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Heterophils and Macrophage-Like Cells in the Caeca of Chicks after Experimental Infection with *Salmonella enteritidis* PT4

Z. ŠEVČÍKOVÁ[1], A. A. ASHEG[1], L. KOLODZIEYSKI[1], V. CIGÁNKOVÁ[2], T. KOMOROVÁ[2], M. LEVKUT[1]

[1]Department of Pathological Anatomy, [2]Department of Anatomy, Histology and Physiology, University of Veterinary Medicine, Košice, Slovak Republic

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Abstract

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In order to study the interaction between heterophils and monocyte-macrophage system cells with salmonella, one-day-old White Plymouth Rock chicks were inoculated with 2×10^8 colony forming unit (CFU) of *Salmonella enteritidis* phage type 4. Samples from the caeca were taken for light and electron microscopy study.

Groups of birds were euthanized by cervical dislocation at 6 hours (h) post inoculation and after 3, and 10 days (d) post inoculation. Histological examination at 3 d after infection showed heterophilic and mononuclear infiltration into the lamina propria mucosae and tela submucosa. The accumulation of macrophage-like cells accompanied this process at 10 d after infection. Electron microscopy revealed the presence of heterophils in the lamina propria at 3 d after infection and their migration between mucosal epithelial cells to intestinal lumen at 10 d after infection. Some of them were smaller with gap formation between intercellular space and heterophils. There was a decrease of the number of specific granules inside. Bacteria were not found within their cytoplasm. Oedema, condensation of chromatin, disruption of nuclear membrane and degradation of cytoplasmic organelles were seen in macrophage-like cells in the lamina propria. Formations of intracellular inclusions with cytoplasmic organelles and bacteria in various stages of degeneration were also observed in these cells. The cytoplasmic membrane was not disrupted.

Our results showed that infection of one-day-old chickens with *Salmonella enteritidis* PT4 caused the interepithelial accumulation of heterophils. The observed morphological changes of heterophils indicated their degranulation. It seems to be caused by the release of antimicrobial peptides.

Intracellular changes of monocyte-macrophage system cells without any injury of their cytoplasmic membrane show their survival of the *Salmonella enteritidis* PT4 infection. We suppose the rupture of their cell membrane in the later stage and the release of dead material with bacteria into the surrounding. In this way the monocyte-macrophage system cells serves in this condition as a vehicle and source of later infection.

Salmonella, ultrastructure, heterophils, macrophages

The recent recognition of the importance of chickens infected with *Salmonella enteritidis* PT 4 as a potential source of human infection has led to several studies on the pathogenesis of the infection in laying hens (Humphrey et al. 1989; Timoney et al. 1989; Chart et al. 1990). The crop and the caecum are the major sites of *Salmonella* colonization in chickens after oral infection (Brownell et al. 1970). The more detailed descriptions of lesions after experimental infection with *Salmonella enteritidis* PT4, however, are scarce (Desmith 1999).

Interaction between *Salmonella* and epithelium triggers the chemotaxis of phagocytic cells to the infection site. This cellular response involves both heterophils and macrophages migrating to the luminal surface where they begin eradication the bacterial pathogen (Henderson et al. 1999).

Address for correspondence:
MVDr. Zuzana Ševčíková, PhD
Department of Pathological Anatomy
University of Veterinary Medicine
Komenského 73, 041 81 Košice, Slovak Republic

Phone: +421 556 338 191
Fax: +421 556 338 191
E-mail: sevcik@uvm.sk
http://www.vfu.cz/acta-vet/actavet.htm

Phagocytic cells are a critical line of defence against infection. The ability of a pathogen to survive and even replicate within phagocytic cells is a potential method of evading the defence mechanisms of the host. A number of pathogens survive within macrophages after phagocytosis and this contributes to their virulence. *Salmonella* is one of these pathogens. It resides in large vacuoles and causes the death of these cells (Lindgren et al. 1996).

The mechanism of *Salmonella*-induced macrophage lysis is not clear. Several studies have shown that apoptosis is a form of *Salmonella*-infected macrophage death (Monack et al. 1996; Schwan et al. 2000) but others describes a different form of cell death, very similar to necrosis (Popiel and Turnbull 1985; Watson 2000; Raupach 2001).

Salmonella enteritidis was shown to interact with avian leukocytes. Histological reports on experimental *Salmonella* infection in mammalian and avian models confirmed that intestinal colonisation by *Salmonella* initiates an inflammatory response characterised by infiltration of the infected site by PMNs. The interaction of PMNs with salmonella have been studied in great detail by using mammalian models but only a few studies have evaluated the ability of avian leukocytes to kill *Salmonella* (Henderson et al. 1999).

The aim of this work was to study the histological and electron microscopic lesions of the chicken caecum caused *Salmonella enteritidis* PT4 infection. The work was focused on the extension of the knowledge about some aspects of heterophils and monocyte-macrophage system cells function and interaction with *Salmonella enteritidis* PT4 agent.

Materials and Methods

Chickens

A total of 24 one-day-old White Plymouth Rock chicks were randomly divided into two groups. Group 1 (experimental) contained 18 birds (6 per each experimental time), Group 2 (control) contained 6 chickens. The birds were kept in isolation in floor pens of 1 m^2 per group on wood shavings that were not changed during the experiment. The pen was lit continuously. The temperature was maintained at that required for the age of the birds. Water and feed were available *ad libitum*.

Salmonella infection

Experimental infection was carried out with *Salmonella enteritidis* PT4. Bacterial culture and doses were previously described (Asheg et al. 2001).

Experimental design

Experimental chicks were orally inoculated with $2 \cdot 10^8$ CFU/ml of *Salmonella enteritidis* PT4. Chicks of Group 2 were used as controls and obtained a placebo (1 ml PBS per orally). The birds (n = 6) of the infected group and two of control groups were euthanized by cervical dislocation 6 hours post inoculation and after 3, and 10 d post inoculation. During necropsy, sections of caecum and liver were taken aseptically for bacteriological examination and sections from the central part of the caecum were taken for histological and electron microscopy examinations.

Histopathological examination

Samples from caecum were fixed in 10% phosphate-buffered formaline, paraffin-embedded, sectioned at 5 μm, and stained with haematoxylin and eosin.

Electron microscopy examination

For electron microscopy, small pieces of caecum were immediately immersed in a fixative solution consisting of a mixture of 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1% M cacodylate buffer (pH 7.2). Samples were postfixed in 0.1% O_sO_4, dehydrated by an increasing ethanol series, and embedded in Durcupan. The ultrathin sections were prepared on an ultramicrotome LKB Nova, mounted on copper grids and contrasted with uranyl acetate (Watson 1985) and lead citrate (Reynolds 1963). The sections were viewed under an electron microscope (JOEL: 1200 MX) with an accelerating voltage of 80 KV.

Results

Light microscopy examination

In chicks infected with *Salmonella enteritidis* PT4, 6 h after infection hyperaemia and the infiltration of heterophils and mononuclear cells in the caecal lamina propria and tela

submucosa (Plate VIII, Fig. 1) was shown. Infiltration by macrophages was seen at 3 and 10 d post inoculation (Plate VIII, Fig. 2).

The presence of bacteria was confirmed by immunohistochemical staining (Asheg et al. in press). Bacteria that were in close contact with brush border caused degeneration of microvilli and protruding cytoplasm much like the small "ruffles". Intestinal luminal contents contained numerous necrotic heterophils and cellular debris (Plate IX, Fig. 3).

Electron microscopy

The entry of bacteria into the epithelial cells was associated with a series of pathological changes. Six hours post infection (p.i.) there was the appearance of active Golgi apparatus and the production of a variety of lysosomal vesicles and secretory granules in epithelial cells. Shortened microvilli and damage to the microvillous zone with interacting bacteria were found 6 h after infection (Plate IX, Fig. 4). There was an evagination of the apical surface that appeared to resemble endocytosis. Three days p.i. the presence of inflammatory heterophils was also seen in the lamina propria (Plate X, Fig. 5).

The changes found at 10 d p. i. showed marked heterophilic infiltration between mucosal epithelial cells (Plate X, Fig. 6). Some heterophils were smaller with the formation of a gap between intercellular space and heterophils (Plate XI, Fig. 7). There was a decrease in the number of specific granules. Their nucleus, nuclear, and cytoplasmatic membrane were not changed. Destruction of macrophage-like cells in the lamina propria was observed. There was also oedema of cells and disintegration of the nucleus but without rupture of the nuclear membrane. Mitochondria and granular endoplasmic reticulum were preserved. The presence of membrane-bound structure with bacteria inside was found (Plate XI, Fig. 8). More severe changes accompanying the oedema were also observed in some cells. Condensation of chromatin was seen with rupture of the nuclear membrane and injury, and even disappearance of cytoplasmatic organelles (Plate XII, Fig. 9). The individual bacteria and injured cytoplasmic organelles in various stage of degeneration occurred in the cytoplasm of macrophage-like cells free or within membrane-bound vesicles. Disruption of the cytoplasmic membrane was not seen (Plate XII, Fig. 10).

Discussion

Recently, *Salmonella enteritidis* has been a considerable threat for public health. Poultry is regarded as the main reservoir of this pathogen (Roberts 1988; Rodrigue et al. 1990). In Europe, *Salmonella enteritidis* PT 4 is widely present (Desmith 1999).

Light and electron microscopic examinations of intestines from chickens experimentally infected with various *Salmonella* species demonstrate similar cellular responses to these organisms, including the influx of heterophils and macrophages to the luminal surface of the intestine (Henderson et al. 1999).

Heterophils are considered to be the avian counterpart to mammalian neutrophils in their action as tissue macrophages and their importance in the host defence against bacterial infection (Henderson et al. 1999). They are critical effector cells in the innate defence of the host against *Salmonellae* infections. Heterophils appear to control both initial salmonella infections and subsequent disease pathogenesis (Kogut et al. 1999).

The infection of one-day-old White Plymouth Rock chicks with 2×10^8 CFU of *Salmonella enteritidis* PT4 in our experiment was accompanied by marked heterophil infiltration. Although many heterophils migrated towards the lamina propria and the intestinal lumen, bacteria were not observed intracellularly within the heterophils. This observation indicates the ability of heterophils to kill *Salmonella* organisms by releasing of antimicrobial peptides.

Five bactericidal peptides (chicken heterophil peptides CHP1 and CHP2; turkey heterophil peptides THP1, 2 and 3) were purified from avian heterophil granules. All peptides were cationic and rich in cysteine, arginine and lysine. Both chicken peptides and THP1 shared sequence homology at 22 residues and a cystein motif, which was similar to that of bovine beta-defensins (Evans et al. 1994). The beta-defensins found in heterophil granules can kill a wide variety of bacterial pathogens and are a major component of the heterophil antimicrobial arsenal (Harmon 1998).

In this work there was a gap seen in the surroundings of some heterophils, which indicated a decrease in the size of these cells. This morphological appearance supported by marked reduction in the number of specific granules indicates heterophil degranulation. It seems to be caused by release of antimicrobial peptides. This assumption correlates with the study where the evaluation of heterophils for *in vitro* microbicidal activity against selected avian pathogens was done. The ability of these peptides at 16 mass unit (mu) g/ml concentration was proved in the reduction of *Bordetella avium*, *Escherichia coli*, and *Salmonella enteritidis* survival (Evans et al. 1995).

After penetration of the intestinal epithelium, Peyer's patches are the first line that *Salmonella* are confronted with. The very early interactions between the host and microbe determine the three possible outcomes: first, whether the phagocyte functions as the effector and kills the ingested organisms, second whether the bacteria induce cell death of the phagocyte, and third whether the microorganisms adapt to the hostile environment and turn the phagocyte into their host cell to establish infection (Raupach and Kaufman 2001).

In this work, there were observed cells of different shapes and sizes than epithelial ones in the lamina propria. They were identified as macrophage-like cells.

Invasion of macrophages by *Salmonella typhimurium* through a specific pathway associated with membrane ruffling signals the mammalian cell to undergo programmed cell death or apoptosis (Monack 1996). But some of the evidence that bacteria-induced cell death is due to apoptosis is controversial (Watson 2000). In our study there were remnants of condensed chromatin and intact cytoplasmic membrane but no morphology typical of apoptosis was found. This indicates other mechanism of injury of the infected cells. Our data correlate with the results of *Salmonella* infection of macrophages with the formation of TUNEL-positive cells, but it was not determined that the TUNEL-positive cells were apoptotic. There was an absence of typical apoptotic morpholgy also in bovine alveolar macrophages and immortalized J774.2 macrophage-like cells after *Salmonella enterica* infection (Watson et al. 2000).

Oedema of macrophage-like cells, condensation of chromatin, disruption of nuclear membrane and degradation of cytoplasmic organelles, formations of intracellular inclusions with cytoplasmic organelles and bacteria in various stages of degeneration indicate severe injury of these cells. It has to be noted that there were no changes in the plasma membrane of these cells. Neither vacuolisation, for apoptosis typical budding phenomena, nor its rupture was found. There is evidence if there is the persistence of the plasma membrane in injured cells, it provides the scaffold for migration, positioning, and attachment of new cells during recovery (Cheville 1982). In our case, *Salmonella enteritidis* PT4 caused only intracellular to necrosis resemble injury of macrophage-like cells. There was no complete lysis of these cells. It is known that free bacteria or macrophages infected bacteria move to other organs and cause the generalisation of the process. (Henderson et al. 1999). On the basis of these facts we suppose that there will be an ability of these cells to move and in later stages underwent the rupture. After release of the dead material with bacteria into the surrounding these cells serve as a source of later infection.

Finally the experimental infection of one-day-old chickens by *Salmonella enteritidis* PT4 induced an inflammation of chicken caecum with the antimicrobial activity of accumulated heterophils, phagocyting and non-lethal injury of monocyte-macrophage system cells.

Štúdium heterofilov a buniek monocytárno-makrofágového systému v slepých vakoch kurčiat po experimentálnej infekcii *Salmonella* enteritidis PT4

Výskyt, funkcia a interakcia heterofilných granulocytov a buniek monocytárno-makrofágového systému boli študované v slepých vakoch jednodňových kurčiat po experimentálnej infekcii 2×10^8 cfu *Salmonella enteritidis(SE)* PT4.

Histologickým vyšetrením bola 3 deň po infekcii (dpi) preukázaná mononukleárna infiltrácia a prítomnosť heterofilných granulocytov v lamina propria mucosae a v tela submucosa. Proces bol na 10. dpi doprevádzaný prítomnosťou buniek monocytárno-makrofágového systému.

Elektrón-mikroskopické vyšetrenie potvrdilo na 3 di prítomnosť heterofilných granulocytov v lamina propria mucosae a na 10 dpi ich migráciu medzi epiteliálne bunky. Niektoré z heterofilov boli menšie, bolo pozorované formovanie „haló zóny" v ich okolí a redukcia počtu špecifických granúl v ich cytoplazme. Prítomnosť baktérií zistená nebola.

Edém, kondenzácia chromatínu, ruptúra jadrovej membrány, deštrukcia cytoplazmatických organel, ako aj formovanie intracelulárnych bunkových inklúzií a prítomnosť baktérií v rôznom štádiu deštrukcie boli pozorované v bunkách monocytárno-makrofágového systému v lamina propria mucosae. Cytoplazmatická membrána však porušená nebola.

Naše výsledky poukazujú na to, že infekcia jednodňových kurčiat *Salmonella enteritidis* PT4 vyvolala v slepých vakoch akumuláciu heterofilných granulocytov. Morfologické zmeny, ktoré boli na nich pozorované poukazujú na ich degraduláciu, čo môže byť zapríčinené vylučovaním antimikrobiálnych peptidov.

Intracelulárne zmeny buniek monocytárno-makrofágového systému bez poškodenia ich cytoplazmatickej membrány nepoukazujú na smrť týchto buniek ako následok experimentálnej infekcie *Salmonella enteritidis* PT4. V neskorších štádiách infekcie dôjde pravdepodobne k ruptúre cytoplazmatickej membrány a k uvoľneniu ich obsahu do okolitého prostredia. Bunky monocytárno-makrofágového systému sa tak stanú zdrojom pre šírenie sa infekčného procesu aj do statných orgánov, v dôsledku čoho dochádza k generalizácii procesu.

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Plate VIII
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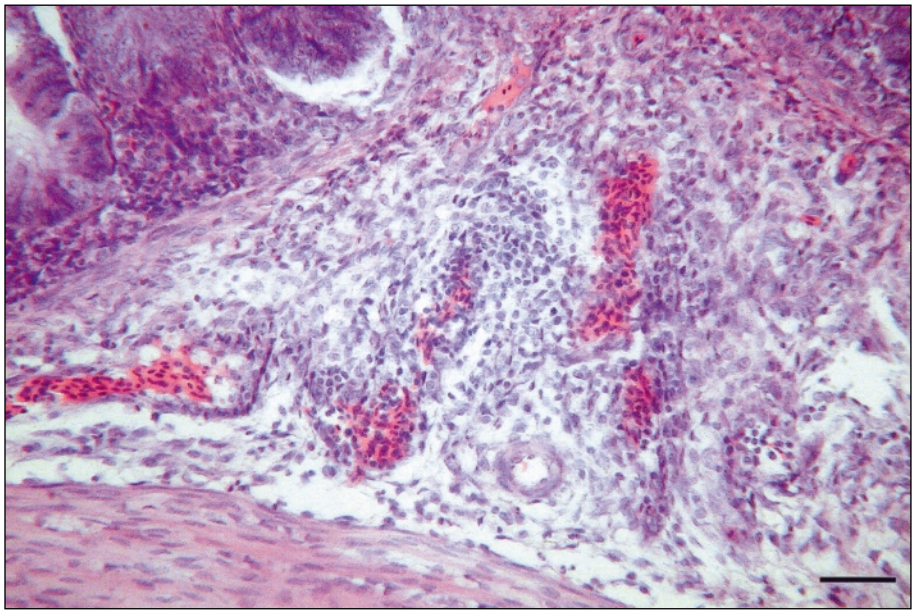

Fig. 1. Six hours after infection there is hyperemia and infiltration of heterophils and mononuclear cells in caecal tela submucosa and lamina propria. HE, × 20 (bar 2 μm).

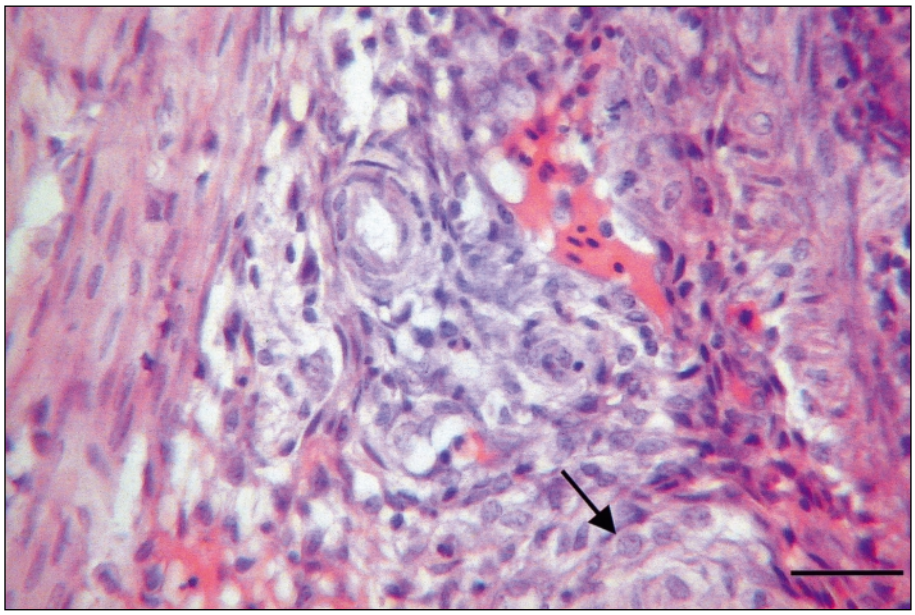

Fig. 2. Ten days after infection a marked infiltration of macrophages (arrow) was observed. HE, × 40 (bar 5 μm)

Plate IX

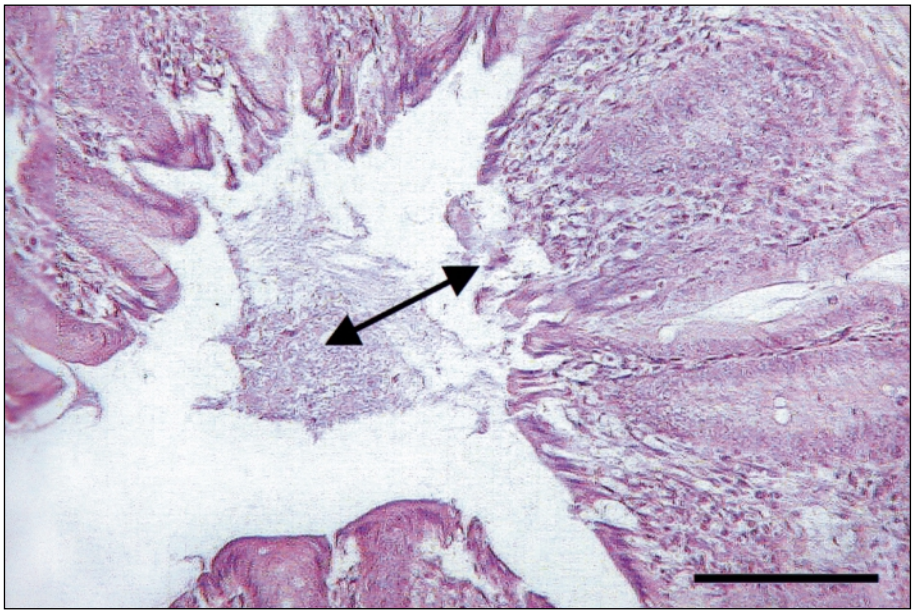

Fig. 3. Three days after infection the intestinal luminal contents with numerous necrotic heterophils and cellular debris was found. The degeneration of microvillous zone with its typical ruffles was also seen (arrows). HE, × 10 (bar 10 µm)

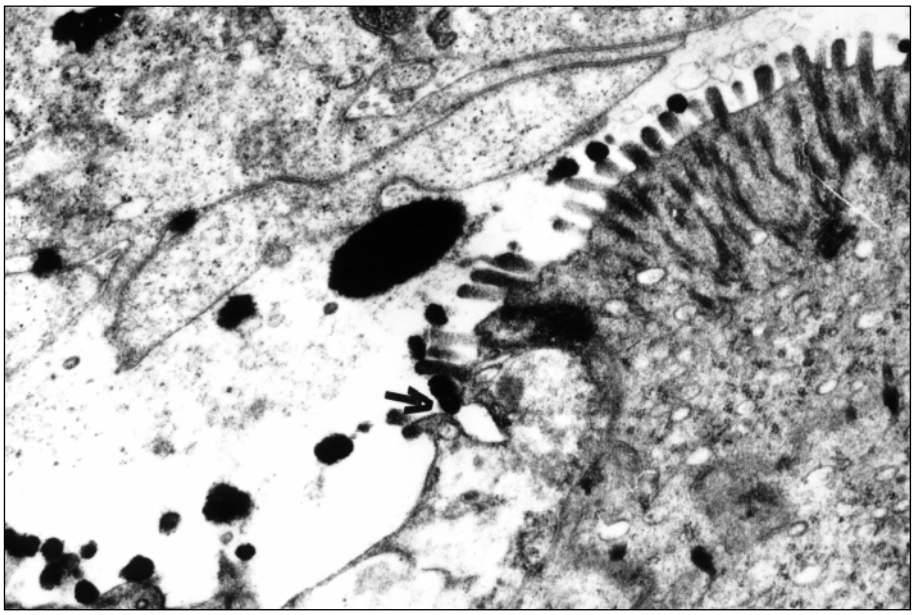

Fig. 4. Transmission electron micrograph of caecal microvilli and lumen in experimental chicken 6 hours after infection. Arrow shows the damage of microvillous zone with interacting bacteria. There was evagination of apical surface (× 18 200)

Plate X

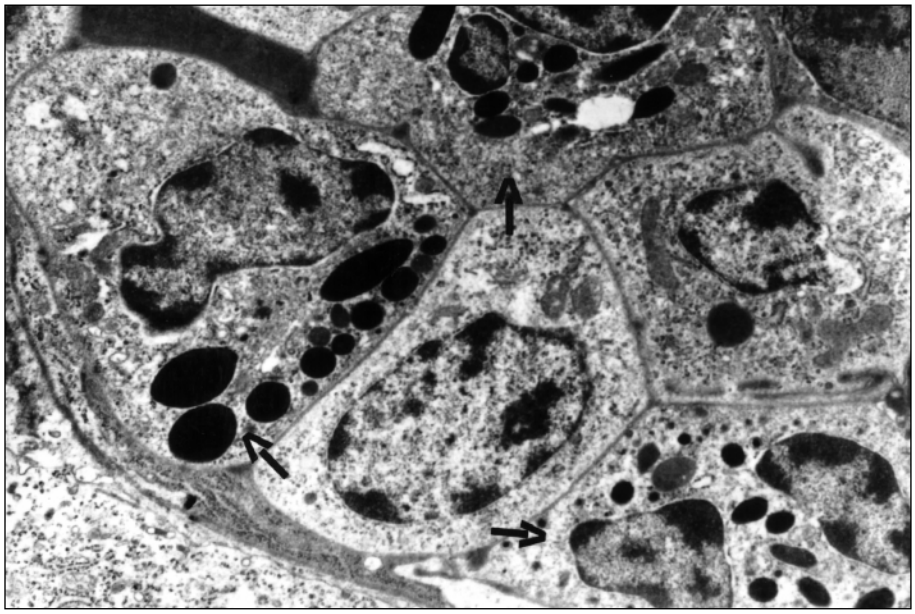

Fig. 5. Transmission electron micrograph of caecal lamina propria in experimental chicken 3 days after infection. Arrows show the presence of inflammatory heterophils in this layer (× 2 800)

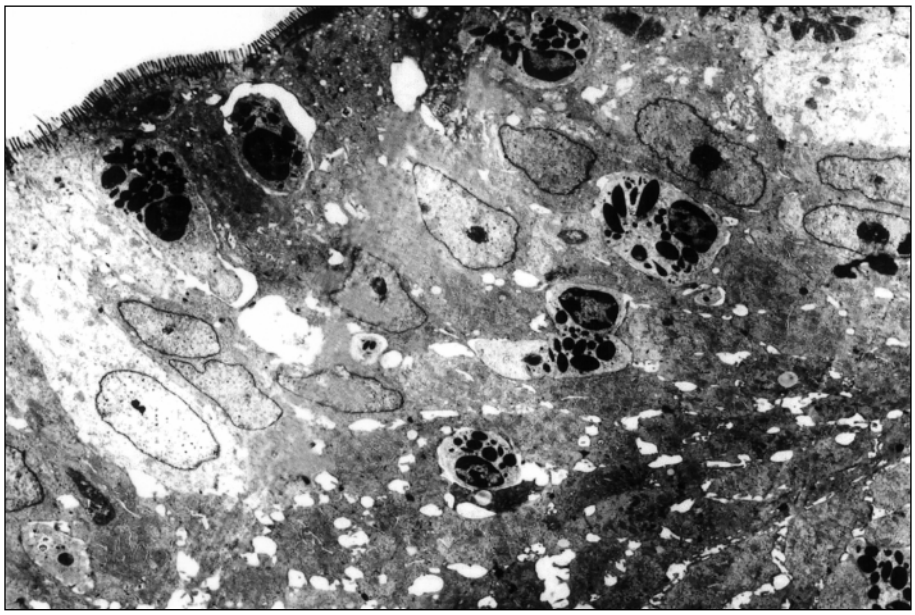

Fig. 6. Transmission electron micrograph of caecal epithelial layer in experimental chicken 10 days after infection. There is marked heterophilic infiltration between mucosal epithelial cells (× 1 600)

Plate XI

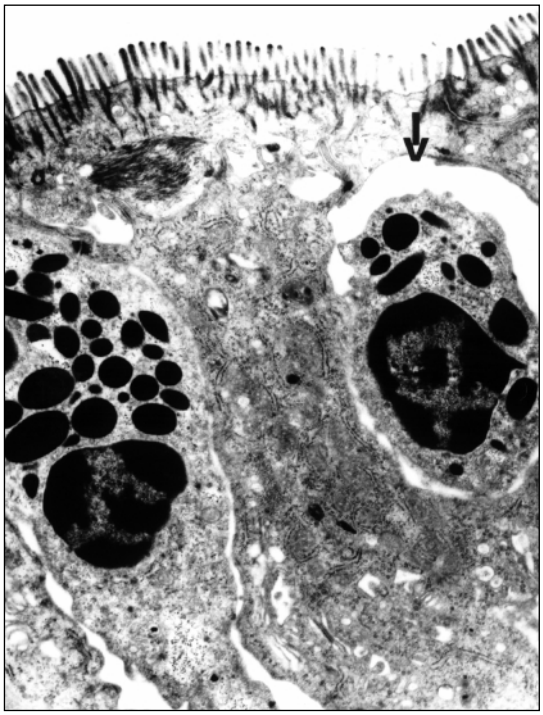

Fig. 7. Transmission electron micrograph of caecal epithelial layer in experimental chicken 10 days after infection. Arrow shows the smaller heterophil with the decreasing of the number of specific granules inside, with the gap formation in surrounding of cell. The nucleus, nuclear and cytoplasmic membrane were not changed (× 4 200)

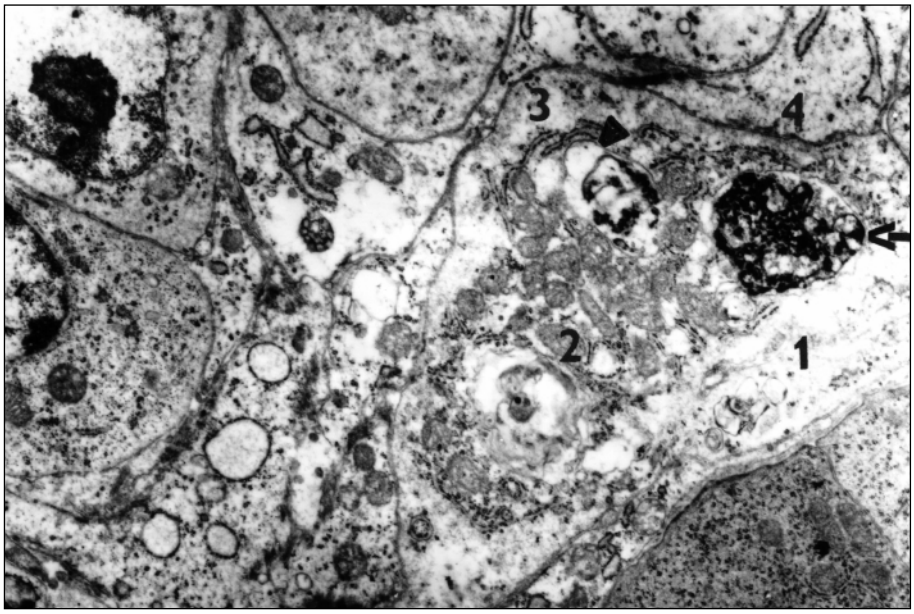

Fig. 8. Transmission electron micrograph of injured macrophage-like cell in experimental chicken 10 days after infection. There is an oedema (1), disintegration of nucleus but without the nuclear membrane rupture (arrow). Mitochondria (2), granular endoplasmic reticulum (3) and cytoplasmic membrane (4) were preserved. The presence of membrane bound structure with bacteria inside was found (arrowhead) (× 4 200)

Plate XII

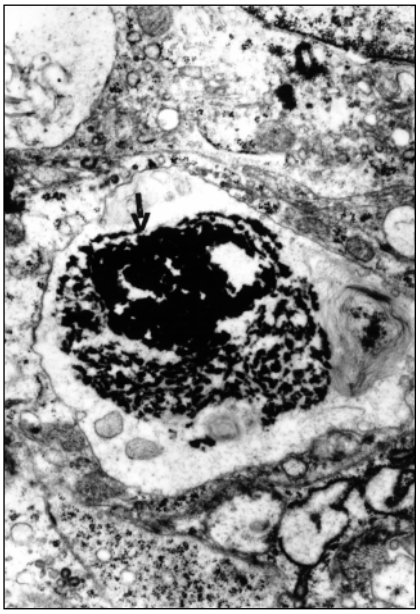

Fig. 9. Transmission electron micrograph of macrophage-like cell in experimental chicken 10 days after infection. Some cells showed more severe changes accompanying oedema. There was condensation of chromatin (arrow) with rupture of nuclear membrane and injury, even disappearance of cytoplasmic organelles (× 10 000)

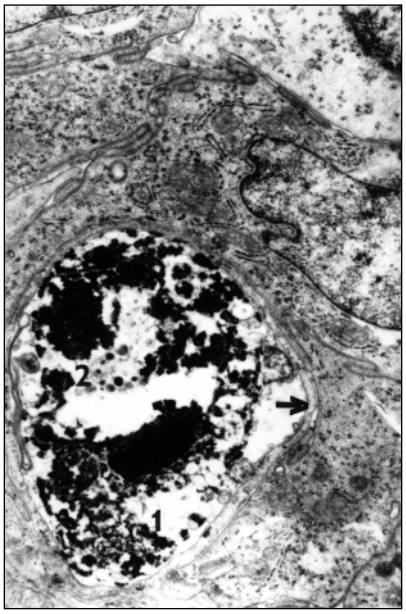

Fig. 10. Transmission electron micrograph of macrophage-like cell in experimental chicken 10 days after infection. There are individual bacteria and injured cytoplasmic organelles in various stage of degeneration free (1) or within membrane-bound vesicles (2). Disruption of cytoplasmic membrane was not seen (arrow) (× 8 000).