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Levels of immunoglobulin A1 and messenger RNA for interferon γ and tumor necrosis factor α in total saliva from patients with diabetes mellitus type 2 with chronic periodontal disease

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Background: Diabetes mellitus and periodontal disease have high incidence in the general population and are associated with various degrees of dysfunction in the immune system. It has been shown that diabetic patients with severe periodontal disease have more complications of diabetes and less effective metabolic control compared with diabetic patients with healthy gingiva. Patients with diabetes and severe periodontal disease present higher levels of serous immunoglobulin A (IgA). Elevation of the IgA1 isotype is thought to contribute to this phenomenon. Another important event in the diabetes–periodontitis association is the disturbance in local and systemic production of inflammatory cytokines.

Objective: In this study we tested the hypothesis that type 2 diabetic patients with chronic moderate periodontal disease have differences in salivary IgA1 titers and cytokine expression when compared with the chronic severe periodontal disease cases.

Methods: We utilized a jacalin–IgA capture assay to determine the IgA1 titers in total saliva and reverse transcriptase–polymerase chain reaction to detect mRNA for interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) in total saliva samples of 13 patients with chronic moderate periodontal disease and 10 with chronic severe periodontal disease.

Results and conclusions: We observed a predominance of IgA1 titers of 64 (45.5%) in saliva samples from chronic severe periodontal disease patients and titers averaging 512 (30.8%) in chronic moderate periodontal disease patients. We detected mRNA for IFN-γ in six out of 10 chronic severe periodontal disease subjects and in two out of 13 chronic moderate periodontal disease patients. TNF-α expression was similar in both groups. Our data suggest that higher levels of

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IgA1 may exert partial protection of the periodontal tissue in chronic moderate periodontal disease diabetic patients when compared to severe periodontal disease. Despite the small number of patients, IFN-γ expression had a trend association with severity of periodontitis and TNF-α gene expression did not correlate with severity of periodontal disease.

Periodontitis is a chronic inflammatory disease that results in gingival inflammation, destruction of periodontal tissues, alveolar bone loss, and, in severe cases, tooth loss (1–3). It is one of the most prevalent diseases in humans and affects mainly individuals above 35 years of age. Diabetes mellitus consists of a group of disorders that include reduction or absence of insulin production (4, 5). Both diseases have high prevalence rates: it is estimated that 1–6% of the general population suffer from diabetes and that periodontal disease affects over half of the adult population in the US.

Microbial components are thought to play an important role in periodontal disease. Hundreds of species compose the microbial population in the mouth. Among them, Gram-negative bacteria, strict anaerobic species (*Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticula*) and facultative anaerobic species (*Actinobacillus actinomycetemcomitans*) are recognized as putative pathogens for the periodontal tissue (6).

Periodontitis has been considered one of the major complications of diabetes (7). Diabetic patients have increased susceptibility to periodontal disease, probably due to a disruption of the microbial equilibrium maintained in healthy dental plaque (8). In addition, periodontal disease influences the glucose control in diabetic patients, making the maintenance of glycemia more challenging (9).

The highly vascular inflamed periodontal tissue can serve as a source for systemic release of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and other inflammatory mediators (10). The destruction of tissue, often seen in periodontal disease, can be aggravated by recruitment and activation of systemic inflammatory cells (11). In addition, the production and release of high levels of TNF-α systemically interferes with lipid metabolism due to TNF-α antagonism to insulin (12, 13). On the other hand, production of gamma interferon (IFN-γ) can reverse the imbalance promoted by TNF-α by enhancing the phagocytic activity of neutrophils and macrophages (14).

Polyclonal activation of B cells and increased IL-4 production are typically seen in Th2 driven immune responses, which result in antibodies production and specific isotype switch (15, 16). The response in advanced stages of periodontitis in adults is significantly Th2 driven; however, Th1 cells have an important role in the infection (17, 18).

Secretory immunoglobulin A (IgA) has the ability to promote antigen-specific agglutination of oral bacteria, which prevents microbial adherence to the mucosal surfaces (19). Salivary antibodies can also be used for diagnosis and prognosis in inflammatory and infectious diseases (20–22). Type 2 diabetic patients present elevated levels of serous IgA when compared to healthy controls (23), and the increase in the levels of IgA1 subclass seems to contribute to this event (24). It has been shown that patients with aggressive periodontitis have lower salivary concentrations of secretory IgA and IgA1 when compared to healthy controls (25). Imbalance on production or secretion of IgA caused by either type 2 diabetes or peridontitis can potentially alter the mucosal defenses against infection.

The present work evaluated the association between levels of IgA1, cytokines expression in total saliva and clinical status of type 2 diabetic patients with chronic moderate periodontal disease or chronic severe periodontal disease.

Material and methods

Patients

Type 2 diabetic patients were recruited from the outpatient Endocrinology service at Hospital de Clinicas – Universidade Federal de Uberlandia, Brazil. Enrollment criteria for this study included previous established diagnosis of type 2 diabetes, adherence to the prescribed treatment for diabetes and complete physical and laboratory assessment. Cigarette-smoking patients, patients with infections or systemic inflammatory diseases, history of antimicrobial therapy in the last 6 months, as well as those who had received any periodontal treatment in the same period were excluded from this study. All the enrolled patients were subjected to a periodontal clinical examination, radiographic documentation (Figs 1A–D) and gingival probing using Hufriedy™ periodontal probe. Periodontal status was classified according to the American Academy of Periodontology guidelines (1999). Twenty-four selected patients were divided in to two groups: 13 type 2 diabetic patients with chronic moderate periodontal disease and 11 type 2 diabetic patients with chronic severe periodontal disease. The clinical characteristics of the selected patients are shown in Table 1. Biological specimens were collected and manipulated according to biosafety guidelines (26). Clinical and laboratory procedures were approved by the Committee for Human Research at Universidade Federal de Uberlândia, Brazil.

Saliva samples

Sample collection was performed always between 8 AM and 10 AM, with fasted patients. Whole saliva (minimal volume of 4 ml) was collected without

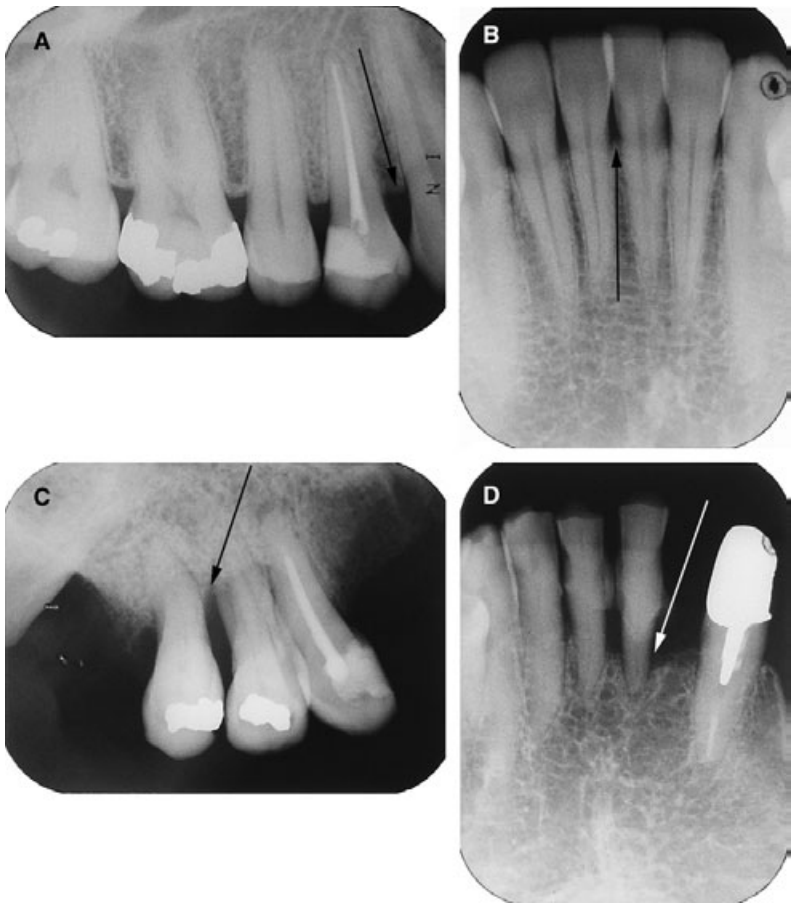

Fig. 1. Socket of a tooth change in diabetes mellitus type 2 patients with chronic moderate (A and B) or severe (C and D) periodontal disease. Arrows show moderate (A and B) or severe osseous loss (C and D).

Table 1. Clinical characteristic of patients with diabetes type 2

	CMPD[a]	CSPD[b]
Male	6	5
Female	7	6
Age	54–46 ± 9.47	53–63 ± 14.23
Insulin dependence	6/13	6/11
Glucose control[c]	10/13	5/11

[a]Patients with chronic moderate periodontal disease.
[b]Patients with chronic severe periodontal disease.
[c]Patients with glucose control.

stimulation, into a plastic recipient, during a period of 5 min. Cells and sediments were separated from supernatant by centrifugation at 1000 *g* for 5 min. The supernatant was used for IgA measurement and the sediment was ressuspended in 50 µl of RNA *later*™ (Qiagen Inc., Valencia, CA, USA) and used for gene expression assays. Samples were stored at −20°C until processing.

Measurement of total immunoglobulin A1 in saliva

Total IgA1 measurement in saliva was standardized using a capture enzyme-linked immunosorbent assay (ELISA). Jackfruit lectin (jacalin), a molecule with a well-studied property to bind IgA, was used to capture IgA1 in the solid phase of the assay (27). High-binding 96-well microtiter plates (Costar, Corning Inc., Corning, NY, USA) were coated (50 µl/well) with 1 µg/ml of jacalin lectin (Sigma Chemical Co., St Louis, MO, USA) in carbonate/bicarbonate buffer, 0.06 M, pH 9.6, for 18 h at 4°C. After rinsing to remove unbounded lectin, each well received 50 µl of saliva samples in doubling serial dilutions (1:8–1:1024) or 0.10–200 µg/ml of purified human IgA (Sigma) in phosphate-buffered saline containing 0.05% Tween 20 and 2% Gelatin (Difco Laboratories, Detroit, MI, USA) and incubated for 1 h at 37°C. Goat IgG anti-human IgA conjugated to peroxidase was used as detection system. The complex IgA + Anti-IgA/Peroxidase was detected by the addition of 50 µl of chromogenic peroxidase substrate (*o*-phenylenediamine, 1 mg/ml, in 0.1 M citrate buffer, pH 5.0 and 0.03% H_2O_2). After 15 min of incubation, 25 µl of 2 N H_2SO_4 was added to the wells to stabilize the reaction. Absorbance at 492 nm was taken using a micro-ELISA reader, and measurement of total IgA1 in saliva samples was established by comparison with the IgA1 standard curve.

Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from saliva sediment using RNA kit (Rneasy Mini Kit, Qiagen) according to the manufacturer's instructions. The cDNA was synthesized with iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Polymerase chain reaction (PCR) amplification was performed with 8 µl of master mix [0.2 mM dNTP, 1 µl PCR buffer 10 ×, 0.2 µM of each primer (sense and antisense from each analyzed cytokine, IFN-γ 5′-GTAACTTTTGACTTGAATGTCCAA-3′ and 5′-GTAACTGACTTGAATGTCCAA-3′, TNF-α 5′-CTGGTAATGAGCCCATCTTATCTGG-3′ and 5′-TTGGATGTTCGTCCTCCCAC-3′, IL-4 5′-GGGTCTCACCTCCCAACTGC-′3 and 5′-TGTCTGTTACGGTCAACTCGGT-′3, IL-13 5′-TCGAAGTAGCCCACTTTATAACAAAA-3′ and 5′-GAAAATGAGTCCACAGCTCAGATG-3′), 4.4 µl water, 1 U Taq polymerase,

1.5 mM $MgCl_2$ (Invitrogen life Technologies, Carlsbad, CA, USA)]. For each reaction, 2 μl of cDNA was added to the master mixture. The reactions were run in a thermocycler (Applied Biosystems) programmed to 3 min at 95°C, following 37 cycles of 30 s at 95°C (denaturation), 30 s at 57°C (annealing) and 1 min at 72°C (extension). The PCR products were analyzed by electrophoresis in 2% agarose gel in Tris–borate–EDTA buffer 0.05 M, pH 8.0 containing 0.4 μg/ml of ethidium bromide and documented in Polaroid film type 667 (FisherBiotech, Cambridge, MA, USA) under UV light.

Statistical analysis

Results were analyzed by ANOVA test (GraphPad Prism, version 3 for Windows). Values with $p < 0.05$ were considered statistically significant.

Results

Immunoglobulin A1 titer in saliva

We found a statistically significant difference in the IgA1 titers between diabetic patients with chronic moderate periodontal disease and chronic severe periodontal disease. Our results revealed that 61.5% of the diabetic patients with chronic moderate periodontal disease had IgA1 titers ≥128, and in nearly half of the diabetic patients with chronic severe periodontal disease (54.5%) the IgA1 titers were ≤ 64 ($p < 0.05$: Table 2). We observed a wide range of IgA titers within each group. The lowest titer observed was 16, in only one sample. None of the samples reached the titer of 1024 (Fig. 2).

Table 2. Percentage of patients in immunoglobulin A1 (IgA1) titer

	Patients (%)	
IgA1 titer	CMPD[a]	CSPD
16	7.7	9.1
32	15.4	0.0
64	15.4	45.5*
128	23.0	18.2
256	7.7	0.0
512	30.8*	27.3
1024	0.0	0.0

[a]CMPD, chronic moderate periodontal disease; CSPD, chronic severe periodontal disease.
*$p > 0.05$.

Analysis of messenger RNA cytokines expression

We evaluated IFN-γ, TNF-α, IL-4 and IL-13 mRNA expression by reverse transcriptase–polymerase chain reaction (RT–PCR). mRNA for IFN-γ was detected in two out of 13 patients with diabetes mellitus and chronic moderate periodontal disease (Fig. 3A). In the group of diabetic patients with chronic severe periodontal disease, five out of 10 patients had high levels of IFN-γ expression (Fig. 3B). The TNF-α expression was detected in seven patients from the chronic moderate periodontal disease group and six from the chronic severe periodontal disease group (Figs 3A and B, respectively). We used glyceraldehyde-3-phosphate dehydrogenase as a housekeeping reference gene for normalization purposes and as an internal control for our reactions (Fig. 3). IL-4 and IL-13 had no detectable expression in the samples utilized in this study (data not shown).

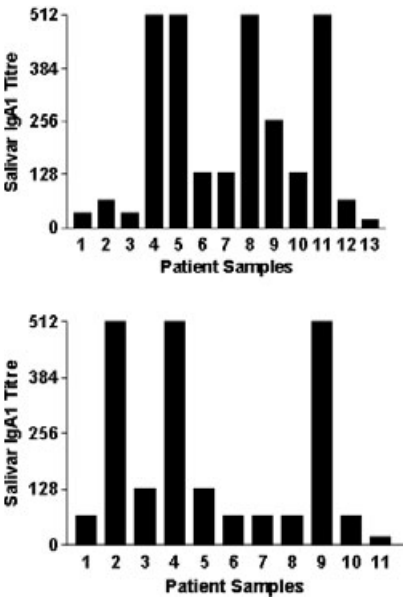

Fig. 2. Immunoglobulin A1 (IgA1) titer in total saliva samples from diabetes mellitus type 2 patients with chronic moderate (A) or severe (B) periodontal disease determined by reactivity with jacalin lectin measured by enzyme-linked immunosorbent assay.

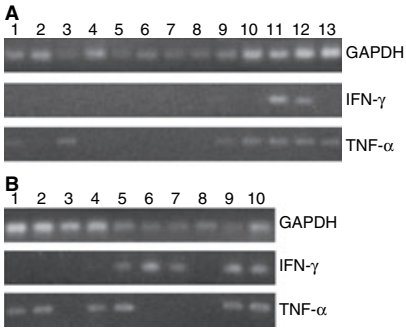

Fig. 3. mRNA cytokine expression by reverse transcriptase–polymerase chain reaction in total saliva sample. Diabetes mellitus type 2 patients with chronic moderate (A) and severe (B) periodontal disease. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α.

Association between the immunoglobulin A1 titers and cytokines expression

There was no association between the mRNA expression for TNF-α and the IgA1 levels in saliva samples in both the chronic moderate periodontal disease and chronic severe periodontal disease groups. TNF-α expression was detectable in samples with titers ranging from 32 to 512 (Table 3). On the other hand, the mRNA expression for IFN-γ was observed only in samples from patients who had IgA1 titers ≥64 (Table 3).

Discussion

The association of diabetes mellitus and periodontal disease is synergic and demonstrates inflammatory response mediated by different factors (28). Immunological and microbiological factors, as well as genetic background, are important in the determination of clinical index applicable to diagnosis in periodontal disease. These multifactor components could be important to precisely indicate the stage of the periodontal disease, as well as being a prognostic and diagnostic tool for evaluation of active or latent inflammatory disease (29, 30).

Table 3. Association between immunoglobulin A1 (IgA1) titer and messenger RNA cytokine expression

			mRNA cytokine expression			
	IgA1 titer		IFN-γ		TNF-α	
Patient no.	CMPD	CSPD	CMPD	CSPD	CMPD	CSPD
1	32	64	ND	ND	D	D
2	64	512	ND	ND	D	D
3	32	128	ND	ND	D	ND
4	512	512	ND	ND	ND	D
5	512	128	ND	D	ND	D
6	128	64	ND	D	ND	ND
7	128	64	ND	D	ND	ND
8	512	64	ND	ND	D	ND
9	256	512	ND	D	D	D
10	128	64	ND	D	D	D
11	512	32	D	NR	D	NR
12	64	NR	D	NR	D	NR
13	16	NR	ND	NR	ND	NR

mRNA, messenger ribonucleic acid; CMPD, chronic moderate periodontal disease; CSPD, chronic severe periodontal disease; D, detected; ND, none detected; NR, none realized.

Secretory IgA is one of the most important immunological components in salivary secretion. IgA has the ability to inhibit the bacterial adherence to the mucosal surface and to neutralize microbial enzymes and thus control oral microorganisms (25). IgA has two subclasses, IgA1 and IgA2, and IgA1 constitutes 40–50% of total IgA in salivary secretions (31).

In the present study we evaluated the IgA1 levels and mRNA for inflammatory cytokines in saliva samples from type 2 diabetic patients with chronic moderate or severe periodontal disease. Marder *et al.* in 1972 (32) showed that patients with diabetes mellitus present more alterations in salivary composition from submandibular and parotid glands than healthy patients. Furthermore, it has been observed that there are increased levels of IgA1 in serum of patients with diabetes mellitus (31). The analysis of antibodies levels, proteins and other mediators associated with patients' immunological response to periodontal disease has been performed in saliva samples (25), gingival crevicular fluid (33), serum (34), periodontal tissue biopsies (35) and even urine (36). In the present work we chose to use saliva collected without stimulation. This choice was based on claims that induction of saliva production can alter the concentration of salivary immunoglobulins (25, 37). Moreover, we utilized the ability of jacalin to bind IgA1 molecules as a tool to determine the levels of those antibodies present in the saliva samples. The assay with jacalin presents a methodological alternative to immunoenzymatic tests that use monoclonal antibodies to capture IgA subclasses. The described method can be a useful tool to evaluate IgA1 concentration which is an immunological parameter associated with periodontal disease.

In this study, we detected higher IgA1 titers in saliva samples from the chronic moderate periodontal disease group, which suggests that this antibody subclass could be exerting partial protection against pathogenic microorganisms, as the severity in periodontal disease is directly associated to more pathogenic subgingival flora (11). On the other hand, the lower IgA1 levels detectable in the chronic severe periodontal disease group could be related to decreased synthesis of the IgA in these patients or with proteolytic degradation triggered by microorganisms' secreted proteases (25, 38, 39).

Besides immunoglobulins, other soluble mediators may exert an important role in the inflammation process. The tissue damage observed in periodontal disease is mediated by locally produced inflammatory cytokines, and several molecules are potentially involved, such as IL-1β, TNF-α, IFN-γ, IL-12, IL-6, IL-4 and IL-13 (29, 40, 41). The elevated production of IL-1β and TNF-α in patients with diabetes mellitus and periodontal disease leads to an increased severity of the symptoms in the periodontal tissue. The secretion of high levels of these cytokines can also result in glucose metabolism dysfunction (13, 42). The detection of mRNA expression for TNF-α in 62% of patients from the chronic moderate periodontal disease group and 60% from the chronic severe periodontal disease group suggests an active role for this cytokine in maintenance of the inflammatory response. It has been demonstrated that TNF-α and IL-1β are increased in periodontal active lesion sites (41). Furthermore, the inflamed periodontal tissue serves as a source, similar to the endocrine, of TNF-α production and other inflammatory mediators (11, 28, 43), which has been associated with cellular apoptosis and bone remodeling (40). Considering the limitations of a relatively small number of patients analyzed in our study, TNF-α expression did not correlate to severity of periodontal disease, suggesting that TNF-α participates in both phases of the disease processes (moderate and severe). The negative results on mRNA detection for IL-4 and IL-13 in this work could be due the short half-life of the cytokines' message in saliva or its presence may not be in detectable levels in salivary secretion (41).

Our study had shown high levels of IFN-γ mRNA for only two out of 13 patients (15.4%) from the chronic moderate periodontal disease group, which suggests that the cellular immune response has been suppressed in these patients. It has been demonstrated that, when stimulated with mitogen, mononuclear cells from peripheral blood of patients with periodontal disease show lower expression of IFN-γ than healthy controls. Ebersole and Taubman (44) demonstrated that mRNA for IFN-γ was increased in periodontal diseased tissue. Our data shows a lack of IFN-γ production in chronic moderate periodontal disease patients, suggesting suppression of the Th1 response in chronic moderate periodontal disease (45). On the other

hand, in chronic severe periodontal disease patients, mRNA for IFN-γ was detected in 50% of samples analyzed, suggesting that Th1 profile may be involved in the exacerbation of the disease and the periodontal destruction seen in these patients. The presented data do not yield strong correlations between the presence of mRNA for inflammatory cytokines and the severity of periodontal disease.

This work points to the fact that the etiology and pathology of periodontal disease is complex. It involves host and microbial factors, many of those yet unknown.

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