Autoantibodies against Lacrimal Gland M₃ Muscarinic Acetylcholine Receptors in Patients with Primary Sjögren’s Syndrome

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PURPOSE. The authors demonstrated that immunoglobulin G, present in the sera of patients with primary Sjögren syndrome (pSS), could recognize and activate muscarinic acetylcholine receptors (mAChRs) of rat exoborial acral gland.

METHODS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and radioligand binding and biologic assays were used to demonstrate autoantibodies against mAChRs.

RESULTS. These autoantibodies recognized by means of SDS-PAGE and immunoblotting assay a band of approximately 70 kDa expressed on lacrimal gland membranes that comigrated with the peak of labeled mAChRs. Moreover, pSS IgG were able to inhibit, in an irreversible manner, the binding of [³H]quinuclidinyl benzilate to mAChRs of rat exoborial lacrimal glands and to simulate the biologic effect of mAChR agonists, because they trigger the activation of phosphoinositide turnover. Atropine and 4-diphenylacetoxy-V-methylpiperidine methiodide blocked the effect and carbachol mimicked it, confirming that the M₃ subtype mAChRs mediated pSS IgG action. As control, IgG from sera of women without pSS gave negative results on immunoblotting, binding, and biologic assays, thus demonstrating the specificity of the reaction.

CONCLUSIONS. Autoantibodies against mAChRs may be considered among the serum factors implicated in the pathophysiology of the development of pSS dry eyes. (Invest Ophthalmol Vis Sci. 1998; 39:151-156)

Sjögren syndrome (SS) is classically defined as the presence of at least two of the clinical triad consisting of dry mouth, dry eyes, and autoimmune disease.¹ The most comprehensive diagnostic criteria for SS are those proposed by Fox and associates²; the clinical confirmation of keratoconjunctivitis sicca as indicated by abnormally low results of a Schirmer test and the presence of rose bengal and fluorescein staining, objective evidence of decreased salivary gland flow, proof of lymphocytic infiltration of the labial salivary gland, and verification of the systemic autoimmune process as manifested by the presence of serum autoantibodies.

Patients with the ocular manifestations of SS generally report severe and occasionally disabling eye irritation, especially while reading. The majority have a foreign body sensation, burning, itching, redness, photophobia, and intermittent blurring of vision. They cannot produce tears and have excessive mucus secretion.²

The lymphoproliferation in the SS lacrimal gland consists predominantly of B and CD4 T lymphocytes.³ More than 90% of patients with primary Sjögren syndrome (pSS) have circulating antibodies that recognize widely distributed human tissue antigens, including anti-Ro/SS-A and anti-La/SS-B antibodies, antinuclear antibodies, antissalivary gland antibodies, rheumatoid factor,⁴ and antibodies against neurotransmitter receptors.⁵

Lacrimal glands are critically important in the production of the aqueous component of tear film, and autoimmune and age-related decrements in lacrimal gland function are major causes of ocular morbidity.⁶⁻⁷

In the rat exoborial lacrimal gland, exocrine protein secretion is mainly controlled by acetylcholine acting through muscarinic acetylcholine receptors (mAChRs).⁸⁻⁹ Cholinergic agonists stimulate protein secretion by interacting with muscarinic receptors on the acinar cell basolateral surface membranes,¹⁰ and these receptors are, in part, responsible for mediating the parasympathetic stimulation of fluid, electrolyte, and macromolecular product secretion by the lacrimal gland.¹¹ Muscarinic AChRs are coupled to the activation of the phosphatidylinositol 4,5-bisphosphate (PIP₂) specific phospholipase C (PLC) pathway and to the mobilization of both intracellular and extracellular calcium.¹²⁻¹³ Carbachol significantly increased the ratio of inositol phosphates to inositol¹⁴ through activation of M₃ mAChRs in the rat exoborial lacrimal gland.¹⁵

In view of the presence of circulating antibodies against salivary gland M₃ mAChRs in pSS¹⁶ and the important involvement of the parasympathetic system in the secretory function of the lacrimal gland,¹¹ we have investigated the presence of autoantibodies against M₃ mAChRs that interact with the rat exoborial lacrimal gland in the sera of patients with pSS. Here we report that these antibodies recognize the mAChRs and...
Two groups of patients (aged 25–35 years) from metropolitan Buenos Aires were selected: six control women without pSS and six women with pSS. All six patients fulfilled four or more of the preliminary criteria for SS.17 The syndrome was diagnosed if the following were present as well: keratoconjunctivitis sicca (abnormal Shimer test results and rose bengal staining), symptomatic xerostomia associated with parotid enlargement, and anti-Ro(SS-A) and anti-La(SS-B) antibodies in the absence of other connective tissue disorders. Rheumatoid factors were investigated by using the latex fixation and rose ragan test and by enzyme-linked immunosorbent assay (immunoglobulin-M-specific); antinuclear antibodies were investigated by using indirect immunofluorescence over cryostat sections of rat liver and mouse kidney. Anti-Ro(SS-A) and anti-La(SS-B) were studied by double diffusion using a human spleen extract and a rabbit calf thymus extract, respectively.

Both antibodies were also investigated by enzyme-linked immunosorbent assay using bovine-purified antigens. Among the six patients, five were positive for RF (rheumatoid factor) and ANA (anti nuclear antibody), six were positive for anti-Ro/SS-A, and two were positive for anti-La/SS-B.

All the studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki. Informed consent was obtained, and the research was approved by the institutional human experimentation committee of CEMIC.

**METHODS**

**Patients**

Two groups of patients (aged 25–35 years) from metropolitan Buenos Aires were investigated by using the latex fixation and rose ragan test and by enzyme-linked immunosorbent assay (immunoglobulin-M-specific); antinuclear antibodies were investigated by using indirect immunofluorescence over cryostat sections of rat liver and mouse kidney. Anti-Ro(SS-A) and anti-La(SS-B) were studied by double diffusion using a human spleen extract and a rabbit calf thymus extract, respectively.

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**Purification of Human Immunoglobulin G**

Antibodies were purified from sera of control and patient groups described above. Immunoglobulin G (IgG) was obtained by precipitation with ammonium sulfate at 50%; this was followed by three washes and reprecipitation with 33% ammonium sulfate. The resulting precipitate was submitted to chromatography on DEAE-cellulose, equilibrated with 10 mM phosphate buffer, pH 8. The eluted peaks were concentrated by ultrafiltration (Minicon B15 concentrator; Amicon, Lexington, MA) to 10 µg protein/ml. Control immunoelectrophoresis with goat anti-human IgG (Cappel Labs, Cochranville, PA) showed only one precipitin line.

**Preparation of Microsomal Fractions**

Exorbital lacrimal gland membranes were prepared from adult female Wistar strain rats. All animals were used according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The glands were dissected away from fat, connective tissue, and lymph nodes and immersed in a tissue chamber containing Krebs-Ringer bicarbonate (KRB) solution gassed with 5% CO2 in oxygen and maintained at pH 7.4 and 30°C. All subsequent steps were performed at 4°C. Lacrimal glands were homogenized for 10 seconds twice in 50 mM buffer phosphate, pH 7.4 (buffer A), in an Ultra-Turrax (IKA, Germany) (setting 5). The homogenate was centrifuged for 10 minutes at 1000g. The pellets were discarded, and the supernatants were centrifuged (10,000g) at 4°C for 10 minutes and then at 40,000g for 1 hour. The resulting pellets were resuspended in buffer A supplemented with 0.1 mM phenylmethyl-sulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid, 5 µg/ml leupeptin, and 1 µM pepstatin A (buffer B) as described previously18 and used as a membrane source for competitive

**Radioligand Binding Assay**

Exorbital lacrimal gland membranes were incubated in a final volume of 0.5 ml of buffer B for 75 minutes at 37°C with 100 nM [3H]QNB (specific activity, 43 Ci/mmol) with shaking. The reaction was stopped with ice-cold buffer A and filtered through Whatman (Maidstone, UK) glass fiber filters (GF/c) under suction. After washing with 12 ml of buffer A, filters were placed in vials, dried, and counted in 8 ml of scintillation cocktail (Triton/toluene) with approximately 60% efficiency. Nonspecific binding (measured in the presence of 10 µM atropine) did not exceed 15%. To obtain the Kd of the antagonistic drugs, a range of 10–10 to 10–4 M of each drug was incubated with exorbital lacrimal gland membranes with 0.4 nM [3H]QNB. For competition assays, membranes were incubated with normal IgG (nIgG) and with IgG obtained from sera of women with pSS (pSS IgG) in 50 mM buffer phosphate, pH 7.4, during 30 minutes at 30°C with shaking, and then 100 µl of the membrane solution (100 µg of protein) was used for the binding assay with 0.4 nM [3H]QNB. For the saturation assay, membranes were incubated with different concentrations of [3H]QNB (0.05–1.00 nM). The equilibrium dissociation constant (Kd, nanomolar) and the number of binding sites (Bmax, femtomol/mg protein) were taken from plots according to the method of Scatchard,19 taking into account the total incubation volume (500 µl/tube) and the milligrams of tissue protein used (100 µg/tube).

**SDS–PAGE and Immunoblotting Assay**

Membranes (1–2 mg/ml protein) were incubated with 5 nM propylbenzylcholine mustard ([3H]PBCM) for 30 minutes at 30°C with or without 50 µM atropine. The reaction was stopped by dilution with buffer A. After extensive washing, the pellets were solubilized in 2% SDS, 1% 2-mercaptoethanol, 0.01% bromphenol blue, 10% (vol/vol) glycerol, and 0.05% N-Tris-HCl, pH 6.8 (sample buffer). SDS–PAGE was performed by using the discontinuous system of Laemmli20 on 8.5% polyacrylamide separation gel. The fractionated proteins were stained with Coomassie Blue R-250 (Sigma), cut into 2-mm slices and counted in a liquid scintillation counter or electrophotically transferred to nitrocellulose by the method described by Towbin et al.21 The nitrocellulose was incubated overnight at 4°C with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.05 (vol/vol) Tween 20, and 1% bovine serum albumin. Once blocked, the nitrocellulose was incubated at 4°C overnight with control sera (normal) or pSS sera diluted 1/100 for 18 to 22 hours at 4°C with constant shaking in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.05% (vol/vol) Tween 20 (TBST). After washing in TBST, the nitrocellulose strips were incubated for 1 hour at room temperature with a 1:6000 dilution of goat anti-human IgG (y-chain-specific)-alkaline phosphatase at the same temperature. Once the strips were thoroughly washed, they were incubated with a mixture of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) at a 1:1 molar ratio under alkaline conditions for 30 minutes.
Inositol Phosphate Measurement

Female adult rats were killed by decapitation, and exorbital lacrimal glands were excised and transferred to vessels containing 0.5 ml of KRB solution gassed with 5% CO₂ in oxygen with 1 μCi [³H]myoinositol ([³H]MI) and 10 mM LiCl for inositol monophosphate accumulation, according to the method of Berridge et al.²² Exorbital lacrimal glands were incubated at 37°C with shaking for 120 minutes. The preparation was washed three times with KRB and 10 mM LiCl. Carbachol and IgGs were added for 45 minutes, and when blockers were used they were added 15 minutes before the addition of carbachol or IgGs. After incubation, water-soluble inositol phosphates (IPs) were extracted according to the method of Berridge et al.²² Briefly, lacrimal glands were washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml of chloroform/methanol (1:2 vol/vol) for stopping reactions. Samples were centrifuged at 2000g for 10 minutes, and the upper aqueous phase (1.8 ml) was applied to a 0.7-ml column (AG-50X8, Bio-Rad, Richmond, Calif) suspended in 0.1 M formic acid and previously washed with 10 mM Tris-formate, pH 7.4. The resin was washed with 20 ml of 5 mM myo-inositol and then with 5 ml of water, and the IPs were eluted with 1 M ammonium formate in 0.1 M formic acid. Fractions of 1 ml were recovered, and radioactivity was determined by scintillation counting. Results were expressed as criteria of Simpson’s equation, and, to determine the absence of [³H]MI in the IP-eluted phase, chromatography was performed on Silica Gel 60 F₂₅₄ sheets (Merck, Darmstadt, Germany) as described previously.⁵²³²⁴

Drugs

Carbachol, pirenzepine, atropine, 2-nitrocarboxyphenyl-N,N-diphenylcarbamate (NCDC) were obtained from Sigma. 11-[(2-Diethylamino)ethyl]-1-piperidinyldiacetyl-5,11-dihydro-6H-pirido[2,3-b]-1,4-benzodiazepine-6-one (AFD-X116) and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) were kindly provided by Boehringer Ingelheim Pharmaceuticals. Other chemicals used were of analytical grade. Stock solutions of the drugs were dissolved in the corresponding solvents and freshly prepared.

Statistical Analysis

Student’s t-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Dunnett test was applied. Differences between means were considered significant if P < 0.05.

Results

Competition Binding Assay

Figure 1 shows the inhibition of [³H]QNB binding to rat exorbital lacrimal gland membranes by primary Sjögren syndrome (pSS) immunoglobulin G (IgG). Increasing concentrations of human pSS IgG (•) and normal IgG (nlgG) (□) were incubated with exorbital lacrimal gland membranes, and a binding assay with 0.4 nM [³H]QNB was carried out. The results are mean ± SEM of six nlgG and six pSS IgG performed in duplicate. P < 0.001 between pSS IgG and nlgG. Control binding of 100% refers to the value of [³H]QNB specifically bound to exorbital lacrimal gland membranes without any reagent added.

Differential Immunoreactivity of SDS–PAGE Fractionated Proteins with pSS and Normal Sera

Differential patterns of recognition were obtained with pSS sera that were positive in binding assays by means of SDS–PAGE and immunoblotting analysis with respect to normal sera. Figure 3 (upper panel) shows a peak of [³H]PBCM-labeled protein in sliced gels of approximately 70 kDa, which disappeared when membranes were labeled in the presence of 5 × 10⁻⁵ M atropine and corresponds to mAChRs expressed on gland membranes. Figure 3 (lower panel) shows nitrocellulose membranes immunoblotted with pSS or normal sera. A diffuse band is revealed by pSS sera with a mobility coincident with that of labeled receptor, whereas no bands were seen on nitrocellulose strips incubated with normal sera.

Effect on Phosphoinositide Turnover

To analyze whether pSS IgG interaction with M₃ mAChRs activates some M₃ mAChR-mediated intracellular signal, the production of phosphoinositides (PIs), by rat exorbital lacrimal glands were studied. Glands were incubated with [³H]MI and LiCl for inositol monophosphate accumulation. Figure 4 shows water-soluble PIs formed in the presence of

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933426/)

**Figure 1.** Inhibition of [³H]quinuclidinyl benzilate ([³H]QNB) binding to rat exorbital lacrimal gland membranes by primary Sjögren syndrome (pSS) immunoglobulin G (IgG). Increasing concentrations of human pSS IgG (•) and normal IgG (nlgG) (□) were incubated with exorbital lacrimal gland membranes, and a binding assay with 0.4 nM [³H]QNB was carried out. The results are mean ± SEM of six nlgG and six pSS IgG performed in duplicate. P < 0.001 between pSS IgG and nlgG. Control binding of 100% refers to the value of [³H]QNB specifically bound to exorbital lacrimal gland membranes without any reagent added.
pSS IgG, carbachol, and nlgG. As can be seen in Figure 4a, pSS IgG increased significantly the PI turnover with respect to nlgG. This effect mimicked that of carbachol, even though it was lower. The stimulatory action of pSS IgG and carbachol on PI turnover was concentration dependent. Normal IgG, used at the same concentrations as indicated for pSS IgG, had no effect at all points tested (Fig. 4a). To assess whether mAChRs participate in the stimulatory action of pSS IgG on PI turnover, the influence of cholinergic antagonists was determined. Atropine and 4-DAMP, both at $1 \times 10^{-5}$ M, inhibited the stimulatory effect of either pSS IgG or carbachol (Fig. 4b). To demonstrate that pSS IgG-mediated hydrolysis of PIP$_2$ depends on PLC activation, the action of a PLC inhibitor, NCDC, was studied. Figure 4b shows that the effect of both cholinergic agonist and pSS IgG were abrogated by NCDC and elucidates the participation of this enzyme in the phenomenon.

The same concentration of primary Sjögren (pSS) immunoglobulin G (1 $\times 10^{-6}$ M) (V), normal IgG (1 $\times 10^{-6}$ M) (V), and membranes alone (white) incubated with different concentrations of [H]quinuclidinyl benzilate. (See Methods.)

**DISCUSSION**

Sjögren syndrome is a chronic autoimmune disease with a marked preponderance among female patients. It occurs mainly in the fourth and fifth decades and is described as primary Sjögren syndrome when it appears independently of other autoimmune disorders. The mechanisms involved in the pathogenesis of pSS have not yet been defined.

We examined the possible role of altered human immunity by studying how mAChRs participate in the effect of antibodies in the sera of patients with pSS on the exorbital lacrimal gland. In this study, we show evidence that one or more components of the IgG fraction obtained from sera of patients with pSS can bind and recognize the mAChRs of rat exorbital lacrimal glands, thereby activating the PLC pathway—an effect associated with the M$_3$ muscarinic receptor population.

In previously published studies, the binding of labeled antagonists to membranes from rat lacrimal glands has been described to be saturable and of high affinity. The rank order of potency and the affinities of antagonists in the rat lacrimal gland agree with those reported previously for the M$_3$ mAChR subtype. In this study, we demonstrate that patients with pSS have autoantibodies that inhibit the binding of [H]QNB to muscarinic cholinergic receptors. This is supported by the noncompetitive manner of inhibition of the antibodies observed in the [H]QNB binding assay, whereby IgG from patients with pSS decreased the number of binding sites without affecting the equilibrium dissociation constant. In addition, pSS
sera recognized a band of approximately 70 kDa expressed on lacrimal gland membranes that comigrate with the peak of [3H]PBCM-labeled mAChRs. The specificity of pSS IgG against mAChR is demonstrated, because it was absent in patients without pSS. The fact that IgG from patients with pSS reacted with mAChRs among other proteins suggests that the anti-mAChR antibodies are another serum factor to be considered in the pathophysiology of lacrimal gland autoimmunity. A similar profile of protein recognition has been described in the submaxillary gland.27

Studies of mAChR-mediated phosphoinositide turnover have been shown in cultured human retina epithelium cells28 and ciliary muscle cells29 and also in rat lacrimal acinar cells12 through coupling M3 mAChRs to PLC.15 Carbachol, but not α-adrenergic agonists such as phenylephrine, increased the ratios of inositol phosphate to inositol, indicating that only the cholinergic agonist activates PI turnover in the lacrimal gland.14 The findings reported here demonstrate that pSS IgG increased PI turnover and mimicked the effect of carbachol. Atropine and 4-DAMP blunted the biologic effect induced by pSS IgG, confirming the participation of M3 mAChR in the response.

Patients with keratoconjunctivitis sicca attributable to SS exhibit a number of characteristic clinical ocular signs, with the eyes as one of the most important organs involved in this disease.2 A great spectrum of autoantibodies is commonly described in ss, mAChR antibodies could be involved in the pathogenesis of pSS. It is possible that chronic interaction of pSS IgG on lacrimal and salivary gland M3 mAChRs, behaving as a muscarinic cholinergic agonist, would induce desensitization, internalization, and/or intracellular degradation of receptors. Moreover, these autoantibodies could alter intracellular mAChR pools that are known to be in an extensive recycling traffic.31 This in turn could lead to a progressive blockade of mAChRs inducing dry eyes and dry mouth, classically described signs in the course of pSS. Alternatively, destruction of the tubuloacinar architecture of the lacrimal glands by T cells, by apoptotic and perforin-related mechanisms, could lead to secondary production of mAChR antibodies and may also be related to pSS pathogenesis.

Additional work is required to elucidate the possible relationship between the specificity of IgG with muscarinic cholinergic activity and the clinical manifestation of pSS. Correlation of basal and reflex tearing with these autoantibodies should also be determined.

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References