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Kurtyak B.M., Maslyanko R.P., Levkivsky D.M., Pundyak T.O., Sobko G.V. ©
Lviv national university of veterinary medicine and biotechnology named of S. Z. Gzitskyj

MUCOSAL IMMUNITY OF THE RESPIRATORY TRACT IN CHICKENS

Abstract. *This review article presents fundamental mechanisms of the local mucosal immunity m selected regions of the respiratory tract in healthy birds and in some pathological conditions. The respiratory system whose mucosa come into direct contact with microorganisms contaminating inhaled an, has some associated structures, such as Harderian gland (HG), conjunctive-associated lymphoid tissue (CALT) and paranasal glands (PG), whose participation in local mechanisms of the mucosal immunity has been corroborated by numerous scientific studies.*

The nasal mucosa, with structured clusters of lymphoid tissue (NALT - nasal-associated lymphoid tissue) is the first to come into contact with microorganisms which contaminate inhaled air Lymphoid nodules made up of B cells with frequently developed germinal centres (GC), surrounded by a coat of $CD4^+$ cells, are the major NALT structures m chickens, whereas $CD8^+$ cells are situated in the epithelium and in the lamina propria of the nasal cavity mucosa.

Studies into respiratory system infections (e.g. Mycoplasma gallisepticum) have shown the reactivity of the tracheal mucosa to infection, despite a lack of essential lymphoid tissue.

Bronchus-associated lymphoid tissue (BALT) takes part in bronchial immune processes and its structure, topography and ability to perform defensive function in birds is largely age-dependent. Mature BALT is covered by a delicate layer of epithelial cells, called follicle-associated epithelium (FAE). Germinal centres (GC), surrounded by $CD4^+$ cells are developed in most mature BALT nodules, while $CD8^+$ lymphocytes are dispersed among lymphoid nodules and m the epithelium, and they are rarely present in GC.

Macrophages make up the first line of defence mechanisms through which the host rapidly responds to microorganisms and their products in the respiratory mucosal system. Another very important dement are polymorphonuclear cells, with heterophils being the most important of them. Phagocytic cells obtained from lung lavages in birds are referred to as FARM (free avian respiratory macrophage). Their number in chickens and turkeys is estimated to be 20 times lower than that m mice and rats, which indicates a deficit in the first-line of defence in the birds' respiratory system.

There are numerous B cells and antibody secreting cells (ASC) present throughout the respiratory system in birds. Their role comes down to perform antigen-specific protection by producing antibodies (IgM, IgG or IgA class) as a result of contact with pathogenic factors.

***Keywords**: chicken, turkey, respiratory system, local immunity*

Introduction. Upper airways are known to be the entrance for many pathogens, also they are preferred - more often as a more effective method - in immunoprophylaxis of infectious diseases in birds. This review article presents the complexity of immune mechanisms in the respiratory system in chickens and turkeys. Immune structures in the respiratory system, their topography, construction and basic functions are also considered.

The respiratory system in birds in general is unique and its anatomy shows a lot of differences in comparison with the respiratory system in mammals. The presence of air sacs, different bronchial tree structure and breathing system are just a few examples of its disparity in this animal phylum.

There are differences in the structure and function of the immune system in birds in comparison to mammals. Central organs of the lymphatic system in birds are the bursa of Fabricius (responsible for maturation and differentiation of B lymphocytes) and thymus (responsible for T lymphocytes maturation). Beside central organs there are numerous peripheral organs (also referred to as secondary) of the lymphatic system: Harderian gland (HG), conjunctiva - associated lymphoid tissue (CALT), head - associated lymphoid tissue (HALT), gut - associated lymphoid tissue (GALT), bronchus - associated lymphoid tissue (BALT), spleen, cecum tonsils and others [1]. These structures are frequently involved (directly or indirectly) in the immune response taking place in the respiratory mucosal system in birds.

The following work is split into two thematic parts: (i) description of the immune structures in the respiratory system; (ii) basic functional dependencies of immunocompetent cells and others, involved in the local immunity of the respiratory mucosal system in chickens and turkeys.

Intensive poultry breeding facilities are places where dust and microorganisms accumulate, which makes the birds' respiratory systems exposed to particular stress. The respiratory mucosal system is covered by epithelial cells, which make a tight barrier separating the rapidly changing environment from the highly stable internal environment of the body. Coexistence of these two environments is made possible by the mucosal immune system [2]. The following structures are associated with birds' respiratory system, whose mucosa is in direct contact with microorganisms: HG, CALT and paranasal gland (PG). Though these structures are not directly connected (in anatomic way) with the respiratory system, they are functionally important part of the local immunity, especially in the upper airways. The local immunity of the respiratory mucosal system is ensured by non-specific defensive reactions (e.g. by mechanical removal of impurities by movements of the respiratory epithelial cilia system) on one hand, and by precise mechanisms employed by immunocompetent cells (B and T lymphocytes) on the other.

Immune structures in the respiratory system in chickens and turkeys

The nasal cavity mucosa with clusters of nasal-associated lymphoid tissue (NALT) is the first to come into contact with microorganisms that enter the body with inhaled air. However the lymphocytes are present both in the nasal mucosa

(intraepithelially and under the respiratory epithelium), as well as in the paranasal gland and in their secretory ducts [3].

Major NALT structures in chickens are lymphoid nodules made up by B cells, frequently with developed Germinal Centres (GC), surrounded by a coat of $CD4^+$ cells. The surface of the nodules are covered by the unciliated epithelium. The majority of B cells are IgG^+ cells, whereas IgA^+ and IgM^+ are not so numerous. $CD8^+$ are deployed in the epithelium and in the lamina propria of the nasal cavity mucosa [3].

Given the fact the way that pathogens, responsible for respiratory system diseases (as well as diseases with general disorders) and functioning of the upper airways mucous membrane, enter macroorganisms (conjunctiva, nasal cavity), to describe immune mechanisms in this part of the body, besides NALT, we should consider HG, CALT and PG as structures involved in the local immunity of the upper airways.

A secretory duct leads out of the Harderian gland (also referred to as the deep gland of the third eyelid) to the conjunctival sac, from which its excretion returns the beak cavity through the nasolacrimal duct. The lymphoid tissue of the HG is organised into two histologically separate structures - the body of the gland and the lymphoid structures of the head of the gland. The body is filled with plasmatic cells at different stages of maturation. After they contact with an antigen, they migrate into the epithelium of the head central canal and produce immunoglobulins. Central canal's epithelium of the head of the gland can therefore be classified as lympho-epithelial tissue, where small and medium-sized lymphocytes are accompanied by dendritic-like cells and scarce macrophages. Moreover, there are high endothelial venules (HEV) in the gland, which indicate intensive T cell migration taking place there, as well as germinal centres (GC) of B cells [4]. B cells in HG are Bursa-dependent, and they migrate to the gland before hatching [5-7]. Some primed B cells differentiate to plasma cells whose proliferation is governed by factors derived from the HG stroma. Populations of these s produce large amounts of immunoglobulins and refore they are called antibody-secreting cells *[8, 9]*. Antibodies they produce, suspended in HG's excretion, are transported to the nasal cavity (through the naso-lacrima1 duct) where they cap the mucous membrane of proximal parts of upper airways leading to so called “immune exclusion”.

[10] have shown that local immunisation with a vaccine containing the toxoid of *Clo*stridium tetani results in production of high levels of specific IgA and IgY with very small amounts of IgM. The results show that HG can initiate the immune response, in which plasmatic cells quickly switch from IgM^+ isotype to IgA^+ and IgG^+, although no gene conversion or somatic mutation in HG germinal centres have been shown to take place so far.

The paranasal gland is a structure associated with e nasal cavity. The PG's excretion is exuded to the nasal cavity through secretory ducts. The lymphoid structures present in the paranasal gland in chickens id in their secretory ducts are localised similarly as in e nasal atrium epithelium [11, 3]. Numerous T cells (with

smaller number of B cells) have been shown to be present in the nasolacrimal duct, which suggests a certain relationship of NALT with HG [3].

Various immune structures have been shown to exist in the lymphoid tissue of the chickens' conjunctiva, from randomly clustered lymphocytes (in 1 week old chicks) to BALT-like structures with GC and plasmatic cells in birds over 4 weeks old [12, 13] have shown that absorption of iron oxide and carbon monoxide molecules by CALT structures increases with age, with a peak between weeks 3 and 5 of the chicks' lives. This may suggest that CALT (due to the presence of immunocompetent cells) can be considered as **l** structure neutralizing pathogen factors at their entrance gate (to the macroorganisms), and that the importance of CALT to the immunity of the respiratory mucosal system in birds is age-related.

Studies into respiratory system infections have shown the reactivity of the tracheal mucosa to infections, despite the lack of essential lymphoid tissue. [14] compared the reaction of the tracheal mucosa to Mycoplasma gallisepticum infection and found significant differences between SPF birds vaccinated against the pathogens and the unimmunised animals. Unvaccinated birds showed evident pathological lesions, both in the trachea and in air sacs, while these were much weaker in the immunised birds throughout the study period (until week 6). It has been - shown that the tracheal mucosa was mainly infiltrated by B cells in birds in both groups. Birds in the vaccinated groups reacted by producing secondary lymphoid nodules as B cell clusters covered by a coat of $CD4^+$ cells. The nodules first appeared on day 4, and they were fully formed on day 12 post infection. They were accompanied by a mild lymphocytic infiltration without heterophils [14, 15]. Unvaccinated birds reacted to infection with thickening of the tracheal mucosa, caused by infiltration of a large number of lymphocytes, histiocytes, plasmatic cells and a considerable number of heterophils in the lamina propria. They were accompanied by deciliation as well as degeneration of epithelial cells of the mucosa [15]. Tests conducted by the ELISPOT technicks have shown a much higher number of ASC cells, both IgA^+ and IgG^+, in the trachea of the vaccinated birds [15].

The bronchial immunity is mediated by lymphoid tissue called BALT (bronchus-associated lymphoid tissue). Mature BALT is covered by the follicle-associated epithelium (FAE) that harbours numerous lymphocytes. FAE is made up of ciliated and unciliated cells (no goblet cells) and some of them have irregular microvilli on their luminal surface, being in close contact with lymphocytes. FAE cells are believed to mediate antigen contacts with lymphocytes [16, 17] have shown the endocytosis taking place in epithelial cells in BALT with the use of live and ultraviolet-killed Bordetella avium and ferritin. Ferritin and B. avium were taken up by both ciliated and non-ciliated cells of the epithelium overlying BALT (BALT epithelium). Ferritin was found in organelles associated with endocytosis and was apparently transported across epithelial cells, since it was also found in intercellular spaces. Bacteria (B. avium) were found in vacuoles within BALT epithelial cells, but not free in intercellular spaces.

GC, surrounded by cells with the expression of CD4 molecules on their surface, developed in most mature BALT nodules. $CD8^+$ cells are dispersed among lymph

nodules and in the epithelium, and they rarely occur in GC. Plasmatic cells have been found directly under FAE [18].

BALT structures developed especially at the junctions of the primary bronchi with caudal secondary bronchi [6, 19], at the ostia of the air sacs [20]. They also frequently occur in the cranial part of the primary bronchi in turkeys [14]. There are no B cells in these places upon hatching. They appear two weeks later in chickens, and the numbers of IgG^+, IgA^+ and IgM^+ cells are then equal. The proportions soon change in favour of IgG^+ and IgM^+, which start to dominate over IgA^+. First, populations of T cells appear between week 1 and 2 after hatching [16, 18, 21]. Fully developed BALT is not observed until birds are 6 weeks old, and the number of lymph nodules in chickens and turkeys increases with age [16, 18, 21].

Lymphocytes of BALT nodules are known to posses melatonin receptors and melatonin in known to play an immunomodulatory role in vertebrates including humans [22]. Since there have been shown that administration of *melatonin results in improvement of both humoral and cell-mediated immune responses in growing chicks (e.g. 53* during Aflatoxin Bl-contaminated diets) the expression of melatonin receptor types [23] have been demonstrated in order to propose an immunomodulatory role of melatonin in lung associated immune system in the tropical bird (Perdicula asiatica - the Indian jungle bush quail) so far. Mel 1b receptor was found in the bronchial region of the lungs in lymphocytes in the BALT nodule as well as in free form. In contrast, immunolocalization for Mel 1a receptor was observed in various areas of the lung instead of in the bronchial region. Sufficient distribution of melatonin receptor types in lung tissue suggests that the neuroendocrine system (the pineal gland and melatonin) is certainly involved in maintenance of lung-associated immunity of wild birds [22]. Studies into melatonin receptors expression in the respiratory system in chickens and turkeys have not been conducted so far.

APC (antigen presenting cells) and macrophages account for the majority of immune cells present in the parenchymal tissue of the lungs in 5-day-old chickens (21, 22). The highest percentage of T cells show the $TCR\alpha\beta^+$ $CD8^+$ immunophenotype, whereas T $CD4^+$ and TCR1 γδ cells are rare [24].

Basic functional dependencies of cells involved in the local immunity of the respiratory mucosal system

Local immunity of the respiratory mucosal system is ensured by non-specific defensive reactions (e.g. by mechanical removal of impurities by movements of the respiratory epithelial cilia system) on one hand, and by precise mechanisms employed by immunocompetent cells (B and T lymphocytes) on the other.

Macrophages are the first line of defence in the respiratory mucosal system. Their primary functions include phagocytosis, killing cells with neoplastic changes, secretion of prostaglandins and cytokines and regulating the activity of lymphocytes and other macro-phages [25]. Moreover, macrophages play a vital role in presentation of antigens' to T cells within the context of MHC (major histocompatibility complex) I and MHC II proteins.

Another very important element of non-specific immunity in birds are polymorphonuclear cells (PMN represented mainly by heterophils. Their functions

include chemotaxis, adherence, phagocytosis and killing bacteria, with the activity level being age-dependent in many birds [26].

Lung lavages in chickens and turkeys contain a small number of phagocytic cells [13, 27-28] are compared to mice or rats, where the number is 2 times larger [29], which indicate a first-line defence deficiency in the birds' respirator' system. Phagocytic cells obtained in this manner are called FARM (free avian respiratory macrophages [27, 30].

Macrophages have been shown to be present on the epithelial lining in atria and in infundibula of tertiary bronchi [28-30]. They are also present in the connective tissue, which is situated under the epithelium near the atria [28], and in the interatrial barrier [31], which indicates that phagocytic cells are situated at a strategic site of gas exchange.

[32] have shown that heterophil account for the majority of non-epithelial cells in air sacs, with their number being greater than that of macrophages and lymphocytes.

Macrophages and heterophils are quickly attracted to the respiratory surface in inflammatory condition and test results show that they migrate from outsiding the respiratory system even before the clinical form of disease develops, which has been proven experimentally by making use of various factors stimulating; chemotaxis [27]. Introduced directly to the abdominal air sacs, incomplete Freund's adjuvant stimulated the inflow of a large number of activated FARM to the lungs and air sacs. The cell showed phagocytic and cytotoxic activity towards E. coli in in vitro experiments [13, 29]. The peak of the cells' activity was recorded 6-8 hours after inoculation [33]. The extent of FARM cells inflow was illustrated by Toth and Siegel (1988) based on the results of experiments with the use of live E. coli bacilli introduced to the trachea. The number of FARM cells was found to have increased 50-100 times after 24 hours post inoculation a: compared to the control group.

Neutral iron-containing aerosol particles of 0,18 μm in a diameter were used to illustrate the cellular mechanisms involved in the process of removal of molecules which were outside the reach of the ciliated epithelium (trachea, primary bronchi). They were shown to be phagocytosed by FARM in the gaseous exchange zone and that they are absorbed by epithelial cell; which line the atria and proximal parts of infundibula Iron particles were also detected in macrophages situated under the atrial epithelium [28, 34]. Their subsequent fate has not been fully elucidated. In theory, they can be presented as antigens locally processed in BALT or in structured lymphoid nodules. In the view of these facts we can assume that macrophages of FARM and epithelial cells (e.g. FAE, unciliated epithelial cells of the mucous membrane in the nasal cavity) are the cells responsible for antigen presentation to immunocompetent cells in the respiratory mucosal system.

B and T cells are equipped with receptors on their surface which allow them to recognize antigens. B cells have BCR (B-cell receptor) on their surface, made up of membrane immunoglobulins (mlg), which enables them to recognize both natural antigens and those processed by APC. TCR (T-cell receptor) of T lymphocytes can recognise antigens which have been pre-processed and subsequently presented on an

APC. The receptors (TCR) are made up of two chains - α and β or γ and δ. This difference in the TCR structure is the basis for categorising T cells into TCRlγδ, TCR2 and TCR3αβ groups. They differ by their distribution in the body and by expression of co-receptors (CD4, CD8), and, what follows, by their function [35-36]. Apart from presenting an antigen on their surface, APC also send TCR-in-dependent (co-stimulating) signals, which are necessary to prime T cells. One of mediators of these signals is CD28 - a T cell surface molecule, which is associated with cytokine production [35], T cells are additionally stimulated by cytokines secreted by APC, e.g. IL-1 (interleukin 1) induces them to express a CD25 molecule - an IL-2 receptor, and to secrete the interleukin [25]. Operation of the thus-activated T $CD4^+$ cells results in modulation of the immune response. The cytokines they produce (IL-2, INF-y and TNF-P) affect APC (e.g. boost their phagocytic activity), and they can stimulate B cells (by secreting IL-3, 4, 5, 6, 10 and 13). T $CD8^+$ cells are primed by their receptor (TCR) being stimulated by antigens presented in a complex with MHC I proteins. The presentation leads to proliferation with resulting differentiation of T $CD8^+$ cells, which show cytotoxic activity.

B cells, which for example produce IgA in the respiratory system, can be activated in two ways, and their activation path depends on the type of antigens which stimulate the immune system. T-independent antigens (as a rule, they are bacterial polysaccharides) can by themselves induce immunoglobulin (IgM) production by B cells. In that case, recurrent antigen epitopes can be the signals which induce humoral response. After being recognised by BCR, T-dependent antigens are degraded in a B cell and are further presented (in combination with MHC II) to T $CD4^+$ cells.

Activated T cells stimulate the processes which take place in B cells through surface molecules and secreted cytokines. Antigen-stimulated B cells in clusters of lymphoid tissue make up GC. Three major classes of antibodies are produced in humoral response in birds: IgM, IgG and IgA. IgG, in birds referred to as IgG because of a slightly different structure than that found in mammals (longer molecules), are functionally homologous with mammals IgG [36]. The antibodies are also present in the respiratory mucosal system.

There are numerous B cells and ASC present in the entire respiratory system of birds. Antigen-specific protection in the respiratory system is provided by Bursa-dependent lymphocytes which produce and secrete polymeric IgA and IgM [37-38]. These immunoglobulins are transported between epithelial cells by basolaterally expressed protein receptor, also known as poly-Ig receptor [32]. Secretory Ig couses a kind of "immune exclusion" by inhibiting the absorption of soluble antigens, by blocking adhesion sites and by blocking invading microorganisms inside the epithelium [39-40, 52].

Mucosal production of IgA is T-dependent (in vitro reduction of TCR αβ cells population decreases IgA , production) [41]. The protective role of-secretive (s) IgA has been shown based on several types of vaccinations and infections with respiratory pathogens. Introducing an anti-NDV vaccine to the conjunctival sac or to the nasal cavity stimulated the production of antigen-specific IgA, which were found in tears and tracheal washings [42-44, 50-51]. Comparable effects have been achieved in

birds infected with M. gallisepticum, in which antigen-specific IgA, IgM and IgG were produced, found in the upper and lower respiratory tract [45, 49]. It has been revealed in in vitro experiments that the antibodies provided protection against infection, but the extent of protection did not correlate with the IgA titre, indicating the role of IgG and IgM in the mucosal immunity [15, 39]. On the other hand, viruses glycoproteins (e.g. ILTV gG - glycoprotein G) can bind to chemokines, with high affinity and inhibit leukocyte chemotaxis. During this process, gG may have evolved to direct the host immune response away from a cell-mediated immune response and towards an antibody-mediated immune response to create conditions that are favourable for virus survival. This may be the mechanism behind observations showing that vaccination with ILTV gG deficiency is more effective at preventing disease following challenge with virulent virus [46-48], showing the relevance of cell-mediated mechanisms for protection of the respiratory mucosal system.

It can be claimed in conclusion that despite being highly complex, both in its anatomy and function, the drastic conditions of intensive rearing of birds make their mucosal immune system the most frequently used gateway for infiltration of pathogens which result in diseases and serious economic losses in poultry rearing.

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Рецензент – д.вет.н., професор Гуфрій Д.Ф.