Clinical and acquired immunologic responses to West Nile virus infection of domestic chickens (*Gallus gallus domesticus*)

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ABSTRACT Numerous bird species are highly susceptible to North American strains of West Nile virus (WNV), and although domestic chickens are relatively resistant to WNV-associated disease, this species currently represents the most practical avian model for immune responses to WNV infection. Knowledge of the immunomodulation of susceptibility to WNV in birds is important for understanding taxonomic differences in infection outcomes. While focusing on immunophenotyping of $CD3^+$, $CD4^+$, $CD8^+$, and $CD45^+$ lymphocyte subpopulations, we compared lymphocyte subpopulations, blood chemistries, cloacal temperatures, IgM and IgG antibody titers, and differential whole-blood cell counts of WNV-infected and uninfected hens. Total blood calcium and lymphocyte numbers were lower in WNV-infected chickens compared with uninfected chickens. The heterophil-to-lymphocyte ratio increased over time from 2 to 22 d postinoculation (DPI) in uninfected chickens and from 2 to 8 DPI in WNV-infected chickens, although levels declined from 8 to 22 DPI in the latter group. No significant differences were found in the remaining immunological and hematological variables of the WNV-infected and uninfected groups. Our results reaffirm that chickens are resistant to WNV infection, and demonstrated that the heterophil-to-lymphocyte ratio differed between groups, allowing for sorting of infection status. Similar patterns in immune responses over time in both infected and uninfected hens may be related to age (i.e., 10 wk) and associated immune development.

Key words: West Nile virus, chicken, immunophenotyping, hematology, antibody, heterophil:lymphocyte ratio

2011 Poultry Science 90:328–336
doi:10.3382/ps.2010-00809

INTRODUCTION

West Nile virus (**WNV**) is a mosquito-borne flavivirus that has emerged as an important cause of viral encephalitis among humans and horses in North America (McLean, 2006). West Nile virus is maintained in an enzootic cycle among mosquitoes and birds, with variable susceptibility among bird species, ranging from absence of clinical signs and low-titered viremia, such in the Rock Pigeon [*Columba livia* (Allison et al., 2004; Deegan et al., 2005; Gibbs et al., 2005)], to species that suffer high mortality rates, such as the common raven [*Corvus corax* (Potter, 2004)] and American crow [*Corvus brachyrhynchos* (Caffrey et al., 2005; Ward et al., 2006; Yaremych et al., 2004)]. Breeding Bird Survey data revealed large-scale reductions in population effects on some avian species [e.g., American crow, blue jay (*Cyanocitta cristata*), and American robin (*Turdus migratorius*)] associated with the arrival of WNV to the United States (LaDeau et al., 2007). The virulence of North American WNV strains in birds may be related to genetic alterations in the virus (Brault et al., 2004), whereas differences in susceptibility among avian hosts may be due to underlying differences in genetics and immunocompetence. The mechanisms that drive the differences in the susceptibility to WNV infection among avian species remain unknown.

The reasons underlying the variation in host responses to WNV infection may be primarily immunologically based. In humans, the incidence of neuroinvasive WNV disease and associated mortality increases with age, and severe disease has also been observed in immunocompromised patients (Hayes et al., 2005). Similarly, wild birds may be immunocompromised because of environmental or physiological stresses (Jankowski et al., 2010; Owen et al., 2010) and because of preexisting infection with various pathogens leading to immunosuppression; these factors may affect their response to subsequent WNV infection.

West Nile virus infections are limited by humoral immunity because animals lacking antibodies develop

Received May 27, 2010.
Accepted October 27, 2010.
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high-grade viremia, early movement into the brain, and higher mortality (Diamond et al., 2008). Through murine models, T and B cell lymphocytes have been shown to protect against WNV infection (Halevy et al., 1994; Diamond et al., 2003a,b). Cellular immunity is critical to infection clearance by virulent North American WNV isolates. Mice deficient in $CD4^+$ or $CD8^+$ T cells develop persistent WNV infection in the brain (Shrestha and Diamond, 2004; Sitati and Diamond, 2006), and humans with hematological malignancies and impaired T cell function are at increased risk of severe WNV infection (Murray et al., 2006). Nemeth et al. (2009) found in birds that humoral immunity to West Nile virus is long-lasting and protective in house sparrows (*Passer domesticus*).

The overall purpose of the present research was to characterize immunological and clinical responses in chickens after experimental WNV infection, and to compare these responses with uninfected chickens (*Gallus gallus domesticus*). Specifically, we measured peripheral blood lymphocyte ratios ($CD3^+$, $CD4^+$, $CD8^+$, and $CD45^+$), neutralizing IgM and IgG antibody titers, complete white blood cell counts, select blood chemistry values, viremia titers, and cloacal temperatures. We measured WNV-specific antibodies by the plaque reduction neutralization test (**PRNT**).

MATERIALS AND METHODS

Birds and Bird Care

Nineteen 10-wk-old White Leghorn laying hens were acquired from the Hudson Pullet Farm in Hudson, Colorado. Younger hens were selected to test the effects of WNV on developing chickens, and chickens from a commercial source were used to represent a heterogeneous population with varied immune responses. On arrival, all chickens were confirmed as seronegative for WNV by PRNT. The hens were housed at Colorado State University in Fort Collins within biosafety level-3 rooms (3.7 m high × 3.7 m wide × 5.5 m long), were provided Family Farm Egg Maker 16 crumbles (Manna Pro Corporation, St. Louis, MO) and fresh water ad libitum, and were exposed to natural lighting for 14 h/d at a relatively constant temperature and humidity (approximately 21°C and 20% RH, respectively). Chickens were allowed approximately 2 wk to adjust to their new surroundings before experimental inoculation. All hens appeared in good health before initiation of the study. Ten hens were randomly assigned to the WNV-inoculated group and 9 hens were randomly assigned to the uninfected control group; these groups were housed in separate rooms. The care of all birds in this study was in compliance with National Institutes of Health guidelines for the humane use of laboratory animals, and all protocols were approved by the Institutional Animal Care and Use Committees at Colorado State University and Los Alamos National Laboratory. At the end of the study, WNV-infected birds were killed by sodium pentobarbital overdose delivered intravenously.

All hens (including negative controls) were bled immediately before inoculation (0.5 mL) and again (approximately 3.0 mL) on 2, 8, and 22 d postinoculation (**DPI**). Cloacal temperatures were recorded from each bird before blood collection at each of the prescribed time points. Postinoculation sampling time points represented various phases of infection, with 2 DPI as the early phase of infection, when birds are most likely to be viremic (Komar et al., 2003), and later time points (i.e., 8 and 22 DPI) representing the phase soon after seroconversion and the recovery phase, respectively (Langevin et al., 2001). Blood was collected from either the jugular or brachial vein into heparinized syringes and immediately transferred into EDTA and lithium heparin tubes. Blood for virological and serological analyses was collected in nonheparinized syringes. A portion of the blood was placed into serum or plasma separator tubes, allowed to clot for approximately 60 min at room temperature, centrifuged at 6,000 × *g* for 5 min at room temperature, and frozen to −62°C until assayed for virus or to −28°C until assayed for antibodies. In addition, blood samples from each time point were transported at room temperature to Los Alamos National Laboratory in Los Alamos, New Mexico, for additional analyses, which occurred within 12 h of blood collection.

Virus Inoculation, Virus Detection, and Virus Neutralization Assays

West Nile virus strain NY99-4132 (originally isolated from crow brain) was used for all bird inoculations and virus neutralization assays (Bunning et al., 2002; Langevin et al., 2005). Ten hens were inoculated subcutaneously over the breast muscle with approximately 18,000 plaque-forming units (**PFU**) of WNV. Sera collected on 2 DPI were assayed for infectious virus by Vero cell plaque assay as described previously (Bunning et al., 2002). Briefly, Vero cell monolayers in 6-well plates were inoculated in duplicate with 0.1 mL of sample per well. After 1 h incubation at 37°C, cells were overlaid with 3 mL/well of 0.5% agarose in minimum essential media supplemented with 2% fetal bovine serum and antibiotics. Two days later, cells were overlaid with a second 3-mL overlay containing 0.004% neutral red dye. Viral plaques were counted on the third and fourth days of incubation. The minimum threshold for WNV detection by virus isolation was approximately 50 PFU/mL of serum or plasma.

Sera or plasma were heat inactivated (56°C for 30 min) before testing for neutralizing antibodies to WNV by PRNT as described previously (Beaty et al., 1995). Sera or plasma collected before WNV inoculation and from uninfected hens (d 8 and 22 postinoculation) were screened at a 1:10 dilution, whereas serial 2-fold dilutions were performed on postinoculation samples from

experimentally inoculated chickens to determine end point 90% neutralization titers (**$PRNT_{90}$**). Sera or plasma with ≤60% neutralization of WNV at a 1:10 dilution were considered negative for anti-WNV antibodies, and those with $PRNT_{90}$ titers of ≥10 were considered antibody-positive.

Lymphocyte Isolation

Venous blood samples were diluted 1:1 with sterile PBS (1×, Gibco, Carlsbad, CA) with 1% BSA (ICN Biomedicals, Aurora, OH) to a 2-mL total volume. This mixture was then layered on 3 mL of Ficoll (Histopaque-1077, Sigma, St. Louis, MO) in a 15-mL tube. The samples were centrifuged using a Centra GP8 instrument (IEC, Needham Heights, MA) at 890 × *g* for 30 min with no brake. The lymphocyte-containing monolayer of cells above the Ficoll layer was then collected with a sterile transfer pipette and washed twice with 4 mL of PBS with 1% BSA. These samples were centrifuged at 480 × *g* for 7 min. Final pellets were resuspended in 1 mL of PBS with 1% BSA and counted using a Coulter Multisizer Z1 instrument (Beckman Coulter, Fullerton, CA).

Monoclonal Antibodies

Monoclonal antibodies (**mAb**) were purchased from Southern Biotechnology Associates (Birmingham, AL). The following antibodies were used: mouse anti-chicken CD8α fluorescein isothiocyanate (**FITC**) conjugate clone; CT-8 Ig isotype: mouse $IgG_1κ$ spec: α chain (34 kDa) of chicken $CD8^+$; mouse anti-chicken $CD4^+$ FITC conjugate clone: CT-4Ig isotype: mouse $IgG_1κ$ spec: chicken $CD4^+$ (M_r 64 kDa); mouse anti-chicken $CD3^+$ FITC conjugate clone: CT-3 Ig isotype: mouse $IgG_1κ$ spec: chicken CD3 and mouse anti-chicken $CD45^+$ R-phycoerythrin (**R-PE**) conjugate clone LT40 Ig isotype: mouse IgMκ spec: chicken CD45 (M_r 190 to 215 kDa).

Staining

Working dilutions and conditions for the above mAb conjugates were purchased from Southern Biotechnology Associates. The FITC-stained mAb were diluted 1:10 in PBS. The R-PE stained mAb were diluted 1:40 in PBS. These working solutions (20 μL total of each) were then added to approximately 10^6 lymphocytes. The lymphocytes were incubated for 20 min at room temperature in the dark and washed once with 1× PBS. The cells were centrifuged at 480 × *g* for 7 min. The pellet was resuspended in 100 μL of Cal-Lyse (Caltag Laboratories, Burlingame, CA) and incubated at room temperature for 10 min. Next, 2 mL of double-distilled H_2O was added, followed by an additional 10 min of incubation. The resulting mixture was centrifuged at 480 × *g* for 7 min and washed once with PBS. The final pellet was resuspended in 250 μL of PBS. Lymphocytes were single-stained with $CD3^+$ FITC and $CD4^+$ FITC and dual stained with $CD8^+$ FITC and $CD45^+$.

Flow Cytometry

Flow cytometry analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with a 488-nm argon laser. Green fluorescence (from FITC) was detected on the FL1 channel (530/30 nm band pass filter), and orange fluorescence (from R-PE) was detected on the FL2 channel (585/42 nm band pass filter). Cells were analyzed at up to 20,000 events. Flow cytometry was repeated for each sample to ensure consistent results.

ELISA Antibody Detection

The IgG and IgM sandwich ELISA assays (protocols and supplies) were performed using the ELISA Starter Kit (Cat. No. EA101, Bethyl Inc., Montgomery, TX). We examined total IgG and IgM antibodies to investigate antibody responses. The capture antibodies used were affinity-purified goat anti-bird IgG antibody-heavy and light chain (A140-110A, Bethyl Inc.) and rabbit anti-bird IgM (Cat. No. RBM-65A, ICL Inc., Newberg, OR). All steps were performed at room temperature. To coat the wells (9 μg/mL of IgG and 1.9 μg/mL of IgM in 100 μL), coating buffer was used (0.05 *M* carbonate-bicarbonate, pH 9.6) for each well and incubated at 27°C for 60 min. After incubation, the solution was removed by aspiration and the wells were washed 3 times with wash solution (50 m*M* Tris, 0.14 *M* NaCl, 0.05% Tween 20, pH 8.0). Next, 200 μL of blocking solution (50 m*M* Tris, 0.14 *M* NaCl, 1% BSA, pH 8.0) was added to each well for 60 min and the wells were washed 3 times with wash solution. Bird serum or plasma was diluted in sample-conjugate diluent (50 m*M* Tris, 0.14 *M* NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) at 1:12,500 for IgG and 1:5000 for IgM; 100 μL was placed in each well and incubated at 27°C for 60 min. After incubation, samples were removed and each well was washed 7 times with wash solution. The detection antibodies, horseradish peroxidase-conjugated goat anti-bird IgG heavy and light chain antibody (Cat. No. A140-110P, Bethyl Inc.; 0.035 μg/mL) and rabbit anti-bird IgM (Cat. No. RBM-65P, ICL Inc.; 1.8 μg/mL), were diluted with sample-conjugate diluent, and 100 μL was placed in each well and incubated for 60 min. Wells were washed 7 times and 100 μL of tetramethylbenzidine peroxidase substrate was added to each well for 6 min. The reaction was stopped with 100 μL of 2 *M* H_2SO_2. The plates were read at 450 nm on a BioTek Synergy 2 multidetection microplate reader (BioTek, Winooski, VT). Chicken reference serum (Cat. No. RS10-102, Bethyl Inc.) was used as a standardized control.

Hematology

Whole-blood smears were made immediately after blood collection, and leukocytes were classified (Dein, 1986) and counted on the slides fixed with methyl alcohol and stained with Wright-Giemsa stain. Leukocyte counts were performed using a 40× objective (Zeiss Axioskop) microscope (Carl Zeiss Ltd., Welwyn Garden City, Herts, UK) by randomly selecting 5 fields from all areas of the slide. Identity of granulocytes was confirmed using a 100× objective, and basophils were omitted from analysis because of their relative rarity. Results were analyzed both as total leukocytes and by cell type, which included monocytes, lymphocytes, and granulocytes (i.e., heterophils and eosinophils). The ratio of heterophils to lymphocytes (**H:L**) was calculated. This sampling design has been deemed reliable, with 82% of observed variation attributed to differences between birds and 18% attributable to sampling error (Apanius, 1991). Blood chemistries of plasma (125 μL) were analyzed using an automated dry chemistry system (Spotchem EZ, Heska Corp., Loveland, CO).

Data Analyses

The Statistical Analysis System (SAS Institute, 1987) was used for all statistical analyses, and assumptions for parametric statistics were examined. All results were compared among individual birds with a repeated mixed GLM (PROC MIXED) and an unstructured covariance model that best fit the data. We considered the among-individual variance components to represent repeatability. Data were tested and found to be normally distributed, with equal variances between groups (Shapiro-Wilk and Levene's test, respectively).

RESULTS

No morbidity or mortality was observed in either WNV-infected or uninfected chickens, and no significant difference was found in body temperature between the 2 groups (Table 1). The average cloacal temperature for uninfected controls throughout the study was 41.5°C (range 41.5 to 42.1°C), whereas average cloacal temperatures for preinoculation and 2, 8, and 22 DPI for inoculated chickens were 41.3, 41.4, 41.4, and 41.6°C, respectively. Viremia was detected on 2 DPI in all but one of the WNV-infected chickens, with an average of $10^{2.9}$ PFU/mL of serum (range $10^{<1.7}$ to $10^{3.2}$). All experimentally inoculated chickens seroconverted by 8 DPI ($PRNT_{90}$ antibody titers from 40 to 320), and antibody titers increased ≥4-fold in most (8/10) chickens between 8 and 22 DPI. No uninfected chickens had detectable viremia or were seroconverted during the study. Total lymphocytes of the WNV-infected group did not differ from those of the uninfected group (Table 1) over time but were lower in the WNV-infected group on the day of infection. The H:L increased over time, from 2 to 22 DPI in noninoculated chickens and from 2 to 8 DPI in WNV-inoculated chickens, although levels declined from 8 to 22 DPI in the latter group (Figure 1). No significant difference was found in the other immunological and hematological variables of WNV-infected and uninfected groups. The IgM antibody titers increased significantly for both groups over time with a similar pattern in both groups, increasing on d 8 and then decreasing (Figure 1). Total IgG antibodies and $CD4^+$:$CD8^+$ ratios decreased over time but did not vary between WNV treatments (Table 1). All variables, except total protein, varied with time for both groups of chickens. The percentage of $CD4^+$ cells decreased by 47 and 38% for the WNV-infected and uninfected groups, respectively (Figure 2). However, the percentage of $CD3^+$ cells increased by 20 and 33% for the WNV-infected and uninfected control groups, respectively (Figure 2). Percentages of $CD8^+$ cells increased between 2 and 8 DPI for the WNV-infected group, and then decreased (although not significantly) from 8 to 22 DPI; the latter pattern was also observed in the uninfected group (Figure 2). Blood calcium declined between 2 and 8 DPI in both infected and uninfected hens and returned to preinoculation levels by 22 DPI in the former. All other blood chemistry variables did not vary between the 2 groups.

DISCUSSION

Depending on susceptibility, microbial infection within a given host would be expected to elicit a variety of clinical responses that may be evident hematologically, immunologically, or both. In the present study, the increased H:L ratios observed in infected chickens from 2 to 8 DPI that subsequently decreased by 22 DPI may have reflected systemic inflammatory responses to acute WNV infection that normalized after this stage. These ratios increased linearly throughout the study period in the uninfected chickens. The pattern of H:L ratio increase (i.e., low-grade steady increase over time) among uninfected chickens may have reflected endogenous glucocorticoids resulting from handling stress; in contrast, the H:L ratios in infected chickens exhibited a sharp increase (and relatively higher), followed by a decrease after approximately 8 DPI. Although the H:L ratios are a recognized measure of stress in birds (Gross and Siegel, 1983), increasing evidence suggests that lymphocyte populations relative to one another are also sensitive indicators for infection status (Leitner et al., 2000; Huff et al., 2005). However, the decreases in percentages of T lymphocyte subpopulations (i.e., $CD8^+$ T cells) in both WNV-infected and uninfected chickens may have been due to study (stress)-related immunosuppression in both groups; the decrease was more rapid in WNV-infected birds between 2 and 8 DPI, possibly again associated with the combination of handling stress and WNV infection. Measures of lymphocyte subpopulations were limited to percentage composition and were

Table 1. Statistical comparison (GLM) of flow cytometry results, cloacal temperature, blood chemistry, and white blood cell counts between West Nile virus (WNV)-infected and uninfected chickens

Variable	Effect	*F*-value	*P*-value
Cloacal temperature	Time	9.18	0.002
	WNV inoculation	0.48	0.50
	Time × WNV inoculation	0.23	0.80
IgM	Time	573.43	<0.0001
	WNV inoculation	0.35	0.56
	Time × WNV inoculation	3.28	0.05
IgG	Time	4.03	0.04
	WNV inoculation	0.06	0.81
	Time × WNV inoculation	1.43	0.27
$CD3^+$	Time	16.64	<0.0001
	WNV inoculation	0.07	0.79
	Time × WNV inoculation	0.93	0.41
$CD4^+$	Time	27.47	<0.0001
	WNV inoculation	0.83	0.37
	Time × WNV inoculation	0.29	0.75
$CD8^+$	Time	3.69	0.04
	WNV inoculation	2.38	0.14
	Time × WNV inoculation	0.77	0.48
$CD45^+$	Time	3.95	0.04
	WNV inoculation	2.42	0.14
	Time × WNV inoculation	0.78	0.47
$CD4^+$:$CD8^+$ ratio	Time	8.00	0.004
	WNV inoculation	1.36	0.26
	Time × WNV Inoculation	1.99	0.17
Thrombocytes	Time	1.83	0.19
	WNV inoculation	3.42	0.08
	Time × WNV inoculation	0.49	0.62
Monocytes	Time	0.21	0.81
	WNV inoculation	0.00	0.96
	Time × WNV inoculation	0.05	0.95
Total lymphocytes	Time	2.51	0.11
	WNV inoculation	0.86	0.37
	Time × WNV inoculation	5.87	0.01
Glucose	Time	5.17	0.02
	WNV inoculation	0.11	0.75
	Time × WNV inoculation	0.28	0.76
Alkaline phosphatase	Time	3.46	0.05
	WNV inoculation	0.04	0.85
	Time × WNV inoculation	0.27	0.77
Protein	Time	0.24	0.79
	WNV inoculation	0.02	0.88
	Time × WNV inoculation	0.04	0.96
Calcium	Time	25.31	<0.0001
	WNV inoculation	8.04	0.01
	Time × WNV inoculation	6.89	0.006
Phosphorus	Time	4.42	0.03
	WNV inoculation	0.27	0.61
	Time × WNV inoculation	2.07	0.16

not estimates of cell numbers. Blood calcium declined between 2 and 8 DPI in both infected and uninfected hens and returned to preinoculation levels by 22 DPI in the former. Transient hypocalcemia could have been associated with WNV-induced changes in renal physiology (Vig and Kinet, 2009); renal pathology has been observed in chickens experimentally inoculated with WNV (Senne et al., 2000) as well as in other bird species (Nemeth et al., 2006). Because WNV is not highly virulent in chickens (Langevin et al., 2001; Nemeth and Bowen, 2007), the normalization of calcium blood levels in infected birds may have reflected recovery.

Numerous components of the immune response have proven important in the outcome of WNV infection in both birds and mammals. The antibody-mediated immune response to WNV is an important factor in controlling the early course of infection, as well as in determining survival outcomes (Diamond et al., 2003a,b; Dauphin and Zientara, 2007). All inoculated chickens in the present study seroconverted within 8 d, consistent with previous studies (Langevin et al., 2001; Weingartl et al., 2003). Humoral immunity mediated by B cells is a critical component of the immune response to WNV for both IgG (Diamond et al., 2003a) and IgM (Diamond et al., 2003b), and until recently, was thought to play a more important role than T cell responses. Indeed, T lymphocytes are a critical component of immune defense in murine models of WNV infection (Kesson et al., 1987; Wang et al., 2003b; Shrestha and Diamond, 2004; Shrestha et al., 2006). Mice deficient in T-cell receptor β have increased WNV-associated mortality (Wang et al., 2003a) and

the $CD4^+$ cell response occurs primarily in peripheral blood (Kulkarni et al., 1991), whereas $CD8^+$ cells are located in the spleen and brain after WNV infection (Liu et al., 1989). The $CD8^+$ lymphocyte-deficient mice had prolonged survival times but increased mortality after WNV infection; inflammatory cell infiltrates in the brain were primarily $CD8^+$ lymphocytes (Wang et al., 2003b). The $CD4^+$ T cells contribute to the control of WNV infection through multiple mechanisms, including $CD8^+$ T-cell priming, cytokine production, B-cell activation and priming, and direct cytotoxicity (Samuel and Diamond, 2006).

Chickens are considered poor amplifying hosts of WNV because of their low peak viremia titers, which are generally not considered infectious to mosquitoes (Senne et al., 2000; Langevin et al., 2001; Nemeth and Bowen, 2007). Chickens in the present study followed this pattern of low viremia titers of relatively short duration. Limited WNV replication in the blood is consistent with the apparent lack of variation in immunological variables observed between infected and uninfected chickens in the present study. However, numerous other avian species experience high viremia titers that apparently contribute to morbidity and mortality [e.g., various corvid species, including the American crow, blue jay (*Cyanocitta cristata*), and black-billed magpie (*Pica hudsonia*)] (Komar et al., 2003), and characterization of the immune response in these species may provide insight into differential susceptibility to WNV infection among avian taxonomic groups as well as individual birds. For example, house sparrows (*Passer domesticus*) that reached higher WNV titers in blood and other tissues appeared to be more likely to succumb to infection (Nemeth et al., 2009).

In the present study, time had the most predominant effect on immune function and hematological parameters, and the observed patterns were generally consistent between infected and uninfected chickens.

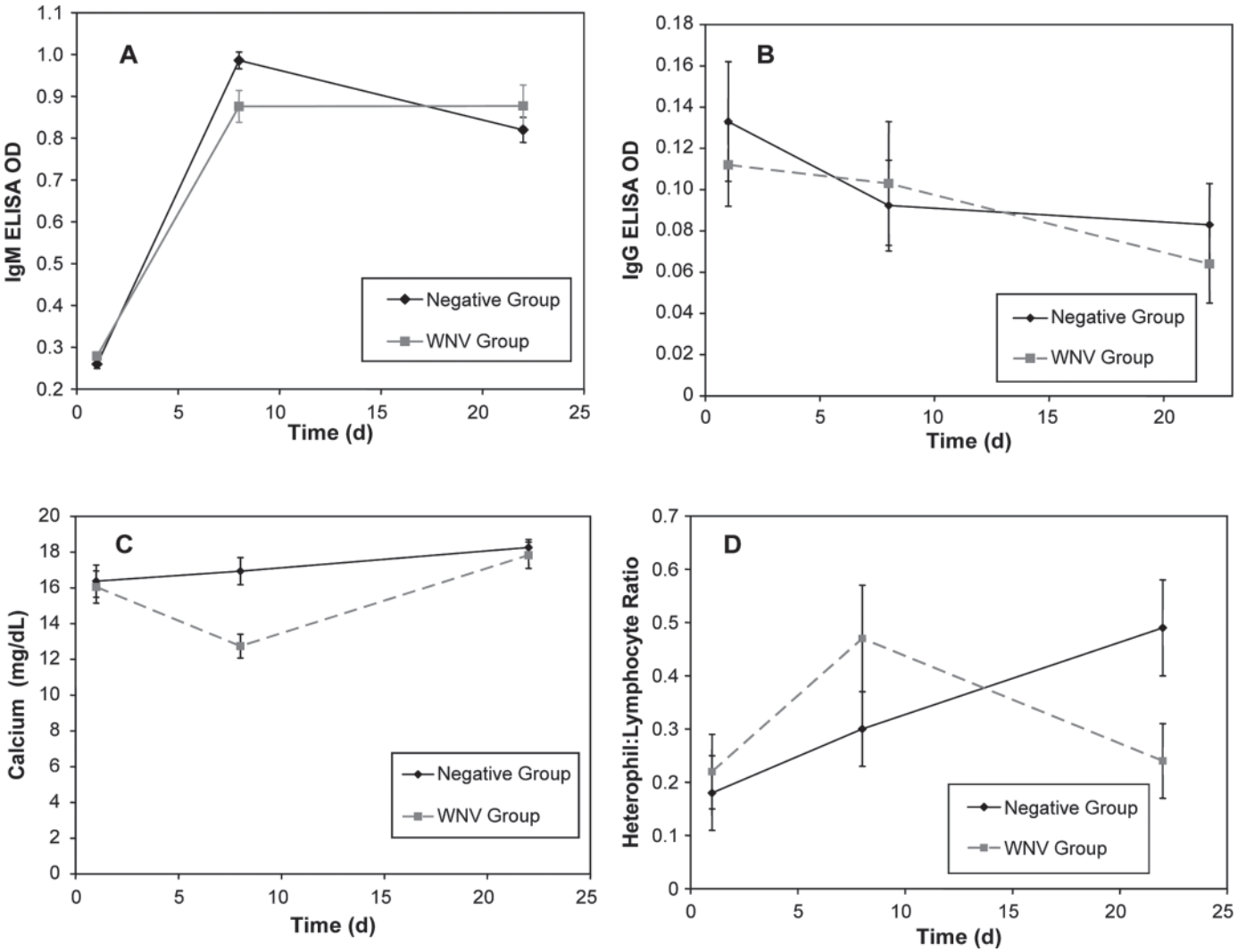

Figure 1. Select variables (panels A to D) in West Nile virus (WNV)-inoculated and noninoculated chickens at 2, 8, and 22 d postinoculation. A) IgM ELISA values, B) IgG ELISA values, C) calcium values, and D) heterophil:lymphocyte ratios from blood smears. OD = optical density units at 450 nm.

Therefore, most of these responses were not likely related to infection. Development of the immune system is thought to be initiated during embryogenesis but may be not complete until weeks or months after hatch (Dibner et al., 1998). Immunosuppression observed in chickens during the first 2 wk posthatch was evidently due to a lack of T-cell function (Lowenthal et al., 1994). Further, Lowenthal et al. (1994) observed that reactivity of T cells to mitogens gradually developed between d 2 and 4 posthatch, and by 1 wk of age, this level of responsiveness was equivalent to that observed in adult-derived T cells. Although the chicken immune system is thought to be mostly developed at 10 wk of age (Lowenthal et al., 1994), immunological development may not have been complete in the hens during the present study (i.e., from 10 to 13.5 wk of age) and may reflect the changes in immunological parameters over time in both infected and uninfected chickens. The ability of the immune system to respond to a specific pathogen may vary with development, and additional research is needed to determine the development of immune function in poultry accurately.

The susceptibility of a host to viral infection is driven by many factors, including the ability of the virus to enter and replicate host cells, as well as the ensuing immune response. For example, the major host range determinant for influenza viruses is the receptor binding site (e.g., sialic acid) for entry of the virus into the host cell (Suzuki et al., 2000). For WNV, a candidate host cell receptor is thought to be $\alpha_v\beta_3$ integrin (Chu and Ng, 2004); integrins are membrane proteins that play a role in the attachment of the cell to the extracellular matrix and in signal transduction.

Overall, we observed no significant differences in most of the variables we examined between WNV-infected and uninfected chickens. Perhaps aspects of the immune response not included in the present study (e.g., cytokines such as interferon-α/β and interferon-γ; Shrestha et al., 2006) are important for resistance to the effects WNV infection in poultry. Furthermore, as

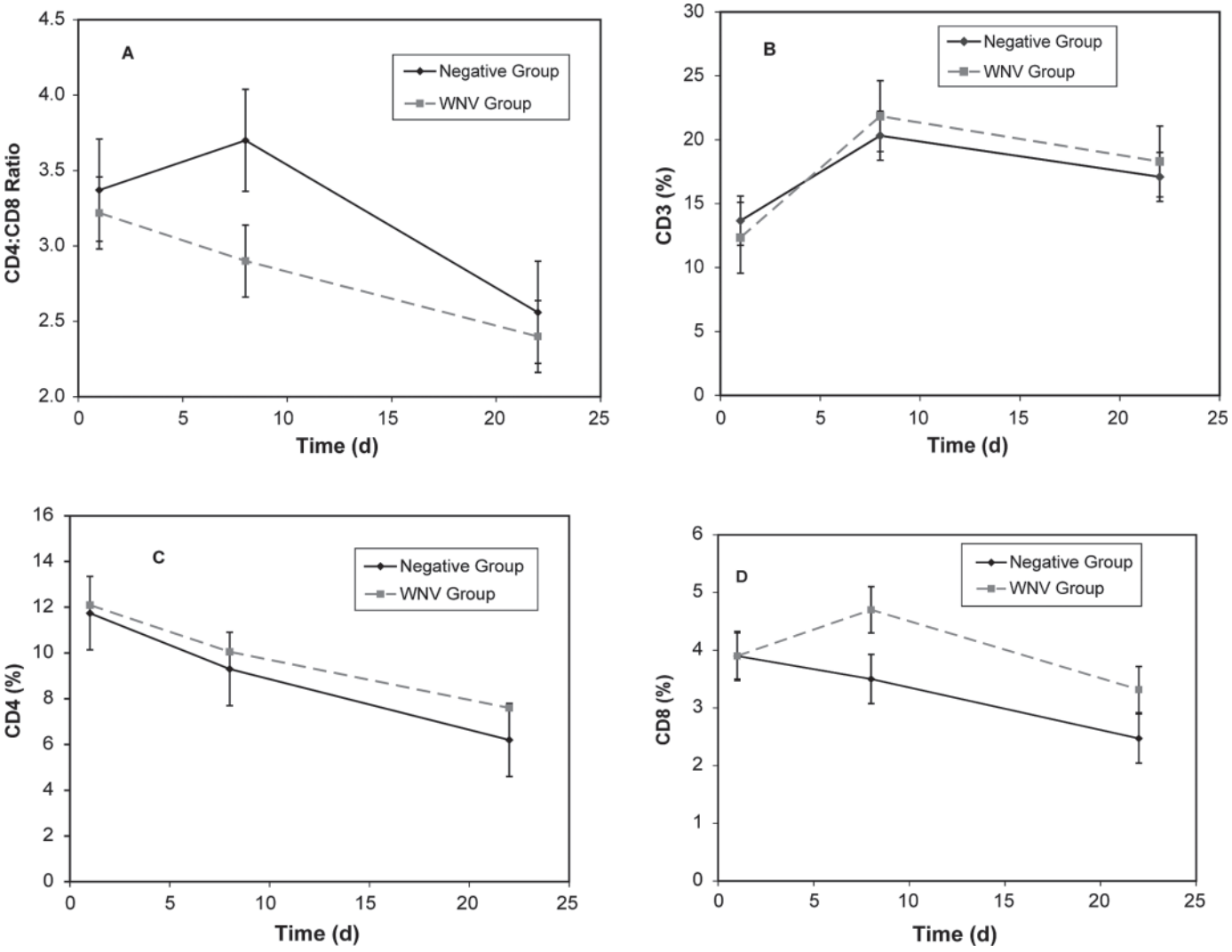

Figure 2. Flow cytometry variables for blood (panels A to D) in West Nile virus (WNV)-inoculated and noninoculated chickens on 2, 8, and 22 d postinoculation. A) $CD4^+$:$CD8^+$ ratios, B) $CD3^+$ percentage of lymphocytes, C) $CD4^+$ percentage, D) $CD8^+$ percentage.

additional immunological tools become available, examination of a wider array of avian species compared with poultry, including those that are more susceptible to WNV infection, will be possible.

ACKNOWLEDGMENTS

We thank the following people for excellence in bird care and laboratory assistance: Chuck Hathcock, David Keller, Lucus Bare, Rhonda Robinson, and Sherri Sherwood [Los Alamos National Laboratory (LANL)]. In addition, we thank Richard Bowen for providing laboratory space and supplies, as well as biosafety level-3 bird space (Colorado State University). We thank Hector Hinojosa and Mark Jankowski (LANL) for comments on an early manuscript draft. This research was supported by the Laboratory Directed Research and Development Program through Los Alamos National Security LLC, operator of the LANL under Contract No. DE-AC52-06NA25396 with the US Department of Energy (Washington, DC). The National Flow Cytometry and Sorting Research Resource at LANL is supported by grant P41-RR01315 from the National Institutes of Health National Center for Research Resources (Bethesda, MD).

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