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ACIDIC MAMMALIAN CHITINASE (AMCASE): A NEW TARGET FOR OCULAR DISEASES

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SUMMARY

Chitin, the second most abundant polysaccharide in nature, is not expressed in mammalian systems, but is found in the structural coatings of fungi, the exoskeleton of many arthropods and parasitic nematodes. The host defense against chitin-containing pathogens includes the production of chitinases. An acidic mammalian chitinase (AMCase) is produced by human epithelial cells of the lower airways and conjunctiva via a Th2-specific, IL-13-dependent pathway, and appears to be associated with asthma and inflammatory ocular pathologies. Animal studies showed that AMCase activity was increased in tears of rabbits with endotoxin-induced uveitis, and that this activity was attenuated and inhibited by chitinase inhibitors. The level of AMCase activity was found to be elevated in the tears of patients with vernal keratoconjunctivitis and seasonal allergic conjunctivitis, and specific mRNA, extracted by conjunctival epithelial cells, correlated with AMCase activity. An increase in AMCase activity was found in the tears of patients affected by dry eye syndrome, which correlated with specific mRNA. Therefore, it appears that AMCase represents an important mediator in the pathogenesis of Th2-driven inflammatory ocular diseases.

INTRODUCTION

Chitinases have a basic role in the defense of organisms against chitin-containing parasites (1, 2). Since chitin, the second most abundant polysaccharide in nature, is found in the structural coatings of fungi (3), the exoskeleton of many arthropods, it provides protection for pathogens (4) and parasitic nematodes (5) from harsh conditions inside the host. Although chitin and chitin synthase do not exist in mammals, chitinases are expressed in mammals. They belong to the glycosyl hydrolase (GH-18) family, including chitotriosidase (Chit), acidic mammalian chitinase (AMCase), YKL-39 and YKL-40, Ym1 and Ym2, oviduct-specific glycoprotein (oviductin) and stabilin-1-interacting chitinase-like protein (SI-CLP) (6).

Chit and AMCase show chitinase enzymatic activity, whereas other mammalian chitinases do not possess this activity. Chit expression can vary from species to species; it is a 50-kDa protein that contains a 39-kDa *N*-terminal catalytic domain that can hydrolyze chitin and is exclusively produced by macrophage cells during inflammation. Characteristically, the resistance to acidic pH distinguishes AMCase from Chit, which has its optimum at pH 6 (7). On the contrary, AMCase has its optimum at pH 2 and 4 in rodents and humans, respectively; AMCase is expressed in different tissues (stomach, lung, salivary gland, etc.) and appears to be associated with inflammatory diseases (8).

AMCase does not play a direct role in Th2 cytokine induction, but rather mediates pathogenesis by contributing to the effector responses of IL-13 (9). AMCase has also been proposed as a potential therapeutic target in Th2-mediated inflammation (10). In fact, the inhibition of AMCase activity by the chitinase inhibitor allosamidin (11) decreased the number of inflammatory cells in the bronchoalveolar lavage (BAL) fluid of ovalbumin-sensitized and -challenged mice, and reduced asthma symptoms (9). Interestingly, the same response was obtained using antisera against AMCase given in the airways by aerosol (9).

The role of AMCase in chronic airways pathologies was also demonstrated by Ramanathan et al. (12), who showed that AMCase mRNA was significantly more overexpressed in the nasal mucosa of patients with severe sinus inflammation than in control subjects, attributing to AMCase the role of a marker for chronic inflammation. Clearly, this finding supports the concept that severe and persistent sinusitis may be a consequence of a misplaced immune response against parasites that are not really present, and the chitinolytic activity of AMCase represents only a functional characteris-

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tic of this protein, which in other organs (e.g., stomach) can be involved in the digestion of chitin (13). This observation is important, since AMCase appears to play a key role as first responder of the immune system (12), both in the epithelial cells lining the nasal mucosa and other contiguous conjunctival cells. When the epithelial cells are stimulated against nonexistent parasites through a mechanism that induces an IL-13 response, they can produce chitinases, which represent a marker of both innate immunity and inflammatory response. In addition to the role in innate immune response, the modulation of AMCase expression by specific inhibitors suggests a new pathway for controlling ocular inflammation. These observations allow us to hypothesize that AMCase could also be a potential key enzyme in the pathogenesis of allergic conjunctivitis (Fig. 1).

Another chitinase, Chit, has been shown to be expressed together with lysozyme in the human lacrimal gland (14). This chitinase produced by macrophages and neutrophils also has a role in innate immunity (15). Since this chitinase was considered more active in the control of chitin-containing pathogens, the presence of measurable Chit activity in tears appears to support the protection of the eye in both humans and mice, where the chitotriosidase (*CHIT*) gene is evolutionarily conserved (16). In this context, it is interesting to note that Chit, unlike bacterial chitinases, does not appear to have any mucolytic activity (17), and therefore the lacrimal film is not modified in its structure, maintaining the integrity of visual function. Moreover, Hall et al. (14) found that recombinant Chit does not inhibit bacterial growth and does not synergize with lysozyme on the growth of Gram-positive and Gram-negative bacteria, and the antimicrobial activity appears to be limited only to fungi.

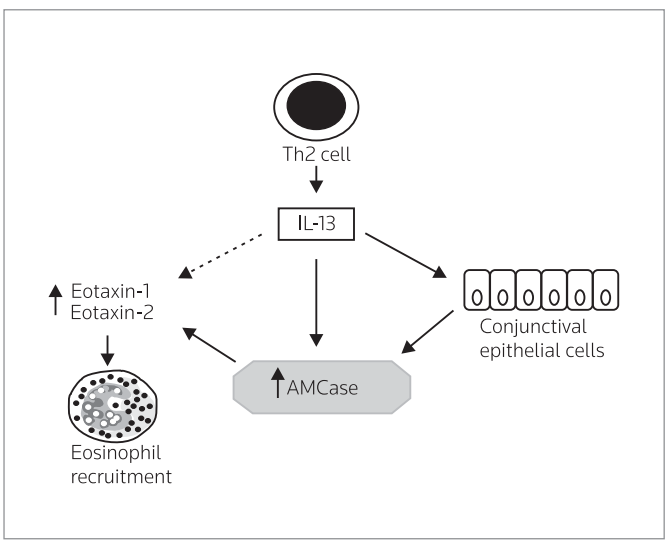

Figure 1. Hypothetical mechanism of acidic mammalian chitinase production in allergic eye pathologies. Adapted and reprinted from Binomium Chitin-Chitinase Recent Issues, Role of AMCase in the allergic and non allergic ocular pathologies, 263-80, ©2008, Musumeci, M., Musumeci, S., with permission from Nova Science Publishers, Inc.

The peculiar role of AMCase in inflammation has induced us to study AMCase in different ocular inflammatory conditions with the aim of proposing a novel treatment using a chitinase inhibitor (9). This article seeks to provide a succinct review of a rapidly changing area of visual science, focusing on the role of AMCase in the pathogenesis of allergic and nonallergic ocular inflammatory diseases.

ALLERGIC CONJUNCTIVITIS

Although there are several types of ocular allergy, seasonal and perennial allergic conjunctivitis (PAC) represent the majority of all ocular allergy cases, whereas the severe conditions of atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) affect a smaller group of patients. The allergic response in conjunctivitis is typically elicited by ocular exposure to allergens, which causes cross-linkage of membrane-bound IgE, triggering mast cell degranulation and releasing a cascade of allergic and inflammatory mediators. One such mediator, histamine, is the primary contributor to the development of early-phase signs and symptoms of seasonal allergic conjunctivitis (SAC), usually in distinct waves that reflect allergen exposure. VKC and SAC are Th2-driven diseases with a Th2 cytokine-derived pattern and overexpression of eotaxin-1 and -2, RANTES, MCP-1 and matrix metalloproteinases (MMPs). Th2-associated cytokines and chemokines have been identified in the tears of VKC and SAC patients (19, 20), along with an overexpression of AMCase in both children and adults. An AMCase assay showed a sensitivity and specificity of 100%, suggesting the potential utility of AMCase measurement as a biomarker of VKC and SAC (Fig. 2) (21). The activity of AMCase was significantly increased in VKC and the peculiar nature of the chitinolytic activity in tears of allergic ocular pathologies was supported by the acid stability of this enzyme at pH 4, and it was confirmed by mRNA measurement obtained by conjunctival impressions. The source of AMCase from the conjunctival epithelial cells was confirmed by RT-PCR of RNA. The RT-PCR measurement demonstrated a correlation between mRNA expression and tear AMCase activity (Fig. 2). No remarkable chitotriosidase expression was observed (data not shown).

The results obtained in allergic conjunctivitis (21) confirmed that AMCase is involved in the pathogenesis of allergy, as demonstrated by other groups in asthmatic patients (9). However, we cannot rule out that AMCase expression in allergic conjunctivitis is due to antigens cross-reacting with homologous sequences of the original parasites for which the chitinase have been designated. In this context, it must be considered that another cytokine, IL-9, largely regarded as a Th2 cytokine, makes multifocal contributions to allergic disease. In fact, recent data suggest that a distinct population of IL-9-producing "Th9" helper T cells can exist in certain conditions, where they may regulate chronic allergic disease through IL-4 and transforming growth factor-β (TGF-β) (22), but our study shows that AMCase is produced by epithelial cells and mediates allergic conjunctivitis pathogenesis by contributing to the effector responses of IL-13, according to the proposed model (Fig. 1). These results allowed us to investigate the effects of inhibitors of chitinases in experimental uveitis, which recapitulates much of the immune alteration of allergic conjunctivitis.

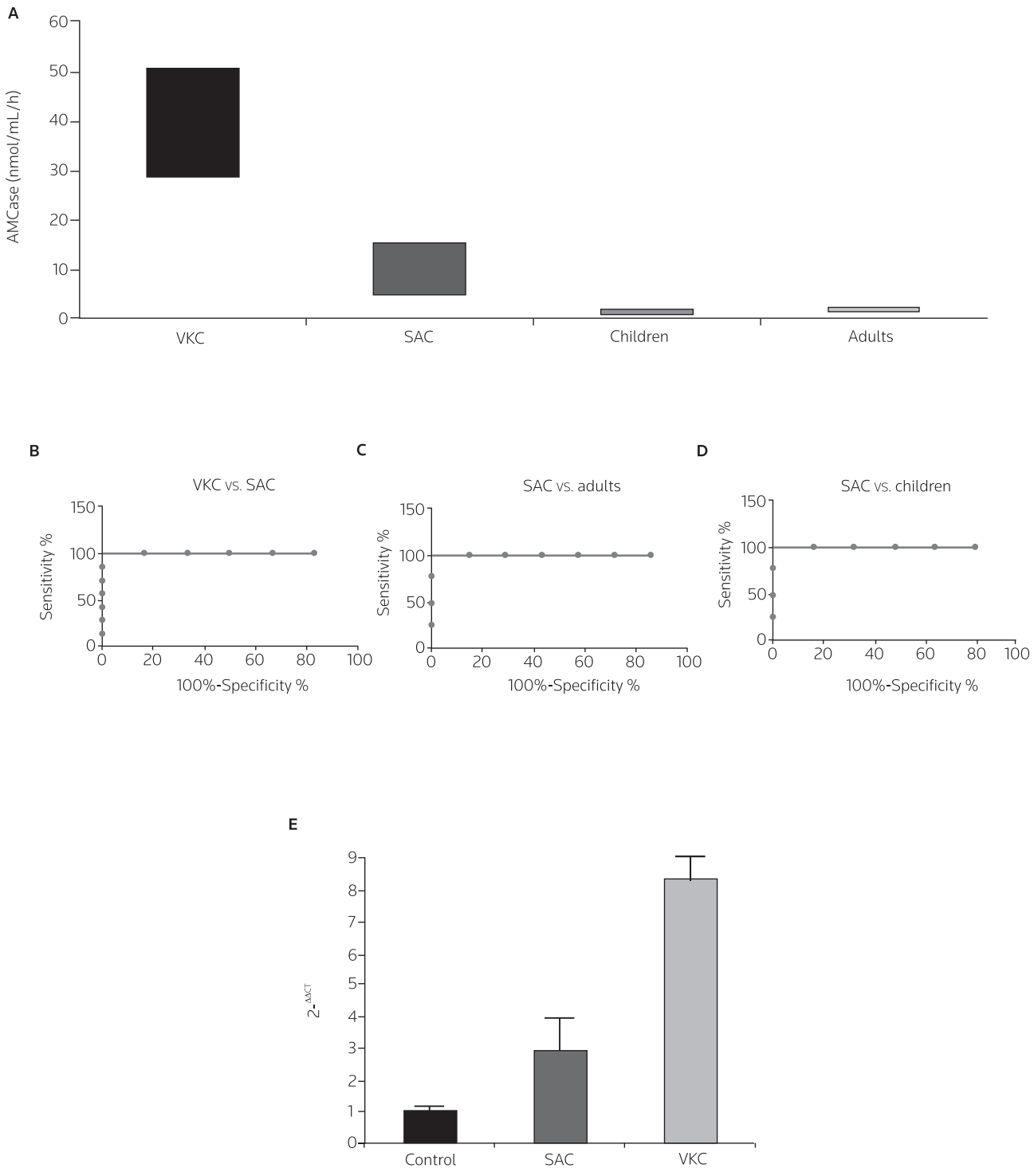

Figure 2. Acidic mammalian chitinase (AMCase) activity (panel **A**) and receiver–operator characteristic (ROC) analysis (panel **B**, **C**, **D**) in vernal keratoconjunctivitis (VKC), seasonal allergic conjunctivitis (SAC), children and adult controls. AMCase expression (panel **E**) by quantitative RT-PCR of RNA (2-ΔΔCT) in adult controls, SAC and VKC. Adapted and reprinted from Binomium Chitin-Chitinase Recent Issues, *Role of AMCase in the allergic and non allergic ocular pathologies*, 263-80, ©2008, Musumeci, M., Musumeci, S., with permission from Nova Science Publishers, Inc.

EXPERIMENTAL UVEITIS

A recent study from our lab, where experimental uveitis was induced by lipopolysaccharide (LPS) in the rabbit eye, confirmed that the chitinolitic activity in tears collected at 0, 6 and 24 hours after LPS injection was due to AMCase, as demonstrated by acidic resistance (pH 2) (23). No remarkable Chit expression was observed (data not shown). Uveitis is comprised of a group of heterogeneous diseases characterized by an intraocular inflammatory process that involves many complex immune pathways that have not yet been completely elucidated. It appears that the Th1 system dominates in intraocular inflammation, whereas the Th2 immune response is present during an ocular surface inflammatory response (24). According to this observation, a hypothetical model of AMCase mediating the pathogenesis has been proposed (Fig. 3).

Figure 4 shows the kinetics of AMCase activity in the tears of rabbits treated with phosphate-buffered saline before and after LPS injection. Before the induction of endotoxin-induced uveitis by LPS injection, the AMCase activity (basal) was very low in the tears. LPS injection caused a significant increase in AMCase activity. Similar to in the asthma model (9), we confirmed that the inflammatory reaction induced by LPS was controlled by topical treatment of chitinase inhibitors, such as allosamidin or caffeine, and dexamethasone at different concentrations ranging from 0.001 mM to 0.1 mM. A significant

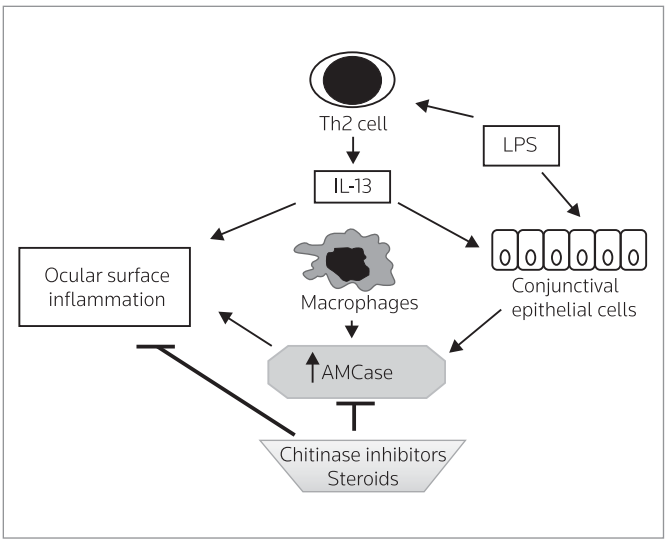

Figure 3. Hypothetical mechanism of acidic mammalian chitinase and IL-13 control of Th2-driven ocular surface inflammation in rabbit endotoxin-induced uveitis. Effect of chitinase inhibitors and steroids. LPS, lipopolysaccharide; AMCase, acidic mammalian chitinase. Adapted and reprinted from Binomium Chitin-Chitinase Recent Issues, *Role of AMCase in the allergic and non allergic ocular pathologies*, 263-80, ©2008, Musumeci, M., Musumeci, S., with permission from Nova Science Publishers, Inc.

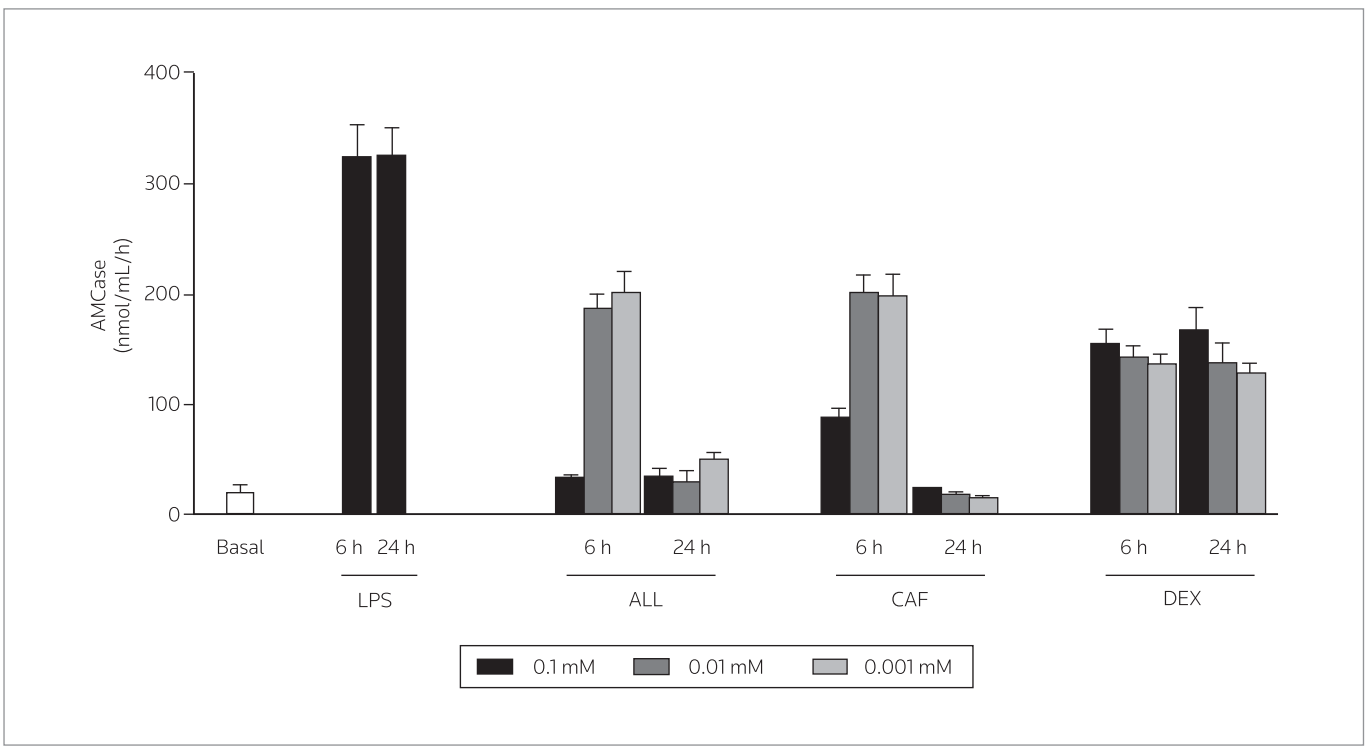

Figure 4. Acidic mammalian chitinase (AMCase) activity (nmol/mL/h) before (basal) and at 6 and 24 hours after intravitreal lipopolysaccharide (LPS) injection at 6 and 24 hours after conjunctival instillation of different concentrations (0.1, 0.01 and 0.001 mM) of allosamidin (ALL), caffeine (CAF) and dexamethasone (DEX). Adapted and reprinted from Binomium Chitin-Chitinase Recent Issues, *Role of AMCase in the allergic and non allergic ocular pathologies*, 263-80, ©2008, Musumeci, M., Musumeci, S., with permission from Nova Science Publishers, Inc.

dose-related inhibition of AMCase activity was observed at 6 and 24 hours in the allosamidin-treated group, as well as in the caffeine-treated group, compared to the LPS group. Likewise, the group treated with dexamethasone showed a significant inhibition of AMCase activity that was not dose-dependent at 6 and 24 hours. Topical instillation of allosamidin or caffeine caused a remarkable dose-dependent reduction of ocular inflammation. Also, the group treated with dexamethasone showed a significant dose-dependent decrease in ocular inflammation at all responder doses. In this study, caffeine was used because it is able to bind to the active site of chitinase in the same manner as the well-known chitinase inhibitor allosamidin (25). Noteworthy, caffeine, among its multiple mechanisms of action (adenosine receptor antagonist, phosphodiesterase inhibitor, histone deacetylase inducer), also acts via chitinase inhibition (26).

Recently, chitinase has been proposed as a target for glucocorticoids. Zhao et al. (27) demonstrated that in mice sensitized and challenged with ovalbumin, dexamethasone downregulated AMCase in the bronchial tissue. In our study, dexamethasone inhibited the AMCase expression in conjunctival epithelial cells of rabbits with uveitis. The inhibition of ocular inflammation in the group treated with dexamethasone was significantly greater compared to the group treated with allosamidin, probably because corticosteroids act through multiple mechanisms (26, 27). Interestingly, a comparable effect was observed with caffeine, which determined a significant inhibition of AMCase activity. This was likely due to the various mechanisms of action of this methylxanthine (26). The inhibition of AMCase by caffeine is more important than its effects on other pathways and it could be used in the treatment of allergic conjunctivitis. Since chitin is not naturally present in mammals, the abnormal production of the corresponding enzyme (i.e., AMCase) in allergy and inflammatory diseases, and its role in the buildup of mucus and other fluid or in polyp formation in human chronic sinusitis, opens the way to legitimate this enzyme as a target for drugs to block its action or, where possible, its production.

In conclusion, from our data, AMCase appears to be a marker of the early inflammatory process, but the early inhibition of AMCase by allosamidin and caffeine at higher concentrations is due to a direct inhibition of chitinolytic activity rather than reduced secretion, unless AMCase is already preformed after the induced stimulation and ready to be secreted upon early activation. On the contrary, the effect of dexamethasone is due to blockade of AMCase expression. These considerations appear to contrast with the observations of Reese et al. (28), who demonstrated that the inoculation of chitin into mice determines recruitment of inflammatory cells (eosinophils and basophils), characteristic of an allergic response, and that the preincubation of chitin with AMCase precluded this effect, raising the possibility that inherent deficits in human chitin degradation could underlie airways inflammation and favor allergic reactions. However, other studies suggest that chitin has quite a relevant role in the immune response. Oral administration of chitin, for instance, has been shown to downmodulate allergic airways inflammation in a murine model (29).

Another study by Strong et al. (30) demonstrated that direct application of chitin microparticles to the respiratory tract can alleviate allergic symptoms in a mouse model of allergy. In these studies, the importance of the administration route (oral, nasal or parenteral) appears to be a key point for the ability of chitin to induce a Th1 antiallergic effect or a Th2-specific IL-13 mechanism. The size of the chitin particles that people are exposed to may result in different biological effects and may clarify some of the mystery associated with chitin and chitinase. For example, depending on the size of chitin particles, these may or may not be phagocytized and displayed by dendritic cells to T cells to stimulate an immune response. Moreover, chitin preparations used in murine models of allergy could mimic the effect of chitinase inhibitors because of the high affinity for AMCase, supporting our observations in the rabbit model of endotoxin-induced uveitis using allosamidin and caffeine (23). Our results in allergic human eye pathologies and in LPS-induced uveitis point to a role for chitinases in other ocular pathologies and the use of chitinase inhibitors as innovative pharmacological tools.

DRY EYE SYNDROME

Other types of nonallergic ocular inflammation may be associated with AMCase secretion in tears and conjunctival cell expression. Since the chitinase is thought to be related to innate immunity, increased AMCase expression could be suspected in dry eye syndrome caused by lacrimal film alterations due to different mechanisms. Dry eye syndrome is an ocular disease characterized by reduced lacrimal film stability due to decreased aqueous component production or increased evaporation. Dry eye syndrome is also a frequent side effect of many systemic and topical drugs and medical conditions (31). Ocular discomfort and visual impairment are the possible consequences of dry eye syndrome and the standard treatment for dry eye syndrome is the use of artificial tears, which is often accompanied by the use of antiinflammatory eye drops containing corticosteroids and/or ciclosporin (32).

Recently, several reports associated dry eye syndrome with an inflammatory condition of the ocular surface. ICAM-1, synthesized by epithelial cells, may represent a signaling molecule for the predisposition to ocular surface inflammation and facilitate the presentation of potential antigens by epithelial cells (33). This could serve as a clue to understanding the pathogenic mechanisms underlying the association between dry eye manifestations and allergic ocular diseases (34). In fact, experimental dry eye in mice stimulates the expression and production of IL-1α , IL-6, TNF-α and MMP-9, and activates MAPK signaling pathways on the ocular surface, which could play an important role in the induction of those inflammatory mechanisms implicated in the pathogenesis of dry eye syndrome (35). Moreover, the expression of Th1 cytokines such as IL-1α, IL-6 and TNF-α transcripts was higher in the corneal epithelium and conjunctiva of C57BL/6 mice, and the Th2 cytokines such as IL-4 and IL-10 were significantly greater in BALB/c mouse tears (36). These observations obtained in the dry eye mouse model are important and could be translated to humans, where the continuous physical trauma induced by alteration of tear composition activates an inflammatory response with the same mediator characteristics as Th1 and Th2 immune responses (37).

Since the immunomechanisms involved in allergic conjunctivitis and dry eye syndrome appear to be very different at the effector stage, it is possible that these two conditions may share common molecular pathways at the early stage, particularly those involving mucosal immunity. In fact, IL-13 can be commonly expressed at the ocular surface as part of innate immunity, modulating AMCase

expression by macrophages and epithelial conjunctival cells. Recently, a new cytokine, IL-17, produced by Th17 cells, also appears to be involved in the pathogenesis of dry eye syndrome and the blockade of this cytokine by regulatory T cells (Tregs) reduced the severity and progression of the disease in a mouse model. The presence of dysfunctional Tregs and the resistance of pathogenic T cells maintain an inflammatory autoimmune disease affecting the ocular surface (38). However, if these intricate interactions of Th1, Th2 and Th17 cytokines in dry eye syndrome are sustained by experimental data, the role of innate immunity was never considered sufficiently.

The mechanism of AMCase is completely different because this enzyme is produced by epithelial cells and expressed by conjunctival cells. In fact, the epithelial cells lining the nasal and conjunctival surfaces could play an important role as first responders of the immune system. They are capable of actively participating in immune reactions via the expression of surface antigens such as adhesion molecules and cytokines (35). In patients with meibomian gland dysfunction (MGD) and Sjögren's syndrome (SS) dry eye, and in healthy subjects (all adults), we collected tears to measure AMCase activity and conjunctival epithelial cells to measure specific mRNA by RT-PCR (39). AMCase activity was significantly higher in patients with MGD dry eye compared to SS dry eye ($P < 0.0001$), while in healthy controls AMCase was very low. RT-PCR analysis by conjunctival cytology impression showed a consistent upregulation of AMCase in the MGD dry eye patients with respect to SS dry eye patients and normal controls (Fig. 5), providing data on its epithelial origin. The quantitative real-time evaluation of AMCase indicated a strong increase in MGD dry eye compared to controls, whereas in SS dry eye the increase was less evident, but still statistically significant. In patients with SS dry eye the pathogenic mechanism is linked to an immune process that involves the ocular surface, with a marked reduction in the aqueous layer of tears as a hallmark of the disease (aqueous-deficient dry eye). In the MGD form, the pathogenic mechanisms are different (evaporative dry eye): the altered tear film is characterized by a primary alteration of the lipid component, which can be accompanied by a modified bacterial flora in the conjunctiva (40). This suggests a stronger innate response, as demonstrated by higher levels of AMCase expression. The results support the hypothesis that similar mechanisms are associated with allergic ocular pathologies with dry eye. In Figure 6 a model combines the two pathologies (allergy and dry eye syndrome), suggesting a common pathogenic mechanism(s) and hypothesizing a new treatment with chitinase inhibitors.

CONCLUSIONS

From the above considerations, AMCase appears to be a marker of the early inflammatory process, and at the same time this enzyme is a potential therapeutic target that can be manipulated to control ocular inflammation. Currently, the role of AMCase in the immune response is mainly derived from experimental observations. The transition from basic to clinical studies will certainly solve doubts about its immunoregulatory function, which remains unresolved to date. Thus, additional investigations are needed to define the roles of AMCase in ocular allergic inflammatory diseases and other Th2- and Th1-mediated ocular responses. Furthermore, chitinase inhibitors (e.g., allosamidin, methylallosamidin, methylxanthine) have received partic-

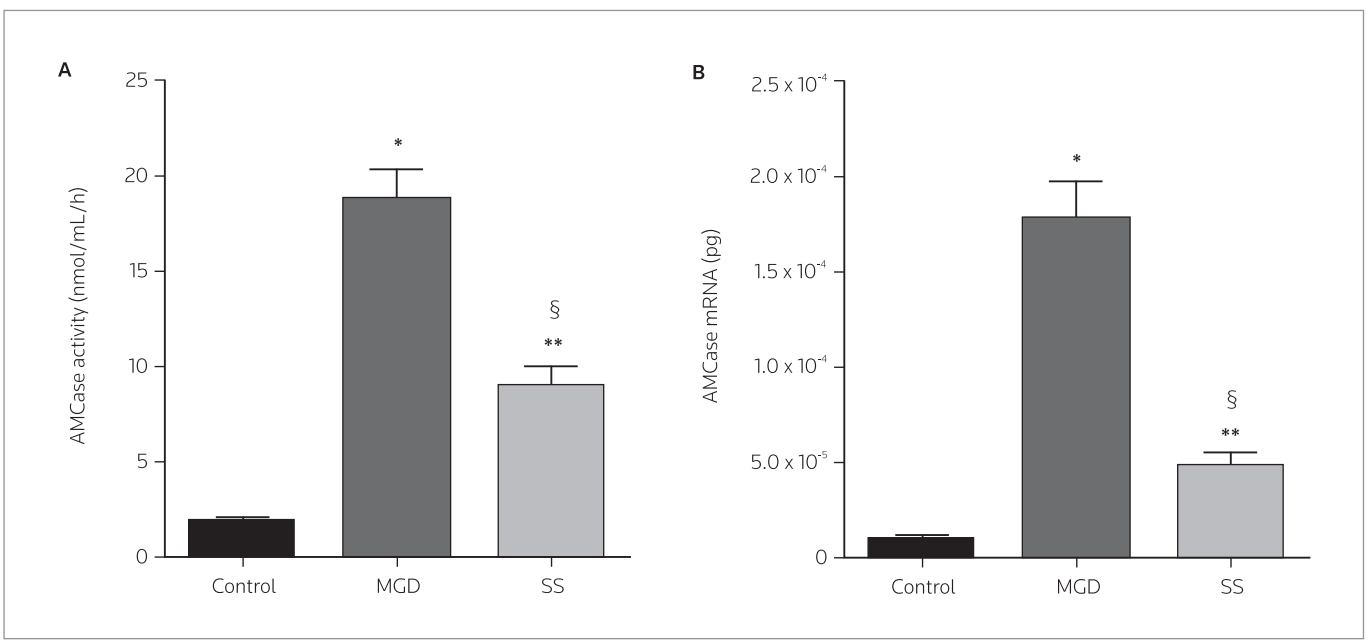

Figure 5. Levels of acidic mammalian chitinase (AMCase) activity (panel **A**) and quantity (pg) of mRNA (panel **B**) in healthy controls and patients with meibomian gland dysfunction (MGD) and Sjögren's syndrome (SS) dry eye. *$P <$ 0.001 vs. control; **$P < 0.05$ vs. control; §$P < 0.001$ vs. MGD group. Adapted and reprinted from Binomium Chitin-Chitinase Recent Issues, *Role of AMCase in the allergic and non allergic ocular pathologies*, 263-80, ©2008, Musumeci, M., Musumeci, S., with permission from Nova Science Publishers, Inc.

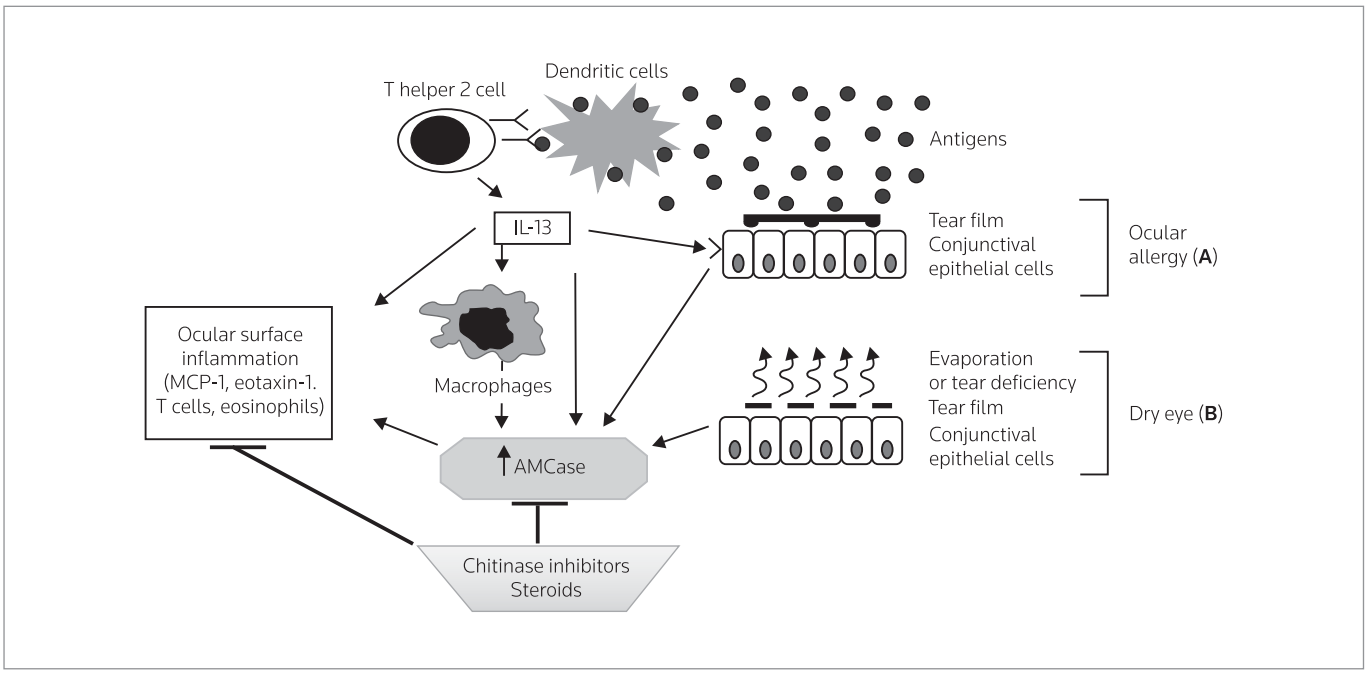

Figure 6. Model of acidid mammalian chitinase (AMCase)-mediated ocular allergy and dry eye inflammation. (**A**) Dendritic cells actively uptake antigens (chitin ?) and present antigen to Th2 cells producing IL-13, which plays a role in inducing AMCase by conjunctival cells and macrophages, both expressing the IL-13 receptor (IL-13R) on their surface. Noteworthy, AMCase stimulates MCP-1 and eotaxin-1 production, which induce the recruitment of T cells, eosinophils and macrophages, sustaining ocular surface inflammation. (**B**) Alteration of tear film (e.g., excessive evaporation or tear deficiency) stimulates conjunctival cells to express AMCase, which induces ocular surface inflammation by the same mechanism. Chitinase inhibitors may suppress ocular surface inflammation by blocking AMCase enzymatic activity, while steroids inhibit its expression. Adapted and reprinted from Binomium Chitin-Chitinase Recent Issues, *Role of AMCase in the allergic and non allergic ocular pathologies*, 263-80, ©2008, Musumeci, M., Musumeci, S., with permission from Nova Science Publishers, Inc.

ular attention in the context of pharmacological approaches designed to develop new molecules useful in clinical practice. Therefore, future studies may be warranted in order to evaluate the possibility of using AMCase inhibitors in the treatment of ocular inflammatory diseases.

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DISCLOSURES

The authors state no conflicts of interest.

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