Leukotriene Synthesis by Epithelial Cells of Human Mucosa-Associated Lymphoid Tissue

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Citation

Abstract

Lymphocyte activation and immunoglobulin synthesis are key events in acquired immunity. Leukotrienes are lipid mediators known to modulate these events in vitro, but how they might directly interact with lymphocytes in vivo has been unclear. The enzyme 5-lipoxygenase catalyzes the rate-limiting steps in leukotriene synthesis. Here we report that 5-lipoxygenase protein was found in abundance in epithelial cells of human mucosa-associated lymphoid tissue. In addition, leukotriene A4 hydrolase and the 5-lipoxygenase activating protein were also present in lymphoid tissue epithelium, indicating that these cells should be able to independently metabolize arachidonic acid to leukotrienes. Indeed, epithelium-rich tissue fractions were capable of synthesizing both leukotriene B4 and 5-hydroxyeicosatetraenoic acid from arachidonic acid. The abundance of 5-lipoxygenase protein, the amount of leukotriene B4 secreted, and the extent of epithelium stratification differed between samples from different donors. Together, these results place leukotriene synthesis in an unexpected site, mucosa-associated lymphoid tissue epithelium, where secreted leukotrienes may affect lymphocyte activation and immunoglobulin synthesis. Dysregulation of leukotriene synthesis in this context, in turn, may be relevant to asthma, allergic response, and autoimmune disorders.

Abbreviations: AA, arachidonic acid; DAB, 3,3'-diaminobenzidine; FLAP, 5-lipoxygenase activating protein; HETE, hydroxyeicosatetraenoic acid; Ig, immunoglobulin; 5-LO, 5-lipoxygenase; LT, leukotriene; MALT, mucosa-associated lymphoid tissue; RBL, rat basophilic leukemia.

INTRODUCTION

Leukotrienes (LTs) are lipid mediators that play pivotal roles in normal immune defense. However, the overproduction of LTs contributes to several diseases, including asthma [1,2] and allergic response [3, 4, 5]. In particular, the cysteinyl LTs, LTC4 and LTD4, trigger the classic symptoms of asthma and allergic hyperresponsiveness, including bronchoconstriction, edema, and mucus secretion [6,7]. LTB4 is best known as a promoter of leukocyte recruitment [8] and activation [9] and is thought to contribute to the chronic inflammation that can occur in the asthmatic patient. Pharmaceuticals that modify the action of LTs, particularly of the cysteinyl LTs, are efficacious in ameliorating these symptoms and are currently used in the treatment of asthma.

LTs are synthesized enzymatically from arachidonic acid (AA). The synthesis of LTs is initiated by the action of 5-lipoxygenase (5-LO) [10] in concert with the 5-lipoxygenase activating protein (FLAP) [11,12]. Leukotriene A4, generated by the oxygenation of AA by 5-LO, may be converted either to LTB4 by the enzyme LTA4 hydrolase, or to LTC4 via LTC4 synthase.

As suggested by their name, LTs are synthesized primarily by leukocytes, especially those of the myeloid lineage. Non-leukocytes, including epithelial cells, may contain FLAP protein and either LTA4 hydrolase or LTC4 synthase, but characteristically lack 5-LO protein [13, 14, 15]. As a result, these cells cannot independently synthesize either LTB4 or LTC4. However, it is well established that epithelial cells can synthesize LTs by a transcellular pathway by metabolizing LTA4 released from neighboring leukocytes [16, 17, 18].

Several studies have demonstrated that LTB4 can stimulate B lymphocytes and enhance IL-4-mediated IgG, IgM and IgE synthesis [19, 20, 21], as well as modulate T cell proliferation [22, 23, 24, 25, 26, 27] and IL-2 synthesis [28,29]. More recently, Irvin and colleagues found that 5-LO knockout mice showed a diminished capacity to generate immunoglobulin (Ig) E or antigen-specific IgG following antigen exposure [30]. Also, the spleens of 8-week-old mice lacking a functional 5-LO gene were 20% smaller than those of wild type mice [31]. Although LTs can clearly affect

lymphocytes in vitro, it is unclear how lymphocytes might directly encounter these paracrine mediators in vivo.

The upper airway is a critical site for the introduction of inhaled particles into the body. Mucosa-associated lymphoid tissue (MALT) in the upper airway, including the tonsils and adenoids, are organized tissues where non-lymphoid cells interact with lymphocytes for antigen presentation and to modulate lymphocyte activation. Thus, MALT is a potential site where LTs might be able to regulate lymphocyte function and Ig production. In this study, we asked whether LT synthesis occurs in human MALT. We now demonstrate that the stratified epithelium of both palatine and pharyngeal tonsils (adenoids) contains the enzymes 5-LO, FLAP and LTA4 hydrolase and can synthesize LTB4 and 5-HETE. The amounts of 5-LO protein and the extent of epithelium stratification vary significantly between tissue samples from different donors. These results indicate that MALT epithelium may be a source of secreted LTs, suggest that LT secretion may vary significantly between individuals, and place LT generation directly in mucosa-associated lymphoid tissue.

METHODS

Materials. Tonsil and adenoid tissues were obtained from anonymous patients undergoing surgery for reasons unrelated to this study (tonsillitis or airway obstruction). All available tissues, obtained from 23 different donors, were accepted into this study, and included tissues from both children and adults. Upper airway tissues from anonymous patients with mild asthma, obtained by transbronchial biopsy, were from archived pathology samples. The use of human tissue for this study was approved by the University of Michigan Medical Center Institutional Review Board.

Immunohistochemical Analysis of Enzyme Expression. Freshly-harvested tissues were routinely washed extensively with ice-cold PBS to remove blood and foreign debris, sectioned in half longitudinally, with one half processed for immunohistochemistry and the other half used for metabolic analyses. Immunohistochemistry was performed as described ($_{32}$), using tissue specimens fixed in neutral-buffered formalin, embedded in paraffin, sliced, mounted, de-paraffinized (Americlear), and rehydrated in descending concentrations of ethanol. Tissue was blocked (0.1% BSA in PBS with 25% non-immune goat serum), probed with primary antibody (37 oC, 60 minutes), washed and then incubated with biotinylated goat anti-rabbit IgG (1:200, 37 oC, 60 minutes). After washing, sections were incubated

with avidin-conjugated horseradish peroxidase reagent for 45 minutes, washed, incubated with peroxidase substrate, and then counterstained. Primary antibodies were rabbit polyclonal antibodies raised against (1) purified human leukocyte 5-LO (a generous gift of Dr. J. Evans, Merck Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada ($_{33}$), (2) amino acid residues 41-52 of the human FLAP sequence (also a gift from Dr. J. Evans, Merck Frosst) ($_{34}$), and (3) human recombinant LTA4 hydrolase (Cayman Chemical, Ann Arbor, MI). Peroxide substrates were 3,3'-diaminobenzidine (DAB) with or without nickel. Counterstains included Harris' hematoxylin and neutral red. Images were captured using a Zeiss Aristoplan microscope with the capacity for differential interference contrast microscopy.

Tonsil Cell Fractionation. Freshly-harvested tonsils were divided into "lymphocyte-rich" and "epithelium-rich" cell populations: saline-washed tissues were thinly sliced and mechanically manipulated in ice-cold saline to release loosely-adherent cells. The released cells were recovered by centrifugation and analyzed as the "lymphocyte-rich" fraction. Tissue slices were further washed and manipulated two additional times to release additional loose cells, then washed and recovered by centrifugation and analyzed as the "epithelium-rich" fraction.

Analysis of LT Synthesis. Leukocyte-rich and epithelium-rich cell populations were resuspended in Medium 199 containing 10 mM calcium ionophore A23187 and 1 mM AA supplemented with 0.5 mCi [3H]-AA (specific activity 76-100 Ci/mmol, NEN Life Science Products), for 30 minutes, 37 oC. Radiolabeled eicosanoids in conditioned media were assayed as described ($_{35}$). Briefly, eicosanoids were extracted from conditioned culture medium using Sep-Pak C18 cartridges (Waters Associates, Milford, MA), dried under nitrogen, resuspended in water:acetonitrile (2:1), and separated by reverse-phase HPLC using a Waters mBondapak C18 column with a mobile phase of acetonitrile/water/trifluoroacetic acid. Radiolabeled products were identified by their co-elution with authentic standards and quantitated by on-line radiodetection.

Specific quantitation of immunodetectable LTB4 in conditioned media was performed by enzyme immunoassay, performed according to the supplier's instructions (Cayman Chemicals). Following cell stimulation and media retrieval, cells were sonicated and total cellular protein of sonicated cells was determined by a modified Coomassie blue dye-

binding assay (Pierce Chemical Co.). Immunodetectable LTB4 was divided by total cellular protein to standardize for variation in cell numbers between samples. For this analysis, duplicate determinations were made from samples from 3 different donors, and results were expressed as the mean and standard deviation from the mean.

Reproducibility. In all cases, experiments were repeated at least three times, using different human donors.
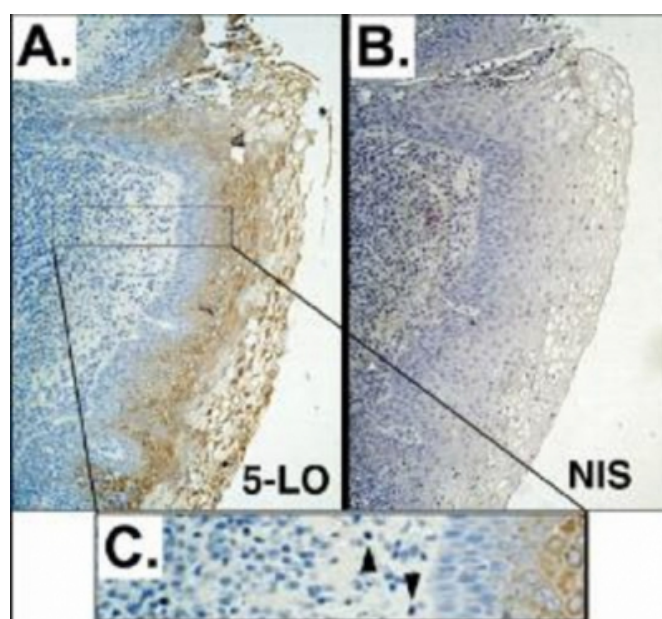
RESULTS

5-LO, FLAP and LTA4 Hydrolase in MALT Epithelium. Given that LTs could promote lymphocyte activation and function, we sought to determine if there was a cellular source of LTs in human MALT, using palatine tonsils removed for tonsillitis. We chose infected tissue, expecting to find abundant, 5-LO-positive recruited leukocytes, the typical LT source. Surprisingly, abundant positive staining for 5-LO was obtained in the stratified epithelium (Fig. 1A). A serial section stained with non-immune serum did not show staining of the epithelium (Fig. 1B). Scattered leukocytes, presumably recruited, also stained positive for 5-LO in this preparation (arrowheads, Fig. 1C); the positive brown staining was superimposed on the nuclear counterstain, since 5-LO is intranuclear in these cells ($_{36}$). Lymphocytes, the bulk of the cells in the interior of MALT, were negative for 5-LO.

Figure 1

Figure 1: Localization of 5-LO protein to the stratified squamous epithelium of human palatine tonsils. A.) Tonsil sections, donor #109, probed with anti-5-LO (original magnification: x60). B.) Tonsil sections probed with non-immune serum (NIS). C.) Detail from (A), indicating positively stained leukocytes (arrowheads). 5-LO-positive leukocytes have dark blue nuclei, since 5-LO is intranuclear in recruited leukocytes (32,36), resulting in an overlap of the positive stain with the counterstain; negative cells have light blue nuclei. Positive staining is brown (from the peroxidase substrate DAB); sections are counterstained blue with HarrisÂ' Hematoxylin

Experiments with palatine tonsil tissues from additional donors gave similar results (Fig. 2). In each case, the cytoplasm of epithelial cells stained positive, whereas the nuclei were non-staining. Staining for 5-LO was absent from the file of dividing cells that give rise to the epithelium (arrows, Fig. 2), indicating that the expression of 5-LO begins during epithelial cell differentiation.

Figure 2

Figure 2: Positive staining for 5-LO protein in palatine tonsils from additional patients. A) Donor #110 (original magnification: x100). B) Donor #98 (original magnification: x55). Arrows indicate the plane of cell division giving rise to the tonsil epithelium. Staining as in Fig. 1
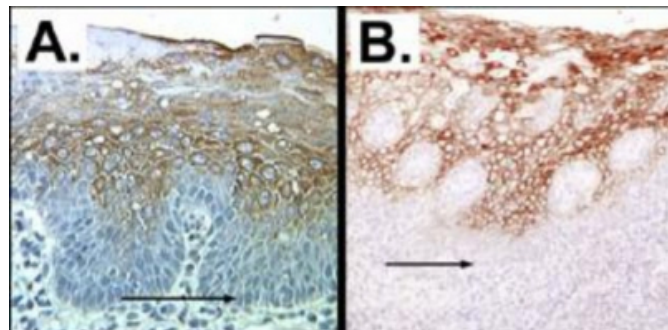
Additional studies examined the pharyngeal tonsils (adenoids), as well as airway tissue that was not associated with MALT. Again, the stratified epithelium of pharyngeal tonsils was positive for 5-LO, as were scattered leukocytes (Fig. 3A). The epithelium of the upper airways, in contrast, was negative (Fig. 3B), as is typical of most normal epithelium. Leukocytes in airway tissue stained intensely for 5-LO, providing a positive control for 5-LO staining (arrow, Fig. 3B). Thus, 5-LO expression in epithelium is a characteristic of, and unique to, at least some MALT tissues.

Figure 3

Figure 3: Staining for 5-LO in other airway epithelium: MALT vs. non-MALT sites. Adenoid (A) and upper airway (B) tissues were probed for 5-LO protein; positive staining is brown (DAB). A) Donor #110, counterstained with neutral red to allow visualization of numerous, scattered infiltrating leukocytes (original magnification: x40). B) Faintly counterstained with hematoxylin (gray-blue) and imaged with differential interference contrast optics (original magnification: x225). Basement membrane (bm) underlies the epithelium (E); a positively-stained leukocyte is indicated (arrow)
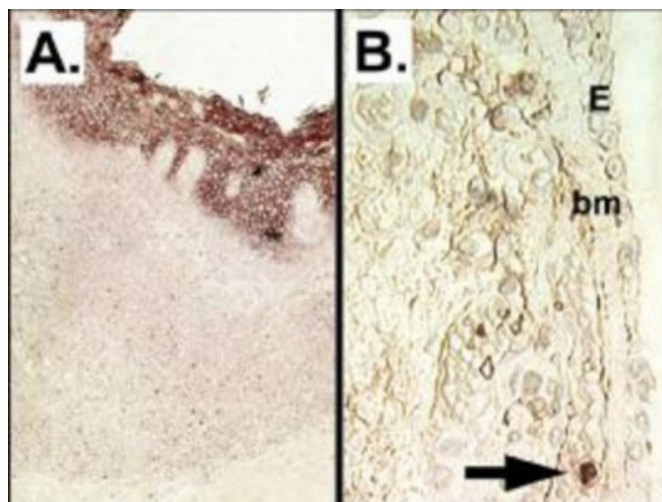
As noted above, epithelial cells typically contain FLAP and LTA4 hydrolase. MALT tissue was positive for both enzymes, with the greatest staining in the epithelium (Fig. 4). Thus, with 5-LO, these cells have the enzymes necessary for the independent synthesis of LTB4 from AA.

Figure 4

Figure 4: Detection of FLAP and LTA4 hydrolase in human palatine tonsils. A) Donor #06, probed for FLAP (brown, DAB), counterstain: hematoxylin (blue) (original magnification: x45). B) Donor #55, probed for LTA4 hydrolase (brown, DAB), counterstain: neutral red (original magnification: x55)
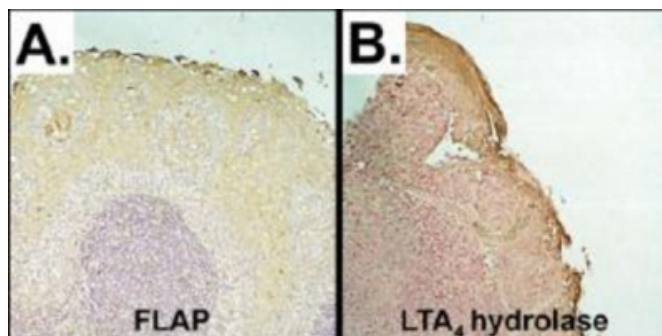

LT Synthesis by MALT Epithelium. We next sought to determine whether the 5-LO in MALT epithelium was

functional. Typically, neither epithelial cells nor T lymphocytes are capable of independent LT synthesis, and B cells make LTs only under unusual in vitro conditions ([37,38]). Since the above results indicated that recruited myeloid cells, a potential source of LTs, were scattered among the lymphocytes, we separated the lymphocytes, and presumably the majority of the recruited myeloid cells, by thinly slicing tonsil tissue and extensively washing and mechanically manipulating these slices, as described above. The epithelium-rich and lymphocyte-rich fractions were then tested for LT synthetic capacity. Epithelium-rich fractions secreted abundant amounts of the 5-LO products LTB4 and 5-hydroxyeicosatetraenoic acid (5-HETE), as well as 12-hydroxyheptadecatrienoic acid (a metabolite of prostaglandin H2), and 15-HETE (Fig. 5A) when stimulated with 10 mM calcium ionophore A23187 in the presence of 1 mM AA supplemented with 0.5 mCi [3H]-AA. In contrast, lymphocyte-rich fractions released more prostaglandin E2 and unmetabolized AA and less LTB4 (Fig. 5B).

Figure 5

Figure 5: Metabolism of arachidonic acid by tonsil tissue: HPLC analysis of all AA metabolites. Freshly isolated tonsil tissue (donor #35) was thinly sliced and washed with saline to release lymphocytes (Â"lymphocyte-richÂ" fraction). The remaining tissue was washed further to remove additional lymphocytes and subsequently analyzed as the Â"epithelium-richÂ" fraction. Epithelium-rich (A) and lymphocyte-rich (B) fractions were incubated with radiolabeled arachidonic acid and calcium ionophore A23187 for 30 min. Lipids were extracted from the conditioned medium and separated by HPLC. Products, based on co-elution with authentic standards, include the 5-LO products LTB4 (B) and 5-HETE (5), as well as PGE2 (E), 12-hydroxyheptadecatrienoic acid (H), 15-HETE (15), and unmetabolized arachidonic acid (AA)
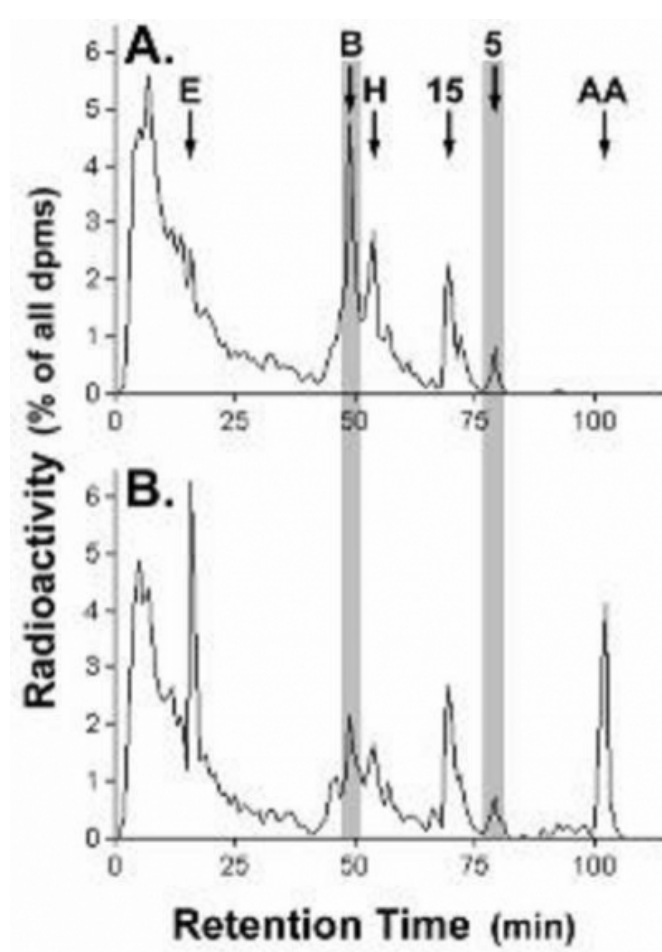

As a second approach to characterizing the 5-LO enzymatic activity in tonsil epithelium, LTB4 secreted from epithelium-rich fractions was quantitated by enzyme immunoassay. Samples from three different donors released abundant LTB4 upon stimulation (Fig. 6). The amount of LTB4 was significant, well above what might be expected from any contaminating leukocytes. In fact, this amount was comparable to that synthesized by a million peripheral blood

neutrophils stimulated in a similar manner (36). There also appeared to be significant variability in LTB4 synthetic capacity between different donors.

Figure 6

Figure 6: Metabolism of arachidonic acid by tonsil tissue: EIA measurement of LTB4. Epithelium-rich fractions from palatine tonsils from three donors were prepared and stimulated as in Fig. 5. LTB4 in conditioned media was quantitated by EIA and standardized for total cellular protein. Bars indicate standard deviations from duplicate determinations
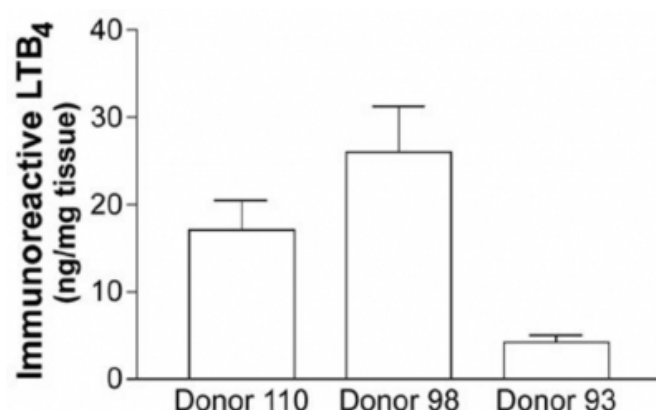
Variability Between Donors. Two significant sources of variability in LT synthetic capacity between donors were evident. First, there were significant differences in the degree of stratification of the epithelium. In tonsils from some donors, the epithelium appeared reduced or absent, with no clear layer of dividing cells separating the dense lymphoid region from the epithelium (Fig. 7A). In others, the epithelium was heavily stratified and the germinal layer was evident (Fig. 7B). The non-staining regions interdigitating the positively-stained stratified epithelium (Fig. 7B; see also Figs. 2A and 3A) have been described as dermal papillae ([39]), stain positive for keratins, and appear to be sites of epithelial cell proliferation.

Figure 7

Figure 7: Variability in tissues from different donors: thickness of epithelium and 5-LO protein abundance. A) An example of a tonsil where the stratified epithelium is negligible, with limited staining for 5-LO. Positive staining is brown (DAB) (donor #54, original magnification: x75). B) A highly stratified epithelium, with intense staining for 5-LO. The arrow, indicating the epithelial germinal layer, is used to help visualize the thickness of the epithelium. Positive staining is black (DAB complexed with nickel) (donor #110, original magnification: x75)
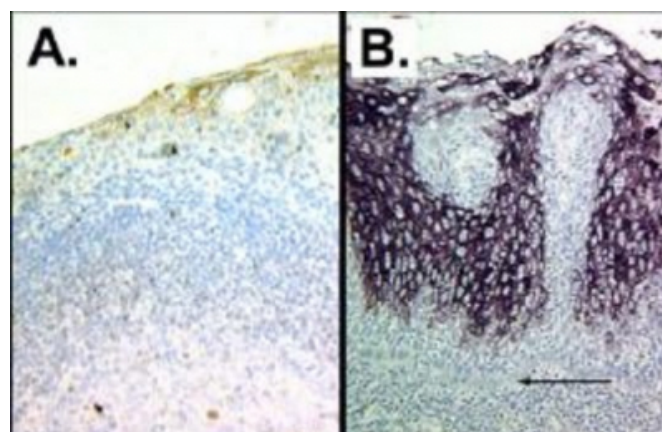
Second, staining for 5-LO was variable, both in amount, as evidenced in Fig. 7, and in location. In some cases, staining was particularly pronounced deep in crypts (Fig. 8A). In others, staining was greatest in the surface regions between crypts (Fig. 8B). Although some of the variability in the intensity of staining could be artifactual, it is interesting that variability in staining also could be observed within a given region on a single slide, with some groups of epithelial cells clearly staining more than adjacent groups (Fig. 8B).

Figure 8

Figure 8: Variability in tissues from different donors: 5-LO protein abundance. A) Positive staining for 5-LO in the multi-layered epithelium deep in the crypt of a tonsil (donor #64, original magnification: x35). B) Variable staining for 5-LO between different cells within the epithelium of a single donor (donor #55, original magnification: x85). Positive staining is brown (DAB)
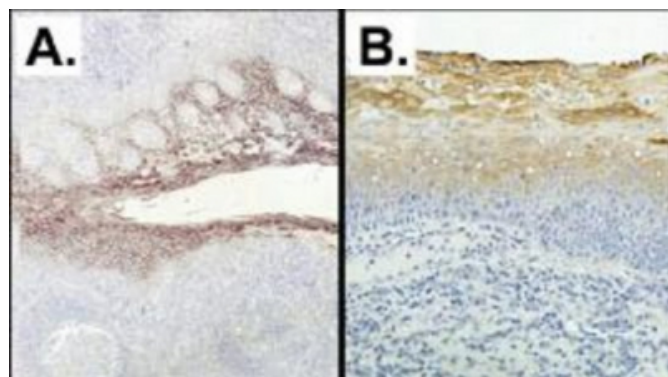
DISCUSSION

This study demonstrated for the first time that MALT epithelium is able to metabolize AA to the 5-LO products LTB4 and 5-HETE, and that 5-LO, as well as FLAP and LTA4 hydrolase, is expressed in epithelial cells of human MALT. We also have found that the amount of 5-LO protein in MALT epithelium, as well as the degree of epithelium stratification, can vary between individuals, and these may contribute to a variable capacity to elaborate LTs. These results are significant because they place a variable and significant amount of LT secretion at a site of lymphocyte development and action. This completely novel and unexpected site for LT production may help explain previously unexplainable findings and have significant implications toward pathogenesis.

LTs may play several roles in MALT. As noted above, LTB4 can activate B cells and enhance IL-4-mediated IgG, IgM and IgE synthesis (19-21), as well as modulate the function of some T cell subsets (25-27). Thus, epithelium-derived LTs may play an integral role in MALT function, regulating lymphocyte activation and immunoglobulin production. LTB4 is also recognized as a potent chemoattractant and activator of neutrophils (8,9) and may play a role in inflammation in MALT and neighboring tissues. For example, the secretion of LTs by adenoids may be relevant to the clinical treatment of otitis media with effusion. Adenoidectomy has long been used in the treatment of otitis media with effusion ($_{40}$), although the reason for its effectiveness is not known ($_{41,42}$). The adenoids

may, under as yet unknown conditions, become a persistent source of LTs that drive chronic or recurrent inflammation in neighboring tissues (e.g., the Eustachian tubes). Similarly, LTs may be secreted from gut-associated lymphoid tissues and be relevant to inflammatory bowel disease ($_{43}$), or from bronchial-associated lymphoid tissues and contribute to the chronic inflammation of the airways that is characteristic of asthma and allergic rhinitis ($_{44}$).

MALT is a specialized tissue, involved in antigen sampling from the airway and gut. As a result, the epithelium of MALT is a point of entry for pathogens ($_{45,46}$), rather than the barrier presented by other epithelia. Another distinction appears to be the expression of 5-LO: most epithelial cells do not contain 5-LO protein or message (13-15). The combination of pronounced expression of 5-LO and hyperplasia observed in epithelia from some, but not all, tonsil samples indicates an impressive potential for LT generation. This situation may represent the extreme end of a spectrum of potential for LT synthesis: the tissues used in this study were surgically removed, presumably, because they were not normal. Some tissues had been removed because of recurrent infection, whereas others were removed because the tissues were enlarged and obstructive. Future studies will examine the correlation between 5-LO expression and MALT pathology, as well as seek to evaluate 5-LO expression in normal MALT.

In sum, this study places LT synthesis within MALT. The results support independent LTB4 generation by MALT epithelium. Variability of 5-LO expression and degree of epithelium stratification are proposed as mechanisms that lead to variability in LT generation. LT generation by MALT epithelium may contribute both to lymphocyte activation and function and to inflammation in MALT and neighboring tissues.

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