Muscarinic Acetylcholine Type-3 Receptor Desensitization Due to Chronic Exposure to Sjögren's Syndrome-Associated Autoantibodies

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ABSTRACT. ***Objective.*** Sjögren's syndrome (SS) is an autoimmune disease characterized by salivary and lacrimal gland dysfunction leading to dry mouth (xerostomia) and dry eyes (xerophthalmia). Anti-muscarinic acetylcholine type-3 receptor (anti-M3R) autoantibodies have been shown to inhibit M3R-mediated responses by both *in vitro* contractility and fura-2 microfluorimetry analyses of intracellular calcium mobilization, suggesting an important role for anti-M3R autoantibody in secretory dysfunction. We investigated whether chronic stimulation of M3R by anti-M3R autoantibodies and/or the agonist pilocarpine results in a general M3R desensitization.

Methods. Carbachol-evoked responses of mouse-bladder smooth muscle strips were measured following exposure to anti-M3R-positive and/or anti-M3R-negative sera from either NOD/Lt mice, a model of SS-like disease, or human patients with primary SS.

Results. Bladder smooth muscle strips isolated from NOD/Lt mice with circulating anti-M3R autoantibodies exhibited lower carbachol-evoked responses than smooth muscle strips from anti-M3R autoantibody-negative NOD/Lt mice and control C57BL/6 mice. Repeated pilocarpine injections of NOD mice for 6 days also revealed M3R desensitization in the agonist-evoked contractile assay, whereas age and sex matched C57BL/6 mice injected with pilocarpine for the same period showed a 2-fold higher response. Incubation of smooth muscle strips with sera obtained from patients with primary SS resulted in both stimulated and inhibited responses.

Conclusion. These results support the hypothesis that chronic stimulation of membrane-bound M3R can result in receptor desensitization, and raise questions about repeated use of pilocarpine by patients positive for anti-M3R autoantibodies. (J Rheumatol 2006;33:296–306)

Key Indexing Terms:
SJÖGREN'S SYNDROME
NON-OBESE DIABETIC MOUSE
ANTI-MUSCARINIC ACETYLCHOLINE TYPE-3 RECEPTOR
AUTOANTIBODY
BLADDER STRIP CONTRACTILE ASSAY
SALIVARY GLAND DYSFUNCTION

Sjögren's syndrome (SS) is a systemic autoimmune disorder displaying organ-specific manifestations in the salivary and lacrimal glands, leading to significant loss of fluid secretion[1,2]. Other characteristic features include the presence of mononuclear lymphocytic infiltration within target organs and a high prevalence of autoantibodies, including antinuclear autoantibodies (ANA) such as anti-SSA/Ro and SSB/La antibodies. Despite considerable attention on these antibodies in SS, none is specific for SS, and they are commonly detected in other systemic autoimmune diseases like systemic lupus erythematosus. The pathological implications of these antibodies in the suppression of secretory function (the hallmark of SS) remain unclear.

One interesting observation with regard to the identification of organ-specific autoantibodies has been the recognition of antibodies reactive with muscarinic acetylcholine type-3 receptors (M3R) expressed on the surface of exocrine epithelial cells. The presence of anti-M3R antibodies was first proposed in studies by Bacman, *et al*[3] and Yamamoto, *et al*[4] using IgG from human patients with SS and sera from disease-prone non-obese diabetic (NOD) mice, respectively. The former indicated that IgG present in the sera of patients with primary SS inhibited the binding of ^{3}H-quinuclidinyl benzilate (QNB) to M3R of purified rat parotid gland membranes *in vitro* in a noncompetitive manner. Similarly, Yamamoto, *et al* showed that sera from diabetic NOD, but not normal BALB/c, mice

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immunoprecipitated radiolabeled M3R. A subsequent study indicated that either loss or stimulation of saliva flow could be induced when purified IgG from SS patients was transfused into B cell-deficient mice[5].

The potential implication of anti-M3R autoantibodies in inducing loss of secretory function is thought to be due to the role of M3R in regulating fluid secretion in salivary and lacrimal glands. Recent studies[6-8] on the functional roles of anti-M3R autoantibodies clearly indicate that these antibodies can inhibit carbachol-evoked responses. When M3R, G-protein-coupled 7 transmembrane receptors, are stimulated by parasympathetic neurotransmitters, they trigger intracellular calcium release that mediates multiple calcium-stimulated cellular functions, e.g., bladder smooth muscle contraction or activation of chloride channels in salivary glands for fluid secretion. Thus, the presence of anti-M3R autoantibodies interfering with ligand-M3R interactions is assumed to disrupt subsequent alterations of normal intracellular signaling pathway mediated by M3R.

The disease-prone NOD mouse model of SS has been studied extensively in attempts to define the pathogenesis of SS. Despite the general belief that mouse models do not provide a complete replication of human diseases, the NOD mouse model exhibits focal lymphocytic infiltration and inflammatory cytokine profiles similar to human patients, plus the production of autoantibodies such as ANA and anti-M3R autoantibodies. Further, NOD mice lose secretory function following lymphocyte infiltration of the glands[9,10]. Other strains such as NFS/sld or BAFF transgenic mice, although known to develop reduced saliva function, do so only after 13–15.5 months of age in BAFF transgenic mice[11] and 18–20 months of age in NFS/sld mice[12], suggesting an age-related loss of tolerance as a potential confounding factor.

We analyzed various serum samples from NOD mice and human patients with SS to identify if sera positive for anti-M3R autoantibodies can alter carbachol-induced signaling through M3R, leading to pathological loss of secretory function. In general, sera from diseased mice or human patients were incubated with bladder smooth muscle strips from disease-free mice to investigate any acute effects of the autoantibodies. The chronic effects of the autoantibodies were examined by analyzing the contraction curves of the bladder strips from the diseased NOD mice that were positive for the antibodies. In addition, pilocarpine, a secretagogue commonly used in clinical settings, was injected over various lengths of time to investigate if chronic exposure to pilocarpine would accelerate receptor desensitization initiated by anti-M3R autoantibodies. Results indicate that M3R desensitization can occur in the presence of continued stimulation. What this might mean in the clinical treatment of patients with SS is discussed.

MATERIALS AND METHODS

Animals. NOD/Lt and C57BL/6 mice used for this study were bred and maintained in the SPF Mouse Facility of the Department of Pathology at the University of Florida. Female and male mice between the ages of 8 and 28 weeks were utilized. The studies were approved by the University of Florida IACUC.

Patient serum and saliva samples. Patient serum samples were generously provided by Dr. Roland Jonsson (University of Bergen, Bergen, Norway). Patients were diagnosed with SS using the modified European criteria proposed by the European-American Consensus Group[13]. Informed consent was approved by the ethics committee of the University of Bergen and signed by each patient before the samples were collected. Table 1 lists clinical and laboratory characteristics of each patient with SS from which the serum samples were derived.

Detection of anti-mouse M3R autoantibodies. Detection of anti-M3R autoantibodies in the sera of individual mice was carried out using 3×10^5 Flp-In CHO cells transfected with the mM3R gene plated on collagen type I coated plates (MatTek Corp., Ashland, MA, USA) without fixation. Generation of the mM3R transfected Flp-In CHO cells is described elsewhere[14,15]. Cells were incubated in duplicate with individual mouse sera for 1 h at a dilution of 1:50, followed by 40 min incubation with FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich Co., St. Louis, MO, USA) at a dilution of 1:100. Positive staining of the cells was visualized with a fluorescence microscope under a 40× objective. Control reactions included mM3R-transfected cells incubated with secondary antibody alone, mM3R-transfected cells incubated with control C57BL/6 sera, and nontransfected Flp-In CHO cells.

Detection of anti-human M3R (anti-hM3R) autoantibodies. Detection of anti-hM3R autoantibodies in the sera of individual SS patients was carried out using Flp-In CHO cells transfected with the hM3R gene[14]. Prior to testing, each test serum was preabsorbed on nontransfected Flp-In CHO cells (1×10^5 cells/µl serum for 2 h) to eliminate any natural antibodies reactive with CHO cells. Ten microliters of each serum were then incubated for 1.5 h at 4°C with 1×10^5 transfected Flp-In CHO cells that had been washed, centrifuged, and resuspended in FACS buffer [1× phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.07% NaN_3] at 1×10^6 cells/ml. The cells were washed in 1 ml FACS buffer, resuspended in 50 µl of buffer, and incubated 30 min at 4°C with 2 µl FITC-conjugated goat anti-human IgG Fab-specific antibody (Sigma-Aldrich). After a final wash, the cells were suspended in FACS buffer and analyzed using a FACSCalibur flow cytometer equipped with Cell Quest software (BD Biosciences, San Jose, CA, USA). Control reactions included hM3R-transfected cells with secondary antibody alone, hM3R-transfected cells incubated with control sera, and nontransfected Flp-In CHO cells. An increase in intensity of fluorescence with hM3R-transfected versus nontransfected Flp-In CHO cells was considered a positive reaction for anti-M3R autoantibodies.

In vivo injection of mice with pilocarpine. Pilocarpine-HCl (Sigma-Aldrich) was dissolved in 1× PBS and injected (0.2 mg per injection of 100 µl) into 20-week-old female C57BL/6 and NOD mice (n = 3 per strain) 3 times per day at 6 h intervals for 1, 6, or 12 days. The mice were euthanized 2 h before the functional assays were carried out.

In vitro contractile studies. The effects of anti-M3R autoantibodies on smooth muscle cell contraction were tested using urinary bladder strips. Ring-shaped urinary bladder strips were prepared from the bladders of mice, as described[16]. In brief, bladder strips were placed in oxygenated Krebs solution (118.2 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 24.8 mM $NaHCO_3$, and 10.0 mM dextrose). Each tissue strip was mounted in one of 4 chambers of a tissue bath system (700 MO, Danish Myo Technology, The Netherlands) and covered with 10 ml of Krebs solution. The baths were constantly aerated with 95% O_2/5% CO_2 (pH 7.4) at 37°C. The bladder strips were maintained at a resting tension of 1 g during an equilibrium period of 60 min and washed every 15 min. At the beginning of each experiment, the contractile response to KCl (60 mM) was assessed to determine the viability of each tissue. After washing and stabilization, concentration-effect curves to agonist stimulation (i.e., carbachol ranging from 1 nM to 0.1 mM) were generated for each tissue. To test the affinity for the antagonist, atropine, the tissues were equilibrated in either the absence (time-control) or presence of antagonist (10 nM) for 60 min, followed by generation

Table 1. Summary of clinical and laboratory characteristics of patients with primary SS.

Serum Sample	Disease State	Serology (anti-Ro/anti-La antibody)	Lip Biopsy Grade†	Unstimulated Saliva Flow (cutoff value 1.5 ml/15 min)	Facs analysis for anti-M3R antibody††	*In vitro* Contractile Assay
1	pSS	P	P4	0.0	P	Inhibitory*
2	pSS	N	P1	NA	P	Inhibitory
3	pSS	N	P4	1.4	P	Stimulatory**
4	pSS	P	P5	6.0	N	No change
5	pSS	P	P4	15.0	P	Stimulatory
6	Blood donor	NA	NA	NA	N	No change
7	Blood donor	NA	NA	NA	N	No change
8	Blood donor	NA	NA	NA	P	Stimulatory

† Number of foci in 4 mm^2 tissue. †† Detected by flow cytometry with patient sera as a primary antibody (1:10 dilution) and FITC-conjugated anti-human M3R antibody as a secondary antibody (1:100 dilution). * $p < 0.05$, ** $p < 0.01$. pSS: primary Sjögren's syndrome; NA: not available; P: positive; N: negative.

of second concentration-effect curves to the same agonist. Once standard curves were completed, mouse or human serum samples at a 1:50 dilution were placed in the chambers and incubated 1 h. Without washing, a concentration-effect curve following carbachol stimulation was generated in comparison with bladder muscle strips incubated with normal serum from control subjects or with no added serum. The degree of contraction is expressed as a percentage of the KCl contraction.

Statistical analyses. Carbachol concentration response was calculated for each preparation using GraphPad Prism software (GraphPad, San Diego, CA, USA). The shifts of response by antagonists were determined from EC_{50} values (molar concentration of agonist producing 50% of the maximum response) in the presence and absence of antagonist. For statistical analysis, Student's t test was used and $p < 0.05$ was considered to be significant.

RESULTS

Detection of anti-M3R autoantibodies in sera collected from disease-prone NOD mice and patients with primary SS. To detect the possible presence of anti-M3R autoantibodies in NOD/Lt mice, serum samples prepared from individual NOD/Lt and C57BL/6 mice were incubated at a 1:50 dilution first with either nontransfected or mM3R-transfected Flp-In CHO cells, and second with a FITC-conjugated goat anti-mouse IgG antibody. Visualization using a fluorescence microscope revealed that sera from C57BL/6 mice incubated with either nontransfected or mM3R-transfected Flp-In CHO cells showed no positive staining for M3R (Figures 1A, 1D, respectively), while sera from NOD/Lt mice exhibiting SS-like disease showed positive staining with mM3R-transfected Flp-In CHO cells (Figure 1E), but not with nontransfected cells (Figure 1B). Neither nontransfected nor mM3R-transfected Flp-In CHO cells treated with secondary antibody alone showed any positive staining (Figures 1C, 1F, respectively). Six NOD/Lt sera that were identified as positive for anti-mM3R autoantibody and six C57BL/6 sera of age and sex matched mice identified as negative were pooled and used in subsequent functional assays.

To identify human sera positive for anti-hM3R autoantibodies, 8 individual sera, 5 from patients diagnosed with primary SS and 3 from healthy blood donors, were screened at a dilution of 1:10 on nontransfected and hM3R-transfected Flp-In CHO cells using flow cytometric analyses. As illustrated in Figure 2, 4 of the 5 serum samples derived from the SS patients (samples 22, 29, 30, and 36) showed a FACS profile indicating the presence of anti-hM3R autoantibodies. One sample (no. 35) proved to be negative for anti-M3R antibodies despite having been found to contain anti-SSA and anti-SSB autoantibodies (Table 1). Of the serum samples obtained from healthy blood donors, 2 sera (samples 6 and 7) showed the absence of anti-hM3R autoantibodies; however, one serum sample (sample 8) tested positive for anti-hM3R antibodies by flow cytometry. As described below, this serum was also able to alter calcium-mediated contraction of bladder smooth muscle strips, suggesting the distinct possibility that this donor may actually have an undiagnosed autoimmune condition, even though this is only speculative.

Stimulation of mouse-bladder smooth muscle strips by acute exposure to anti-M3R autoantibodies. To test the effects of anti-M3R autoantibodies on subsequent carbachol-evoked calcium-mediated muscle contraction, C57BL/6 bladder muscle strips isolated from 10–12-week-old mice were incubated with either NOD/Lt serum shown to be positive for anti-mM3R autoantibodies or C57BL/6 serum shown to be negative for anti-mM3R autoantibodies for 1 h prior to receptor stimulation. As illustrated in Figure 3A, bladder strips exposed to anti-mM3R autoantibody-positive NOD/Lt serum exhibited upregulated responses to carbachol stimulation compared to bladder strips exposed to autoantibody-negative C57BL/6 serum (maximum relative contractions of 47.54 ± 4.02 vs 32.51 ± 2.94, respectively; $p < 0.05$). This result suggests that a short initial exposure of naive bladder smooth muscles to anti-M3R autoantibodies can provide an additive stimulatory effect that enhances agonist-evoked M3R responses.

In a similar study, the effects of anti-hM3R autoantibodies in human sera on subsequent carbachol-evoked calcium-mediated muscle contraction were examined. C57BL/6 bladder muscle strips isolated from 10–12-week-old mice were incubated 1 h with either control sera shown to be negative for

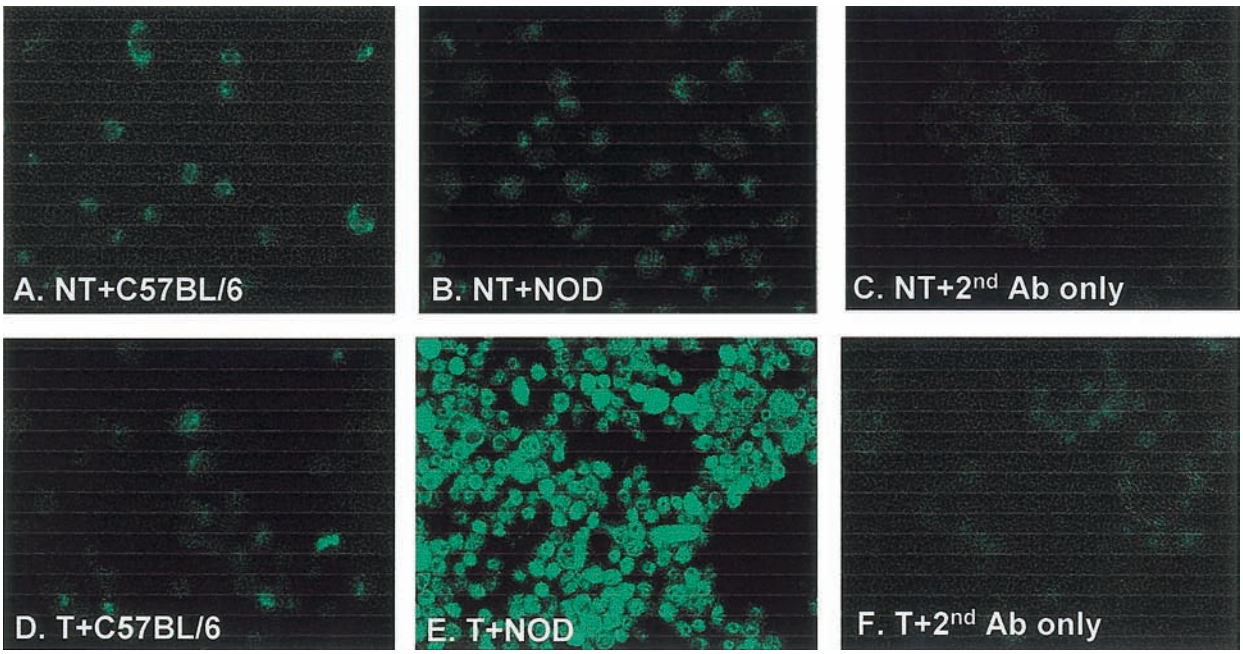

Figure 1. Identification of anti-mouse M3R autoantibody-positive mouse serum samples by immunofluorescent staining. Nontransfected (NT) and mouse M3R-transfected (T) Flp-In CHO cells were incubated for 1 h with individual serum samples at a dilution of 1:50, followed by 40 min incubation with FITC-conjugated anti-mouse IgG secondary antibody diluted 1:100. Reactions were controlled using cells incubated with secondary antibody (2nd Ab) only. Staining was visualized and photomicrographs were taken with a fluorescence microscope, 40× objective.

anti-hM3R autoantibodies or SS patient sera shown to be positive for anti-mM3R autoantibodies prior to bladder strip stimulation. As illustrated in Figure 3B, incubation of the bladder strip with normal serum prior to stimulation with carbachol resulted in a response that did not differ from a response obtained when the bladder strip was incubated with buffer alone, indicating that serum *per se* has little if any effect on M3R activation by M3R agonists. In contrast, incubation of bladder strips for 1 h with sera containing anti-hM3R autoantibodies, as detected by flow cytometry, resulted in mixed responses, as shown in Figures 3C to 3H. For example, serum from SS patient 30 showed a stimulatory response (Figure 3G; $p < 0.01$), while sera from SS patients 22 and 29 showed inhibitory responses (Figures 3E, 3F; $p < 0.05$ for 22). Interestingly, serum from SS patient 35, which proved negative for anti-hM3R autoantibody by flow cytometry, did not alter subsequent carbachol-evoked contractile responses (Figure 3D), whereas antibody-positive control serum did alter the response (Figure 3C). A correlation between the absence and presence of anti-hM3R autoantibodies and muscle contraction responses is presented in Table 1. Overall, these observations are in accord with our previous studies in which human serum samples injected into B cell-deficient NOD.Igμ^{null} mice either stimulated or inhibited saliva secretion[5].

Responses of bladder smooth muscle strips from NOD/Lt mice chronically stimulated by circulating anti-mM3R autoantibodies. Carbachol-evoked responses by bladder muscle strips prepared from NOD/Lt mice that exhibited SS-like disease and tested positive for the presence of circulating anti-mM3R autoantibodies were measured to determine the effects of chronic exposure to anti-M3R antibodies. The maximum responses of such bladder strips isolated from C57BL/6 mice (8 and 20 weeks of age) or pre-disease NOD/Lt mice proved almost equivalent (46 ± 7, 43 ± 5, and 41 ± 3, respectively), as shown in Figure 4A; however, significantly reduced stimulation ($p < 0.05$) was observed (33 ± 4) when the bladder strips from NOD/Lt mice with SS-like disease were tested (Figures 4A, 4B). That lower responses were observed in the strips isolated from older diseased NOD/Lt mice but not from younger pre-disease NOD/Lt mice suggests that the observation is not dependent on strain differences. Further, females exhibited reduced carbachol-evoked responsiveness compared to males (Figure 4C), an observation that is consistent with other pharmacological studies[17].

To identify how an antagonistic reagent affects the influence of anti-M3R autoantibodies in this assay, bladder muscle strips derived from 20-week-old C57BL/6 and NOD/Lt mice were treated for 1 h with 10 nM atropine prior to stimulation with carbachol. As shown in Figures 4D and 4E, the presence of atropine resulted in a slightly diminished contractile response exhibited by NOD/Lt-derived bladder strips (172-fold at EC_{50}) compared to C57BL/6-derived bladder strips (326-fold at EC_{50}) ($p < 0.05$). Overall, our data in Figure 4 suggest that despite a heterogeneity of antibody function during acute exposure (shown in Figure 3), repeated chronic stimulation of M3R by anti-M3R autoantibodies appears to lead to receptor desensitization, consistent with studies with

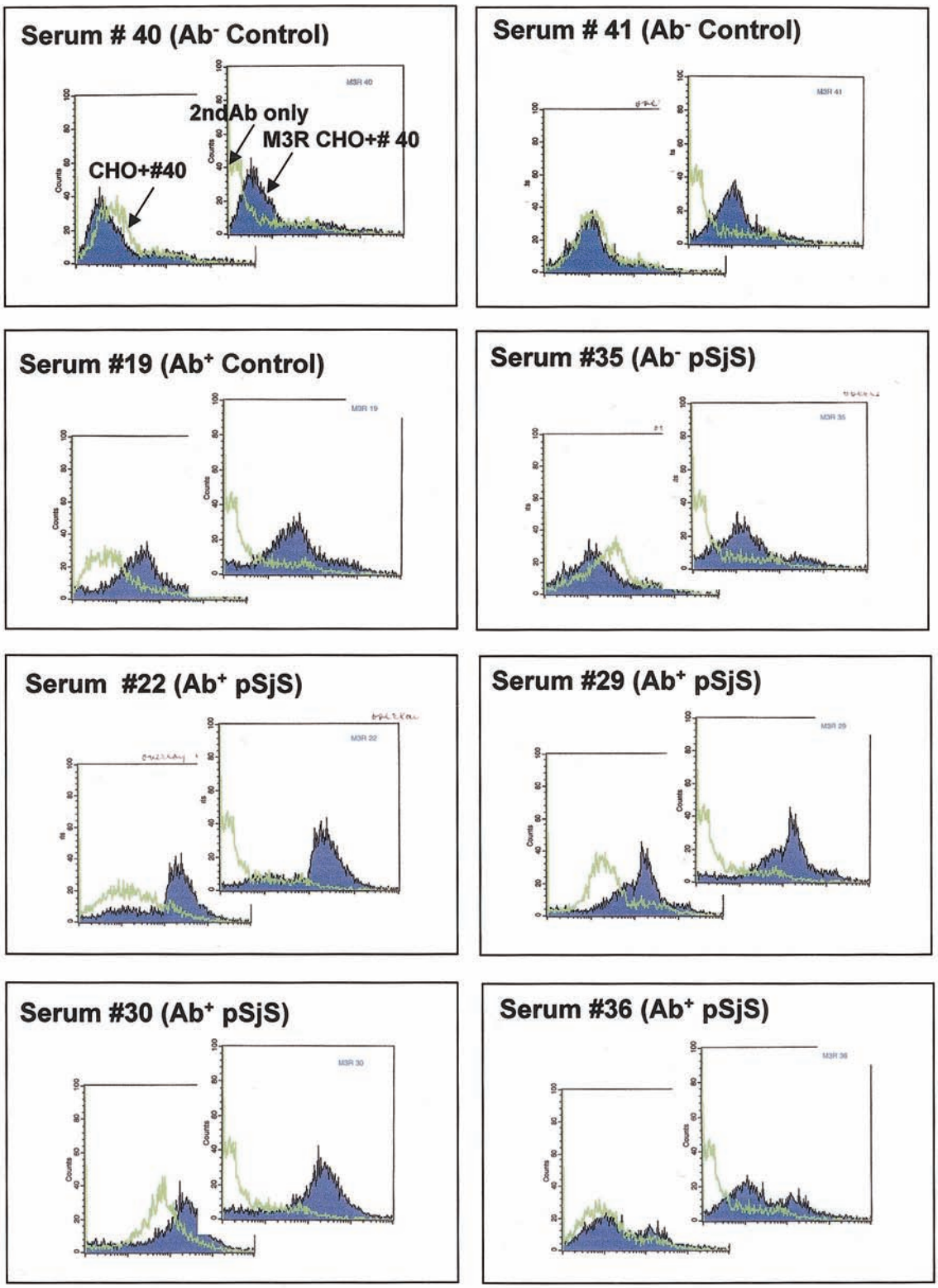

Figure 2. Identification of anti-human M3R autoantibodies in human serum samples by flow cytometric analyses. Nontransfected and human M3R-transfected Flp-In CHO cells were incubated for 1.5 h with individual patient serum samples at a dilution of 1:10, followed by 30 min incubation with FITC-conjugated goat anti-human IgG Fab-specific antibody diluted 1:50. Negative reactions consisted of the CHO cell populations incubated with secondary antibody only. Solid blue histograms: hM3R-transfected CHO cells incubated with individual serum samples. Left column, green histograms: nontransfected cells incubated with serum samples. Right column, green histograms: hM3R-transfected CHO cells incubated with secondary antibody only. Ab+/–: anti-M3R antibody-positive or negative; pSjS: primary Sjögren's syndrome.

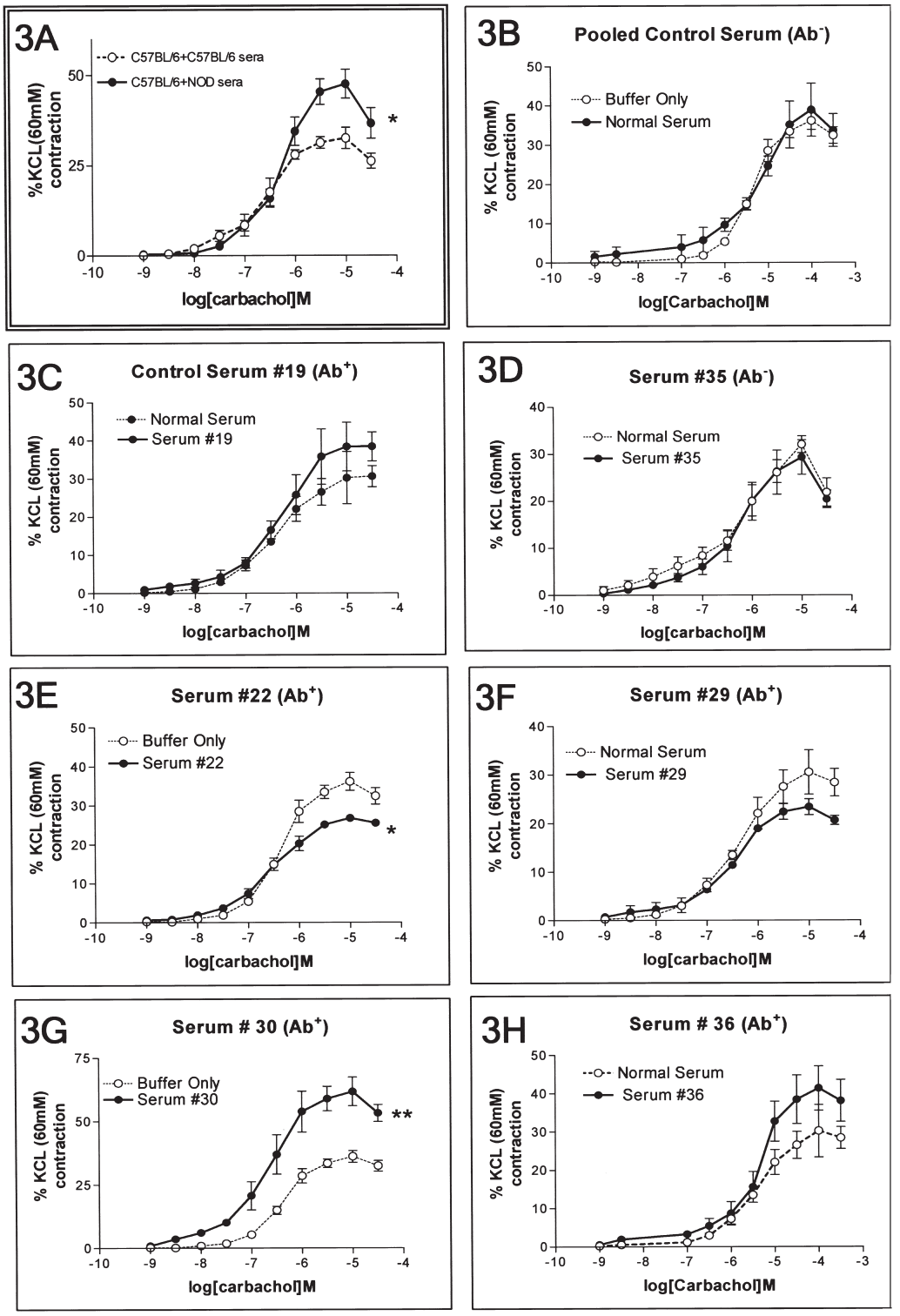

Figure 3. Effects of mouse or human serum anti-M3R autoantibodies on mouse bladder smooth muscle strips from C57BL/6 mice. A: Smooth muscle strips prepared from 10-week-old C57BL/6 mouse bladders were incubated 1 h in mouse sera (diluted 1:50) pooled from either anti-mM3R autoantibody-positive NOD/Lt mice (n = 6) aged 20–28 weeks or anti-mM3R autoantibody-negative age and sex matched disease-free C57BL/6 mice (n = 6), then stimulated with various concentrations of carbachol. The carbachol-evoked responses, measured by intracellular calcium release, are presented as percentage of calcium release by muscle strips after stimulation with 60 mM solution of KCl. B-F: Smooth muscle strips prepared from 10-week-old C57BL/6 mouse bladders were incubated 1 h in individual human serum samples (diluted 1:10), followed by stimulation with carbachol. Carbachol-evoked responses are presented as percentage of contractile responses in KCl. Each serum sample was tested between 4 and 8 times. *$p < 0.05$, **$p < 0.01$.

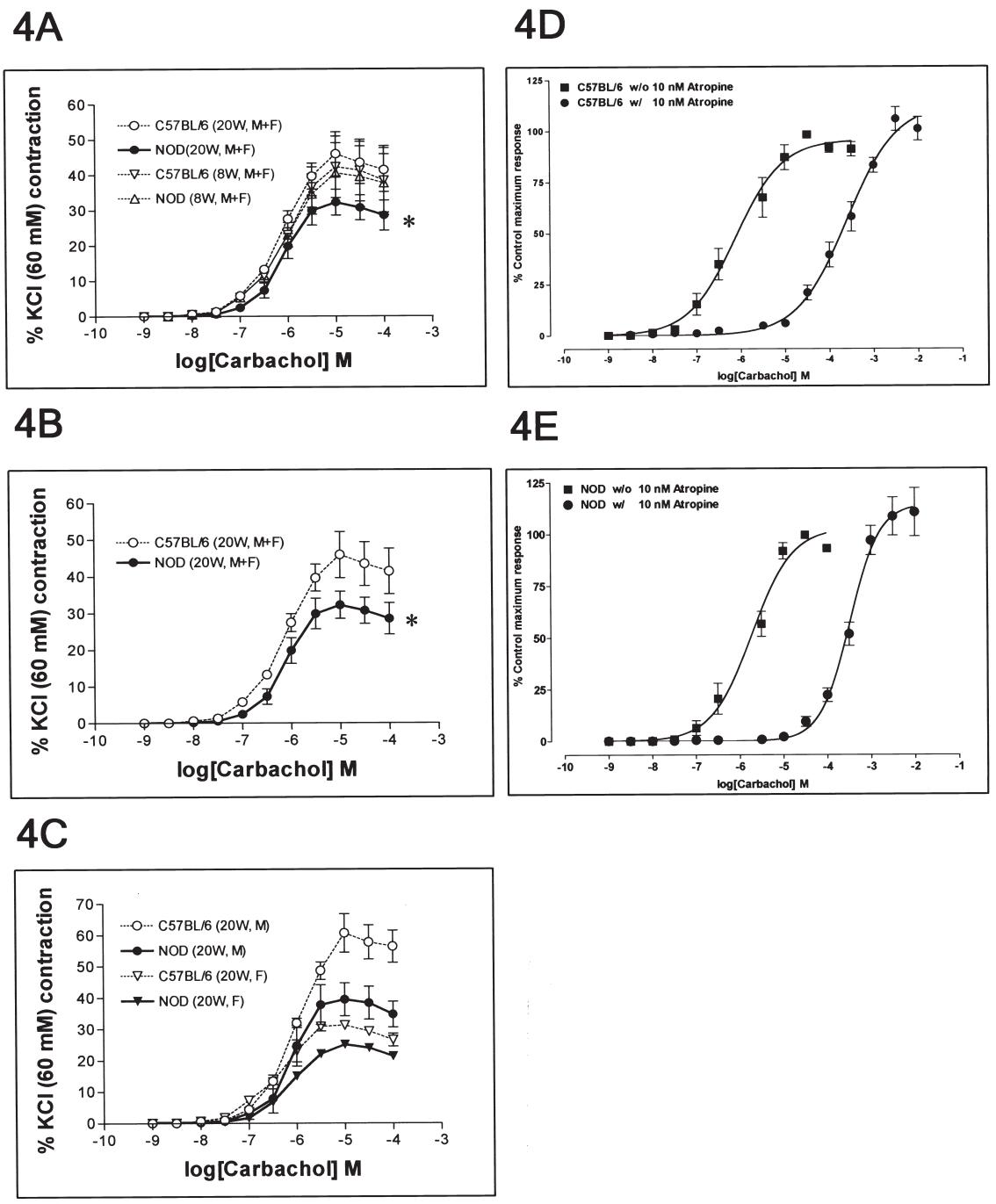

Figure 4. Downregulated carbachol-evoked responses in the NOD mouse as a result of chronic circulation of anti-M3R autoantibodies. A: Downregulation of carbachol-induced muscle contraction was observed in 20-week-old NOD/Lt mice compared to 8-week-old NOD/Lt or to 8 and 20-week-old C57BL/6 mice. Concentration-response curves were generated from carbachol stimulation ranging from 1 nM to 0.1 mM. Contractile effects were expressed as percentage of contractions in the presence of 60 mM KCl. Values shown are mean ± SE for 8 sets of data from 4 mice (2 males, 2 females per strain). *$p < 0.05$. B: Values from 20-week-old mice were plotted separately to show receptor desensitization in the NOD/Lt mice in comparison with C57BL/6 (*$p < 0.05$). C: Values from 20-week-old mice were separated to indicate gender differences in carbachol-stimulated bladder smooth muscle contractions. D and E: Altered affinity of muscarinic antagonist (10 nM atropine) to the receptor was observed in the 20-week-old NOD compared to C57BL/6 (C57BL/6 vs NOD: 326-fold shift vs NOD: 172-fold shift; $p < 0.01$). Contractile effects were expressed as percentage of maximum response of the control curve. Values shown are mean ± SE for 4 mice (2 males, 2 females) per strain.

human serum samples that interfere with calcium-mediated downstream signal transduction[6,7,18,19].

Pilocarpine treatment exacerbates M3R desensitization by chronic exposure to anti-M3R autoantibody. Assuming that the chronic presence of anti-M3R autoantibodies induces downregulation of M3R activity as measured by carbachol-evoked responses in bladder smooth muscle strips, one might assume that addition of a M3R agonist, e.g., pilocarpine, would exacerbate this effect. To test this concept, 20-week-old NOD/Lt and C57BL/6 female mice were injected 3 times each day with pilocarpine, a common secretagogue used in clinics to relieve dry mouth symptoms, for 1, 6, or 12 days. Following the various treatment periods, bladder strips were prepared from the individual mice and stimulated with carbachol. As shown in Figure 5A, the carbachol-evoked responses observed in bladder strips of NOD/Lt mice were significantly lower ($p < 0.01$) by Day 6 of treatment (34.21 ± 3.92) than in their C57BL/6 counterparts (61.70 ± 4.29), a nearly 50% drop, despite showing a strong response to pilocarpine stimulation on Day 1 of treatment. Even after 12 days of treatment, the responses by NOD/Lt bladder strips were less (38.15 ± 1.05) than those observed with C57BL/6 bladder strips (48.33 ±

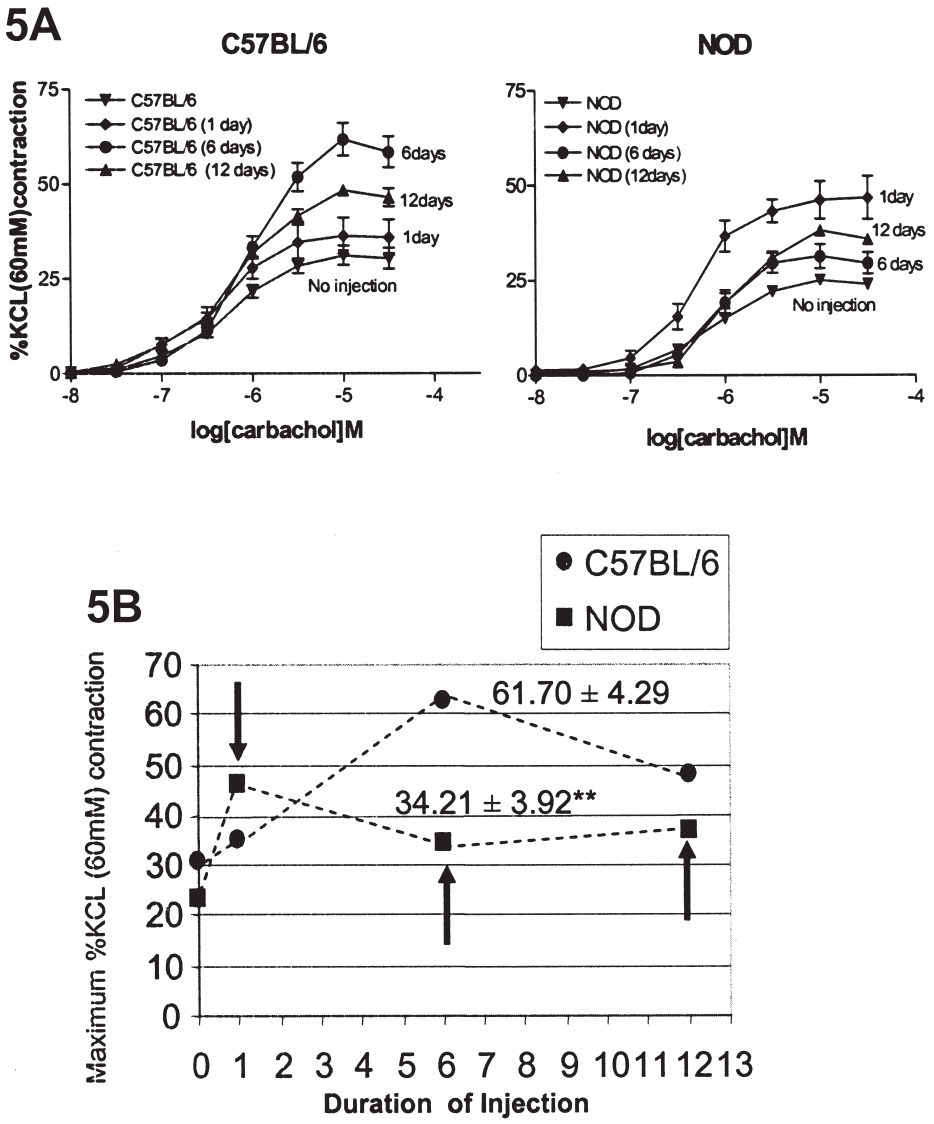

Figure 5. Rapid receptor desensitization observed in NOD mouse caused by the synergistic effect of chronic pilocarpine injection and presence of anti-M3R autoantibodies *in vivo*. Pilocarpine (0.2 mg/100 µl saline per injection) was administered intraperitoneally to 20-week-old female C57BL/6 and NOD/Lt mice (n = 3 per group). Each mouse received 3 injections per day for 1 day, 6 days, or 12 days with 6 h intervals. Bladder strip tensions were measured 2 h after a last injection at 9 AM on the day of measurement (A). Maximum contractile responses from each contraction-dose curve shown in A are replotted in B to show temporal changes. Arrows indicate days of analysis. Statistical values shown at 6 days are significant (**$p < 0.01$).

0.77) ($p < 0.01$), although there was a decline in responses in C57BL/6 mice after 12 days of treatment (Figure 5B). These data indicate that receptor desensitization occurs faster in the anti-M3R antibody-positive NOD mice than in anti-M3R antibody-negative C57BL/6 mice, suggesting a synergistic effect between autoantibodies and pilocarpine stimulation of the M3R.

DISCUSSION

SS-like disease in the NOD mouse model has been shown to closely resemble SS disease in humans. The chronic inflammatory process that targets the salivary and lacrimal glands results in a 75% and 33% loss of saliva flow and tear flow, respectively, by 16 weeks of age, irrespective of sex; as well, inflammation in the lacrimal glands (i.e., dacryoadenitis) is more severe in males, whereas inflammation in the salivary glands (i.e., sialoadenitis) is more severe in females[20]. Although the loss of saliva and tear flow was initially believed to be a consequence of acinar cell apoptosis elicited by cytotoxic T lymphocytes, an interesting paradigm shift was proposed following studies showing the requirement for B lymphocytes and immunoglobulin. First, B cell deficient NOD.Igμ^{null} mice fail to develop secretory dysfunction, and second, IgG from SS patients can induce a reversible stimulation or inhibition of salivary function when infused into NOD-*scid* mice[5].

Accumulating evidence suggests that lymphocytic disturbances, including ectopic germinal center formation in the target tissue and/or aberrations of cellular signaling regulated by B cell activating factor (BAFF), are present in SS[11,21-27]. As shown in transgenic mice overexpressing BAFF and in patients with SS, B cell hyperactivity and abnormalities may lead to excessive immunoglobulin production and prolonged B cell expansion, which eventually lead to monoclonal expansion of B cells and transformation to B cell lymphoma in a subset of patients[28,29]. Additionally, intrinsic defects in the B cell compartment may play a role in generation of SS autoantibodies with diversified prevalence and specificity, as evidenced by the wide array of autoantigens targeted in SS. Thus, identification of autoantibodies that cause dryness in patients with SS is essential for development of strategies to relieve this major discomfort.

Of the many autoantibodies identified in SS patients to date, the association between anti-M3R autoantibodies and secretory dysfunction seems most obvious, since M3R is the major receptor-mediating secretion in the salivary and lacrimal glands in response to parasympathetic stimuli. Studies strongly indicate that serum or purified IgG from SS patients downregulates carbachol-evoked bladder muscle contraction by 50%, while antiidiotypic antibodies neutralize this inhibition of cholinergic neurotransmission[6-8]. Similarly, studies using the human salivary gland ductal cell line HSG showed that pretreatment of the cells with SS IgG for 12 or 24 h reduced the magnitude of subsequent carbachol-induced intracellular calcium release by 62% and 45%, respectively[18].

We tested our hypothesis that frequent use of pilocarpine by patients who have already progressed to M3R desensitization induced by anti-M3R autoantibodies will be less effective than in patients without anti-M3R antibody because of a synergy between pilocarpine and anti-M3R autoantibodies. We took advantage of the NOD mouse model in which anti-M3R autoantibodies are produced over time and provide a continuous stimulation of the M3R expressed on various tissues, including acinar cells of the salivary and lacrimal glands as well as bladder smooth muscle. This model permitted testing of the efficacy of pilocarpine, a drug commonly used in clinical settings to relieve dry mouth, to stimulate M3R in the presence or absence of chronic exposure to the effects of anti-M3R autoantibodies. Results indicate that the presence of anti-M3R autoantibodies in NOD mice resulted in (1) a desensitization of the M3R, as measured by direct carbachol-evoked responses; (2) a greater sensitivity of M3R expressed on C57BL/6 upon short-term incubation with NOD sera; and (3) an accelerated loss of responses to repeated pilocarpine injections. In addition, human sera containing anti-M3R autoantibodies revealed mixed responses after 1 h exposure, similar to results from previous studies in which human IgG from patients with SS was infused into mice[5].

Our data strongly suggest that M3R desensitization occurs only in mice with anti-M3R autoantibodies, as revealed in the comparison of carbachol-evoked responses in NOD mice > 20 weeks of age versus either age and sex matched C57BL/6 or antibody-negative 8–10-week-old NOD mice. These observations, therefore, would be consistent with the hypothesis that chronic stimulation by anti-M3R antibody induces an inhibitory effect on M3R. Nevertheless, the ability of anti-M3R autoantibody to induce contrasting effects, as first shown in our infusion studies in NOD.Igμ^{null} mice[5], was also observed in the current study, where muscle strips from C57BL/6 mice incubated with anti-M3R antibody-positive NOD sera for just 1 h prior to carbachol stimulation resulted in a stimulated response.

Results showing that chronic exposure of M3R to circulating autoantibody leads to a reduced sensitivity to agonist stimulation suggest the function of the antibody is antagonistic. This could be from a direct occupation of the ligand binding site of M3R by the autoantibodies, or alternatively, a modulation of receptor expression on the membrane; but in either case this results in a reduction in the amount of intracellular calcium concentration, as shown in the reduced contractile responses of muscle strips (dose/response) by carbachol stimulation. A possible explanation derived from the results in this mouse model is that direct binding of anti-M3R autoantibodies to the receptor induces conformational changes in the receptor, subsequently altering the threshold for, or directly blocking, the activation of downstream signaling molecules. These changes in turn indirectly alter responses to neurotransmitter-induced fluid secretions from acinar cells. In the long term this leads to desensitization, a phenomenon observed

when tested by agonist stimulation. Interestingly, the experiments using serum samples from human patients revealed both potentiated (agonistic) and reduced (antagonistic) responses to carbachol-induced stimulation. These differences may be explained by differences in autoantibody titers, isotypes of autoantibodies, and duration and severity of the patients' disease states.

A similar dichotomy was demonstrated with chronic versus acute stimulations with pilocarpine. Initially, NOD mice with overt SS-like disease responded well to pilocarpine-induced stimulation; however, this stimulation was downregulated following chronic injections. We interpret these results as an augmented desensitization of M3R in the presence of anti-M3R autoantibodies. Extrapolating these data to a clinical setting, chronic intake of pilocarpine might enhance saliva secretion initially, yet may eventually induce a more rapid desensitization in patients positive for anti-M3R autoantibodies. Development of drug tolerance by receptor desensitization has been reported for other drugs, e.g., salbutamol, a $ß_2$-adrenergic drug used for asthma, and nitrate used for patients with coronary heart disease[30,31]. Thus, frequent use of pilocarpine should be reevaluated, especially when patients develop stimulatory/inhibitory anti-M3R autoantibodies. Intermittent or short-term pilocarpine intake in place of continuous use could prove to be more beneficial when anti-M3R autoantibodies are present.

Another interesting observation was the mixed response profiles obtained when bladder strips isolated from C57BL/6 mice were incubated with various human sera. While a few sera enhanced the response, other sera inhibited smooth muscle contraction in comparison with either normal sera or Krebs physiological buffer. This could represent effects of different antibody titers, length of incubation time, and/or different titers of a pathogenic subset of anti-M3R autoantibodies. In light of our recent studies indicating that SS-like disease may be dependent on anti-M3R autoantibodies of a specific isotype, i.e., IgG1[32], it may be necessary to identify the presence of each IgG anti-M3R autoantibody isotype in each serum sample to fully understand the differential activities observed. Further, it might be informative to follow patients over time to identify how changes in their antibody profiles and titers affect the degree of salivary flow and results from this *in vitro* functional test. This would certainly be of interest in studying healthy blood donors that were asymptomatic for SS disease, yet showed the presence of anti-M3R autoantibodies in their serum.

Our study using the NOD mouse model showed downregulated muscle contractions associated with exposure to anti-M3R autoantibodies, results that are similar to other studies testing human sera[6,7,18]. In addition, repeated injection of pilocarpine resulted in more pronounced and rapid M3R desensitization in the NOD mouse than in the C57BL/6 mouse, indicating an additive effect between pilocarpine and anti-M3R autoantibodies. Finally, although only a limited number of human sera were examined, the acute exposure of mouse bladder muscle strips to anti-M3R autoantibodies altered responses mediated through M3R, in either a stimulatory or an inhibitory manner, suggesting that antibody titers, antibody isotype, and/or duration of the disease may be more directly associated with the severity of dryness rather than merely the presence or absence of autoantibodies. Thus, the mechanisms by which M3R are desensitized by autoantibody need to be studied further, focusing particularly on the downstream regulatory mechanisms and signaling pathways.

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