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Male NOD mouse external lacrimal glands exhibit profound changes in the exocytotic pathway early in postnatal development

Silvia R. da Costa^{1,#}, Kaijin Wu^{1,#}, Michelle MacVeigh², Mike Pidgeon³, Chuanqing Ding³, Joel E. Schechter³, and Sarah F. Hamm-Alvarez^{1,4,5,*}

¹Departments of Pharmaceutical Sciences,

³Cell and Neurobiology,

⁴Physiology and Biophysics and

⁵Ophthalmology and

²USC Center for Liver Diseases, University of Southern California, Los Angeles CA 90033

Abstract

The lacrimal glands of male NOD mice exhibit many of the features of the human lacrimal gland in patients afflicted with the autoimmune disease, Sjögren's syndrome, including loss of secretory functions and lymphocytic infiltration into the lacrimal gland. To elucidate the early changes in the secretory pathway associated with development of Sjögren's syndrome, we investigated the organization of the exocytotic pathway in lacrimal glands of age-matched male BALB/c and NOD mice. Cryosections from lacrimal glands from 1 and 4 month male BALB/c and NOD mice were processed for confocal fluorescence and electron microscopic evaluation of different participants in exocytosis. No changes in apical actin filaments were noted in glands from NOD mice, but these glands exhibited thickening of basolateral actin relative to that seen in the BALB/c mice. Rab3D immunofluorescence associated with mature secretory vesicles was distributed abundantly in a continuous vesicular network concentrated beneath the apical plasma membrane in glands from 1 and 4 month BALB/c mice. In glands from 1 month NOD mice, rab3D immunofluorescence exhibited marked discontinuity and irregularity in the vesicular labeling pattern. While this change was also detected in glands from 4 month NOD mice, many of these glands exhibited an additional extension of rab3D labeling through the cell to the basolateral membrane. Electron microscopic analysis confirmed the formation of irregularly-shaped, unusually large secretory vesicles in lacrimal glands from NOD mice. Quantitation of multiple secretory vesicles from electron micrographs revealed a significant ($p \leq 5$) increase in the percentage of secretory vesicles incorporated into multivesicular aggregates in lacrimal glands from 1 and 4 month NOD mice compared to BALB/c mice. The M3 muscarinic receptor, a key signaling effector of exocytosis, was redistributed away from its normally basolateral locale in glands from BALB/c mice, with concomitant enrichment in intracellular aggregates in glands from NOD mice. These findings show that lacrimal glands in NOD mice as young as 1 month contain aberrant secretory vesicles with altered effector composition that undergo premature cytoplasmic fusion, and that changes in the distribution of the M3 muscarinic receptor occur within the same time frame.

*Address correspondence to: Sarah F. Hamm-Alvarez, Ph. D., USC School of Pharmacy, 1985 Zonal Avenue, Los Angeles CA 90033, 323-442-1445 O, 323-442-1390 F,shalvar@usc.edu.

#The first two authors contributed equally to this study

Keywords

Actin; rab3D; exocytosis; M3 muscarinic receptor; lacrimal gland; confocal fluorescence microscopy; NOD mouse; BALB/c mouse

Abbreviations

SjS, Sjögren's syndrome; non-obese diabetic, NOD; SV, secretory vesicle; EM, severe combined immune deficient, scid; electron microscopy; DPBS, Dulbecco's phosphate buffered saline; M3MR, M3 muscarinic receptor

Introduction

The aqueous tear film is enriched in a variety of proteins responsible for maintaining the integrity of the ocular surface by nurturing the corneal epithelium and protecting against pathogens. Numerous proteins have been detected in the tears such as lysosomal hydrolases (van Haeringen and Glasius, 1980), secretory IgA (van Haeringen, 1981), lactoferrin (Yoshino et al., 1997), transferrin (Salvatore et al., 1999) and growth factors (Van Setten et al., 1996). These tear proteins are largely produced by the acinar epithelial cells of the lacrimal gland. Dry eye, a disorder affecting over ten million Americans, is associated with decreased tear protein secretion by the lacrimal gland (Pflugfelder et al., 2000). In its most severe form, it is present as the autoimmune disease, Sjögren's Syndrome (SjS), which affects two to four million Americans. Diagnosis of SjS is based on presenting symptoms of dry eye and dry mouth accompanied by a characteristic focal lymphocytic infiltration of the lacrimal and salivary glands (Daniels and Fox, 1992; Haga, 2002). One key unresolved issue in the study of SjS is the relationship between the loss of secretory functions and the inflammatory responses.

One established mouse model for SjS is the non-obese diabetic or NOD mouse. This mouse, although normoglycemic, spontaneously develops insulin-dependent diabetes mellitus, as well as lymphocytic infiltrates in submandibular (sialoadenitis) and lacrimal (dacryoadenitis) glands associated with reduced flow (Nguyen et al., 2000; van Blokland and Versnel, 2002; Barabino and Dana, 2004). The two autoimmune processes that develop in these mice, e.g., diabetes and SjS, occur independently in the same animal. For reasons not yet understood, male mice are more prone to dacryoadenitis than female mice (Robinson et al, 1998; van Blokland and Versnel, 2002). Lymphocytic infiltration into the lacrimal gland is typically detected in male mice from ~6-10 weeks but develops later in female mice, from ~30 weeks (Hunger et al., 1998; van Blokland and Versnel, 2002). The resulting dacryoadenitis is accompanied by decreased production of lacrimal fluid. Clinical manifestations of SjS are detectable in males by ~4 months.

Some of the effectors and pathways involved in lacrimal acinar secretion have been elucidated, primarily from in vitro studies in rabbit and rat lacrimal acini. In lacrimal acini, secretory products are sorted into immature secretory vesicles in the trans-Golgi network and acquire external proteins characteristic of mature secretory vesicles (SVs) as they move towards the apical membrane. Mature SVs localized in the subapical cytoplasm of lacrimal acini are enriched in the small GTPase, rab3D (Ohnishi et al., 1996; Wang et al., 2003). Although the precise function of rab3D in acinar secretion is still in question, its association with mature SVs is decreased concomitant with apical exocytosis (Wang et al., 2003). Dominant negative forms of rab3D reduce amylase secretion in pancreatic acini (Chen et al., 2003), while overexpression of wild type rab3D can enhance pancreatic acinar exocytosis (Ohnishi et al., 1997), suggesting its direct participation in acinar exocytosis. Actin filaments in lacrimal acini can be detected in association with peripheral membranes, with considerable enrichment

beneath the apical membrane due to actin concentration in the dense cortical apical actin array which includes microvilli (da Costa et al., 1998). Apical actin filaments in acini play a regulatory role in governing accessibility of SVs to the apical membrane and facilitating discharge of their contents (Perrin et al., 1992; Muallem et al., 1995; Valentijn et al., 1999).

Several signaling effectors serve key roles in the transmission of extracellular cues into internal signals that result in mobilization of exocytotic pathways. Key amongst these is the M3 muscarinic receptor (M3MR) which is localized to the basolateral membrane of lacrimal acinar cells (Mauduit et al., 1993). M3MR activation by cholinergic agonists promotes exocytosis of tear proteins in lacrimal gland (Nakamura et al., 1997). The signaling pathway activated upon agonist binding has been well-characterized, involving initial activation of the G protein, G α_q , which in turn activates phospholipase C β (Dartt, 2001). Production of diacylglycerol and 1,4, 5-inositol triphosphate from phosphatidylinositol-bisphosphate catalyzed by activated phospholipase C results in mobilization of signaling pathways that then influence the activity of the trafficking effectors. The organization of these constituents of the acinar secretory pathway is shown in Scheme 1 in resting acini and acini stimulated through activation of M3MR.

In addition to activation of the major signaling cascade that culminates in lacrimal acinar exocytosis, the M3MR is directly implicated in the pathogenesis of SjS (van Blokland and Versnel, 2002). Autoantibodies to M3MR have been identified in serum from SjS patients (Bacman et al., 2001). In some cases, these autoantibodies were able to trigger the downstream signaling effects normally associated with M3MR activation (Bacman et al., 1998). Introduction of human serum containing M3MR autoantibodies was also able to confer loss of secretory functions in NOD-severe combined immune deficient or scid mice (Robinson et al., 1998).

Although the severe dysfunction of the lacrimal gland associated with dry eye and SjS is characterized by loss of acinar secretory capacity, very little is known about the changes in exocytotic pathways in the lacrimal gland that must occur during the development of SjS and their relationship to the ensuing inflammatory changes. Some work implicates lymphocytic infiltration and exposure to autoantibodies (including pathogenic autoantibodies to M3MR) directly in the loss of secretory function (Nguyen et al., 2000; van Blokland and Versnel, 2002). Other studies suggest that early changes in lacrimal gland function, specifically in the secretory pathway, may precede infiltration; for instance, NOD-scid mice exhibit differences in protein composition in saliva compared to controls (Robinson et al., 1996). The latter work suggests that there may be fundamental changes in the NOD mouse acinar secretory pathways that may predispose these mice to autoimmune disease.

Our goal in these studies was to investigate the changes in the organization of key effectors, early and late, of exocytosis that occur in NOD mice lacrimal glands compared to age-matched controls (BALB/c mice). We hypothesize that such information may lead to new research avenues which can identify mechanisms involved, particularly at the trafficking level, which initiate SjS. The mice used in the study were sampled at 1 month, prior to any reported lymphocytic infiltration or loss of function, and at 4 months, when lymphocytic infiltration of the gland and clinical manifestations have been reported.

Methods

Reagents

Goat anti-rabbit secondary antibody conjugated to FITC and rhodamine-phalloidin were obtained from Sigma Chemical Co (St. Louis, MO). Rabbit polyclonal antibody to rab3D was generated against recombinant mouse rab3D (Antibodies, Inc., Davis CA) and purified using

protein A/G Agarose. The rabbit polyclonal antibody to M3MR was obtained from Biodesign International (Saco, ME). ProLong antifade mounting medium was from Molecular Probes (Eugene, OR). Goat anti-rabbit IRDye™800-conjugated secondary antibody was purchased from Rockland (Gilbertsville, PA).

Isolation and processing of mouse lacrimal glands

Male NOD (Taconic Farms, Germantown, NY) and BALB/c mice (Jackson Labs, Bar Harbor, ME) were used throughout our studies. The mice were maintained in our vivarium facility on a 12-hour light-dark cycle, and given food and water ad libitum. Animals were sacrificed by cervical dislocation and/or with carbon dioxide. Exorbital lacrimal glands were removed and some tissue fragments were fixed and processed for epoxy embedment for electron microscopy (EM) as previously described (Schechter et al., 2002). Additional fragments of lacrimal glands were fixed in 4% paraformaldehyde/4% sucrose in Dulbecco's phosphate buffered saline (DPBS) at room temperature for 3-4 hours and then transferred to 30% sucrose in DPBS at 4°C overnight. The next morning the fragments were dried, immersed in Optimum Cutting Temperature (O.C.T.) solution (Sakura Finetek USA, Torrance, CA), rapidly frozen with liquid nitrogen and cryo-sectioned at 5 µm with a Mikrom cryostat. These sections were processed with appropriate antibodies and affinity label as described below for confocal fluorescence microscopy. Finally, some lacrimal gland fragments were directly suspended in lysis buffer for preparation of cell lysates, SDS-PAGE and Western blotting. All studies conformed to the standards and procedures for the proper care and use of animals as described in the Declaration of Helsinki, the Guiding Principles in the Care and Use of Animals (DHEW publication, NIH 80-23) and the ARVO Resolution on the Use of Animals in Ophthalmic Research. Male 1-month and 4-month NOD and 1-month and 4-month BALB/c mice were used in the present study.

EM

Processing of exorbital lacrimal glands for EM analysis included use of 1% tannic acid in 0.1 M cacodylate buffer, pH 7.0, as a stain enhancer following osmification. Tissue samples were embedded in LR White (London Resin, London, UK) for sectioning. Samples were analyzed using a JEOL 1200 EX transmission electron microscope. Electron micrographs of mouse lacrimal glands were utilized to quantify SVs. All images were chosen at random and were acquired at equal magnification. A total of 8168 SVs were counted (n = 1432 to 3026 per data point) and labeled as (a) "single" (if it was clearly distinguished from other SVs or, if it was adjacent to another SV, but it still maintained an intact and distinguishable membrane); (b) "double" if the SVs was clearly fused with an adjacent vesicle and exhibited discontinuity in the membrane or (c) "≥3" if three or more SVs were fused.

Confocal fluorescence microscopy

Cryosections placed on glass coverslips were rinsed with DPBS, incubated with 50 mM NH₄Cl in DPBS for 5 min, rinsed with DPBS, exposed to 0.1% Triton X-100 in DPBS for 5 minutes, rinsed with DPBS, and blocked with 1% bovine serum albumin. Cryosections were then incubated with appropriate primary and fluorophore-conjugated secondary antibodies and/or rhodamine phalloidin. Most confocal images were acquired with a Zeiss LSM 510 Meta NLO imaging system equipped with Argon, red HeNe and green HeNe lasers mounted on a vibration-free table. A few images were acquired on a Nikon PCM Confocal System equipped with Argon ion and green HeNe lasers attached to a Nikon TE300 Quantum inverted microscope. All panels were compiled in Adobe Photoshop 7.0 (Adobe Systems Inc, Mountain View, CA).

Confocal micrographs of mouse lacrimal glands were also used to quantify the changes in rab3D labeling. All images were chosen at random and acquired at equal magnification from

3-4 lacrimal glands at each age and strain. For designation of rab3D labeling as “regular” or “irregular”, a total of 732 acini from 1 month mice were scored (n=317-432 per data point). For designation of rab3D labeling as “apical” or “dispersed”, a total of 791 acini from 4 month mice were scored (n=267-524 per data point).

Analysis of rab3D content by Western Blotting

Lacrimal glands from 1 and 4 month BALB/c and NOD mice were homogenized in MESP buffer (125 mM mannitol, 40 mM sucrose, 1 mM EDTA-Tris, and 5 mM PIPES [Piperazine-N,N'-bis(2-ethanesulfonic acid)]-Tris, pH 6.7) using a Dounce homogenizer (10 passes), incubated on ice for 30 minutes, and then centrifuged at 11,000 RPM in an Eppendorf 5415 C centrifuge at 4°C. Protein content of the supernatant was assayed using the Bio-Rad protein assay according to the manufacturer's instructions. Samples containing 50 µg of protein were mixed with 6x sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose for Western blotting using rabbit polyclonal anti-rab3D and goat anti-rabbit secondary antibody conjugated to IRDye™800. Signal intensity was quantified using an Odyssey Scanning Infrared Fluorescence Imaging System (Li-Cor, Lincoln, Nebraska).

Results

Cortical actin exhibits age- and strain-related changes in lacrimal acini

We examined the cellular organization of actin filaments, as an indicator of the relative dimensions of apical and basolateral membranes in lacrimal acini, and of the cellular organization of individual cells within the acini (Figure 1). BALB/c mice lacrimal glands at 1 month exhibited intense labeling of apparent actin-enriched luminal regions, along with fainter labeling of actin filaments associated with basolateral surfaces. The intensity of the basolateral labeling of actin filaments was noticeably discontinuous; higher magnification examination gave the impression of beads along a string. These regions of relative actin accumulation or the “beads” at the basolateral surface may be related to large (1-2 µm) aggregates of cell membrane interdigitations noted also by EM (data not shown). In the BALB/c mice glands, some lumina appeared distinct, small and circular while others appeared larger and appeared to extend throughout the acini. Comparison of actin filament organization in lacrimal glands from 4 month BALB/c mice revealed comparable labeling patterns, except that extended lumina appeared more frequently in these sections.

In lacrimal glands from 1 month NOD mice, actin filament organization was markedly altered relative to its organization in BALB/c mice lacrimal glands, with the most noticeable change being the tremendous increase in the intensity of labeling of basolateral actin. Glands from 4 month NOD mice exhibited this change as well. The overall amount of actin filament labeling detected in 4 month NOD mice lacrimal glands appeared diminished in sections from some mice relative to that seen in either BALB/c or 1 month NOD mice glands, particularly in areas of extensive tissue destruction or lymphocytic infiltration.

Confocal fluorescence microscopy reveals altered distribution of rab3D in lacrimal glands from NOD mice

Mature SV organization in BALB/c and NOD mice lacrimal glands was evaluated by analyzing the immunofluorescence labeling associated with rab3D by confocal fluorescence microscopy (Figure 2). In lacrimal glands from 1 month BALB/c mice, rab3D was detected in association with large vesicular organelles. Analysis of the morphology of the labeling revealed a continuous circular labeling pattern around vesicles sized ~1 µm, consistent with that of SVs in this tissue. These vesicles were enriched abundantly beneath the apical plasma membrane, while the intensity of labeling decreased towards the cell interior. In these mice, rab3D labeling was very rarely detected extending completely to the basolateral membrane, nor could it be

detected preferentially associated with the basolateral membrane. A similar rab3D labeling pattern was seen in lacrimal glands from 4 month BALB/c mice, with some minor variation in the uniformity of rab3D staining suggestive of increased heterogeneity in SV size associated with aging.

The rab3D labeling pattern in glands from 1 month NOD mice exhibited a substantial increase in the discontinuity of rab3D labeling, evident as large regions free of labeling that, in many cases, were bounded by an intense area of rab3D labeling. This increased discontinuity of labeling was also seen in lacrimal glands from 4 month NOD mice, but was accompanied by an additional prominent change. In 4 month NOD mice, particularly those exhibiting significant lymphocytic infiltration, we noted a marked increase in rab3D labeling towards the basolateral membrane where actin aggregates were particularly prominent. This was associated with a general cellular accumulation of rab3D-enriched membranes as well as some general swelling. These changes in rab3D labeling in lacrimal glands from 1 and 4 month NOD mice are shown in Figure 2.

To determine the prevalence of these changes across individual glands and between different mice, we randomly sampled multiple fields from lacrimal glands under each condition and scored the most prominent changes in rab3D distribution in the gland at 1 month and at 4 months. Our results are shown in **Table 1**. In the lacrimal glands from 1 month NOD mice, a marked increase in the percentage of total acini exhibiting “irregular” (discontinuous) labeling rather than “regular” or continuous labeling was seen. Likewise, in the 4 month NOD mouse glands, a marked increase in the percentage of total acini exhibiting a dispersed rab3D labeling pattern rather than an “apical” distribution was also seen.

The altered vesicular labeling patterns for rab3D in the 1 month and 4 month NOD mice lacrimal glands were not associated with altered protein expression of rab3D in whole gland lysates, since SDS-PAGE analysis and Western blotting of lacrimal gland lysates revealed no significant differences in rab3D protein content in BALB/c and NOD mice lacrimal glands relative to the total protein at 1 and 4 months (Figure 3).

EM analysis confirms increased SV heterogeneity and premature cytoplasmic fusion of SVs in NOD mice lacrimal glands

Examination of lacrimal glands from 1 and 4 month BALB/c and NOD mice by EM revealed substantial changes in overall morphology and further evidence for profound alterations in SVs (Figure 4). Lower magnification images of glands from 4 month BALB/c mice revealed acinar cells enriched in regularly-shaped SVs of heterogeneous size (1-2 μm) and content (mucous and serous); images of glands from age-matched NOD mice exhibited a complex array of irregularly shaped SVs, many of which had undergone apparent cytoplasmic fusion and appeared as multivesicular aggregates. Examination of SV morphology in lacrimal glands from 1 month and 4 month BALB/c and NOD mice at higher magnification revealed further evidence for the transition from regularly-shaped, distinct individual SVs in BALB/c mice, to a state characterized by formation of multivesicular aggregates in NOD mice. We documented this change by quantifying SV categories in randomly acquired images from multiple mouse lacrimal glands under each condition (Figure 5). The majority of SVs in lacrimal glands from 1 and 4 month BALB/c mice fell within the “individual” SV category. However, lacrimal glands from 1 and 4 month NOD mice displayed characteristics of premature cytoplasmic fusion of SVs, with a significant ($p \leq 0.05$) increase in aggregated (>3) vesicles to almost half of total SVs. Although the percentage of SVs present as multivesicular aggregates in lacrimal glands from 4 month NOD mice was not significantly different than that in glands from 1 month NOD mice, the SVs in some of the 4 month NOD glands exhibited additional visible evidence of deterioration characterized by the detection of malformed and irregularly shaped smaller SVs (Figure 4).

The appearance of SVs as lighter or darker by EM is related, to a certain extent, to their contents, with lighter vesicles containing more mucous content and darker vesicles containing more serous content. When we quantified SVs in lacrimal glands from a minimum of two mice per experimental group into “light” and “dark” categories”, we found that the percentage of total SVs designated as “dark” was 34% of total in 1 month BALB/c mice lacrimal glands (n=632 SVs), 31% of total in 1 month NOD mice lacrimal glands (n=466 SVs), 20% of total in 4 month BALB mice lacrimal glands (n=492 SVs) but 59% of total in 4 month NOD mice lacrimal glands (n=929 SVs). Although this number suggests a shift towards a more serous SV population in glands from 4 month NOD mice, it should be viewed with some caution since the relative contrast of the individual SVs in each image is influenced by fixation conditions and the quality of the section. Because of this issue, the quantitative analysis was not expanded. Additional studies using techniques which can more directly measure and quantify changes in specific SV content proteins such as immunofluorescence or immunogold/EM will be necessary to confirm this apparent shift.

M3MR distribution is substantially altered in NOD mice lacrimal glands relative to BALB/c mice lacrimal glands

Figure 6 shows the distribution of immunofluorescence associated with the M3MR in frozen sections from lacrimal glands of age-matched BALB/c and NOD mice. Lacrimal glands from either 1 month or 4 month BALB/c mice exhibited M3MR immunofluorescence at or adjacent to the basolateral cell periphery. In contrast, lacrimal glands from 1 month or 4 month NOD mice exhibited very little basolateral labeling associated with M3MR; stores were instead detected intracellularly in punctuate spots. Extensive tissue damage and infiltration of immune cells is also evident in one of the 4 month NOD mouse lacrimal gland sections shown here, reflective of our general findings. The redistribution of M3MR appeared to be more remarkable in lacrimal glands exhibiting more tissue damage and infiltration of immune cells in 4 month NOD mice, similar to our previous findings on the magnitude of the effects on rab3D labeling.

Figure 7 depicts M3MR immunofluorescence in 1 month BALB/c and NOD mouse lacrimal glands in reconstructed image stacks obtained by summing serially-acquired confocal images throughout the entire frozen section; this technique enabled us to better visualize M3MR labeling which was quite diffuse in single plane images. The images depicted are also rotated from 0° at intervals through to 180°, since the changes in orientation enable additional features of the labeling to be visualized. The rotated, compressed stacks very clearly show M3MR immunofluorescence at the basolateral membrane of the lacrimal acini from the 1 month BALB/c mouse, with fainter immunofluorescence detected in a gradient of decreasing intensity toward the apical membrane. In sections from 1 month BALB/c mice lacrimal glands, the M3MR receptor immunofluorescence was regularly distributed in all focal planes. In sections from 1 month NOD mice lacrimal glands, an immediate difference in the pattern of M3MR immunofluorescence was detected, consisting of a primarily punctate labeling pattern in the interior of the acinus. The labeling intensity was likewise diminished in 1 month NOD mice glands, relative to the profile seen in the age-matched BALB/c mouse. In the age-matched NOD mice, the punctate spots of M3MR immunofluorescence were asymmetrically localized towards one side of the cell. This is evident when comparing images obtained from 0-45° in the 1 month NOD lacrimal gland sample, which shows abundant punctate immunofluorescence, and images obtained between 135-180°, when the punctate signal diminishes tremendously. This phenomenon was highly reproducible and distinguishable from the more homogeneous M3MR labeling seen in BALB/c mice lacrimal glands, although the significance of this is unclear.

Discussion

It is clear that the loss of ocular surface fluid associated with SjS must be associated at some level with changes in the exocytotic pathway responsible for release of tear proteins and fluid from the lacrimal gland. Despite this, there has been a surprising lack of research focused on discerning mechanistic changes in lacrimal acinar exocytosis in SjS disease models. In this study we show that SVs in lacrimal glands from male NOD mice exhibit profound morphological changes as early as 1 month of age. These changes included an increased irregularity in the association of the effector, rab3D, with SVs concomitant with significantly increased premature cytoplasmic fusion of individual SVs into multivesicular aggregates. In addition to the evidence for premature cytoplasmic fusion seen in the 1 month NOD mouse lacrimal glands, glands from 4 month NOD mice additionally exhibited a remarkable redistribution of the normally apical rab3D stores towards the basolateral membrane. These changes in rab3D labeling in NOD mice lacrimal glands are generally suggestive of altered fusogenic properties of these acinar SVs, likely contributing to consequent changes in acinar secretory functions. The time course of these events suggested that early deterioration of the SVs typified by premature cytoplasmic fusion and loss of rab3D was followed by a gradual accumulation of altered SVs which accumulated within the cytoplasm, ultimately reaching the basolateral membrane by 4 months.

Other changes detected in NOD mice glands at 1 month which persisted or increased in magnitude in the 4 month NOD mice glands included increased accumulation of basolateral actin and a remarkable redistribution of M3MR immunofluorescence from the basolateral membrane to aggregates in the cell interior. All of the changes in SV organization and in M3MR distribution observed in the 1 month NOD mice lacrimal glands occurred before notable immune cell infiltration in the majority of the mice, consistent with previous reports that immune cell infiltration is typically detected between 6-10 weeks in male NOD mice lacrimal glands (Hunger et al., 1998).

The changes in vesicular rab3D labeling in lacrimal glands from NOD mice occurred without significant changes in total rab3D protein expression relative to BALB/c mice lacrimal glands. Although the total intensity of labeling in the 4 month NOD mice glands appears increased by confocal fluorescence microscopy, largely due to the increased rab3D immunofluorescence detected at the basolateral membrane, this apparent increase in labeling intensity could be accomplished by changes in the partitioning between soluble and membrane-bound rab3D. Membrane-associated rab3D might appear as a more intense immunofluorescence because of its concentration on cellular membranes. Alternatively, because of the extensive tissue destruction in 4 month NOD mice, a smaller percentage of total cells in the gland are represented by the acini while immune cells represent an increasing percentage of total cells and, consequently, of total protein. Rab3D expression/protein in the gland therefore may remain unaltered in 4 month NOD mice lacrimal glands while rab3D expression/acini is increased.

The precise function of rab3D in acinar secretion is still unclear although studies overexpressing active and dominant negative forms in pancreatic acini showed changes in exocytosis (Ohnishi et al., 1997; Chen et al., 2003). Surprisingly, recent generation of a rab3D knockout mouse resulted in no detectable phenotype in release kinetics in the exocrine pancreas or parotid gland (Riedel et al., 2002). However, the size of the secretory granules in the pancreas and parotid gland of this knockout mouse were significantly increased, suggesting that rab3D may act by preventing premature homotypic fusion of mature SVs. Unfortunately, the organization and function of lacrimal glands were not examined in this study of the rab3D knockout mice. NOD mice lacrimal glands also exhibited more severe degeneration of exocytotic pathways than did the other exocrine glands examined in the rab3D knockout mouse

(pancreas and salivary glands), suggesting the existence of more complicated changes than a simple effect on rab3D function. However, the observation of shared increases in homotypic fusion in these two models suggests that defective rab3D activity may be one factor contributing to the phenotype seen in the NOD mouse secretory pathway. Rab3D, like other rab proteins, exists in GTP and GDP bound forms with the cycling between these forms modulated by other proteins including GDP dissociation inhibitors and membrane receptors. Changes in rab3D function independent of altered protein expression could be elicited in a variety of ways including altered prenylation through CHM/Rep (Alory and Balch, 2001), phosphorylation by rab3D kinase (Pombo et al., 2001) or altered expression of effectors that modulate its activity such as GDP dissociation inhibitors or GTPase activators (Wu et al., 1996). General changes in the signaling environment could also be responsible for altered functioning of rab3D and other effectors of the exocytotic pathway.

Evidence in support of an altered signaling environment in the NOD mouse lacrimal gland includes our observation of redistribution of M3MR from the basolateral membrane to interior compartments. This is consistent with a previous report that signaling responses are diminished in NOD mouse salivary glands (Humphreys-Beher et al., 1998). Exposure to unusually high amounts of agonist released by parasympathetic nerves might cause downregulation of basolateral M3MR through internalization by endocytosis and sorting to degradative compartments such as lysosomes. This model is consistent with our observation of the accumulation of basolateral actin seen in the NOD mice lacrimal glands which can occur as a consequence of sustained secretagogue exposure in vitro (data not shown).

The relationship between the altered M3MR distribution and the observed degeneration of the terminal exocytotic compartments (SVs) is of major interest. Both changes are detected at same age in early postnatal development, so it is not clear yet whether they occur in parallel or whether one is the cause of the other. Our data best support a model in which M3MR redistribution, perhaps triggered by altered agonist stimulation of the receptor itself, leads to a change in the cellular signaling environment which is conducive to SV deterioration. This working model is depicted in Scheme 2. Basal signaling through M3MRs may normally be essential for maintenance of exocytotic pathways. Changes in intracellular signaling would result in gradual deterioration of SVs, from an early stage characterized by premature cytoplasmic fusion and loss of rab3D (1 month NOD mice), to a later stage characterized by intracellular accumulation of multivesicular aggregates extending to the basolateral membrane (4 month NOD mice).

Some contents of the altered SVs that accumulate beneath the basolateral membrane may be released at this domain, representing misdirected exocytosis. SV exocytosis normally occurs at apical actin-enriched domains in lacrimal acini, while actin filament remodeling is also thought to be an essential component of acinar exocytosis. The significant accumulation of basolateral actin filaments in NOD mouse lacrimal glands is consistent with a possible increase in SV fusion at this domain. However, the generalized cellular swelling and apparent SV accumulation in 4 month NOD mice lacrimal glands suggests that the overall rates of exocytosis are decreased, although some apical and/or basolateral exocytosis may occur at a reduced level. Subsequent autoimmune responses directed at the lacrimal gland may result directly from the alteration in secretory pathways and/or low levels of misdirected exocytosis of secretory products in the interstitium.

Alternatively, if SV deterioration precedes M3MR redistribution, primary changes in secretory trafficking may also result in reflux of materials that are normally released apically through basolateral pathways and into the interstitium where they can initiate immune responses; subsequent exposure of lacrimal gland to lymphocytes may then induce M3MR receptor internalization and aberrant processing. This latter scenario is inconsistent with our observation

that most of the lacrimal glands from 1 month NOD mice that showed changes in exocytotic pathways and in M3MR distribution did not exhibit detectable lymphocytic infiltration. However, we cannot rule out the possibility that low levels of immune cell activation are present in the 1 month NOD mouse lacrimal glands that are not microscopically evident in the thin sections chosen for study.

Our data suggest, in the NOD mouse, that early changes in SV morphology, composition and apparent fusogenic properties may contribute to the development of SjS-type symptoms. These changes may be driven by an altered intracellular signaling environment due to early redistribution of M3MR. Primary changes in exocytotic membrane trafficking may represent a first step in initiation of disease, with subsequent inflammatory responses toward the gland caused by initial changes in early and late effectors of the exocytotic pathway.

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Figure 1. Actin filament organization in BALB/c and NOD mouse lacrimal glands

Confocal fluorescence micrographs of frozen sections from lacrimal glands from BALB/c and NOD mice labeled with rhodamine-phalloidin to detect actin filaments. (a) 1 month BALB/c mouse lacrimal gland. (b) Inset from 1 month BALB/c mouse lacrimal gland magnified from (a). (c) 1 month NOD mouse lacrimal gland. (d) Inset from 1 month NOD mouse lacrimal gland magnified from (c). (e) 4 month BALB/c mouse lacrimal gland. (f) Inset from 4 month BALB/c mouse lacrimal gland magnified from (e). (g) 1 month NOD mouse lacrimal gland. (h) Inset from 4 month NOD mouse lacrimal gland magnified from (g). *, regions adjacent to small lumena or within extended luminal regions; arrows, basolateral actin accumulation; arrowheads, regions of discontinuous basolateral actin labeling; bars, 10 μ m.

Figure 2. Rab3D association with SVs in BALB/c and NOD mouse lacrimal glands
 Confocal immunofluorescence microscopy of rab3D (green) and actin filaments (red) in lacrimal glands from BALB/c and NOD mice. (a) 1 month BALB/c mouse lacrimal gland. (b) 4 month BALB/c mouse lacrimal gland. (c) 1 month NOD mouse lacrimal gland. (d) 4 month NOD mouse lacrimal gland. arrows, region of discontinuous rab3D labeling in NOD mice; arrowheads, accumulation of basolateral actin; bars, 10 μ m.

Figure 3. Rab3D levels in lacrimal glands from age-matched BALB/c and NOD mice

(a) Representative Western blot showing rab3D signal in 50 μ g protein from lacrimal gland lysates from 1 and 4 month BALB/c and NOD mice. (b) Quantitation (signal/protein) of rab3D content from lacrimal glands from 3 separate mice under each experimental condition. No significant differences in expression were detected.

Figure 4. EM analysis of SV morphology in BALB/c and NOD mouse lacrimal glands
 Lower magnification images of (a) 4 month BALB/c mouse lacrimal gland and (b) 4 month NOD mouse lacrimal gland depict the overall organization in glands from these mice. The higher magnification EM micrographs from (c) 1 month BALB/c mouse lacrimal glands, (d) 4 month BALB/c mouse lacrimal glands, (e) 1 month NOD mouse lacrimal glands and (f) 4 month NOD mouse lacrimal glands reveal differences in SV size, shape and extent of premature cytoplasmic fusion. L, lumen.

Figure 5. Premature cytoplasmic fusion of SVs in NOD mice revealed by quantitative EM analysis
EM images of lacrimal glands of 1 and 4 month BALB/c and NOD mice were acquired at equal magnification. SVs were classified as “individual”, “double” (two fused SVs) or >3 (three or more fused SVs). SVs in lacrimal glands from 1 and 4 month BALB/c mice were generally uniform in size and distribution with the majority (>50%) considered “individual”. 1432-3026 SVs quantified per category; lacrimal glands were obtained from 3-5 mice per condition; *, significant from age-matched control at $p \leq 0.05$

Figure 6. M3 muscarinic receptor (M3MR) immunofluorescence in lacrimal glands from BALB/c and NOD mice

Cryosections of lacrimal glands from age-matched BALB/c and NOD mice were processed as described in **Methods** to label M3MR (green) and actin filaments (red) and sections were imaged by confocal fluorescence microscopy. (a) and (b), 1 month BALB/c mouse lacrimal gland; (c) and (d), 1 month NOD mouse lacrimal gland; (e) and (f), 4 month BALB/c mouse lacrimal gland; (g) and (h), 4 month NOD mouse lacrimal gland. *, representative luminal regions; bars, 10 μ m.

Figure 7. M3 muscarinic receptor (M3MR) distribution in 3D images of lacrimal glands from 1 month BALB/c and NOD mice

3D reconstruction of XY images of lacrimal gland frozen sections from 1 month BALB/c (a) or 1 month NOD (b) mouse lacrimal glands labeled to detect the M3MR (green) and actin filaments (red) as described in **Methods**. Images were acquired at Z intervals of 0.4 μm from the bottom to the top of the entire 5 μm section and were reconstructed into a 3D movie file which could be rotated to demonstrate the structure of the acini from different angles. To do this, individual Z-stack images were converted into a projection file using the Zeiss LSM 510 operating software, V3.2. The information from the whole series of images in a Z-Stack file was combined and reorganized into a series of XYZ three dimensional images which were

displayed from gradually changed angles of view. Both the total image number and the difference angle between every two adjacent images for display were selectable using the feature setting function of the software. For these reconstructions, 9 images and 22.5° difference angles were selected to constitute the image panels shown here. The first image corresponds to the view at 0° and the subsequent views show angles increasing by 22.5° up to 180° in each panel. The rotation is made around the Y axis of the images. Curved arrows indicate the direction of the rotation; bars, $10\ \mu\text{m}$. (c). Schematic diagram indicating the X, Y and Z orientations relative to an acinus, and showing the projection of the acinus at 0° and 90° .

Scheme 1. Model depicting selected effectors of the secretory pathway in lacrimal acini

The organization of major effectors of the secretory pathway in lacrimal acini is shown including rab3D-enriched SVs, immature SVs (ISV), actin filaments, and the M3MR. These constituents are shown in resting acini and in stimulated acini in which the M3MR has been activated by ligand binding, resulting in exocytosis of secretory products. APM, apical plasma membrane and BLM, basolateral membrane.

Scheme 2. Working model depicting relationship between changes in effectors of the secretory pathway in NOD mouse lacrimal glands

(1) The early internalization of the M3MR, and its accumulation within an intracellular lysosomal compartment is elicited by altered agonist stimulation, either directly by activation of the M3MR as shown here or indirectly through changes in other signaling effectors. (2) Consequent changes in the intracellular signaling environment associated with altered M3MR trafficking lead to changes in SV organization and function, including the premature cytoplasmic fusion of rab3D-enriched SVs beneath the APM which occurs first in 1 month NOD mouse lacrimal glands, and the subsequent accumulation of SVs including prematurely-fused SVs at the basolateral membrane (BLM) that is prominent in 4 month NOD mouse lacrimal glands. (3) Misdirected exocytosis of the contents of altered SVs at the BLM may occur (dashed arrows), contributing to inflammation. The accumulation of actin filaments at the BLM may reflect increased actin remodeling associated with misdirected exocytosis at this domain. APM, apical plasma membrane.