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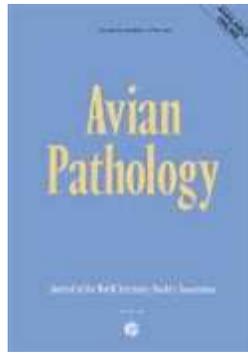
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Impact of heterophil granulocyte depletion caused by 5-fluorouracil on IBDV infection in SPF chickens

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Abstract

The purpose of this study was to investigate the influence of the cytostatic drug, 5-fluorouracil (5-FU), that causes depletion of heterophil granulocytes, on clinical symptoms and histological lesions during the progress of infectious bursal disease virus (IBDV) infection in chickens. The aim was to disclose the mechanism behind the clinical disease symptoms. Three groups of specific pathogen free chickens were used for the experiment. Chickens in groups 1 and 3 were pretreated with 5-FU, while chickens in group 2 were treated with a placebo. After five days, the chickens in groups 2 and 3 were inoculated with the classical IBDV strain F52/70. Bursae of Fabricius were sampled at fixed intervals, and the progress of the infection was monitored by various histological techniques and RT-PCR. We found correlation between histological observations and RT-PCR results. In the 5-FU pretreated chickens, IBDV caused only mild clinical symptoms, even though histological alterations, similar to alterations caused by IBDV were still observed. The 5-FU pretreatment resulted in severe heterophil granulocyte depletion by days 2 and 3 after infection (*p.i.*) and increased numbers of bursal secretory dendritic cells in the medulla of the follicles. IBDV infection seemed to induce fusion of secretory dendritic cells, resulting in formation of multinucleated giant cells, loaded with apoptotic B cells and virus particles associated with granules of bursal secretory dendritic cells. Our results indicate that the heterophil granulocytes together with the bursal secretory dendritic cells contribute to the

outbreak and/or progress of clinical symptoms.

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Introduction

Infectious bursal disease virus (IBDV) is the prototype of the genus *Avibirnavirus* belonging to the family *Birnaviridae* (Fauquet *et al.*, 2005). Two serotypes are recognized, serotype 1 and 2 (Mc Ferran *et al.* 1980), but only serotype 1 is pathogenic to chickens. Serotype 1 strains may cause severe clinical symptoms in young chickens, result in immunosuppression and increase flock mortality to 80% (Bumstead *et al.*, 1993; Sharma *et al.*, 2000; van den Berg, 2000). The chicken's susceptibility depends on several factors including breed (Nielsen *et al.*, 1998) and age (Allan *et al.*, 1972). Domesticated chickens at the age of three to six weeks are most susceptible. Clinical symptoms seem to be related to the development of the primary target organ, the bursa of Fabricius (Okoye & Uzoukwu, 1990), which reaches its maximum size at the age of three to six weeks (Hoffmann & Lade 1972). Chemical or surgical bursectomy prevents chickens from responding with clinical disease to IBDV infection (Hiraga *et al.*, 1994; Okoye *et al.*, 1992; Okoye and Uzoukwu, 1990). In bursa-intact chickens, viral particles replicate in lymphoid cells of the bursa causing lymphocyte depletion (Nieper & Muller, 1996), and possibly in macrophages and other cells (Khatri *et al.*, 2005), but the exact mechanism by which the virus causes disease is not yet known. Young chickens, less than one week old, do not respond to infection with clinical signs, but the immune response is compromised (Saif, 1991; Sivanandan & Maheswaran, 1980; Rosenberger & Gelb, 1978). At this age, the function of the innate immune system is hampered by the functionally immature heterophil granulocytes (Seto, 1981; Kogut *et al.*, 1994; Wells *et al.*, 1998). Heterophil granulocytes belong to the polymorphonuclear leucocytes circulating in the blood and involved in elimination of pathogens. Granulocytes and macrophages have a common myeloid progenitor (Inaba *et al.*, 1993), and both are capable of phagocytosis and degranulation (Lam, 1998). In the blood of normal, adult chickens 15-40% of the white blood cells are heterophil granulocytes (Jain, 1993). Probably

activated by T-cells, granulocytes cause release of cytokines or oxidative burst (Kogut *et al.*, 2001, 2003). Release of cytokines and nitric oxide has been suggested as part of the IBD syndrome resembling septic shock (van den Berg, 2000).

Epithelial cells of endodermal origin form the fine, supporting structure of the follicular medulla in the bursa of Fabricius (Oláh & Glick 1992a). In addition to epithelial cells, four different cell types are normally present in the follicles: B-cells, T-cells, dendritic cells and macrophages. Dendritic or secretory cells (BSDCs) are present in mature and immature forms (Oláh & Glick, 1987). Contrary to B cells, BSDCs express vimentin intermediate filaments (Oláh *et al.*, 1992b; Oláh & Glick 1995). During IBDV infection, the normal cell population changes, as B-cells are depleted (Ramm *et al.*, 1991), the number of macrophages decreases (Khatri *et al.*, 2005), the BSDCs leave the medulla (Oláh *et al.*, 1997) and the number of heterophils and T-cells is increased (Sharma, 1989; Tanimura & Sharma, 1997).

A model for heterophil depletion of chickens by treatment with the cytostatic drug 5-fluorouracil (5-FU) has been described (Kogut *et al.*, 1993) and used for investigation of the influence of heterophil granulocytes on bacterial (Bojesen *et al.*, 2004) and viral (Raj *et al.*, 1997) infections in chickens. In a previous, unpublished experiment, chickens treated with the recommended dose of 5-FU and subsequently infected with IBDV showed mild clinical symptoms, but survived the infection. Based on this study, the present experiment was designed to make IBD-viraemia occur at the time when blood contents of heterophile granulocytes were at its lowest level.

Aiming at obtaining basic information on the role of heterophile granulocytes in the complex disease manifestations of IBD, we investigated the impact of 5-FU induced heterophil depletion on clinical symptoms and bursa lesions during IBDV infection. The distribution of B-cells, T-cells, dendritic cells and virus particles in the bursa of Fabricius were investigated by

immunocytochemistry and light- and transmission electron microscopy (TEM). In addition, the presence of virus RNA was monitored by RT-PCR.

Materials and methods

Chickens. White Lohmann chickens from SPF eggs (Lohmann Tierzucht, Cuxhaven, Germany) were hatched at the laboratory. One-day-old chickens were reared in isolators (HM 1500, Andersen BV, Sevenum, the Netherlands) with a filtered airflow. The chickens had unlimited access to water and commercial chicken feed. Light supply was programmed to 8 hours of darkness every 24 hours after the first week. All chickens, including the terminally ill, were humanly killed in accordance with Article 2(1) in Directive 86/609/European Economic Community of 24 November 1986.

Experimental design. Three groups of 15 chickens were used in the experiment. Before treatment, heparin stabilised blood samples were collected from the ulnar vein of each chicken. Chickens in groups 1 and 3 were treated with 5-FU at the age of 15 days, while chickens in group 2 were given placebo. Five days later, at 20 days, groups 2 and 3 were inoculated with IBDV, while group 1 was similarly inoculated with placebo. Three chickens from each group were killed on days 20, 21, 22, 23 and 30, corresponding to 0, 1, 2, 3 and 10 days after IBDV infection.

Before the chickens were killed, heparin stabilised blood samples were collected from the ulnar vein. Autopsy was performed immediately. Each bursa of Fabricius was sampled, divided into four pieces, that were frozen for RT-PCR, placed in 4% formaldehyde for evaluation of bursa lesions, or in 4% buffered glutaraldehyde for light and transmission microscopy, and snap-frozen in liquid nitrogen for immunohistochemistry, respectively.

Virus. The classical IBDV strain, Faragher 52/70 (F52/70) (Lohmann Tierzucht, Cuxhaven, Germany) was obtained from bursa tissues from previously infected chickens. The bursa tissues were homogenised in equal weight of sterile sand using a sterile mortar and pestle. The homogenate was eluted in phosphate buffered saline (PBS) containing 10,000 U/ml Penicillin, 10,000 µg/ml Streptomycin, 250 µg/ml Gentamycin, 500 U/ml Nystatin and 5% FBS (Invitrogen, Carlsbad, California). This eluate was centrifuged for 15 minutes at 3000 rpm. The resulting supernatant was diluted in Hank's buffered saline solution (HBSS) (Gibco™, Paisley, Scotland) to 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and inoculated via the chorio-allantoic membrane (CAM) into embryonated hen's eggs to determine virus titres (Busby *et al.*, 1964). The inoculate had a titre of $10^{4.2}$ ELD₅₀. Experimental chickens were inoculated with 0.2 ml of the dilution as drops in the eyes, beak and nostrils.

Heterophile depletion. The cytostatic drug, 5-fluorouracil (5-FU) (Sigma, Copenhagen, Denmark) is used in cancer treatment. The effect of 5-FU is related to its replacement of uracil in RNA (Ardalan & Glazer, 1981). A dose of 200 mg/kg was injected once into the *vena ulnaris* (Kogut *et al.*, 1993). Sterile saline was used as placebo.

Blood. Heparinized blood samples were collected from the ulnar vein and refrigerated. One drop from each sample was smeared onto a glass slide, air dried and stained with Haemacolor (Malinckrodt Baker B.V. Deventer, Holland). One hundred cells were counted under a microscope. All polymorphonuclear cells were regarded as heterophil granulocytes when the percentage of the total blood cell count was calculated.

Light and electron microscopy. Bursa tissue samples were fixed in formaldehyde overnight and embedded in paraffin wax. The paraffin-embedded bursa tissue samples were cut in 2 μ sections, mounted on Super Frost Plus slides (Hounisen, Risskov, Denmark), stained by haematoxylin-eosin (HE) (Swayne *et al.*, 2000) and studied under a Leica DMRB microscope. Bursa lesions were evaluated quantitatively according to the lesion scoring system recommended by a working group under COST839 (Mundt, 2002).

Bursa tissue samples were fixed in glutaraldehyde overnight. After washing in PBS, the tissue samples were postfixed in 1% OsO₄ for two hours. Graded alcohol was used for dehydration of the tissue blocks prior to embedding in Polybed®Araldite mixture (Polysciences Inc. Eppelheim, Germany). These samples were cut into semithin 1 μ m, and thin sections. The one-micron semithin sections were stained with toluidin blue for light microscopy, while thin sections were contrasted by 1% uranyl acetate and lead citrate and studied by Hitachi type H-7600 electron microscope.

Immunohistochemistry. A bursa tissue sample was completely enveloped in liver tissue to protect from the damage caused by sudden freezing. This “package” was placed on a small piece of carton and frozen on polystyren, floating in liquid nitrogen, and stored at -80°C.

Immunostaining was performed on 8-micron cryostat sections, which were collected on poly-L-lysine coated slides (Sigma-Aldrich Kft., Budapest, Hungary). Briefly, the acetone-fixed and air-dried sections were re-hydrated in PBS and incubated with primary antibodies for 45 minutes.

The primary antibodies were: Clone Lu5 anticytokeratin mAb (Boehringer-Mannheim GmbH, Indianapolis, USA), Clone 5-11G2 (Mouse IgG1) Bu-1b for B cell staining (Southern Biotechnology Associates Inc., Birmingham, USA), Rabbit Anti-human T cell, CD3 (rabbit IgG) for T cell staining (Dako Cytomation, Aarhus, Denmark), Clone Amf-b17 (mouse IgG1), Vimentin intermediate filament for staining of avian dendritic cells and heterophil granulocytes

(DSHB, Iowa, USA), Clone 74.3 staining intracellular antigen in the BSDCs and FDCs (Jeurissen *et al.* 1992), Clone CG-106 (mouse IgG1) surface IgG staining BSDCs (Sigma-Aldrich Kft., Budapest, Hungary), Rabbit polyclonal serum (rabbit IgG) virion staining IBDV (produced in our laboratory), 5A-10 (mouse IgM) VP2 staining IBDV (gift from Dr. Palya, Ceva-Phylaxia Veterinary, Hungary) . Biotinylated horse anti-mouse IgG, biotinylated horse anti-mouse IgM and biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, California) were followed by quenching of endogenous peroxidase activity by 1.5 % hydrogen peroxide (Sigma-Aldrich Kft., Budapest, Hungary) for 10 minutes. After applying the avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Burlingame, California) the binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma Aldrich Kft., Budapest, Hungary).

RT-PCR. RNA was extracted as previously explained (Kabell *et al.*, 2005). The QIAGEN OneStep RT-PCR Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions. Briefly, 10 µl of a 5 x reaction buffer, 2 µl dNTP mix, 2 µl enzyme mixture, 100 pmol of each oligonucleotide and 5 µl of RNA were mixed with RNase-free water to a final volume of 50 µl. All ingredients were kept on ice during handling.

The PCR reaction was performed in a Biometra T3 Thermocycler (Biometra GmbH, Goettingen, Germany) as follows: 30 minutes at 50°C (RT reaction), 94°C for 15 minutes (initial PCR activation), 39 three-step cycles of 94°C for 30 sec, 58°C for 1 minute and 68°C for 2 minutes, and 68°C for 7 minutes (final extension).

Results

Clinical signs. The only 5-FU treated chickens (group 1) did not show any signs of disease, but ten days after 5-FU treatment, they began to shed more feathers than normally expected. The virus inoculated chickens (group 2) developed severe clinical symptoms on day three after virus inoculation, including anorrexia, depression, diarrhoea and prostration. Consequently, remaining birds were euthanized. The 5-FU treated and virus inoculated chickens (group 3) showed mild signs of depression three to four days after virus inoculation, and they shed feathers like group 1.

Blood. Chickens from group 1 showed relatively high percentages of heterophil granulocytes in the blood before treatment, decreasing on the seventh day after 5-FU treatment, remaining low during the rest of the experiment, as shown in Table 2. Heterophil granulocytes from the non-5-FU-treated group 2 remained within the value range of 12-50% during the experiment. The average number of heterophil granulocytes from groups 2 and 3 were still comparable five days after 5-FU treatment. The next day, one day *p.i.*, the number of heterophil granulocytes in the 5-FU treated and virus inoculated group 3 increased to an average of 33%, and two and three days after infection, it decreased to subnormal levels, to increase again ten days after virus inoculation.

Histology of the bursa of Fabricius. Five days after 5-FU treatment, the size of the bursal follicles from groups 1 and 3 was moderately decreased, and the centers of the follicles seemed mildly depleted. The overall picture of the effect of 5-FU treatment was a mild histological alteration of the bursa at the time of the IBDV inoculation. From two days *p.i.*, lesions observed in samples from the 5-FU treated and IBDV inoculated group 3 included lymphocyte depletion,

as seen by HE-staining (not shown) and folding of the interfollicular epithelium, illustrated by anticytokeratin immunostaining of epithelial cells (Fig. 1a), comparable with the lesions seen in the only IBDV inoculated group 2 (Table 2). Cell alterations, observed during the first three days of the IBDV infection are summarised in table 3. By day two, staining by Bu-1b in groups 2 and 3 showed that the B cell depletion was significant in the center of the medulla and close to the cortico-medullary border of the cortex (Fig. 1b). One day later, B cell staining of the medulla appeared as a non-homogeneous mass, encircled by a strongly depleted cortical ring. The non-homogenous B cell staining in the medulla consisted of irregularly shaped bodies of 40-50 μm in size.

Heterophile granulocytes were observed in the toluidine blue stained sections, being present in large numbers in the vessels and follicles of group 2, contrary to group 3 (Figs 1c, 1d). Although it is difficult to make a quantitative estimation on immunohistochemical and histological slides, sections stained with toluidine blue as well as sections stained with anti vimentin showed that the number of granulocytes was clearly decreased on day 2 *p.i.* in the 5-FU and IBDV inoculated group 3, remaining low during the experiment, as compared to the only IBDV inoculated group 2 (Figs 1c/1d and 1e/1f). On day 3, vimentin positive cells in group 3 were few, but the majority was small and heavily stained, and proved to be heterophil granulocytes, while enlarged, pale-stained and ring-shaped cells were identified as giant cells. Transmission electron micrographs did not show virus particles in the heterophile granulocytes. However, the transmission electron micrographs indicated, that cell clusters described above as inhomogenous B cell staining, represented single- or multi-nucleated giant cells, which were loaded with virus and cell debris (Figs. 2a,b).

CD3 staining showed that the number of T cells increased in the follicular cortex of both groups of IBDV inoculated chickens, while the medulla contained only scattered T cells (Fig. 2c).

Besides the granulocytes, the number of BSDCs differed in the IBDV inoculated groups. The number of BSDCs seemed to increase in both groups at day two *p.i.*, as evaluated by the toluidin blue staining. This elevation may not be absolute, possibly relative, as B-cells were depleted. In group 3, the shape of the BSDCs was bulky, not elongated as generally seen in mature BSDCs, their cytoplasm contained only few granules around the Golgi zone, and lipid droplets were scattered over the cytoplasm. Mature forms of BSDC(s) were seen infrequently in the 5-FU pretreated and IBDV inoculated group 3, unlike the only IBDV inoculated group 2. Two days *p.i.*, the 74.3 mAb staining revealed follicles of highly variable appearance. In most follicles, the 74.3 positive BSDCs were aggregated close to the inner surface of the cortico-medullary border (Fig. 2d). The 74.3 mAb staining revealed similar staining pattern as the B cell staining three days *p.i.*, but the size of the bodies appeared smaller (Fig. 2e). IgG staining confirmed that the observed rearrangement was due to BSDCs (Fig. 2f).

Progress of the IBDV infection was monitored by polyclonal anti-IBDV, and monoclonal anti-virus protein, VP2. Two days *p.i.* the virus and virus protein in both virus inoculated groups was concentrated mainly in the follicular medulla, while one day later, the cortex was also heavily loaded with virus. Virus particles were packed in the cytoplasm of large bulky cells in both the medulla and the cortex (not shown).

RT-PCR. All bursa tissue samples from IBDV inoculated chickens were positive, and all bursa tissue samples from uninoculated chickens were negative.

Discussion

The aim of this study was to disclose a possible impact of 5-FU depletion of heterophil granulocytes on the complex pathogenesis and onset of clinical symptoms of IBDV infection.

The 5-FU pre-treated and IBDV inoculated group 3 showed only mild clinical disease symptoms, and the chickens survived the infection, contrary to the placebo pretreated and IBDV inoculated group 2, in spite of similar histological lesions in tissues from the bursa of Fabricius. These observations are in accordance with Raj *et al.* (1997), who reported reduced clinical response to bronchitis virus infection after 5-FU pre-treatment. The shedding of feathers, observed in both 5-FU treated groups of chickens 10 days after treatment, might indicate an impact of 5-FU possibly similar to alopecia in humans during cancer treatment.

The blood contents of heterophil granulocytes from 5-FU treated and placebo treated chickens were within the normal range five days after 5-FU treatment, when the IBDV inoculation took place. After one day, the heterophil counts rose unexplicably in the only 5-FU treated group 1, while the two other groups showed normal values. However, the number of heterophil granulocytes was drastically reduced in both treated groups seven and eight days after 5-FU treatment, two and three days after virus inoculation, when viraemia was expected. The results of the blood cell counts were too few to be statistically evaluated, but the effect of 5-FU on blood cells seemed already discussed in detail by Kogut *et al.* (1993) and Bojesen *et al.* (2004). The histological studies of bursa tissues confirmed the results of lymphocyte counts, revealing cellular depletion five days after 5-FU treatment. Fifteen days after 5-FU treatment, a marked difference in blood contents of heterophils between groups 1 and 3 was observed. The increase in the number of heterophil granulocytes in group 3 was probably stimulated by the IBDV infection, while the non-infected chickens may require more time to restore the normal blood content of heterophil granulocytes.

According to the bursa lesion scores, 5-FU seemed to moderately reduce the number of B cells. However, the histological alterations two and three days *p.i.* corresponded to the general picture characterizing IBDV infections, and the 5-FU treatment did not seem to influence the development of pathological lesions. The IBDV infection reportedly stimulates the heterophil

granulocytes entering the bursa (Sharma *et al.*, 1989; Lam, 1998), but this reaction was markedly decreased after 5-FU treatment. Except for the low number of heterophil granulocytes, and an increased number of BSDCs in group 3, the histological alterations of the bursa caused by IBDV were comparable between groups 2 and 3. The observed histological differences may indicate that the heterophil granulocytes and the BSDCs play crucial roles in the onset of clinical symptoms. In the 5-FU pretreated group 3, the cytological features of the BSDCs indicated their immature condition, as compared to the mature BSDCs, that have two long cytoplasmic processes, one of which is occupied by cytoplasmic granules (Oláh & Glick, 1987; Nagy *et al.*, 2001). The immature, newly formed BSDCs were lined up along the cortico-medullary border, while the mature BSDCs were scattered over the medulla. When the 5-FU pre-treatment reduced the heterophil-invasion, it may have turned the disease from acute to subacute. This induced, subacute form of the IBDV infection did not impair the BSDCs as severely as the acute form, resulting in increase in the total number of BSDCs.

Phagocytosis of the cell debris and engulfing of virus particles was evidenced by light and transmission microscopy and by immunohistochemistry. In the medulla of the follicles, only the BSDCs may be charged with phagocytosis (Nagy *et al.*, 2004). In group 2, the BSDCs disappeared, as shown by a decreased number of vimentin positive cells in the follicular medulla, substantiated by the faded IgG staining. However, transmission microscopy showed that their cytoplasmic granules seemed to fuse with virus particles. The virus attack on the BSDCs may transform them rapidly to phagocytic cells. Under physiological conditions, the turnover of the BSDCs is slow. The senescent BSDCs enter the follicle-associated epithelium (FAE), and are eliminated in the lumen of the bursa (Nagy *et al.*, 2004). This turnover is highly facilitated by IBDV (Oláh *et al.*, 1997), and the BSDCs may leave the medulla not only towards the FAE but also through the cortico-medullary border into the cortex, carrying the virus and the digested apoptotic cells. Thus, the terminal maturation of the BSDC might be a phagocyte, distinct for the

bursa of Fabricius (Nagy *et al.*, 2004). The multinucleated giant cells appeared first in the medulla and about one day later in the cortex. The light- and electron microscopy and immunocytochemistry revealed only epithelial reticular cells, lymphocytes and BSDCs in the medulla. We propose that BSDCs form the multinucleated giant cells.

In groups 2 and 3, T-cells were recognized in the cortex of the follicles and in the interfollicular connective tissue from the second day after IBDV inoculation. T-cells may reportedly influence the development of bursa lesions (Rautenschlein *et al.*, 2002). According to the bursa lesion score evaluation, major differences were not observed between bursa lesions after IBDV infection in groups 2 and 3, suggesting that T-cell influence was not compromised by 5-FU.

In conclusion, the impact of 5-FU, appearing as a depletion of blood heterophil granulocytes did not seem to alter the course of the IBDV infection in the bursa even though clinical symptoms were drastically reduced. We suggest, that the decrease of heterophil granulocytes induced a subacute form of infection that maintained an increased number of BSDCs. The reactions of the heterophil granulocytes and possibly the BSDCs may be crucial for the development of clinical disease. As both cells are known to produce cytokines (Kogut *et al.*, 2003, Swaggerty *et al.*, 2004, Oláh & Glick 1993), these observations add credibility to theories on cytokine involvement in clinical symptoms (Sharma *et al.*, 2000). Further investigations into the function of heterophil granulocytes and BSDCs may add information to explanations of how IBDV causes clinical disease.

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Figure captions

Figure 1. Reactions to IBDV infection in 5-FU pretreated and non-pretreated chickens

Figure 2. BSDCs and T-cells during IBDV infection

Figure legends

Fig. 1a. 5-FU and IBDV inoculation (group 3): Three days after IBDV inoculation (p.i.), folded surface epithelium was observed (arrows); the medullary epithelial reticular cells form “empty holes”. Anticytokeratin immunostaining. Magnification: 40X

Fig. 1b. IBDV inoculation (group 2): Two days p.i., B cell depletion. Depletion started in the center of the medulla and inner part of the cortex. Bu-1b staining. Magnification: 35X

Fig. 1c. IBDV inoculation (group 2): Two days p.i., the follicular cortex flooded by heterophil granulocytes (arrows). Semithin section stained with toluidin blue. Magnification: 500X

Fig. 1d. 5-FU and IBDV inoculation (group 3): Two days p.i., reduction in the number of heterophil granulocytes (arrows). Compare with Fig. 1c. Semithin section stained with toluidin blue. Magnification: 500X

Fig. 1e. IBDV inoculation (group 2): Two days p.i., huge numbers of vimentin positive cells in the medulla and surface epithelium of the follicles. Magnification: 140X

Fig. 1f. 5-FU and IBDV inoculation (group 3): Two days p.i., the number of vimentin positive cells in the medulla reduced as compared to group 2 (See Fig. 1e). Magnification: 140X

Fig. 2a. 5-FU and IBDV inoculation (group 3): Two days after IBDV infection. The cell contains apoptotic lymphocytes (A) and two demarcated bodies, in which the virus particles are associated with electron dense substance (arrow). Part of one of the demarcated bodies is outlined and shown in Fig. 2b. Lipid droplets (L) in the cytoplasm. Magnification: 10000X

Fig. 2b. Detail from Fig. 2a. In the demarcated body, the virus particles are intermingled with electron dense substance. Magnification: 40000X

Fig. 2c. IBDV inoculation (group 2): Three days p.i., CD3 positive T cells aggregate in the follicular cortex. Medulla contains only scattered T cells. Magnification: 140X

Fig. 2d. IBDV inoculation (group 2): Two days p.i., the 74.3 positive BSDCs aggregate along the cortico-medullary border in several follicles. Magnification: 160?X

Fig. 2e. IBDV inoculation (group 2): Three days p.i., the 74.3 positive BSDCs seem to be completely aggregated in the medulla. Magnification: 140X

Fig. 2f. *IBDV inoculation (group 2): Two days p.i., the anti-IgG staining confirm observations obtained by 74.3 mAb. The IgG positive BSDCs aggregate along the cortico-medullary border. Compare with Fig. 2d. Magnification: 70X*

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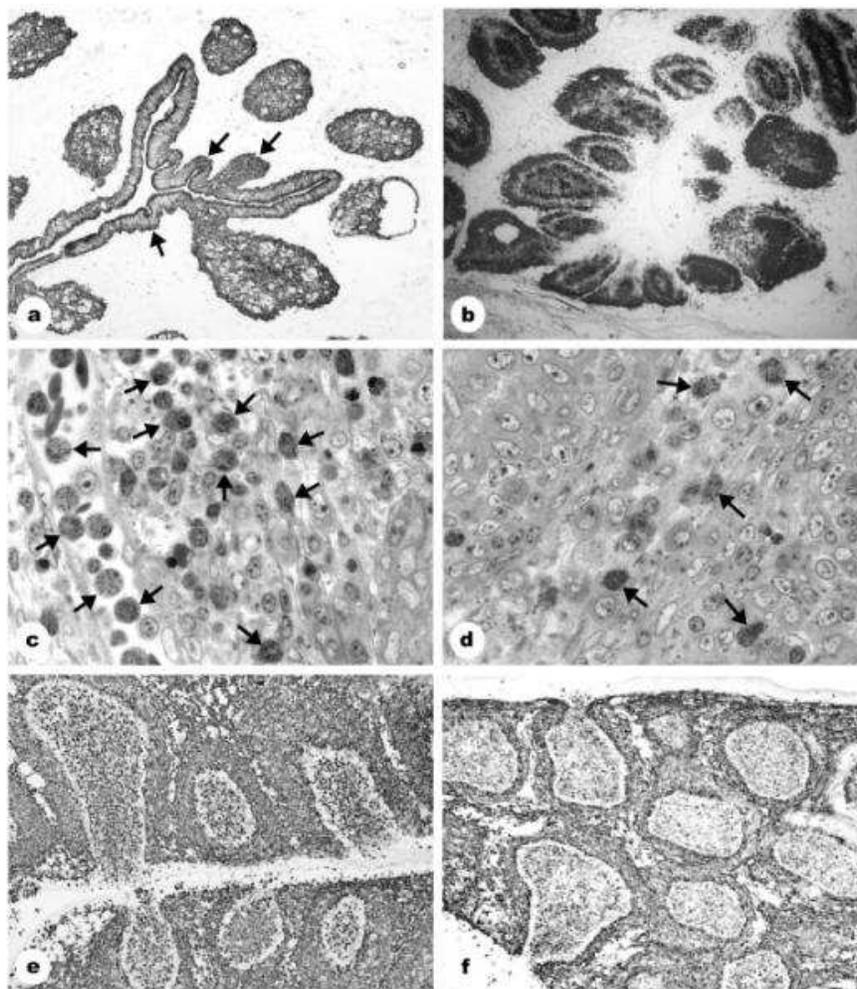


Figure 1. Reactions to IBDV infection in 5-FU pretreated and non-pretreated chickens
190x254mm (72 x 72 DPI)

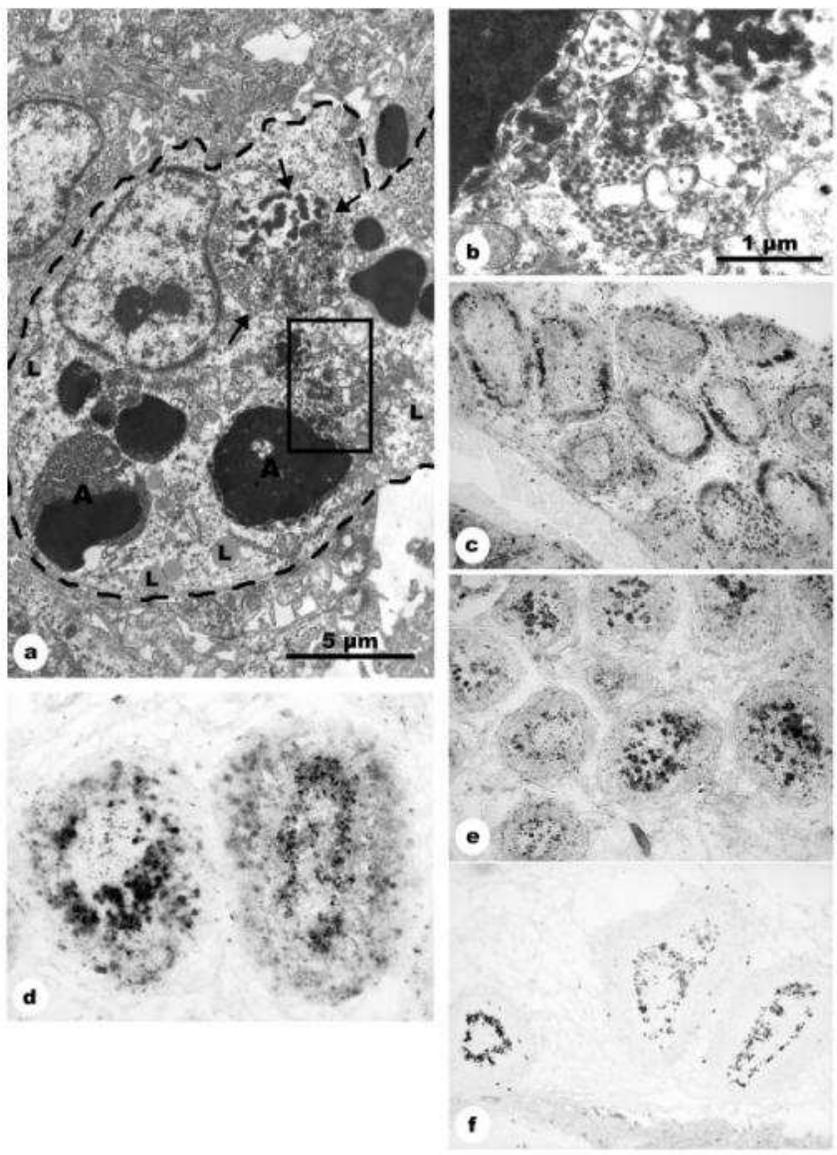


Figure 2. BSDCs and T-cells during IBDV infection
190x254mm (72 x 72 DPI)

Table 1. *Heterophile granulocytes (HGC) in blood and bursa lesion score (BLS). Three chickens were examined on each sampling day.*

Days after 5-FU treatment	Days after IBDV inoculation	HGC			BLS		
		Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
5	0	29,31,41	26,18,15	35,15,20	0,1,1	0,0,0	2,1,1
6	1	49,44,*	16,19,18	37,25,38	1,3,1	0,0,0	3,2,2
7	2	20,10,10	50,41,12	0,0,10	1,1,1	3,4,4	4,4,3
8	3	4,1,0	24,23,50	8,1,1	2,2,1	4,4,4	4,4,4
15	10	3,2,0	ND	40,6,14	0,0,0	ND	4,4,4

BLS: bursa lesion score, HGC: heterophile granulocyte count, ND: not done, * sample missing.

The BLS values for groups 1-3 indicate the percentage of bursa tissue involved in changes as follows: 0 = 0%, 1-25% = 1, 25-50% = 2, 51-75% = 3, 76-100% = 4. The HGC are indicated as percentage of blood heterophiles.

Table 2. *Histological development in the bursa of Fabricius 5 to 8 days after 5-FU treatment = 0 to 3 days after IBDV inoculation*

Cell type	Group 1 5-FU	Group 2 IBDV	Group 3 5-FU + IBDV
B-cells	Mild depletion	Increasing depletion starting from medulla and inner part of cortex on day 2 <i>p.i.</i>	Mild depletion in medulla from day 0 <i>p.i.</i> increasing in medulla and cortex
BSDCs	Not done	Present in mature form in medulla and along the cortio-medullary border 2 days <i>p.i.</i> Most of the cells had disappeared 3 days <i>p.i.</i>	Elevated number of immature forms, aggregated at the cortico-medullary border from 2 days <i>p.i.</i> Few mature BSDCs in medulla
Epithelial cells	Normal surface epithelium	Folded surface epithelium 3 days <i>p.i.</i>	Folded surface epithelium 3 days <i>p.i.</i>
Heterophile granulocytes	Mild depletion	Increased number, mostly in cortex, 2 days <i>p.i.</i>	Depletion from 2 days <i>p.i.</i>
Multinucleated giant cells	Not done	Virus containing cells in cortex 3 days <i>p.i.</i>	B-cell- and virus containing cells in medulla on day 2 <i>p.i.</i> and virus containing cells in cortex 3 days <i>p.i.</i>
T-cells	Not done	Increase and aggregation, mainly in cortex 2 days 2 <i>p.i.</i>	Increase and aggregation, mainly in cortex 2 days <i>p.i.</i>