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Immunohistochemistry and ultrastructure of the Tubal Tonsil in Bactrian Camel

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ABSTRACT

The tonsils from 6 adult (5–10 years) healthy Bactrian camels of both sexes without symptoms of clinical disease were examined by immunohistochemistry, scanning and transmission electron microscopy. The camel tubal tonsil was covered by pseudostratified columnar ciliated epithelia which were randomly interrupted by the lymphoid follicles. Follicle associated epithelium demonstrated morphology characteristic of microvillus cells. The inter-follicular and parafollicular areas contained diffusely localized lymphocytes. The bright germinal centre of the lymphoid follicles appeared less electron dense than the rest of the follicle, which contained lymphoblasts, follicular dendritic cells, apoptotic cells, plasma cells and tingible body macrophages. High endothelial venules, a few inter-digitating cells with processes of varying length and thickness, macrophages, and plasma cells were observed among lymphocytes. CD20+ B lymphocytes, CD3+ T lymphocytes and a few CD68+ macrophages were identified in the follicle associated epithelium, lamina propria mucosae, and parafollicular and inter-follicular areas. The positive follicular dendritic cells were only found in germinal centers. These findings suggested that the camel tubal tonsil contains all the elements necessary to function as an effector and inductor immunological organ.

Key words: Bactrian camel, Immunohistochemistry, Tubal tonsil, Ultrastructure

The tonsils belong to the pharyngeal mucosa-associated lymphoid tissue representing a first line of defense against ingested and inhaled foreign antigens (Casteleyn *et al.* 2010). They consist of aggregations of lymphoid cells that are present in the mucosae of the oropharynx, nasopharynx and laryngopharynx (Cesta 2006; Cocquyt *et al.* 2005, Perry and Whyte 1998). Six tonsils can be present in domestic animals, i.e. the lingual tonsil, palatine tonsil, paraepiglottic tonsil, pharyngeal tonsil, tubal tonsil and the tonsil of the soft palate. The presence and location of these tonsils vary, however, according to the animal species. Only in the sheep, goat and Bactrian camel (*Camelus bactrianus*), all six tonsils are present (Casteleyn *et al.* 2011, Yang *et al.* 2011). All tonsils together form the so-called Waldeyer's ring (Perry and Whyte 1998). Its location at the crossing of the digestive and respiratory tracts plays a key role in immunity as this is the site where vast amounts of foreign antigens enter the body during feeding and breathing (Casteleyn *et al.* 2011).

The tonsils have gained much scientific interest during the past decade because of their role in immune responses and in the pathogenesis of several diseases including prion diseases (Casteleyn *et al.* 2011). However, the larger amount of immunological investigations are limited to those tonsils that are most easily identified. Large variations in histological characteristics exist between the several tonsils. Although the tubal tonsil has been reported in humans, ruminants, pig and the horse, very little information on its structure has been recorded (Casteleyn *et al.* 2011, Kumar and Timoney 2005b; Mair *et al.* 1987, Perry and Whyte 1998). The tubal tonsil is macroscopically visible after fixation in 2% acetic acid and located near the opening of the eustachian tube in the Bactrian camel (*Camelus bactrianus*), where they may help guard against infection spreading from the pharynx towards the inner ear. The tubal tonsil consisted of secondary lymph nodules, scattered lymph nodules, aggregations of lymphocytes and diffuse lymphoid tissue. Secondary lymph nodules comprised follicle-associated epithelium (FAE), dome area (DA), follicular area (FA) and parafollicular area (PFA) (Yang *et al.* 2011).

The aims of the present study were to explore the immunohistochemical and ultrastructural features of the tubal tonsil in the Bactrian camel. The results presented in our study could possibly contribute to a better understanding of the initiation of immune responses in the camel tonsils.

MATERIALS AND METHODS

The heads of 6 adult (5–10 years) healthy Bactrian camels of both sexes without symptoms of clinical disease (daily

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inspection by a veterinarian) were obtained from the slaughterhouse of the Right Alasan Banner Food Company in Inner Mongolia Autonomous Region, China, after the animals had been killed by exsanguination for human consumption.

Immunohistochemistry

The fragment from the tubal tonsils were fixed in 10% formaldehyde, dehydrated, cleared and embedded in paraffin for immunohistochemical process. Serial tissue sections (8 μm thick) with a 100 μm interval were made from all tissues. The sections were dewaxed and rehydrated, and antigen retrieval was performed by microwaving the sections for 20 min (4×5 min) at medium power in 0.01 M sodium citrate buffer (pH 6.0) and were allowed to cool for 10 min. Then, the endogenous peroxidase was inhibited with 3% H_2O_2 in PBS for 30 min. After non-specific binding was blocked with 10% normal goat serum in PBS, the sections were rinsed three times in PBS containing 0.02% Triton X-100. Then the sections were incubated separately with monoclonal mouse anti-human antibodies, i.e. CD20, CD3, CD68, and follicular dendritic cells (FDC) overnight at 4°C. Again, sections were rinsed with PBS and then were covered with the biotinylated goat anti-mouse IgG. After washing in PBS, sections were incubated with streptavidin-labeled peroxidase complex for 3 h at room temperature. After 2 further washes with PBS, the immunolabeling was visualized using diaminobenzidine (DAB) with hematoxylin counterstaining. Negative control sections were performed in a similar manner, except the primary antibody was substituted with PBS. Images were captured using a digital camera. The positively reacting cells in different areas were counted with × 400 magnification in 3 different fields. Relative number of positive cells was assessed as follows: +++, >50% of cells strongly stained; ++, 10–50% of cells distinctly stained; +, <10% were stained; -, negative.

Scanning and transmission electron microscopy

Fresh tissues were thoroughly washed and processed for scanning and transmission electron microscopy (Yang *et al.* 2009).

RESULTS

Immunohistochemistry

Secondary lymph nodules with the cytomorphological and immunophenotypic features of MALT was identified in the tubal tonsil specimens from 6 camles. The CD20+ B lymphocytes, CD3+ T lymphocytes, CD68+, and FDC+ were indicated by the presence of cytoplasmic and/or membrane brown staining in different areas of the secondary lymph nodules, while no positive labelling was observed in negative control sections. CD20+ B lymphocytes, CD3+ T lymphocytes and a few CD68+ macrophages were identified in the FAE, lamina propria mucosae, and parafollicular and inter-follicular areas (Fig.1-A~E). CD3+ T lymphocytes and a few CD68+ seen infiltrating the epithelium, but overall the extent of infiltration was low. The parafollicular areas were more heavily populated with CD3+ T lymphocytes. CD68+ macrophages were mainly localized in the parafollicular and inter-follicular areas. Germinal centres have been shown to generate a variable number of CD20+ B lymphocytes and few FDC (Fig.1-F). The distributions of subsets of lymphoid cells, FDC and macrophages in different lymphoid compartments were summarized in Table 1.

Table 1. Relative numbers of lymphoid cells, FDC and macrophages in different regions of the tonsil of the soft palate of the camel.

	Phenotype epithelium	Laminapropria mucosae	Inter-follicular area	Parafollicular area	Germinal center
CD^{20+}	++	+	+	+	+++
CD^{3+}	+	++	++	+++	++
CD^{68+}	+	++	++	+	+
FDC^{+}	–	–	–	–	++

–, not detected; +, few; ++, moderately frequent; +++, very frequent.

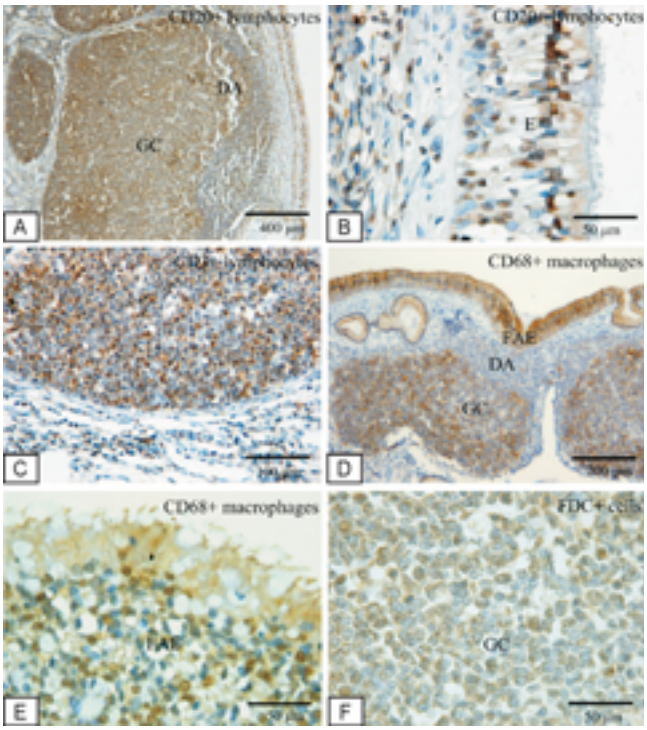

Fig. 1 (A-F). Immunohistochemical staining for **A** and B: CD20+ **B** lymphocytes; **C**: CD3+ T lymphocytes; **D** and **E**: CD68+ macrophages; **F**: FDC+ cells in secondary lymph nodules of the tubal tonsil in the camel. FAE: follicle-associated epithelium; E: epithelium; DA: dome area; FA: follicular area.

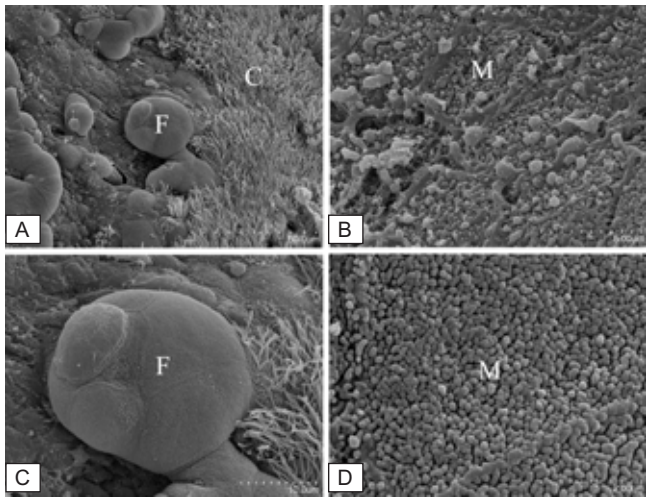

Fig. 2 (A-D). SEM views of the camel tubal tonsil. **A** and **B**: The tubal tonsil is mainly covered by a ciliated epithelium that is interrupted by patches of FAE. **C**: Follicles (F) closely aggregated prominent round to oval hemispheres. **D**: SEM images at higher magnification showing regular microvillus of M cells (M).

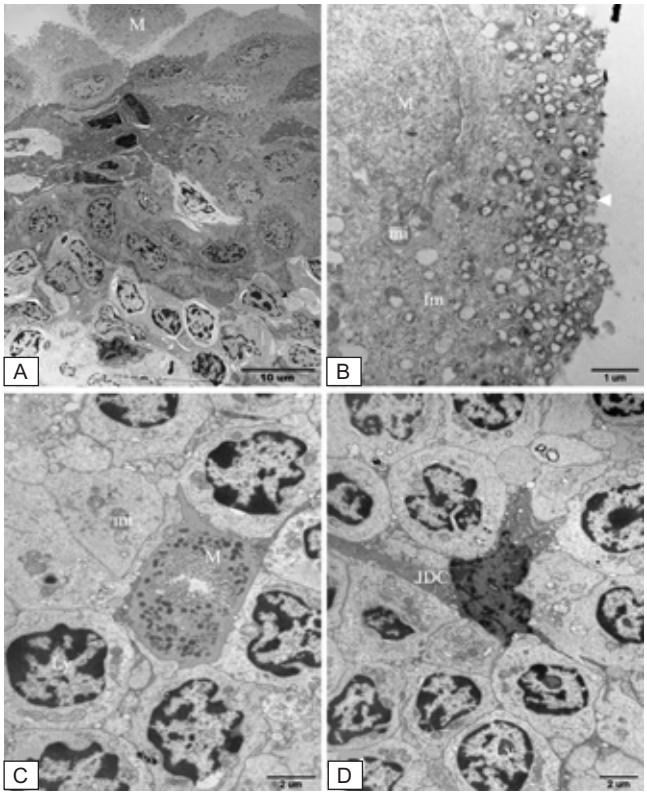

Fig. 3 (A-D). TEM views of different cells in the FAE and parafollicular area (PFA). **A**: The pseudostratified columnar ciliated epithelium was often infiltrated by lymphoid cells in regions without exposed lymphoid cells. **B**: Higher magnification of M cell (M) in the FAE region showing irregular nucleus and distribution of mitochondria (mi), a fine filament meshwork (fm), small vesicles (arrowheads) and other organelles. **C** and **D**: TEM images of lymphoid cells(Ly), inter-digitating cells (IDC) and macrophage (m) in PFA.

Scanning electron microscopy

Scanning electron microscopy (SEM) of the tubal tonsil showed that a ciliated surface with small islands of interspersed M cells (Fig.2-A). M cells with very few short microvilli and microridges were mainly located along the cell borders (Fig.2-B). The ciliated epithelium was randomly interrupted by the lymphoid follicles. They closely aggregated prominent round to oval hemispheres (Fig.2-A, C), 100–300 µm in diameter, of follicle associated epithelium consisting of cells with densely packed short microvilli (Fig.2-D). The reticular epithelium was composed of either loosely arranged squamous epithelial cells with densely packed microvilli or small knobs, or columnar epithelial cells lacking cilia with interspersed superficial lymphoid cells in some places.

Transmission electron microscopy

Transmission electron microscopy (TEM) images from the similar areas revealed that the pseudostratified columnar ciliated epithelium was often infiltrated by lymphoid cells

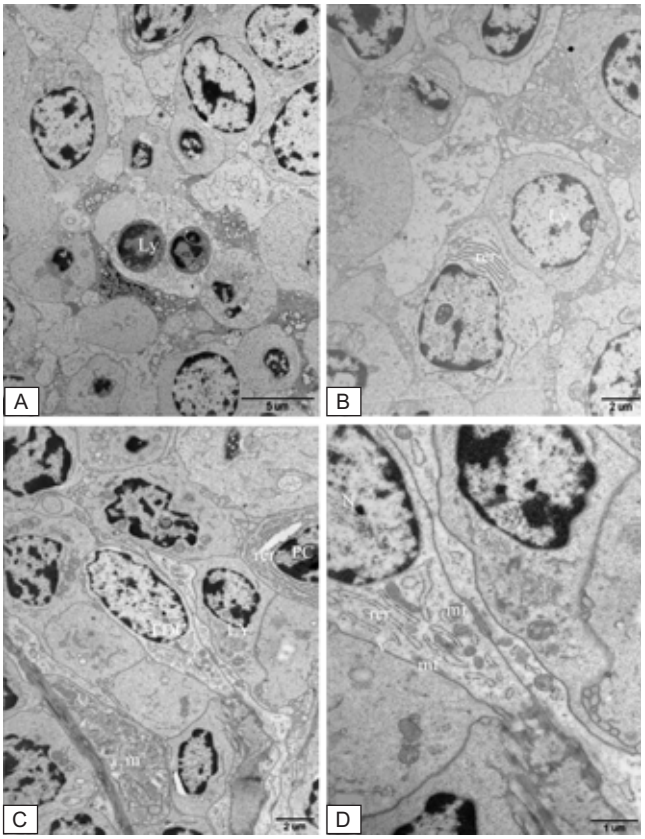

Fig. (A-D) 4. TEM views of different cells in the germinal centre. **A–C**: The germinal centre is occupied by densely packed lymphoid cells(Ly), FDC, plasma cell (PC) and apoptotic lymphocytes (al) with a small dark nucleus. **D**: TEM images of the process of the FDC, closely adjacent to the lymphoblast, is seen to contain microfilaments (mf), mitochondria (mi), rough endoplasmic reticulum (rer) and multiple vesicles (v).

in regions without exposed lymphoid cells (Fig.3-A).FAE overlying the secondary lymph nodules which consisted the tubal tonsil demonstrated morphology characteristic of M cells, permeated by groups of lymphoid cells (Fig.3-A). M cells were generally isolated, cuboidal, and with eccentric nucleoli and clumps of chromatin in their irregular nuclei. The M-cell luminal cytoplasm contained oval to elongated mitochondria, free ribosomes and few cisternae of the rough endoplasmic reticulum were also present. Abundant together with numerous small sized vesicles towards the luminal surface were predominantly supranuclear (Fig.3-B).The bright germinal centre (GC) of the lymphoid follicles contained lymphoblasts, FDC, apoptotic cells, plasma cells and tingible body macrophages, and appeared less electron dense than the rest of the follicle. Apoptotic lymphocytes with a small dark nucleus filled by peripheral dark masses of heterochromatin were interspersed (Fig.4-A, B). FDC extend among the lymphoblasts of the germinal centers, which had a highly euchromatic nucleus of roundish or oval shape and occasional large nucleoli, sent long cytoplasmic processes between the lymphoid cells (Fig.4-C, D). The cytoplasm of macrophages contained phagosomes, lysosomes, vacuoles and cellular debris and their nuclei were irregular. The inter-follicular and parafollicular areas contained diffusely localized lymphocytes. High endothelial venules, a few inter-digitating cells (IDC) with processes of varying length and thickness, macrophages, and plasma cells were observed among these lymphocytes (Fig.3-C, D).

DISCUSSION

The tubal tonsil were similar to other tonsils in the camel (Yang *et al.* 2011) and in accordance with ruminants, pig and the horse (Kumar and Timoney 2005b). The lymphoid nodules in the camel tubal tonsil are characteristic of the simple follicular areas traditionally described in mucosal lymphoid tissue (Stanley *et al.* 2001), and are similar in structure to the secondary lymphoid follicles observed ovine tubal tonsil. CD20+ B lymphocytes, CD3+ T lymphocytes and a few CD68+ macrophages were identified in the FAE, lamina propria mucosae, and parafollicular and inter-follicular areas. Intra-epithelial T and B-lymphocytes and some macrophages in the equine nasopharyngeal and tubal tonsils (Kumar and Timoney 2005b, Kumar *et al.* 2001) and NALT of the rat are of unknown origin, movement and function (Kuper *et al.* 1992). FDC positive cells in GC of the camel tubal tonsil are in accordance with the observation in the bovine lingual tonsil, which was further proved by TEM. As typical, specific, histological components of lymph nodules in secondary lymph organs (Rebmann and Gasse 2008), FDC bind antigen-antibody complexes to their surface for long periods and are essential for generation of effective humoral antibody responses (Heinen *et al.* 1995).

The ultrastructure of the camel tubal tonsil epithelial lining is resembles that of the horse and sheep, which could be explained by their closely adjacent position in the nasopharynx (Casteleyn et al. 2010). The tubal tonsil was covered by pseudostratified columnar ciliated epithelia which were randomly interrupted by the lymphoid follicles. FAE demonstrated morphology characteristic of M cells, as reported for M-cells in general (Gebert and Posselt 1997). M cells rank among the most important epithelial cell types that facilitate the adherence, uptake, and sampling of foreign antigens and micro-organisms (Kraehenbuhl and Neutra 2000). Some vesicles in the M cell apical cytoplasm are reported to contain cathepsin E, an endosomal protease, which generates an acidic milieu (Allan *et al.* 1993). Different maturation stages of M cells are present on the ovine tubal tonsil (Kumar and Timoney 2005b). Tonsillar lymphoid cells could also get access to the nasopharyngeal cavity through very small epithelial channels that were lined by electron dense cuboidal Mv1 cells (Casteleyn *et al.* 2010). Epithelial channels that were also observed in the pharyngeal tonsil of sheep seem to form another route for luminal antigens to gain access to the tonsillar lymphoid tissue (Chen *et al.* 1991). Similar epithelial channels need to be investigated in the camel tubal tonsil.

Luminal antigens are transported by the lymphoid cells, or by dendritic cells from the M-cell pockets to the follicular zones, which can result in the formation of a bright germinal centre (Wolniak *et al.* 2004) of proliferating lymphocytes, as was indeed found here in secondary lymph nodule of the camel. Proliferating lymphoblasts and FDC with prominent vesicles in their cytoplasm continuously provide antigens on their surface, as observed here and in other lymphoid tissues (Szakal *et al.* 1983), and considered to support the differentiation of the proliferating lymphoblasts (Wolniak *et al.* 2004). When differentiating lymphocytes exhibit inappropriate antigen specificity or binding affinity (Knop and Knop 2005), they are induced to die to form apoptotic lymphocytes in the GC of camel tubal tonsil. Macrophages are dispersed throughout the camel tubal tonsil, and serve for phagocytosis, processing and presentation of foreign antigens (Yamamoto et al. 1988). HEVs are a special morphologicaland functional adaptation of post-capillary venules to the requirements of efficient, regulated lymphocyte migration in lymphoid tissues (Knop and Knop 2005). HEVs, IDC, macrophages, and plasma cells were observed among lymphocytes in the parafollicular area of the tubal tonsil in the camel, as described for these cells in horse lingual and tubal tonsils (Kumar and Timoney 2005a, b). IDC are thought to be involved in antigen presentation to lymphocytes (Yamamoto *et al.* 1988).

The present study confirmed that the camel tubal tonsil contains all the elements necessary to function as a potent antigen sampling site in upper respiratory tract.

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