1. Introduction

Waldeyer’s ring is composed of the nasopharyngeal tonsil (adenoid), paired tubal tonsils, paired palatine tonsils, lingual tonsils and tonsils of the soft palate (Nickel et al., 1979; Perry and Whyte, 1998). The palatine and nasopharyngeal tonsils, composed primarily of follicles, are known as follicular tonsils (Nickel et al., 1979). The mucosal layer of follicular tonsils contains numerous invaginations known as fossae or crypts that greatly expand surface area. In the human palatine tonsil it is estimated that the crypt epithelial surface area is 295 cm² (Perry and Whyte, 1998), while in sheep it is estimated that the epithelial surface area of the pharyngeal tonsil and paired palatine tonsils is 23.5 cm² and 7.14 cm², respectively (Casteleyn et al., 2008). Crypts are surrounded by nodular lymphoid tissue creating tonsilar lymphoid follicles (Nickel et al., 1979). In cattle, palatine tonsilar crypts open into a larger tonsilar sinus that communicates with the oral cavity. The paired palatine tonsils are located at the junction of the nasopharynx and oropharynx; ideally positioned to sample antigens entering through either the nasal or oral cavities.

Epithelium not associated with tonsilar lymphoid follicles (non-lymphoepithelial tonsilar epithelium or non-reticular epithelium) of the palatine tonsil is characterized as stratified non-keratinized or parakeratinized epithelium. Within this epithelium there are rare non-epithelial cells or vascular structures. In contrast, epithelium overlying lymphoid follicles (lymphoepithelium, follicle-associated epithelium, or reticular epithelium) (Perry and Whyte, 1998), is characterized by epithelial cells altered in shape and cellular content, infiltrative non-epithelial cells (lymphoid cells, macrophages and dendritic
cells), intraepithelial vasculature, and a disrupted underlying basement membrane (Perry and Whyte, 1998). Reticular epithelium provides a venue for intimate contact between antigens, specialized epithelial cells, intraepithelial lymphocytes and antigen presenting cells, with subsequent antigen uptake and transport. Reticular epithelium in tonsils of some species also contains specialized epithelial cells (M-cells) that allow for selective sampling through endocytosis and transport of luminal antigens with exocytosis to immune cells within or below the mucosal layer. M-cells have been identified in bovine nasopharyngeal tonsils (Schuh and Oliphant, 1992), but not bovine palatine tonsils.

A detailed understanding of the bovine tonsil is important as it represents the first line of defense against foreign antigens entering by either the respiratory or digestive routes. Bovine palatine tonsils play a role in important diseases such as bovine viral diarrhea (Liebler-Tenorio et al., 1997), Mannheimia haemolytica induced pneumonia (Briggs et al., 1998), bovine tuberculosis (Cassidy et al., 1999), and bovine spongiform encephalopathy (Wells et al., 2005). The importance of lymphoepithelial in the pathogenesis of ruminant diseases such as paratuberculosis and tuberculosis has been reviewed (Lugton, 1999). Additionally, the palatine tonsil has been used as a route of exposure in models of tuberculosis in cattle and deer species (Griffin et al., 2006; Palmer et al., 1999a; Palmer et al., 1999b). Detailed studies of bovine tonsillar reticular epithelium have been limited to the nasopharyngeal tonsils (Schuh and Oliphant, 1992), but not bovine palatine tonsils.

2. Materials and methods

2.1. Animals

Six, 7-month-old, healthy Holstein steers were humanely euthanized and palatine tonsils collected and preserved both by snap-freezing in liquid nitrogen-cooled isopentane as well as by immersion in 10% neutral buffered formalin. Formalin-fixed tissues were further processed by routine paraffin-embedding techniques, cut in 5 μm sections, stained with hematoxylin and eosin (HE) or periodic acid Schiff stain (PAS) and examined by light microscopy.

2.2. Immunohistochemistry and morphometry

Frozen sections of palatine tonsil were cut by cryostat in 6 μm sections and processed for immunohistochemistry using primary antibodies to the cell markers B-B7, CD4, CD8, γ/δ T cell receptor (TCR), CD172a, CD11c, CD68, and CD14 (Table 1) as described previously (Frink et al., 2002; Kunkle et al., 1995) using HistoMark Biotin Streptavidin HRP system (Kirkegaard and Perry, Gaithersburg, MD, USA) and 3’,3’-diaminobenzidine-nickel (DAB-Ni peroxidase substrate, Vector Laboratories, Burlingame, CA, USA) as a peroxidase substrate. Non-specific protein binding was blocked using normal goat serum and endogenous peroxidase activity was quenched using 0.3% H2O2 in methanol prior to application of the primary antibody. Digital images of sections from all palatine tonsils were obtained with a light microscope (Nikon Eclipse E800; Nikon Co., Tokyo, Japan) and digital camera (Spot RT, Diagnostic Instruments Inc., Sterling Heights, MI, USA).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primary/secondary antibodies, suppliers and conditions used in immunohistochemical identification of cell markers.</th>
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<tbody>
<tr>
<td><strong>Cellular expression</strong></td>
<td><strong>Isotype</strong></td>
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<tr>
<td>Primary antibody</td>
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<tr>
<td>CD4</td>
<td>T Platt</td>
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<td>CD8</td>
<td>T Platt</td>
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<tr>
<td>Gamma/delta TCR</td>
<td>γ/δ T cells</td>
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<td>CD172a</td>
<td>Mono, gran</td>
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<tr>
<td>CD11c</td>
<td>Mono, mac, NK</td>
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<tr>
<td>CD68</td>
<td>Mono, mac</td>
</tr>
<tr>
<td>CD67</td>
<td>Mono, mac</td>
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<tr>
<td>Secondary antibody</td>
<td>Biotinylated goat anti-mouse IgM</td>
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<tr>
<td>Biotinylated goat anti-mouse IgG (H&amp;L)</td>
<td>Kirkegaard and Perry</td>
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<td>Biotinylated goat anti-mouse IgM</td>
<td>Kirkegaard and Perry</td>
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<tr>
<td>Immunofluorescence</td>
<td>Alexa Fluor 488 goat anti-mouse IgG (H&amp;L)</td>
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<tr>
<td>Alexa Fluor 488 goat anti-mouse IgM</td>
<td>Molecular Probes</td>
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<tr>
<td>Alexa Fluor 488 goat anti-mouse IgG1</td>
<td>Molecular Probes</td>
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<tr>
<td>Alexa Fluor 594 goat anti-mouse IgG (H&amp;L)</td>
<td>Molecular Probes</td>
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RT, room temperature; RTU, ready to use, no dilution required; T Platt, T helper lymphocyte; T Platt, cytotoxic T lymphocyte; Mono, monocytes; Mac, macrophage; NK, natural killer cells; TCR, T cell receptor.
Images were analyzed using Image Pro-Plus (Media Cybernetics, Silver Springs, MD, USA). Ten random regions of reticular epithelium and 10 random regions of non-reticular epithelium were evaluated from each animal. Using image analysis software the area of epithelium was measured as well as the number of stained cells within the measured region of epithelium. The number of cells per unit area was then calculated. Distances were calibrated for each microscope objective using image analysis software and a standard micrometer bar.

Additionally samples of frozen tissue were processed for single or dual labeling immunofluorescence, using primary antibodies to CD11c, CD14 and CD172a and Alexa Fluor (Molecular Probes, Eugene, OR, USA) conjugated secondary antibodies (Table 1). To improve cell visibility, fluorescently labeled sections were coverslipped using mounting media with DAPI (Vectorsheld mounting media with DAPI, Vector Laboratories). Fluorescent images were analyzed using the above Nikon microscope equipped with the VFM Epi-fluorescent attachment with xenon lamp (Nikon, Co.).

2.3. Electron microscopy

Samples for transmission electron microscopy (TEM) were immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4 °C. After 2 h fixation, tissue samples were rinsed in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin. Sections of tonsil were cut at 0.5 μm, stained with toluidine blue, and examined by light microscopy. Ultrathin sections of appropriate areas were cut, stained with uranyl acetate and lead citrate, and examined with a FEI Tecnai 12 Biotwin (FEI company, Hillsboro, OR) transmission electron microscope. Samples for scanning electron microscopy (SEM) were fixed as described above for TEM, rinsed in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in alcohols, critical point dried, mounted on stubs and coated with gold in a sputter-coater. Samples were examined on JEOL 5800LV scanning electron microscope.

2.4. Statistical analysis

Mean values for cell density (cells/1000 μm²) of cells immunoreactive for various surface markers by immunohistochemistry were compared using an unpaired Student’s t-test with Welch’s correction (GraphPad Prism, GraphPad Software, San Diego, CA). A p-value <0.05 was considered significant.

3. Results

3.1. Histology and ultrastructure

Epithelium of the palatine tonsilar crypts ranged from a continuous layer of non-keratinized stratified squamous epithelium (non-reticular epithelium) to areas of epithelium overlying lymphoid follicles which were disrupted by the infiltration of numerous non-epithelial mononuclear cells extending from the lymphoid follicle through the basement membrane effacing normal epithelial architecture (reticular epithelium). Reticular epithelium consisted of epithelial cells separated by wide intercellular spaces forming pockets containing many lymphocytes and lesser numbers of macrophages, plasma cells, neutrophils and cells morphologically compatible with dendritic cells (Fig. 1A). In some regions of reticular epithelium, non-epithelial cell infiltrates extended superficially to the crypt lumen. In these regions the most apical lymphoid cells communicated freely with the lumen or were separated from the lumen by an attenuated cell layer often representing the long cytoplasmic extension of a single cell (as thin as 0.1 μm) (Fig. 1B). Some cytoplasmic extensions spanned up to 50 μm across the luminal surface. Cells forming the luminal border of such pockets were less electron dense than surrounding cells, with a single elongate to angular nucleus, moderate numbers of cytoplasmic microvesicles (0.1–0.4 μm diameter), multivesicular bodies (up to 1.5 μm diameter), endoplasmic reticulum, polyribosomes, mitochondria, irregularly arranged microfilaments (8 nm diameter), and variable numbers of short irregular microvilli (0.1–0.3 μm in length) on the luminal surface (Fig. 1C). These cells often formed desmosomal attachments with surrounding cells of similar morphology or morphology consistent with squamous epithelial cells.

Periodic acid Schiff stained sections as well as electron microscopic imaging demonstrated an intact basement membrane in regions of non-reticular epithelium; however, a highly interrupted and discontinuous basement membrane was present in regions of reticular epithelium (Fig. 2). Lymphocytes and macrophages were numerous in regions of discontinuous basement membrane, presumably migrating from subjacent lymphoid structures to the reticular epithelium. Small blood vessels were seen within reticular epithelium lined by plump rounded epithelial cells (Fig. 3).

Examination of the palatine tonsil by SEM revealed an epithelial surface formed mostly by flat, angular squamous cells. Intermittently other cells, smaller than squamous cells, were found on the epithelial surface. These cells were round to oval and covered by closely packed, knob-like, microvilli (Fig. 4A). These cells were demarcated from surrounding cells by a shallow furrow. Microvillous covered cells were found singly and other times as multiple cells connected by microvillus covered extensions forming a lattice work around squamous cells. Also evident were regions of squamous epithelium disrupted by complete loss of surface cells leaving the underlying non-epithelial cells exposed. These areas likely corresponded to regions seen by light microscopy of reticular epithelium where leukocytes extended through the entire epithelial layer to the crypt lumen (Fig. 4B).

3.2. Immunohistochemistry

Labeling for various surface markers demonstrated reticular epithelium containing moderate to large numbers of CD4+, B-B7+, γ/δ TCR+, CD11c+, CD14+ and CD68+ cells and low numbers of CD8+ and CD172a+ cells. Morphometric analysis revealed differences between reticular and
non-reticular epithelium of greatest magnitude for CD4+, B7-B7+ and CD11c+ cells (Fig. 5), although reticular epithelium also contained significantly more γ/δ TCR+,

CD172a+ cells than did non-reticular epithelium. In contrast, reticular epithelium contained fewer CD8+ cells than non-reticular epithelium. Cells immunopositive for the above surface antigens were generally scattered in varying numbers within all levels of reticular epithelium (Fig. 6). In slight contrast, γ/δ TCR+ cells, while present in low to moderate numbers throughout the epithelial layer, were most numerous in the basal layer of reticular epithelium (Fig. 7).

Low numbers of round to stellate cells within the reticular epithelium stained positive for CD68 (Fig. 8A), a marker that has been associated with macrophages. In contrast, numerous cells within the reticular epithelium including cells with squamous morphology stained positive for the marker CD14 (Fig. 8B), also associated with macrophages (Fig. 8). Interestingly, CD14 staining of squamous cells was more pronounced in reticular epithelium compared to non-reticular epithelium (Fig. 8C). The markers CD172a and CD11c have been previously asso-

![Fig. 1. Section of palatine tonsil from a 7-month-old steer. (A) Note reticular epithelium characterized by infiltrates of lymphocytes and other non-epithelial cells. Flattened and angular cells form pockets (asterisks) that contain non-epithelial cells. In some cases non-epithelial cells are separated from the crypt lumen only by a thin cytoplasmic process (arrow). H/E. Bar = 10 μm. (B) Electron micrograph of palatine tonsil showing pockets (asterisks) within reticular epithelium, the luminal surface of which is covered by flattened electron lucent cells (arrows). (C) Higher magnification of flattened cell with elongate nucleus, intracytoplasmic vesicles (small arrows) and numerous microvilli (large arrows). Bar = 2 μm.](attachment:image1.png)

![Fig. 2. Section of palatine tonsil from a 7-month-old steer. Junction of reticular and non-reticular epithelium demonstrates intact PAS positive basement membrane of non-reticular epithelium (arrows) and interrupted basement membrane of reticular epithelium (left half of image) and numerous infiltrative mononuclear non-epithelial cells. PAS. Bar = 25 μm.](attachment:image2.png)

![Fig. 3. Section of palatine tonsil from a 7-month-old steer. Within reticular epithelium are small caliber blood vessels lined by plump endothelial cells (arrows). PAS. Bar = 10 μm.](attachment:image3.png)
associated with dendritic cells (Miyazawa et al., 2006). Within reticular epithelium, more cells stained positive for CD11c than CD172a; however, double labeling revealed that many cells staining for CD172a also stained positive for CD11c (Fig. 9A and B). CD172a+ cells occasionally were arranged in clusters within reticular epithelium (Fig. 9C). Dual labeling using antibodies to CD172a and CD14 or CD11c and CD14 revealed cells within the reticular epithelium that stained for CD172a, CD11c and CD14 singly as well as both CD172a and CD14 or CD11c and CD14 (data not shown).

4. Discussion

The intestinal Peyer’s patches represent the epitome of follicle-associated epithelium where reticular epithelium is composed of M-cells, lymphoid cells and normal epithelium. Luminal antigens, including microbes, are endocytosed and transported through M-cells and discharged into the intercellular space where dendritic cells, macrophages, T and B-lymphocytes, plasma cells and neutrophils are found. Luminal antigens are then transported by lymphoid cells or dendritic cells to the underlying lymphoid follicle for antigen presentation and initiation of an immune response. Other characteristics of lymphoepithelium include disrupted basement membrane, desquamation of upper epithelial layers, and infiltration of small intraepithelial blood vessels (Perry, 1994).

Histology and ultrastructure of bovine palatine and nasopharyngeal tonsils has been said to be similar to humans (Rebelatto et al., 2000). Most studies of the bovine palatine tonsils have described the tonsil as a whole and not focused specifically on the epithelium (Cocquyt et al., 2007, 2008; Manesse et al., 1998; Rebelatto et al., 2000; Velinova et al., 2001). The present study demonstrates that bovine palatine tonsilar reticular epithelium has many of the same characteristics of reticular epithelium in other anatomic locations such as intestinal Peyer’s patches. Similar to findings in other species, bovine palatine tonsilar reticular epithelium contains large numbers of B-B7+ B-cells and CD4+ T-cells (Gebert and Pabst, 1999). Moreover, proportions of key immune cells such as B-cells, CD4+, CD8+ and γ/δ TCR+ T-cells were found to be similar in palatine tonsilar reticular epithelium to that described for bovine Peyer’s patches and follicle-associated epithelium of ovine jejunum (Press et al., 1991), and is in agreement with other studies of the bovine palatine tonsil as a whole (Rebelatto et al., 2000). Additionally, the present study demonstrates that bovine palatine tonsilar reticular epithelium is characterized by an interrupted basement membrane and intraepithelial vasculature, characteristics of reticular epithelium in other tissues.

CD172a and CD11c have been demonstrated on a subset of bovine dendritic cells (Brooke et al., 1998; Renjifo et al., 1997), although macrophages may also express CD172a and CD11c, while some T-cells and B-cells may also express CD11c (Miyazawa et al., 2006). Bovine dendritic cells in peripheral blood are positive for both CD11c and CD172a expression, as are cells within the bovine thymus postulated to be dendritic cells of myeloid lineage (Miyazawa et al., 2006). In the present study, although the reticular epithelium contained greater numbers of CD11c+ cells compared to CD172a+ cells, dual labeling identified cells that expressed both markers, similar to putative dendritic cells in the bovine thymus (Miyazawa et al., 2006).

CD172a, also known as signal regulatory protein (SIRP) α, macrophage fusion receptor, or SHPS-1 is a transmembrane regulatory protein expressed primarily by myeloid cells (i.e., macrophages, monocytes, dendritic cells, granulocytes, myeloid progenitors), hematopoietic stem cells, and neurons (Barclay and Brown, 2006; van Beek et al., 2005). CD172a is essential for leukocyte trafficking through functional binding to the cell-associated ligand, CD47 (Liu et al., 2002; Vignery, 2005; Zen and Parkos, 2003). A population of cells of myeloid origin, with morphologic characteristics of monocytes, has been identified in both bovine spleen and peripheral blood. These cells express CD172a, CD11c and CD14, and have
been shown to produce nitric oxide in response to stimulation with heat killed *Mycobacterium bovis* BCG or *Babesia bovis* merozoites (Bastos et al., 2007).

CD14 and CD68 are both associated with macrophages; however, in the current study immunolabeling for these two markers yielded dissimilar patterns of expression. Although CD68 labeling was seen among scattered cells within the follicle, interfollicular regions, and epithelium, as would be expected of macrophages, CD14 expression was largely limited to polygonal or flattened cells of the reticular epithelium with little or no labeling of cells within the non-reticular epithelium. Distinct staining patterns for these two markers is not unexpected. CD68 is a glycoprotein associated with lysosomes in macrophages, myeloid cells and some neoplastic mononuclear cells and has previously been shown to label bovine macrophages including those found in tonsilary lymphoid follicles (Ackermann et al., 1994). In contrast, CD14 is a receptor for bacterial envelope constituents such as LPS (Pugin et al., 1994). Given the constant exposure of tonsilar epithelium to bacteria, labeling of cells within the reticular epithelium may represent expression of CD14 in response to exposure to bacterial constituents.

The presence of increased numbers of γδ TCR+ cells in palatine tonsilar reticular epithelium in the present study is consistent with previously held beliefs that γδ TCR+ cells localize preferentially to mucosal tissues and skin serving as a first line of defense (Hein and Mackay, 1991). In contrast to the present study, previous work with bovine tonsils demonstrated many CD8+ and γδ TCR+, and few CD4+ cells in tonsilar epithelium (Manesse et al., 1998). A separate study in adult cattle, showed >50% of all CD2+ cells to be CD8+ in the nasopharyngeal tonsil epithelium (Schuh and Oliphant, 1992). The authors hypothesize that increased numbers of CD8+ T-cells in the tonsil epithelium suggests a role for such cells that could include destruction of cells that have bound or ingested antigens (Manesse et al., 1998). A possible explanation for the
discrepancies between these studies may be different states of activation based on different antigen exposure in calves from the two studies, different ages of calves, or the focus on different tonsils, nasopharyngeal versus palatine. Alternatively, the present study and those by Manesse et al. and Schuh and Oliphant all used different antibodies to the CD8 surface marker to identify CD8+ cells. It is possible that each of the three antibodies labeled a different subset of CD8+ cells.

Typically, M-cells have characteristic short microvilli or microfolds on their luminal surface, lack cilia and mucus containing vesicles. Ultrastructural descriptions of M-cells include characteristics such as large euchromatic nuclei, prominent nucleoli, fine cytoplasmic filaments, and endocytic vesicles (Knop and Knop, 2005). Microvillus surface projections, the structural feature from which M-cells derive their name, usually differ from that of surrounding cells; however, their exact morphology varies in different organs and can vary in different regions of the same organ. Therefore, the presence of microvilli is not sufficient to identify cells as M-cells (Liebler-Tenorio and Pabst, 2006; Paar et al., 1992). Indeed, evidence of endocytosis and antigen transport is required to definitively identify cells as M-cells (Gebert and Pabst, 1999).
One possible reason M-cells have not been identified in bovine palatine tonsil is the variable morphology of M-cells between different anatomic sites. M-cells in the bovine nasopharyngeal tonsil, with its ciliated pseudostatified columnar epithelium, resemble classic M-cells of ileal dome follicle-associated epithelium. M-cells with similar classic morphology have not been identified in the bovine palatine tonsil with its associated stratified squamous epithelium. M-cells have been identified in other species in mucosal sites with a stratified squamous epithelium such as conjunctiva (Knop and Knop, 2005; Latkovic, 1989; Liu et al., 2005; Meagher et al., 2005). Light and electron microscopic descriptions of those M-cells are similar to those reported in the present study (Knop and Knop, 2005). M-cells of the rabbit and human palatine tonsil have been shown to form pockets that contain intraepithelial lymphocytes and are separated from the crypt lumen by an attenuated extension of M-cell apical cytoplasm (Gebert, 1997; Howie, 1980), morphologically similar to the arrangement seen in the present findings. The formation of a thin rim of apical cytoplasm separating lymphocytes from the crypt lumen shortens the passage of transcytosed antigen.

Human tonsils have been described as the “Peyer’s patches” of the upper respiratory tract (Bernstein et al., 1999), where they serve as a site for antigen uptake and processing leading to the generation of antigen specific T and B cell responses. According to the dogma of the common mucosal immune system, these antigen specific lymphocytes migrate to other mucosal sites or back to the tonsil. Demonstration of bovine palatine tonsilar reticular epithelium with cells morphologically consistent with M-cells of other species, and infiltrated with a population of lymphoid and other mononuclear cells similar to that seen in Peyer’s patches, suggests that the bovine palatine tonsil is an important site for antigen uptake and initiation of immune response. A better understanding of palatine tonsilar epithelium will be important in elucidating the pathogenesis of various bovine disease where tonsils play a critical role as well as aiding in the development of vaccines that could target palatine tonsilar M-cells for efficient vaccine uptake and processing.

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