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Peripheral blood leukocyte response and macrophage function during *Eimeria adenoeides* infection in turkey poults

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ABSTRACT

Intestinal coccidiosis, caused by various species of *Eimeria*, is an economically important disease of chickens and turkeys. The peripheral blood leukocyte response and macrophage functions during a coccidial infection in turkeys have not been defined. To examine these aspects of innate immunity during primary *Eimeria* infection in turkeys, 4-week-old poults were orally inoculated with either 50,000 *E. adenoeides* oocyst (24 infected poults) or water (24 control poults). To monitor the concentrations and proportions of white blood cells (WBC) throughout the course of infection, heparinized blood was collected from 12 infected and 12 control poults prior to inoculation (day 0), and on days 4, 7, and 11 post-inoculation (PI). To study macrophage function, Sephadex-elicited abdominal exudate cells (macrophages) were collected on day 7 PI from 12 infected and 12 control poults. Macrophages were used to study phagocytosis of unopsonized and antibody-opsonized sheep red blood cells (SRBC), production of nitric oxide, and production of cytotoxic factors. *E. adenoeides* infection was associated with alterations in the concentration of WBC, including a decrease in the numbers of circulating lymphocytes on day 4 and a rise in lymphocytes and heterophils on day 11. Although phagocytic activity was not different in macrophages from infected and control poults, macrophages from infected poults exhibited greater cytotoxic activity. Data from these studies strongly suggest that components of innate immunity were recruited and activated during this primary infection of turkey poults with *E. adenoeides*. Further investigations are needed to determine the role of these components in limiting primary infection by *E. adenoeides*.

[*] Freshmen- and sophomore-level students who conducted this interdisciplinary team research project during Spring 2000 as part of the laboratory rotations in the agricultural research course. Amanda Drake is the primary author.

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Meet the Student-Authors

The student authors were first year and sophomore students in the experimental "Laboratory Rotations in Agricultural Research" course funded by a USDA higher education challenge grant. After having learned techniques and approaches used by faculty members David Chapman, Gisela Erf, and Mark Parcells in structured laboratory sessions, this group of students decided to conduct an interdisciplinary team research project combining skills learned in immunology and parasitology under the tutelage of Erf and Chapman. The project culminated in an oral research presentation and a written abstract, describing the objectives, methods, results, and conclusions.

The primary author of this paper is Amanda Drake, who reevaluated the data prior to preparation of the manuscript.

"I thought the class was a really neat experience," Drake said. "When I came to school, I already knew I was interested in doing research, I just didn't know I'd have the opportunity to participate in it so soon." A native of Pine Bluff, and a graduate of Sheridan High School, Drake is now a junior poultry science major.

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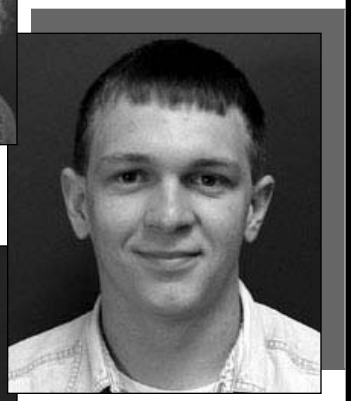
Timothy G. Kimball, now a sophomore poultry science major, is from Combs, and graduated from St. Paul High School.



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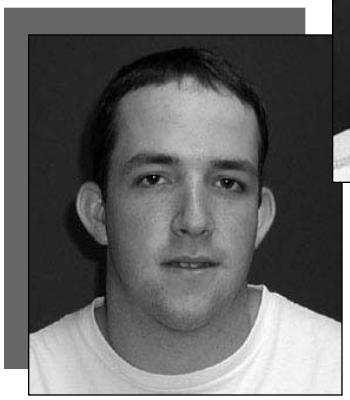
Mena native Timothy Peters, a graduate of Mena High School, is a senior poultry science major.



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INTRODUCTION

Coccidiosis is a disease of chickens and turkeys caused by various species of *Eimeria*. The intestinal infection caused by this intracellular protozoan parasite seriously impairs the growth and feed utilization of infected birds, thus coccidiosis has become an economically important disease of poultry throughout the world (McDougald and Reid, 1991). Host immune responses to coccidial infection are complex and not fully understood (Rose, 1996). Although parasite-specific antibodies are produced during the adaptive immune response to *Eimeria*, cell-mediated rather than antibody-mediated responses appear to play an important role in protection against coccidiosis. At this time, direct parasite-specific killing of infected cells by cytotoxic T cells is believed to be a major mechanism of cell-mediated immunity in the elimination of the parasite (Lillehoj and Trout, 1996; McDonald, 1999). During initial exposure to *Eimeria*, components of innate immunity are likely to be important until adaptive immunity has had time to develop. In chickens, immune responses in primary *Eimeria* infections involve changes in white blood cell (WBC) concentrations, production of oxidative radicals including nitric oxide, and production of cytokines such as interleukin-1 and tumor necrosis-like factor (TNLF) (Byrnes et al., 1993; Rose et al., 1979; Zhang et al., 1995). Moreover, macrophages are known to play an important role in reducing oocyst numbers in the feces during a primary infection (Lee and Al-Izzi, 1981).

Eimeria adenoeides develops in the ceca of young turkey poults. Pathological signs of the disease include severe enteritis of the lower small intestine, ceca, and rectum, watery stools (containing mucus or blood), and yellow, cheesy droppings. For *E. adenoeides*, the period from the initial infection to the appearance of oocysts in the feces is between 4 and 6 days (Clarkson, 1958). Depending on the numbers of oocysts ingested (e.g., 100,000 to 200,000), *E. adenoeides* may cause up to 100 % mortality 5 days after infection (Clarkson, 1958). Poults recovering from infection have developed immunity to *E. adenoeides* and the resolution of the infection in the intestine is associated with lymphocyte infiltration into the submucosa and the epithelium of the villi.

The objective of this research project was to examine aspects of innate immunity in response to a primary infection with *E. adenoeides* in 4-week-old turkey poults. Aspects of innate immune activity examined included assessment of WBC concentrations, WBC profiles, and macrophage function over an 11-day period following primary *E. adenoeides* infection.

MATERIALS AND METHODS

Experimental Animals: Forty-eight 4-week-old Nicholas turkey poults were reared at the University of Arkansas Poultry Health Laboratory in a HEPA-filtered environment maintained under biosecurity level 2. At 3 weeks of age, the poults were moved from the Poultry Health Laboratory to a battery cage facility at the University of Arkansas Poultry Veterinary Farm. Two groups of 24 poults were used for these studies; Group 1 was used for a hematology study, and Group 2 was used to study macrophage function. For each group of birds, 12 poults were randomly selected and inoculated orally with 50,000 *E. adenoeides* oocysts (infected birds). The other 12 poults in each group were sham-inoculated with water (control birds). Throughout the duration of the experiments, food and water were available ad libitum and standard rearing, lighting, and temperature protocols were followed. To monitor coccidia infection in Group 1, feces were collected daily from all infected and control poults for determination of the number of oocysts excreted. Similarly, feces from Group 2 were collected and oocyst counts conducted on a daily basis until birds were euthanized on day 7 post-inoculation (PI). Additionally, poults in Group 2 were subjected to post-mortem examination to determine the severity of coccidia infection based on a lesion score that ranged from 0 (no lesion) to 5 (maximal lesion).

Hematology Study: Prior to and 4, 7 and 11 days PI, all poults in Group 1 were weighed to the nearest gram, and a 1.5 mL blood sample was taken from a wing vein using a heparinized syringe. Total WBC concentration was determined using an automated hematology analyzer (CELL-DYN). Blood smears were also prepared and stained with Wright stain to determine the proportions of the various WBC populations (lymphocytes, heterophils, monocytes, eosinophils, and basophils). For each poult, at least 300 WBC were examined using a bright field microscope and 1000x magnification (Lucas and Jamroz, 1961). The concentration of each type of WBC was then calculated based on the total WBC concentration and on the proportion of a type of WBC within the total WBC population. Body-weight

measurements were used to calculate total body-weight gain over the 11-day experimental period (day 0-11), body-weight gain between day 4 and day 11 (day 4-11), and body-weight gain between day 7 and day 11 (day 7-11).

Abdominal-Exudate Cell Elicitation and Preparation of Macrophage Cell Suspensions: Five days PI, birds in Group 2 were weighed and injected intra-abdominally with a 3% solution of Sephadex (G-50) beads (1 mL/100 g body-weight). Forty-two to 44 hours post-Sephadex injection, the birds were euthanized with pentobarbital and the abdominal exudate cells were harvested. Abdominal exudate cells (macrophages) were then washed with Dulbecco's phosphate-buffered saline (PBS) and the cell concentrations were adjusted to 4 x 106 cells/mL with LM Hahn medium. Macrophages were used to determine phagocytosis as well as production of soluble factors such as nitric oxide (nitrite assay) and TNLF (cytotoxicity assay).

Phagocytosis: Macrophages from each poult were allowed to adhere to glass coverslips during a 45-min incubation at 41ºC. Glass coverslips with adherent macrophages were then incubated with sheep red blood cells (SRBC) or with antibody-opsonized SRBC (Ab-SRBC) for 45 minutes at 41ºC. After incubation, the coverslips were washed with PBS, stained with Wright stain, and placed on microscope slides (one for SRBC and one for Ab-SRBC per poult). For each slide, 900 macrophages were examined using a microscope. The numbers of macrophages with and without internalized SRBC as well as the number of SRBC within a phagocytically active macrophage were recorded.

Nitrite Assay and Cytotoxicity Assay: To assess the release of soluble factors by macrophages, macrophages were plated in 24 well culture plates (2 x 106 cells/well) and incubated with and without E. coli LPS (10 mg/culture) for 24 hours at 37ºC with 5% CO_2. Following incubation, the supernatant fluid was collected. For the nitrite assay, 100 mL of each supernate were plated in duplicate in 96-well plates. Greiss reagent was added to each of the wells and the plates were read at 540 nm with an automated microplate reader. Standard concentrations of nitrite ranging from 1.25 mM to 90 mM were included in each plate to establish the relationship between nitrite concentration and absorbance units (a.u.). The equation describing the linear relationship between a.u. and nitrite concentration was then used to determine the concentration of nitrite produced by unstimulated and LPS-stimulated macrophages from infected and control poults.

For the cytotoxicity assay, 50 mL of each macrophage supernate were added to 2 x 106 RP-9 cells/well (tumor cell line) in 96-well culture plates. The cultures were then incubated for 18 hours at 37ºC with 5% CO_2 . Following incubation, MTT colorimetric assay was used to detect surviving RP9 cells (Mosmann, 1983). The plates were read at 540 nm with an automated microplate reader. RP9 cells incubated with medium alone or with a solution of 0.02% Triton-X (detergent) were used as the negative (no cytotoxicity) and positive (complete cytotoxicity) controls, respectively.

Statistical Analyses: For each aspect examined in this study, data were analyzed for the effect of treatment by one-way ANOVA using the Systat Statistical Analysis software (SPSS Inc., Chicago, Ill.). Data were presented as means ± SEM. Differences between means with a P-value of ≤0.05 were considered significant.

RESULTS AND DISCUSSION

Pathology of Infection: In *E. adenoeides*-infected poults from Group 1, oocysts were first recovered in the feces on day 5 PI (4 x 106/bird). The number of oocysts recovered in the feces of Group 1 poults was highest on day 6 PI (20.2 x 106/bird), decreased to 14.7 x 106/bird by day 9 PI, and then dropped drastically to 1.4 x 106/bird by day 11 PI. No oocysts were recovered from the feces of controls throughout the 11-day study. Total weight gained by infected poults between day 0 and day 11 PI tended to be less than that of controls (Table 1). However, day 4-11 and day 7-11 body-weight gain of infected poults was significantly (P

Table 1. Body weight gain (g) in turkey poults inoculated with *Eimeria adenoeides* or water.

Growth period	Body-weight gain (mean ± SEM) Infected	Control
Day 0 - 11 post-infection[z]	745.7 ± 59.1	847.5 ± 38.8
Day 4 - 11 post-infection	414.3 ± 30.4 **b**[,y]	542.8 ± 26.2 **a**
Day 7 - 11 post-infection	231.7 ± 20.3 **b**	354.8 ± 17.1 **a**

[z] When the poults were 4 weeks old, 12 poults were infected with 50,000 *Eimeria adenoeides* oocysts/poult administered orally; 12 poults were uninfected (controls).

[y] Different letters within a row indicate significant (P < 0.05) differences between infected and control poults.

< 0.05) lower than that of controls (Table 1). The reduction in body-weight gain of infected poults may be explained by the pathogenic nature of the *Eimeria* infection, which resulted in lesions and associated enteritis of the lower small intestine, ceca, and rectum, and impaired digestion and absorption of food. The presence of lesions in infected poults was confirmed when poults from Group 2 where euthanized on day 7 PI and lesion scores were determined. In this group of birds, as in Group 1, oocysts were first recovered from the feces on day 5 PI (9.1 x 106/bird), with higher numbers of oocysts recovered on day 6 and 7 PI (27.6 and 25.0 x 106/bird, respectively). At termination of the macrophage function study (day 7 PI, Group 2), all infected poults had developed cecal lesions with lesion scores ranging between 1 and 4 on a 0 to 5 point scale. Lesion scores in poults from the control group were zero.

In summary, the large number of oocysts isolated from the feces, the reduction in body-weight gain, and the lesions observed in infected poults attest to the success of the induction of coccidiosis in all poults that had been infected orally with *E. adenoeides* oocysts.

Effect of *E. adenoeides* Infection on the Concentrations and Proportions among White Blood Cells: The *Eimeria* infection in the gastrointestinal tract resulted in altered concentrations of WBC (Table 2). Compared to controls, infected poults had reduced (P = 0.09) concentrations of WBC on day 4 PI, and

Table 2. White blood cell (WBC) concentrations (x103/mL) in blood from turkey poults inoculated with *Eimeria adenoeides* or water.

	WBC concentration (mean ± SEM)		
Time[z]	Infected	Control	P-value
Day 0	42.31 ± 3.26	40.05 ± 2.97	0.631
Day 4	35.11 ± 1.39	42.38 ± 3.86	0.090
Day 7	40.28 ± 1.93	45.92 ± 3.93	0.212
Day 11	46.39 ± 3.02 [a, y]	37.14 ± 1.86 [b]	0.015

[z] When the poults were 4 weeks old, 12 poults were infected with 50,000 *Eimeria adenoeides* oocysts/poult administered orally; 12 poults were uninfected (controls).

[y] Different letters within a row indicate significant (P < 0.05) differences between infected and control poults.

increased (P < 0.05) concentrations of WBC on day 11 PI (Table 2). The reduction in the concentration of WBC detected on day 4 PI was primarily due to lower levels (P < 0.05) of lymphocytes in infected poults compared to levels in poults from the control group (Table 3). The increase in WBC concentrations in infected poults compared to poults from the control group was due to an increase (P < 0.05) in both the number of lymphocytes and the number of heterophils (Table 3). Similar observations have been made in broiler chickens where a drop in the concentration of blood lymphocytes was associated with maximal output of oocysts in the feces (Rose et al., 1979). The drop in blood lymphocyte concentrations observed here also coincided with maximal excretion of oocysts in the feces. Based on histological examination of intestinal tracts from chickens and turkeys (Clarkson, 1958; Rose et al., 1979, 1984) infected with *Eimeria*, large infiltrations of lymphocytes into the intestinal submucosa and epithelial tissues occurred during the same period of time following primary infection. Hence, the drop in the amount of WBC can be explained by the migration of lymphocytes from the blood to infected tissues. The elevated concentrations of blood lymphocytes and heterophils on day 11 PI are in accordance with a similar rise observed in *Eimeria* infected rats and chickens (Rose et al., 1979) which has been attributed to the establishment of a protective response and the resolution of infection. The elevated eosinophil concentrations on day 4 PI and reduced monocyte concentrations on day 11 PI observed in the blood of infected poults can also be explained by recruitment of these cell types to the site of infection. Eosinophils constitute a first line of defense against large parasites. Similarly, monocytes, called macrophages after they leave the blood to enter other tissues, are cells that are specialized in killing intracellular parasites. As reported by Clarkson (1958), eosinophils were present in large numbers in the cecal submucosa of turkeys with *E. adenoeides* infection on day 1-6 PI, whereas, macrophages were most abundant in infected tissues during the resolution of the *Eimeria* infection (Rose et al., 1979). Overall, the alterations in the concentrations of WBC in *E. adenoeides* infection in turkeys are similar to those reported for *Eimeria* infections in other species and can be explained by altered production and recruitment of cells required to resolve the infection and develop protective immunity to the infective agent.

Effect of *E. adenoeides* Infection on Macrophage Function: An important role of macrophages in primary infection with *Eimeria* became apparent when the number of oocysts excreted in the feces of infected chickens was four times higher when their macrophages had been selectively killed in vivo (Lee

Table 3. Concentrations and proportions among white blood cells in turkey poults inoculated with *Eimeria adenoeides* or water.

Day[z]	Treatment	Lymphocytes	Heterophils	Monocytes	Eosinophils	Basophils
Concentration (# of cells/mL of blood) [y]						
0	infected	23.47 ± 2.09	14.43 ± 2.04	1.92 ± 0.43	0.56 ± 0.15	2.14 ± 0.49
0	control	21.61 ± 1.66	13.79 ± 1.50	1.98 ± 0.24	0.54 ± 0.16	2.18 ± 0.49
4	infected	19.64 ± 0.57 **b**,[x]	11.59 ± 1.29	1.79 ± 0.32	0.46 ± 0.12 **a**	1.73 ± 0.30
4	control	22.13 ± 1.31 **a**	15.18 ± 2.24	2.51 ± 0.38	0.17 ± 0.04 **b**	1.93 ± 0.33
7	infected	22.64 ± 0.19	11.53 ± 1.60	3.20 ± 0.48	0.67 ± 0.15	2.29 ± 0.31
7	control	21.90 ± 1.96	13.39 ± 1.00	4.03 ± 0.53	0.46 ± 0.13	2.44 ± 0.40
11	infected	22.56 ± 1.39 **a**	18.99 ± 2.88 **a**	2.32 ± 0.22 **b**	0.65 ± 0.16	1.61 ± 0.20
11	control	18.76 ± 0.98 **b**	12.07 ± 1.10 **b**	3.56 ± 0.33 **a**	0.33 ± 0.07	1.37 ± 0.20
Proportions (% of total leukocytes) [w]						
0	infected	52.82 ± 1.83	33.78 ± 3.53	4.41 ± 0.76	1.35 ± 0.37	5.43 ± 1.56
0	control	52.45 ± 2.51	34.08 ± 2.85	5.02 ± 0.58	1.36 ± 0.46	4.93 ± 0.87
4	infected	56.76 ± 2.36	32.30 ± 2.70	4.96 ± 0.82	1.29 ± 0.31a	4.95 ± 0.83
4	control	52.49 ± 2.47	34.54 ± 2.94	5.79 ± 0.64	0.48 ± 0.15b	4.52 ± 0.63
7	infected	56.24 ± 4.01	28.50 ± 3.67	7.84 ± 1.04	1.63 ± 0.36	5.28 ± 0.74
7	control	52.24 ± 3.43	34.02 ± 3.32	8.71 ± 0.86	0.99 ± 0.26	5.07 ± 0.63
11	infected	51.09 ± 3.89	38.05 ± 4.06	5.63 ± 0.55 **b**	1.32 ± 0.26	4.10 ± 0.63
11	control	53.35 ± 2.32	32.35 ± 2.42	9.62 ± 0.81 **a**	0.86 ± 0.17	3.77 ± 0.59

[z] When the poults were 4 weeks old, 12 poults were infected with 50,000 *Eimeria adenoeides* oocysts/poult administered orally; 12 poults were uninfected (controls).

[y] For each blood sample, total leukocyte concentration was determined using an automated hematology analyzer (CELL-DYN). The concentration of various leukocyte populations was then calculated using total leukocyte concentration and the manual estimate of the proportion of each cell type (see footnote 3).

[x] Different letters within a day and cell type indicate significant ($P < 0.05$) differences between infected and control poults.

[w] For each blood sample, the proportion among leukocyte populations was estimated by identifying 300 leukocytes within a Wright-stained monolayer of blood cells using a bright-field microscope (1000 x magnification).

Table 4. Macrophage phagocytic activity in turkey poults inoculated with *Eimeria adenoeides* or water[z].

Activity[y]	Infected	Control
Percentage of macrophages phagocytozing unopsonized SRBC	9.53 ± 0.99	8.81 ± 1.27
Percentage of macrophages phagocytozing Ab-opsonized SRBC	82.09 ± 3.62	83.46 ± 4.02
Number of unopsonized SRBC phagocytozed per phagocytic macrophage	1.49 ± 0.18	1.63 ± 0.22
Percentage of Ab-opsonized SRBC phagocytozed per phagocytic macrophage	3.16 ± 0.16	3.09 ± 0.14

[z] When the poults were 4 weeks old, 12 poults were infected with 50,000 *Eimeria adenoeides* oocysts/poult administered orally; 12 poults were not infected (controls).

[y] Five days post-infection, Sephadex beads were injected into the abdominal cavity of 12 infected and 12 control poults. Sephadex-elicited abdominal macrophages were harvested 42 hours later and incubated with SRBC or antibody-opsonized SRBC. For each activity, 900 macrophages per bird were examined using a microscope.

and Al-Izzi, 1981). Additionally, factors such as nitric oxide and TNLF produced by macrophages, have been shown to be important in the reduction of oocyst excretion during primary *Eimeria* infection, further supporting an important role of macrophages in coccidiosis. On day 7 PI, macrophages obtained from infected and control poults had similar abilities to carry out lower-order functions, including phagocytosis of unopsonized SRBC and Fc-receptor-mediated phagocytosis of antibody-opsonized SRBC (Table 4). Although macrophage production of nitric oxide was not affected by *E. adenoeides* infection (Fig. 1), nitric oxide production by LPS-activated macrophages from infected poults tended to be higher (P = 0.121) than

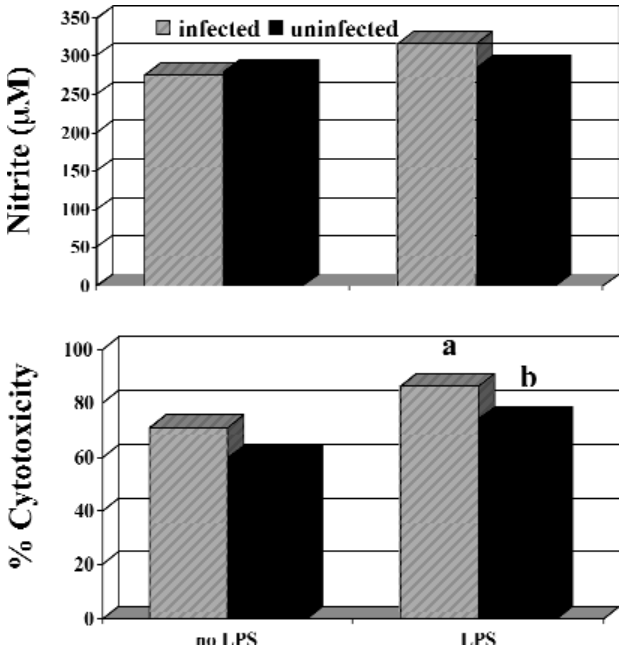

Fig. 1. Nitric oxide production (mM) and cytotoxicity (%) by macrophages from turkey poults inoculated with *Eimeria adenoeides* or water at 4 weeks of age. Abdominal exudate cells (macrophages) were elicited from 12 infected and 12 uninfected poults on Day 5 post-infection by injection of Sephadex beads into the abdominal cavity. Forty-two hours later, macrophages were harvested and cultured with or without lipopolysaccharide (LPS). Culture supernatant fluid from LPS-activated and unactivated macrophage cultures was collected 24 hours later. Culture supernatants were assayed for nitric oxide production and cytotoxicity by nitrite assay and killing of RP9 tumor cells, respectively.

that by LPS-activated macrophages from controls. Similarly, the production of cytotoxic factors (i.e., TNLF) by macrophages from infected poults tended to be higher than that from controls, although this trend was only significant ($P < 0.05$) when macrophages were further activated in vitro with LPS (Fig. 1). These differences in higher-order functions of macrophages, especially in the production of cytotoxic factors, suggest that the internal environment of infected birds already provides signals for macrophages to become more responsive to stimuli (e.g., LPS, *Eimeria*). Nitric oxide and cytotoxic factors like TNLF have been shown to be beneficial during primary *Eimeria* infection in chickens, although TNLF tended to exhibit both protective and pathological effects (Allen and Lillehoj, 1998; Byrnes et al., 1993; Zhang et al., 1995). Pathological effects of TNLF included primarily metabolic effects such as body-weight reduction, whereas, TNLF did not appear to contribute to the intestinal lesions observed during *Eimeria* infections (Zhang et al., 1995). Overall, macrophages obtained from poults with primary *E. adenoeides* infection exhibited a higher level of responsiveness to LPS stimulation than macrophages from controls, suggesting a priming effect of the internal environment in infected birds. This heightened responsiveness to environmental stimuli (e.g., signals from components of adaptive immunity) and the resulting increase in macrophage activity are likely to be important in the resolution of *E. adenoeides* infection.

In summary, data from these studies strongly suggest a response to an initial *E. adenoeides* infection by components of innate immunity in infected turkey poults.

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