Defective signalling in salivary glands precedes the autoimmune response in the non-obese diabetic mouse model of sialadenitis

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Summary
The spontaneous non-obese diabetic (NOD) mouse model of Sjögren’s syndrome provides a valuable tool to study the onset and progression of both the autoimmune response and secretory dysfunction. Our purpose was to analyse the temporal decline of salivary secretion in NOD mice in relation to the autoimmune response and alterations in various signalling pathways involved in saliva secretion within each salivary gland. A progressive loss of nitric oxide synthase activity in submandibular and parotid glands started at 12 weeks of age and paralleled the decline in salivary secretion. This defect was associated with a lower response to vasoactive intestinal peptide in salivary flow rate, cAMP and nitric oxide/cGMP production. No signs of mononuclear infiltrates or local cytokine production were detectable in salivary glands in the time period studied (10–16 weeks of age). Our data support a disease model for sialadenitis in NOD mice in which the early stages are characterized by defective neurotransmitter-mediated signalling in major salivary glands that precedes the autoimmune response.

Keywords: autoimmune response, nitric oxide signalling, NOD mice, sialadenitis, Sjögren’s syndrome

Introduction
Sjögren’s syndrome (SS) is a chronic autoimmune disorder of unknown aetiology characterized by severe dryness of the mouth and the eyes [1–3]. The mild infiltration of salivary glands correlates poorly with the severe loss of secretory function, suggesting that neural rather than immune mechanisms have a role in the pathogenesis of this disease [4]. Aberrant activation of glandular epithelial cells has been proposed in the induction and perpetuation of the inflammatory response [3]. The spontaneous non-obese diabetic (NOD) mouse model of SS provides a valuable tool to study the onset and progression of both the autoimmune response and secretory dysfunction which are almost impossible to monitor in humans. As reported in patients, there is a poor correlation between moderate sialadenitis and reduction in saliva secretion in NOD mice [5]. It has been suggested that the initial trigger of autoimmune sialadenitis of NOD mice may reside in a defect in salivary gland homeostasis and the autoimmune response would promote further glandular damage [6]. In line with this, we described a loss of nitric oxide synthase (NOS) activity in salivary glands of NOD mice developing SS-like symptoms [7,8]. This signalling alteration reflected differential regulation of the neural isoform of the NOS by calcium calmodulin kinase II [9]. Similarly, evidence of altered expression of metalloproteases [10] and autonomic receptors [11] in salivary glands of NOD mice has been provided. Based on these observations, we hypothesized that early modifications in cellular signalling might precede the autoimmune response against the salivary glands. Here we present evidence to indicate that altered neurotransmitter-mediated response and signalling in salivary glands of NOD mice underlie the failure of salivary secretion and precede the autoimmune response against the glands.

Materials and methods
Animals
NOD and BALB/c female mice were bred and maintained in the Central Animal Care facility at the School of Exact and Natural Sciences, University of Buenos Aires. Mice aged 10, 12, 14, 16 and 20 weeks were fasted overnight with water ad libitum before being used. They were tested routinely for blood glucose levels (Wiener Laboratory, Rosario, Argen-
tina) and considered pre-diabetic as their values of serum glucose on two occasions over a 24-h period did not significantly differ from those of control mice (1.0 ± 0.1 g/l, n = 23). All studies were conducted according to standard protocols of the Animal Care and Use Committee of the School of Exact and Natural Sciences, University of Buenos Aires.

Salivary flow rate measurement

As mice do not have basal spontaneous secretion of saliva, it was evaluated after stimulation with the muscarinic agonist pilocarpine (Sigma Chemical Co, MO, USA) (50 µg pilocarpine/100 g weight) injected intraperitoneally. The effect of vasoactive intestinal peptide (VIP) was evaluated after injection of pilocarpine plus VIP (Sigma Chemical Co., St Louis, MO, USA) (10 µg VIP/100 g weight). Animals were handled firmly after injection and saliva accumulated in the oral cavity was driven to microtubes on ice by means of a micropipette for 12 min. The flow rate was calculated as the volume of saliva collected in microlitres per minute and per 100 g of body weight [7,8].

Nitric oxide synthase activity and cyclic nucleotide determination

Nitric oxide synthase (NOS) activity was measured in submandibular and parotid glands using L-[U-14C]-arginine as substrate as described previously [9,12]. Whole glands were incubated with 0.2 µCi L-[U-14C]-arginine (Amersham Biosciences, Buckinghamshire, UK, 300 mCi/mmol) in Krebs–Ringer bicarbonate (KRB) solution pH 7.4 gassed with 5% CO2 in O2 at 37°C for 30 min. Tissues were homogenized and 4°C-citrulline was separated on a Dowex AG 50 W-X8 resin (Bio-Rad). NOS activity was calculated as total activity minus that measured in the presence of 500 µM L-Nω-monomethyl arginine (LNMMA) (Sigma).

Intracellular adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) accumulation was determined in parotid and submandibular glands by radioimmunoassay [8,9]. The anti cAMP antibodies was kindly provided by Dr A. F. Parlow from the National Hormone and Pituitary Program (USA) and anti-cGMP from Chemicon Int. [125I]-cAMP and [125I]-cGMP (>2200 Ci/mmol) were labelled by Dr Omar Pignataro (IBYME, Buenos Aires, Argentina). Samples were prepared by incubating whole glands for 30 min at 37°C in KRB with 100 µM 3-isobutyl-1-methyl xanthine. When used, VIP was added during the last 15 min.

Histological studies and detection of serum autoantibodies

In most experiments, each mouse was used both for cytokine measurements and histological studies in submandibular or parotid glands by fixing one gland in 4% paraformaldehyde overnight at 4°C and homogenizing the contralateral gland for cytokine determination. Parotid or submandibular glands from each mouse were whole-fixed and embedded in paraffin wax and at least six sections of 3–5 µm were placed on siliconized glass slides and stained with haematoxylin–eosin [12]. Slices were observed at 250× and the number of ducts quantified in a survey of 20 fields for each slice. Immunohistochemistry was performed on similarly obtained sections of each gland with antimouse CD3 antibody (BD) and revealed as described previously [9]. The presence of circulating autoantibodies against glandular structures was evaluated by immunoblotting with the sera from NOD mice (1/100 dilutions) incubated with proteins extracted from salivary glands from BALB/c or NOD mice of the ages indicated that were fractionated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and revealed with biotinylated antimouse IgG and streptavidin–horseradish peroxidase (HRP) (Dako, Carpinteria, CA, USA) as described previously [9,13].

Cytokine measurements

As stated above, the other freshly isolated gland from each animal was homogenized at 4°C in 50 mM Tris-HCl buffer pH 7.5 with protease inhibitors [7]. After centrifugation at 10 000 g 10 min at 4°C, supernatants were frozen at –80°C until used for cytokine and protein determination. Cytokines were determined with a capture enzyme-linked immunoabsorbent assay (ELISA) assay as described previously [14]. Briefly, microtitre plates (Corning Inc., New York, USA) were coated with a capture monoclonal anti-mouse interleukin (IL)-10, tumour necrosis factor (TNF)-α, IL-12 or interferon (IFN)-γ antibody (Pharmingen, San Diego, CA, USA) at 2 µg/ml at 4°C. After washing and blocking with phosphate-buffered saline containing 3% bovine serum albumin, tissue samples or sera were added for 12 h. Unbound material was washed off and biotinylated monoclonal anti-IL-10, TNF-α, IL-12 or IFN-γ antibodies (Pharmingen) were added at 2 µg/ml for 45 min and revealed with avidin–peroxidase and azino-bis(3ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate solution (Sigma). The intra- and interassay variability for cytokine determination was <5%. The detection limits were 15 pg/ml for IL-10 and TNF-α and 30 pg/ml for IL-12 and IFN-γ; the values were expressed in pg/mg protein for glandular extracts and pg/ml for sera.

Statistical analysis

Statistical significance of differences was determined by the two-tailed t-test for independent populations. When multiple comparisons were necessary, the Student–Newman–Keuls test was used after analysis of variance. Differences between groups were considered significant at P < 0.05.
Progression of secretory and signalling dysfunction in NOD salivary glands

NOD mice showed a progressive decrease of saliva flow rate that was maximal from 14 weeks on (Table 1). VIP stimulates and in some conditions even potentiates muscarinic-mediated pathways for salivary secretion in normal mice, thus we investigated whether VIP-stimulated secretion was also progressively lost in NOD mice. As can be seen in Table 1, VIP stimulated salivary secretion over pilocarpine only in 10-week-old NOD mice and the effect of VIP decreased thereafter. The values of salivary flow rate obtained in 10-week-old NOD mice were similar to those obtained in BALB/c normal mice of 10–16 weeks (Table 1).

Nitric oxide participates directly in salivary secretion and through cGMP signalling pathways activated by VIP [15,16]. As shown in Fig. 1a, the progressive decrease of basal nitric oxide synthase (NOS) activity in both salivary glands from NOD mice is even more pronounced in submandibular glands, where at 14 weeks the activity is almost lost. In accordance with the effect in salivary flow rate, VIP also failed to stimulate NOS activity in NOD mice of 14 and 16 weeks (Fig. 1b, upper panel) compared to the control 10-week-old NOD mice. Accordingly, Fig. 1b (lower panel) shows the lack of response to VIP in cGMP assays in both glands of 14- and 16-week-old NOD mice. The values of NOS activity and cGMP of 10-week-old NOD mice presented here were similar to those of normal BALB/c mice aged 10–16 weeks (Fig. 1).

In addition to the l-arginine/NO/cGMP pathway, VIP also signals through cAMP in a NO-independent manner in

### Results

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### Table 1. Time-course of the salivary flow rate decrease in non-obese diabetic (NOD) mice.

<table>
<thead>
<tr>
<th>NOD mouse age</th>
<th>Pilocarpine</th>
<th>Pilocarpine + VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 weeks</td>
<td>69 ± 1</td>
<td>76 ± 9</td>
</tr>
<tr>
<td>12 weeks</td>
<td>57 ± 6</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>14 weeks</td>
<td>42 ± 4*</td>
<td>23 ± 5*</td>
</tr>
<tr>
<td>16 weeks</td>
<td>45 ± 3*</td>
<td>26 ± 5*</td>
</tr>
<tr>
<td>20 weeks</td>
<td>44 ± 4*</td>
<td>42 ± 4*</td>
</tr>
<tr>
<td>BALB/c mice</td>
<td>62 ± 7</td>
<td>89 ± 8</td>
</tr>
</tbody>
</table>

Total saliva was collected from the oral cavity of NOD or BALB/c mice (16 weeks of age) after stimulation of secretion with either the muscarinic agonist pilocarpine (50 µg pilocarpine/100 g weight) injected intraperitoneally or pilocarpine plus vascular endothelial peptide (VIP) (10 µg VIP/100 g weight). By means of a micropipette, saliva accumulated during the following 12 min was collected and flow rate was calculated as the volume of saliva in microlitres per minute and per 100 g of body weight. Values are mean ± s.e. of at least five separate experiments.* P < 0.05 versus the value of the same treatment of NOD mice at 10 weeks of age.

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**Fig. 1.** Progressive decrease of nitric oxide synthase and vascular endothelial peptide (VIP)-activated signalling through NO/cGMP in non-obese diabetic (NOD) mice glands. (a) Basal NOS activity was determined in submandibular and parotid glands of NOD mice at different ages from 10 to 20 weeks and glands of BALB/c mice of the ages indicated (insert) as described in Materials and methods. Values represent the mean ± s.e. of at least six different glands. * P < 0.05 versus basal of the corresponding gland from 10-week-old NOD mice. (b) The effect of VIP on NOS activity (10 nM VIP, upper panel) and cGMP accumulation (100 nM VIP, lower panel) was assessed in submandibular or parotid glands of 10-, 14- and 16-week-old NOD mice and BALB/c mice of 16 weeks (insert). Each value represents the mean ± s.e. of at least four determinations. * P < 0.05 versus basal of the corresponding gland at the same age.
Table 2. Effect of vascular endothelial peptide (VIP) on cAMP accumulation in non-obese diabetic (NOD) salivary glands.

<table>
<thead>
<tr>
<th>NOD mice age</th>
<th>Submandibular glands</th>
<th>Parotid glands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP (fmol/mg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basal VIP</td>
<td>Basal VIP</td>
</tr>
<tr>
<td>10 weeks</td>
<td>171 ± 33</td>
<td>274 ± 19*</td>
</tr>
<tr>
<td></td>
<td>211 ± 3</td>
<td>442 ± 22*</td>
</tr>
<tr>
<td>14 weeks</td>
<td>238 ± 32</td>
<td>228 ± 21</td>
</tr>
<tr>
<td></td>
<td>202 ± 37</td>
<td>296 ± 21</td>
</tr>
<tr>
<td>16 weeks</td>
<td>222 ± 16</td>
<td>207 ± 34</td>
</tr>
<tr>
<td></td>
<td>240 ± 99</td>
<td>288 ± 55</td>
</tr>
<tr>
<td>BALB/c mice</td>
<td>233 ± 19</td>
<td>499 ± 69*</td>
</tr>
<tr>
<td></td>
<td>179 ± 19</td>
<td>528 ± 83*</td>
</tr>
</tbody>
</table>

Samples from NOD mice of the ages indicated and BALB/c mice (16 weeks of age) were prepared by incubating whole glands for 30 min in Krebs–Ringer bicarbonate (KRB), gassed with 5% CO_2 in O_2 and vascular endothelial peptide (VIP) (100 nM) was added during the last 15 min. Once homogenized in ethanol and dried, residues were dissolved in sodium-acetate buffer for subsequent cyclic nucleotides measurement by radioimmunoassay (RIA). Values are mean ± s.e. of at least six separate determinations. *P < 0.05 versus basal value of the corresponding gland at the same age.

Submandibular and parotid glands showed a constant ductal to acinar cell ratio along the age period studied (Fig. 2). In the case of NOD submandibular glands it was higher compared to age-matched normal BALB/c glands at 10 weeks (ducts 18 ± 1) (Fig. 2). This increased ducts/acini ratio in NOD submandibular glands is common in ageing normal mice, but it appears to occur from early ages in this strain. Figure 2, upper panel, shows that despite the higher number of ducts in submandibular glands of 10- and 14-week-old mice, their morphology was not altered. At 16 weeks we could observe that some acini showed a mild vacuolization, duct morphology was also becoming slightly altered with a loss of homogeneity in the cytoplasm and a few picnotic nuclei probably corresponding to morphological apoptotic images were found (Fig. 2, upper panel). Immune cell infiltrates were absent in the eight mice aged 10 weeks taken as the control group as assessed by histological studies and immunostaining for CD3. We observed a few isolated and small foci of CD3 positive mononuclear cells in two of eight mice of 14- and 16-week-old NOD mice, respectively. However, BALB/c mice of 14 and 16 weeks of age monitored for mononuclear infiltrates also presented this kind of incipient focal infiltration and the incidence was similar (12.5% and 33.3% for 14- and 16-week-old mice). In parotid glands we...
could not find infiltrates during the whole period studied and morphology of ducts and acini remained unchanged (Fig. 2, lower panel). We detected a higher incidence of infiltrates in NOD submandibular glands from 20 weeks onwards that consisted of small nests of CD3 positive mononuclear cells with periductal localization. BALB/c mice of 20 weeks remained with a 33% incidence. In contrast, NOD parotid glands never did show infiltrating cells.

Cytokine levels in NOD salivary glands and detection of serum autoantibodies

The Th1 and Th2 cytokines measured in gland extracts of NOD mice did not change with age in either submandibular or parotid glands and were similar to normal BALB/c mice (Table 3). Two Th1 cytokines, IFN-γ and IL-12, were determined in the serum of NOD mice of 10, 14 and 16 weeks and while IFN-γ was undetectable, IL-12 increased at 16 weeks (IL-12, pg/ml serum: 10 weeks: 121 ± 14; 14 weeks: 106 ± 10; 16 weeks: 189 ± 18*, *P < 0.05 versus 10 weeks). Immunoblotting assays were run with sera of NOD mice to investigate the appearance of circulating autoantibodies recognizing glandular structures. Sera from NOD mice did not recognize normal gland proteins when assayed on SDS-PAGE-fractionated extracts from salivary glands of NOD mice. The pilocarpine plus VIP-induced secretion started to decline at 12 weeks, paralleling the onset and progression of salivary dysfunction of NOD mice could induce an autoimmune response, we also tested the serum of each NOD mice by immunoblotting on the fractionated proteins of its own glands and we could see that only at 16 weeks of age did NOD mice show a band pattern characteristic of parotid secretory protein (PSP) recognized by circulating antibodies of the same mice (Fig. 3b). As can be seen in the figure, parotid gland extracts from both BALB/c and NOD mice contain Igs that are revealed with the secondary antibody.

Discussion

An intriguing question in the study of autoimmune disorders is whether functional disabilities within the target organ might injure certain cell types and predispose them to an inflammatory reaction. These abnormalities, probably arising in the silent or asymptomatic period, would be detectable only in patients’ tissues long after the disease outbreak. This is the case with SS, as most women are frequently diagnosed after complaining for many years of sicca symptoms [17] and the organ-localized failure co-exists – but seems not to correlate – with a mild local inflammatory reaction [4,18]. Attempts to disclose such mechanisms in chronic autoimmune disorders are limited mainly to experimental models and, although no single animal model mimics a human disease perfectly, the NOD mouse model of SS is especially useful to monitor early biochemical alterations in the target tissues.

Results presented here support the hypothesis that functional alterations in salivary glands precede the autoimmune response in NOD mice by providing evidence of several signalling pathways involved in saliva secretion that appear severely affected before the autoimmune response against salivary glands has become evident. Our conclusion is based on two main observations. First, the onset and progression of salivary dysfunction parallels the progressive loss of nitric oxide synthase activity and the decrease of two independent VIP-stimulated pathways involved in saliva secretion. Secondly, the effects are seen both in submandibular and parotid glands in the absence of locally expressed cytokines or immune infiltrates. The impact that a defect in the nitric oxide/cGMP pathway might have in the pathogenesis of salivary dysfunction in this model is evident in the light of the growing number of reports on its prominent role in saliva secretion. Hence, the inhibition of NOS activity reduced the parasympathetic nerve-evoked salivary flow and the agonist-stimulated secretion in various species [15,19–21]. Together with a defective NOS, we presented evidence of a reduced response to VIP in major salivary glands of NOD mice. The pilocarpine plus VIP-induced secretion started to decline at 12 weeks, paralleling the decrease in nitric oxide synthase activity and cyclic nucleotide accumulation. As the most active peptide in the parasympathetic control of aqueous salivary secretion, VIP acts by coupling to different signalling cascades [9,12,16]. The failure of VIP to activate nitric oxide/cGMP pathway as well as cAMP accumulation, two independent signals activated

### Table 3. Cytokine levels in salivary glands of non-obese diabetic (NOD) mice.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Submandibular glands</th>
<th>Parotid glands</th>
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</thead>
<tbody>
<tr>
<td>TNF-α (pg/mg prot)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD 10 weeks</td>
<td>0.3 ± 0.0</td>
<td>6.8 ± 2.1</td>
</tr>
<tr>
<td>14 weeks</td>
<td>0.4 ± 0.0</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>16 weeks</td>
<td>0.2 ± 0.0</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>BALB/c 16 weeks</td>
<td>0.3 ± 0.0</td>
<td>7.0 ± 2.0</td>
</tr>
<tr>
<td>IFN – (pg/mg prot)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD 10 weeks</td>
<td>2.9 ± 1.1</td>
<td>16.0 ± 1.9</td>
</tr>
<tr>
<td>14 weeks</td>
<td>2.6 ± 0.0</td>
<td>15.0 ± 2.2</td>
</tr>
<tr>
<td>16 weeks</td>
<td>3.2 ± 0.0</td>
<td>26.6 ± 8.7</td>
</tr>
<tr>
<td>BALB/c 16 weeks</td>
<td>2.8 ± 1.4</td>
<td>15.7 ± 1.8</td>
</tr>
<tr>
<td>IL-10 (pg/mg prot)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD 10 weeks</td>
<td>1.0 ± 0.0</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td>14 weeks</td>
<td>2.2 ± 0.5</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>16 weeks</td>
<td>2.0 ± 0.6</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>BALB/c 16 weeks</td>
<td>0.9 ± 0.3</td>
<td>7.8 ± 1.8</td>
</tr>
</tbody>
</table>

Freshly isolated glands from each animal were homogenized individually in Tris-HCl buffer with protease inhibitors as described in Materials and methods. Aliquots of each extract were separated for cytokine determination by a sandwich enzyme-linked immunosorbent assay kit. Values are the mean ± s.e. of at least four separate determinations.
by the neuropeptide, points clearly to the potential contribution of this defect to the pathogenesis of salivary dysfunction in NOD mice.

The absence of detectable levels of cytokines in the same glands, where signalling abnormalities have reached their maximal degree, together with the observation that autoantibodies raised against glandular aberrant proteins did not appear before 16 weeks of age, suggests a mechanism of gland dysfunction that is not secondary to the autoimmune response. The abnormal cleavage of PSP in parotid glands and the aberrant expression of these forms in submandibular glands have been described in NOD mice of 18 weeks and older [6]. The possible relationship between these abnormal forms of PSP as emerging autoantigens and the defects in NOS signalling shown here are not addressed by the present results. However, it seems that if these events were related one to another, the temporal sequence better supports a disease mechanism where dysfunction predisposes the tissue to an autoimmune response rather than the converse. Autoantibodies that recognize glandular proteins [22] and muscarinic receptors of salivary glands have been described in NOD mice of 18 weeks and older [13,23], although they were detected long after disease outbreak. These antibodies interfered with glandular secretion when transferred or exposed to the glands in vitro, supporting the view that the autoimmune response exacerbates dysfunction. On the other hand, the increase in serum IL-12 at 16 weeks and the absence of serum IFN-γ confirm the latency of Th1 cytokine induction at the time when the signalling dysfunction is overt. Several cytokines have been detected in salivary glands of NOD mice with a typical Th1 profile, although at later stages (20 weeks), and associated mainly with lymphocyte infiltrates [24,25]. These observations support collectively the hypothesis of a pre-existing functional defect in the target organ that contributes to confer susceptibility to develop an autoimmune process, which in turn promotes further damage. While the disease mechanisms underlying sialadenitis in NOD mice and patients are unknown at present, we hypothesize that modified enzyme or receptor molecules necessary for signalling may alter secretion at a first stage and arise as immunogens later. The presence of antimuscarinic receptor antibodies in the sera of both NOD mice and patients support this proposal. In line with this, defective iodine processing machinery of epithelial cells has been proposed as a trigger to autoimmune thyroiditis in susceptible strains [26]. Finally, the higher ductal to acinar cell ratio observed in submandibular glands of young 10-week-old NOD mice compared with normal BALB/c mice seems not to be related to the functional alterations observed, because functional parameters were similar in both mice. This higher duct number did not vary with age in NOD mice, while signalling alterations progressed, suggesting that they are independent events. Consistent with this, parotid glands of NOD mice showed signalling alterations with a normal and constant duct number and the absence of apoptotic images. The observed expression of apoptosis-related proteins in salivary glands of NOD-scid mice at 18 weeks suggests a minor relevance of the immune response in the programmed cell death [27]. The involvement of apoptotic cell death in the loss of acinar epithelium has been also reported in labial glands of

![Fig. 3. Circulating autoantibodies against salivary gland proteins.](image_url)

(a) Submandibular (SM) and parotid (P) glands of BALB/c mice were homogenized and fractionated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins transferred to nitrocellulose membranes were incubated with 1/100 dilutions of sera from BALB/c or non-obese diabetic (NOD) mice of the ages indicated and revealed as described in Materials and methods. (b) Fractionated proteins from submandibular (SM) and parotid (P) glands of NOD mice of the ages indicated obtained as in (a) were incubated with 1/100 dilution of the sera from the same NOD mice and revealed as described in Materials and methods. Each blot shown is representative of three others assayed similarly; the arrowhead indicates relevant proteins revealed by NOD sera at 16 weeks of age.
Sjögren’s patients [28,29]. On the other hand, high constitutive CD40 expression was found in ductal cells of labial glands from SS patients, suggesting their intrinsic activation and proliferation status [30].

Kong and co-workers have proposed a two-phase model for the pathology of NOD mice in which initiation of the disease process occurs via a lymphocyte-independent phase characterized by apoptosis of acinar epithelial cells followed by a second phase with infiltration and tissue damage [27]. The authors suggest that the initial trigger may reside in a defect in salivary homeostasis and provide evidence of an altered pattern of protein secretion [6]. Studies in Sjögren’s patients showing intrinsic activation of gland epithelial cells support this notion [3,30]. Our data are in line with these observations and extend such defects to neurotransmitter receptor activation and signalling pathways that are directly involved in the secretory process. Early biochemical alterations as those reported here are far from being detected in clinical studies in the case of Sjögren’s patients, but their identification could help to develop more specific diagnostic tools and more rational therapeutic approaches.

Acknowledgements

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