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Autoantibodies against cerebral muscarinic cholinoceptors in Sjögren syndrome: functional and pathological implications

Silvia Reina[a], Leonor Sterin-Borda[a,b], Betina Orman[a], Enri Borda[a,b,*]

[a] *Pharmacology Unit, School of Dentistry, University of Buenos Aires, Buenos Aires, Argentina*
[b] *Argentine National Research Council (CONICET), Buenos Aires, Argentina*

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Abstract

Previous studies have demonstrated that antibodies against muscarinic acetylcholine receptors (mAChRs) from exocrine glands, correlates with Sjögren syndrome (SS) in the majority of patients. The aim of the present investigation was to establish if serum IgG antibodies present in SS interacts with cerebral mAChRs. Results show that anti-cerebral IgG are present in the sera of 40% SS patients studied. Autoantibodies were able to interact with mAChRs of cerebral frontal cortex membranes inhibiting the [^{3}H]QNB binding to its specific receptor. Moreover, tested by ELISA and dot blot they recognized the synthetic peptides corresponding to the second extracellular loop of human M_1 and M_3 mAChR. In addition, the corresponding affinity-purified anti-M_1 and anti-M_3 peptide IgGs displayed an agonistic activity, stimulating phosphoinositide hydrolysis. The results support the notion that serum IgG autoantibodies in SS patients target cerebral mAChRs may have some role in the pathogenesis of higher cognitive dysfunction present in SS patients.

Keywords: Cerebral antibodies; mAChRs; Cerebral PIs; Cerebral NOS; ELISA; M_1 mAChR IgG; M_3 mAChR IgG

1. Introduction

Sjögren syndrome (SS) is an autoimmune disease with exocrine glands as the site of intense immunological activity (Moutsopoulos and Talal, 1987). It is also the second most common autoimmune rheumatic disease, exceeded only by rheumatic arthritis and systemic lupus erythematosus (Tomsic and Rozman, 1999), although to date, SS is underdiagnosed (Lemp, 1999).

Symptoms of SS refer to kerotoconjuntivitis sicca and xerostomia resulting from immune lymphocytes that infiltrate the lacrimal (Vitali et al., 1993; Tsubota et al., 1999) and salivary (Ferguson, 1999) glands. Xerostomia unrelated to SS are increased in the elderly. They may be due to age-related glandular atrophy and/or hormonal disturbances that reduces basal salivary flow rates (Astor et al., 1999) associated with higher rate of dental caries (Ravald and List, 1998) and accelerated periodontal disease (Gonzales and Coleman, 2000). The severity of keratoconjuntivitis sicca correlates fairly well with the degree of lacrimal glandular pathology (Anaya and Talal, 1997).

Aside from the SS-glandular manifestations, a large number of extraglandular lesions have been observed. Different types of skin infiltrates and skin dryness may result from lymphocytic infiltration into exocrine skin glands (Sais et al., 1998), abnormal local expression of chemokines (Magro and Crowson, 1999) and frequent association with anti-SS antibody (Kawakami and Saito, 1999).

Cardiovascular manifestations may also include parasympathetic dysfunction in approximately 15% of SS patients, while changes in sympathetic function have not been detected (Barendregt et al., 1999; Martinez-Lavin and Hermosillo, 2000; Calkins and Rowe, 1998). Infants with neonatal heart block from SS mothers may develop this disease as a result of autoantibodies directed against the neonatal heart muscarinic M_1 cholinoceptor (Borda et al., 1999).

The level of IgG antibodies against SS-A or SS-B was not observed to correlate with SS disease activity patients

* Corresponding author. Pharmacology Unit, School of Dentistry, University of Buenos Aires, Marcelo T. de Alvear 2142 - 4to. "B", 1122AAH Buenos Aires, Argentina. Fax: +54-11-4963-2767.
E-mail address: enri@farmaco.odon.uba.ar (E. Borda).

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(Praprotnik et al., 1999). However, the distribution of IgG antibodies against exocrine glands M_3 and M_1 mAChRs was found to correlate well with SS disease activity (Bacman et al., 1996, 1998, 2001; Perez Leiros et al., 2002). Moreover, the titer of antibodies of IgA subclass also correlated with SS disease activity (Anaya and Talal, 1997; Halse et al., 2000). Berra et al. (2002) demonstrated high prevalence of saliva IgA with mAChR activity in SS patients.

Central nervous system (CNS) alterations in SS patients were reported (Lafitte, 2000; Belin et al., 1999). Neuropsychometric testing showed a high frequency of short-term memory defects, and tomography scan analysis indicated abnormalities in the frontal lobes (Belin et al., 1999). On neuropsychiatric testing, patients with primary SS (pSS) had significantly higher scoring rates for clinical anxiety and clinical depression compared with age- and sex-matched reference groups (Valtysdottir et al., 2000). Immune-mediated hearing loss may occur in SS patients (Stone and Francis, 2000). Patients with demyelinating lesions and myasthenia gravis with sicca symptoms were reported (Ito et al., 1999). There is a well-documented association between muscarinic receptor activity and the modulation of certain central nervous system functions in SS patients, such as memory and other cognitive functions (Tandon et al., 1991; Zom et al., 1994).

Hence, we considered it relevant to investigate whether anti-frontal cerebral IgG antibodies are present in SS patients and whether these antibodies are able to bind and activate frontal cerebral mAChRs of M_3 and M_1 subtypes.

2. Materials and methods

2.1. Subjects and serological test

Women (aged 35–55 years) were selected from the metropolitan area of Buenos Aires. The subjects were divided into three groups: group I, 48 primary Sjögren Syndrome (pSS); group II, 24 rheumatoid arthritis (RA) and group III, 35 normal control subjects. The diagnosis of SS followed four or more criteria of Vitali et al. (1993). Serologic tests were performed: anti-Ro/SS-A and anti-La/SS-B antibodies, rheumatoid factor (RF) and antinuclear antibodies (ANA) (Table 1). All studies involving human subjects had informed consent and were conducted according to the tenets of the Declaration of Helsinki.

Table 1
Serological tests performed on different groups

Serological Test	Group I	Group II	Group III
ANA	36/48 (75%)	10/24 (40%)	2/35 (5%)
Anti-Ro/SS-A	22/48 (44%)	10/24 (40%)	0/35 (0%)
Anti La/SS-B	18/48 (37%)	10/24 (40%)	0/35 (0%)
RF	15/48 (32%)	20/24 (83%)	2/35 (5%)

2.2. Rat cerebral frontal cortex membranes preparations

Male Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires) were housed in our colony in small groups and kept in automatically controlled lighting (lights on 08:00–19:00) and uniform temperature (25 °C) conditions. All animals were used at 3–4 months of age. The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Neural cell membranes from cerebral frontal cortex were prepared as previously described (Borda et al., 1998). In brief, tissues were homogenized in an Ultraturrax at 4 °C in 5 volumes of 10 mM potassium phosphate buffer, 1 mM $MgCl_2$, 0.25 M sucrose (buffer A) pH = 7.5, supplemented with 0.1 mM phenylmethylsulphonyfluoride (PMSF), 2 μg ml^{-1} leupeptin and 1μM pepstatin A. The homogenate was centrifuged twice for 10 min at $3000 \times g$, then at $10{,}000 \times g$ and $40{,}000 \times g$ at 4 °C, for 15 and 90 min, respectively. The resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitors at pH 7.5 (buffer B).

2.3. Peptides

A 24-mer peptide E-R-T-L-A-G-Q-C-Y-I-Q-F-L-S-Q-P-I-I-T-F-G-T-A-M and 25-mer peptide K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I corresponding to the aminoacid sequence of the second extracellular loop of the human M_1 and M_3 mAChRs, respectively, were synthesized by F-moc-aminoacids activated using 1-hydroxy benzo triazole/dicyclo hexyl carbodimide (HOBt/DCC) strategy with an automatic peptide synthesizer Applied Biosystems Model 431A. The peptide was desalted, purified by HPLC, and subjected to amino-terminal sequence analysis by automatic Edman degradation with an Applied Biosystems 470 A Sequence.

2.4. Purification of human IgG

The serum immunoglobulin G (IgG) fraction from patients of groups I and II and from normal subjects (group III) was isolated by protein G affinity chromatography, as described elsewhere (Borda et al., 2002) for protein A and standardized for protein G. Briefly, sera were loaded on the protein G (Sigma, Saint Louis, MO, USA) affinity column equilibrated with 1 M Tris–HCl, pH = 8.0, and the columns were then washed with 10 volumes of the same buffer. IgG fraction were eluded with 100 mM glicine–HCl, pH = 3.0, and immediately neutralized. IgG concentrations were determined by radial immunodifusion assay.

2.5. Purification of anti-peptide antibodies by affinity chromatography

The IgG fraction of pSS patients was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA). The IgG fraction was loaded on the affinity column equilibrated with phosphate-buffered saline (PBS), and the non-peptide fraction was first eluted with the same buffer. Specific anti-peptide autoantibodies were then eluted with 3 M KSCN, 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of both non-anti-peptide antibodies and specific anti-muscarinic receptor peptide antibodies were determined by radial immunodifusion assay, and their immunological reactivity against the muscarinic receptor peptide was evaluated by enzyme immunoassay (ELISA) (Borda et al., 2002).

2.6. Enzyme immunoassay (ELISA)

Fifty microliters of peptide solution (20 μg/ml) in 0.1 M Na_2CO_3 buffer pH = 9.6 was used to coat COSTAR microtiters plates at 4 °C overnight. After blocking the wells with 2% bovine serum albumin in PBS for 1 h at 37 °C, 100 μl of 1/30 dilution of sera or different concentrations of purified IgG from patients of groups I and II and normal subjects (group III), were allowed to react with peptide for 2 h at 37 °C. Wells were then thoroughly washed with 0.05% Tween in PBS and 100 μl of 1:6000 goat anti-human IgG alkaline phosphates conjugate antibodies (Sigma) were added and incubated for 1 additional hour at 37 °C. After extensive washing, *p*-nitrophenylphosphate (1 mg/ml) was added as substrate. After 30 min, optical density (OD) values were measured at 405 nm with an ELISA reader (Uniskan Labsystem, USA) (Borda et al., 2002). As negative controls, non-antigen paired wells and wells with no primary antiserum, were also tested.

2.7. Radioligand binding assay

Receptor ligand binding was performed as previously described (Borda et al., 1998). Aliquots of the membrane suspension (50 μg protein), pretreated or not with increasing concentrations of IgG from normal subjects or pSS patients for 30 min at 30 °C, were incubated with increasing concentrations of ^{3}H-quinuclydinyl benzilate ([^{3}H]QNB, New England Nuclear, Sp.Act. 85.6 Ci/mmol) for 60 min at 25 °C in a total volume of 150 μl of buffer B. Binding was stopped by adding 2 ml ice-cold buffer followed by rapid filtration (Whatman GF/C). Filters were rinsed with 12 ml of ice cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Non-specific binding was determined in the presence of 10^{-5} M atropine (Sigma) and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts. Radioactive binding was analyzed with the computer-assisted curve fitting program LIGAND.

2.8. Measurement of total labeled phosphoinositides (PIs)

Rat slices from cerebral frontal cortex were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 mCi [*myo*-^{3}H]inositol ([^{3}H]MI) (Sp.Act. 15 Ci/mmol) from Dupont/New England Nuclear, and LiCl (10 mM) was added for determination of inositol monophosphate accumulation, according to the technique previously described (Borda et al., 1998). IgG were added 30 min before the end of the incubation period and the muscarinic receptor blockers (4-DAMP and pirenzepine) and the inhibitor of phospholipase C (PLC) (U-73122) were added before the addition of IgG. Water-soluble PIs was extracted after a 120-min incubation. Tissue samples were washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reaction. Next, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 × *g* for 10 min, and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7-ml column of Bio-Rad (Formate Form) 1 × 8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid that had been previously washed with 10 mM Tris-formic pH 7.4. The resin was then washed with 20 volumes of 5 mM *myo*-inositol followed by 6 volumes of water and PIs were eluted with 1 M ammonium formate in 0.1 M formic acid. One-milliliter fractions were recovered and the radioactivity was determined by scintillation counting. Peak areas were determined by triangulation and results corresponding to the second peak were expressed according to previous criteria (Borda et al., 1998).

2.9. Indirect immunofluorescence technique

Rat frontal brain slides were incubated for 60 min with pSS or normal IgG (1×10^{-6} M) at room temperature in a wet chamber. After washing three times with PBS, they were further incubated with rabbit anti-human IgG FITC-conjugated F(ab′)2 fragment (1:100) (DAKO) for 30 min at room temperature in a humidified chamber. After three additional washes with PBS, slides were mounted in PBS–glycerol and observed with a Nikon photomicroscope equipped with epi-illumination (Colbum et al., 2001).

2.10. Dot blot assay

Nitrocellulose discs were dotted with 2 μg M_1 and M_3 mAChR synthetic peptides. They were then diluted in Tris–HCL 25 mM, NaCl 150 mM pH 7.4 (TBS), and were blocked with TBS 5% skimmed milk (TBSM) and incubated with a 2-fold dilution of sera from pSS patients—normal subjects or the corresponding IgG

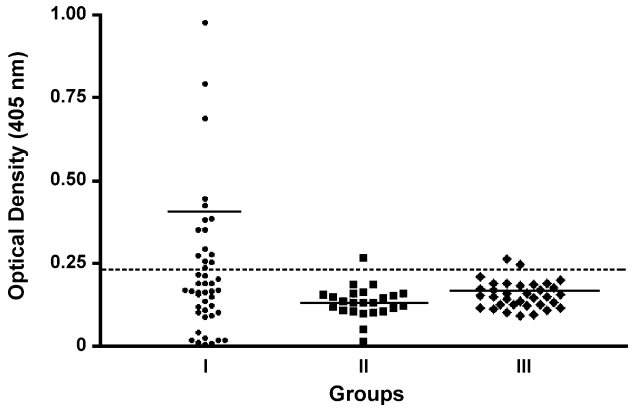


Fig. 1. Immune reactivity of anti-cerebral frontal cortex membrane antibody of sera from different groups: 48 pSS patients (group I), 24 RA without SS patients (group II) and 35 normal subjects (control) (group III). Serum (1/30 dilution) was assayed on sensitized microplates with 50 μg/ml membranes. Dotted/dashed line, cutoff value 0.24 (mean optical density ± 3 S.D. for group III); solid lines, median optical density values. $P < 0.001$ between group I and groups II and III.

$(1 \times 10^{-6}$ M) alone or preincubated with 1×10^{-5} M M_1 and M_3 mAChR synthetic peptides in TBSM for 2 h at room temperature. After three washes with TBS 0.05% Tween 20 (TBST), the immune complexes were revealed with alkaline phosphate-labeled goat anti-human immunoglobulin (Sigma, USA) (1:1000 dilution), followed by the addition of the chromogenic substrate mixture, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) at 1:1 ratio under alkaline conditions (Colbum et al., 2001).

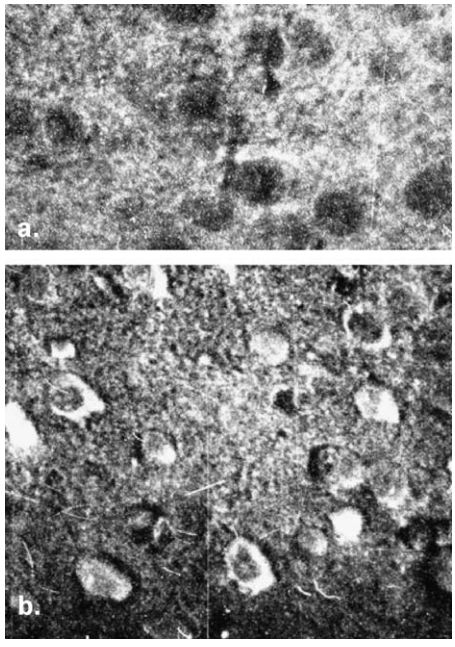
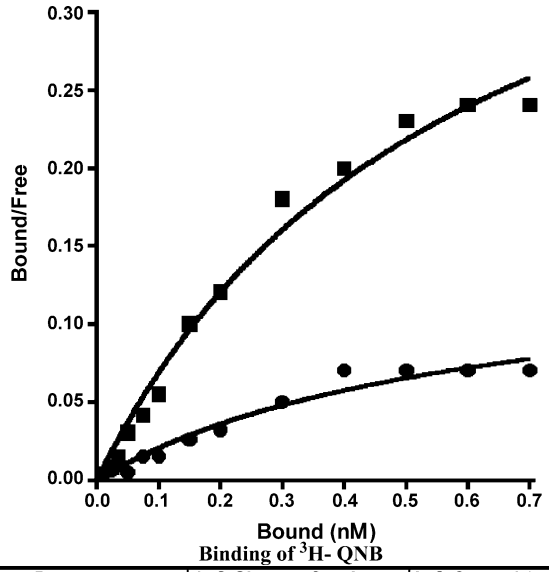


Fig. 2. Indirect immunofluorescence stained of cerebral frontal cortex slices. Slices were incubated with 1×10^{-6} M IgG from normal (a) or SS (b) patients, washed with phosphate buffer solution and stained with anti-human IgG FITC conjugate $F(ab')_2$. The preparations were photographed with a Nikon photomicroscope equipped with epi-illumination × 450.



Parameters	IgG Sjögren Syndrome	IgG Control (normal)
Bmax (fmol/mg protein)	186.1 ± 20.2	563.6 ± 42
Kd (nM)	0.74 ± 0.13	0.85 ± 0.16

Fig. 3. Inhibition of [^{3}H]QNB binding on rat cerebral frontal cortex membranes by IgG from SS patients. Cerebral membranes (0.50 mg/protein) were incubated with 1×10^{-6} M IgG from pSS patients (●) or normal individuals (■) in the presence of increasing concentrations of [^{3}H]QNB. Values are calculated from linear regression analysis. Results are the mean ± S.E.M. of eight subjects in each group performed in duplicate. The parameters values of cerebral membranes alone were: B_{max}: 574.5 ± 45; K_d: 0.72 ± 0.26. *$P < 0.001$ between group I and group III.

2.11. Drugs

Carbachol, 4-DAMP, pirenzepine, AF-DX 116 and tropicamide and U-73122 were purchased from Sigma. Stock solutions were freshly prepared in the corresponding buffers.

2.12. Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA)

Table 2
Inhibition of [^{3}H]QNB binding to rat cerebral frontal cortex membranes

Cholinoceptor Antagonist	K_i ($\times 10^{-7}$ M)
Atropine	1.7 ± 0.02
Pirenzepine	3.4 ± 0.03
4-DAMP	2.1 ± 0.03
AF-DX 116	68.4 ± 0.35
Tropicamide	47.1 ± 0.35

The inhibition constants (K_i) for the competing agents were calculated from the equation of Chen and Prussoff: $K_i = IC_{50}/1([^3H]QNB)/K_d$, where IC_{50} is the concentration of the competing drug to inhibit 50% of the specific radioligand binding present at a concentration of 0.6 nM. IC_{50} values were obtained from competition experiments performed in duplicate at several concentrations of each drug.

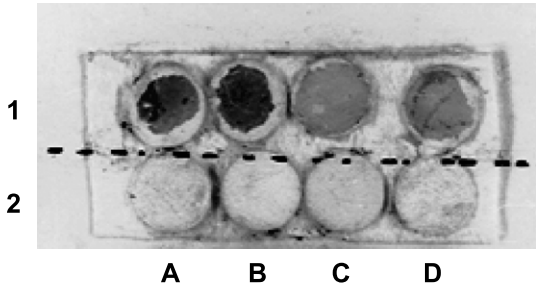


Fig. 4. Dot blot analysis of 1×10^{-6} M IgG from group I (1) and group III (2) patients on M_1 synthetic peptide (A), M_3 synthetic peptide (B) and cerebral frontal cortex membranes (C). The reaction with serum (1/30 dilution) from group I (1) and group III (2) upon cerebral frontal cortex membranes (D) is also shown. Results are representative of 19 separated assay using antibodies from 19 pSS patients and 19 control (normal) subjects with similar results.

and post-hoc test (Dunnett's Method and Student–Newman–Keuls test) were employed when a pairwise multiple comparison procedure was necessary. Differences between means were considered significant if $P < 0.05$.

3. Results

3.1. Detection of serum antibodies

To demonstrate the presence of serum IgG directed against cerebral frontal cortex in pSS patients, we performed an ELISA assay using cerebral membranes as a coating antigen. Fig. 1 shows the immune reactivity of sera from different groups against cerebral frontal cortex membranes. One can see that the OD values for sera from pSS patients (group I) was 3 S.D. higher than those from rheumatic patients (group II) and normal control (group III).

These results were assessed using an indirect immunofluorescence (IFI) assay. Fig. 2a shows positive staining of neuronal cells when sections were incubated with IgG from pSS patients. A negative image was obtained when IgG from normal controls were tested (Fig. 2b).

To test the ability of the antibodies from pSS patients to interact with cerebral frontal cortex mAChRs, radioligand assay, dot blot and ELISA were performed. The radioligand

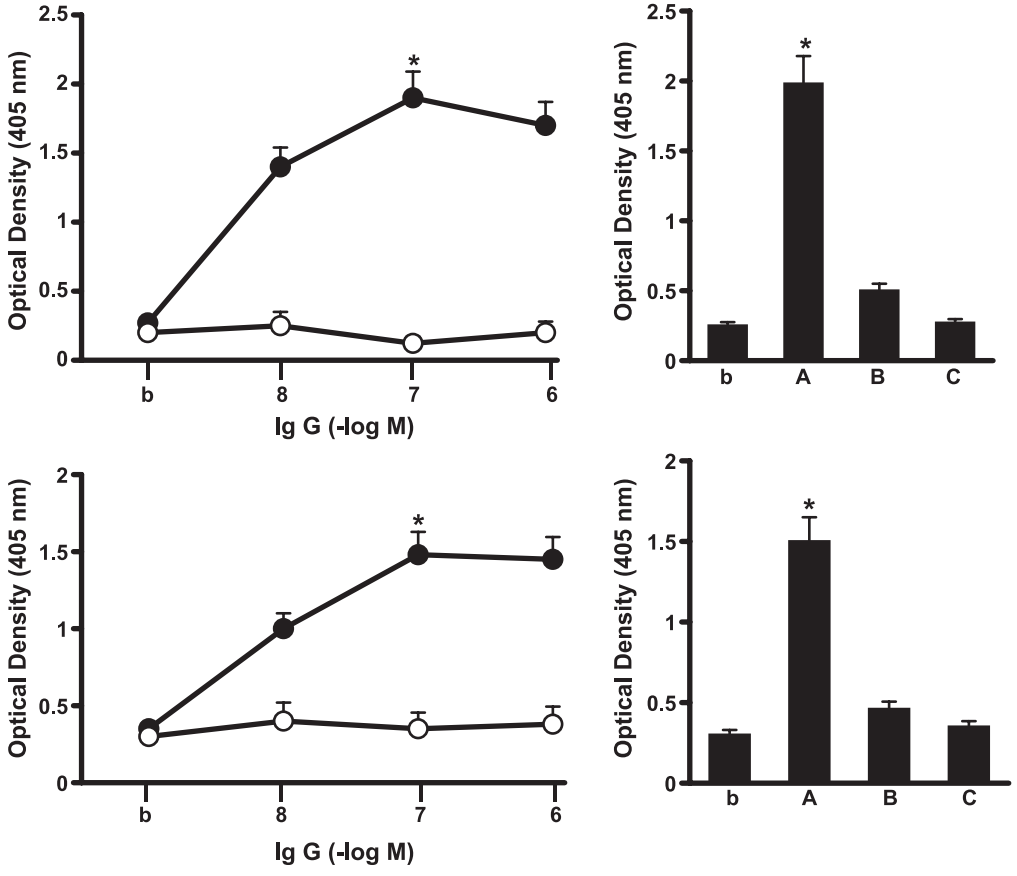


Fig. 5. Immunoreactivity of anti-M_1 and anti-M_3 mAChR antibodies of pSS patients sera directed against the second extracellular loop of M_3 and M_1 mAChR peptides tested by ELISA. Effect of increasing concentrations of anti-M_3 (TOP) and anti-M_1 (BOTTOM) peptide IgGs from pSS patients (●) and IgG from normal (control) subjects (○). b: Non-antigen paired control wells substracted from the antigen containing wells. Histogram shows—b: basal values, A: 1×10^{-7} M affinity purified anti-M_3 IgG (TOP) and 1×10^{-7} M affinity purified anti-M_1 IgG (BOTTOM) alone or B: in the presence of 1×10^{-5} M M_3 peptide (TOP) or M_1 peptide (BOTTOM) and C: none anti-M_3 (TOP) or none anti-M_1 (BOTTOM) peptides fraction eluted from the column. Results are mean ± S.E.M. of 10 independent pSS patients and normal individuals performed in duplicate. *$P < 0.001$ different from normal individuals.

Table 3
Distribution of serum IgG from different groups

Antibodies	Group I	Group II	Group III
IgG anti-membrane	19/48 (40%)	1/24 (4.2%)	2/35 (5.7%)
IgG anti-peptide M_1	40/48 (83%)	1/24 (4.2%)	1/35 (2.8%)
IgG anti-peptide M_3	45/48 (93%)	2/24 (8.3%)	3/35 (9.5%)

The results were expressed as the number of positives per the total with percentages in parenthesis.

competition binding assay was carried out by using [^{3}H]QNB as the specific radioligand agent for mAChRs. In saturation studies and linear regression analysis, an irreversible interaction was established in cerebral frontal cortex membranes expose to IgG from group I. Thus, Fig. 3 shows that IgG inhibited the binding of muscarinic cholinergic radioligand to its receptors, thus reducing the number of binding sites (B_{max}) without causing changes in the equilibrium dissociation constant (K_d). On the contrary, normal IgG did not affect B_{max} or K_d.

Values of K_i calculated from competitive binding assay showed that cerebral frontal cortex preferentially expressed M_1 and M_3 mAChR subtypes (Table 2).

By dot blot and ELISA, we determined the molecular interaction between antibodies and human M_1 and M_3 mAChRs, testing whether the IgG from group I could recognize the human M_1 and M_3 synthetic peptides. Fig. 4 shows that both sera and IgG from group, I but not sera or IgG from group III, reacted positively when M_1 and M_3 mAChR peptides or membrane from cerebral frontal cortex were used as the coating antigens.

By means of ELISA with M_1 and M_3 synthetic peptides as antigens, we confirmed the presence of anti-M_1 and anti-M_3 autoantibodies in the sera of pSS patients. Fig. 5 shows the concentration-dependent increase in OD values with the IgG from group I with OD values always >3 S.D. of those from group III when they were reacted with M_3 (TOP), and M_1 (BOTTOM) peptides. Histogram of Fig. 5 also shows the comparative increase in OD values triggered by the affinity-purified anti-M_3 (TOP), and anti-M_1 (BOTTOM) peptide IgGs. The specificity of the anti-M_1 and anti-M_3 peptide IgGs was assessed by the ability of the corresponding M_1 and M_3 peptides (10-fold concentrated) to inhibit the reaction. The non-anti-M_1 or anti-M_3 peptide fraction eluted from the column showed OD values similar to normal IgG. As expected, the IgG fraction from normal subjects purified by affinity chromatography with the synthetic peptides, yielded negative results (data not shown).

Table 3 shows the distribution of autoantibodies against frontal cortex membranes, M_1 and M_3 synthetic peptides in pSS patients. It can be observed that the frequency of anti-membrane, anti-M_1 and anti-M_3 mAChR autoantibodies was higher in group I compared to those from groups II and III. Moreover, we can also see in Table 3 that in group I, the frequency of anti-membrane IgG was less than those of anti-peptide M_1 and M_3 IgGs.

The OD values for each of the 19 patients of group I that gave positive reactions on cerebral cortex membranes are shown in the scattergram of Fig. 6. The immunoreactivity of IgG anti-M_3 was 100% positive and IgG anti-M_1 was 94% positive. Thus, there was a high correlation between pSS patients' serum IgG against cerebral membranes and those against synthetic peptides.

3.2. Muscarinic cholinergic M_1 and M_3 receptors mediated effect of serum autoantibodies

As already shown, the anti-M_1 and anti-M_3 peptide antibodies reacted with rat cerebral frontal cortex. Knowing that the aminoacid sequence of rat and human M_1 and M_3 mAChRs displays strong homology (93% and 89%, respectively), we studied a M_1 and M_3 mAChR-mediated effect of autoantibodies from group I on rat cerebral frontal cortex. The modifications of PIs accumulation were measured as intracellular signal coupled to M_1 and M_3 mAChR activation. As shown in Fig. 7, there was a significant increase in PIs production by rat cerebral frontal cortex preparations exposed to IgG and the corresponding affinity-purified anti-M_1 and anti-M_3 peptide IgGs. The effects of antibodies mimicked the carbachol (cholinergic agonist) action, and could be abolished by pretreating cerebral tissues with pirenzepine or 4-DAMP, respectively. Furthermore, the antibodies' effects could be blocked by U-73122—indicating that PLC-mediated hydrolysis of PIs was involved in these effects. Also, the M_1 and M_3 synthetic peptides neutralized the

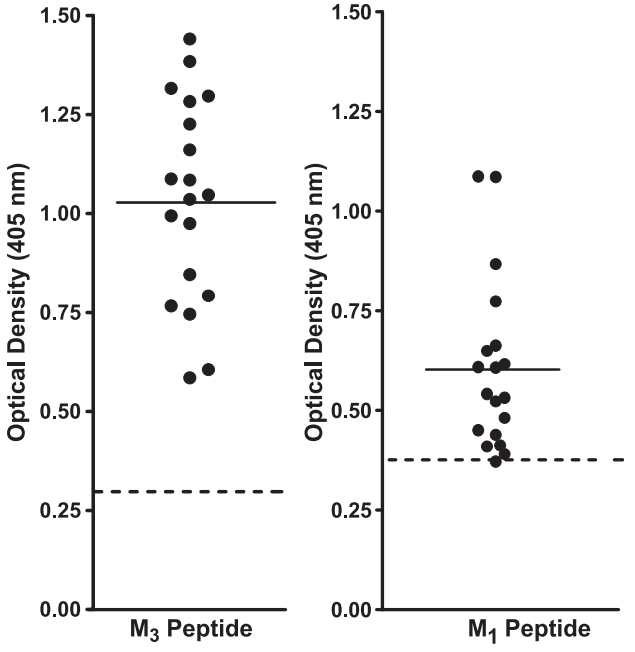


Fig. 6. Immune reactivity of anti-M_3 and M_1 peptide IgGs from 19 pSS patiens. Dotted/dashed line represent cutoff values and solid lines represent mean optical density values.

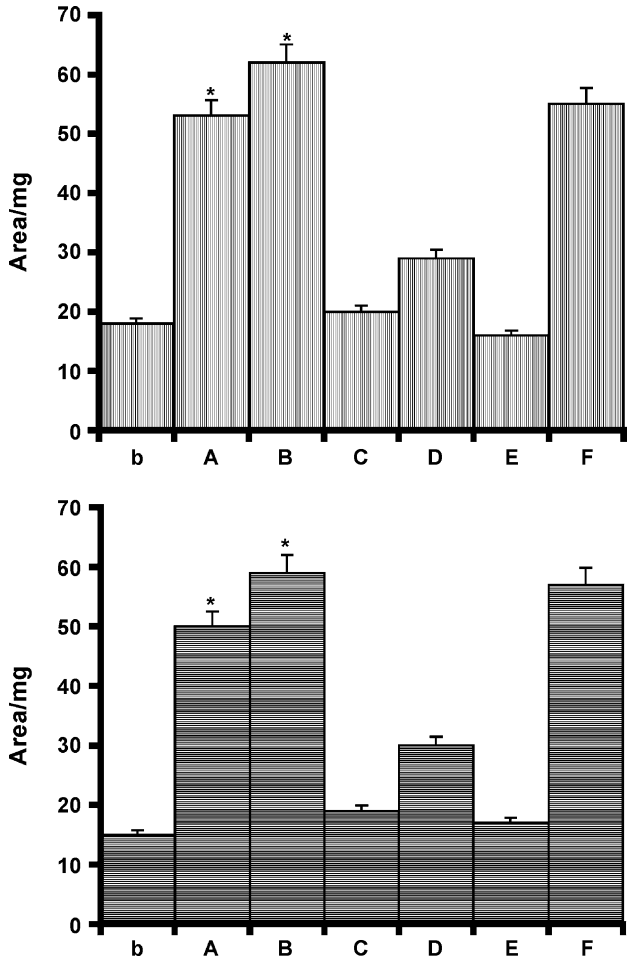


Fig. 7. Increase of phosphoinositide hydrolysis (PIs) in rat cerebral frontal cortex slices by antibodies from pSS patients. Slices were incubated for 30 min with [^{3}H]MI and for an additional 30 min with or without blockers. Tissues were then left for further 30 min in the absence (b) or in the presence of IgGs or carbachol. Cerebral frontal cortex slices were preincubated before the addition of 1×10^{-6} M IgG (A, TOP and BOTTOM) or 1×10^{-7} M affinity purified anti-M_3 peptide IgG (C, TOP) or affinity purified anti-M_1 peptide IgG (C, BOTTOM) alone or in the presence of 1×10^{-7} M 4-DAMP (D, TOP) or pirenzepine (D, BOTTOM). The action of 1×10^{-6} M U-73122 plus affinity purified anti-M_3 peptide IgG (E, TOP) or affinity-purified anti-M_1 peptide IgG (E, BOTTOM) and 1×10^{-7} M carbachol (F, TOP and BOTTOM), are also shown. Results are mean ± S.E.M. of 10 independent pSS patients and normal individuals performed in duplicate. *Significantly different from basal (b) values $P < 0.001$.

corresponding IgG action, indicating the specificity of the reaction. Normal IgG had no effects on the system studied.

4. Discussion

Immune-mediated neurological manifestations in SS have been described (Praprotnik et al., 1999; Bacman et al., 1996; Stone and Francis, 2000), but the precise immune mechanisms remain unclear. Here, we demonstrated the possible role of altered humoral immunity by exploring the cholinergic muscarinic activity of IgG from SS patients.

Indirect immunofluorescence and dot blot assays provided evidence that certain components of the IgG fraction from SS patients can recognize rat cerebral frontal cortex neural cells. The presence of serum anti-neural and anti-brain antibodies in autoimmune diseases especially those with overt CNS manifestations has also been detected (Colbum et al., 2001; Weiner et al., 2000).

Among the extraglandular manifestations of SS patients, alterations in cognitive function with subtle abnormalities in the frontal lobes have been demonstrated (Lafitte, 2000; Belin et al., 1999). It is interesting that many of the cognitive aspects of diseases linked to attention deficits display cholinergic dysfunction of brain tissue (Bysmaster et al., 1999; Faraone et al., 2000; Lawrence and Sahakian, 1998; Von der Kammer et al., 2001). Therefore, in an attempt to elucidate the nature of the cholinergic central nervous system dysfunction in SS patients, we characterized the effect of the anti-brain antibodies on the muscarinic cholinergic system. In this sense, we conducted binding, dot blot and ELISA. The antibody binds irreversibly to muscarinic receptors, thereby decreasing the available binding sites without affecting receptor affinity. These data suggest that the alteration on cholinergic activity could be the result of antibody fixation to mAChR. In accord with this observation, serum mAChR antibodies in schizophrenic patients are known to be related with cognitive function alterations (Borda et al., 2002). Also, in Alzheimer's disease patients, the cholinergic system is severely damaged (Von der Kammer et al., 2001). Brainstem, basal forebrain and cortical mAChRs have also recently been implicated in the etiology and protracted expression of several anxiety-subtype disorders, although this hypothesis remains controversial in humans (Wall et al., 2001; Wall and Messier, 2002).

Five different mAChR subtypes are expressed in different brain regions and they have excitatory and inhibitory effect on cholinergic synapsis by modulating the conductance of ionic channels (Fukuda et al., 1988) and by coupling to several intracellular second messengers. The M_1 and M_3 mAChR subtypes are coupled to PLC and PI turnover (Kovacs et al., 2000), and they are associated with long-term memory and learning (Maes et al., 1997; Albrecht et al., 2000). Our results establish that M_1 and M_3 mAChR are expressed in rat cerebral frontal cortex and their presence may be a target for the anti-brain autoantibodies described in SS patients.

From dot blot and ELISA, the antibodies reacted molecularly against the second extracellular loop of the human M_1 and M_3 mAChRs. It has been shown that the second extracellular loop of mAChR appears to be the main immunogenic region of the receptor (Fu et al., 1993). The specificity of these interactions was assessed by the fact that corresponding affinity-purified anti-peptide antibodies behaved similarly to the corresponding total IgG. These anti-peptide antibodies were not only able to interact molecularly

with the human M_1 and M_3 peptides, but also displayed agonistic activity which triggered PIs hydrolysis.

Recently, we have determined (Sterin-Borda et al., 2003) that agonist activation of cerebral frontal cortex mAChRs induced early transcriptor factors having the capacity to modulate the m1 muscarinic receptor gene expression. The mechanism appears to occur secondarily to stimulation of PIs turnover via PLC activation. The immediate early genes under the control of mAChRs play an important role in coupling receptor stimulation to long-term neuronal responses (Albrecht et al., 2000), by the activation of transmission as well as controlling cellular functions in post-synaptic cholinergic target cells (Von der Kammer et al., 2001). In the pathogenesis of SS, a defect in mAChR signalling has been proposed (Kovacs et al., 2000).

Thus, on the basis of our results, we postulate that early agonistic-promoting activation in M_1 and M_3 mAChR initiated by antibodies bind to persistently activate cerebral frontal cholinoceptors. Later, the agonistic activity displayed by these autoantibodies might induce desensitization, internalization and/or intracellular degradation of the mAChR, leading to a progressive decrease of cerebral M_1 and M_3 mAChR expression and activity. Also, IgG antibodies binding to mAChRs might modify a spare receptor affinity, sensitivity and expression in brain tissue. Therefore, it could be hypothesized that the central nervous system manifestations, which are apparent during SS, might be induced by an impaired response to cholinergic stimuli by mAChR-antibody-specific interactions.

However, further evidence is required to show persistent abnormal levels of immunoglobulins in forebrain tissues from SS patients to further understand the implications of these autoantibodies on the cognitive deficit in these patients.

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