Role of Salivary IgA in the Pathogenesis of Sjögren Syndrome

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Saliva IgA autoantibodies against M₃ muscarinic acetylcholine receptors (mAChRs) could be a new marker for the diagnosis for Sjögren syndrome (SS) dry mouth. Saliva IgA from dry mouth primary SS (pSS) or secondary SS patients tested by ELISA recognized membrane parotid gland acinar cell antigens and the synthetic 25-mer peptide corresponding to the second extracellular loop of human M₃ mAChRs. Moreover, the IgA fraction was able to inhibit the [³H]QNB binding to parotid acinar membrane mAChRs. In addition, the IgA prevented carbachol stimulation of protein secretion by the parotid gland. As controls, IgA and saliva from women without dry mouth and from normal control subjects gave negative results on ELISA, binding, and biological assays, thus demonstrating the specificity of the reaction. IgA autoantibodies against mAChR may be considered among the immunoglobulin factors implicated in the pathophysiology of the development of pSS dry mouth and could be a new marker for differentiating SS dry mouth from non-SS dry mouth.

Key Words: antibodies; Sjögren syndrome; parotid gland; saliva; IFI; binding assay.

INTRODUCTION

Sjögren syndrome (SS) is an autoimmune disease with exocrine glands as the site of intense immunological activity (1). This may occur in association with well-characterized connective tissue diseases (secondary SS; sSS) or in their absence (primary SS; pSS) (2). Lymphoproliferation and B cell activation are common and result in two important pathogenetic mechanisms: production of circulating autoantibodies and lymphocytic infiltration of the exocrine glands (3). Both of these processes could contribute to the wide range of clinical manifestations and to the immune-mediated destruction of the lacrimal (4) and salivary (5) glands, resulting in the loss of function which determines the defining criteria of the syndrome (5).

Thus, SS phenotype expression comprises keratoconjunctivitis sicca and xerostomia. Joint pain Raynaud’s phenomenon and fatigue are the most common systemic manifestations of SS. Sicca symptoms have been attributed to infiltration and destruction of the lacrimal and salivary glands by T lymphocytes (5). The severity of the sicca symptoms, however, do not always correlate with the degree of glandular destruction (6) and could result in diminishing function of the acinar and ductal cells due to the actions of different cytokines or due to parasympathetic dysfunction (6).

In an experimental model of SS, salivary gland dysfunction preceded lymphocyte infiltration of the gland (7). However, antibody production correlated with abnormal function of the gland, suggesting that antibodies are necessary for gland dysfunction in this model (6). Indeed, autoantibodies to muscarinic acetylcholine receptors (mAChRs) have been described in the sera of pSS and sSS patients (8–10) and in experimental models (11).

We recently proposed a role for these circulating immunoglobulin G (IgG) autoantibodies that recognized and activated mAChRs in submandibular, parotid, and lacrimal glands, participating in their physiological response (8, 10, 12). Acetylcholine acting on muscarinic receptors of the M₃ subtype mainly control exocrine protein secretion (13, 14). Cholinergic agonists stimulate protein secretion by interacting with mAChRs on the acinar cell basolateral surface membranes (15), and these receptors are, in part, responsible for mediated parasympathetic stimulation of fluid, electrolyte, and macromolecular products (16). We proposed that patients with SS produce autoantibodies able to be fixed in these receptor subtypes and capable of causing glandular autonomic dysfunction (9, 10).

Based on these observations, we considered it of special relevance to investigate whether salivary secretory IgA from SS patients could act on salivary gland M₃ mAChRs and whether they are able to interfere with the biological effect of the cholinergic agonists associated with these receptors, causing autonomic glandular dysfunction.

MATERIALS AND METHODS

Subjects and Serological Tests

Women (aged 35–55 years) free of treatment for 6 months, with 5 to 15 years since diagnosis, were se-
lected from the metropolitan area of Buenos Aires. The subjects were divided into four groups: group I, 16 primary Sjögren syndrome dry mouth patients; group II, 18 secondary Sjögren syndrome dry mouth patients with rheumatoid arthritis (RA); group III, 21 postmenopausal dry mouth patients without SS; group IV, 20 normal control subjects. The diagnosis of SS followed four of more criteria of Vitale et al. (labial biopsy, 95% positive; xerostomia, 100% positive; keratoconjunctivitis sicca, 89% positive; and two or three serologic tests) (17). The following serologic tests were performed: anti-Ro/SS-A and anti-La/SS-B antibodies, rheumatoid factor (RF), and antinuclear antibodies (Table 1). All of the studies involved human subjects who gave their informed consent, and the studies were conducted according to the tenets of the Declaration of Helsinki.

Preparation of Rat Parotid Gland Acini

Parotid gland acini were prepared from adult female Wistar-strain rats. Animals were used according to “The Guide to the Care and Use of Experimental Animals” (DHEW Publication, NIH 80-23). 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. Parotid glands were minced and incubated in KRB supplemented with 10 mM Hepes and 5.5 mM glucose (KRB-Hepes) and 0.5% bovine serum albumin (BSA), pH 7.4, containing collagenase (150 U/ml). Gland lobules were subjected to gentle pipetting. The preparation was then filtered through nylon mesh (150-μm pore size) and the acini were pelleted with 2 min centrifugation at 50g. The pellet was then washed twice by centrifugation (50g for 2 min) through a 4% BSA solution and the acini were pelleted with 2 min centrifugation at 1000g. The pellets were discarded, and the supernatants were centrifuged (10,000g) at 4°C for 10 min and then at 40,000g for 60 min. The resulting pellets were resuspended in the same buffer as described previously (19) and used as a membrane source for the ELISA test.

Preparation of Microsomal Fractions

Parotid gland acini were homogenized for 10s twice in 50 mM phosphate buffer, pH 7.4, in an Ultra-Turrax (setting 5). The homogenate was centrifuged for 10 min at 1000g. The pellets were discarded, and the supernatants were centrifuged (10,000g) at 4°C for 10 min and then at 40,000g for 60 min. The resulting pellets were resuspended in the same buffer as described previously (19) and used as a membrane source for the ELISA test.

Purification of Saliva IgA

The IgA fractions of 10 patients from groups I, II, III and 10 normal individuals from group IV were independently purified by standard DEAE chromatography. Briefly, saliva samples were dialyzed against 0.01 M, pH 8.0, phosphate buffer for 18 h and then applied to DEAE cellulose columns equilibrated in the same buffer. The pass through IgG-rich fractions was discarded and IgA-rich fractions were eluted with 0.05 M NaCl in 0.01 M phosphate buffer, pH 8. The IgA concentration in the enriched fractions was determined by radial immunodiffusion after concentration by ultrafiltration with PM-30 filtering membranes (Amicon, Beverly, MA) (cutoff molecular weight, 30,000 Da). The concentration of IgA was 35.8 ± 15.2 mg/dl in the IgA-enriched fractions. IgA was also purified by affinity chromatography of different sera on Jacalin–agarose beads following the recommendations of the supplier (ICN Pharmaceuticals, Irvine, CA) and previously described methods (20).

Purification of Human IgG

IgG was obtained by precipitation with ammonium sulphate at 50%, followed by three washes and reprecipitation with 33% ammonium sulphate from patient groups I, II, and III and group IV, the normal individuals. 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 to 10 μg protein/ml. Control immunoelectrophoresis with goat anti-human total serum and goat monospecific anti-human IgG showed only one precipitin line.

ELISA

Fifty microliters of peptide solution (20 μg/ml) in 0.1 M Na2CO3 buffer, pH 9.6, was used to coat microtiter plates (NUNC, Kastrup, Denmark) at 4°C overnight. After blocking the wells, diluted sera from patients of groups I, II, III, and IV were added in triplicate and allowed to react with the peptide for 2 h at 37°C. After the wells were thoroughly washed with 0.05% Tween 20 in a phosphate buffer solution (PBS), 100 μl of 1:6000 biotinylated goat anti-human IgA antibodies (Sigma Chemical Co., St. Louis, MO) was added and incubated for 1 h at 37°C. Then, a 1:6000 dilution of extravidin–alkaline phosphatase (Sigma) was allowed to react an extra 30 min at 37°C. After extensive washings, p-nitrophenylphosphate (1 mg/ml) was added as the substrate, and the reaction was stopped at 30 min. In addition, 50 μl of parotid gland acinar cell membranes (50 μg/ml) in 0.1 M Na2CO3 buffer, pH 9.6, was
used to coat microtiter plates at 4°C overnight, and the ELISA procedure was performed as described above. Finally, the plates were read at 405 nm and the results for each sample were expressed as the means ± SD of triplicate values.

Binding Assay

Parotid gland acinar membranes (0.50 mg/ml) were incubated in a final volume of 0.15 ml of Tris–HCl (buffer A), pH 7.4, for 90 min at 37°C with [³H]quinuclidinyl benzilate ([³H]QNB) (Dupont/New England Nuclear, Boston, MA) with a specific activity of 48 Ci/mmol (1 Ci = 37 GBq) with shaking. The reaction was stopped with ice-cold buffer A and filtered through Whatman glass fiber filters (GF/c) under suction. After a washing with 12 ml buffer A, filters were placed in vials, dried, and counted in an 8-ml scintillation cocktail (Triton–toluene) with about 40% efficiency. Non-specific binding (measured in the presence of 10 μM atropine) did not exceed 15%. For competition assays, membranes were incubated with IgA from groups I to IV in 50 mM phosphate buffer, pH 7.4, for 60 min at 30°C with shaking; then, 100 μl of the membrane solution (50 μg protein) was used for the binding assays with 0.5 nM [³H]QNB. For the saturation assay, membranes were incubated with different concentrations of [³H]QNB (0.1–1.5 nM). The equilibrium dissociation constant (Kₐ: nM) and the number of binding sites (Bₘax: fmol/mg protein) were taken from plots according to the methods of Scatchard (21), taking into account the total incubation volume (150 μl/tube) and the milligrams of tissue protein used (50 μg/tube).

Immunofluorescence Procedures

For direct immunofluorescence staining, samples were incubated for 45 min at room temperature with FITC-conjugated affinity-purified donkey anti-human IgA of 1:100 dilution (Jackson ImmunoResearch, West Grove, PA).

For indirect immunofluorescence staining, cryosections (8 μm) of human labial salivary glands were mounted on glass slides (Superfrosted/Plus, Fischer Scientific, Pittsburgh, PA), kept at room temperature for 30 min, fixed in cold acetone for 5 min and air dried for 10 min. Minor human salivary gland tissue was obtained from biopsies of the mucosa of the lower lip of patients from groups I and III.

With indirect immunofluorescence, pSS IgA were preincubated with or without synthetic M₃ peptide for 30 min at 30°C. The primary incubation was done for 45 min with pSS IgA, pSS IgA + synthetic M₃ peptide, normal subject IgA, or rabbit IgA anti-M₃ mAChR (R and D Systems, Berkeley, CA) at a 1:50 dilution for 45 min at room temperature. Samples were then washed with PBS and the second antibody, either FITC-conjugated affinity-purified donkey anti-human IgA at a 1:100 dilution for human IgA or FITC-conjugated affinity-purified donkey anti-rabbit IgG at a 1:50 dilution for rabbit IgG anti-M₃ mAChR (Jackson ImmunoResearch), for 45 min at room temperature.

In both immunofluorescence procedures (direct or indirect), after PBS washes the specimens were mounted with PBS–glycerol and observed with a Nikon photomicroscope equipped for epi-illumination.

Salivary Test

Parotid saliva was collected at least 2 h after a meal with a cup which was placed over the opening of Stensen’s duct, and the secretion from both glands was collected simultaneously. Also, parotid saliva was collected with gustatory stimulation provided by lemon juice under the tongue every 30 second for 10 min. Saliva was stored at −70°C until further processing.

Salivary lysozyme was performed with the commercially available kit Lysozyme Test Kits (Kallestad Diagnostics, Inc., Chaska, MN), and total salivary IgA was performed with commercial plates for radial immunodiffusion containing anti-IgA.

Drugs

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 sequence of the second extracellular loop of the human M₁ mAChR was synthesized by Peptido Genetic Research Company (Livermore, CA) as previously described (10). Carbachol, atropine, and 4-DAMP were provided by

<table>
<thead>
<tr>
<th>Serological test</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>12/16 (75%)</td>
<td>15/18 (83.3%)</td>
<td>1/21 (4.8%)</td>
<td>1/20 (5%)</td>
</tr>
<tr>
<td>Anti-Ro (SS-A)</td>
<td>7/16 (43.7%)</td>
<td>7/18 (38.9%)</td>
<td>0/21 (0%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>Anti-La (SS-B)</td>
<td>6/16 (37.5%)</td>
<td>7/18 (38.9%)</td>
<td>0/21 (0%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>RF</td>
<td>5/16 (31.6%)</td>
<td>15/18 (83.3%)</td>
<td>1/21 (4.8%)</td>
<td>1/20 (5%)</td>
</tr>
</tbody>
</table>

TABLE 1
Serological Tests Performed on Different Groups
Sigma. Stock solutions were freshly prepared in the corresponding buffer.

Statistical Analysis

ELISA optical density values from anti-M₃ peptide antibodies were distributed in four groups. Prevalence values between groups were compared by $H$ tests. All statistically significant values were justified at $P < 0.05$. Student's $t$ test for unpaired values was used to determine the level of significance. Differences between means were considered significant at $P < 0.05$.

RESULTS

Figure 1 shows the immunoreactivity of saliva from different groups against parotid gland membranes. One can see that the optical density values for saliva from both pSS (group I) and sSS (group II) patients were similar. Saliva from non-SS dry mouth patients (group III) showed optical density values that did not differ from those of the normal control (group IV).

To test the ability of antibodies from SS patients to interact with salivary gland membrane mAChR, radioligand competition assays were performed. Figure 2 shows that IgA from pSS or sSS patients but not from non-SS dry mouth patients or normal individuals inhibited specific binding of the cholinergic radioligand to rat salivary gland membranes in a concentration-depended manner. Saturation assay and Scatchard plots indicated that the inhibition was caused by a decrease in the number of binding site ($B_{max}$) with no significant changes in the equilibrium dissociation constant ($K_d$) (Table 2). Therefore, the SS IgA might bind irreversibly to those receptors.

With ELISA we determined the molecular interaction between IgA and human M₃ mAChR, testing whether the SS IgA could recognize the human M₃ mAChR synthetic peptide. In Fig. 3 one can see that both saliva and IgA from pSS and sSS patients but not from group III and IV patients gave a concentration-depended increase in optical density values when the M₃ mAChR peptide was used as a coating antigen. These values of optical density were always greater than 3 SD of those from normal individuals. The specificity of the reaction was assessed by the ability of the M₃ synthetic peptide (10-fold concentration) to inhibit the reaction when saliva or IgA was incubated with the synthetic peptide for 30 min at 37°C and then added together in the microtiter plates (histograms in Fig. 3).

The optical density values for each of the 75 subjects studied are shown in the scatterogram (Fig. 4). The immunoreactivity of saliva from pSS patients was similar to that of sSS patients. Furthermore, the immunoreactivity of saliva from pSS and sSS patients was significantly higher than that of saliva from group III and group IV patients ($P < 0.0005$). The optical density of saliva from groups I and II was always at least 3 SD from that of saliva from normal individuals.

The frequency of IgA antimembrane salivary gland and IgA anti M₃ mAChR autoantibodies in groups I and II was significantly higher than those observed with other groups (Table 3), which implies a strong

![FIG. 1. Immunoreactivity of antimembrane parotid gland antibodies of saliva from different groups: (●) 16 pSS patients (group I); (○) 18 sSS patients (group II); (▲) 21 RA postmenopausal without SS patients (group III); and (●) 20 normal subjects (control, group IV). Saliva (1/50 dilution) was assayed on sensitized microplates with 50 μg/ml parotid gland membranes. Values are means ± SD. *$P < 0.001$ vs group III or IV.]

![FIG. 2. Inhibition of [³H]QNB binding on rat parotid gland membranes by SS IgA. Increasing concentrations of saliva IgA from group I (●), II (○), III (▲), or IV (□) patients were incubated with membranes, and binding assays with 0.4 nM [³H]QNB were carried out. B, basal values without IgA. The results are the means ± SEM of six representative patients in each group. $P < 0.0001$ between group I or II and group III or IV. Control binding of 100% refers to the values of [³H]QNB specifically bound to parotid membranes without any reagent added.]}
association between the existence of secretory IgA anti-membrane and IgA anti-M₃ mAChR. Moreover, there was a high correlation between the patients' saliva IgA and serum IgG of different groups. It is important to note that no significant differences in patient ages among the different groups were demonstrated (data not shown) and that only women were selected for this study.

As already shown, the saliva IgA from SS patients is able to react with rat parotid membrane mAChR. Knowing that the amino acid sequences of rat and human M₃ synthetic peptides corresponding to the second extracellular loop of mAChR have a great homology (84%), we studied the muscarinic cholinergic receptor-mediated effect of IgA from SS patients on rat parotid gland. In order to evaluate whether the IgA binding with the same receptor domain could interfere with the effect of the cholinergic agonist, the action of carbachol on protein secretion in the presence of SS IgA was tested. Figure 5 shows that preincubation of SS IgA (1 × 10⁻⁷ M) for 30 min before the addition of carbachol (1 × 10⁻⁶ M) inhibited the incremental protein secretion triggered by the cholinergic agonist. This effect mimicked the action of the specific M₃ mAChR antagonist. Normal IgA had no effect.

Furthermore, salivary tests from different groups were performed and showed that basal and stimulated salivary flow were decreased, accompanied by decreased lysozyme and total protein in pSS and sSS patients compared with other groups (III and IV). However, there were no differences in the total salivary IgA concentrations (Table 4). These results suggest functional differences between secretory acinar cells and plasma cells.

Figure 6A shows the direct immunofluorescence staining of human biopsies of minor salivary glands taken from the mucosa of the lower lip of pSS patients. One can see deposits of IgA in the basal pole of the acinar membrane and in the interstice around infiltrated tissue. Labial salivary glands from group III patients had negative staining (Fig. 6B).

When an indirect immunofluorescence test was performed on human normal gland slices exposed to salivary IgA from pSS patients, the same deposit of IgA in
the basal pole of the acinar membrane was observed (Fig. 7A). This deposit of IgA was not observed either with saliva from pSS patients incubated with synthetic M₃ peptide prior to tissue addition (Fig. 7C) or using saliva from normal subjects (Fig. 7D). One also can see in Fig. 7B that mAChRs were localized on human labial salivary glands, preferentially on the basal pole of the acinar membrane, as was revealed using a commercial anti-M₃ mAChR antibody.

### DISCUSSION

We have demonstrated that subsets of patients with pSS or sSS produce functional salivary IgA autoantibodies that interact with the M₃ mAChRs and interfere with parasympathetic neurotransmitters to acinar cell membranes of salivary glands. Saliva and IgA from these patients were able to abolish protein secretion by the salivary gland in response to the authentic agonist carbachol. The high prevalence of these autoantibodies in pSS and/or sSS patients provides new evidence for a common pathogenic link between primary and secondary SS with regard to their sicca and autonomic nervous system dysfunction features.

The possibility that anti-mAChR IgG antibody may play a role in the pathogenesis of dry eye SS has been proposed (10). The presence of IgG autoantibodies against mAChR in the serum of pSS and/or sSS patients has provided new evidence for a common pathogenic link between primary and secondary SS with regard to their sicca and autonomic nervous system dysfunction features.

Thus, pSS and sSS IgA, similarly to monoclonal anti-human M₃ mAChRs, recognized the apical region of parotid acinar cell membranes. The specificity of interaction was assessed by inhibiting the IgA binding by the human M₃ synthetic peptide. Moreover, the indirect immunofluorescence study on human biopsies from pSS parotid gland patients gave similar patterns of IgA distribution, indicating that the in vitro situation could be applied to the in vivo situation.

The most important feature of this article relating to the autoimmune nature of SS is the presence of secre-
IgA in saliva that, acting on M₃ mAChR, resulted in a primary organ-specific dysfunction. Possibly, the IgA recognizing a synthetic peptide corresponding in amino acids sequence to the second extracellular loop of the human M₃ mAChR induces tissue damage by impairment of the neurotransmitter parasympathetic activity frequently observed in pSS/sSS patients.

Muscarinic receptors of the M₃ subtype are present in salivary glands, where they mediate the secretomotor effect of acetylcholine (22); also, M₃ regulates protein secretion, electrolytes, and water (23). The fact that SS IgA inhibited protein secretion induced by carbachol indicates the possibility that, in SS patients, the sicca syndrome is an autoimmune disorder produced by IgA fixation to acinar M₃ mAChRs.

We have also shown in this article an association between the existence of IgA anti-mAChR and the presence of xerostomia accompanied by a decrease in saliva, lysozyme, and protein secretions. All of these were dependent on acinar cells, while the concentration of IgA in saliva, depending on plasma cell activity, was not modified. Thus, these secretory IgA, together with a selective number of antibodies that are commonly detected in SS, make these IgA autoantibodies a valuable marker for dry mouth associated with both

**FIG. 6.** Direct immunofluorescence procedure of minor human salivary gland tissue obtained from a biopsy of pSS patients (A) or postmenopausal dry mouth patients without SS (B). Arrowheads show deposits of IgA in the basal poles of acinar membranes.

**FIG. 7.** Indirect immunofluorescence of normal minor human salivary gland tissue incubated with salivary IgA from pSS patients alone (A) or salivary IgA from pSS incubated with synthetic M₃ peptide (C). As a positive control, commercial anti-M₃ mAChR (B) and, as negative control, salivary normal human IgA (D) were employed. Arrowheads indicate positive staining in the basal pole of acinar membranes, revealed using salivary IgA from pSS patients (A) or commercial anti-M₃ mAChR antibody (B).

**TABLE 3**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
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<tbody>
<tr>
<td>IgA antimembrane</td>
<td>15/16 (93%)</td>
<td>17/18 (94%)</td>
<td>3/21 (14%)</td>
<td>2/20 (10%)</td>
</tr>
<tr>
<td>IgA antipeptide M₃ and mAChR</td>
<td>14/16 (87%)</td>
<td>15/18 (83%)</td>
<td>2/21 (9.5%)</td>
<td>1/20 (5%)</td>
</tr>
<tr>
<td>IgG antimembrane</td>
<td>14/16 (88%)</td>
<td>16/18 (88%)</td>
<td>4/21 (19%)</td>
<td>3/20 (15%)</td>
</tr>
<tr>
<td>IgG antipeptide M₃ mAChR</td>
<td>15/16 (99%)</td>
<td>16/18 (88%)</td>
<td>2/21 (9.5%)</td>
<td>2/20 (10%)</td>
</tr>
</tbody>
</table>

Note. Distribution of saliva IgA and serum IgG from the patients of different groups. The results were expressed as the number of positives per the total, with percentages in parentheses.

IgA AND SJO GREN SYNDROME
In addition, we have shown that serum IgG had a good correlation and high prevalence of saliva IgA anti-M₃ mAChR in pSS and sSS patients that was not observed in the other groups. In SS, sicca symptoms have been attributed to a T-lymphocyte-mediated infiltration and destruction of the lacrimal and salivary glands. However, it is well known that the severity of the symptoms correlates fairly well with the degree of glandular pathology (24). Another factor(s), such as anti-M₃ mAChR autoantibodies, could participate in the parasympathetic dysautonomia observed in SS (25). It is possible that anti-M₃ mAChR IgA chronically interacting with membrane M₃ receptor acinar cells could lead to a progressive blockade of these receptors, eliciting a loss of secretory response by altering water channel protein translocation of the apical membrane of acinar cells (26).

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