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PATHOGENESIS OF *Eimeria colchici* IN THE INTESTINE OF CHICKENS AND THE RELATED IMMUNE RESPONSE

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Abstract

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To study the immune response in poultry, a non-specific host for pheasant *Eimeria colchici* coccidium, ten-day-old chickens were each orally inoculated with 10^6 oocysts. For determination and comparison of coccidia invasion and development, histological examinations in poultry and pheasant chickens were made. In histological sections of four intestinal regions (duodenum, jejunum, ileum and caeca), first generation schizonts were found in epithelial cells, 12, 36 and 60 h post-infection (p.i.). Invasion of chicken intestine was non-specifically localized in the duodenum and caecum. By 60 h p.i. the numbers of schizonts of *E. colchici* in chicken caecum were significantly lower compared to the numbers of schizonts in pheasant caecum ($p < 0.005$). We suppose that the size of the parasite was smaller in the fowl chickens as a result of a slower development of the first generation schizonts of *E. colchici.*

In order to estimate the immunological response after administration, non-specific coccidia to the chickens, subpopulations CD3+, CD4+, CD8+, and BU1b+ in the peripheral blood and spleen were measured by flow cytometry. The indirect immunofluorescence method, mouse anti-chicken monoclonal antibodies, and goat anti-mouse secondary antibody were used. A significant ($p < 0.05$) increase of leukocytes and CD4 positive T cells was observed at 60 h post infection in chickens. We found that increase of CD4+ cells in a studied non-specific host infected with pheasant coccidia is similar to increase of these cells in host specific coccidia infection. It indicates participation of cellular immune response and one of the defence mechanisms during the invasion and development of coccidia in a non-specific host.

Eimeria colchici, immunity, T-cell subsets, flow cytometry, intestine, experimental infection

Marked host and characteristic colonisation site specificity defines the development of *Eimeria* species in the digestive tract. It is rare for this protozoan parasite to occur naturally in more than one host (Aly 1993). Doran (1978), however, was able to produce an experimental, patent infection in Leghorn chickens, the chuckar partridge, ring-necked pheasant and bobwhite quail using a turkey coccidium, *Eimeria dispersa*. The prepatent period was 6 h shorter in the quail and partridge than in chicks and pheasants. Furthermore, the oocyst shedding in chicks and pheasants was much lower than in partridge and quail. The only similarity found among the four hosts was the size and the nature of the second and third generation schizonts. Norton (1976) noted that *E. colchici* from a pheasant produced an infection in turkeys when a large number of oocyts was given to them.

Infection with *Eimeria* induces an immune reaction in the host, including both humoral and cell-mediated responses that lead to acquired protection. Although it is generally accepted that acquired immunity is parasite-host species-specific, Augustine and Danforth (1990) showed that chickens repeatedly inoculated with *E. adenoeides* develop a measure of immunity that protected them at least partially from a subsequent moderate challenge with *E. tenella*. Reciprocal infection studies, in which turkeys were immunised with *E. tenella* or *E. acervulina* failed to show protection against challenge with *E. adenoeides* (Augustine

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and Danforth 1995). Mechanisms preventing the intracellular development of *Eimeria* in the non-specific hosts are not fully understood. One of the mechanisms likely to be involved is the immune system of the host. A dominant role of the cell-mediated immunity in the host-protective response to *Eimeria* infection has been shown (Wakelin and Rose 1990; Rose and Hesketh 1982; Lillehoj and Trout 1993).

To study the infection of *E. colchici* in a non-specific host we investigated whether *E. colchici* could successfully colonize the intestine of the chicken host. To quantify the effects of a reciprocal "cross infection", we measured the elicited longitudinal changes in the blood and spleen lymphocyte subsets during infection.

Materials and Methods

Experimental design

Thirty-six coccidia-free (1-day-old) White Leghorn chicks were raised in standard poultry cages and given non-medicated feed and water *ad libitum*. At 10 d of age, the birds were distributed randomly into treatment and control groups (n = 18 in each). A pure culture of pheasant coccidium, *Eimeria colchici*, was obtained by isolation of a single oocyst on agar and infecting pheasant chicks and the oocysts collected from faeces were sporulated in 2% potassium dichromate (Goldová et al. 1998). Chicks of the treatment group were orally inoculated with 10^6 oocysts per bird, while the control group was sham-infected with inoculum buffer only. Six chicks of both groups were killed at 12, 36 and 60 h post infection and blood samples, and tissues were collected from spleen and intestine.

Thirty two-week-old coccidia-free pheasant chicks were infected with a pure suspension of sporulated *E. colchici* oocysts (5 000 oocysts/per chicks).

Histological examination

Intestinal samples were fixed in 10% neutral formalin and subjected to routine paraffin processing. From the sample blocks, 5 µm thick histological sections were cut and stained with haematoxylin-eosin. The sizes and numbers of schizonts in epithelial cells of the small intestine were measured after calibration of eyepiece micrometer in 50 microscopic fields.

Counting of leukocytes and isolation of lymphocytes

Blood samples for flow cytometry examinations and counting of leukocytes were collected from all animals by cardiac puncture into EDTA containing tubes. Lymphocytes were separated by Ficoll-Hypaque gradient sedimentation (Boyum 1974). Leukocytes were counted by routine laboratory method using Fried - Lukáčová solution (475 µl solution plus 25 µl blood) (Fried and Jantošovič 1961).

The spleen cells for flow cytometry were harvested by teasing and passing the splenic tissue through a 70 µm - mesh screen (Heller 1987).

Antibodies

Primary antibodies: The primary mouse anti-chicken monoclonal antibodies (MoAbs) used are summarized in Table 1. They were obtained from Scandic (Czech Republic) and are produced by Veterinary Medical Research and Development Inc. (USA). The BU1b antibody was a generous gift of Prof. Heller.

Secondary antibody: FITC conjugated goat anti-mouse IgG (Dakopatts, Germany).

Table 1
Primary monoclonal antibodies

Specificity	MoAbs	Isotype	Diluted
CD3	RTMCA1378	mouse IgG1	1:25
CD4	SRTMCA1473	mouse IgG1	1:25
CD8	SRTMCA1377	mouse IgG1	1:25
BU-1b	8370-01	mouse IgG1	1:25

Flow cytometry analysis

After the Ficoll-Hypaque separation, lymphocytes were twice washed with phosphate buffer saline (PBS) and once in a tissue cultured medium (TCM – RPMI 1640 supplemented by 2 % of foetal calf serum). Fifty µl of cellular suspension (1 x 10^6 lymphocytes in TCM) and 50 µl of specific or control MoAbs were mixed and incubated at 4 °C for 30 min. After incubation the cells were washed twice in the TCM, the resulting pellets were re-suspended and mixed with 25 µl of secondary antibody and incubated in the dark as described above. After staining the cells were washed twice in the TCM and once in PBS, then fixed in 1 % paraformaldehyde in PBS. Prepared samples

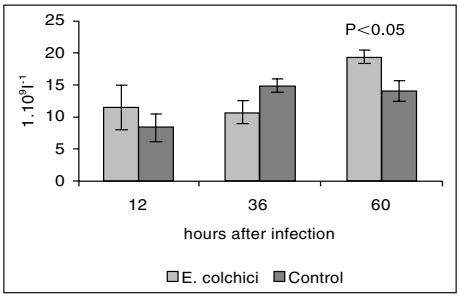

Fig. 1. Total number of leukocytes in the peripheral blood of domestic chicken after *E. colchici* infection

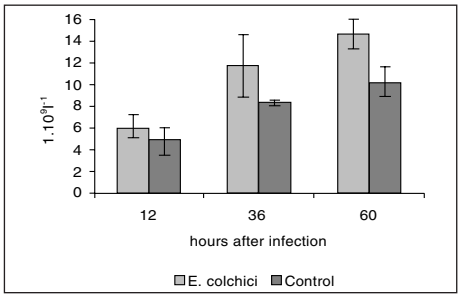

Fig. 2. Absolute number of lymphocytes in the peripheral blood of domestic chicken after *E. colchici* infection

were analysed on a Becton Dickinson FACScan flow cytometer (Germany). Data on 10 000 cells were collected using the Becton Dickinson Cell Quest program. Absolute lymphocyte counts were computed as follows:

WBC count × % of the relative lymphocytes × % lymphocyte subpopulations

Statistical analysis

Results were evaluated by Student's *t*-test and expressed as mean ± SD. A confidence level of $p < 0.05$ was considered significant.

Results and Discussion

Histological examinations of Leghorn chick intestines showed that the pheasant coccidium *E. colchici* invaded the epithelial cells of intestinal lamina propria mucosae of caecum and all parts of small intestine. Invasion of the intestine chickens was non-specifically localized and was not equal to that observed in the intestinal cells of pheasant (Goldová et al. 1998). By sixty hours post infection (p.i.) the number of *E. colchici* schizonts in the chick caeca was very low (1.4 ± 1.2). It was significantly lower ($p < 0.005$) when compared to pheasant caecum infested with *E. colchici* (1653.0 ± 663.5) evaluated at similar time p.i. (Goldová et al. 2001).

Measurement and comparison of the schizont size revealed further differences between the specific and non-specific host, i.e. pheasants versus chicks. In the non-specific host, schizonts found in the duodenum, jejunum and ileum measured 5.7 × 3.8 µm at 12 h p.i. and 9.5 × 7.6 µm at 36 h p.i.

A much lower number of schizonts were found 60 h p.i. These were identical in size (5.7 × 3.8 µm) to those found in the 12 h p.i. localized mainly in the duodenum. In contrast, examination of the pheasant host, infected with its host-specific parasite species, *E. colchici*, showed a number of marked differences. Many of them were noted in the classical works on Eimeria by Pellérdy (1974) and Norton (1976). They found the first generation schizonts in the lower part of the small intestine having a significantly larger dimension (18

Table 2
CD3 and CD8 positive T cells in the peripheral blood (absolute numbers; $1.10^9 \cdot l^{-1}$ – $G \cdot l^{-1}$) and in the spleen (percentage) in the chickens after *E. colchici* infection (Means ± SD)

	CD3		CD8		CD3		CD8	
	blood ($G \cdot l^{-1}$)		Blood ($G \cdot l^{-1}$)		Spleen (%)		Spleen (%)	
h p.i.	Control	*E. colchici*	Control	*E. colchici*	Control	*E. colchici*	Control	*E. colchici*
12	0.89 ± 0.5	2.21 ± 0.9	0.45 ± 0.4	0.60 ± 0.4	33 ± 5	43 ± 11	39 ± 6	30 ± 9
36	1.87 ± 0.5	1.29 ± 0.1	1.20 ± 0.6	1.45 ± 0.3	43 ± 10	36 ± 17	48 ± 2	43 ± 3
60	1.23 ± 0.7	2.11 ± 0.5	0.84 ± 0.6	1.22 ± 0.7	52 ± 11	59 ± 18	48 ± 2	47 ± 11

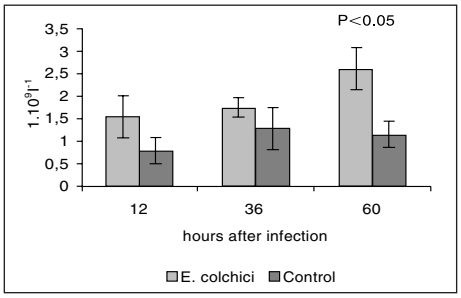

Fig. 3. Absolute number of CD4 positive cells in the peripheral blood of domestic chicken after *E. colchici* infection

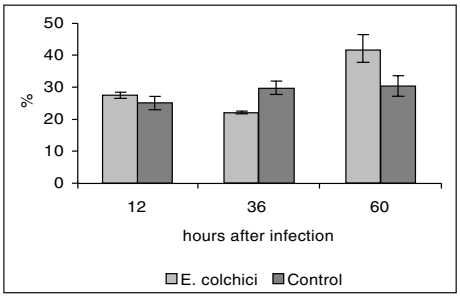

Fig. 4. Relative percentage of CD4 positive cells in the spleen of domestic chicken after *E. colchici* infection

× 13 μm). Goldová et al. (1993) described the *E. colchici* localisation in the caecal crypts, where they reached 18.7 × 15.1 μm. The second generation schizonts appeared as colonies in the lamina propria of the caecal mucosa and measured 28 × 21 μm (Pellérdy 1974; Norton 1976; Goldová et al. 1993).

We are not aware of reports dealing with the developmental stages of *E. colchici* that show diminished ability to colonize, develop and thrive in non-specific or foreign host birds. The diminished ability of a turkey-specific *Eimeria* spp. to colonize the chicken digestive tract was shown by Augustine et al. (1991). In an experiment using parasite-specific monoclonal antibodies, they showed that the sporozoites of the turkey coccidia survived within the chicken intestinal cells for up to 3 days but failed to develop.

Evaluation of the total number of the peripheral blood leukocytes (Fig. 1) and lymphocytes (Fig. 2) showed significant increase of leukocytes ($p < 0.05$ at 60 h p.i.) and mild increase of lymphocytes in the infected group as compared to the control. Absolute number of CD3 (Table 2), CD4+ (Fig. 3 and 4), CD8+ (Table 2), BU1b+ (Fig. 5 and 6) cells and ratio of CD4/CD8 (Table 3) cells in both, the peripheral blood and spleen reflected a statistically significant increase of CD4+ cells at 60 h p.i. in the peripheral blood.

The results demonstrated that during sporozoite invasion of a non-specific host, the antigen-specific T cells were activated. An increased number of CD4 should have contributed cytokine support for an antibody response and possible for phagocytic killing of *E. colchici*. CD4+ T cells are also important in controlling primary infection with *E. tenella* in natural host-chicken (Trout and Lillehoj 1996). T lymphocytes have a direct effect on preventing the development of *Eimeria* sporozoites once they invade the intestinal mucosa of a non-specific host. On the other hand, suppression of T lymphocyte activity in a non-specific host

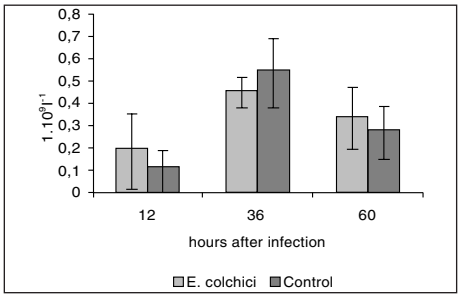

Fig. 5. Absolute number of Bu1b positive cells in the peripheral blood of domestic chicken after *E. colchici* infection

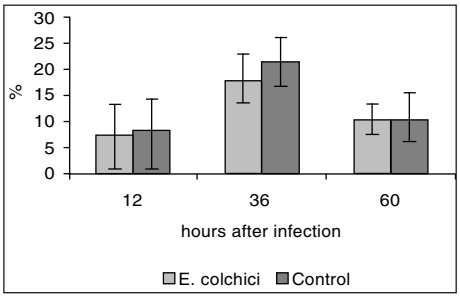

Fig. 6. Relative percentage of Bu1b positive cells in the spleen of domestic chicken after *E. colchici* infection

Table 3
CD4/CD8 ratio in the peripheral blood and in the spleen after *E. colchici* infection (Means ± SD)

	Peripheral blood		Spleen	
h p.i.	Control	*E. colchici*	Control	*E. colchici*
12 h	2.00 ± 0.63	2.98 ± 0.88	0.63 ± 0.04	0.96 ± 0.30
36 h	1.11 ± 0.64	1.21 ± 0.33	0.58 ± 0.08	0.50 ± 0.02
60 h	1.18 ± 0.15	2.79 ± 1.60	0.63 ± 0.08	0.92 ± 0.30

early during an infection with a heterologous species of *Eimeria* permitted the complete intracellular development of the parasite (Kogut and Eirmann 1991).

In conclusion, infection of chicks with pheasant host-specific coccidia resulted in dispersed, impotent invasion of the caeca and small intestine of the chicks. Schizonts in the intestinal mucosa failed to develop and were also significantly smaller than those in natural hosts. These findings taken together support the notion that the measured changes in WBC's in general and CD4+ cells changes in particular indicate participation in the defence of the intestinal mucosa during the invasion and development of coccidia in a non-specific host.

Patogenéza *Eimeria colchici* v čreve kurčiat a imunitná odpoveď

Za účelom štúdia imunitnej odpovede hydiny, ktorá je nešpecifickým hostiteľom pre bažantie kokcídie *E. colchici,* kurčatá vo veku 10 dní boli infikované *per os* oocystami bažantích kokcídií (*Eimeria colchici)*. Kvôli stanoveniu a porovnaniu invázie a vývoja kokcídií bolo vykonané histologické vyšetrenie kurčiat a bažantov. Histologické rezy čreva (duodenum, jejunum, ileum a cecum) 12, 36 a 60 h po infekcii, v epiteliálnych bunkách sliznice demonštrovali vývoj po prvú generáciu schizontov. Invázia čreva kurčiat bola nešpecificky lokalizovaná v duodéne a céku. Šesťdesiat hodín po infekcii počty schizontov *E. colchici* v céku kurčiat boli signifikantne nižšie v porovnaní s počtami schizontov *E. colchici* v céku bažantov ($p < 0.005$). Predpokladáme, že veľkosť parazita u kurčiat bola menšia ako u bažantov z dôvodu spomaleného vývoja prvej generácie schizontov *E. colchici*.

Imunologickú odpoveď u kurčiat po aplikácií bažantích kokcídií sme sledovali prietokovou cytometriou v periférnej krvi a slezine. Využili sme metódu nepriamej imunofluorescencie s použitím primárnych myšacích antikuracích monoklónových protilátok (CD3, CD4, CD8 a BU1b) a sekundárnej kozej antimyšacej protilátky konjugovanej s FITC. Šesťdesiat hodín po infekcii kurčiat sa v periférnej krvi pozorovalo signifikantné ($p < 0.05$) zvýšenie leukocytov a CD4 pozitívnych buniek. Zistili sme, že podobne ako po infekcii špecifickými kuracími kokcídiami, došlo k zvýšeniu CD4 pozitívnych buniek aj u nešpecifického hostiteľa infikovaného bažanťou kokcídiou. To poukazuje na účasť bunkovej imunitnej odpovede a jeden z možných obranných mechanizmov v priebehu invázie a vývoja bažantích kokcídií u nešpecifického hostiteľa.

Acknowledgements

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