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Anti-M₃ Muscarinic Acetylcholine Receptor Antibodies in Systemic Lupus Erythematosus

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Abstract

Background: Evidences have shown that anti-M₃ muscarinic acetylcholine receptor IgG (anti-M₃ mAChR IgG) are clinically useful autoantibody that exert a cholinergic pharmacologic effect binding and interacting with M₃ mAChR at the level of exocrine gland (salivary and ocular). **Aims:** The aim of this study was to determine the associations between serum level of anti-M₃ mAChR IgG in patients with systemic lupus erythematosus (SLE) and other autoantibodies, serum prostaglandin E₂ (PGE₂), and clinical manifestations. **Methods:** Serum autoantibodies against M₃ mAChR synthetic peptide were measured by enzyme-linked immuno absorbent assay (ELISA) using, as an antigen, a 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 amino acid sequence of the second extracellular loop of the human M₃ mAChR. Serum levels of antinuclear antibodies (ANA), anti-Smith (Sm) antibodies, anti-phospholipid (APL) antibodies, and PGE₂ were determined by ELISA in patients with SLE. **Results:** We found significantly enhanced titers of anti-M₃ mAChR IgG in sera from SLE patients compared with healthy individuals (control). In addition, serum levels of PGE₂ were significantly higher in SLE patients than in control patients and were significantly higher in active than in non-active SLE. No correlation was found with other autoantibodies present in SLE. By contrast, a positive correlation was found between anti-M₃ mAChR IgG and PGE₂ serum levels in SLE. **Conclusions:** As anti-M₃ mAChR antibodies present in the sera of SLE patients may be another factor in the pathogenesis of this disease, and the increment of PGE₂ in the sera of SLE has a modulatory action on the inflammatory process, suggesting that the presence of these autoantibodies against M₃ mAChR may contribute to sustained immune deregulation and the strong inflammatory component observed in SLE.

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Keywords

Anti-M₃ mAChR Antibodies, Systemic Lupus Erythematosus, Prostaglandin E₂

1. Introduction

The onset and development of autoimmune disease (AID) are the consequence of interactions between genetic and environmental factors, which result in dysregulation of the immune system, and are characterized by the presence of autoantibodies and autoreactive T cells. Under these circumstances, immune system antimicrobial defenses react against normal components of the body and result in organ-specific or systemic immunopathology. Autoantibodies may also appear in the blood of healthy individuals or in some special situations, such as infection and the pre-clinical phase of infectious diseases [1]-[3].

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of multiple autoantibodies [4]-[8], with an inflammatory/necrotic phenomenon in different tissues [9] [10]. This condition is associated with hyperactivity of B cells, and different immuno-regulatory abnormalities [11] [12]. In addition, T cells from SLE patients exhibit defects in early activation events as well as an impaired proliferative response to mitogenic lectins [12] [13]. Furthermore, anti-nuclear (ANA), anti-Smith (anti-Sm), anti-phospholipid (APL), and other autoantibodies are detected in patients with SLE [4] [14] [15]. One early study, in which 23 asymptomatic pregnant women with positive anti-Ro or anti-La titers were followed for many years, reported that four subjects developed SLE, which suggested that anti-Ro or anti-La antibodies preceded the development of SLE [16]. In another study, Aho *et al.* reported that SLE patients were positive for ANA before the onset of SLE and the percentage was much higher than that of controls which was a much higher rate than controls [17]. Moreover, the results from the United States Department of Defense Serum Repository showed that the presence of ANA, anti-Ro, anti-La, anti-double strain deoxyribonucleic acid (anti-ds DNA), anti-Sm, APL, anti-ribonucleo protein (anti-nRNP) antibodies and rheumatoid factor (RF) preceded the onset of SLE [18]-[20]. Among SLE patients, 88% were positive for at least one of these autoantibodies, which was a much higher percentage than healthy controls, and the prevalence of these autoantibodies increased after diagnosis [21] [22]. Anti-Ro, anti-La, and APL antibodies were the earliest detectable autoantibodies during the pre-clinical phase of rheumatoid arthritis (RA) [18]-[22]. In addition, the presence of these autoantibodies was associated with incipient severe SLE. For example, patients who were positive for anti-ds DNA antibodies often developed renal disease [22] [23], patients who were positive for IgG RF were more likely to develop arthritis [18], and positive APL was associated with malar rash and photosensitivity [19]. In addition, regular patterns exist among these autoantibodies. The majority of La-positive pre-clinical SLE patients were also Ro-positive, and a significant overlap was observed between patients positive for anti-ds DNA antibody and those positive for anti-chromatin antibody [20].

A recently published nested case-control study showed that 66% of Sjögren Syndrome (SS) patients were positive for ANA, RF, and anti-La or anti-Ro antibodies approximately 5 years before the onset of SS. A maximum of 18 years elapsed between positivity for these antibodies and the onset of SS in these subjects [23]. The seropositive rate for these antibodies and the anti-M₃ muscarinic acetylcholine receptor (mAChR) autoantibodies increased as the onset of SS neared [24]-[26]. In conclusion, the evidence suggests that these autoantibodies are predictors of SS. Moreover, the presence of these autoantibodies in the serum of patients with SLE is another topic of discussion in SLE pathogenesis.

The autoimmune nature of lupus and its predominant inflammatory component was accompanied by the expression of cyclo-oxygenase-2 (COX-2) in the inflammatory areas, with the subsequent release of arachidonic acid via membrane-bound phospholipase A₂. The biosynthesis of arachidonic acid by COX-2 led to an enhancement of prostanoid production of PGE₂ serie, which conduct to the dysregulation in the production of proinflammatory cytokines (IL-6, IL-10, and nitric oxide) [27] [28]. Therefore, we focused on the family of prostaglandins (PG), which are the result of the oxidative modification of arachidonic acid and its cascade products through COX-2 expression and activation in patients with SLE.

2. Aim

The aim of our preliminary study was to investigate the inflammatory status in SLE patients compared to active and non-active groups by assessing the generation of PGE₂ and its association with the presence or absence of

anti M₃ mAChR autoantibodies and SLE disease activity index (SLEDAI).

3. Methodology

3.1. Patients

Blood samples from 30 patients with SLE, according to the classification criteria of the American College of Rheumatology (ACR) [29] were obtained. A total of 26 women subjects and four men subjects with a mean age of 41.4 ± 11.9 years were included in the study. Also, blood samples of 30 healthy women subjects with a mean age of 39.6 ± 10.2 years were used as controls. Fifteen patients had non-active disease (<3) and 15 were considered to have active disease (≥ 3), according to the SLEDAI measured [30] [31]. Most patients were receiving low to moderate doses of glucocorticoids as well as immunosuppressive drugs-mainly cyclophosphamide, methotrexate, azathioprine, and chloroquine. No patients with renal failure or conditions different from SLE were included in the study. The demographic and clinical characteristics of the study population (SLE patients) and normal individuals (controls) are shown in **Table 1**. All of the patients signed an informed consent form to participate in the study, and the investigation was conducted according to the tenets of the Declaration of Helsinki of 1975, as revised in 2000.

3.2. Autoantibody Detection

ELISAs were performed to measure ANA, anti-Sm, and APL using commercially available ELISA kits (INOVA Diagnostic, Inc., San Diego, CA, USA). ELISAs were performed according to the manufacturers' instructions. Values of optical density (OD) at 450 nm were obtained, and the IgG antibody concentrations were calculated by extrapolating OD₄₅₀ values to a standard curve.

3.3. M₃ mAChR Autoantibodies

The IgG fraction from 30 patients with SLE and 30 normal subjects was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described previously [25]. Briefly, the IgG fraction was loaded onto the affinity column equilibrated with phosphate-buffered saline (PBS). The non-peptide fraction was first eluted with the same buffer. Specific anti-

Table 1. Demographic and clinical characteristics of SLE patients and healthy individuals.

Clinical characteristics		
	SLE patients	Normal individuals
Number	30	30
Current age, mean years \pm SD	41.4 ± 11.9	39.6 ± 10.2
Disease duration, mean years \pm SD	7.88 ± 10.6	N/A
Female gender, n (%)	26 (86.7)	30 (100)
Organ/system involved		
Malar rash, n (%)	18 (60.0)	N/A
Photosensitivity, n (%)	17 (56.7)	N/A
Hematological, n (%)	23 (76.6)	N/A
Renal, n (%)	13 (43.4)	N/A
Current antibodies		
ANA positive, n (%)	30 (100)	N/A
Anti-Sm positive, n (%)	5 (16.6)	N/A
Anti-phospholipid (APL) positive, n (%)	6 (20.0)	N/A
Anti-M3 synthetic peptide IgG, n (%)	13 (43.3)	3 (10.0)

peptide antibodies were then eluted with 3 M potassium thiocyanate (KSCN) and 1 M sodium chloride (NaCl), followed by immediate extensive dialysis against PBS. The IgG concentrations of non-anti-peptide antibodies and specific anti-muscarinic receptor peptide IgG were determined by a radial immunodiffusion assay, and their immunological reactivity against muscarinic receptor peptides was evaluated by ELISA. The concentration of the affinity-purified anti-M₃ peptide IgG (1×10^{-7} M) that maximally increased optical density (OD: 2.4 ± 0.2) corresponded to a total IgG concentration of 1×10^{-6} M (OD: 2.2 ± 0.2). The normal IgG fraction purified by affinity column chromatography gave a negative result (OD: 0.24 ± 0.03).

3.4. ELISA Assay

Fifty microlitres of M₃ mAChR peptide solution in 0.1 M sodium carbonate (Na₂CO₃) buffer (pH = 9.6) was used to coat microtiter plates (Corning Costar, Tewksbury, MA, USA) at 4°C overnight as described [25]. After blocking the wells, varying concentrations of purified IgG from patients with SLE and healthy individuals were allowed to react with the antigens for 2 h at 37°C. The wells were then thoroughly washed with Tween[®] 20 in PBS. Goat anti-human IgG avidin-alkaline phosphatase (50 µl) was added and incubated for 1 h at 37°C. After several washing steps, p-nitrophenyl phosphate ($1 \text{ mg} \cdot \text{mL}^{-1}$) was added as the substrate; the reaction was terminated at 30 min. OD values were measured using an ELISA reader (Uniskan Laboratory System, Helsinki, Finland). As negative controls, non-antigen paired wells and wells with no primary antiserum were also tested.

3.5. PGE₂ Procedure

Serum PGE₂ was measured by ELISA according to the manufacturer's protocol (PGE₂ Biotrack Enzyme Immune Assay System; Amersham Bioscience, Piscataway, NJ, USA). The OD cutoff value for PGE₂ was 4.4 ± 0.33 ng/ml. All serum samples were frozen promptly after collection and kept at -80°C until used for PGE₂ determination. The PGE₂ results are expressed as ng/mL.

3.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad, San Diego, CA, USA). Statistical significance was determined by the two-tailed *t* test for independent populations. Analysis of variance (ANOVA) and Dunn's and Kruskal-Wallis tests were employed for multiple comparisons. Pearson's analysis was applied to establish correlation. Differences between means were considered significant at $P \leq 0.05$.

3.7. Ethical Approval of the Study Protocol

The study was approved by the Ethics Committee of the School of Dentistry at Buenos Aires University (Buenos Aires, Argentina). The studies were conducted according to the tenets of the Declaration of Helsinki. All participants provided written informed consent to participate in the study.

4. Results

ELISA assays were performed to determine whether a correlation exists between serum IgG against M₃ mAChR synthetic peptide (**Figure 1(a)**) as well as serum PGE₂ levels (**Figure 1(b)**) in SLE patients compared with normal individuals. **Figure 1(a)** shows the optical density (OD) values for each studied serum from SLE patients and normal subjects. Also, **Figure 1(b)** shows serum PGE₂ levels in SLE patients and normal subjects. The cut-off values obtained with SLE patient sera were always greater than two standard deviations (SD) from those from normal individuals. In **Figure 1(c)**, a positive correlation is shown between serum PGE₂ levels and anti-M₃ synthetic peptide titers (see table insert) of the individual sera from SLE patients.

Additionally, **Table 2** shows the comparison and a statistical analysis of different autoantibodies from sera of SLE patients, showing that anti-M₃ synthetic peptide IgG was significantly different from anti-Sm antibodies and APL antibodies. No significant values were obtained when we compared anti-M₃ synthetic peptide IgG with ANA.

The possible association between SLE disease activity, according to the SLEDAI and levels of anti-M₃ synthetic peptide IgG and serum PGE₂ levels (expressed as optical density values or ng/ml, respectively) was tested. Accordingly, when SLE patients were grouped on the basis of the disease status (active or non-active), no sig-

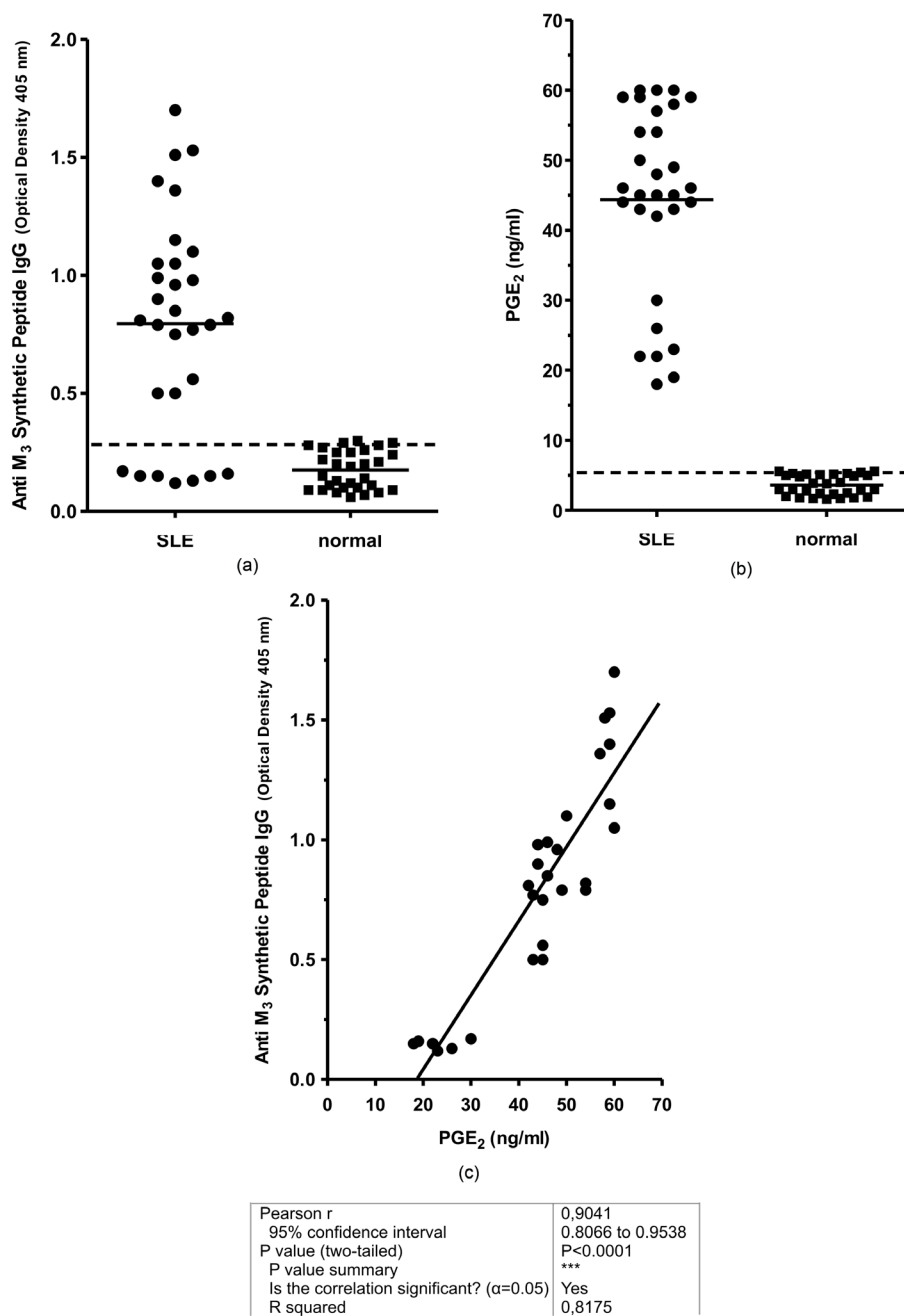


Figure 1. Scattergram showing immunoreactivity of circulating IgG antibodies against M₃ mAChR synthetic peptide (a) and serum PGE₂ (b). The individual optical density (OD) values for each serum sample (1:30 dilution) from 30 SLE patients and 30 normal individuals. Dotted/dashed line: cutoff values of OD 0.24 and 4.40 for anti-M₃ mAChR synthetic peptide IgG and serum PGE₂, respectively. Solid lines, median OD values. $P < 0.0001$ between groups, and (c) correlation between the anti-M₃ synthetic peptide IgG and serum PGE₂ levels.

nificant differences were found for the anti-M₃ synthetic peptide IgG (**Figure 2(a)**). On the contrary, a significant association between active or non-active status of SLE patients was observed when we analyzed serum PGE₂ levels (**Figure 2(b)**).

Finally, when we examined the possible association among the three parameters tested (SLEDAI, anti-M₃ synthetic peptide IgG levels, and serum PGE₂ levels) in this study, a highly significant association was found

Table 2. Comparison between anti-M₃ synthetic peptide IgG and different antibodies in SLE patients.

Variable	Dunn's multiple comparison test	Number of patients
Anti-M3 synthetic peptide IgG versus ANA	ns	30
Anti-M3 synthetic peptide IgG versus anti-Sm	Yes ($P < 0.05$)	30
Anti-M3 synthetic peptide IgG versus APL	Yes ($P < 0.05$)	30

Results were analyzed by one-way ANOVA followed by Kruskal-Wallis tests ($P < 0.0001$).

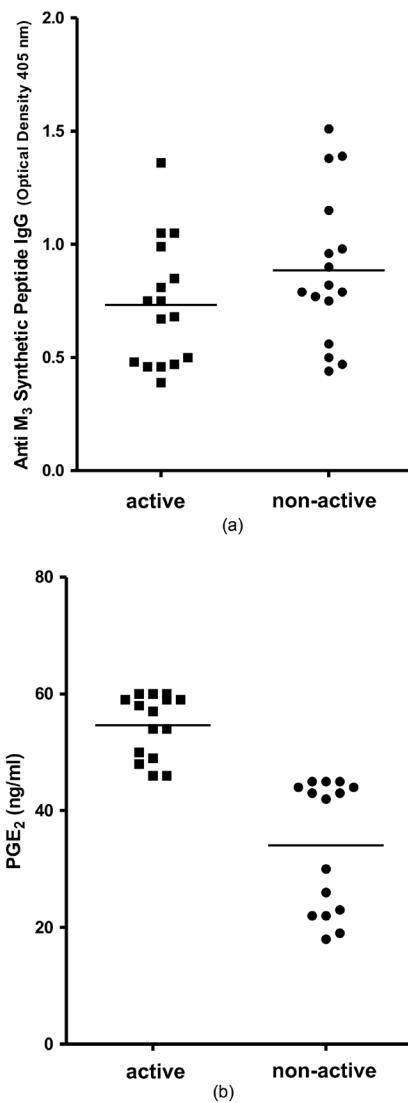


Figure 2. Scattergram showing immunoreactivity of circulating IgG antibodies against M₃ mAChR synthetic peptide (a) and serum PGE₂ (b) in active and non-active forms of SLE disease. Values are the individual OD values for each serum sample from 30 SLE patients and 30 healthy volunteers. Dotted/dashed line: cutoff values of OD 0.24 and 4.40 for anti-M₃ mAChR synthetic peptide IgG and serum PGE₂, respectively. Solid lines, median OD values. $P < 0.0001$ between groups.

only between the levels of PGE₂ and the active and non-active SLE disease status (SLEDAI) (Table 3).

5. Discussion

Systemic lupus erythematosus (SLE) is a challenging disease to assess and manage. Much progress in our un-

Table 3. Association between disease activity (SLEDAI) and levels of autoantibodies and serum PGE₂.

Conditions	Active SLE	Non-active SLE	P values
SLEDAI	6.51 ± 0.90	0.53 ± 0.23	<0.0001
Anti-M ₃ synthetic peptide IgG (optical density 405 nm)	0.73 ± 0.10	0.88 ± 0.22	0.0876
PGE ₂ (ng/mL)	54.60 ± 1.39	34.07 ± 2.89	<0.0001
Number of patients	15	15	N/A

Values represent the mean ± SEM of 30 patients grouped according to disease activity.

Understanding of its etiopathogenesis has been made. Moreover, differences in the clinical interpretation of the signs and symptoms are attributable to the complexity of the disorder and the likely diverse mechanisms that contribute to its clinical expression. This extra challenge complicates the work of scientists who seek to understand the disease more fully and clinicians treating patients with SLE.

SLE is an autoimmune disease that not only can have very different manifestations, but may also share some pathogenic mechanisms that may help to identify therapeutic targets.

In this study, we have shown the differential expression of some autoantibodies, such as ANA, anti-Sm, and APL [18] [19], is present at a much higher percentage in sera from SLE patients than in sera from healthy individuals. In addition, we detected for the first time the presence of anti-M₃ synthetic peptide IgG in the serum of SLE patients. This autoantibody was significantly associated with anti-Sm and APL antibodies but was unassociated with ANA. In previous studies was reported that ANA has the highest sensitivity in patients with SLE [32]. Moreover, the anti-M₃ synthetic peptide IgG was unassociated with malar rash, photosensitivity, or hematologic and renal alterations.

All of these autoantibodies are also associated with the clinical characteristics in the development of different clinical forms of SLE detected both in initial and chronic disease.

SLE is characterized not only by a dysregulatory immune response including overactive B-cells but is also always accompanied by an inflammatory process with T cell hyperactivity, where PGE₂ is increased in the serum of SLE patients. That, in turn, not only modulates the immune processes at the sites of inflammation but also has the ability to generate other inflammatory cytokines [28]. However, the increased serum levels of PGE₂ in SLE patients reveal that this prostanoid mediates the inflammatory process observed in this disease; under identical experimental conditions, a significant correlation between PGE₂ levels and anti-M₃ synthetic peptide IgG was found.

These results appear to be a pivotal factor mediated at the level of inflammatory site, maintaining and increasing the production of other proinflammatory cytokines, such as IL-6, IL-10, and nitric oxide [33]. Therefore, PGE₂ and the anti-M₃ synthetic peptide antibody could be jointly responsible for the dysregulated production of proinflammatory cytokines that maintains the immune-inflammatory process characteristic of this disease.

In previous works patients with active discoid SLE were shown to have a strong expression of COX-2 in the inflammatory areas [27].

When SLE patients were grouped on the basis of disease activity (active and non-active disease), no significant differences were found for anti-M₃ synthetic peptide IgG titers, though a significant difference in serum PGE₂ levels was observed. Perhaps the increase in PGE₂ is necessary to maintain the active inflammatory process in the course of SLE disease.

Although a possible association exists between the presence of elevated levels of anti-M₃ synthetic peptide IgG and many different clinical and laboratory parameters, we failed to find a significant correlation with most of these parameters. However, a significant correlation between the titer of this cholinergic autoantibody and the production of PGE₂ was detected. These data suggest that, first, the anti-M₃ synthetic peptide IgG may participate in the onset of the disease, and second, these autoantibodies may contribute to the generation of other proinflammatory cytokines.

6. Conclusion

On the basis of our results, we have demonstrated significantly enhanced titers of anti-M₃ synthetic peptide IgG

in patients with SLE, as well as significantly higher levels of serum PGE₂. We consider that PGE₂ and these cholinergic autoantibodies may contribute, in part, to the pathogenesis of the autoreactive and inflammatory phenomenon described here and observed in SLE patients.

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Conflict of Interest

The authors declare that they have no vested interest that could be constructed to have inappropriately influenced this study.

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