ARTHRITIS & RHEUMATISM
Vol. 56, No. 7, July 2007, pp 2216–2222
DOI 10.1002/art.22738

Proteomic Study of Salivary Peptides and Proteins in Patients With Sjögren's Syndrome Before and After Pilocarpine Treatment

G. Peluso,[1] M. De Santis,[1] R. Inzitari,[1] C. Fanali,[1] T. Cabras,[2] I. Messana,[2] M. Castagnola,[3] and G. F. Ferraccioli[1]

Objective. **To investigate the effect of pilocarpine on the salivary peptide and protein profile in patients with primary Sjögren's syndrome (SS) and to study the differences between patients with primary SS, patients with SS associated with other rheumatic diseases, and healthy control subjects.**

Methods. **Saliva specimens were obtained from 9 primary SS patients, 9 secondary SS patients, and 10 healthy controls. Samples were analyzed for levels of 62 different salivary proteins using high-performance liquid chromatography coupled with mass spectrometry using a spectrometer equipped with an electrospray ionization source. In 6 of the primary SS patients, saliva was collected at 30 minutes, 60 minutes, and 24 hours after taking 5 mg of pilocarpine.**

Results. **Before pilocarpine, ~60% of salivary proteins in samples from primary SS patients were not identifiable or showed lower levels than those in healthy controls. After 30–60 minutes following pilocarpine treatment, approximately one-third of the less represented proteins was found in a similar percentage of primary SS patients and controls. Almost all of the proteins that were detectable at lower levels in primary SS patients compared with controls reached levels similar to those in controls at 30–60 minutes after pilocarpine. The parotid gland proteins had the best response to pilocarpine. Primary SS patients were characterized by higher α-defensin 1 levels and by the presence of β-defensin 2. Secondary SS patients showed an intermediate protein profile between that of the primary SS patients and the controls.**

Conclusion. **Pilocarpine partially restored the levels and numbers of identifiable proteins in saliva from patients with primary SS. Higher levels of α-defensin 1 and the presence of β-defensin 2 in the saliva of patients with primary SS could be markers of oral inflammation in this patient group.**

Saliva is a complex fluid composed of a variety of electrolytes, metabolites, nucleotides, polynucleotides, and proteins. This fluid plays an important role in the maintenance of oral health. The rate of salivary protein secretion is controlled mainly by noradrenaline, which is released from the sympathetic terminals and acts through the β-adrenergic receptors. The rate of fluid and electrolyte secretion is controlled by acetylcholine, which is released from the parasympathetic terminals and acts through the muscarinic cholinergic receptors.

A large number of systemic agents have been proposed as secretagogues, but only a few have shown consistent salivary secretion–enhancing properties in well-designed trials. Among cholinergic agonists, studies in rats have shown that pilocarpine is the most effective for protein secretion (1) and has mild β-adrenergic stimulatory properties; however, few data in humans have been reported. Pilocarpine has been shown to improve symptoms of oral dryness and to increase salivary output in patients with primary Sjögren's syndrome (SS) (2), a chronic autoimmune disorder of the exocrine glands with associated lymphocytic infiltrates and consequent dryness of the mouth and eyes (3).

The composition of saliva in patients with primary SS has been found to be different from that in

[1]G. Peluso, MD, M. De Santis, MD, R. Inzitari, PhD, C. Fanali, PhD, G. F. Ferraccioli, MD: Catholic University, Rome, Italy; [2]T. Cabras, PhD, I. Messana, PhD: Cagliari University, Cagliari, Italy; [3]M. Castagnola, PhD: Catholic University, Rome, Italy, and Institute for the Chemistry of Molecular Recognition, CNR, Rome, Italy.

Address correspondence and reprint requests to Professor G. F. Ferraccioli, MD, Catholic University, Rheumatology Department, Via G. Moscati No. 31, 00168 Rome, Italy. E-mail: gf.ferraccioli@rm.unicatt.it.

Submitted for publication October 5, 2006; accepted in revised form April 3, 2007.

Table 1. Demographic and clinical characteristics of the SS patients and healthy control subjects*

	Primary SS patients (n = 9)	Secondary SS patients (n = 9)	Controls (n = 10)
Age, mean ± SD years	55.8 ± 13	48.5 ± 16.4	56.6 ± 12.5
Sex, no. female	9	9	10
Disease duration, mean ± SD years	7.9 ± 4.2	5.7 ± 5.1	–
No. with xerostomia	9	9	0
No. with keratoconjunctivitis sicca	9	9	0
No. with unstimulated whole salivary flow <1.5 ml in 15 minutes	9	9	0
No. with positive Schirmer's test result	9	9	0
No. with anti-SSA antibodies	9	7	0
No. with anti-SSB antibodies	6	4	0
No. with salivary gland focus score ≥1	6	–	–
Therapy (dosage)	AMs in 9 (4–6 mg/kg)	AMs in 3 with SLE (4–6 mg/kg); ilopr. in 3 with SSc (1 infusion/month); MTX in 3 with RA (15–20 mg/week)	–

* The 9 patients with secondary Sjögren's syndrome (SS) had either systemic lupus erythematosus (SLE; n = 3), systemic sclerosis (SSc; n = 3), or rheumatoid arthritis (RA; n = 3). AMs = antimalarial agents; ilopr. = iloprost; MTX = methotrexate.

normal subjects (4). However, the pattern of salivary gland proteins in primary SS patients has not yet been completely defined with regard to its composition, mainly in relation to low molecular weight components, such as acidic and basic proline-rich proteins (PRPs), statherins, histatins, and cystatins, as well as defensins, which are immunopeptides of epithelial and neutrophilic origin. There are no data concerning the effects of pilocarpine on the salivary protein profile in primary SS patients. Moreover, there are no studies on the possible differences in salivary protein profiles between patients with primary SS and patients with SS associated with other rheumatic diseases.

In the present study, we investigated differences in the salivary protein profiles of primary SS patients, secondary SS patients, and healthy control subjects. We also examined the effects of pilocarpine on the salivary peptide and protein profile in a subgroup of patients with primary SS.

PATIENTS AND METHODS

Patients and controls. Patients were enrolled at the Rheumatology Clinic, Catholic University of Rome, and the study was approved by the local Ethics Committee. After obtaining informed consent, saliva specimens were collected from 9 patients with primary SS, 9 patients with secondary SS (3 with systemic sclerosis [SSc], 3 with systemic lupus erythematosus [SLE], and 3 with rheumatoid arthritis [RA]), and 10 healthy age- and sex-matched subjects who had no signs or symptoms of SS and had negative findings on immunologic and serologic tests (Table 1). Saliva specimens were also collected from a subset of 6 of the primary SS patients at 30 minutes, 60 minutes, and 24 hours after a single oral dose of 5 mg of pilocarpine. All patients were diagnosed according to the revised international classification criteria for SS (3).

Collection and preparation of saliva samples. Whole saliva was collected with a very soft plastic aspirator between the hours of 2:00 PM and 4:00 PM in order to reduce variations in concentrations associated with circadian rhythms of secretion. Samples were collected at least 30 minutes after any food or beverage had been consumed and tooth brushing had been performed. A total of 0.5 ml of saliva was collected from each subject. Immediately after collection, samples were placed in an ice bath, and 0.5 ml of an acidic solution (0.2% trifluoroacetic acid [TFA]) was added at a 1:1 ratio (volume/volume). The solution was then centrifuged at 8,000*g* for 5 minutes at 4°C. The supernatant was removed, and the precipitate was discarded. The supernatant was immediately analyzed by high-performance liquid chromatography (HPLC) in conjunction with mass spectrometry (MS), using a spectrometer equipped with an electrospray ionization (ESI) source. HPLC-ESI-MS was performed within 30 minutes of collection of the saliva samples.

HPLC-ESI-MS analysis of salivary proteins. A total of 62 salivary proteins were analyzed. The HPLC-ESI-MS apparatus was a Thermo Finnigan Surveyor HPLC instrument (Thermo Finnigan, San Jose, CA) connected by a T splitter to a photo diode array detector and to an Xcalibur LCQ Deca-XP Plus mass spectrometer equipped with an ESI source. The chromatography column was a Vydac C-8 column (Vydac, Hesperia, CA) with a 5-μm particle diameter (column dimensions 150 mm in length × 2.1 mm inner dimension). The following solutions were used for reverse-phase chromatography: eluent A consisted of 0.056% (v/v) aqueous TFA, and eluent B consisted of 0.050% (v/v) TFA in acetonitrile/water 80/20 (v/v). The gradient applied was linear, from 0% to 55% of eluent B over 40 minutes, at a flow rate of 0.30 ml/minute. The T splitter addressed a flow rate of ~0.20 ml/minute toward the diode array detector and a flow rate of ~0.10 ml/minute toward the ESI source. The diode array detector was set at 2 wavelengths: 214 nm and 276 nm. Mass spectra were collected

Table 2. Levels and frequencies of 62 salivary proteins in healthy control subjects, patients with secondary SS, and patients with primary SS, as well as in a subset of primary SS patients before and after pilocarpine treatment*

			Primary SS patients			
	Controls (n = 10)	Secondary SS patients (n = 9)	Before pilocarpine (n = 9)	30 minutes after pilocarpine (n = 6)	60 minutes after pilocarpine (n = 6)	24 hours after pilocarpine (n = 6)
Basic PRPs						
P-F	16 ± 27 (10)	3.7 ± 6.3 (5)	2.4 ± 3.7 (4)†	8.4 ± 7.0 (4)‡	5.0 ± 3.5 (5)‡	4.4 ± 3.6 (5)‡
P-J	13 ± 20 (10)	4.9 ± 8.2 (5)	2.9 ± 4.2 (5)	11 ± 7.4 (5)§	6.7 ± 7.5 (5)§	4.9 ± 3.5 (5)
P-E	NA (1)	1.6 ± 3.4 (4)	NA (1)	5.8 ± 9.0 (2)	NA (1)	NA (1)
P-D	9.5 ± 16 (8)	13 ± 20 (8)	9.2 ± 13 (8)	27 ± 21 (6)§	25 ± 29 (6)§	13 ± 9.5 (6)
P-H	17 ± 25 (10)	5.6 ± 7.8 (7)	4.7 ± 5.6 (7)	14 ± 11 (5)	11 ± 11 (6)§	7.6 ± 5.7 (5)
IB-8a	12 ± 19 (6)	NA (0)†	NA (0)†	NA (0)	NA (0)	NA (0)
IB-6	NA (1)	4.3 ± 6.6 (5)†	NA (0)	3.8 ± 5.8 (2)	NA (1)	NA (1)
IB-1	29 ± 41 (10)	11 ± 17 (8)†	3.3 ± 5.3 (6)†	14 ± 14 (6)¶	12 ± 13 (6)	6.9 ± 5.5 (5)
II-2	67 ± 13 (10)	14 ± 22 (7)	5.8 ± 9.2 (7)†	33 ± 28 (6)¶	26 ± 19 (6)	14 ± 8.3 (6)
IB-1 DesR	1.6 ± 2.8 (7)	NA (1)†	0.9 ± 2.5 (2)†	2.1 ± 4.7 (2)	2.3 ± 4.9 (2)	0.1 ± 2.7 (1)
II-2 DesR	2.8 ± 4.1 (10)	3.5 ± 4.9 (5)	3.9 ± 4.1 (7)	2.3 ± 5.9 (5)	3.2 ± 6.3 (5)	3.1 ± 3.9 (5)
IB-7	0.07 ± 0.1 (2)	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)
Acidic PRPs						
PRP-1 3P	1.0 ± 1.3 (10)	3.0 ± 7.5 (5)	0.2 ± 0.5 (3)†	0.4 ± 0.6 (3)	0.3 ± 0.4 (2)	0.4 ± 0.5 (3)
PRP-1	29 ± 26 (10)	11 ± 16 (7)	1.7 ± 2.3 (6)†	3.5 ± 2.3 (5)¶	4.8 ± 2.8 (6)§	3.0 ± 3.5 (6)
PRP-1 1P	2.9 ± 2.1 (9)	2.8 ± 6.6 (7)	0.3 ± 0.4 (3)†	0.8 ± 0.8 (5)‡	0.9 ± 0.5 (6)‡	0.5 ± 0.7 (5)‡
PRP-3	17 ± 18 (10)	15 ± 16 (9)	1.5 ± 2.1 (6)†	4.6 ± 3.6 (5)§	5.9 ± 5.0 (6)¶	3.2 ± 3.1 (5)§
PRP-3 1P	1.8 ± 1.3 (10)	1.4 ± 1.5 (8)	0.3 ± 0.4 (4)†	0.6 ± 0.5 (5)‡	5.9 ± 12 (6)‡	0.5 ± 0.5 (4)‡
PRP-3 0P	0.06 ± 0.08 (5)	0.9 ± 1.5 (5)	0.4 ± 0.8 (3)	0.7 ± 0.9 (3)	0.8 ± 0.6 (5)§	0.5 ± 0.7 (3)
PC	32 ± 29 (10)	13 ± 17 (7)	7.8 ± 9.5 (9)†	26 ± 19 (6)¶	29 ± 22 (6)¶	15 ± 10 (6)
PC DesQ	0.4 ± 0.4 (9)	0.2 ± 0.3 (4)†	0.4 ± 0.5 (5)	0.8 ± 1.1 (4)	1.0 ± 1.4 (4)	0.3 ± 0.4 (3)
PC DesPQ	0.8 ± 0.9 (9)	NA (1)†	0.5 ± 1.1 (2)†	0.6 ± 1.0 (3)‡	NA (1)	0.5 ± 0.8 (2)
Statherin						
Statherin	45 ± 26 (10)	16 ± 23 (8)†	5.8 ± 13 (7)†	12 ± 13 (5)	13 ± 16 (5)¶	12 ± 22 (5)
Statherin 1P	0.9 ± 0.7 (10)	0.3 ± 0.5 (3)†	0.4 ± 0.5 (5)	0.3 ± 0.2 (4)	0.2 ± 0.3 (3)	0.2 ± 0.3 (2)
Statherin 0P	0.03 ± 0.06 (3)	0.3 ± 0.4 (5)	0.3 ± 0.3 (5)	0.09 ± 0.1 (3)	NA (1)	NA (1)
SV1	2.3 ± 2.3 (10)	2.8 ± 3.4 (8)	1.5 ± 2.2 (7)	2.3 ± 3.0 (6)	1.7 ± 1.3 (6)	2.9 ± 4.8 (6)
Statherin DesTF	1.2 ± 1.3 (10)	0.8 ± 1.1 (7)	0.6 ± 0.8 (6)	0.5 ± 0.7 (3)	2.6 ± 3.5 (4)	0.7 ± 0.8 (4)
Statherin DesD1	0.9 ± 0.8 (10)	0.3 ± 0.4 (6)	0.8 ± 0.2 (4)†	0.3 ± 0.3 (3)	0.5 ± 0.7 (3)	0.5 ± 0.7 (3)
SV2	0.4 ± 0.3 (9)	0.09 ± 0.2 (2)†	NA (0)†	NA (0)	NA (0)	NA (0)
SV3	NA (1)	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)
Statherin $Des^{1–9}$	1.4 ± 1.0 (10)	0.9 ± 1.5 (5)	0.2 ± 0.4 (3)†	0.6 ± 0.8 (3)	0.4 ± 0.3 (4)‡	0.5 ± 0.7 (3)
Statherin $Des^{1–10}$	0.4 ± 0.3 (10)	0.6 ± 0.7 (6)	0.3 ± 0.7 (3)†	0.6 ± 0.8 (3)	0.6 ± 0.8 (4)‡	0.6 ± 0.8 (3)
Statherin $Des^{1–13}$	0.3 ± 0.2 (10)	0.4 ± 0.4 (6)	0.3 ± 0.4 (5)	0.3 ± 0.4 (3)	0.5 ± 0.5 (4)‡	0.3 ± 0.4 (3)
P-B peptide	24 ± 15 (10)	18 ± 20 (8)	8.9 ± 11 (9)†	14 ± 12 (6)¶	9.6 ± 20 (6)	12 ± 16 (6)
P-B $Des^{1–5}$	0.8 ± 0.9 (9)	1.2 ± 1.8 (7)	2.4 ± 3.1 (7)	1.4 ± 1.8 (5)	3.2 ± 4.5 (5)	1.9 ± 3.4 (4)
P-B $Des^{1–4}$	NA (1)	0.6 ± 1.1 (3)†	0.9 ± 1.1 (6)†	0.8 ± 0.8 (5)	1.1 ± 1.7 (4)	0.8 ± 1.2 (4)
P-B $Des^{1–7}$	2.1 ± 1.4 (9)	3.7 ± 3.9 (8)	1.6 ± 2.3 (7)	3.5 ± 2.8 (6)	5.1 ± 4.1 (6)	3.5 ± 3.3 (5)
Cystatins						
Cystatin A	1.0 ± 1.2 (10)	1.7 ± 1.8 (9)	5.0 ± 7.3 (8)†	2.3 ± 2.9 (5)	1.7 ± 1.8 (4)	2.0 ± 2.4 (4)
Cystatin C	0.7 ± 0.4 (9)	NA (0)†	NA (0)†	NA (0)	NA (0)	NA (0)
Cystatin D	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)
Cystatin S	0.5 ± 0.4 (10)	NA (0)†	NA (0)†	NA (0)	NA (0)	NA (0)
Cystatin S1	5.9 ± 4.6 (10)	3.9 ± 6.1 (5)	1.9 ± 3.2 (4)†	3.4 ± 5.4 (3)	4.5 ± 3.0 (3)	4.8 ± 1.3 (3)
Cystatin S2	2.7 ± 2.5 (10)	NA (0)†	NA (1)†	NA (1)	4.6 ± 4.1 (2)	5.4 ± 3.2 (2)
Cystatin SA	2.7 ± 4.5 (10)	NA (0)†	NA (1)†	NA (1)	NA (1)	NA (1)
Cystatin SN	18 ± 21 (10)	NA (0)†	2.8 ± 4.0 (4)†	4.7 ± 6.4 (3)	5.3 ± 1.4 (3)	5.4 ± 3.0 (3)
Histatins						
Histatin 1	11 ± 26 (9)	4.6 ± 7.5 (8)	1.1 ± 2.0 (5)†	1.6 ± 1.6 (5)	2.2 ± 2.5 (5)	4.0 ± 8.3 (4)
Histatin 2	1.3 ± 1.3 (9)	0.5 ± 1.3 (3)†	NA (1)†	NA (1)	0.4 ± 0.6 (3)	0.4 ± 0.8 (2)
Histatin 3	4.1 ± 5.7 (7)	NA (1)†	NA (1)†	NA (1)	0.8 ± 1.3 (2)	2.4 ± 5.2 (2)
Histatin 4	0.3 ± 0.3 (10)	0.7 ± 2.0 (2)†	NA (0)†	NA (1)	NA (0)	NA (0)
Histatin 5	8.5 ± 8.6 (9)	2.8 ± 4.3 (7)	0.6 ± 0.9 (4)†	0.7 ± 1.1 (3)	1.5 ± 1.7 (3)	2.6 ± 4.3 (3)
Histatin 6	2.2 ± 2.3 (9)	1.0 ± 1.5 (5)	0.2 ± 0.2 (4)†	0.3 ± 0.4 (3)	0.3 ± 0.5 (3)	0.7 ± 1.6 (2)
Histatin 7	0.5 ± 0.6 (10)	NA (1)†	NA (0)†	NA (1)	NA (1)	NA (0)
Histatin 8	0.4 ± 0.2 (9)	0.3 ± 0.5 (5)	0.1 ± 0.1 (2)†	0.1 ± 0.1 (3)	0.1 ± 0.2 (3)	0.03 ± 0.1 (1)
Histatin 9	0.1 ± 0.2 (7)	NA (0)†	NA (0)†	NA (0)	NA (0)	NA (0)
Histatin 11	0.08 ± 0.1 (10)	NA (0)†	NA (0)†	NA (0)	NA (0)	NA (0)
Histatin 12	0.1 ± 0.1 (9)	NA (0)†	NA (0)†	NA (0)	NA (0)	NA (0)

Table 2. (*Cont'd*)

			Primary SS patients			
	Controls (n = 10)	Secondary SS patients (n = 9)	Before pilocarpine (n = 9)	30 minutes after pilocarpine (n = 6)	60 minutes after pilocarpine (n = 6)	24 hours after pilocarpine (n = 6)
Lysozyme	0.5 ± 0.3 (10)	NA (1)†	0.3 ± 0.6 (2)†	0.6 ± 0.6 (4)‡	0.6 ± 0.7 (3)	0.3 ± 0.4 (2)
Defensins						
α-defensin 1	1.6 ± 1.5 (9)	6.0 ± 9.0 (8)	13 ± 14 (9)†	11 ± 12 (6)	13 ± 12 (6)	10 ± 12 (6)
α-defensin 2	1.3 ± 1.3 (8)	4.8 ± 7.8 (8)	9.8 ± 9.3 (9)	7.5 ± 9.0 (6)	8.9 ± 9.3 (6)	7.5 ± 9.4 (6)
α-defensin 3	0.7 ± 0.9 (8)	4.6 ± 9.1 (8)	8.5 ± 9.4 (7)	5.6 ± 9.8 (5)	6.1 ± 9.3 (6)	6.0 ± 11 (6)
α-defensin 4	0.3 ± 0.4 (9)	1.4 ± 2.4 (7)	2.6 ± 3.4 (6)	2.0 ± 2.7 (4)	2.4 ± 2.7 (4)	1.4 ± 1.8 (4)
β-defensin 1	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)
β-defensin 2	NA (0)	0.1 ± 0.4 (3)	1.9 ± 4.6 (6)†	0.5 ± 0.7 (3)	0.4 ± 0.6 (2)	0.5 ± 0.5 (3)

* A single oral dose of 5 mg of pilocarpine was administered to 6 of the 9 patients with primary Sjögren's syndrome (SS), and protein levels were determined at 30 minutes, 60 minutes, and 24 hours thereafter. Values are the mean ± SD protein levels in the extracted ion current area ($\times 10^8$) (see Patients and Methods for details). Values in parentheses are the number of subjects in whom the protein was identified. NA = not applicable.
† $P < 0.05$ versus controls.
‡ Detection frequency significantly increased versus baseline (before pilocarpine).
§ $P < 0.05$ versus before pilocarpine.
¶ $P < 0.05$ versus before pilocarpine and $P > 0.05$ versus controls.

every 3 msec in the positive ion mode. The MS spray voltage was 4.50 kV, and the capillary temperature was 220°C. All common chemicals and reagents for the HPLC-MS analysis were of analytical grade and were purchased from Farmitalia Carlo Erba (Milan, Italy), Merck (Darmstadt, Germany), and Sigma-Aldrich (St. Louis, MO).

Deconvolution of the average ESI mass spectra was automatically performed by the software provided with the Deca-XP instrument (Bioworks Browser) or by MagTran 1.0 software (5). Experimental mass values obtained from the analysis were compared with average theoretical values available from the Swiss-Prot (available at http://www.expasy.org/tools) and EMBL (available at http://www.embl-heidelberg.de) databases.

The relative abundance of the different salivary proteins was approximated by performing a multiple extracted ion current (XIC) strategy for any protein. The XIC procedure for each protein was based on the extraction from the total ion current (TIC) profile to 3 mass/charge (m/z) values selected from among the most relevant, provided that these did not overlap with the m/z values of nearly eluting proteins. For peptides with a mass in the detection range of the ESI-MS apparatus (i.e., 300–2,000), the monoisotopic m/z values of the monocharged ion, the bicharged ion, or both ions was chosen for the XIC procedure. Taking into account that constant analytical conditions were used for each sample, the numerical value corresponding to the integrated peak area of the XIC strategy was used for the statistical analyses and for rough estimations of the relative abundance of peptides/proteins.

Statistical analysis. Statistical analysis was performed with SPSS software version 13.0 (SPSS, Chicago, IL). Categorical and quantitative variables were respectively described as numbers and percentages, as well as the mean ± SD. The Mann-Whitney U test and Wilcoxon's matched pairs signed rank test were used to compare continuous variables. Categorical variables were analyzed by chi-square test or Fisher's exact test, depending on sample size restrictions.

RESULTS

Frequencies and levels of salivary gland proteins in primary SS and secondary SS patients versus healthy controls. In the 9 patients with primary SS, 28 of the 62 salivary proteins analyzed (45.2%) were identifiable in a significantly lower percentage of primary SS patients than in the 10 healthy controls, 8 (12.9%; IB-1, II-2, PRP-1, PRP-3, PC, statherin, P-B peptide, and histatin 1) showed significantly lower levels than in the controls, 2 (P-B Des^{1-4} and β-defensin 2) were identifiable in a significantly higher percentage of primary SS patients than in the controls, and 2 (cystatin A and α-defensin 1) showed significantly higher levels than in the controls.

In the 9 patients with secondary SS, 18 of the 62 salivary proteins analyzed (29%) were identifiable in a significantly lower percentage of secondary SS patients than in the controls, 2 (IB-1 and statherin) showed significantly lower levels than in the controls, and 3 (IB-6, P-B Des^{1-4}, and β-defensin 2) were identifiable in a significantly higher percentage of secondary SS patients than in the controls.

In particular, salivary cystatins (C, S, S2, SA, and SN) and histatins (2, 3, 4, 7, 9, 11, and 12) were less frequently identifiable in primary and secondary SS patients versus controls. Moreover, β-defensin 1 was not identifiable in any of the patients or the controls, whereas β-defensin 2 was found in 6 of the 9 primary SS patients (66.7%), in 3 of the 9 secondary SS patients (33.3%), and in none of the controls (Table 2).

Based on these data, 36 of the 62 proteins

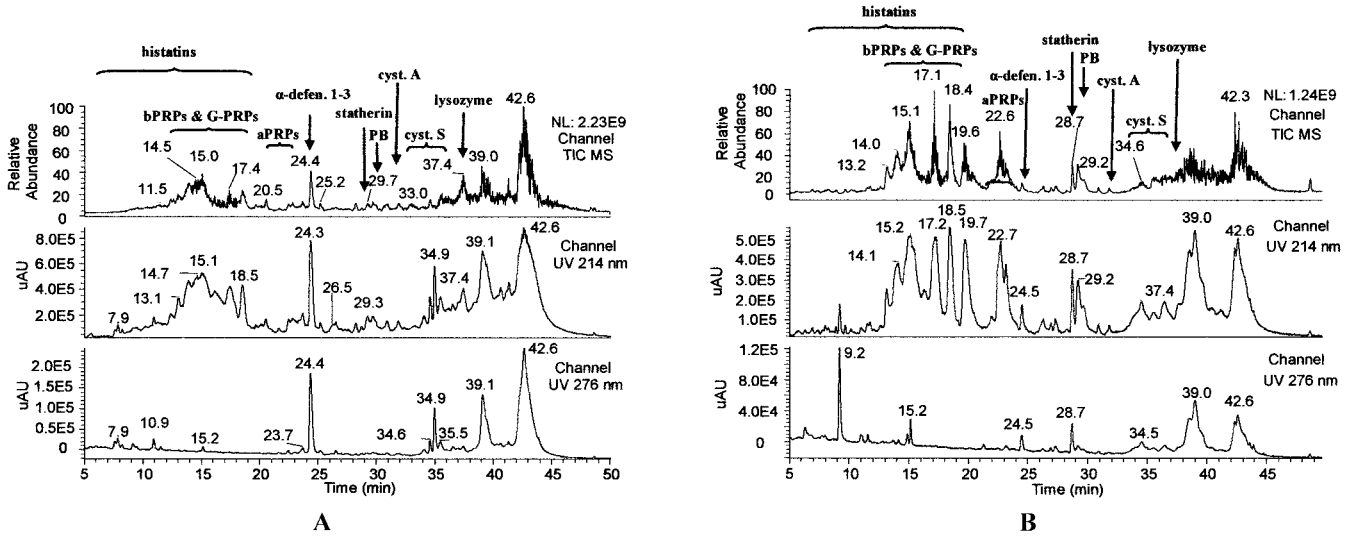

Figure 1. Profile of salivary proteins in a patient with primary Sjögren's syndrome, determined before **(A)** and after **(B)** a single oral dose of 5 mg of pilocarpine. Salivary proteins were analyzed by high-performance liquid chromatography in conjunction with mass spectrometry, using a spectrometer equipped with an electrospray ionization source. The relative abundance of proteins and the ultraviolet absorbance units (uAU) at 214 nm and 276 nm were determined. bPRPs = basic proline-rich proteins; G-PRPs = glycosylated PRPs; aPRPs = acidic PRPs; α-defen. = α-defensin; PB = statherin P-B peptide; cyst. A = cystatin A; NL = normal; E = exponential; TIC = total ion current; MS = mass spectrometry.

analyzed (58%) were either not identifiable or showed lower levels in primary SS patients than in the controls.

Protein profile after taking pilocarpine. Six of the 9 primary SS patients were treated with a single oral dose of 5 mg of pilocarpine, and salivary proteins were evaluated before and after treatment (Figure 1). Eight of the 62 proteins analyzed (12.9%) were found in a significantly higher percentage of patients after pilocarpine: 5 after 30 minutes, 6 after 60 minutes, and 3 after 24 hours, and they were found at a similar frequency as in the control group (Table 2). Moreover, the levels of 11 proteins (17.7%) increased significantly after pilocarpine as compared with basal specimens: 8 at 30 minutes after pilocarpine and 8 at 60 minutes after. Seven of the 8 proteins (87.5%) that were significantly less abundant at baseline as compared with controls reached levels not statistically different from the controls (Table 2).

The salivary cystatin and histatin protein classes were modified less by pilocarpine treatment as compared with the other classes. In fact, both the number of patients in which cystatins and histatins were detectable and the levels of cystatins and histatins were similar before and after pilocarpine.

The basic and acidic PRPs and the statherins showed the best response to pilocarpine treatment. One of the basic PRPs (P-F), 3 of the acidic PRPs (PRP-1 1P, PRP-3 1P, and PC DesPQ), and 3 of the statherin fragments (statherins $Des^{1–9}$, $Des^{1–10}$, and $Des^{1–13}$) were found in a higher number of the primary SS patients at 30 and 60 minutes after pilocarpine (Table 2). Moreover, 5 proteins of the basic PRP group (P-J, P-D, P-H, IB-1, and II-2), 4 of the acidic PRP group (PRP-1, PRP-3, PRP-3 0P, and PC), and 2 of the statherin group (statherin and P-B peptide) showed significantly increased levels at 30 minutes and 60 minutes as compared with basal specimens. PRP-3 levels were also increased at 24 hours after pilocarpine (Table 2).

Thus, the secretion of 8 species among the 28 proteins that were less frequently identifiable in basal saliva specimens from patients with primary SS (28.6%) seems to be stimulated by pilocarpine. Moreover, pilocarpine was shown to be able to restore protein levels to nearly normal for almost all of the proteins that were less abundant in the basal saliva specimens.

DISCUSSION

Recent studies suggest that sicca syndrome in patients with primary SS is due to functional inhibition of autonomic neurotransmission to lacrimal and salivary glands, rather than to infiltration and destruction of the glands by lymphocytes, because there is a poor correlation between the degree of glandular destruction or the focus score and the degree of dysfunction (4). Moreover, antimuscarinic receptor antibodies have been demon-

strated in the serum of patients with primary SS. These autoantibodies can inhibit parasympathetic neurotransmission (6). Since pilocarpine has been shown to increase the secretion of salivary gland fluid, we examined protein levels before and after pilocarpine in a subset of patients with primary SS. We found that pilocarpine can also increase the amount and the number of salivary proteins detectable in primary SS patients.

About 60% of salivary proteins analyzed in primary SS patients were not identifiable or showed lower levels than in healthy controls. About one-third of the proteins that were less represented in primary SS patients were identified in a similar percentage of patients and controls at 30 minutes and 60 minutes after pilocarpine. Almost all of the proteins present at lower levels in primary SS patients than in healthy controls before pilocarpine treatment reached levels comparable to those in the controls, most of them between 30 and 60 minutes.

These data suggest that pilocarpine could partially overcome the salivary gland dysfunction in SS. A recent study showed that long-term stimulation of membrane-bound type 3 muscarinic acetylcholine receptor (M3R) by circulating anti-M3R autoantibodies or pilocarpine exposure results in receptor desensitization (7). Further studies will be necessary to confirm our hypothesis concerning pilocarpine and to study the effects of long-term treatment with this agent.

The basic and acidic PRP groups and the statherin group showed the best response to pilocarpine. The salivary cystatin and histatin groups were the less represented classes in primary SS and secondary SS patients and showed the worst response to pilocarpine. Since salivary cystatins and histatins are chiefly of submandibular/sublingual origin, these data suggest that the submandibular and sublingual glands may be less susceptible to the effects of pilocarpine than are the parotid glands. Although the role of acidic PRPs and statherins is not completely understood, it seems to be related to oral calcium metabolism and to oral bacterial flora. Basic PRPs, together with acidic PRPs and statherins, seem to be involved in the formation of the protein net that interacts with the buccal epithelium and with dental enamel. Indeed, the higher incidence of caries and periodontal diseases in patients with primary SS could be due to the reduction in these protein classes and may benefit from therapies that can restore the normal protein profile, as pilocarpine has been shown to partially do.

We found a higher level of defensins in saliva from patients with primary SS. Defensins are a family of small (3,000–4,000 daltons) cationic proteins that are involved not only in innate immunity against infectious microbes, but also in adaptive immunity, inflammation, and wound repair (8). Alpha-defensins 1–4 are present in azurophil granules of neutrophils (8). HPLC and MS have shown the presence of α-defensins 1–3 in saliva from normal subjects; moreover, our group of investigators has recently found low levels of α-defensin 4 in gingival crevicular fluid from normal subjects (9). This study is the first to show the presence of α-defensins 1–4 in the saliva of patients with primary and secondary SS, with a significantly higher amount of α-defensin 1 in patients with primary SS. The increased amount of α-defensin 1, together with cystatin A, another neutrophilic protein, in primary SS patients as compared with healthy subjects could be the result of the periodontal diseases that are common in patients with primary SS, thus suggesting a possible role of α-defensin 1 as a marker of oral inflammation in these patients.

Beta-defensins 1 and 2 are mainly expressed by keratinocytes (10): β-defensin 1 is constitutively and heterogeneously expressed in epithelial cells and is only poorly induced by cytokines, whereas β-defensin 2 expression can be induced by a local cytokine imbalance in which there is a predominance of Th1 cytokines, such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 (11), as in oral inflammatory diseases or in the presence of infectious agents such as lipopolysaccharide. However, significant individual heterogeneity in β-defensin gene expression has been reported, resulting from genetic polymorphisms, the number of copies of defensin genes and the multiple regulatory pathways and mediators involved in their expression, the different grades of tissue atrophy, or replacement of the innate or natural defense system by mechanisms of the acquired immune response in chronically inflamed tissues. In fact, some studies have suggested that there is increased expression of β-defensins 2 and 3 in inflamed tissues (12), whereas others have shown decreased expression in inflamed oral tissue samples (13). Kawasaki et al (14) reported a significant up-regulation of the β-defensin 2 gene, but not other β-defensin genes, in conjunctival epithelial cells from patients with primary SS. We found that β-defensin 2 was identifiable in the majority of patients with primary SS (66.7%), but in none of the healthy subjects, thus suggesting that β-defensin 2 can be considered a marker of inflammation in primary SS, perhaps reflecting high levels of cytokines, such as IL-1β, IL-6, and TNFα (15,16).

In conclusion, patients with primary SS are characterized by a different number of identifiable proteins

and different levels of salivary gland proteins as compared with healthy subjects. Pilocarpine treatment restored the protein levels and partially restored the protein numbers that were found to be decreased in primary SS patients, with the parotid gland proteins showing the best response to the drug. High levels of α-defensin 1 and the presence of β-defensin 2 in the saliva of patients with primary SS may represent markers of inflammation in SS. Patients with secondary SS showed a protein profile that was intermediate between that of the primary SS patients and the healthy subjects. Taken together, these data should be of help when considering the possible analysis of secretagogue function and the composition of saliva substitutes.

AUTHOR CONTRIBUTIONS

Dr. Ferraccioli had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Peluso, De Santis, Inzitari, Messana, Castagnola, Ferraccioli.

Acquisition of data. Peluso, De Santis, Inzitari, Fanali, Cabras.

Analysis and interpretation of data. Peluso, De Santis, Inzitari.

Manuscript preparation. Peluso, De Santis, Inzitari.

Statistical analysis. Peluso, De Santis, Inzitari.

REFERENCES

1. Baba A, Taniguchi K, Motokawa W, Abe K. Fluid and protein secretion by the submandibular glands of weanling rats in response to various agonists. Arch Oral Biol 1994;39:979–84.
2. Fox PC. Salivary enhancement therapies. Caries Res 2004;38: 241–6.
3. Fox RI. Sjögren's syndrome. Lancet 2005;366:321–31.
4. Ryu OH, Atkinson JC, Hoehn GT, Illei GG, Hart TC. Identification of parotid salivary biomarkers in Sjögren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. Rheumatology (Oxford) 2006;45:1077–86.
5. Zhang Z, Marshall AG. A universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. J Am Soc Mass Spectrom 1998;9:225–33.
6. Waterman SA, Gordon TP, Rischmueller M. Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. Arthritis Rheum 2000;43: 1647–54.
7. Cha S, Singson E, Cornelius J, Yagna JP, Knot HJ, Peck AB. Muscarinic acetylcholine type-3 receptor desensitization due to chronic exposure to Sjögren's syndrome-associated autoantibodies. J Rheumatol 2006;33:296–306.
8. Chen H, Xu Z, Peng L, Fang X, Yin X, Xu N, et al. Recent advances in the research and development of human defensins. Peptides 2006;27:931–40.
9. Pisano E, Cabras T, Montaldo C, Piras V, Inzitari R, Olmi C, et al. Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. Eur J Oral Sci 2005;113:462–8.
10. Dunsche A, Acil Y, Siebert R, Harder J, Schroder JM, Jepsen S. Expression profile of human defensins and antimicrobial proteins in oral tissue. J Oral Pathol Med 2001;30:154–8.
11. Joly S, Organ CC, Johnson GK, McCray PB, Guthmiller JM. Correlation between β-defensin expression and induction profiles in gingival keratinocytes. Mol Immunol 2005;42:1073–84.
12. Liu L, Wang L, Jia HP, Zhao C, Heng HH, Schutte BC, et al. Structure and mapping of the human β-defensin HBD-2 gene and its expression at sites of inflammation. Gene 1998;222:237–44.
13. Dunsche A, Acil Y, Dommisch H, Siebert R, Schroder JM, Jepsen S. The novel human β-defensin-3 is widely expressed in oral tissues. Eur J Oral Sci 2002;110:121–4.
14. Kawasaki S, Kawamoto S, Yokoi N, Connon C, Minesaki Y, Kinoshita S, et al. Up-regulated gene expression in the conjunctival epithelium of patients with Sjögren's syndrome. Exp Eye Res 2003;77:17–26.
15. Fox RI, Kang HI, Ando D, Abrams J, Pisa E. Cytokine mRNA expression in salivary gland biopsies of Sjögren's syndrome. J Immunol 1994;152:5532–9.
16. Tishler M, Yaron I, Shirazi I, Yossipov Y, Yaron M. Increased salivary interleukin-6 levels in patients with primary Sjögren's syndrome. Rheumatol Int 1999;18:125–7.