

Distribution of Dendritic Cells in Normal Human Salivary Glands

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Received March 3, 2011; accepted June 13, 2011; published online July 20, 2011

Dendritic cells (DC) are believed to contribute to development of autoimmune sialadenitis, but little is known about their distribution in normal salivary glands. In this study, DC were identified and their distribution was determined in normal human parotid and submandibular glands. For light microscopy, salivary gland sections were stained with H&E or immunocytochemically using antibodies to DC markers. Transmission electron microscopy (TEM) was used to evaluate the ultrastructural characteristics of DC. In H&E sections, elongated, irregularly shaped nuclei were occasionally seen in the striated and excretory duct epithelium. Immunolabeling with anti-HLA-DR, anti-CD11c and anti-S100 revealed DC with numerous processes extending between ductal epithelial cells, often close to the lumen. Morphometric analyses indicated that HLA-DR-positive DC occupied approximately 4–11% of the duct wall volume. Similar reactive cells were present in acini, intercalated ducts and interstitial tissues. TEM observations revealed cells with indented nuclei containing dense chromatin, pale cytoplasm with few organelles, and lacking junctional attachments to adjacent cells. These results indicate that DC are abundant constituents of normal human salivary glands. Their location within ductal and acinar epithelium suggests a role in responding to foreign antigens and/or maintaining immunological tolerance to salivary proteins.

Key words: antigen presenting cells, immunocytochemistry, electron microscopy, parotid gland, submandibular gland

I. Introduction

Dendritic cells (DC) are antigen presenting cells (APC) that play a major role in the control of immune responses. DC affect immune responses via secretion of cytokines and exposure of naïve B- and T-lymphocytes to antigens. They process and display foreign antigens on their cell surface with the major histocompatibility complexes I and II (MHC I and MHC II). Antigen exposure along with stimulation by various cytokines and other factors induces immature DC to undergo phenotypic and functional changes from efficient antigen-capturing cells to APC, and triggers their migration to secondary lymphoid organs, where they recruit and

stimulate T-cells by releasing cytokines and chemokines such as interleukin (IL)-12, IL-8, and macrophage inflammatory protein (MIP)-1 α and β , and by upregulating a variety of accessory and costimulatory molecules. Dendritic cells also play an important role in the induction of T-cell tolerance to self-antigens. Central tolerance occurs in the thymus by deletion of developing T-cells. Peripheral tolerance occurs in lymphoid organs by the induction of anergy or deletion of mature T-cells [3, 4, 10, 47].

The function of the salivary glands in the mucosal immune system is well established, especially their role in the secretion of immunoglobulins produced by local plasma cells [6, 31]. In addition to immunoglobulin producing plasma cells, macrophages, DC and T-cells are present in the salivary glands of rodents and humans [8, 18, 24, 38, 44, 51]. In rat salivary glands, macrophages and DC are present in the interstitial tissues [8, 44] and within the epithelium of the intra- and interlobular ducts [44]. Macrophages isolated from rat salivary glands are able to

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efficiently present antigens to naïve CD4⁺ T-lymphocytes [37]. Organized lymphoid tissue is present around the ducts of minor salivary glands [32] and the main excretory duct of monkey parotid gland [26]. In the latter study, lymphocytes and DC were the predominant intraepithelial immune cells, located in close proximity to other DC, intraepithelial lymphocytes and epithelial cells. DC previously have been shown to be present in the interstitial connective tissue of normal human major [51] and minor [18] salivary glands.

The possible role of APC and T-cells in the development of Sjögren's syndrome (SS) has generated considerable interest [57]. Previous studies have shown that DC infiltrate the submandibular gland of nonobese diabetic mice, a model of SS, before lymphocytic infiltration and may play a role in the initiation of SS [55]. Dendritic cells also have been demonstrated in salivary glands of humans with SS and other autoimmune diseases [16, 25, 27, 35, 36, 56, 58, 59], and both DC and macrophages have been implicated in the pathogenesis of SS [25, 36, 56, 58, 59].

The objectives of this study were to determine the distribution of DC in normal human major salivary glands using immunohistochemistry, and to characterize the morphology of DC in the glands by transmission electron microscopy (TEM). We show that DC are abundant constituents of normal glands, and are present within the epithelium of ducts and acini, in addition to the interstitial connective tissue.

II. Materials and Methods

Tissue samples

Normal human salivary gland tissues were obtained from 17 consenting patients undergoing surgery in the Otorhinolaryngology Clinic at the University of Cagliari, Italy. All procedures were approved by the Human Experimentation Committee at the University of Cagliari, and the use of the tissues at the University of Connecticut Health Center was approved by the Institutional Review Board. The samples were fixed in the operating suite and subsequently shipped by express carrier to Connecticut for further processing. In some cases processing and embedding for TEM was completed in Cagliari before shipping.

Light microscopy and immunohistochemistry

Small pieces of glandular tissue were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and stored in 1% buffered paraformaldehyde. The tissues were processed for paraffin embedding by standard methods and 5 μ m sections were cut and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed manually using sections collected on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Antigen retrieval with 5% urea/50 mM β -mercaptoethanol or 10 mM citrate buffer, pH 6.0, containing 0.05% Tween 20, at 95° was performed following paraffin removal. The sections were treated with 0.3% H₂O₂ in 85% methanol, and non-specific binding was blocked with 1% bovine serum albumin

(BSA)/5% normal goat serum (NGS) in phosphate buffered saline (PBS). Sections were incubated with the following primary antibodies: mouse monoclonal anti-human HLA-DR, specific for the MHC II α -chain (DakoCytomation, Carpinteria, CA, USA); rabbit polyclonal anti-human CD74, which recognizes the MHC II invariant chain (Sigma Prestige Antibodies, St. Louis, MO, USA); rabbit monoclonal anti-human CD11c, a marker for cells of the myeloid lineage (Epitomics, Burlingame, CA, USA); CD123, a marker for plasmacytoid dendritic cells (R&D Systems, Minneapolis, MN); and rabbit polyclonal anti-bovine brain S100, a calcium binding protein found in DC and other cells (Serotec, Raleigh, NC, USA). The bound primary antibodies were detected with a biotin-labeled secondary antibody, and visualized using the VectaStain avidin-biotin-complex (ABC) reagent (Vector Laboratories, Burlingame, CA, USA) and peroxidase substrate solution (either diaminobenzidine-H₂O₂ or Vector NovaRed substrate [Vector Laboratories]). The sections were counterstained with hematoxylin, dehydrated, and coverslips were mounted with DPX (Electron Microscopy Sciences, Hatfield, PA, USA). Immunocytochemical controls included omission of the primary antibody or substitution of the primary antibody with non-immune IgG or an irrelevant antibody.

The sections were photographed in a Leitz Orthoplan brightfield microscope using a Nikon Coolpix 5000 digital camera. Images were transferred to a Macintosh Power PC G4 computer, and background highlight levels and contrast were adjusted in Adobe Photoshop (versions 6.0.1 or 7.0.1; Adobe Systems Inc., San Jose, CA, USA).

The volume percentage of DC in the epithelium of the striated and excretory ducts was determined by a point counting method. Images of ducts in sections of 3 different submandibular and 2 different parotid glands immunostained with anti-HLA-DR antibody were opened in Photoshop and a grid pattern was superimposed on the image. The number of grid intersections lying over HLA-DR-positive DC divided by the total number of intersections lying over the duct wall, multiplied by 100, equaled the volume percentage of the duct wall occupied by DC.

Transmission electron microscopy (TEM)

Morphological characteristics of DC were evaluated in thin sections of plastic embedded human salivary gland samples examined by TEM. Two different submandibular gland samples and 2 parotid gland samples were utilized. The tissues were fixed in 1.25% glutaraldehyde/1.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, postfixed in 2% osmium tetroxide, treated with 0.25% aqueous uranyl acetate, dehydrated in acetone and embedded in Epon resin. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined and photographed in a JEOL 100CX or Philips CM10 TEM. The developed negatives were scanned with an Epson Perfection V750 Pro scanner, and levels and contrast were adjusted in Photoshop.

III. Results

Light microscopy

Light microscopic examination of striated and excretory ducts in H&E stained sections of parotid and submandibular glands revealed the presence of occasional elongated or irregularly shaped nuclei within the simple columnar or pseudostratified epithelial lining of the duct wall (Fig. 1). These nuclei typically were located basal to the nuclei of the columnar epithelial cells, but above the basal surface of the duct, and often extended horizontally along the duct wall. Distinct cell borders were not visible and the cytoplasm of these cells was not discernable from that of the surrounding epithelial cells. Myoepithelial cells also may be present in striated and excretory ducts of human salivary glands [7, 11]; their nuclei typically were located along the basal surfaces of the ducts (Fig. 1).

Immunohistochemistry

Dendritic cells express high levels of MHC II [3], therefore immunohistochemistry with anti-HLA-DR antibody was used to identify DC in the salivary gland sections. Numerous reactive cells with a dendritic morphology were seen in the ducts, acini and interstitial tissues of the parotid and submandibular glands (Fig. 2A–C; Fig. 3A–C). In sections incubated with the anti-CD11c antibody (Fig. 3D), a marker of cells derived from the myeloid lineage, reactive cells were distributed similarly, although in general, fewer cells were labeled. Like the HLA-DR-positive cells, many of the CD11c-positive cells had a dendritic morphology. In several instances, incubation of one section with anti-HLA-DR and the adjacent section with anti-CD11c revealed labeling of the same cell with both antibodies (Fig. 3E and F; Fig. 3G–I). In the striated and excretory ducts, the processes of the DC were located in between the epithelial cells, occasionally extending more than 20 μm along the duct wall (Fig. 2A and B). The DC processes often were located basally in the duct epithelium, but processes also could be found apical to the duct cell nuclei (Fig. 2A and C; Fig. 3A–C), sometimes close to the luminal surface. Occasionally, multiple processes extended toward the lumen, creating a “pitchfork” appearance (inset, Fig. 2A and B). Dendritic cells also were present in intercalated ducts, but these were observed less frequently. In the acini, DC were located in the intercellular spaces between adjacent acinar cells (Fig. 2A and B). The size, shape, location and reactivity of these cells clearly distinguished them from the acinar and myoepithelial cells. Dendritic cells also were distributed throughout the interstitial tissues (Fig. 2A and B; Fig. 3B, D and F).

Occasionally, some acinar and duct epithelial cells showed reactivity with the anti-HLA-DR and anti-CD11c antibodies, and blood vessel endothelium was frequently labeled by the anti-HLA-DR antibody (Fig. 2A and B; Fig. 3F).

The distribution of DC in the submandibular and parotid glands was similar, although intraepithelial DC were more

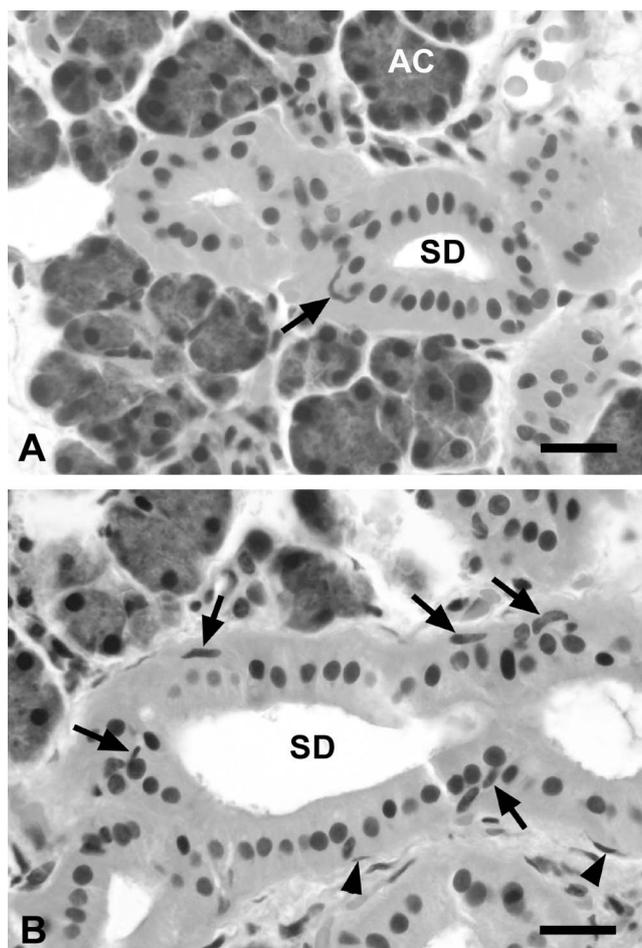


Fig. 1. Light microscopy of human major salivary gland striated ducts (SD) stained with H&E. Bars=20 μm . **A**, parotid gland. **B**, submandibular gland. Arrows indicate nuclei of probable DC within the duct epithelium. Arrowheads indicate nuclei of possible myoepithelial cells at the basal surface of the duct. AC, acini.

frequent in the submandibular gland. The volume percentage of cells labeled with the anti-HLA-DR antibody in striated and excretory ducts was determined using a point counting method. In the submandibular gland, HLA-DR-positive DC constituted $10.6 \pm 1.9\%$ of the volume of the duct wall. In the parotid gland, the proportion of the duct wall occupied by DC was $4.5 \pm 0.9\%$.

Anti-S100 reactivity was present in cells with a dendritic morphology in the striated duct epithelium (Fig. 3J–L). Only a few S100-reactive cells were observed in the excretory ducts. As noted by other investigators, peripheral nerves and serous acinar cells also labeled with the anti-S100 antibody [12, 17, 33]. In sections incubated with the anti-CD74 (MHC II invariant chain) antibody, fewer reactive DC were observed, and duct epithelial cells frequently were labeled (Fig. 3M). Labeled DC were present in the duct epithelium (Fig. 3M) and in the acini (Fig. 3N), as well as scattered throughout the interstitial tissues (Fig. 3N). Occasional small inflammatory foci, consisting mainly of lymphocytes, were seen near small ducts in some

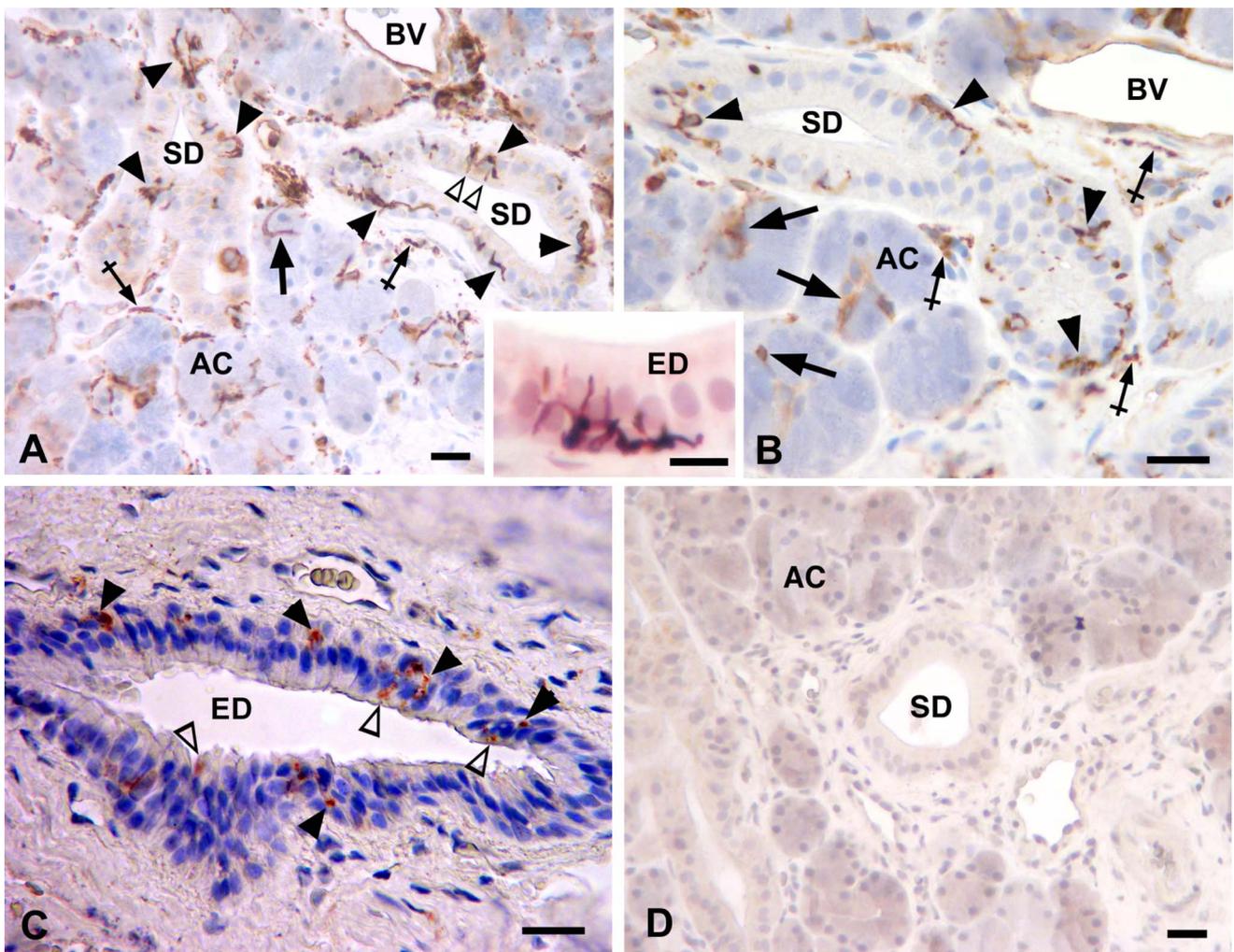


Fig. 2. Light microscopic immunohistochemistry with anti-HLA-DR. Bars=20 μ m; 10 μ m (inset). **A** and **B** and inset, submandibular gland; **C**, parotid gland. Black arrowheads indicate examples of labeled DC in the epithelium of striated (SD) and excretory (ED) ducts. Open arrowheads indicate labeled DC processes near the luminal surface of the ducts. Black arrows indicate DC within acini (AC). Basally located DC often extend multiple processes toward the lumen (inset, panels **A** and **B**). Numerous labeled DC are present in the interstitial tissue (small crossed arrows); around the acini it often is difficult to tell if these cells are located in the interstitium or within the acini. Note reactivity of blood vessel (BV) endothelium. **D**, submandibular gland, non-immune IgG control.

of the otherwise normal glands. These foci also contained cells labeled with anti-CD74, as well as with anti-HLA-DR and anti-CD11c (data not shown).

Antibody to CD123, a marker of plasmacytoid DC, was used in an attempt to further characterize the reactivity of the salivary gland DC. Plasmacytoid DC recognize nucleic acids and produce large amounts of type I interferon in response to viral infections [9]. No reactive DC were seen in sections of normal glands incubated with antibody to CD123. A few labeled cells were observed in a degenerating area of one gland, suggesting that plasmacytoid DC may have entered the gland in response to autoimmune sialadenitis (data not shown).

In control sections, no labeling was seen when the primary antibody was omitted from the labeling sequence, or when non-immune IgG or an irrelevant antibody was substituted for the primary antibody (Fig. 2D).

Electron microscopy

Examination of thin sections of the parotid and submandibular glands revealed the presence of small cells with the characteristics of DC within the epithelium of the ducts and acini (Figs. 4 and 5). The nucleus typically was indented and had dense peripheral heterochromatin. The cytoplasm contained relatively few organelles, i.e. mitochondria, short cisternae of endoplasmic reticulum, a few dense bodies, and cytoskeletal filaments. Birbeck granules, characteristic of Langerhans cells [5], were not seen in the salivary gland DC. No intercellular junctions, such as tight junctions, desmosomes or gap junctions, were observed between the DC and adjacent epithelial cells. The intercellular space surrounding the cell bodies of DC often appeared somewhat widened, possibly due to the lack of intercellular junctions and subsequent shrinkage of the cell during fixation and processing. Occasionally, apparent proc-

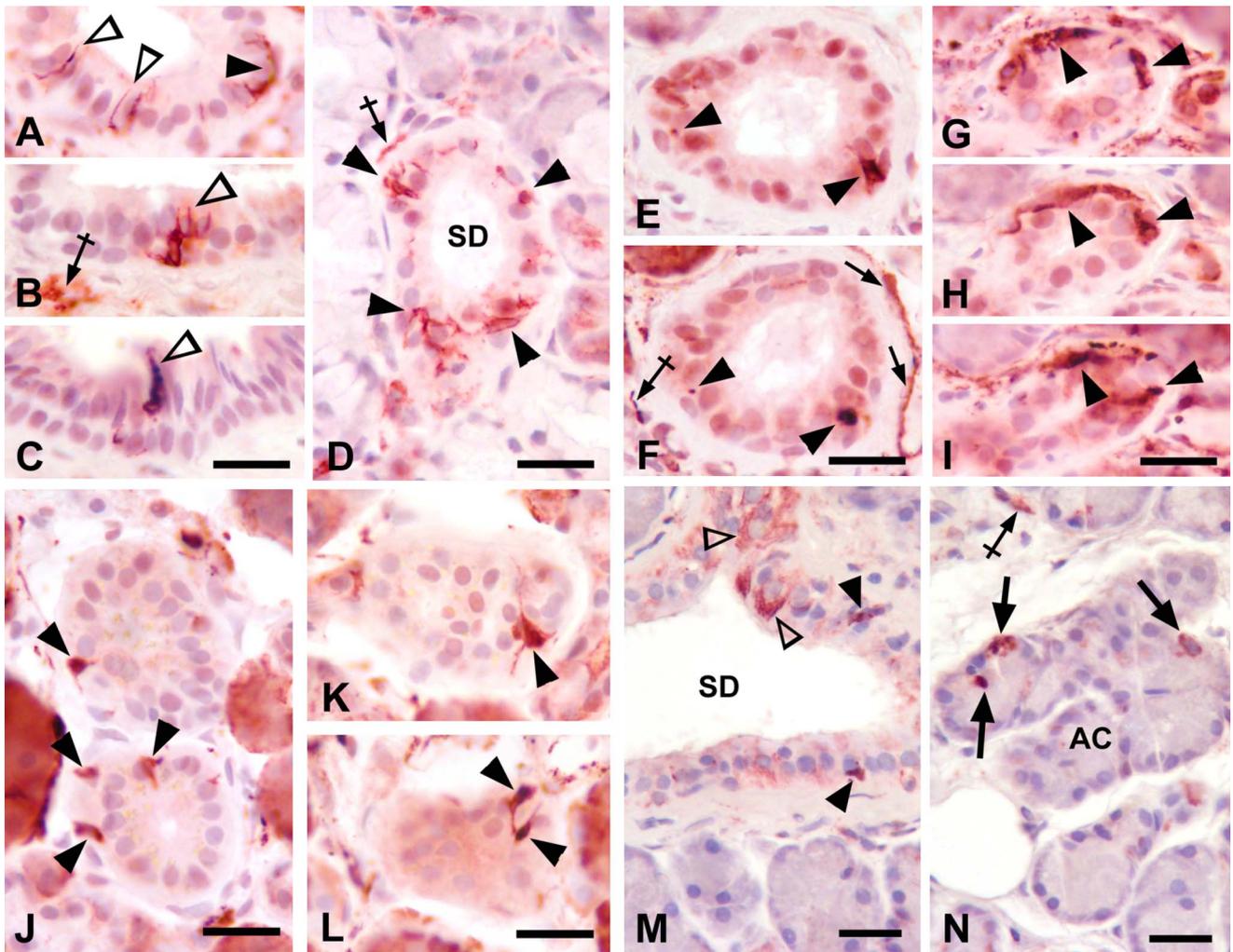


Fig. 3. Light microscopic immunohistochemistry. Bars=20 μ m. **A** and **C**, parotid gland, **B**, submandibular gland; anti-HLA-DR. Labeled processes (open arrowheads) of DC extend toward the lumen of the ducts. A basally located DC (black arrowhead) also is seen in panel **A**, and interstitial DC are seen in panel **B** (small crossed arrow). **D**, submandibular gland, anti-CD11c. Several labeled DC are present within the epithelium of a striated duct. A few labeled DC are present in the interstitium (small crossed arrow). **E** and **F**, submandibular gland. A striated duct is seen in adjacent sections incubated with anti-CD11c (**E**) and anti-HLA-DR (**F**). The same cell (right arrowheads) and process (left arrowheads) are positive for both CD11c and HLA-DR. The small arrows in **F** indicate HLA-DR-positive endothelium of a small blood vessel. The small crossed arrow indicates a labeled DC in the interstitium. **G**, **H**, and **I**, submandibular gland. A small striated duct is seen in three adjacent sections incubated with anti-HLA-DR (**G**), anti-CD11c (**H**) and anti-HLA-DR (**I**). The same cells (arrowheads) show reactivity for both HLA-DR and CD11c. **J**, **K** and **L**, submandibular gland, anti-S100. Reactive cells, some with long processes, are present in the epithelium of striated ducts. Nerve bundles in the interstitial tissue show reactivity, and acinar cells also are labeled. **M** and **N**, submandibular gland, anti-CD74. A few reactive DC are present in the ducts (black arrowheads) and acini (arrows). Some duct epithelial cells (open arrowheads) are labeled. The small crossed arrow indicates a labeled DC in the interstitium.

esses of the DC were seen extending away from the cell body, often along the base of the duct or acinus (Fig. 5).

IV. Discussion

The results of this study indicate that cells with the morphology and immunohistochemical characteristics of dendritic cells are present in substantial numbers within the epithelium and interstitial tissues of normal human salivary glands. Although previous light microscopic studies have

shown that DC are present in the interstitium of normal human glands [18, 51], their presence within the epithelium of the ducts and acini has not been described previously. The DC were consistently labeled with antibody to HLA-DR, indicating the expression of MHC II, a characteristic, although not specific, marker of DC. Both intraepithelial and interstitial DC were labeled. The DC also labeled with antibody to CD11c, a marker of the myeloid lineage that differentiates myeloid or classical DC from plasmacytoid DC [9, 22, 30, 41]. As tissue macrophages also may express

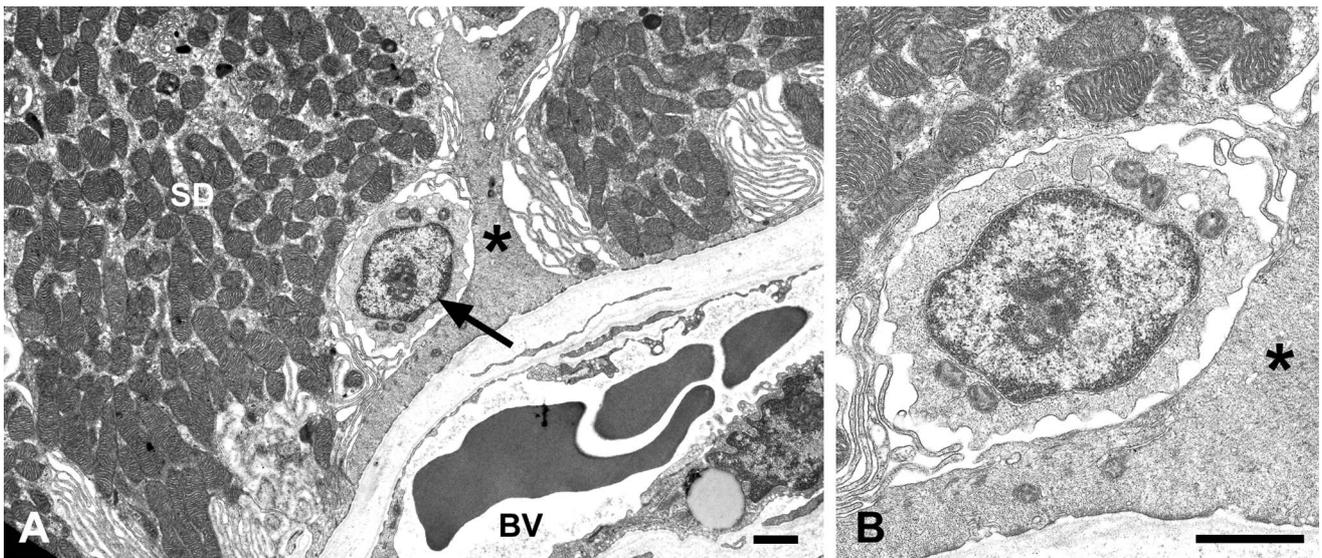


Fig. 4. Electron micrographs of submandibular gland striated duct. Bars=1 μ m. **A**, probable DC (arrow) located in the basal region of the duct epithelium (SD). **B**, higher magnification of cell in panel A showing fewer organelles and mitochondria than adjacent epithelial cells, and absence of intercellular junctions. The cell marked with an asterisk is either a myoepithelial cell or the extended process of a basal duct cell. BV, blood vessel.

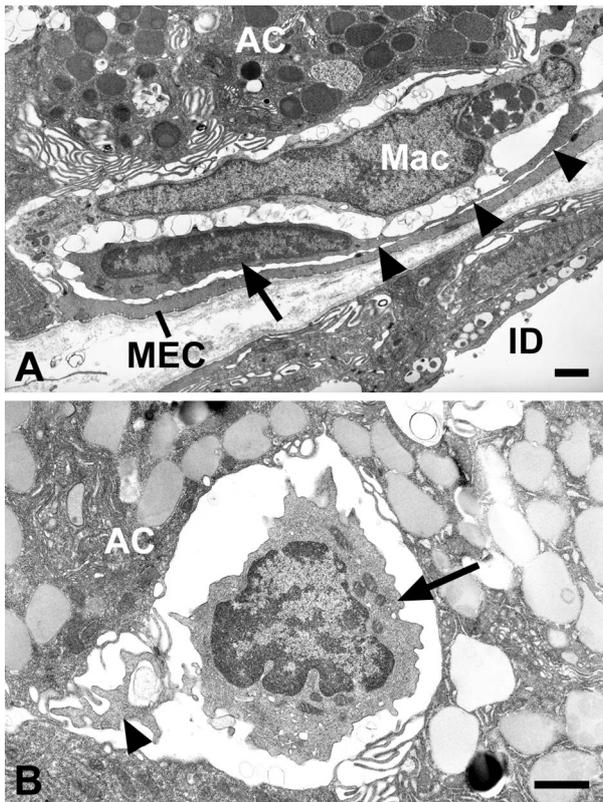


Fig. 5. Electron micrographs of acini. Bars=1 μ m. **A**, submandibular gland. A probable DC (arrow) with an elongated nucleus and a long process (arrowheads), and a macrophage-like cell (Mac) are enclosed by a myoepithelial cell process (MEC) at the base of the acinus. ID, intercalated duct. **B**, parotid gland. A probable DC (arrow) with a dense, indented nucleus, few organelles and a portion of its process (arrowhead), is surrounded by acinar cells (AC).

CD11c, some of the labeled cells may be macrophages rather than DC. However, many of the CD11c-positive cells exhibited a dendritic morphology, and by examination of serial sections were shown to also be HLA-DR-positive. Antibody to CD74 labeled intraepithelial and interstitial cells, but fewer labeled cells were observed than with anti-HLA-DR or anti-CD11c. Cells with a dendritic morphology located in the ductal epithelium also labeled with anti-S100. Abundant labeling for S100 was observed in the interstitium, however most of this appeared to be due to the reactivity of nerve fibers [12]. Although these markers are not specific for DC, and may be expressed by macrophages [14] and other cell types, their expression by cells with typical dendritic morphology strongly suggests that the observed cells are in fact DC.

The presence of interstitial DC in normal rodent [8, 24, 44, 46] salivary glands has been demonstrated previously. We also showed that DC are present within the epithelium of ducts in rat salivary glands [44]. The present results indicate that intraepithelial DC are commonly found in the acini and ducts of normal human major salivary glands. Morphometric analyses indicate that DC may constitute as much as 10% of the cellular volume of the wall of the striated and excretory ducts. This large number of cells suggests that salivary gland DC play a significant role in the normal function of these organs.

Epithelial cells of the glands occasionally exhibited reactivity with the antibodies used in this study. With the anti-HLA-DR and anti-CD11c antibodies, occasional acinar cells were labeled, and a very faint labeling of a few duct cells was seen. Labeling of salivary gland duct cells with antibodies detecting MHC II epitopes has been reported in normal rodent salivary glands [29, 46], and in animal models of SS [43]. More extensive salivary epithelial expression of

MHC II antigens may be induced in various experimental conditions, such as interferon- γ treatment [46] and in estrogen-deficient mice [1]. Epithelial MHC II expression has been described in normal human salivary glands [20, 51, 52] and is increased in glands of SS patients [2, 13, 15, 18, 49, 50, 52] and patients with various connective tissue diseases [28]. It is assumed that MHC II-positive epithelial cells are capable of presenting antigens to T-cells, but it remains uncertain whether the increased epithelial expression of MHC II precedes or is a consequence of autoimmune disease.

We also observed HLA-DR labeling of the endothelium of small blood vessels. Previous studies have demonstrated endothelial expression of MHC II in normal salivary glands [51], and in glands of SS patients [2, 18]. Endothelial cell MHC II expression is upregulated by interferon- γ [39, 46], and is increased in several autoimmune diseases [53].

The ultrastructure of DC in human salivary glands is similar to that described for DC in other tissues, such as the rat kidney [19], incisor enamel organ [48], salivary glands [44] and human bronchi [42]. The nuclei usually are oval or elongated in shape, often with one or more indentations. Their cytoplasm contains fewer organelles than acinar or duct cells, but occasionally includes dense lysosome-like bodies and small phagocytic vacuoles. Unlike Langerhans cells found in skin, oral and vaginal mucosa, salivary gland DC lack Birbeck granules, which have been shown to be part of the endosomal system [54].

Salivary gland DC apparently lack intercellular junctions such as tight junctions and desmosomes, typical of most epithelial tissues. They also lack gap junctions, commonly found between salivary gland acinar cells and between intercalated duct cells. The absence of intercellular junctions most likely accounts for the widened intercellular spaces between the DC and the adjacent epithelial cells. In contrast, DC present in the intestinal epithelium have been shown to extend processes to the luminal surface, where they express tight junction proteins and form tight junction-like structures with the adjacent epithelial cells [40]. These intestinal DC are thought to sample the luminal contents and to bind and internalize pathogenic microorganisms without disruption of the epithelial barrier. The formation and extension of these DC processes requires the expression of the chemokine, fractalkine (CX3CL1), by the intestinal epithelial cells, and the expression of the fractalkine receptor, CX3CR1, by the DC [34]. The ducts and acini of human salivary glands express fractalkine, although variably [23]. It remains to be determined if the recruitment of DC to the ducts and acini of the salivary glands and their migration into the epithelium occurs in response to fractalkine expression. Although we did not observe DC processes exposed at the luminal surface of the salivary gland ducts, HLA-DR-reactive DC processes frequently were seen extending toward the lumen, in some cases within 2–3 μm of the surface.

The location of salivary DC within the glandular epithelium would appear to be optimal for the major

functions of these cells, immune surveillance, T- and B-cell activation to initiate an immune response, and the development and maintenance of tolerance to self antigens. Ascending infections of the salivary gland ducts could expose DC within the epithelium of the main duct and probably large interlobular ducts to microorganisms and their products. The organized, duct-associated lymphoid tissue (DALY) around the oral openings of the excretory ducts [26, 32], as well as a more diffuse distribution of immune cells along the parotid main duct [21], are important in the local response to an infection. Whether or not ascending infectious material would reach the smaller interlobular excretory ducts and intralobular striated ducts, which are well-endowed with DC, is unknown. In these locations and in the acini, maintenance of tolerance to the numerous cellular and secreted molecules produced by the acinar and ductal epithelial cells may be an important function of the DC. Uptake of self-antigens, e.g., from apoptotic cells or from leakage of secretory products through tight junctions to the intercellular spaces, would lead to presentation of the antigens to naïve circulating T-cells, and result in the development of anergy or deletion of autoreactive cells, or the induction of suppressor regulatory T-cells [45, 57]. In the development of autoimmune diseases, such as SS, the local cytokine environment may lead to dysregulation of salivary gland DC, with subsequent maturation and stimulation of autoreactive T-cells [57].

In summary, DC are common constituents of normal human salivary glands. They are present in the epithelium of the acini and ducts, and in the interstitial tissues of the major salivary glands. The DC are characterized by their shape, nuclear and cytoplasmic structure, lack of intercellular junctions with adjacent epithelial cells, and expression of molecules characteristic of DC, such as MHC II and CD11c. The location of DC within acinar and ductal epithelium suggests a role in responding to foreign antigens and/or maintaining immunological tolerance to salivary proteins. Further studies are necessary to completely characterize the various DC subsets present in the salivary glands, and their function in normal and pathological conditions.

V. Acknowledgments

We thank Ms. Maya Yankova for expert technical assistance, Drs. Alessandro Riva and Marco Piludu, University of Cagliari, Italy, for providing the samples of human salivary glands, and Drs. Irving Goldschneider and Maija Mednieks, University of Connecticut Health Center, for helpful discussions and for critically reading the manuscript. This research was supported by the University of Connecticut Health Center and NIH grant DE07302.

VI. References

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