



Distribution of Leucocyte Subsets in the Canine Pharyngeal Tonsil

F. Billen, D. Peeters, S. Dehard, M. J. Day* and C. Clercx

Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20 B44, 4000 Liège, Belgium, and * Division of Veterinary Pathology, Infection and Immunity, School of Clinical Veterinary Science, University of Bristol, Langford BS40 5DU, UK

Summary

This report describes the distribution and nature of lymphoid tissue in the nasopharyngeal mucosa of six puppies (mean age \pm SD, 0.3 ± 0.25 years) and eight adult dogs (mean age \pm SD, 8.8 ± 2.67 years) without respiratory disease. A non-encapsulated area of organized mucosa-associated lymphoid tissue was observed in the caudal part of the posterior wall of the nasopharynx, distal to the openings of the auditory tubes. This structure was consistent with the pharyngeal tonsil and was microscopically more extensive in puppies than in adult dogs. Histochemistry and immunohistochemistry were used to characterize and enumerate the leucocyte subsets in this part of the nasopharynx. Mast cells were found immediately beneath the respiratory epithelium but were also scattered in the glandular and muscular tissue. IgA⁺ plasma cells outnumbered IgG⁺ and IgM⁺ plasma cells, especially in the glandular tissue. All classes of plasma cells were present in significantly greater numbers in adults than in puppies. MHC class II⁺ cells were mainly observed in areas containing diffuse and follicular aggregates of lymphoid cells. Both MHC class II⁺ cells and CD1c⁺ cells with a dendritic morphology were predominantly found immediately beneath or within the epithelium, and cells expressing these markers were more abundant in puppies than in adult dogs. The anti-L1 marker labelled low numbers of cells with a neutrophilic morphology, which were significantly more abundant in puppies than in adult dogs. The majority of lymphoid cells were CD3⁺ T lymphocytes and these were particularly abundant in areas containing aggregates of lymphoid cells; CD4⁺, CD8⁺ and TCR $\alpha\beta$ ⁺ cells had the same distribution as the CD3⁺ cells. CD4⁺ cells were more numerous than CD8⁺ cells. The quantitative and qualitative data obtained will enable comparisons to be made with similar studies in dogs suffering from nasopharyngeal diseases, or when the local immune system needs to be investigated.

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Introduction

The mucous membranes are continuously exposed to various antigens, to which the body must either mount an immune response or maintain immunological tolerance (Hiller *et al.*, 1998; Debertin *et al.*, 2003). As they form a weak mechanical barrier (Kuper *et al.*, 1992), mucosal surfaces are provided with a local immune system (mucosa-associated lymphoid tissue [MALT]) (Davis, 2001; Debertin *et al.*, 2003). The MALT consists of organized, non-encapsulated lymphoid tissue with follicular structures and is found in the gastrointestinal,

respiratory and urogenital tracts and the conjunctival surface of the eye (Moneret-Vautrin *et al.*, 1992; Hellings *et al.*, 2000). One of the main functions of the MALT is to generate and disseminate antigen-sensitized B cells, which differentiate into IgA-producing plasma cells in the lamina propria of the various mucosal sites and the associated secretory tissues (e.g., salivary or lacrimal glands) (Wu *et al.*, 1997; Hellings *et al.*, 2000; Davis, 2001; Zuercher *et al.*, 2002).

Within the respiratory tract, the MALT is represented by the nasal-associated lymphoid tissue (NALT), Waldeyer's ring of the pharyngeal mucosa, and the bronchus-associated lymphoid tissue (BALT). Waldeyer's ring is the term used to describe the MALT

F. Billen and D. Peeters contributed equally to this work.

surrounding the openings of the digestive and respiratory tracts (Hellings *et al.*, 2000). Peeters *et al.* (2005) reported the absence of typical organized NALT and BALT structures in the nasal and bronchial mucosa of dogs without respiratory disease. Waldeyer's ring in the dog has been described as including the lingual tonsil (on the base of the tongue), the palatine tonsils, the soft palate tonsil (on the ventral surface of the soft palate) and the pharyngeal tonsil or adenoid (on the roof of the nasopharynx) (Baron, 1984; Slatter, 2003). The palatine tonsils are the only well-developed component of Waldeyer's ring in the dog, and their histological structure has been studied in detail (Baron, 1984; Belz and Heath, 1995; Dellman and Eurell, 1998; Slatter, 2003). In contrast, the pharyngeal tonsil is described simply as an aggregation of lymphoid tissue localized to the dorsal wall of the nasopharynx; it is almost indistinguishable macroscopically from the surrounding tissue (Baron, 1984; Dellman and Eurell, 1998). To the authors' knowledge, there have been no detailed histological or immunohistochemical studies of the canine pharyngeal tonsil.

The frequency of occurrence of the various respiratory MALT structures varies with age. In man, BALT is nearly always absent in healthy adults but is present in 40% of persons aged ≤ 20 years (Hiller *et al.*, 1998). Similarly, Waldeyer's ring is well-developed during childhood but starts to disappear during adolescence (Davis, 2001). In cattle and horses, BALT is absent in neonatal lungs, increases progressively with age and then declines in adulthood (Anderson *et al.*, 1986; Blunden and Gower, 1999).

The aims of the present study were (1) to investigate the distribution of lymphoid tissue in the nasopharyngeal mucosa of dogs without upper respiratory tract disease, and (2) to characterize immunohistochemically the leucocyte subsets in this part of Waldeyer's ring in the dog. To investigate a possible age effect on the amount of lymphoid tissue and distribution of cell types, both puppies and adult animals were included in the study.

Materials and Methods

Animals

Tissues were collected from 12 male or female dogs of various ages and breeds, submitted for routine post-mortem examination, and from two Beagle puppies previously used in an experimental behavioural study (Table 1). The animals were grouped on the basis of age, group 1 consisting of six puppies aged 0.8 to 0.6 years (mean \pm SD, 0.3 ± 0.25 years) and group 2 consisting of eight adult dogs aged 5.5 to 13 years (mean \pm SD, 8.8 ± 2.67 years). The dogs had no history or clinical

signs of respiratory or immune-mediated disease, and had received no corticosteroids within the month preceding euthanasia.

Sampling and Processing of Material

The dogs were sedated with acepromazine (ACP[®] injection, 0.5 mg/kg; Eurovet, Heusden-Zolder, Belgium) and killed with an intravenous overdose of pentobarbitone sodium (Natriumpentobarbital[®]; Kela nv, Hoogstraten, Belgium). Within 20 min of euthanasia, the lower jaw was removed from the head and the soft palate was opened longitudinally. The macroscopical appearance of the nasopharyngeal mucosal surface was recorded and nine duplicated blocks of mucosal tissue (maximum dimension 2–3 mm) were systematically sampled as illustrated in Fig. 1: areas A, B, C and D were situated on the dorsal wall of the nasopharynx, areas E, F and G on the ventral wall, and H and I on the lateral wall. From each area, one sample was taken for fixation in 10% neutral buffered formalin and one sample for snap-freezing in OCT medium (Sakura, Zoederwouten, The Netherlands) in isopentane cooled over liquid nitrogen. Formalin-fixed samples were processed by routine methods and embedded in paraffin wax before sectioning at 4 μ m. Frozen sections were cut at 8 μ m, air dried for 2 h and then fixed in cold acetone for 10 min.

Histological, Histochemical and Immunohistochemical Procedures

Formalin-fixed sections were stained with haematoxylin and eosin (HE) or with toluidine blue, by standard procedures (Bancroft and Cook, 1984). Frozen sections were stained with HE.

For immunohistochemistry (IHC), both formalin-fixed and frozen sections were used. Details of the antibodies are given in Table 2. Formalin-fixed sections were passed through graded alcohols to phosphate-buffered saline (PBS; pH 7.4, 0.01 M); frozen sections were rehydrated in PBS for 10 min. Endogenous peroxidase activity was blocked by incubation for 30 min with hydrogen peroxide 0.5% (v/v) in 50% (v/v) methanol or with hydrogen peroxide 0.33% (v/v) in 0.1% (w/v) sodium azide. Formalin-fixed sections were then either microwaved in 10 mM citrate buffer (pH 6.0) for 4 min (maximum power with a 750 W microwave), or incubated with 0.1% (w/v) calcium-trypsin solution (pH 7.8) for 30 min, to enhance antigen retrieval. Both formalin-fixed and frozen sections were then incubated for 30 min with 5% (v/v) rabbit or goat serum (depending on secondary antibody) to reduce non-specific background binding.

The sections were then incubated sequentially with primary and secondary antibodies at room

Table 1
Details of animals

<i>Group</i>	<i>Breed</i>	<i>Age (years)</i>	<i>Sex</i>	<i>Reason for euthanasia</i>
Group 1 (puppies)	Beagle	0.08	M	End of behavioural study
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	Shiba Inu	0.58	F	Uncontrollable aggression
	Beagle	0.25	M	Megaesophagus
	Chihuahua	0.66	M	Vertebral fracture
	Beagle	0.25	F	Cerebral disease
Group 2 (adult dogs)	Labrador retriever	5.5	F	Nephrotic syndrome
	Labrador retriever	7	FN	Cerebral disease
	German shepherd	7	FN	Acute renal failure
	Tervueren sheepdog	7	F	Gastric adenocarcinoma
	Labrador retriever	8.5	MN	Intervertebral disk protrusion
	Beagle	10	M	Hypoadrenocorticism
	Bearded collie	12	FN	Intestinal adenocarcinoma
	Labrador retriever	13	MN	Fibrocartilaginous embolism

F, female; FN, female neutered; M, male; MN, male neutered.

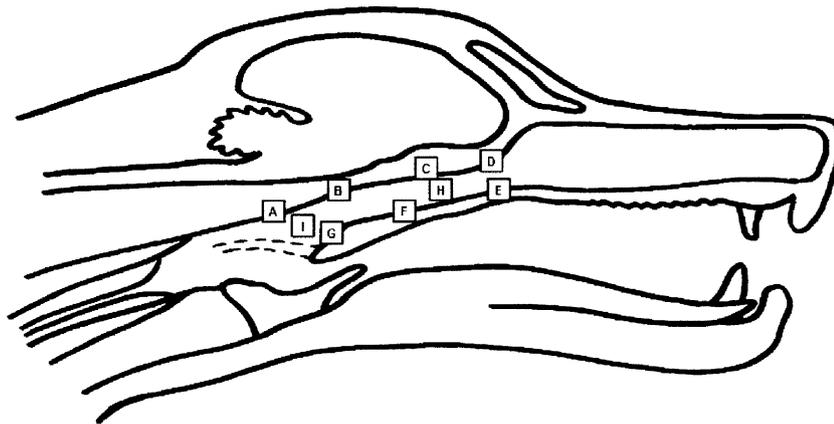


Fig. 1. The nine nasopharyngeal areas sampled. (A) Dorsal wall (caudal); (B) dorsal wall (middle-caudal); (C) dorsal wall (middle-rostral); (D) dorsal wall (rostral); (E) ventral wall (rostral); (F) ventral wall (middle); (G) ventral wall (caudal) (i.e., dorsal surface of the edge of the soft palate); (H) lateral wall (rostral); (I) lateral wall (caudal).

temperature for 30 min. Sections were washed twice in PBS between each incubation stage. All dilutions were made in PBS. For control purposes, primary antibody was replaced by rabbit serum, goat serum or mouse serum at appropriate dilutions. Binding was "visualized" by the addition of 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide (DAB+ Liquid; Dako, Glostrup, Denmark) and sections were counterstained with Mayer's haematoxylin.

Examination of Sections

HE-stained sections were first examined by a veterinary histopathologist (MJD) to assess the quality of the sample and the absence of microscopical abnormal-

ities. All HE-stained sections from fixed tissues were reviewed and compared for the distribution and abundance (+++, high numbers; ++, moderate numbers; +, low numbers; \pm , rare; -, none) of lymphoid cells within the lamina propria, the observer having no knowledge of their origin.

Immunohistochemically labelled and toluidine blue-stained sections were examined with a Nikon Eclipse E800 microscope and Sony CCD-IRIS/RGB colour-video camera. Subjective analysis of the distribution of positive cells was initially made with $\times 10$ and $\times 20$ objectives. Images were then transferred to a television monitor and cell counts were made manually. For each section, the five high-power fields (HPFs) ($\times 63$) with the greatest number of cells were chosen subjectively,

Table 2
Details of immunohistochemical markers

Specificity	Primary antibody		Secondary antibody		Type of sample
	Description	Dilution (1 in)	Description	Dilution (in 1)	
IgA	Goat anti-dog IgA (Fc) *	400	Rabbit anti-goat IgG (HRP) [†]	200	Fixed
IgG	Goat anti-dog IgG (Fc) *	200	Rabbit anti-goat IgG (HRP) [†]	200	Fixed
IgM	Goat anti-dog IgM (Fc) *	400	Rabbit anti-goat IgG (HRP) [†]	200	Fixed
Myelomonocytic antigen	Mouse anti-human LI [‡]	600	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Fixed
MHC Class II	Mouse anti-HLA-DR (α -chain) [‡]	200	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Fixed
Tcells	Rabbit anti-human CD3 [‡]	200	Polymers of goat anti-rabbit Ig and peroxidase [‡]	—	Fixed
CD4	Mouse anti-dog CD4 [§]	400	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Frozen
CD8	Mouse anti-dog CD8 [§]	100	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Frozen
TCR $\alpha\beta$	Mouse anti-dog TCR $\alpha\beta$ [¶]	20	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Frozen
TCR $\gamma\delta$	Mouse anti-dog TCR $\gamma\delta$ [¶]	10	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Frozen
Dendritic cells	Mouse anti-dog CD1c [¶]	100	Polymers of goat anti-mouse Ig and peroxidase [‡]	—	Frozen

HRP, horseradish peroxidase-conjugated; TCR, T-cell receptor; "Fixed", formalin-fixed tissue; "Frozen", frozen tissue.

* Nordic Immunological Laboratories, Tilburg, The Netherlands; [†] Sigma-Aldrich, St-Louis, MO, USA; [‡] Dako, Glostrup, Denmark; [¶] P. Moore, University of Davis, CA, USA; [§] Custom Monoclonals, Sacramento, CA, USA.

and positively labelled cells were counted. The area of one HPF was approximately 13 000 μm^2 . Positive cells within blood vessels and epithelium were not counted, indeed, the epithelium was often damaged or even absent. When the number of positive cells was so high that it prevented reliable counting, the abundance and distribution of cells were recorded. Because many immunohistochemically labelled sections from frozen samples showed some degree of tissue disruption, no manual cell count could be performed on them.

Statistics

The Statistic Analysis System (SAS) was used (Littell *et al.*, 1998). First, the effect of age on the number of positive cells was screened for each cell type by means of analysis of variance (ANOVA), with generalized linear model (glm) procedures (PROC GLM). A two-way ANOVA, with dog nested within age class, was used.

Subsequently, multifactorial glm procedures were performed. In the pharyngeal tonsil, the numbers of IgA⁺, IgG⁺ and IgM⁺ cells were compared. For these analyses, least-squares means (LS MEANS) contrasts were used. The LS MEAN of an effect corresponds to a "worthy of estimation" mean corrected for all other effects in the model. The effects corrected in the present model were the age and the individual variability be-

tween the dogs within the age categories. The associated standard error of the mean (SEM) was the error associated with the estimation of the LS MEAN. For all analyses, $P < 0.05$ was considered significant.

Results

Macroscopical Appearance of the Nasopharynx

The nasopharyngeal mucosa appeared uniform in all dogs. However, careful inspection of the surface of the caudo-dorsal wall behind the openings of the auditory tubes (areas A and B) occasionally revealed an oval-shaped pale-pink to red area. This generally appeared as a flattened plaque or was sometimes elevated with an irregular, micronodular surface.

Histology of the Nasopharynx

Mild neutrophilic infiltration of the mucosa was found in two sections from one dog. These samples were excluded from the study.

The nasopharyngeal mucosa was generally covered by a pseudostratified, ciliated columnar epithelium containing many goblet cells. However, squamous epithelium was frequently observed in the caudo-ventral and caudo-lateral areas (i.e., present in 80% of the dogs in area G and 50% of the dogs in area I).

Transitional epithelium was occasionally detected in the caudo-ventral area (i.e., area G) (Fig. 2). The epithelium was often damaged or even absent in areas A and B, where large numbers of lymphoid cells were present in the underlying lamina propria. No fold or crypt was observed in any section.

The lamina propria consisted of loosely arranged fibrous connective tissue and lymphoid cells in varying numbers. As no smooth muscle fibres (i.e., muscularis mucosae) were found, distinction between lamina propria and submucosa was not clear. Glandular tissue, composed of mixed, predominantly muco-serous tubulo-alveolar glands (occasionally organized into large lobules), was more abundant in the lamina propria and submucosa of areas B, F, G and I than in other areas. In samples in which the lamina propria and submucosa could be distinguished, glandular tissue was mainly observed in the submucosa. Small blood vessels, capillaries and lymphatics were distributed irregularly. They were found in greatest numbers in areas containing large quantities of lymphoid tissue. Striated muscu-

lar fibres orientated in various directions were occasionally observed in areas F, G and I.

Lymphoid tissue was found in the lamina propria immediately beneath the epithelium. Its distribution was similar in all dogs, but the number and organization of lymphoid cells varied between areas (Table 3) and age groups. Overall, lymphoid tissue was particularly abundant in puppies and was found mainly in areas A and B, especially the former. Minimal lymphoid tissue was found in the remaining areas. It was therefore concluded that areas A and B constituted the nasopharyngeal tonsil in the dog.

Lymphoid tissue was present as either follicular aggregates, diffuse aggregates of lymphoid cells, scattered individual lymphoid cells, or intraepithelial lymphocytes.

Follicular aggregates of lymphoid cells. Single or multiple lymphoid follicles were observed exclusively in the lamina propria of areas A and B immediately beneath the epithelium. These were more commonly found in puppies (present in 66% of sections) than in adult dogs (present in 25% of sections). Secondary follicles exhibited a pale central zone (germinal centre) containing a mixture of small and large lymphocytes with a surrounding mantle zone of small lymphocytes and plasma cells (Fig. 3). The epithelium overlying these follicles was occasionally thin, non-ciliated, devoid of goblet cells and infiltrated by small lymphocytes, consistent with typical lympho-epithelium (Fig. 4); in general, however, it retained its columnar and ciliated nature.

Diffuse aggregates of lymphoid cells. Diffuse aggregates of mononuclear cells were observed in all dogs and, like the follicular aggregates, occurred in areas A and B. These aggregates, more abundant in puppies than in adult dogs, were primarily composed of small lymphocytes and plasma cells, but sparse larger lymphocytes and macrophages were also present. Diffuse aggregates ranged from small clusters of lymphoid cells found immediately beneath the epithelium to larger aggregates

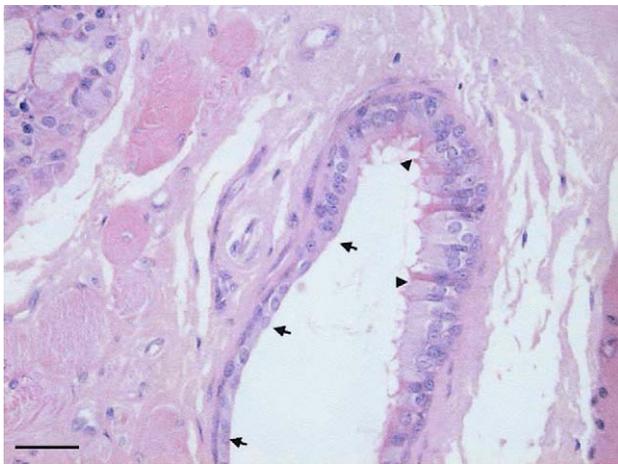


Fig. 2. Transitional epithelium between respiratory and oral epithelium in area G. ►, Respiratory epithelium; ➡, transitional epithelium. HE. Bar, 50 µm.

Table 3
Distribution and amount of different categories of lymphoid tissue in nine areas of the nasopharynx

Area	Follicular aggregates of lymphoid cells	Diffuse aggregates of lymphoid cells	Scattered lymphoid cells	Intraepithelial lymphoid cells
A	++	+++	+++	++
B	+	+++	+++	+
C	-	-	+	+
D	-	-	+	+
E	-	±	++	+
F	-	-	+	+
G	-	-	±	+
H	-	-	±	+
I	-	-	±	+

+++ , Large numbers; ++ , moderate numbers; + , small numbers; ± , rare; - , none.

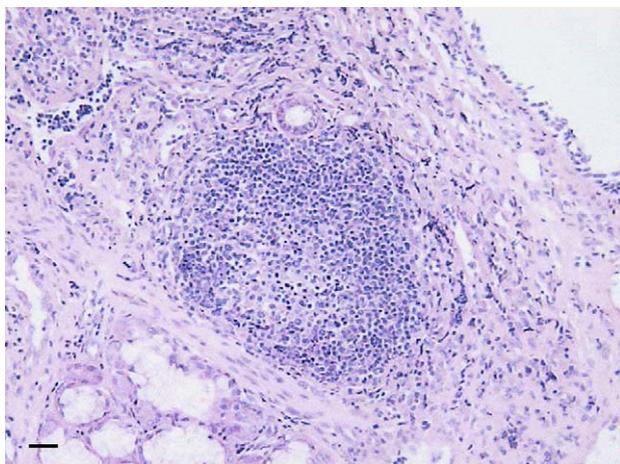


Fig. 3. Secondary follicle, with a germinal centre and mantle zone, within the lamina propria of the pharyngeal tonsil. HE. Bar, 50 μ m.

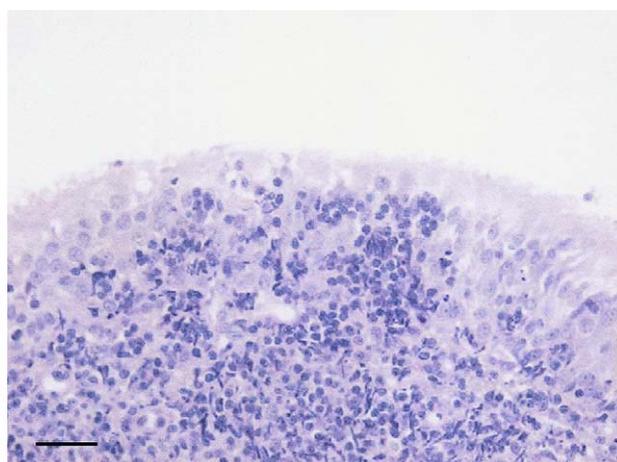


Fig. 5. The lympho-epithelium covering a diffuse aggregate of lymphoid cells is heavily infiltrated by small lymphocytes. HE. Bar, 50 μ m.

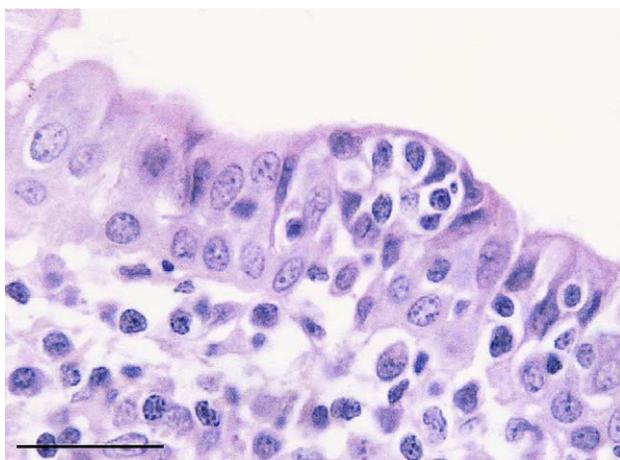


Fig. 4. Lympho-epithelium covering a lymphoid follicle in the pharyngeal tonsil with loss of the ciliated nature of the epithelium, decreased cell height, absence of goblet cells and infiltration of small lymphocytes. HE. Bar, 50 μ m.

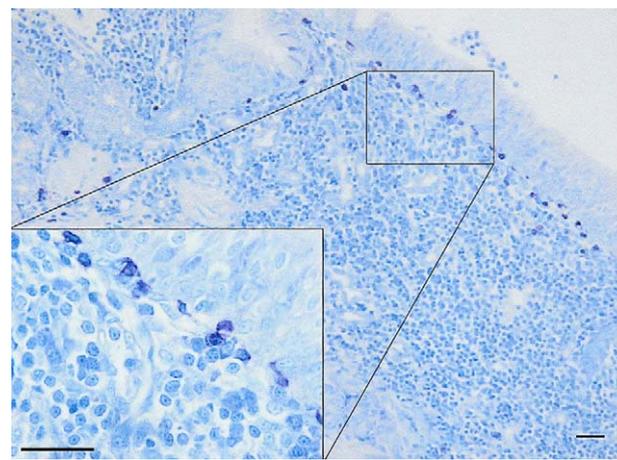


Fig. 6. Lamina propria of the pharyngeal tonsil showing localization of mast cells immediately beneath the epithelium. Toluidine blue. Bar, 50 μ m. Inset: Bar, 50 μ m.

found deeper in the lamina propria and occasionally infiltrating the submucosa and glandular tissue.

Scattered individual lymphoid cells. In all areas of the nasopharynx, scattered isolated small lymphocytes and plasma cells were found throughout the lamina propria and submucosa. Plasma cells were particularly associated with the glandular tissue. In areas A and B, such lymphoid cells were mostly adjacent to follicular and diffuse aggregates of lymphocytes. They were more commonly observed in adult dogs than in puppies.

Intraepithelial lymphocytes. These cells were present in all parts of the nasopharynx in puppies and adult dogs. They were mainly located just above the basement

membrane and were particularly abundant in the epithelium overlying follicular or diffuse lymphoid aggregates (Fig. 5).

Leucocyte Subsets in the Nasopharyngeal Tonsil

Sections from area A were used for the further characterization of leucocyte populations in the nasopharyngeal tonsil.

Mast cells. These were found mainly in the lamina propria immediately beneath the mucosal epithelium (Fig. 6) but were also scattered in the glandular tissue and between muscular fibres. There were significantly more mast cells in adult dogs than in puppies (Table 4).

Table 4
Amount of leucocyte subsets in the lamina propria of the pharyngeal tonsil in puppies and adult dogs

Cell type	Means \pm SE of cells/HPF, or * degree of abundance of T cells in	
	puppies	adults
Mast cells	1.4 \pm 0.16	2.55 \pm 0.14 [†]
L1 ⁺	1.27 \pm 0.26	1.00 \pm 0.22 [†]
IgA ⁺	1.87 \pm 0.60	7.78 \pm 0.52 [†]
IgM ⁺	1.3 \pm 0.19	2.08 \pm 0.17 [†]
IgG ⁺	0.2 \pm 0.22	2.48 \pm 0.19 [†]
CD3 ⁺	+++*	++*
CD4 ⁺	+++*	++*
CD8 ⁺	+*	+*
TCR $\alpha\beta$ ⁺	+++*	+++*
TCR $\gamma\delta$ ⁺	\pm *	\pm *

HPF; high power field.

+++ , large numbers; ++ , moderate numbers; + , low numbers; \pm , rare.

[†]Significant difference in cell count between puppies and adult dogs ($P < 0.05$).

Plasma cells. IgA⁺ and IgM⁺ plasma cells were found beneath the epithelium (Fig. 7), in the glandular tissue (Fig. 8) and in both follicular and diffuse lymphoid aggregates. In follicular aggregates, these cells were found mainly in the dome regions and in the interfollicular areas. IgG⁺ plasma cells were invariably scattered in the lamina propria with no specific localization. Overall, IgA⁺ cells (LSMean \pm SEM of cells per HPF, 4.82 \pm 1.98 cells) were more numerous than IgG⁺ or IgM⁺ plasma cells (LSMean \pm SEM of cells per HPF, 1.34 \pm 1.98 cells and 1.69 \pm 1.98 cells, respectively), but this did not reach significance. The three types of plasma cell were observed in significantly greater numbers in adults than in puppies.

Antigen-presenting cells (APC). MHC class II⁺ cells were mostly observed in the dome regions and in the interfollicular areas of follicular aggregates (Fig. 9), and were homogeneously distributed throughout the diffuse lymphoid aggregates. Positively labelled cells with a dendritic-like morphology were found mainly just beneath or within the epithelium (Fig. 10). Occasionally, the cytoplasm of large lymphoid cells in the germinal centres of secondary follicles was also positively labelled. The MHC class II⁺ cells were present in large numbers and appeared to be more abundant in puppies than in adult dogs.

The CD1c antibody mainly labelled the membrane of cells with a dendritic morphology. Most CD1c⁺ cells were present in the follicular and diffuse lymphoid aggregates. They were also found immediately beneath or within the epithelium and scattered in the lamina propria. CD1c⁺ cells were present in large numbers and were more numerous in puppies than in adult dogs.

The anti-L1 marker labelled cells with a neutrophilic morphology. L1⁺ cells, which were observed, albeit

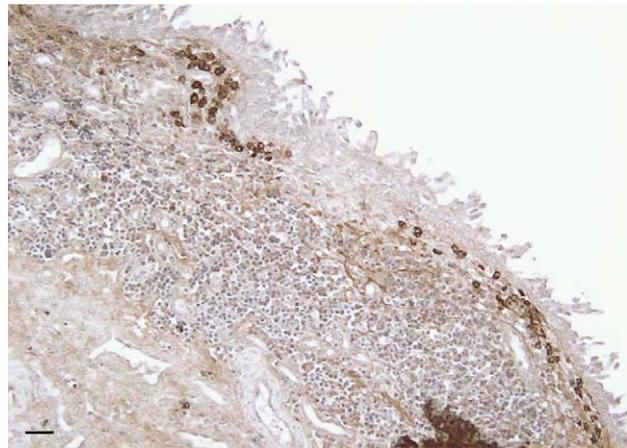


Fig. 7. IgA plasma cells immediately beneath the epithelium of the pharyngeal tonsil. IHC. Bar, 50 μ m.

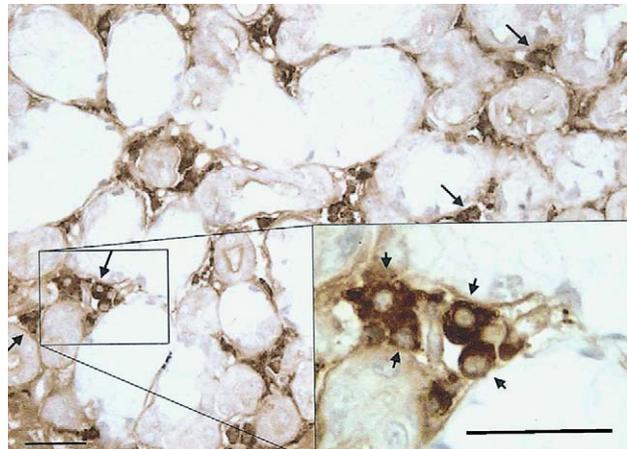


Fig. 8. IgA plasma cells (\blackrightarrow) between the glands. IHC. Bar, 50 μ m. Inset: Bar, 50 μ m.

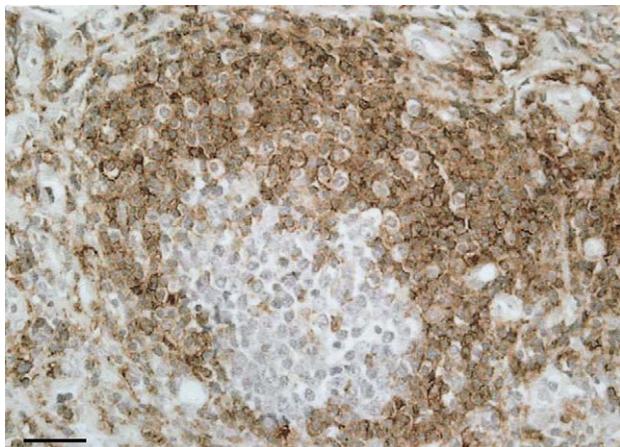


Fig. 9. MHC class II expression within a secondary follicle, showing the typical distribution of positively labelled cells within the mantle zone and interfollicular area. IHC. Bar, 50 μ m.

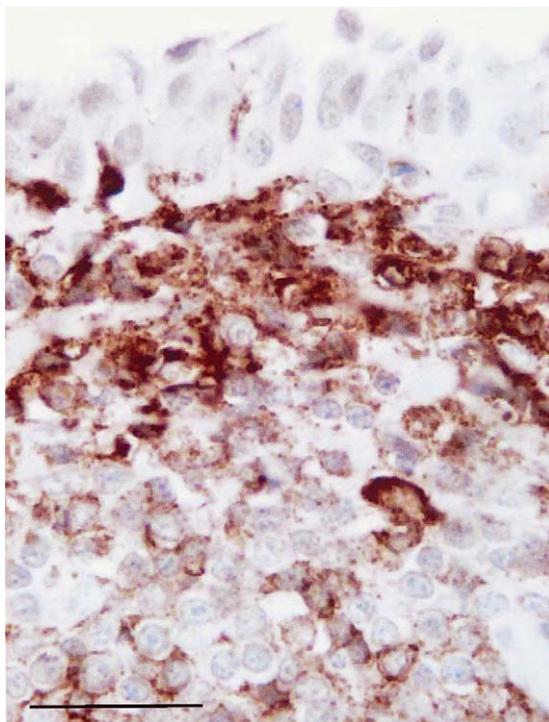


Fig. 10. MHC class II⁺ cells with dendritic morphology immediately beneath the epithelium of the pharyngeal tonsil. IHC. Bar, 50 μ m.

rarely, only in the lamina propria or within blood vessels, were significantly more abundant in puppies than in adult dogs.

T lymphocytes. The majority of lymphoid cells in the sections expressed CD3 and were mainly observed in the follicular and diffuse lymphoid aggregates. Where secondary follicles were present, CD3⁺ cells were typically found in the mantle zone and interfollicular areas, but small numbers were present in the germinal centres

(Fig. 11). CD3⁺ cells were also scattered in the lamina propria and within the epithelium. The number of CD3⁺ cells appeared subjectively to be greater in puppies than in adult animals (Table 4).

CD4⁺, CD8⁺ and TCR $\alpha\beta$ ⁺ T lymphocytes had the same distribution as CD3⁺ cells. CD4⁺ cells were more numerous than CD8⁺ cells, and TCR $\alpha\beta$ ⁺ cells were more numerous than TCR $\gamma\delta$ ⁺ cells, which were observed only occasionally (Table 4). Only CD8⁺ cells were found within the epithelium.

Discussion

The present study represents the first detailed description of the histology and composition of lymphoid tissue within the nasopharynx of dogs without respiratory tract disease. Macroscopically, the nasopharyngeal mucosa appeared uniform and the nasopharyngeal tonsil was not easily identifiable. This contrasts with what is reported in horses, cattle, sheep and pigs, in which the nasopharyngeal tonsil appears as a distinct mass, arising from the surface of the caudal part of the posterior nasopharyngeal wall, with a rough, irregular and deeply furrowed surface (Baron, 1984; Kuper *et al.*, 1992; Schuh and Oliphant, 1992; Kumar *et al.*, 2001). These species breathe mainly through the nose, whereas dogs breathe through both the nose and mouth; as a result, exposure of the canine nasal and nasopharyngeal mucosa to inhaled antigens is reduced. This may explain why the pharyngeal tonsil is less developed in dogs than in other domestic animals.

The distribution of the nasal and oral types of epithelium described in the present study accorded with descriptions in the literature (Bloom and Fawcett, 1966; Baron, 1984; Dellman and Eurell, 1998). The nasopharynx is almost completely covered by respiratory epithelium. However, transitional and stratified squamous



Fig. 11. Distribution of CD3⁺ cells in a secondary follicle of the pharyngeal tonsil. IHC. Bar, 50 μ m.

epithelium has been observed in the areas corresponding to the intrapharyngeal opening represented in the present study by areas G and I. This distribution may be attributable to the fact that the mucosa is covered by a squamous epithelium in the areas where the nasopharyngeal surfaces are brought into contact with each other (e.g., during closure of the intrapharyngeal opening in the pharyngeal phase of deglutition) (Kelly *et al.*, 1984). The intrapharyngeal opening is also close to the laryngopharynx, which is known to be covered by a stratified squamous epithelium (Dellman and Eurell, 1998).

Folds or crypts were not observed in the epithelial surface of the canine nasopharynx, or in the surface of the pharyngeal tonsil, in contrast to descriptions of the epithelial surface of the palatine tonsils in the dog (Belz and Heath, 1995), and the pharyngeal tonsil of horses, sheep and cattle (Baron, 1984; Chen *et al.*, 1989, 1991; Schuh and Oliphant, 1992; Kumar and Timoney, 2001).

The epithelium overlying the observed follicular lymphoid aggregates was occasionally replaced by a lympho-epithelium with decreased epithelial cell height, loss of cilia, absence of goblet cells, and infiltration of small lymphocytes (McDermott *et al.*, 1982; Anderson *et al.*, 1986). In rodents, MALT is invariably covered by a lympho-epithelium (McDermott *et al.*, 1982). In cattle and sheep, however, mucosal lymphoid follicles may also be covered by normal respiratory epithelium and the presence of lympho-epithelium may be induced only by antigenic stimulation (Anderson *et al.*, 1986; Chen *et al.*, 1989).

Intraepithelial lymphocytes were observed throughout the nasopharynx, particularly in lympho-epithelium. Only CD8⁺ cells were observed within the epithelium. This accords with descriptions of epithelium covering the pharyngeal tonsil in cattle (Schuh and Oliphant, 1992) and horses (Kumar *et al.*, 2001), in which CD8⁺ cells predominated. Nevertheless, as the epithelium covering lymphoid aggregates was often damaged or absent in the present study, other positively labelled cells may have escaped notice. The reason for the detachment or loss of the epithelium overlying large aggregates of lymphoid tissue is not clear but has also been reported in the equine pharyngeal tonsil (Mair *et al.*, 1988a). A possible explanation may lie in increased epithelial fragility secondary to the presence of large numbers of lymphoid cells, blood vessels and capillaries beneath the epithelium.

The composition of the lamina propria and submucosa observed in the present study (i.e., fibrous connective tissue, mixed muco-serous tubulo-alveolar glands, blood vessels, capillaries and lymphatics) accorded with descriptions in the veterinary literature (Baron, 1984; Banks, 1993; Dellman and Eurell, 1998). The presence of striated muscle fibres in areas A, F, G and I was

expected, as in these areas the mucosa of the nasopharynx is not in direct contact with underlying bony structures of the skull.

Lymphoid tissue was in general concentrated in the caudal part of the roof of the nasopharynx, distal to the openings of the auditory tubes (i.e., areas A and B). Follicular and diffuse lymphoid aggregates were observed only in these areas, in contrast to scattered lymphoid cells and intraepithelial lymphocytes which were observed throughout the nasopharynx. These localized non-encapsulated accumulations of typical MALT structures are thought to represent the canine pharyngeal tonsil, of which they are reminiscent (McDermott *et al.*, 1982; Junquiera and Carneiro, 2003). Overall, a larger amount of lymphoid tissue, particularly follicular aggregates, was observed in the canine pharyngeal tonsil in young animals than in adults. Similar findings were reported in the human and equine pharyngeal tonsil, in which an age-related regression of lymphoid tissue was observed (Brandtzaeg, 1984; Mair *et al.*, 1988a; Schuh and Oliphant, 1992).

NALT is important for the development of local and systemic immune responses after intranasal deposition of antigen. Indeed, after deposition on the mucosal surface, antigens are sampled, processed and presented to lymphoid cells in the underlying lamina propria or regional lymph nodes, or both. This results in activation of T cells, which help B cells to differentiate into plasma cells (Davis, 2001). In rodents, the nasopharyngeal tonsils play a role in the development of an immune response after intranasal vaccination (Wu *et al.*, 1997; Davis, 2001; Debertain *et al.*, 2003). In man, the pharyngeal tonsil appears to play this role (Davis, 2001). Studies on intranasal vaccination of dogs against *Bordetella bronchiseptica* demonstrated protection and induction of specific serum IgG and mucosal IgA, but the MALT structures associated with such responses were not characterized (Shade and Goodnow, 1979; Bey *et al.*, 1981; Glickman and Appel, 1981; Kontor *et al.*, 1981; Ellis *et al.*, 2001, 2002). A recent study failed to identify MALT structures in nasal mucosa in healthy dogs (Peeters *et al.*, 2005). The results of the present study suggest that the pharyngeal tonsil may play a role in the immune response to intranasal vaccination in dogs.

In the present study, the distribution of mast cells in the canine nasopharynx was similar to that in the canine nasal and bronchial mucosa (Peeters *et al.*, 2005) and in the equine upper respiratory tract (Mair *et al.*, 1988b). Mast cells were found in greater numbers in the nasopharyngeal tonsil of adult dogs than of puppies, in contrast to findings in other parts of the canine respiratory tract (Peeters *et al.*, 2005). The reason for this discrepancy is unknown.

IgA⁺ plasma cells outnumbered IgG⁺ and IgM⁺ plasma cells and were preferentially associated with

the glandular tissue, as in the upper respiratory tract of human beings (Brandtzaeg, 1984), dogs (Peeters *et al.*, 2005), horses (Mair *et al.*, 1988c) and pigs (Bradley *et al.*, 1976). Smaller numbers of all three types of plasma cell were found in puppies than in adult dogs. This accords with findings in other parts of the respiratory tract in pigs and horses (Bradley *et al.*, 1976; Blunden and Gower, 1999). Peeters *et al.* (2005) reported fewer IgA⁺, IgG⁺ and IgM⁺ plasma cells in the nasal and bronchial mucosa of puppies than adult dogs.

APCs expressing MHC class II or CD1c were abundant within the nasopharyngeal tissue, particularly amidst the aggregates of lymphoid cells. The distribution of APCs was similar to that observed in the NALT of rats (Kuper *et al.*, 1990) and cattle (Schuh and Oliphant, 1992). MHC class II⁺ and CD1c⁺ cells were more commonly observed in puppies than in adult dogs, thus contrasting with data obtained from cattle, in which the intensity of MHC class II expression increased with age (Schuh and Oliphant, 1992).

In the present study, CD4⁺ T cells outnumbered CD8⁺ T cells, as also found in the NALT of rats (Hamelers *et al.*, 1989; Kuper *et al.*, 1990) and cattle (Rebelatto *et al.*, 2000). These T cell subsets were concentrated in the mantle zone and interfollicular areas, as has been reported in the pharyngeal tonsil in man (Brandtzaeg, 1984; Richtsmeier and Shikhani, 1987), cattle (Rebelatto *et al.*, 2000), horses (Kumar *et al.*, 2001) and rats (Kuper *et al.*, 1990). CD4⁺ and CD8⁺ cells were homogeneously distributed in areas of diffuse lymphoid aggregation or where individual scattered lymphocytes were present. Slightly more CD3⁺ cells were observed in young dogs than in adults, probably reflecting the numbers of lymphoid follicles. In contrast, T cells were reported by Schuh and Oliphant (1992) to increase with age in the bovine pharyngeal tonsil. TCR $\alpha\beta$ ⁺ and $\gamma\delta$ ⁺ cells were both observed in the lamina propria, the TCR $\alpha\beta$ ⁺ cells greatly outnumbering the TCR $\gamma\delta$ ⁺ cells. This was similar to findings in a recent study of leucocyte distribution in the canine nasal and bronchial mucosa (Peeters *et al.*, 2005).

In conclusion, the canine pharyngeal tonsil, which was flat and not easily identifiable macroscopically, was histologically and immunohistochemically similar to the pharyngeal tonsil of other species except for the absence of epithelial folds and crypts. Its strategic location at the entrance of the respiratory tract suggests a role in regional immune responses to airborne pathogens and, therefore, this structure is a potentially useful target for intranasally delivered immunogens.

References

- Anderson, M. L., Moore, P. F., Hyde, D. M. and Dungworth, D. L. (1986). Bronchus-associated lymphoid tissue in the lungs of cattle: relationship to age. *Research in Veterinary Science*, **41**, 211–220.
- Bancroft, J. D. and Cook, H. C. (1984). *Manual of Histological Techniques*, Churchill Livingstone, Edinburgh pp. 19–99.
- Banks, W. J. (1993). *Applied Veterinary Histology*, Mosby Year Book, St Louis.
- Baron, R. (1984). Splanchnologie I: Appareil digestif et respiratoire. In: *Anatomie Comparée des Mammifères Domestiques*, Vol. 3, R. Baron, Ed., Vigot, Paris.
- Belz, G. T. and Heath, T. J. (1995). The epithelium of canine palatine tonsils. *Anatomica Embryologica (Berlin)*, **192**, 189–194.
- Bey, R. F., Shade, F. J., Goodnow, R. A. and Johnson, R. C. (1981). Intranasal vaccination of dogs with liver avirulent *Bordetella bronchiseptica*: correlation of serum agglutination titer and the formation of secretory IgA with protection against experimentally induced infectious tracheobronchitis. *American Journal of Veterinary Research*, **42**, 1130–1132.
- Bloom, W. and Fawcett, D. W. (1966). *A Textbook of Histology*, W.B. Saunders, Philadelphia.
- Blunden, A. S. and Gower, S. M. (1999). A histological and immunohistochemical study of the humoral immune system of the lungs in young Thoroughbred horses. *Journal of Comparative Pathology*, **120**, 347–356.
- Bradley, P. A., Bourne, F. J. and Brown, P. J. (1976). The respiratory tract immune system in the pig. I. Distribution of immunoglobulin-containing cells in the respiratory tract mucosa. *Veterinary Pathology*, **13**, 81–89.
- Brandtzaeg, P. (1984). Immune functions of human nasal mucosa and tonsils in health and disease. In: *Immunology of the Lung and Upper Respiratory Tract*, J. Bienenstock, Ed, McGraw-Hill, Toronto.
- Chen, W., Alley, M. R. and Manktelow, B. W. (1989). Respiratory tract-associated lymphoid tissue in conventionally raised sheep. *Journal of Comparative Pathology*, **101**, 327–340.
- Chen, W., Alley, M. R., Manktelow, B. W., Hopcroft, D. and Bennett, R. (1991). The potential role of the ovine pharyngeal tonsil in respiratory tract immunity: a scanning and transmission electron microscopy study of its epithelium. *Journal of Comparative Pathology*, **104**, 47–56.
- Davis, S. S. (2001). Nasal vaccines. *Advanced Drug Delivery Reviews*, **51**, 21–42.
- Debertin, A. S., Tschernig, T., Tonjes, H., Kleemann, W. J., Troger, H. D. and Pabst, R. (2003). Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. *Clinical and Experimental Immunology*, **134**, 503–507.
- Dellman, H. D. and Eurell, J. (1998). *Textbook of Veterinary Histology*, William & Wilkins, Baltimore.
- Ellis, J. A., Haines, D. M., West, K. H., Burr, J. H., Dayton, A., Townsend, H. G., Kanara, E. W., Konoby, C., Crichtlow, A., Martin, K. and Headrick, G. (2001). Effect of vaccination on experimental infection with *Bordetella bronchiseptica* in dogs. *Journal of the American Veterinary Medical Association*, **218**, 367–375.
- Ellis, J. A., Krakowka, G. S., Dayton, A. D. and Konoby, C. (2002). Comparative efficacy of an injectable vaccine and an intranasal vaccine in stimulating *Bordetella*

- bronchiseptica*-reactive antibody responses in seropositive dogs. *Journal of the American Veterinary Medical Association*, **220**, 43–48.
- Glickman, L. T. and Appel, M. J. (1981). Intranasal vaccine trial for canine infectious tracheobronchitis (kennel cough). *Laboratory Animal Science*, **31**, 397–399.
- Hameleers, D. M., van der Ende, M., Biewenga, J. and Sminia, T. (1989). An immunohistochemical study on the post-natal development of rat nasal-associated lymphoid tissue (NALT). *Cell and Tissue Research*, **256**, 431–438.
- Hellings, P., Jorissen, M. and Ceuppens, J. L. (2000). The Waldeyer's ring. *Acta Otorhinolaryngologica Belgica*, **54**, 237–241.
- Hiller, A. S., Tschernig, T., Kleemann, W. J. and Pabst, R. (1998). Bronchus-associated lymphoid tissue (BALT) and larynx-associated lymphoid tissue (LALT) are found at different frequencies in children, adolescents and adults. *Scandinavian Journal of Immunology*, **47**, 159–162.
- Junquiera, L. C. and Carneiro, J. (2003). *Basic Histology: Text and Atlas*, Lange Medical Books McGraw-Hill Companies, New York.
- Kelly, D. E., Wood, R. L. and Enders, A. C. (1984). The respiratory system: the nasal cavity and nasopharynx. In: *Bailey's Textbook of Microscopic Anatomy*, T.M. Tracy, Ed., William & Wilkins, Baltimore.
- Kontor, E. J., Wegrzyn, R. J. and Goodnow, R. A. (1981). Canine infectious tracheobronchitis: effects of an intranasal live canine parainfluenza—*Bordetella bronchiseptica* vaccine on viral shedding and clinical tracheobronchitis (kennel cough). *American Journal of Veterinary Research*, **42**, 1694–1698.
- Kumar, P. and Timoney, J. F. (2001). Light and electron microscope studies on the nasopharynx and nasopharyngeal tonsil of the horse. *Anatomy, Histology and Embryology*, **30**, 77–84.
- Kumar, P., Timoney, J. F. and Sheoran, A. S. (2001). M cells and associated lymphoid tissue of the equine nasopharyngeal tonsil. *Equine Veterinary Journal*, **33**, 224–230.
- Kuper, C. F., Hameleers, D. M., Bruijntjes, J. P., van der Ven, I., Biewenga, J. and Sminia, T. (1990). Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. *Cell and Tissue Research*, **259**, 371–377.
- Kuper, C. F., Koornstra, P. J., Hameleers, D. M., Biewenga, J., Spit, B. J., Duijvestijn, A. M., van Breda Vriesman, P. J. and Sminia, T. (1992). The role of nasopharyngeal lymphoid tissue. *Immunology Today*, **13**, 219–224.
- Littell, R. C., Freund, R. J. and Spector, P. C. (1998). *SAS[®] System for Linear Models*, SAS Institute, Cary, NC, USA.
- Mair, T. S., Batten, E. H., Stokes, C. R. and Bourne, F. J. (1988a). The distribution of mucosal lymphoid nodules in the equine respiratory tract. *Journal of Comparative Pathology*, **99**, 159–168.
- Mair, T. S., Stokes, C. R. and Bourne, F. J. (1988b). Distribution and ultrastructure of mast cells in the equine respiratory tract. *Equine Veterinary Journal*, **20**, 54–58.
- Mair, T. S., Stokes, C. R. and Bourne, F. J. (1988c). Immunohistochemical study of the local humoral immune system of the equine respiratory mucosa. *Research in Veterinary Science*, **45**, 160–165.
- McDermott, M. R., Befus, A. D. and Bienenstock, J. (1982). The structural basis for immunity in the respiratory tract. *International Review of Experimental Pathology*, **23**, 47–112.
- Moneret-Vautrin, D. A., Wayoff, M. and Kanny, G. (1992). Local immunologic system of the nose and paranasal sinuses. *Annales d' Otolaryngologie et Chirurgie Cervicofaciale*, **109**, 162–166.
- Peeters, D., Day, M. J., Farnir, F., Moore, P. and Clercx, C. (2005). Distribution of leucocyte subsets in the canine respiratory tract. *Journal of Comparative Pathology*, **132**, 261–272.
- Rebelatto, M. C., Mead, C. and HogenEsch, H. (2000). Lymphocyte populations and adhesion molecule expression in bovine tonsils. *Veterinary Immunology and Immunopathology*, **73**, 15–29.
- Richtsmeier, W. J. and Shikhani, A. H. (1987). The physiology and immunology of the pharyngeal lymphoid tissue. *Otolaryngological Clinics of North America*, **20**, 219–228.
- Schuh, J. C. and Oliphant, L. W. (1992). Development and immunophenotyping of the pharyngeal tonsil (adenoid) in cattle. *Journal of Comparative Pathology*, **106**, 229–241.
- Shade, F. J. and Goodnow, R. A. (1979). Intranasal immunization of dogs against *Bordetella bronchiseptica*-induced tracheobronchitis (kennel cough) with modified live-*Bordetella bronchiseptica* vaccine. *American Journal of Veterinary Research*, **40**, 1241–1243.
- Slatter, D. (2003). *Textbook of Small Animal Surgery*, Elsevier Saunders, St Louis.
- Wu, H. Y., Nguyen, H. H. and Russell, M. W. (1997). Nasal lymphoid tissue (NALT) as a mucosal immune inductive site. *Scandinavian Journal of Immunology*, **46**, 506–513.
- Zuercher, A. W., Coffin, S. E., Thurnheer, M. C., Fundova, P. and Cebra, J. J. (2002). Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *Journal of Immunology*, **168**, 1796–1803.

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