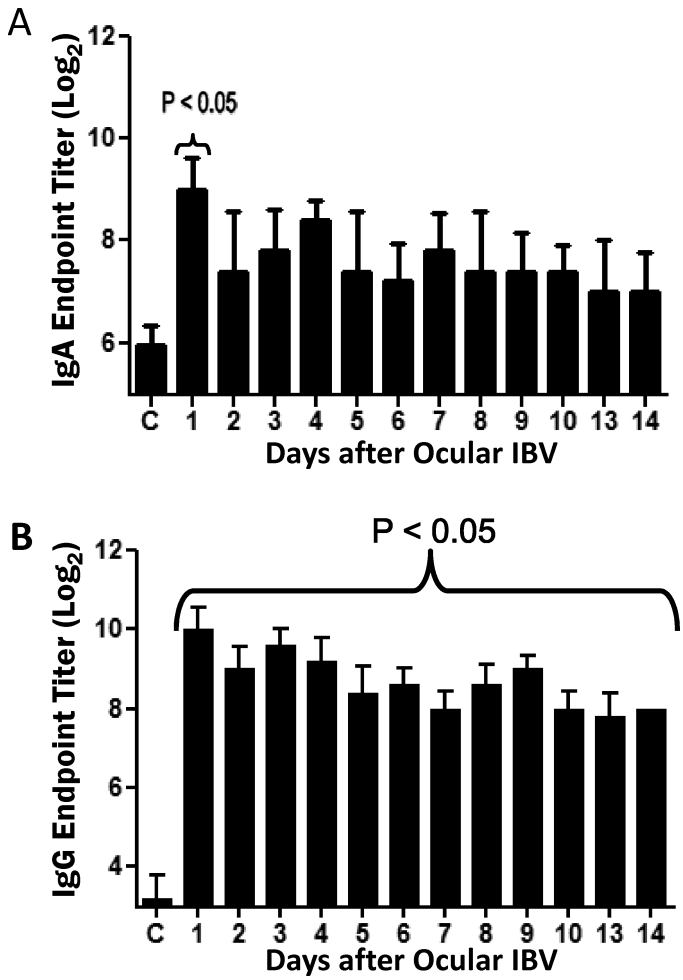

Fig. 6. Secondary IBV-specific IgA and IgG IBV antibody responses in tears after ocular IBV boost. Chickens were ocularly boosted 4 wk after primary IBV vaccination. Tear samples were collected to measure IBV-specific IgA (A) and IgG (B) antibody levels by ELISA. Each data point contains five samples, with the exception of the IgA control, which contains 15 samples. Depicted is the mean (±1 standard error). The one-way ANOVA test with the Dunnett posttest was used for statistical analyses. A significant increase of antibody titers over control values is indicated ($P < 0.05$).

and slowly declines over time. The IgA titers in tears are low and do not significantly differ from those of controls with the exception of day 1 after boosting. The major change is a shift from prevailing IgA-dominant titers in the primary response to prevailing IgG titers in the secondary response in both plasma and tears. Compared to the primary response, the IgA titers are lower in the secondary response, while IgG titers increased compared to the titers observed in the primary response. This is consistent with our recent findings that the secondary interferon-γ response is dominated by a central memory response, while the primary response is almost exclusively observed in HALT based on quantitative reverse transcriptase–PCR (16) and is consistent with a switch of the prevailing immune response from the mucosal to the systemic immune compartment.

In summary, the humoral immune response in tears and plasma in the primary response to a live attenuated Ark DPI IBV vaccine is dominated by IBV-specific IgA, which occurs 3–4 days earlier than IgG and is of higher magnitude. The primary mucosal IgA response is characterized by a very early increase in antibody titer (i.e., day 3 after vaccination), indicative of T-independent mucosal IgA response, which has also been reported in mammals. The mucosal IgA response is somewhat transient. Seventeen days after immunization the IgA titer is no longer significantly higher than that observed in controls. The secondary response to the IBV vaccine is characterized by an increase in IgG antibody titers and a decrease in IgA antibody titers in both tears and plasma, consistent with a shift of the IBV-specific antibody response from the mucosal to the systemic immune compartment. Lower mucosal IgA antibody levels observed upon secondary exposure to IBV may contribute to vulnerability of host epithelial cells to infection by IBV and persistence of the Ark serotype.

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