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NOD Mice Exocrinopathy: Towards a Neuroimmune Link

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NOD mice · Exocrinopathy · Neuroimmune · Nitric oxide · Macrophages · Apoptotic acinar cells

Abstract
Sjögren's syndrome (SS) is a chronic autoimmune disorder of exocrine glands characterized as an autoimmune exocrinopathy and more specifically as an autoimmune epithelitis. An impaired balance of neuroimmune interactions mediated by vasoactive intestinal peptide (VIP) in the target organ at early stages of disease is explored by means of the non-obese diabetic (NOD) mouse model of SS. We have previously described a reduced salivary secretion and signaling upon VIP stimulation. The effect reflected a differential regulation of the neural isoform of nitric oxide synthase by calcium calmodulin kinase II and occurred prior to the appearance of detectable levels of cytokines in NOD glands. VIP acting on NOD macrophages treated with lipopolysaccharide promoted anti-inflammatory effects by inhibiting nitric oxide synthase induction as well as IL-12 and TNF-α production, while stimulating IL-10. Here we present evidence on the ability of apoptotic acinar cells from submandibular glands of NOD mice to stimulate nitric oxide in both peritoneal and glandular macrophage pools to a similar extent as lipopolysaccharide + IFN-γ. VIP was not effective to prevent nitrite accumulation and modestly increased IL-10 levels in macrophages coincubated with acinar cells. An enhanced nitrite response of NOD glandular macrophages in basal and stimulated conditions compared to peritoneal cells is also shown.

Introduction

Sjögren's Syndrome: Nervous and Immune Mechanisms Involved

Sjögren's syndrome (SS) is a chronic autoimmune disorder of exocrine glands characterized by a progressive dryness of the mouth and eyes [1, 2]. SS affects mostly women in a 9:1 relationship, it has an estimated prevalence of 0.5–1% based on the American-European Consensus Group classification criteria (2002) and it is the second most prevalent autoimmune rheumatic disease after rheumatoid arthritis [3]. However, it continues to be seriously underdiagnosed and most SS patients complain of sicca symptoms for many years before they come to an accurate diagnosis, strongly conditioning patients' outcome. While etiopathogenic mechanisms remain unclear, conclusive evidence of an aberrant activation of glandular epithelial cells in the induction and perpetuation of the inflammatory response has characterized SS as an autoimmune exocrinopathy and more specifically as an autoimmune epithelitis [4]. The mild infiltration of exocrine glands cannot fully account for the severeness

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of sicca symptoms. Thus, glandular epithelium might not only be a victim of the autoimmune response, but could provide the glandular milieu with several mediators probably involved in the pathogenesis of SS [4].

Based on the nervous and autoimmune components that characterize SS, the hypothesis of an impaired balance of neuroimmune interactions in the target organ at the onset of the disease seems attractive. These events would be among the earliest events to take place in the course of the disease and several studies on experimental models of SS can be analyzed in line with this approach.

Nonobese Diabetic Mouse as a Suitable Model of SS

The availability of various mouse models of SS offers the possibility of studying early neuroimmune interactions in major salivary glands previous to or during the development of the disease. Nonobese diabetic (NOD) mice have the advantage over other SS models of developing a deep loss of secretory function [5]. Interestingly, as it was reported for SS patients, evidence supporting a nonimmune origin for the secretory dysfunction in this model has been provided; congenic immunodeficient NOD mice lack the ability to produce functional B and T lymphocytes and, nonetheless, they showed a loss of secretory function associated with an increased programmed cell death [6]. As in patients, a mild or absent infiltration of glands correlated poorly with the deep loss of saliva secretion [7]. It has been proposed that the initial trigger of autoimmune exocrinopathy in NOD mice may reside in a defect in salivary gland homeostasis [8]. In keeping with this, defects in metalloproteinase expression [9] and autonomic receptor activation [10] were reported in NOD glands and we described a loss of nitric oxide synthase (NOS) activity as well as a reduced salivary secretion and signaling upon vasoactive intestinal peptide (VIP) stimulation [7, 11]. A differential regulation of the neural isoform of NOS by CaMK II [11] occurred prior to the appearance of detectable levels of cytokines or infiltrating cells in NOD glands [7].

VIP is a neuro- and immunopeptide that promotes secretion, contributes to vasodilation in exocrine glands [12] and promotes anti-inflammatory effects in normal mouse-activated macrophages by inhibiting NOS induction while stimulating IL-10 [13, 14]. It also controlled inflammation in models of rheumatoid arthritis, Crohn's disease and diabetes [13, 15].

On the basis that defects in signaling circuits responsive to VIP in NOD salivary glands and peritoneal macrophages reported previously [7, 16] could underlie an impairment of homeostatic control within the glands, we investigated whether NOD apoptotic acinar cells could activate local (glandular) and systemic (peritoneal) macrophage pools and the anti-inflammatory action of VIP.

We present evidence on the ability of apoptotic acinar cells from submandibular glands of NOD mice to stimulate nitrites in both systemic and local macrophage pools to a similar extent as lipopolysaccharide (LPS) + IFN-γ. VIP was not effective to prevent nitrite accumulation and modestly increased IL-10 levels in macrophages co-incubated with apoptotic acinar cells. We also show an enhanced nitrite response of NOD glandular macrophages in basal and LPS-stimulated conditions compared to peritoneal cells.

Materials and Methods

Animals

Eight- or sixteen-week-old NOD (prediabetic) and BALB/c female mice bred in the Central Animal Care Facility at the University of Buenos Aires were used and studies conducted according to standard protocols of the institution [7, 16].

Macrophage and Acini Isolation

Peritoneal exudate cells were obtained as reported [16] and seeded in 24-well plates at 1×10^6 cells per well. Macrophages (>95%) were stimulated with 10 μg/ml LPS alone or with 100 U/ml IFN-γ in the presence or absence of VIP [16]. Acinar cells were isolated from submandibular glands of NOD mice aged 8 and 16 weeks by enzymatic digestion as reported [17] and glandular macrophages were separated by adherence [16]. After 3 washes, macrophages (>90%) were coincubated with acinar suspensions (3 μg/μl protein) for 90 min, then acini were decanted and macrophages washed another 3 times and fresh medium was added for 24 h. When used, VIP was included from the beginning and readded with the fresh medium.

Detection of DNA Fragmentation

Acinar cells were collected in culture medium, pelleted and homogenized as previously reported [18]. Digestion was allowed to proceed, proteinase K was added and then, after precipitation electrophoresis was carried out on 2% agarose gel, bands were observed under UV transilluminator and digitalized [18].

Fluorescent Nuclear Staining of Apoptotic Cells

Cells fixed with 4% (v/v) paraformaldehyde were exposed to 0.05 g/l Hoechst 33258 dye in PBS, washed and mounted as described [18]. Fluorescent nuclei with apoptotic characteristics were detected by microscopy under UV illumination at 365 nm. The images (×40) were photographed by a Nikon Coolpix 5000 and digitalized. For differential cell counting at least 500 cells were analyzed [18].

RT-PCR and Western Blotting for Bax

Total RNA isolation and reverse transcription were performed using Ready-to-Go (Amersham) [11, 18]. PCR amplification cycles (94°C for 20 s, primer annealing at 62°C for 30 s, extension

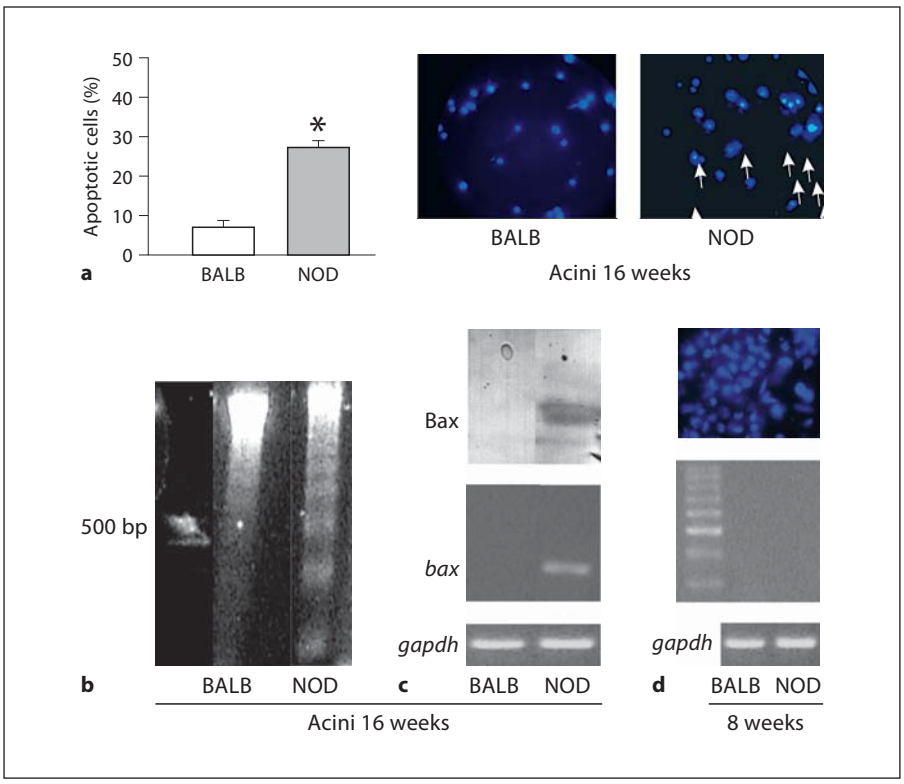

Fig. 1. Apoptotic process in 16-week-old NOD mice submandibular glands acini. To determine apoptosis, 8- and 16-week-old NOD and BALB/c mice acinar cells were isolated from submandibular glands. Bax expression was determined by Western blotting and DNA fragmentation by ladder and Hoechst dye as described in Materials and Methods. Results are means ± SE of at least 3 experiments. * $p < 0.05$ vs. BALB/c.

at 72°C for 40 s) and 30 cycles (94°C for 45 s, 60°C for 30 s, and 72°C for 45 s) with initial incubation at 94°C for 5 min and final incubation at 72°C for 10 min for Bax 2 and for the standard GAPDH were performed.

The proteins were resolved in 10% SDS-PAGE, electroblotted and incubated [11, 18] with anti-Bax antibody overnight, revealed with anti-mouse peroxidase conjugate and detected by ECL (Amersham) using a Fujifilm Dark Box II.

IL-10 and Nitrites Determination

IL-10 was determined in macrophage supernatants with capture ELISA as previously described [7]. Nitrite concentration was determined in the same macrophage supernatants using the Griess method [16].

Statistical Analysis

Statistical significance was determined by the two-tailed t test for independent populations. The Student-Newman-Keuls test after analysis of variance was used for multiple comparisons. Differences were considered significant at $p < 0.05$.

Results and Discussion

Enhanced Apoptosis of NOD Acinar Cells

To determine whether apoptosis of acinar cells paralleled the loss of salivary function and signaling in response to VIP in NOD mice [7], we isolated acinar cells from submandibular glands at 2 ages (8 and 16 weeks). Figure 1 shows that acini from submandibular glands of 16-week-old mice expressed Bax, were positive for Hoechst dye and presented a DNA ladder compatible with an apoptotic process. In contrast, acini coming from 8-week-old mice did not show signs of apoptosis (fig. 1d), no different from acinar cells isolated from normal BALB/c mice. These results add new evidence to previous reports on apoptosis mediated through Fas in NOD salivary glands [6]. The signs of apoptosis were detected in acinar cells isolated from NOD mice at 16 weeks of age when they had already developed a significant saliva flow reduction (more than 40% of reduction in pilocarpine-stimulated saliva flow compared to BALB/c mice) [7]. In contrast, NOD mice at 8 weeks of age showed normal salivary function [7] and there was no detectable apoptosis in acinar cells, suggesting that apoptotic processes, if present, might be incipient and not detectable by the methods used. Of note, we have described apoptotic images in histological slices of submandibular glands from 10-week-old NOD mice prior to the autoimmune response [7]. Similarly, inappropriate apoptosis in salivary glands was observed in NOD-*scid* mice [6], and cas-

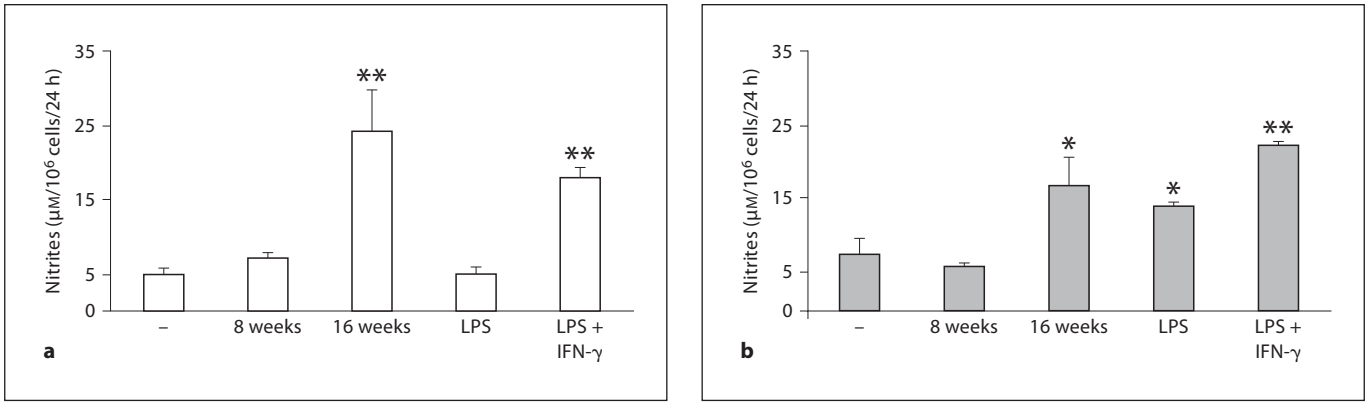

Fig. 2. NOD peritoneal and glandular macrophages presented increased nitrite production when co-incubated with 16-week-old NOD apoptotic acini. NOD peritoneal (**a**) or glandular (**b**) macrophages were co-incubated with 8- or 16-week-old acini, LPS or LPS + IFN-γ. Supernatants were collected for nitrite determination as described in Materials and Methods. LPS only stimulated glandular macrophages nitrite production. Results are means ± SE of at least 3 experiments. * $p < 0.05$; ** $p < 0.001$ vs. basal in each group.

pase – among other genes – appeared differentially expressed in submandibular glands of C57BL6/NOD-*Aec*1*Aec*2 mice, an SS-susceptible strain [19], supporting the notion that early defects in the glands might predispose them to the autoimmune response.

Nitrite Production by NOD Macrophages Co-Incubated with Apoptotic Acinar Cells

We have recently reported that peritoneal macrophages from 16-week-old NOD mice display a differential profile compared to normal cells upon stimulation with LPS + IFN-γ [16]. Due to the fact that LPS is an exogenous inflammatory stimulus, and based on previous evidence on the ability of glandular epithelial cells from SS patients to perpetuate the inflammatory response [4], we investigated the response of peritoneal macrophages coincubated with acini undergoing apoptosis or not. Figure 2a shows that NOD peritoneal macrophages increased nitrite production when coincubated with apoptotic acini from 16-week-old mice, but not with 8-week-old mice acini. The effect was comparable to that of LPS + IFN-γ, a strong inducer of inducible NOS (iNOS) in these cells. As shown earlier [16], figure 2a also shows that LPS alone did not stimulate nitrite production, suggesting that the NOD systemic pool of macrophages requires IFN-γ to potentiate the LPS stimulus, as observed for BALB/c normal macrophages [13, 16].

We wondered whether the local pool of glandular macrophages was also responsive to these stimuli and if there was any difference between the local and systemic macrophage pools. Figure 2b shows that glandular macrophages presented an activation profile similar to that of peritoneal cells. However, LPS alone did stimulate nitrites in this macrophage pool and basal levels of nitrites were higher in glandular vs. peritoneal macrophages ($p < 0.05$, Student's t test). The facts that LPS alone (without IFN-γ) was enough stimulus to increase nitrite levels only in NOD glandular macrophages and that basal levels were already increased suggest that iNOS of the glandular pool is induced and/or more sensitive to inflammatory stimuli. The effect of LPS alone was not seen in control BALB/c macrophages from either source (table 1), supporting that it is not attributable to glandular macrophage isolation protocol. Acinar cells were as potent as

Table 1. Nitrite production in normal macrophages

	Nitrites, μM/10^6 cells	
	peritoneal	glandular
Basal	1.2 ± 0.1	1.5 ± 0.3
LPS	1.4 ± 0.1	1.2 ± 0.1
LPS + IFN-γ	11.3 ± 0.7*	12.6 ± 0.1*

Isolated peritoneal and glandular macrophages from BALB/c mice were treated with LPS or LPS + IFN-γ and supernatants collected for nitrite determination as described in Materials and Methods. Results are means ± SE of at least 3 experiments.

* $p < 0.05$ vs. basal in each group.

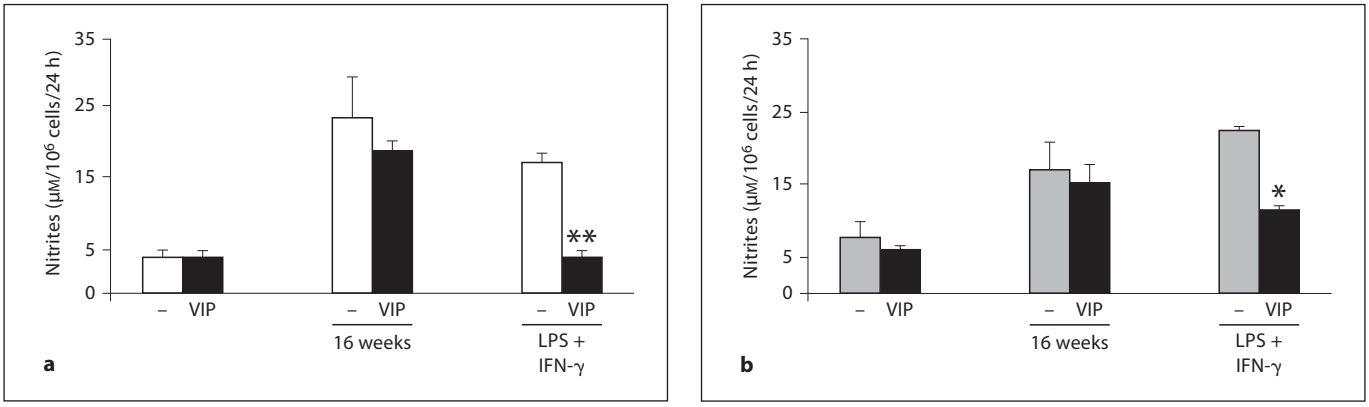

Fig. 3. VIP was unable to inhibit nitrites-increased production in NOD macrophages co-incubated with 16-week-old NOD apoptotic acini. NOD peritoneal (**a**) or glandular (**b**) macrophages were co-incubated with 16-week-old acini, LPS or LPS + IFN-γ in the absence or presence of VIP (10^{-7} M). Supernatants were collected for nitrite determination as described in Materials and Methods. Results are means ± SE of at least 3 experiments. * $p < 0.05$; ** $p < 0.001$ vs. LPS + IFN-γ in each group.

Table 2. IL-10 production by NOD peritoneal macrophages

	IL-10, pg/10^6 cells
Basal	121 ± 12
+ 8 weeks acini	170 ± 16
+ 16 weeks acini	272 ± 24
+ 16 weeks acini + VIP	469 ± 38*
+ LPS + IFN-γ	256 ± 42
+ LPS + IFN-γ + VIP	4,241 ± 362**

Isolated peritoneal macrophages from NOD mice were coincubated with acini of 8 and 16 weeks or treated with LPS and LPS + IFN-γ in the absence and presence of VIP (10^{-7} M) and supernatants were collected for IL-10 determination as described in Materials and Methods. Results are means ± SE of at least 3 experiments.

* $p < 0.05$; ** $p < 0.001$ vs. basal.

LPS + IFN-γ stimulus in both glandular and peritoneal macrophages of NOD mice, probably by reaching the maximum of NOS induction. Figure 2b and table 1 also show that NOD glandular macrophages produce higher nitrites than normal BALB/c cells in basal and LPS-stimulated conditions ($p < 0.05$), similarly to a previous report [16].

Silent clearing of apoptotic cells by macrophages is necessary and involves a downregulation of inflammatory mediator production [20]. In contrast, defective phagocytosis of apoptotic β cells induced an immune reaction, increasing the susceptibility to develop diabetes in the NOD model [21, 22]. Also, pro-inflammatory cytokine production was not reduced in macrophages from prediabetic NOD mice upon encountering apoptotic thymic cells [23]. The results shown here confirm and extend those observations, as local apoptotic signals provided by acinar epithelium seemed to potently stimulate macrophages. Acinar cells did not accumulate detectable nitrite levels in 90 min of incubation, ruling out a contribution from this source in the coincubation experiments.

IL-10 Production in Response to Apoptotic Acinar Cells

We have previously shown that LPS + IFN-γ did not increase IL-10 production in NOD peritoneal macrophages compared to normal BALB/c cells [16]. Noteworthy, as shown in table 2, apoptotic acini were not able to increase IL-10 levels in NOD peritoneal macrophages either. The release or not of the anti-inflammatory cytokine IL-10 following the binding of apoptotic cells to macrophages strongly depends on the participating cells [24] and can probably influence the course to chronic inflammatory and autoimmune diseases. The high nitrite response of NOD macrophages to both LPS + IFN-γ and apoptotic acinar cells and the lack of response with an increase in IL-10 production shown here might reflect a defective regulation upon inflammatory stimuli. NOD glandular macrophages did not show detectable levels of IL-10 in these conditions, probably reflecting the need for further concentration of the cells for this kind of study.

Effect of VIP on NOD Macrophage Response

We have previously shown that VIP prevented nitrite, TNF-α and IL-12 production induced by LPS + IFN-γ in the peritoneal pool of NOD macrophages [16]. VIP was shown to prevent iNOS induction in normal mouse macrophages by inhibiting NF-κB [14] and also through IL-10 in NOD peritoneal macrophages [16]. Here we show that VIP was unable to inhibit iNOS induction in both pools of macrophages stimulated with acinar cells (fig. 3). On the other hand, VIP only slightly increased IL-10 in peritoneal macrophages coincubated with apoptotic acinar cells (table 2). This modest effect of VIP contrasted with the huge increase in IL-10 levels obtained when macrophages were stimulated with LPS + IFN-γ (table 2). Taken together, these results indicate that VIP does not regulate NOD macrophage response to an apoptotic stimulus as it does with LPS stimulation in both systemic and local pools.

Concluding Remarks

VIP has the ability to 'dialogue' in both the immune and nervous systems, thus taking center stage in the NOD model of Sjögren's exocrinopathy. We have profited from the NOD mouse model of SS in an attempt to disclose neuroimmune mechanisms mediated by VIP in salivary glands. In this context, we analyzed VIP nervous and immune profiles and described a reduced secretory and signaling response to VIP in salivary glands and a defect in macrophage regulatory pathways in response to LPS and to NOD apoptotic acinar cells. VIP reduced nitrites in NOD macrophages treated by LPS + IFN-γ, but was ineffective to modulate macrophage activation by acinar cells independently of the local or systemic localization of the cells. It is tempting to speculate that the modulation of local inflammatory stimuli acting on macrophages within the glands might be in part under the homeostatic surveillance of VIP. If so, the aberrant activation of macrophages by apoptotic signals that circumvents VIP control by maintaining high levels of nitrites and low levels of IL-10 shown here would disrupt gland homeostasis and promote further inflammatory/apoptotic damage. Whether VIP has a role as an anti-inflammatory mediator in vivo for NOD exocrinopathy remains unknown, though it reversed salivary dysfunction when given by means of a gene-transfer system [25] and had an anti-inflammatory preventive effect in diabetic NOD mice [15].

Acknowledgments

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