Bulletin UASVM, Veterinary Medicine 67(1)/2010
ISSN 1843-5270; Electronic ISSN 1843-5378

Immunotoxic Action of Ochratoxine A on Lymphocytes from Lymphoid Tissues Associated to Gut Mucosa in Chickens

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Abstract. Investigation were conducted on 2 groups, each of 15 broiler chickens. Ochratoxine A(OTA) was given orally, in sunflower oil suspension, daily , for 21 days in doses of 50 µg/kg g. Control group (of 15 chickens) received only sunflower oil, 5 chickens from each group were killed after 7,14 and 21st day of the experiment. Gut mucosa samples were prevealed from duodenum, jejunum and caecal tonsils. All samples were prepared for paraphin embeding and stained with: monoclonal antibody Tcr1, TCR2, CD3 and CD8. In duodenum, the cryptae from lamina propria showed numerous CD3, with nucleus rich in euchromatine, with 3-4 nucleoli. The enterocytes had a large nucleus rich in euchromatine, with 3-4 nucleoli. Progressively with the age and OTA dosis the lipidic droplets from the axis of the villi and proportion of degenerated enterocytes are increasing and simple epithelium is replaced by stratified epithelium, with many immature cells. In jejunum, the cryptae from lamina propria showed numerous CD8. The coecal tonsils, after 14 days of OTA poisoning showed lamina propria populated by small lymph cells TCR1. Some of the lymph cells showed nuclear lesions like: cariorhexis, cariolysis and other show apoptotic aspects. Into the lymphoid agglomerations some cells with nuclei rich in euchromatine, with 4-5 nucleoli were observed

Key words: Ochratoxine A, chickens, immunotoxicity, lymphoid tissues associated to gut mucosa

INTRODUCTION

Enterocytes form a physical barrier between the host and the intestinal lumen and interact indirectly with bacterial flora of the intestine through the secretion of mucus and antimicrobial molecules. The presence of intraepithelial lymphocytes is explained by enterocytes involvement in the organization and functioning of the immune system. Direct interaction between epithelial cells and TCR γδ + T in chickens may be mediated by CMH molecules or expression of a variety of stress molecules released by the damaged epithelium. The chemokine and defensine repertoire in chickens is limited to β defensine which were identified only heterophiles and macrophages (Fred Davison, et al. 2008).

Mycotoxins are reducing the integrity of intestinal epithelium by modifying proteins in the jonctions. Apoptosis, increased colonization with pathogens, oxidative stress, cytotoxicity, inhibition of protein synthesis and lipid peroxidation are characteristics of toxic effect of mycotoxins. Directly or indirectly affect the host immune response and increase the susceptibility to infection (Al-Anat & Petzinger, 2006).

LT differentiation, is delayed by OTA (Thuvander et all, 1995,. 1996). Some authors conclude that OA has an unselective suppressor effect in various immune reactions (Muller et all. 1995). OA effect on bone marrow and lymphoid cell populations can be explained by the sensitivity of these cells to inhibition of protein synthesis induced by OTA

After hatching chicks intestine is populated with lymphocytes and they increase with age and reach up to 8 weeks. Lymphoid tissue of the intestine may be: diffuse distributed intraepithelial and in lamina propria of mucosa and organized in nodules, grouped or solitary localized in lamina propria of mucosa or submucosa. (Lillehoj and Trout 1996). CD3 and CD8 lymphocyte subpopulations are the first wich appear in caecal tonsils in chickens in the 11th days of incubation and CD4 on the 15th day. CD3 lymphocytes initially appear intrapithelial, CD4 in lamina propria and CD8 both in lamina propria and intraepithelial. All subpopulations note an increase from 21 to 35 days life (Xu Ying 2010).

Lymphocytes after the mRNA level for IL2 and IFNγ are immature immediately after hatching. TCR1 and TCR2 lymphocytes are occasionally present posthatching. At 6 days after hatching both subsets are present, TCR1 predominate intraepithelial and TCR2 in lamina propria (Rothwell et all. 1995). NK lymphocytes mediates cytotoxicity and are detectable after 2 hours posthatching and gradually increase after 18 hours. Intraepithelial lymphocytes are heterogeneous populations including LT, LB and NK. Many lymphocytes are bearing CD8 at surface so called cytotoxic which have an important role in secondary infections (Ilic et al 2003). Of intraepithelial lymphocytes, CD3 occupies a proportion of 21%, CD8-70%, CD4-9%. 30% of CD8 lymphocytes posses NK activity (Göbel et al 2001, Min et al. 2005). Following a detailed immunohistochemical analysis of intestinal lymphoid tissue in chickens, CD3 lymphocytes predominate intraepithelial, but can also be found in the lamina propria. TCR1 are located predominantly intraepithelial. TCR2 lymphocytes predominate in the lamina propria of mucosa but can be also found intrapithelial to a lesser extent.

Functional maturation of LT and LB is made in two stages: a first stage during the first week and the second phase during the second week. These changes seem to be the result of the colonization of the chicken intestine with microbial flora. During the first four weeks are seen important changes in the proportion of various subsets of lymphocytes from the intestine (Davison, et al. 2008).

Aim of this study is to highlight the effect of OTA on the populations of T lymphocytes from the intestinal mucosa.

MATERIALS AND METHODS

Experiment were used 40 Ross 307 broiler chickens, 6 days old, which after a period of one week of accommodation to living conditions provided, were randomly divided in 2 groups: experimental (LE) and control (LM) with an average weight of 79.03 ± 0.73 g. Chicks were reared on sawdust litter, were provided specific microclimate conditions for age, room temperature gradually decreasing from 32 ° C, to 24 ° C. Commercial-type food, free of OTA was administered ad libitum. LE received daily by gavage ochratoxin A (OTA-Sigma Chemicals Co.) 50 mcg/kg bw, eluted in sterilized sunflower oil 50 mcg/kg bw, for 21 days.The control group received only eluent (sterilized sunflower oil). At the end of each week during the experiment, five chickens were selected by random from each group and were killed and samples from duodenum, jejunum and coecal tonsils were prepared for immunohistochemistry: fixed in 10% formalin solution, dehydrated with alcohol for 70%, 80%, 96% and pure (absolute) ethanol, clarified with xylene, impregnated and embedded in paraffin. After cutting preparations were dewaxed in three xylene baths of each of 3 minutes, hydrated with pure ethanol then 80%, 70% and then distilled water, placed in methanol and 3% hydrogen peroxide. To block epitopes the sections were heated at 95°C with 0 10 mmol/L citrate acid buffer, pH 6 for 10 minutes in the microwave and then were left at room temperature for 20 min. Then slides were washed in two baths of PBS solution (pH 7,5) for 5

min. and put in contact with horse serum diluted 1/20 in PBS., then covered with first antibody diluted 1/50 in PBS and kept at room temperature, in humid chamber for 1 hour.

Primary antibodies were mouse anti-chicken and unlabelled: CD3 (clone CT-3) cat no. 8200-01; TCR gd (clone TCR-1) cat.no. 8230-01; TCR VB1 (clone TCR-2) cat.no. 8240-01; CD8α (clone CT-8) cat.no. 82220-01 (Southern Biotech)

Then slides were rinsed in 2 baths of PBS (pH 7,5) for 5 minutes, then were put in contact with secondary detection kit (Vecta Stain ABC kit), peroxidise mouse IgG fro Vector Lab. Cat no. PK-4002, then washed in two baths of PBS each of 5 min., covered with ABC solution for 5 min. then put in contact with the DAB(Substrat kit Vector Nova Red, substrate kit for peroxidise from Vector Lab. Cat no. SK-4800) for 5 min., washed with PBS 3 min. in two containers, passed 3 min in distilled water, stained with Harris hematoxylin (dilution 1/2) 15 min, washed with distilled water, tap water, dehydratated in ethanol 50%, 70%, 80%, 96% and pure ethanol, 3 min in each bath, clarificated in two xylene baths of 3 minutes each and mounted with Xtra Surgipath 26x76x1.0mmm microslide Adhesive (as 1440, 00 211, 1thick)

Lymphocytes populations were counted from 10 microscopical fields (10x100) from each slides. Mean and significance of differences were calculated with Anova test.

RESULTS AND DISCUSSIONS

In the duodenum of the chicks from LE after 7 days of exposure, there is a unsignificant (US) decrease of CD3 intraepithelial and lamina propria lymphocytes, very significant (VS) at 14 days of intraepithelial lymphocytes, a VS increase of lamina propria lymphocytes at 14 days of exposure (Table1). After 21 days of exposure not only intraepithelial lymphocytes but also lamina propria lymphocytes showed a statistically insignificant decrease compared with control group. TCR1 lymphocytes showed a VS reduction in lamina propria after 7 days of OTA exposure. After 14 and 21 days both lamina propria and intraepithelial lymphocytes recorded statistically insignificant variations. TCR2 lymphocytes shows a VS lamina propria and intraepithelial decrease after 7 days of exposure to OTA. Lamina propria CD8 lymphocytes showed a VS reduction after 7days of exposure. Also there have been recorded statistically significant results after 14 and 21 days of exposure (Table 1).

Cytotoxicity of OTA is observed in duodenal mucosa after two and three weeks of exposure to OTA. Epithelium is high, the cells are giant with multiple large nuclei, rich in euchromatin and the brush border is missing (Fig 2, a, b). In some areas the epithelium appears to be detached from basal membrane, due to numerous small vacuoles that appear throughout the cell but mainly in their base, giving a spongy appearance to the epithelium. In the villous lamina propria appear numerous lymphocytes, connective fibers, blood capillary and large vacuoles areas. In the lamina propria of mucosa from the villous base the glands have normal size and present a small number of intraepithelial lymphocytes and an higher number of periglandular lymphocytes. Modified villi alternate with apparently morphological normal ones. Even in the same villous the epithelium may be normal on one side and the other shows the changes described above. Area can be modified at the base, on the medium third or to the apex of intestinal villous. The number of modified villous is less after 7 days of exposure to OTA, but is predominant in chickens exposed to OTA for two and three weeks (Fig.1, a, b). Some of the lymphocytes from in lamina propria have nuclear changes: cortical hyper-chromatosis, cariorexis. Literature studies have shown that OTA the immune system by gradually reducing the number of lymphocytes subpopulations from primary and secondary lymphoid organs (thymus, Fabricius bursa, spleen, lymph nodes and mucosal associated lymphoid tissue) (Al. Anati and Petzinger, 2006, Solcan et all., 2009). Immunotoxic effect in

chickens was studied by administration of OA in the diet in a dosis of 2-4 mg / kg for 20 days in the diet posthatching. Cell-mediated and humoral immune response was altered, accompanied by the reduction of serum globulin (α1, α2, β, γ globuline) (Singh 1990).

Tab. 1

Number of intestinal lymphocytes populations (nr. of cells/field) after OTA exposure

Organ	**Group**	**Markers type**	**IEL Average**	**PPL Average**	**IEL Average**	**PPL Average**	**IEL Average**	**PPL Average**
			OTA exposure 7 days		OTA exposure 14 days		OTA exposure 21 days	
Duodenum	LM	CD3	22,5 ±1,01	8,2 ±2,4	41,2 ±6,7	16,4 ±1,9	35,9 ±3,1	61,3 ±5,1
	LE	CD3	21,5 ±1,9	7,1 ±2,6	10,1*±1,2	40,5*±3,9	27,8* ±2,1	50,1 ±4,9
	LM	TCR1	12,1 ±1,4	22,6 ±1,8	10,8 ±1,4	23,2 ±1,9	10,1 ±1,1	26,2 ±2,1
	LE	TCR1	11,8 ±1,1	10,0* ±2,1	11,3 ±1,2	22,6 ±2,8	9,2 ±1,6	21,0 ±2,2
	LM	TCR2	36,2 ±1,8	28,9 ±1,4	21,0 ±3,1	36,2 ±1,9	8,0 ±1,5	36,1 ±2,4
	LE	TCR2	16,8*±1.1	11,2* ±2,1	19,.2 ±2,1	34,2 ±3,2	9,,6 ±1,8	31,6 ±2,1
	LM	CD8	33,2 ±1,2	16,1 ±1,9	19,2 ±1,9	24,6 ±4,3	19,6 ±1,9	16,4 ±1,4
	LE	CD8	28,6 ±1,7	8,1* ±1,8	12,2**±1,2	14,8* ±1,5	15,4**±2,3	11,2**±0,9
Jejunum	LM	CD3	28,5±1.7	16,3 ±2,1	31,4 ±1,01	10,1 ±0,7	32,6 ±2,1	19,2 ±1,3
	LE	CD3	22,8**±2,1	15,2 ±2,2	12,6* ±0,7	11,4 ±0,9	11,.2*±0,8	16,2 ±0,9
	LM	TCR1	12,0±2,1	28,0 ±1,7	10,5 ±0,8	19,2 ±1.1	9,9 ±2,1	13,8 ±3,6
	LE	TCR1	9,6±2.0	22,2**±1,05	9,3 ±1,7	12,5**±1,07	7,4 ±1.2	10,0 ±2,4
	LM	TCR2	35,2±1,3	12,4 ±2,1	20,0 ±3,9	12,3 ± 1,1	7,8 ±1,3	21,1 ±2,1
	LE	TCR2	28,8±2,2	4,2* ±1,3	24,6 ±2,1	10,0 ±1,15	5,0 ±1,1	18,5 ±1,7
	LM	CD8	31,1±1,3	11,1 ±1,5	42,2 ±4,6	22,5 ±4,5	42,6 ±3,2	46,0 ±3,9
	LE	CD8	28,8±2,2	8,8 ±1,6	89,7* ±8,9	102,6* ±7,7	81,0*±8,6	96,8*±8,4
Caecal tonsils	LM	CD3	32,1±1,5	21,2 ±2,2	34,2 ±2,9	18.0 ±1,3	21,8 ±1,6	22,6 ±1,8
	LE	CD3	29,2±1,9	11,4**±2,7	32,0 ±3,1	14,8 ±0,9	13,6**±1,4	20,6 ±1,7
	LM	TCR1	12,1±2,9	18,9 ±2,7	30,7 ±3,4	17,1 ±1,2	10,4 ±0,9	11,9 ±2,1
	LE	TCR1	3,6*±0,9	16,6 ±2,3	21,4**±1,9	52,6 *±4,9	11,8 ±1,1	56,2*±4,6
	LM	TCR2	31,2±1,3	12,4 ±1,7	29,0 ±3,1	8.9 ±1,5	7,9 ±1,7	12,6 ±1,3
	LE	TCR2	30,5±1,2	3,4* ±,0,9	8,2* ±2.1	7,6 ±2,1	6,0 ±1,9	11,4 ±1,1
	LM	CD8	28,6±1,8	14,8 ±1,1	33,8 ±3,1	36,1 ±2,9	33, 1±3,1	43,9 ±3,1
	LE	CD8	22,4±2,9	12,4 ±1,3	11,0*±3,9	12,8* ±2,1	10,0* ±1,9	11,0* ±3,8

Legend

•* statistically very significant VS, <0.001 LM = control group

•** Statistically significant SS <0.05 LE = experimental group

•IEL - intraepithelial lymphocytes

•PPL - lamina propria lymphocytes

Posthatching, CD3/TCR1 or CD3/TCR2 populations are sequentially generated in the thymus and disseminated through the bloodstream to peripheral lymphoid tissue. First detected in blood were CD3+ LT that does not express any of the two TCR receptors. They appear after the first week from hatching, and are growing in numbers depending on age, representing 15% of circulating lymphocytes in adults. More than 80% of TCR3 lymphocytes express CD4. Significant increase of TCR3 lymphocytes occurs in posthatching performed thymectomy. Only 5% of lymphocytes express CD8. A significant increase occurs after prolonged administration of ochratoxin A being known its immunotoxic effect on thymus lymphocytes.

TCR3 cells seem to represent a new population. A close relationship between TCR2 and TCR3 is suggested by the relatively high frequency of CD4 cells in both subpopulations. Thereafter compensating increase in frequency of TCR3 cells was observed when TCR2 subpopulation was inhibited, suggesting that these T lines are separated (Chen, 1989). In this experiment an increase in the two cell lines was recorded.

Intestinal villous of jejunum have a larger diameter than those of control group. Epithelium is highly prismatic but alternates with modified areas bruch border being now unsteady. In some areas the epithelium is composed of high cells with multiple nuclei, intracytoplasmic vacuoles and bruch border is absent. This epithelium seems detached from basal membrane due to vacuoles which are of varying sizes at the base of these cells (Fig.3). CD3 intraepithelial lymphocytes in the jejunum are significant reduced in number after 7 days of exposure and VS reduced after 14 and 21 days (table1). Lamina propria lymphocytes record US decrease. TCR2 lymphocytes suffer a VS decrease in lamina propria after 7 days of exposure. CD8 lymphocytes show an intraepithelial and lamina propria VS increase after 14 and 20 days of exposure to OTA. Lamina propria lymphocytes can be observed in villous axis, intraepithelial in Lieberckuhn crypts and interglandular.

CD8 lymphocyte influx from the jejunum is related to the presence of bacteria in the intestinal lumen and is consistent with results of Songserm et al. (2002). For these authors, cell-mediated immunity in the intestine is directly related to the elimination of pathogens such as *Pasteurella multocida* and *Eimeira* from intestine. Thus the results obtained by these researchers are in agreement with this work as an increase in CD8+ population was shown. However, as Songserm et al. (2002) stated, in addition to bacterial stimulus, increasing age of the chicken is responsible for the influx of lymphocytes in chicken intestinal epithelium. A study by Lillehoj and Chung (1992) in broilers showed that the percentage of 24.4% CD3+ lymphocytes increased from a week old to 42% by the second week, and 62.6% by the fourth week. These results agree with this study, because in the latter there was also an increased amount of CD3+ lymphocytes as the increased age of chicks but in a lower proportion.

The number of CD8+ lymphocytes obtained by Lillehoj and Chung (1992) deviates from this study because they show a gradual and incremental growth of these cells in older birds. Duodenum was the segment in which the immune response by T, CD3+, CD4+, CD8+ cells, was intense stimulated followed by jejunum and respectively cecum. Number of CD3+ lymphocytes present in the duodenum, jejunum and cecum increases in chicken with age, regardless of determined stimulus.

Caecal tonsils present a VS decrease of CD3 lymphocytes from lamina propria after 7 days and significant decrease of intraepithelial lymphocytes after 21 days of exposure. TCR1 intraepithelial lymphocytes presents a numerical variability, a FS decrease after 7 days and a VS increase of lamina propria lymphocytes after 14 days and 20 days. A VS decrease is also found in the TCR2 lymphocytes population in lamina propria at 7 days and intraepithelial at 14 days (Fig. 4, a, b). The other values are lower but US. CD8 lymphocytes registered VS decrease after 14 and 21 days of OTA exposure both in the lamina propria and intraepithelial. It is known that in chicken the digestive immunosuppression is caused by mycotoxins, nutritional deficiencies and infectious agents. Mycotoxins, T2 trichothecenes, ochratoxins are causing necrosis and tissue lymphocyte depletion of digestive tract and caecal tonsils depending on the dose. Nutritional deficiencies in vitamin E and selenium reduce the immunity of chickens, which are more susceptible to coccidiosis. *E. Maxima, E. Necatrix, E. Tenella* invades lamina propria at various stages (Ilic et al. 2003).

The presence of different predominant populations in those 3 intestinal segments can be explained by the decrease of the defense capacity of the intestine under the influence of OTA. Chicks were exposed in a period of immaturity of intestinal lymphocytes leading to increased susceptibility to certain infectious and parasitic diseases. Insignificant growth of cells after the first and second week of exposure to OTA can be explained by the influence of OTA when in contact with lymphocytes but also with other cells in the body. Lymph nodes were not surprised in any of the studied segments

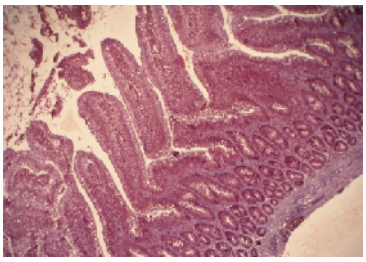

Fig. 1a. Duodenum of. chicken exposed to OTA for 7 days. Numerous intraepithelial and villous lamina propria CD3 lymphocytes Col IHC for LT CD3 x100

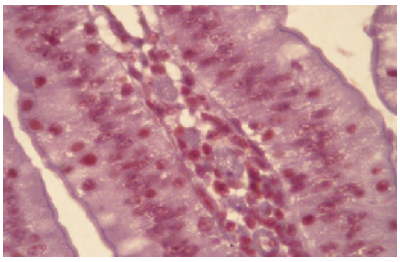

Fig.1b. Duodenum of. chicken exposed to OTA for 7 days. Numerous intraepithelial and villous lamina propria CD3 lymphocytes Col IHC for LT CD3 x1000.

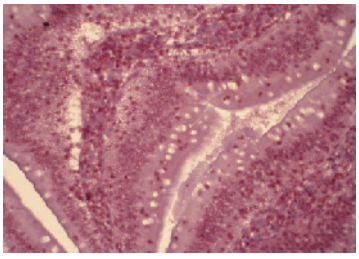

Fig.2a. Duodenum of. chicken exposed to OTA for 2 weeks. Mucosal epithelium with giant cells, without brush border, numerous small vacuoles with large base Col IHC for LT CD3 x100

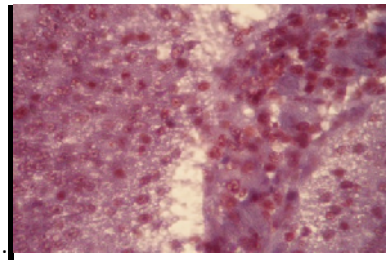

Fig. 2b. Duodenum of. chicken exposed to OTA for 2 weeks. CD3 lymphocytes are present both in lamina propia and intraepithelial with diffuse aspect. Col IHC for LT CD3 x1000

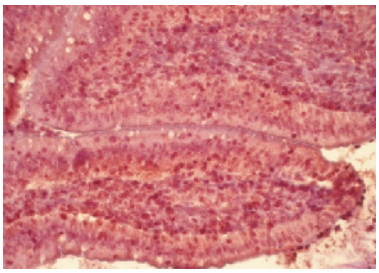

Fig.3a Jejunum of. chicken exposed to OTA for 7 days. CD8. Lymphocytes can be seen as congestions of lamina propia from villous axis and periglandular in small number. Col IHC for CD8 x400

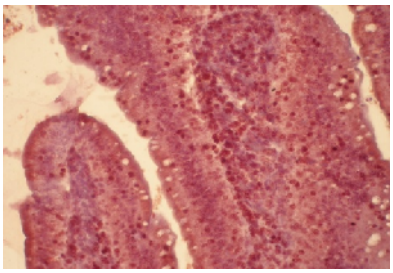

Fig3b.Some villous areas presents duodenum-like changes. Col IHC for CD8 x400

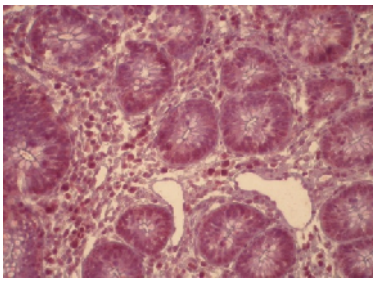

Fig.4a.Caecal tonsils. TCR1 at 2 weeks of exposure to OTA. Col IHC for TCR1 x100.

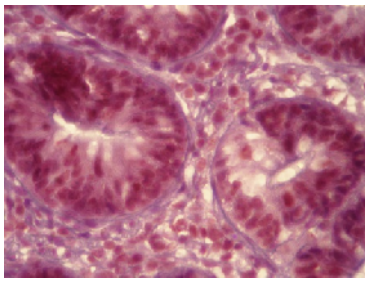

Fig.4b.Lymphocytes in small number both in villous axis and in lamina propria, peri and intraglandular. Col IHC for TCR1 x1000

CONCLUSIONS

- In the duodenum of the chickens from experimental group a decrease of CD3, TCR1, TCR2, CD8 intraepithelial and lamina propria lymphocytes, was registered after 7 days and, a very significant (VS) increase of lamina propria lymphocytes CD3 at 14 days of exposure. After 21 days CD3, TCR1,CD8 decreased unsignificantly in comparison with control group.
- Cytotoxicity of OTA is observed in duodenal and jejunal mucosa after two and three weeks of exposure to OTA. In some areas the epithelium is composed of high cells with multiple nuclei, intracytoplasmic vacuoles and brush border is absent. This epithelium seems detached from basal membrane due to vacuoles which are of varying sizes at the base of these cells..
- Number of CD3 and TCR2 intraepithelial lymphocytes in the jejunum are significant reduced after 7 days of exposure and VS reduced after 14 and 21 days. Intraepithelial and lamina propria CD8 lymphocytes showed a VS increasing after 14 and 21 days of exposure to OTA. CD8 lymphocyte influx from the jejunum is related to the presence of bacteria in the intestinal lumen
- Caecal tonsils showed a VS decrease of CD3 lymphocytes from lamina propria after 7 days and a significant decrease of intraepithelial lymphocytes after 21 days of exposure. TCR1 from lamina propria increased VS after 14 days and 21 days. CD8 lymphocytes registered a VS decrease after 14 and 21 days of OTA exposure both in the lamina propria and intraepithelial.
- The presence of different predominant populations in those 3 intestinal segments can be explained by the decrease of the defense capacity of the intestine under the influence of OTA. Chicks were exposed in a period of immaturity of intestinal lymphocytes leading to increased susceptibility to certain infectious and parasitic diseases.

Aknowlegements. Researches financed by grant PN2 Idei, 1118/2009 by CNCSIS

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