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Three-Dimensional Analysis of the Nasolacrimal Duct and Nasal Cavity and Arrangement of Mucosal Tissue in Chickens

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The nasal mucosa plays an important role in the immune system, with nasal mucous cells secreting mucin that, along with pili, exclude foreign substances from intervening. Nasal mucosal-associated lymphoid tissue (NALT), present in the nasal lamina propria, acts as a local immune system. In birds, the Harderian gland in the orbit also plays an important role in the local immune system. In this study, we analyzed the pathway from the nasolacrimal duct to the nasal cavity in chickens and the distribution of the nasal mucous cells responsible for defense mechanisms against pathogens. To determine the three-dimensional structure of the pathway from the nasolacrimal duct to the nasal cavity, we made casts of the anatomy by injecting an acrylic resin into the area. We then prepared paraffin sections to determine the distribution of the NALT and mucous cells. The mucous gland was clearly seen in the mucosal epithelium of the nasal cavity, suggesting that the pathway along the nasal cavity develops a nonspecific immune system to deal with large foreign substances, such as bacteria, using mucins that are secreted from the mucous glands. Hence, there is not only a physical barrier but also an antibacterial activity. Unlike in other animals, morphologically, the nasolacrimal duct in chicken becomes the ventral nasal meatus and opens into the choanae in the caudal portion of the nasal cavity. NALT was prominently present in the lamina propria of the ventral nasal meatus, suggesting the presence of a specific immune system protecting against avian viruses. Thus, responses to vaccine stimulation could be developed from tissues along the pathway of the ventral nasal meatus via the nasolacrimal duct running from the punctum. These morphological studies suggest that the instillation of eye drops could be used as an efficient vaccination method for avoiding respiratory diseases.

Key words: chicken, nasal cavity, nasolacrimal duct, vaccination

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Introduction

The nasal mucosa is a major entry site for pathogenic organisms because it is constantly exposed to inhaled antigens (Heritage *et al*., 1997). The nasal mucosa has various defense systems, with mucus-covered epithelial cells representing a physical barrier acting in concert with the pili. The main component of the mucus is mucin produced by the mucous glands that consist of mucous cells (e.g., goblet cells). The mucin not only eliminates pathogens but also has a lubricating effect because of its viscosity (Kim *et al*., 1997; Linden *et al*., 2008). Mucosa-associated lymphoid tissue (MALT) exists under the mucosal epithelium. MALT in the nasal mucosa is called nasal-associated lymphoid tissue (NALT) (Brandtzaeg and Pabst, 2004). MALT also includes gut-associated and bronchus-associated lymphoid tissues. These tissues are called inductive tissues because of their importance in the induction of antigen-specific immune response. MALT has B- and T-cell areas, which are covered with M-cell-containing, follicle-associated epithelium through which exogenous antigens are actively transported (Brandtzaeg and Pabst, 2004).

In birds, two structures—the lacrimal gland and Harderian gland—are present in the intraorbital area. The Harderian gland, found on the inner canthus, is larger than the lacrimal

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gland (Survashe and Aitken, 1978). In chickens, this exocrine Harderian gland protects the eye surface and lubricates the eyelid and nictitating membrane in conjunction with the lacrimal gland (Olcese and Wesche, 1989; Mobini, 2012). Because many plasma cells have been observed in its stroma, the Harderian gland has been studied as a peripheral lymphoid organ that offers a local immune response against antigenic pathogens on the ocular surface (Bang and Bang, 1968; Shirama *et al*., 1996; Ohshima and Hiramatsu, 2002).

Recently, highly pathogenic influenza has spread among avian populations in Asian countries. The development of vaccines has been promoted to prevent an epidemic. Because inactivated vaccines must stimulate the NALT, an efficient uptake pathway has been sought. Nose drop or eye drop administration has been considered as an alternative to injection for vaccinating chickens against influenza. Because influenza is a respiratory disease, it is easy to understand that vaccination via the nasal cavity, where NALT is already present, would increase mucosal immunity. In addition, instillations that would stimulate the Harderian gland have been suggested, which can increase the immunity, but there have been no suggestions for stimulating NALT. Because tears enter the nasolacrimal duct, a vaccine initiated at that site might stimulate some of the nasal cavity, but the morphology of the nasal cavity, including the nasolacrimal duct, in birds has not been fully established. Therefore, this study aimed to investigate the anatomical-morphological pathway from the nasal cavity to the nasolacrimal duct, as well as the distribution of the nasal mucous cells responsible for defense against pathogens, using acrylic casts and paraffin sections.

Materials and Methods

The chickens were treated in accordance with the Guideline for Regulation of Animal Experimentation (1997) of the Faculty of Agriculture, Shinshu University. All experimental procedures were reviewed by the Committee for Animal Experiments and approved by the president of Shinshu University (Approval No. 240080).

Experiment 1: Creating Casts of the Nasolacrimal Duct and Nasal Cavity

This experiment was conducted on four 50-day-old carcasses of broiler chickens. To clarify the three-dimensional structure of the pathway from the nasolacrimal duct to the nasal cavity, we prepared a cast using an acrylic resin (Mercox II blue or red; Ladd Research Industries, Williston, VT, USA). After dissecting the upper eyelid, lower eyelid, and lower jaw from the head, nylon guts were placed from the left and right sides of the upper and lower puncta to the choanae and tissues were ligated at the nasolacrimal duct (Fig. 1). Cotton was packed into the lumen of the choanae. Blue acryl resin (5 g) and a catalyst (Ladd Research Industries; 0.1 g) were stirred well for 5−7 min, after which the mixture was injected with a syringe from the upper and lower punctum until overflow was obvious. After removing the gut and the cotton, the injected sample was allowed to settle for 30 min at room temperature to fully cure the resin. Next, the chicken's head was placed with the choanae upward with micropipette chips plugging the left and right nostrils. Red acryl resin (20 g) and the catalyst (0.4 g) were stirred well for 5−7 min. The mixture was injected with a syringe via the micropipette chips plugging the nostrils until overflow from the choanae was confirmed. The sample was then left for 60 min at room temperature to ensure that the resin was fully cured. After curing, the sample was immersed in 20% sodium hydroxide to dissolve the remaining tissue. The area where the blue and red resin parts joined at the choanae was partially modified with acrylic paint.

Experiment 2: Distribution of NALT, Goblet Cells, and Mucous Glands

Two 63-day-old White Leghorn chickens fed a commercial diet and water ad libitum and maintained under con-

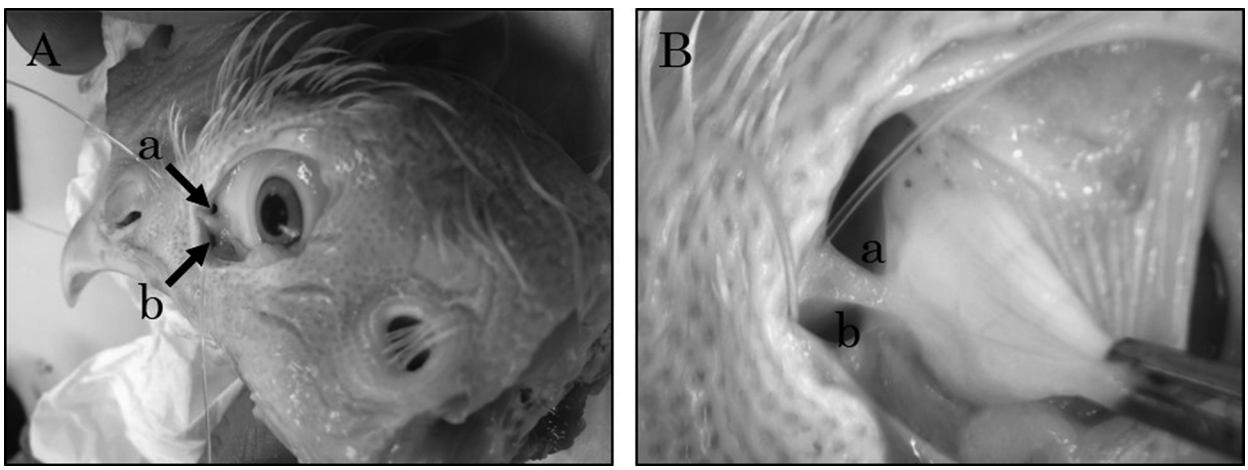

Fig. 1. **Cast preparation.** A: Upper and lower punctum through the nylon gut. B: Higher magnification of A. a: upper punctum; b: lower punctum.

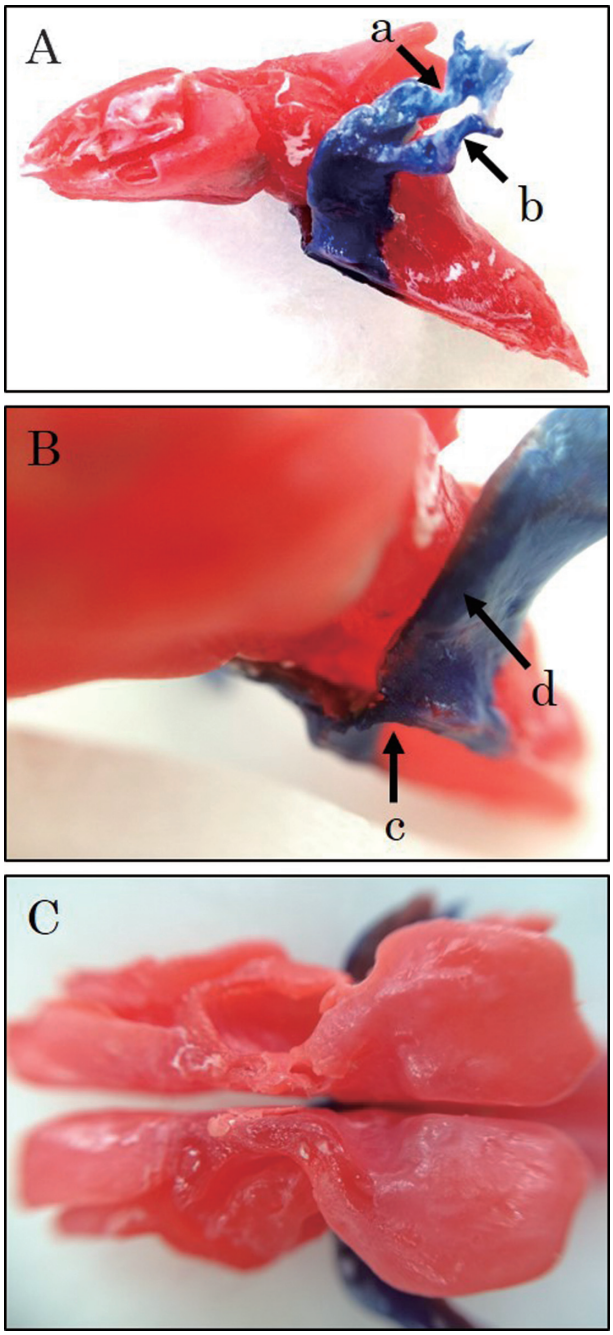

Fig. 2. **Cast sample.** A: Left lateral view of the cast. a: upper lacrimal canaliculus; b: lower lacrimal canaliculus. B: Higher magnification of the left ventrolateral view of the cast. Nasolacrimal duct opens into the choanae (c) as the ventral nasal meatus (d). C: Dorsal view of the cast shows that the closed naris is isolated by the nasal septum.

trolled light conditions (12 h/12 h light/dark cycle) were used for this experiment. Tissue samples obtained from the nostrils to the front of the orbit were immersed in 4% paraformaldehyde fixative for 48 h. The samples were then decalcified using a decalcifying solution (OSTEOSOFT; EMD Millipore Corp., Billerica, MA, USA) for 60 days, after which they were embedded in paraffin wax according to standard procedures. Frontal cross sections were cut at a thickness of 5 μm. To clarify the distribution of NALT, goblet cells, and mucous glands, cross-sections of the nasal cavity were obtained and divided into five (I−V) areas according to a previous report (Kang *et al*., 2013). The sections underwent hematoxylin-eosin and periodic acid–Schiff staining and were evaluated under an optical microscope.

Results

Cast of the Nasolacrimal Duct and Nasal Cavity

Using casts of the nasolacrimal duct and nasal cavity of the chickens, we confirmed the presence of the upper and lower punctum and the upper and lower canaliculus. The lacrimal duct was connected to the ventral nasal meatus via the punctum, canaliculus, and nasolacrimal duct. The chicken's nostril showed a closed naris that was completely separated from the subsequent nasal cavity by the nasal septum (Fig. 2).

Histological Sections of the Nasal Cavity

The nasal cavity was cross-sectioned into five areas (I−V) as follows: I: rostral nasal concha only apparent; II: middle nasal concha apparent; III: rostral nasal concha disappeared and ventral nasal meatus and infraorbital sinus apparent; IV: choanae apparent, and V: caudal nasal concha apparent.

- Area I—The rostral nasal concha extended from the dorsal nasal cavity and caused it to begin to curve. The epithelium covering the nasal cavity was composed of cornified, stratified, squamous epithelium (Figs. 3−I, 4A).
- Area II—The rostral nasal concha extended laterally in the ventral area of the nasal cavity. The middle nasal concha began extending dorsally. The epithelium covering the nasal cavity in the dorsal area and primarily ventral area was composed of pseudostratified, ciliated, columnar epithelium. The respiratory epithelium consisted of simple or compound tubuloalveolar mucosal gland. The epithelium in the rest of the ventral area changed from cornified, stratified, squamous epithelium to respiratory epithelium (Fig. 3-II, 4B,C).
- Area III—The middle nasal concha extended dorsally and had a curled, shell-like structure. The ventral nasal meatus was in the ventral area of the nasal cavity, and the infraorbital sinus was in the ventral area of the ventral nasal meatus. The epithelium covering the nasal cavity was respiratory epithelium, but that of the ventral nasal meatus was cornified, stratified, squamous epithelium (Fig. 3-III).
- Area IV—The middle nasal concha extended laterally and had a curled, shell-like structure. Similar to that in Area III, the ventral nasal meatus was in the ventral portion of the nasal cavity, and the infraorbital sinus was in the ventral position of the ventral nasal meatus. In addition, choanae were seen at the opening of the ventral nasal meatus. Most of the epithelium covering the nasal cavity was the respiratory type, whereas that in the

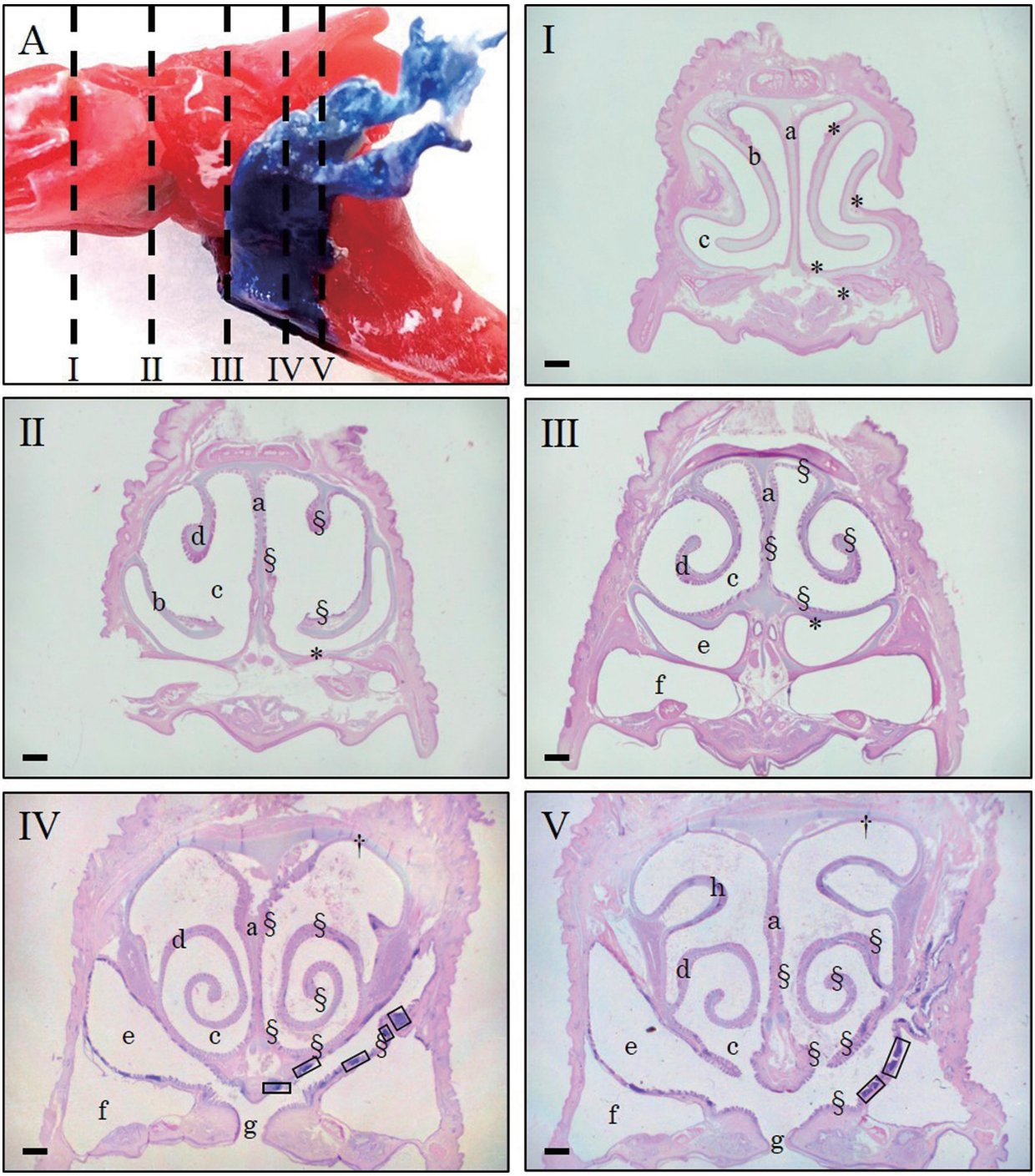

Fig. 3. **Histological sections.** A: Slice positions of the histological section, creating areas I–V. The figures show each section of white leghorn chicken corresponding to the parts of the broiler cast specimen. I: Plane of the dorsal nasal concha. II: Middle nasal concha appears. III: Dorsal nasal concha disappears, and ventral nasal meatus and infraorbital sinus appear. IV: Choanae appear. V: Caudal nasal concha appears. a, nasal septum; b, dorsal nasal concha; c, nasal cavity; d, middle nasal concha; e, ventral nasal meatus; f, infraorbital sinus; g, choanae; h, caudal nasal concha. * Cornified, stratified, squamous epithelium; § respiratory epithelium; † olfactory epithelium; open boxes, nasal-associated lymphoid tissue (NALT). (HE stain; bars＝1 mm)

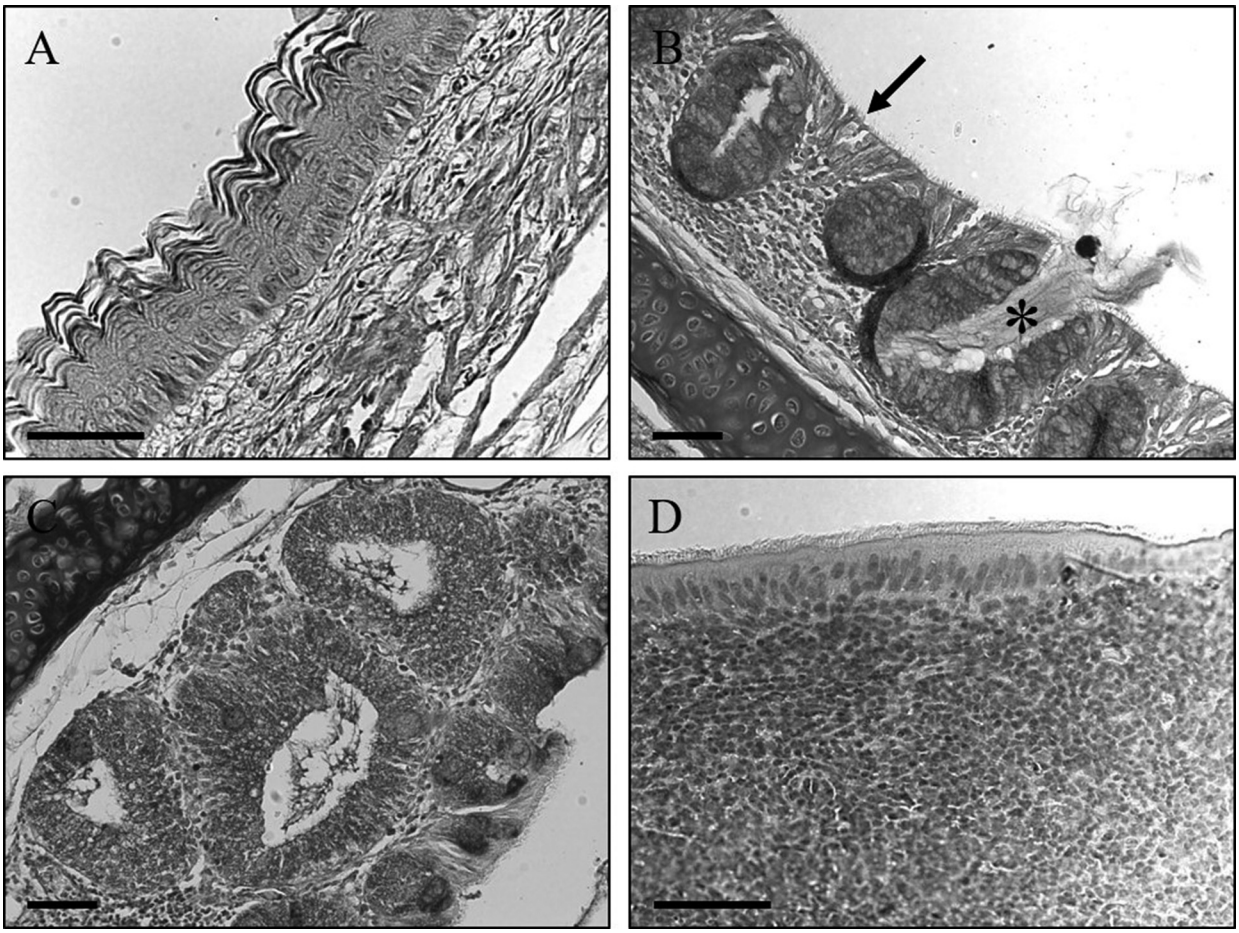

Fig. 4. **Magnified histological sections.** Magnified images of the mucosa at the nasal cavity and the ventral nasal meatus of White Leghorn chicken. A: Cornified, stratified, squamous epithelium is observed in the anterior nasal cavity. B: Respiratory epithelium includes ciliated, pseudostratified epithelium (arrow) and mucous gland (asterisk) consisting of mucous cells. C: Developed compound tubuloalveolar gland from the dorsal part of the nasal cavity to the nasal septum in Fig. 3-II, inside of the middle nasal concha to the dorsal part of the nasal cavity in Fig. 3-III, and ventral part of the nasal cavity in Fig. 3-IV, V. D: NALT, with an accumulation of lymphocytes in the lamina propria of the ventral nasal meatus in Fig. 3-IV, V. (A, D: HE stain. B, C: PAS reaction. Bars＝50 μm)

dorsal area was olfactory epithelium (Fig. 3-IV). The epithelium of the ventral nasal meatus was respiratory epithelium, and NALT was seen in the lamina propria (Fig. 4D).

・ Area V—The protruding caudal nasal concha extended dorsolaterally, whereas the middle nasal concha extended ventrolaterally and had a curled, shell-like structure. Similar to that in Areas III and IV, the ventral nasal meatus was in the ventral nasal cavity and the infraorbital sinus was in the ventral region of the ventral nasal meatus. Similar to Area IV, the choanae were seen at the opening of the ventral nasal meatus. In addition, the nasal cavity and ventral nasal meatus were connected. The epithelium covering the nasal cavity and ventral nasal meatus was similar to that seen in Area IV.

Discussion

Relations of the Structures of the Nasolacrimal Duct and Nasal Cavity

The results derived from the cast showed that the nostril of the chicken was fully separated from the closed naris. In contrast, it has been reported that the duck naris is not separated but is penetrated (Kang *et al*., 2014). Furthermore, it was confirmed that the upper and lower punctum in chickens was on the inner canthus and followed the upper and lower canaliculus. It was also shown that the nasolacrimal duct becomes the ventral nasal meatus, which opens

into the choanae. In humans, the lacrimal duct proceeds toward the nasal cavity through the punctum, upper and lower canaliculus, lacrimal sac, and nasolacrimal duct (Karagulle *et al*., 2002). In dogs and cats, the lacrimal duct consists of the upper and lower punctum, upper and lower canaliculus, lacrimal sac, and nasolacrimal duct on its way to the nostril (Noller *et al*., 2006, Rached *et al*., 2011). In rabbits, the nasolacrimal duct exists independently from the beginning to the ventral medial part of the nasal turbinate adjacent to the incisors (Pereira *et al*., 2011). In snakes, the lacrimal duct runs to the oral cavity adjacent to the opening of the vomeronasal organ (Souza *et al*., 2015). In 10 species of amphibians, the nasolacrimal duct often opens at a site close to the nostril (Nowack and Wohrmann-Repenning, 2010). Compared with these animals, the nasolacrimal duct of the chicken is thought to be short and open into the nostril as the ventral nasal meatus is at a position close to the orbital sinus. Because the ventral nasal meatus opens into the choanae, it is thought that tear fluid flows into the choanae below the nasal cavity, proceeding through the pharynx to the esophagus.

Distribution of the Nasal Mucous Cells

The epithelium covering the forepart of the nasal cavity, called the vestibular region, was composed of cornified, stratified, squamous epithelium. Because the cornified, stratified, squamous epithelium adapts to friction and drying, it is thought that a physical defense mechanism is present in Area I.

The epithelium in Areas II−III was the transition region from cornified, stratified, squamous epithelium to respiratory epithelium. The respiratory epithelium was distributed among the mucous glands consisting of mucous cells. In many animals, mucins are the main component of the mucus and exclude pathogens with their antibacterial action, in addition to providing lubrication due to their viscosity (Kim *et al*., 1997; Linden *et al*., 2008). Mucins also eliminate inhaled foreign substances and bacteria in concert with the ciliated epithelium via mucociliary transport. Thus, not only a physical defense mechanism against friction and drying from the cornified stratified squamous epithelium but also the mucociliary movement are considered the main parts of the immune system after Area II, suggesting that the pathway of the nasal cavity following the nostril had developed a nonspecific immune system (i.e., mucins secreted by mucous cells) to fight off large foreign substances, such as bacteria.

The epithelium of the ventral nasal meatus in Area III was cornified, stratified, squamous epithelium, whereas respiratory epithelium was observed in Area IV. The accumulation of lymphocytes formed the NALT, which was seen extensively in the mucosa covering the ventral nasal meatus. The NALT of birds have been reported to be in the lamina propria around the rift of the choanae and ventral nasal meatus, which is consistent with the results of the present study (Kang *et al*., 2013). Most lymphocytes in the NALT are $CD8^+$ and $CD4^+$ cells that have accumulated around a germinal center. B-cells and immunoglobulin are also observed, but there are fewer B-cells than T-cell subsets (Ohshima and Hiramatsu, 2000).

The avian orbit includes two glandular tissues. The Harderian gland in the inner orbit produces tears as its primary function, whereas peripheral lymphoid organs act as an immunological barrier in the peribulbar region and harbor many immunoglobulin G-containing plasma cells in the interstitial stroma (Shirama *et al*., 1996; Ohshima and Hiramatsu, 2002). Chicken NALT and Harderian gland have been studied extensively as mucosal immune systems that exist in areas close to the outside world. However, to our knowledge, there are no reports on the positional relationship and coordination of these two mucosal lymphoid tissues. Kang *et al*. (2013) have elucidated in detail the pathway of invading antigens from the nostril to NALT using a two-dimensional analysis. However, the pathway from the nasolacrimal duct is not discussed. On the other hand, it has been recognized that the Harderian gland is responsible for immune function for antigen invasion from the eye. However, the invasion pathway of the antigen from the eye to the NALT has not been discussed. We have demonstrated in three dimensions that the two immune systems are located close together via the nasolacrimal duct by using a cast specimen. Moreover, this result suggests that tears may carry antigens to the NALT.

Hikono *et al*. (2013) reported that intra-ocular vaccination with an inactivated avian influenza virus in chicken induced not only systemic but also mucosal antibody responses and conferred protection against highly pathogenic avian influenza. Furthermore, Ducatez *et al*. (2016) pointed out that intra-muscular administration of a recombinant influenza vaccine induces antibodies, but intra-muscular vaccination is not the preferred administration route in the field when dealing with thousands of birds. They also suggested aerosol and eye drop vaccine administrations to achieve better local immunity and induce detectable antibody titers in the serum and tears.

We, therefore, concluded that, in chickens, because the pathway from the area surrounding the eye to the ventral nasal meatus through the punctum and nasolacrimal duct contains B-cells from the Harderian gland and T-cells from NALT, there is a specific immune response system against foreign substances. Thus, it is strongly suggested by the results of the casts that the eye drop vaccination method is effective protection against respiratory diseases in chickens.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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