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Humoral and Cellular Immune Responses in Turkey Poults Infected with Turkey Coronavirus

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ABSTRACT The objective of the present study was to elucidate the kinetics of humoral and cellular immune responses of turkey poults infected with turkey coronavirus (TCV). Turkey poults were orally inoculated with TCV at 10 d of age, and the immune responses were analyzed at 1, 3, 7, 14, 21, 28, 42, and 63 d postinfection (PI) in three different experiments. Total Ig to TCV was initially detected at 7 and 14 d PI in Experiments 1 and 3. In addition, Ig gradually increased from 7 to 21 d PI and remained at 80 immunofluorescent antibody assay (IFA) titers or more thereafter. Lymphocyte proliferation responses of spleen cells to concanavalin A were higher in TCV-infected turkeys than in noninfected control turkeys with significant differences ($P < 0.05$) being noted at 14 and 63 d PI in Experiment 2 and at 3 and 28 d PI in Experiment 3. Strong IFA staining response to TCV antigen was observed in intestines of turkeys at 1, 3, and 7

d PI, and the response declined from 14 to 28 d PI in Experiment 3. In Experiment 3, the IgG isotype antibody response to TCV was markedly increased after 21 d PI and remained high until 63 d PI. The IgM isotype antibody response to TCV was 1.40 and 0.91 at 7 and 14 d PI, respectively. The IgA isotype antibody response to TCV was very low as detected at 7 (0.13), 14 (0.20), and 21 (0.17) d PI. Turkeys infected with TCV had significantly higher ($P < 0.05$) antibody response to sheep erythrocytes than noninfected controls at 7 d PI. Virus-specific lymphocyte proliferation response of spleen cells was significantly stimulated ($P < 0.05$) at 63 d PI in Experiment 3. The proportion of the CD4⁺ subpopulation of T lymphocytes was significantly increased ($P < 0.05$) at 1, 7, and 21 d PI in Experiment 3. The results indicate that humoral and cellular immunities to TCV are elicited in turkeys following infection with TCV.

(Key words: cellular, humoral, immune response, turkey coronavirus, turkey poult enteritis)

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INTRODUCTION

Infection of young turkey poults with turkey coronavirus (TCV) produces a syndrome characterized by acute enteritis, diarrhea, anorexia, ruffled feathers, decreased body weight gain, and uneven flock growth. TCV enteritis, the most costly disease of turkey encountered in Minnesota between 1951 and 1971, is an acute, highly infectious disease, which accounted for 23% of all turkey mortalities and over one-half million dollars in lost income in Minnesota in 1966 (Nagaraja and Pomeroy, 1997). Turkey poult enteritis associated with TCV infection has contributed to significant economic losses for turkey producers in Indiana, North Carolina, and other states for the past several years.

Once the coronaviral enteritis is introduced into areas with high concentrations of turkeys on a year-round basis, it is not easily eliminated and is encountered frequently in turkey poults (Nagaraja and Pomeroy, 1997). Treat-

ments of the disease are often unsuccessful, and there are currently no effective vaccines or medications to prevent the disease. In order to develop strategies for diagnosis, control, and prevention, clarification of immune responses in turkeys infected with TCV is essential. In addition, studies of humoral and cellular immunities may lead to identifying the antigens and epitopes that are responsible for inducing protective immune responses. Such information is important for the development of effective vaccines and vaccination strategies.

Reports of immune responses in turkeys following exposure to TCV are limited. The serum antibodies to TCV were detectable by immunofluorescent antibody (IFA) assay from 9 to 160 d postinfection (PI) (Patel et al., 1976). However, the serum antibodies to TCV were not detectable by immunodiffusion test after 30 d PI in another

Abbreviation Key: BCV = bovine coronavirus; CD = clusters of differentiation; Con A = concanavalin A; EM = electron microscopy; FITC = fluorescein isothiocyanate; IBV = infectious bronchitis virus; IFA = immunofluorescent antibody assay; IL = interleukin; PBL = peripheral blood lymphocyte; PHA-P = phytohemagglutinin-P; PI = postinfection; TCV = turkey coronavirus

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study (Nagaraja and Pomeroy, 1978). In addition, the antibodies to TCV in the intestine and bile were detectable by immunodiffusion test (Nagaraja and Pomeroy, 1978) or a sandwich IFA (Nagaraja and Pomeroy, 1980b) up to 6 mo PI. Thus, the kinetics and magnitudes of antibody responses to TCV infection in turkey poults are not completely known.

The lymphocyte proliferation response of turkey lymphocytes was enhanced in turkeys infected with TCV in a monthly sequential study beginning at 1 mo PI (Nagaraja and Pomeroy, 1980a). However, the infection of TCV in turkey is acute. The clinical signs are evident by 3 d PI and gradually disappear after 7 d PI. Therefore, the interaction between the kinetics and magnitude of cellular immune responses in turkey poults and acute turkey coronaviral infection and subsequent recovery of affected turkeys from the infection is not clear. The present study was undertaken to elucidate the kinetics and magnitude of humoral and cellular immune responses in turkey poults infected with TCV.

MATERIALS AND METHODS

Turkey Eggs and Poults

Turkey eggs and 1-d-old turkey poults (British United Turkey of America, BUTA) of both sexes were obtained.² They were free of recognized pathogens for turkeys, including TCV. Turkey poults were housed in Horsfall-Bauer isolation units for 4 wk and then transferred to isolated floor pens. Feed and water were provided ad libitum.

Virus Isolation and Propagation

Turkey coronavirus was isolated from intestines of 28-d-old turkey poults with outbreaks of acute enteritis. Affected intestines were homogenized with a fivefold volume of PBS, clarified by centrifugation at $3,000 \times g$ for 10 min, and filtered through 0.45- and 0.22- μm membrane filters.³ Filtrates containing only TCV, but not other viruses, were examined by electron microscopy (EM) and immunoelectron microscopy according to procedures previously described (Dea and Garzon, 1991). Twenty-two-day-old embryonated turkey eggs were inoculated with 200 μL of the filtrate via amniotic sac route. Embryo intestines were harvested after 3 d. Harvested embryo intestines were processed and propagated serially as described above for five passages. Intestines of the fifth passaged TCV-infected turkey embryos were prepared as a 20% suspension in PBS. The suspension was homogenized, clarified, filtered as described above, and used as inoculum. The inoculum was also examined by EM and immunoelectron microscopy to confirm the presence of only TCV. The titer of virus was determined by inocula-

tion of the suspension at 10-fold dilutions into seven groups of five 22-d-old embryonated turkey eggs. The 50% embryo infectious dose (EID₅₀) was calculated by the method of Reed and Muench (1938). An inoculum containing 2×10^4 EID₅₀ of TCV/1 mL was prepared and used to experimentally infect turkey poults.

Experimental Design

Three experiments were conducted with three separate hatches of turkey poults. Infection with TCV was confirmed at 3 d PI by examination of intestines of five turkey poults with IFA, histopathology, and EM in each experiment.

Experiment 1. Forty 10-d-old turkey poults were orally inoculated with TCV-containing inoculum prepared from intestines of propagated turkey embryos as previously described. A control group of 40 age-matched birds was sham-inoculated with sterile PBS buffer. Five birds were randomly selected from each group and necropsied at 1, 3, 7, 14, 21, 28, 42, and 63 d PI. Blood was drawn by cardiac puncture from each bird, and the collected serum was stored at -20°C . Virus-specific antibody responses were determined by IFA.

Experiment 2. Thirty 10-d-old turkey poults were orally inoculated with TCV-containing inoculum prepared from intestines of propagated turkey embryos. A control group of 30 age-matched birds was sham-inoculated with sterile PBS buffer. Five birds were randomly selected from each group and necropsied at 7, 14, 21, 28, 42, and 63 d PI. Spleen cells were recovered, and tests were carried out to determine lymphocyte proliferation responses to concanavalin A (ConA).

Experiment 3. Fifty-five 10-d-old turkey poults were orally inoculated with TCV-containing inoculum prepared from intestines of propagated turkey embryos. A control group of 55 age-matched birds was sham-inoculated with sterile PBS buffer. Five birds were randomly selected from each group and necropsied at 1, 3, 7, 14, 21, 28, 42, and 63 d PI. Intestine, blood, and spleen were collected from each bird. The presence of TCV in the intestines was examined by IFA. Virus-specific antibody responses were determined by IFA or antibody-capture ELISA. Spleen cells were recovered, and tests were carried out to determine lymphocyte proliferation responses to ConA or TCV antigen. The proportions of cluster of differentiation (CD)4⁺ and CD8⁺ subpopulations of T lymphocytes in peripheral blood were analyzed by flow cytometry. Five turkey poults each from the experimental and control groups were used for evaluation of antibody responses to SRBC at 1 wk PI and for in vivo mitogenic responses by toe web swelling at 1 and 5 wk PI.

Immunofluorescent Antibody Assay for TCV Antigen in Intestines

The IFA method for detection of TCV was previously described (Patel et al., 1975). Jejunum and ileum were frozen immediately after collection and embedded in em-

²Perdue Farm, Washington, IN 47501.

³Millipore Products Division, Bedford, MA 01730.

bedding medium and sectioned to 6- μ m thicknesses. Tissue sections were incubated with turkey antisera⁴ specific for TCV at 1:40 in dilution buffer containing 150 mM phosphate buffer, 0.85% NaCl, 1% BSA, and 0.02% Tween-20 in a humidifying chamber at room temperature (25 C) for 30 min. After being washed with PBS three times, intestinal sections were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-turkey IgG (H+L) antibodies⁵ at 1:40 in dilution buffer at room temperature for 30 min. The results of IFA were categorized as follows: – (no response), + (weak response), ++ (moderate response), and +++ (strong response).

IFA Assay for Total Immunoglobulins to TCV

The IFA method for detection of antibodies to TCV was previously described (Patel et al., 1976). Frozen intestines of turkey embryos infected with TCV were sectioned and incubated at room temperature for 30 min with twofold serially diluted turkey sera collected at different intervals. For detection of total Ig, intestinal sections were incubated with FITC-conjugated goat anti-turkey IgG (H+L) antibodies⁵ at 1:40 in dilution buffer at room temperature for 30 min in a humidifying chamber. The titer of turkey sera was defined as the reciprocal of the highest dilution of test sample with positive staining.

ELISA for Antibody Isotypes to TCV

The ELISA method for detection of antibody to TCV was previously described (Loa et al., 2000). Ninety-six-well microtiter plates⁶ coated with a pool of MA, JMK, AR, CT, Clone 30, D274, and D1466 strains of infectious bronchitis virus (IBV) antigens were used based on antigenic cross-reactivity between TCV and IBV. Serum samples at 1:40 were added at 100 μ L per well. Two reference wells that contained all reagents except serum samples were included in each plate. After incubation at 37 C for 1 h, plates were washed three times with wash buffer containing 150 mM phosphate buffer, 0.85% NaCl, and 0.02 % Tween-20. For detection of different isotype antibodies, goat anti-chicken IgG (γ -specific), IgM (μ -specific), and IgA (α -specific) horseradish peroxidase- conjugated antibodies⁷ were used at 1:400 in dilution buffer. After incubation, 100 μ L of enzyme substrate, tetramethyl benzidine solution, were added to each well. After incubation at room temperature for 30 min, the reaction in each well was stopped by addition of 2 N HCl solution. The absorbance value of each well was measured at 450 nm with a spectrophotometer.⁸

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⁵Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD 20879.

⁶IDEXX Inc., Westbrook, ME 04092.

⁷Bethyl Laboratories, Inc., Montgomery, TX 77356.

⁸Vmax™ Kinetic Microplate Reader, Molecular Devices Corporation, Sunnyvale, CA 94089.

⁹Sigma, St. Louis, MO 63178.

¹⁰Packard Instrument Company, Meriden, CT 06450.

¹¹Southern Biotechnology Associates, Inc., Birmingham, AL 35209.

Evaluation of Antibody Response to Sheep Erythrocytes

Five turkey poults each, from infected and noninfected groups, were injected intravenously with 1 mL of 7% saline suspension of SRBC at 1 wk PI. Blood samples were collected at 3, 7, and 14 d after injection of SRBC. The anti-SRBC antibody titers were quantified by hemagglutination assay (Dea et al., 1985). The titer was expressed as the log₂ of the reciprocal of the highest dilution at which visible agglutination was observed.

Isolation of Spleen Cells

Spleens were removed, suspended in cold Hank's balanced salt solution, pH 7.0, and crushed by a syringe plunger. Tissue debris was removed by passing the spleen suspensions through 100-mesh stainless screen⁹ to obtain single spleen cell suspensions. The cell suspensions were washed once with Hank's balanced salt solution buffer, and red blood cells were lysed with 7.5% ammonium chloride solution. Spleen cells were washed again, counted, and resuspended in RPMI-1640 medium containing normal turkey serum to a final concentration of 10⁶ cells/mL in Experiment 2, or 5 × 10⁶ cells/mL in Experiment 3.

Lymphocyte Proliferation Assay

Lymphocyte proliferation assay was modified from previously described methods (Nagaraja and Pomeroy, 1980a; Bartaet al., 1992; Sharma and Belzer, 1992). One hundred microliters of 10⁵ (Experiment 2) or 5 × 10⁵ (Experiment 3) spleen cells was placed in a 96-well microtiter plate. One hundred microliters of RPMI medium without mitogen or containing 25 (Experiment 2) or 12.5 (Experiment 3) μ g/mL of Con A or 2 μ g/mL of TCV antigen (Experiment 3) was added to each well of three sequential triplicate cultures in a row. Cell cultures were incubated at 41 C for 48 h for stimulation with Con A or 5 d for stimulation with TCV antigen in a humidified air atmosphere at 5% CO₂. Cultures were pulsed with 1 μ Ci of ³H-thymidine in 50 μ L of RPMI medium per well for a final 5-h incubation. Cells were harvested onto glass filter papers with an automatic cell harvester.¹⁰ The counts per minute (cpm) of individual cell samples were measured in a Packard TopCount Scintillation Counter (Model B9904).¹⁰ The cpm of the triplicate cultures within each sample were averaged. Stimulation index was calculated by dividing the average cpm value in mitogen-stimulated cultures by the average cpm value in unstimulated culture.

Flow Cytometry

Monoclonal antibodies¹¹ CT4 and CT8 specific to chicken CD4 and CD8 molecules, respectively, were used for flow cytometry (Suresh et al., 1993). Peripheral blood lymphocytes (PBL) were prepared from heparinized

blood as previously described (Suresh et al., 1993). Fifty microliters of 10⁶ PBL was incubated on ice for 30 min with the monoclonal antibody at 1:200. The samples were rinsed and incubated with FITC-conjugated goat anti-mouse IgG.¹¹ After a final rinse, staining percentages were analyzed by fluorescence-activated cell sorter.¹² Proportions of CD4⁺ or CD8⁺ cells were determined from 5,000 lymphocytes per sample.

Toe Web Swelling

Response induced in vivo by mitogen was evaluated by injection of phytohemagglutinin-P (PHA-P) into the toe webs between the third and fourth digits of turkey poults. Five turkey poults each from infected and noninfected groups were used at 1 and 5 wk PI. The left foot was injected with 100 µg of PHA-P dissolved in 100 µL of sterile PBS buffer. The right foot was injected with 100 µL of sterile PBS buffer and used as the control. The thickness of toe webs was measured with a constant tension caliper¹³ before injection and 1, 2, and 3 d after injection. Data were calculated as the PHA-P mediated swelling minus the PBS-injected control swelling (mm).

Statistical Analysis

Calculated means from each group were compared by two-tailed *t*-test with significance determined at *P* < 0.05.

RESULTS

Duration of TCV in Intestine

Infection of turkey poults with TCV was confirmed at 3 d PI. The intestines of turkey poults were positive for TCV antigen in IFA and had acute atrophic enteritis as examined by histopathology. Turkey coronaviral particles in the intestinal contents were observed by EM.

The presence of TCV in intestines of turkeys at different times PI, as determined by IFA, is summarized in Table 1. Intestines of noninfected control turkeys from each interval were negative for TCV. Strong IFA response to TCV was observed in intestines of turkeys within 7 d PI. The intensity of IFA response to TCV declined from 14 to 28 d PI. The viral antigen was no longer detectable by IFA at 42 and 63 d PI.

Antibody Responses to TCV

Total immunoglobulins specific to TCV, as determined by IFA, are shown in Table 2. The IFA titer was initially detected at 7 (Experiment 3) or 14 (Experiment 1) d PI. All five turkeys had virus-specific IFA titers after 14 d PI in both Experiments 1 and 3. The noninfected control turkeys remained negative in the IFA assay throughout the experiments.

TABLE 1. Detection of turkey coronavirus (TCV) antigen by immunofluorescent antibody assay (IFA) in turkey intestines at various intervals following infection of turkey poults with TCV¹

Days PI ²	Noninfected				TCV-infected			
	+++ ³	++	+	-	+++	++	+	-
1	0 ⁴	0	0	5	3	1	0	1
3	0	0	0	5	5	0	0	0
7	0	0	0	5	2	3	0	0
14	0	0	0	5	0	2	2	1
21	0	0	0	5	0	3	2	0
28	0	0	0	5	0	0	1	4
42	0	0	0	5	0	0	0	5
63	0	0	0	5	0	0	0	5

¹Turkey poults were infected with TCV at 10 d of age.

²PI = postinfection with TCV.

³The results of IFA were determined as follows: - (no response), + (weak response), ++ (moderate response), and +++ (strong response).

⁴Data are expressed as the number of turkey poults under each IFA category.

The kinetics of antibody isotype responses to TCV as detected by antibody-capture ELISA is shown in Figure 1. The ELISA readings for virus-specific IgG antibody responses were above 3.00 after 21 d PI and persisted throughout the study. Virus-specific IgM antibody responses were 1.40 and 0.91 at 7 and 14 d PI, respectively. Low levels of virus-specific IgA antibody responses were detectable by ELISA in infected turkeys at 7 (0.13), 14 (0.20), and 21 (0.17) d PI.

TABLE 2. Specific humoral antibody responses to turkey coronavirus (TCV) at various intervals following infection of turkey poults with TCV as determined by immunofluorescent antibody assay (IFA)¹

Days PI ²	IFA titer ³						
	0	40	80	160	320	640	1,280
Experiment 1							
1	5 ⁴	0	0	0	0	0	0
3	5	0	0	0	0	0	0
7	5	0	0	0	0	0	0
14	0	3	1	1	0	0	0
21	0	0	3	1	1	0	0
28	0	0	0	2	1	2	0
42	0	0	0	1	3	1	0
63	0	0	1	0	2	2	0
Experiment 3							
1	5	0	0	0	0	0	0
3	5	0	0	0	0	0	0
7	3	2	0	0	0	0	0
14	0	0	5	0	0	0	0
21	0	0	0	0	1	3	1
28	0	0	0	0	1	2	2
42	0	0	0	1	2	1	1
63	0	0	0	0	3	1	1

¹Turkey poults were infected with TCV at 10 d of age.

²PI = postinfection with TCV.

³IFA titer defined the reciprocal of the highest dilution of serum still having positive staining.

⁴Data are expressed as number of turkey poults under each titer category.

¹²Becton Dickinson, Bedford, MA 01730.

¹³Ames, Waltham, MA 02451.

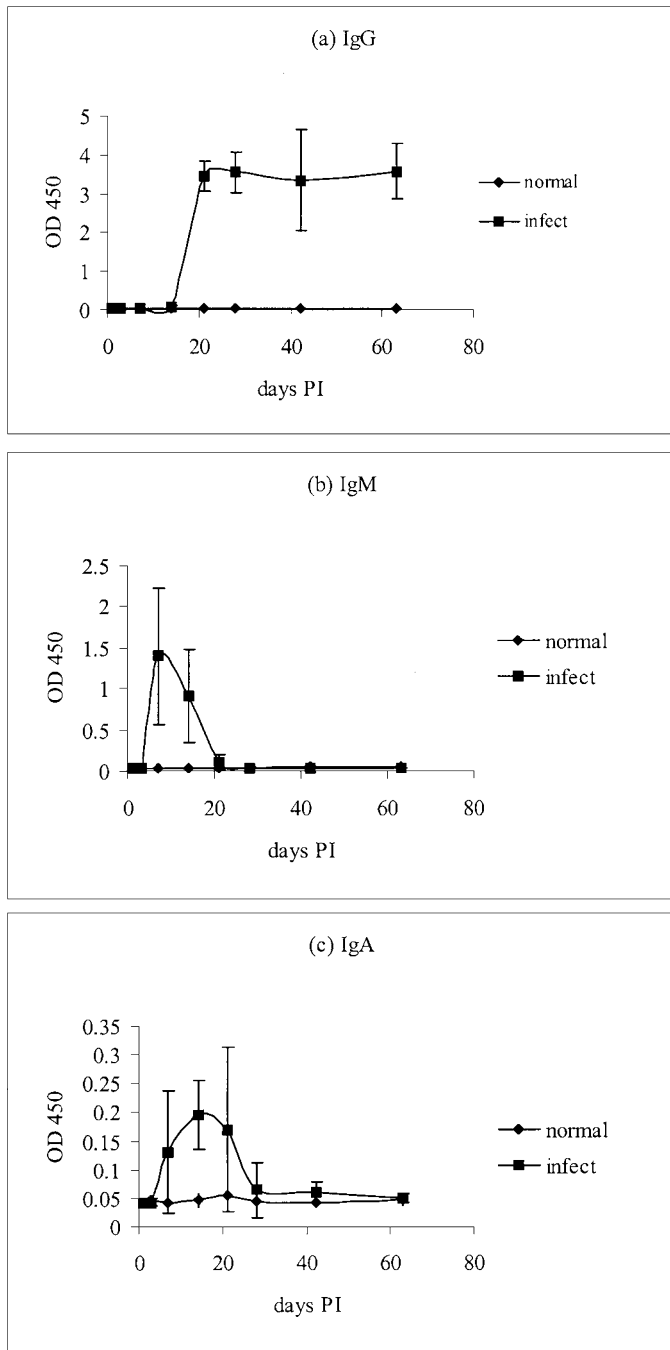


FIGURE 1. Kinetics of virus-specific IgG (a), IgM (b), and IgA (c) antibody responses as determined by an antibody-capture ELISA. Turkey poults were orally infected with turkey coronavirus (TCV) at 10 d of age. Serum samples were randomly collected from turkeys from infected or uninfected control groups at 1, 3, 7, 14, 21, 28, 42, and 63 d postinoculation (PI), and the specific antibody responses to TCV were determined. The error bars represent the SD of samples. OD 450 = optical density read at 450 nm.

Antibody Responses to SRBC

The antibody response to SRBC was significantly increased ($P < 0.05$) at 3 d after injection of SRBC in turkey poults at 7 d PI (Table 3).

Lymphocyte Proliferation Response

The mean of the stimulation index for lymphocyte proliferation response of turkey spleen cells to ConA was higher in the TCV-infected group than in the control group at all intervals in Experiments 2 and 3 (Tables 4 and 5). Statistically significant differences ($P < 0.05$) were observed at 14 and 63 d PI in Experiment 2 and at 3 and 28 d PI in Experiment 3. The lymphocyte proliferation response of turkey spleen cells to TCV antigen was significantly stimulated ($P < 0.05$) at 63 d PI (Table 6).

Flow Cytometric Analysis of CD4⁺ and CD8⁺ T-Cell Subpopulations

Peripheral blood lymphocytes of turkeys infected with TCV had a statistically higher proportion of CD4⁺ cells at 1, 7, and 21 d PI than that of normal controls (Table 7). No significant differences of CD4⁺ cell proportions in the PBL were observed between the two groups after 28 d PI. The CD8⁺ cells in PBL were not detectable in samples at any interval.

Toe Web Swelling

The results of toe web swelling of turkeys infected with TCV for 1 or 5 wk in response to injection of PHA-P are shown in Table 8. The difference of toe web swelling between the infected and control groups was not significant at any interval.

DISCUSSION

The findings that TCV antigen was detectable by IFA in the intestine of turkey from 1 d to 4 wk after infection with TCV were consistent with a previous report (Patelet et al., 1975). The level of TCV antigen was high in the intestine within 1 wk PI as indicated by strong IFA response. The first week following primary infection is the period when the viral antigens are processed and presented to immune cells. Vigorous replication of TCV in the intestine provided abundant viral antigen for presentation and

TABLE 3. Anti-SRBC antibody response of turkey poults infected with turkey coronavirus (TCV)¹

Group	Days after SRBC injection		
	Day 3	Day 7	Day 14
Noninfected	1.0 ± 0.000 ²	2.6 ± 0.894	1.5 ± 0.577
TCV-infected	3.4 ± 1.949 ³	3.8 ± 1.304	1.8 ± 0.837

¹Turkey poults were infected with TCV at 10 d of age. Five poults from the infected or noninfected control group at 7 d after infection with TCV were injected intravenously with 1 ml of 7% SRBC solution. Serum samples were collected from individual poults at 3, 7, and 14 d after SRBC injection. The antibody titer to SRBC was determined by hemagglutination assay.

²Data are expressed as mean ± SD of hemagglutination titers (log₂) from five turkey poults in each group on the day indicated.

³The difference between infected and noninfected control groups was significant ($P < 0.05$).

TABLE 4. Lymphocyte proliferation response of turkey spleen cells to concanavalin A (ConA) at various intervals following infection of turkey poults with turkey coronavirus (TCV)¹ (Experiment 2)

Days PI ²	Noninfected		Infected	
	Background (cpm) ³	SI ⁴	Background (cpm)	SI
7	133.04 ± 15.75	3.00 ± 1.80	47.32 ± 12.58	4.52 ± 1.73
14	176.60 ± 30.72	6.03 ± 2.95	78.37 ± 18.70	18.67 ± 8.49 ⁵
21	136.13 ± 24.78	11.37 ± 2.60	190.75 ± 96.42	13.71 ± 5.88
28	106.04 ± 28.14	23.60 ± 10.33	117.43 ± 71.63	25.93 ± 11.03
42	77.44 ± 18.74	2.33 ± 2.59	169.55 ± 54.81	3.81 ± 4.45
63	28.86 ± 4.91	1.46 ± 0.99	168.08 ± 56.53	128.72 ± 68.94 ⁵

¹Turkey poults were infected with TCV at 10 d of age.

²PI = postinfection with TCV.

³The background counts per minute of cultures without stimulation of ConA are expressed as mean ± SD of counts per minute from five turkey poults in each group on the day indicated.

⁴SI = stimulation index. The SI was calculated by dividing the average counts per minute from mitogen-stimulated cultures by the average counts per minute from unstimulated cultures. Data are expressed as mean ± SD of SI from five turkey poults in each group on the day indicated.

⁵The difference between infected and noninfected control groups was significant (*P* < 0.05).

recognition of immune cells. Presentation of viral antigens resulted in activation of the immune cells—both B and T cells. The enhancement of T-cell immunocompetency was evident by the observation of significantly higher lymphocyte proliferation response to ConA at 3 d PI in Experiment 3 and by significantly higher proportions of CD4⁺ cells in PBL at 1 and 7 d PI in infected turkeys. Given the central role of T cells bearing CD4 molecules (helper T cells) in the immune system, the proportion of CD4⁺ cells was expected to be enhanced immediately following infection at 1 d PI.

The activation of B cells required stimulations of viral antigens and signals from activated helper T cells. After activation, B cells undergo proliferation and differentiation and become specific plasma cells (antibody-secreting cells), which was evident by the initial appearance of virus-specific IgM antibody response at 7 d PI. General humoral immunity was also activated, as shown by the

observation that turkeys infected with TCV could be primed for faster and higher antibody response to an unrelated foreign antigen, sheep erythrocytes, at 7 d PI than control turkeys. Cellular immunity was also activated at 1 wk PI. The lymphocyte proliferation responses to ConA were significantly higher in turkeys infected with TCV at 3 d PI in Experiment 3, which suggests that the functional competent T cells were increased.

The activation of humoral and cellular immunities was reflected by decreased viral antigen from 14 to 28 d PI. During this period, the stimulated specific humoral immunity was further enhanced as shown by decreased specific IgM antibodies and elevated specific IgG antibodies. This result indicates isotype switching and affinity maturation of the virus-specific low-affinity IgM plasma cells to high-affinity IgG plasma cells. The cellular immunity remained high during this period. The proportion of CD4⁺ cells was significantly higher at 21 d PI in infected

TABLE 5. Lymphocyte proliferation response of turkey spleen cells to concanavalin A (ConA) at various intervals following infection of turkey poults with turkey coronavirus (TCV)¹ (Experiment 3)

Days PI ²	Noninfected		Infected	
	Background (cpm) ³	SI ⁴	Background (cpm)	SI
1	28,499.90 ± 12,473.96	19.95 ± 13.33	9,374.41 ± 6,538.34	26.29 ± 21.97
3	2,062.27 ± 1,140.33	13.78 ± 7.12	4,369.63 ± 1,864.19	136.52 ± 31.53 ⁶
7	6181.36 ± 6,779.22	17.13 ± 16.87	2,139.73 ± 393.05	78.51 ± 57.36
14	1,359.93 ± 708.38	145.75 ± 142.22	3,135.25 ± 911.18	157.56 ± 44.65
21	7,735.92 ± 7,677.23	47.77 ± 46.12	11,246.73 ± 5,295.75	68.38 ± 66.59
28	4,893.32 ± 1,254.97	37.96 ± 47.71	3,101.53 ± 1,360.58	150.58 ± 62.81 ⁶
42	242.95 ± 77.66	5.08 ± 4.24	560.74 ± 301.10	108.90 ± 100.29
63	— ⁵	—	—	—

¹Turkey poults were infected with TCV at 10 d of age.

²PI = postinfection with TCV.

³The background counts per minute of cultures without stimulation of ConA are expressed as mean ± SD of counts per minute from five turkey poults in each group on the day indicated.

⁴SI = stimulation index. The SI was calculated by dividing the average counts per minute in mitogen-stimulated cultures by the average counts per minute in unstimulated cultures. Data are expressed as mean ± SD of SI from five turkey poults in each group on the day indicated.

⁵Data not available due to processing error.

⁶The difference between infected and noninfected control groups is significant (*P* < 0.05).

TABLE 6. Lymphocyte proliferation response of turkey spleen cells to turkey coronavirus (TCV) antigen at various intervals following infection of turkey poults with TCV¹ (Experiment 3)

Days PI ²	Noninfected		Infected	
	Background (cpm) ³	SI ⁴	Background (cpm)	SI
1	2,941.05 ± 441.64	4.38 ± 2.59	1,651.91 ± 829.77	5.06 ± 3.17
3	645.97 ± 165.12	17.18 ± 5.24	560.83 ± 100.56	23.07 ± 13.64
7	2,134.11 ± 2,140.10	5.09 ± 3.25	2,098.31 ± 1,008.05	5.85 ± 3.77
14	1,243.47 ± 444.23	15.68 ± 4.30	653.08 ± 402.77	31.35 ± 18.06
21	1,790.53 ± 2,721.51	16.17 ± 9.68	3,054.88 ± 3,339.29	18.25 ± 15.65
28	587.30 ± 481.43	25.32 ± 39.16	1,472.24 ± 2,073.55	38.43 ± 27.12
42	253.89 ± 188.02	24.95 ± 14.40	501.15 ± 185.50	12.23 ± 7.31
63	86.182 ± 15.86	1.64 ± 1.09	76.15 ± 20.75	50.54 ± 21.17 ⁵

Turkey poults were infected with TCV at 10 d of age.

²PI = postinfection with TCV.

³The background counts per minute of cultures without stimulation of TCV antigen are expressed as mean ± SD of counts per minute readings from five turkey poults in each group on the day indicated.

⁴SI = stimulation index. The SI was calculated by dividing the average counts per minute in TCV-stimulated cultures by the average counts per minute in unstimulated cultures. Data are expressed as mean ± SD of SI from five turkey poults in each group on the day indicated.

⁵The difference between infected and noninfected control groups is significant ($P < 0.05$).

turkeys. The lymphocyte proliferation response to ConA was significantly higher in infected turkeys at 14 and 28 d PI in Experiments 2 and 3, respectively. These findings clearly indicate a negative correlation between the immune responses and the duration of TCV in the intestines and that humoral and cellular immunities may be required for recovery of infected turkeys from TCV infection.

After 28 d PI, when the viral antigen was no longer detectable at 42 and 63 d PI, specific humoral and cellular immunities remained high as evidenced by the consistently high level of virus-specific IgG antibody responses and the detection of significantly higher lymphocyte proliferation responses to TCV (Experiment 3) or ConA (Experiment 2) at 63 d PI. On the other hand, general immunity seems to return to normal because the proportions of CD4⁺ cells between infected and noninfected turkeys

were similar at 28, 42, and 63 d PI. Immune responses are antigen-driven. The CD4⁺ cells play an important role for activation of immune system following exposure to foreign antigen. Once antigen is eliminated and specific immune responses are built-up, the helper function of CD4⁺ cells and the machinery for establishment of specific immunities stop. The mechanism for the persistence of TCV-specific immunities was not clear. Long-term antibody responses are believed to be maintained by continuous differentiation of memory B cells into antibody-secreting plasma cells. However, long-term antibody responses have also been reported to be dependent on long-lived plasma cells (Slifka et al., 1998). The persistence of long-term specific cellular immunity in turkeys infected with TCV may also be maintained by continuous differentiation of corresponding memory cells or long-lived effector cells.

Cross-reactivity of chicken-specific anti-immunoglobulins with turkey immunoglobulins has been reported previously (Van Nerom et al., 1997). Cross-reactivity was also observed in the present study as indicated by using commercially available anti-chicken IgG (γ -specific), IgM (μ -specific), and IgA (α -specific) antibodies in the ELISA assay for detection of TCV-specific antibody responses. The observation of low-level specific IgA antibodies is in line with the fact that IgA is only a minor part (10 to 15%) of the Ig in serum and serum IgA has no known biological function. Most IgA is present in surface secretions such as mucus, tears, or saliva, where it serves as an important first-line of defense against infection by microbial pathogens. Activation of resting B cells to TCV-specific plasma cells is likely to occur in the local mucosa-associated immune tissue along the intestine. The observed stimulation of systemic antibody response probably reflected stimulation of local antibody responses. Further studies on antibody responses in the intestines of turkeys infected with TCV are needed to clarify the relationship between the

TABLE 7. Turkey peripheral blood lymphocytes stained with anti-CD4 antibody¹ at various intervals following infection of turkey poults with turkey coronavirus (TCV)²

Days PI ³	% Stained	
	Noninfected	Infected
1	14.94 ± 6.94 ⁴	33.00 ± 15.03 ⁵
3	25.26 ± 17.86	24.08 ± 1.80
7	20.88 ± 4.68	43.52 ± 7.35 ⁵
14	18.22 ± 9.52	22.78 ± 10.21
21	8.94 ± 0.72	15.96 ± 5.69 ⁵
28	10.34 ± 6.61	10.96 ± 4.20
42	12.82 ± 4.23	10.74 ± 2.33
63	8.64 ± 1.71	9.94 ± 1.87

¹Mouse anti-chicken CD4 monoclonal antibody (Southern Biotechnology Associates, Inc., Birmingham, AL).

²Turkey poults were infected with TCV at 10 d of age.

³PI = postinfection with TCV.

⁴Data are expressed as mean ± SD of five turkey poults in each group on the day indicated.

⁵The difference between infected and noninfected control groups is significant ($P < 0.05$).

TABLE 8. Swelling (mm) in turkey toe webs in response to phytohemagglutinin-P (PHA-P) injection at 1 or 5 wk following infection of turkey poult with turkey coronavirus (TCV)¹

Group	Days after PHA-P injection		
	Day 1	Day 2	Day 3
Wk 1 PI ²			
Noninfected	0.864 ± 0.127 ³	0.356 ± 0.102	0.483 ± 0.152
Infected	0.762 ± 0.051	0.406 ± 0.229	0.330 ± 0.152
Wk 5 PI			
Noninfected	0.711 ± 0.102	0.406 ± 0.102	0.914 ± 0.229
Infected	0.787 ± 0.229	0.483 ± 0.152	1.092 ± 0.229

¹Turkey poult were infected with TCV at 10 d of age. A toe web of a turkey poult in each group was injected with 100 µg of PHA-P at 1 or 5 wk PI.

²PI = postinfection with TCV.

³Data are expressed as mean ± SD of swellings from five turkey poult in each group on the day indicated.

systemic and local antibody responses and their contribution to the elimination of TCV antigens.

The results of serum antibody isotype-specific responses to TCV in the present study were similar to those reported for calves experimentally infected with bovine coronavirus (BCV) (El-Kanawati et al., 1996) and for chickens experimentally infected with IBV (Dhinakar Raj and Jones, 1997). The IgM antibody responses to BCV or IBV present only transiently after infection, reach peak concentrations about 7 d PI, and then decline. As the IgM antibody levels declined, the IgG antibodies to BCV or IBV substantially increased and remained the predominant isotype in serum.

In vitro lymphocyte proliferation response to mitogen is widely used to evaluate the integrity of cell-mediated immunity. ConA and PHA are mitogens commonly used in such assay. ConA, a plant-derived lectin, is considered a T-cell-specific mitogen that stimulates predominantly T cells. Lymphocyte proliferation response of spleen cells to ConA was increased in turkeys infected with TCV in the present study. Similar findings were also observed for pigs infected with transmissible gastroenteritis coronavirus (Brim et al., 1995). However, a statistically significant difference of TCV-specific lymphocyte proliferation response between infected and normal control turkeys was found only at 63 d PI, which was largely due to the high lymphocyte proliferation responses of noninfected control turkeys to TCV antigen. The lymphocyte proliferation responses of noninfected control turkeys to TCV antigen were not due to contaminant infection of control turkeys with TCV, because noninfected controls were negative for TCV antigen as well as for antibodies to TCV. The TCV antigen used in the in vitro lymphocyte proliferation assay was prepared from infected turkey embryo intestines. The extraneous materials contained in the TCV antigen preparation might have contributed to such high nonspecific response in spleen cells of control turkeys.

Phytohemagglutinin is a lectin isolated from red kidney bean and stimulates T-cell proliferation with minimal effects on B cells (Tizard, 1996). Therefore, lymphocyte proliferation response to intradermal injection of PHA-P is considered an in vivo measurement of T-cell function.

The toe web swelling in response to injection of PHA-P was increased in turkeys in the present study. However, there was no significant increase associated with TCV infection, although in vitro lymphocyte proliferation responses and the proportions of CD4⁺ T cells were significantly enhanced. Toe web swelling stimulated by PHA-P might not have been sensitive enough to detect the differences of in vivo lymphocyte proliferation response between groups. Similar findings of increased in vitro lymphocyte proliferation response with undetectable difference of toe web swelling response to PHA-P injection has also been reported for chicken infected with *Salmonella typhimurium* (Lessard et al., 1995).

CD4 and CD8 molecules are expressed on two mutually exclusive subsets of T cells. The cells expressing CD4 molecules recognize antigens restricted by MHC II on antigen-presenting cells and provide cytokine-driven expansion of B- and T cell-mediated specific immune responses. Enhancement of CD4⁺ cells proportion was noted in flow cytometric analysis of peripheral blood cells following TCV infection in the present study. This finding is in line with a previous report that the CD4⁺:CD8⁺ ratio in the spleen of coronavirus-positive PEMS poult was higher compared with the uninfected poult (Heggen et al., 1998). The increase in proportion of CD4⁺ cells could account for the observed increase in humoral and cellular immunofunctional abilities. The positive effect of CD4⁺ (helper) T cells on the different aspects of immune system may be due to the increased number of cells as well as their innate functional ability, such as the production and release of cytokines. Secretion of interleukin (IL)-4 and IL-6 by activated helper T cells are crucial for activation, proliferation, and terminal differentiation of resting B cells to specific antibody-secreting plasma cells. IL-2 is required for activation, proliferation, and differentiation of resting CD8⁺ T cells to cytotoxic T effector cells. Two subsets of CD4⁺ T cells can be distinguished by the cytokines they secrete. Type 1 subset secretes IL-2 and interferon-γ and is responsible for the arm of cellular immunity. Type 2 subset secretes IL-4, IL-5, IL-6, and IL-10 and is responsible for the arm of humoral immunity. Further studies on the effects of TCV on levels of cytokines and on the relative levels of Type 1-like and Type 2-like activ-

ity will clarify the interactions between the helper T cells and the immune status of turkey poults.

CD8-defined T cells are crucial in specific cytotoxicity against virally infected target cells (Tizard, 1996). The cells expressing CD8 molecules recognize antigens restricted by MHC I on target cells and play a major role in the specific cell-mediated immune responses. Because antibody specific for turkey CD8 molecule is not available, a monoclonal antibody specific for chicken CD8 was used in the present study for examination of CD8⁺ subproportion in the PBL by flow cytometry. However, the positive peak for CD8⁺ cells was not detectable in all turkey PBL samples, which suggests poor cross-reactivity of the chicken CD8-specific monoclonal antibody with the turkey CD8 molecule.

The results in the present study indicate that humoral and cellular immune responses to TCV were induced in turkey poults infected with TCV. In addition, the negative relationship was present between the immune responses of turkey poults infected with TCV and the presence of TCV in the infected intestines. The elicited humoral and cellular immunities may provide protective immunity for infected turkeys to recover from TCV infection.

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