ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Inflammatory reaction in chronic periodontopathies in patients with diabetes mellitus. Histological and immunohistochemical study

GEORGIANA CRISTIANA CAMEN[1)], O. CARAIVAN[1)], MĂDĂLINA OLTEANU[2)], A. CAMEN[3)], ADINA BUNGET[1)], FLORINA CARMEN POPESCU[4)], ANCA PREDESCU[4)]

[1)]*Department of Histology*
[2)]*Department of Pedodontics*
[3)]*Department of Oral and Maxillo-Facial Surgery*
[4)]*Research Center for Microscopic Morphology and Immunology*
University of Medicine and Pharmacy of Craiova

Abstract

Chronic periodontopathies and diabetes mellitus are two clinical entities, which reciprocally condition one another. The periodontal disease is considered a major complication, which induces an unfavorable evolution of diabetes mellitus. Diabetes mellitus is an endocrine disease which favors the occurrence of periodontopathy through gum's microvascular disorders, the selection and development of an aggressive bacterial plaque and through an exaggerate inflammatory response to the microflora within the oral cavity. Both diabetes mellitus and periodontal disease have an increasing incidence in the whole world. Development of periodontopathy is related to the aggression of bacterial flora in dental plaque, flora that is influenced on its turn by the evolution of diabetes mellitus. In our study, we have evaluated the inflammatory reaction in periodontium in patients with slowly and progressive periodontitis in patients with diabetes mellitus who had diabetes longer than five years. It has been found that all patients presented a chronic inflammatory infiltrate, abundant, with round mononuclear cells of lymphocyte, plasma cells and macrophage type, with non-homogenous arrangement, more intensely where the covering epithelium presented erosions or necrotic areas. Out of the immunity system cells, the most numerous where of T-lymphocytes type.

Keywords: periodontopathy, diabetes mellitus, chronic inflammatory reaction, lymphocytes, macrophages.

Introduction

Diabetes mellitus (DM) is the most frequent chronic endocrine disorder, non-transmissible, characterized by disorders of the entire metabolism, especially of carbohydrate metabolism. Clinical-statistical studies estimate that about 2–6% of the Occidental Europe and North America population suffers from this disease.

In the past 20 years, the association of diabetes mellitus with periodontal diseases received the greatest attention, because the two clinical entities reciprocally influence and aggravate the patient's health condition. Thus, many researchers [1–3] assert that periodontal disease is the a sixth complication of diabetes mellitus, while Taylor GW and Borgnakke WS (2008) [4] have proved that periodontal disease can increase the risk of difficult metabolization of carbohydrates and the occurrence of diabetes, being a risk factor for patients with diabetes mellitus.

Different studies have shown that the prevalence, ampleness, graveness and progression of periodontal disease are increased in patients with DM [5]. On the other hand, prospective studies have proved that periodontopathies in patients with diabetes mellitus is associated with a worse metabolic control of diabetes mellitus and with a bigger number of diabetic, chronic complications [4, 6]. Other studies suggested that the appropriate treatment for periodontal disease in patients with diabetes mellitus may be beneficial in reducing diabetic complications [7–9].

This bidirectional relation between periodontal disease and diabetes mellitus determine diabetes to be a disease with a major importance for stomatologists, in taking certain therapeutic decisions. Because of this reasons, we have proposed to evaluate in this study the inflammatory reaction in the periodontal connective tissue in patients with periodontopathies and diabetes mellitus.

Materials and Methods

The study has been carried out on fragments of periodontium taken from 38 patients, aged between 45 and 72 years, diagnosed with diabetes mellitus type II for a period longer than five years, who presented either slowly progressive periodontitis (16 patients) or rapidly progressive periodontitis (22 patients). Patients with superficial chronic marginal periodontopathy presented odontal and periapical lesions, irrecoverable through conservatory methods and needed extractions or surgical interventions, while those with rapidly progressive periodontitis have presented high dental mobility,

associated or not with periodontal abscesses, reason for which the extraction and alveolar curettage was performed. On surgical interventions, there have been taken fragments of periodontium, which have been immediately fixed in solution of 10% neutral formalin and then processed by wax embedding. There have been carried out seriated sections with thickness of 4 μm, at the paraffin microtome (Microm HM350) equipped with system of transfer of sections on water bath, which have been displayed on simple histology slides or on slides covered with poly-L-lysine (for immunohistochemical studies). For the histological study, the sections were stained with Hematoxylin–Eosin and trichromic Goldner–Szekely; for the immunohistochemical (IHC) study, after paraffin removal and hydration has been carried out the antigenic recovery by boiling in solution of sodium citrate, respectively EDTA seven cycles of 3 minutes at 650 W, after which have been treated with 2% solution of hydrogen peroxide for 30 minutes for the inhibition of endogenous peroxidase. After washing in solution of phosphate buffer (PBS) has been carried out the blocking of unspecific relations with 2% skimmed milk for 30 minutes. Then, the sections have been incubated with the primary antibody for 18 hours at 4^0C, then has been applied the EnVision (Dako) detection system. At the end, the signal has been detected by using 3,3'-diaminobenzidine (DAB, Dako) and the nuclei were counterstained with Mayer's Hematoxylin.

The analysis of the histological preparations has been carried out at Nikon Eclipse 55i Microscope (Nikon, Apidrag, Romania) equipped with automatic system of exposure and 5-megapixels camera. The images have been captured and archived using the Imagine ProPlus 7 AMS soft (Media Cybernetics Inc., Buckinghamshire, Great Britain).

For the immunohistochemical study, we have used the following markers (Table 1).

Table 1 – ***Description of the antibodies utilized***

Markers	Producer	Clonality	Clone	Specificity	Optimal dilution	Antigen recovery
CD3	Dako	IgG1k	MIB-1	T-lymphocytes	1:100	Boiling CB, pH 6
CD20	Dako	IgG2b k	DO-7	B-lymphocytes	1:50	EDTA, pH 8
CD68	Dako	IgG1	KP1	Macrophages	1:100	Boiling CB, pH 6

Results

The histological and immunohistochemical study of periodontium in patients with chronic periodontopathy and diabetes mellitus allowed us to remark in the connective tissue of periodontium, in all cases comprised in the study, the presence of a chronic inflammatory infiltrate with round mononuclear cells of lymphocyte, plasma cells and macrophage type. Very rarely, have been also identified neutrophil polymorphonuclear leukocytes, cellular elements characteristic to acute inflammation.

The intensity of inflammatory reaction has been different from a patient to another and even from an area to another of the periodontium, at the same patient. Mostly, the inflammatory infiltrate has been abundant, with heterogeneous arrangement, diffuse, both in the superficial periodontium and in the profound one (Figure 1). Sometimes, the arrangement of immune system cells has been of insular type, where the inflammatory infiltrate was separate from the connective tissue of periodontium, especially in collagen fibers (Figure 2). Frequently, the inflammatory reaction has been accompanied by vascular modifications, which have varied from congestion of small vessels to micro-hemorrhages (Figure 3). Also, has been frequently noticed that the reaction of immune system cells has been accompanied by a moderate connective reaction manifested by the multiplication of fibroblastic type cells and formation of collagen fibers with inordinate arrangement. In other areas, on the contrary, we have found a reduction of collagen fiber in the gum' ligaments, aspect resulted probably by action of metalloproteinases secreted by the immune system cells (Figure 4).

In patients where the covering epithelium presented erosions or patchy necroses, the inflammatory infiltrate has been very abundant. Epithelial necrosis mostly involved the junctional epithelium located in the gingivodental canal. This epithelial area is less adapted to mechanical stress and to action of bacterial film, having a particular structure, thinner than the rest of the gingival epithelium, being constituted of basal type cells arranged on a single row and several rows of sub-basal cells, elongated, arranged parallel to dental surface. The analysis of histological preparations allowed us to remark the fact that the gingivodental canal represents the most vulnerable area to action of microbial agents in the dental plaque.

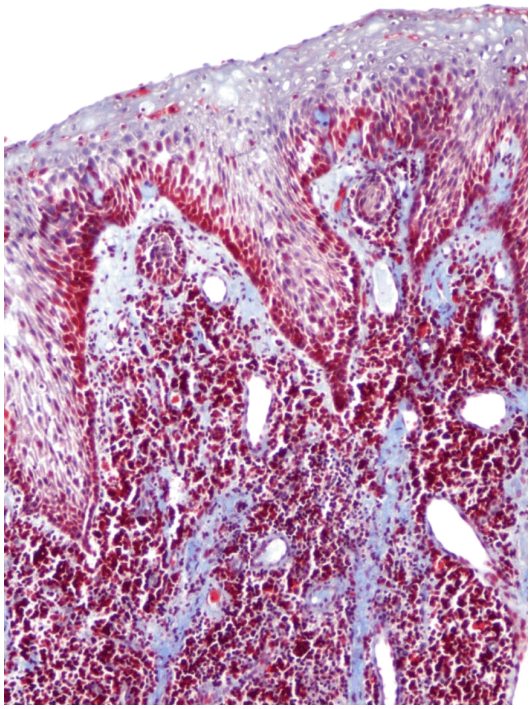

Figure 1 – ***Overview of the periodontium containing a chronic inflammatory infiltrate with non-homogenous arrangement (trichromic Goldner–Szekely technique, ×100).***

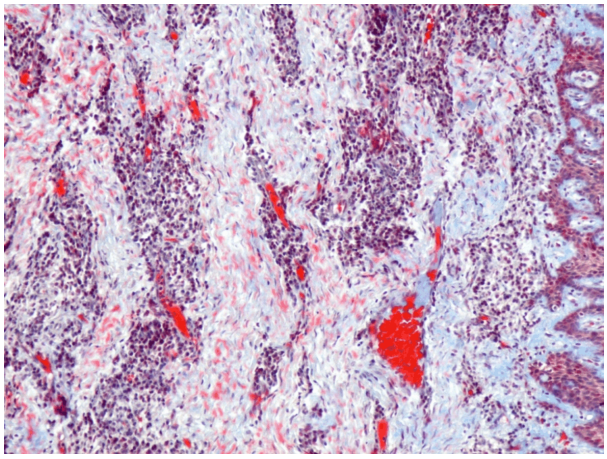

Figure 2 – ***Chronic inflammatory infiltrate with focal arrangement associated with moderate vascular congestion (trichromic Goldner–Szekely technique, ×100).***

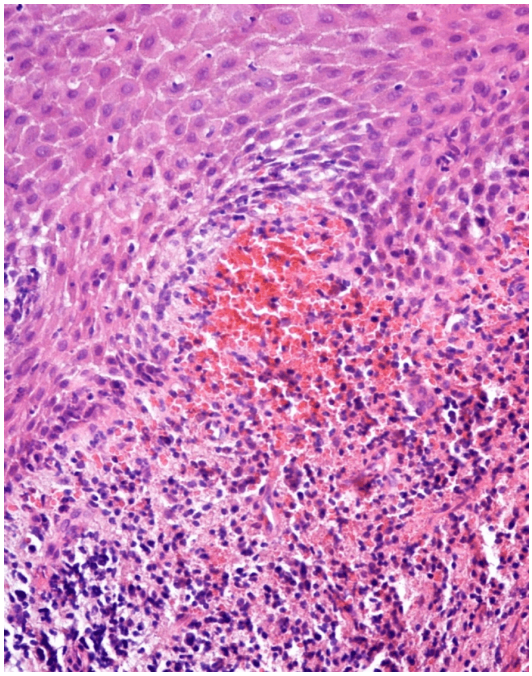

Figure 3 – ***Periodontium with chronic inflammatory infiltrate associated with micro-hemorrhages (HE stain, ×100).***

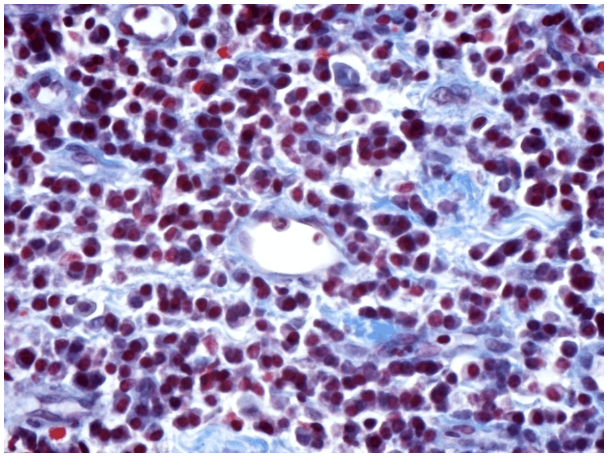

Figure 4 – ***Periodontium strongly infiltrate with lymphocytes and plasma cells (trichromic Goldner–Szekely technique, ×400).***

In the immunohistochemical study, we tried to emphasize the reaction of T-lymphocytes, B-lymphocytes and macrophages' cells, when constituting the inflammatory infiltrate in chronic periodontopathies in patients with diabetes mellitus.

In order to emphasize **T-lymphocytes**, we used anti-CD3 antibody, which is specifically related to CD3 protein complex within the cellular membrane of T-lymphocytes. CD3 antigen is presented in the membrane of all mature T-cells and natural killer lymphocytes (NK) being absent in other types of lymphocytes.

In our study, we have noticed that T-lymphocytes have been arranged in the connective structure of the periodontium, most of the cells being identified under the covering epithelium and around the blood vessels (Figure 5). In the areas where the covering epithelium presented erosions or necroses, the inflammatory infiltrate has been much richer in T-lymphocytes then in the rest of the connective tissue of periodontium. Also, various T-lymphocytes have been noticed in the structure of covering epithelium, not only among the cells of the basal layer, but also among the superficial layers (Figure 6). The occurrence of T-lymphocytes in a large number in the structure of covering epithelium of periodontium is a proof of the positive chemotactic activity of antigens in the oral cavity over the cells of immune system, but also of the own mobility capacity of T-lymphocytes.

Emphasis of **B-lymphocytes** in the periodontium has been made by using anti-CD20 antibody. CD20 is a non-glycosylated phosphoprotein expressed by mature and malign B-lymphocytes which regulate the transmembranar guidance of calcium (probably functioning as a component of calcium channel) and occurs in the progression of cell cycle and proliferation of B-cells. In contrast with T-lymphocytes, B-lymphocytes have been relatively reduced in the inflammatory infiltrate of the periodontium connective tissue, diffusely distributed, being identified rare cell elements disposed in a perivascular manner or at the level of papillary chorion (Figure 7). Although their distribution has been totally non-homogenous, the reaction of B-lymphocytes in the periodontium has been correlated with the local inflammatory reaction, in the sense that if the inflammatory process has been intense, also the number of B-lymphocytes increased. Thus, in patients where covering epithelium presented erosions and discontinuities, the inflammatory reaction has been intense but the number of B-lymphocytes increased (Figure 8).

The appreciation of reaction of **macrophage-type cells** at the level of periodontium in chronic periodontopathies associated with diabetes mellitus has been carried out by immunohistochemistry techniques, using anti-CD68 antibody. CD68 antigen is a glycolysed transmembranar protein, specifically expressed by tissue macrophages, Langerhans cells and in smaller quantities of even dendritic cells. As is noticed in our images, macrophage-type cells have been non-homogenously spread in connective tissue of the inflammed periodontium. This microscopic aspect suggests the fact that

the intensity of inflammatory reaction in periodontopathies is variable from an area to another, probably depending on the quantity of antigens presented at that level (Figures 7 and 8).

The quantitative and qualitative analysis of immune system cells, presented in the periodontal inflammatory infiltrate, allowed us to assert that T-lymphocytes have been the most representative cells of the immune system in periodontopathies associated with diabetes mellitus, as compared to B-lymphocytes and macrophages.

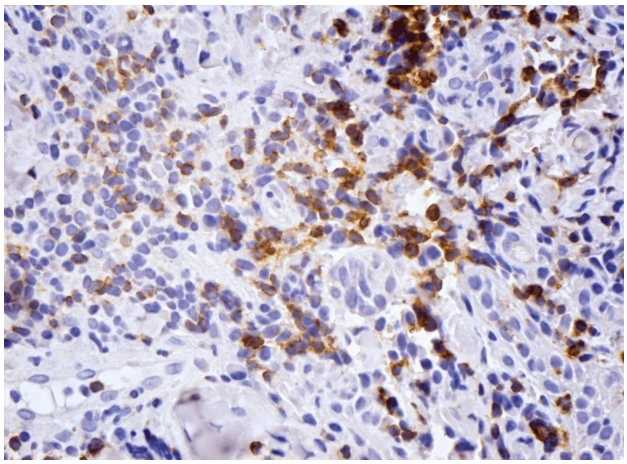

Figure 5 – ***Periodontal chronic inflammatory infiltrate with numerous CD3-positive T-lymphocytes, heterogeneously disseminated (LSAB technique, ×200).***

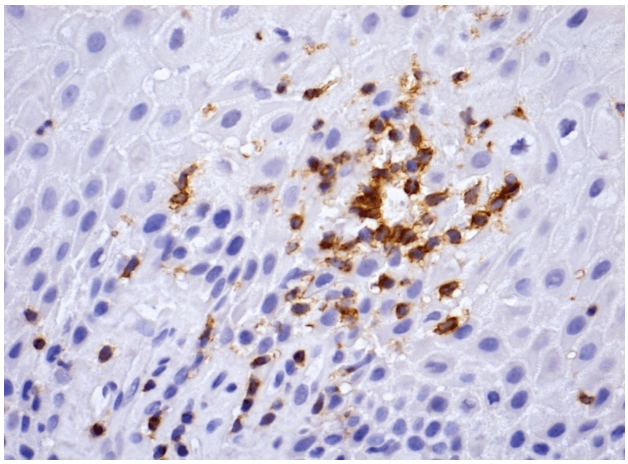

Figure 6 – ***Microscopic image with numerous T-lymphocytes in the covering epithelium, with positive immunostaining for CD3 (LSAB technique, ×200).***

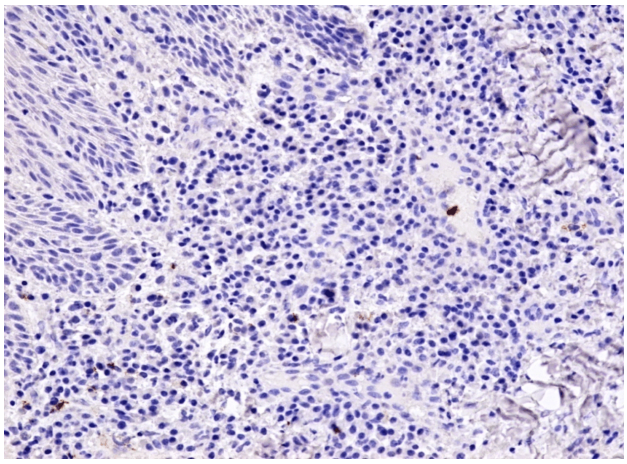

Figure 7 – ***Microscopic image of rare B-lymphocytes with perivascular arrangement or within papillary chorion, with positive immunostaining for CD20 (LSAB technique, ×100).***

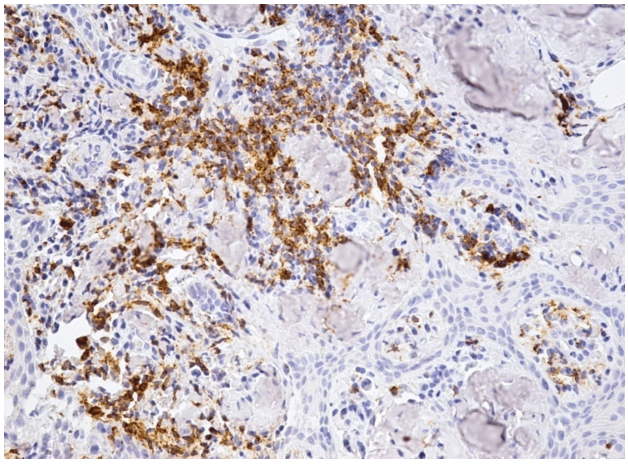

Figure 8 – ***Area of periodontium with erosions of the covering epithelium and with intense reaction of B-lymphocytes with positive reaction for CD20 (LSAB technique, ×100).***

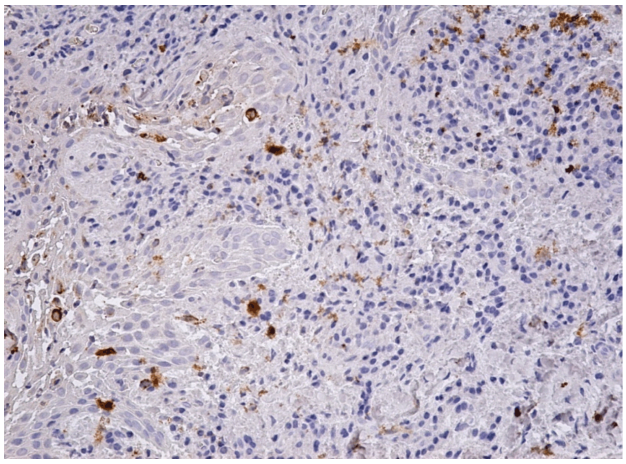

Figure 9 – ***Chronic inflammatory infiltrate with macrophage cells, non-homogenously disseminated within the periodontium, with positive immunoreaction for CD68 (LSAB technique, ×100).***

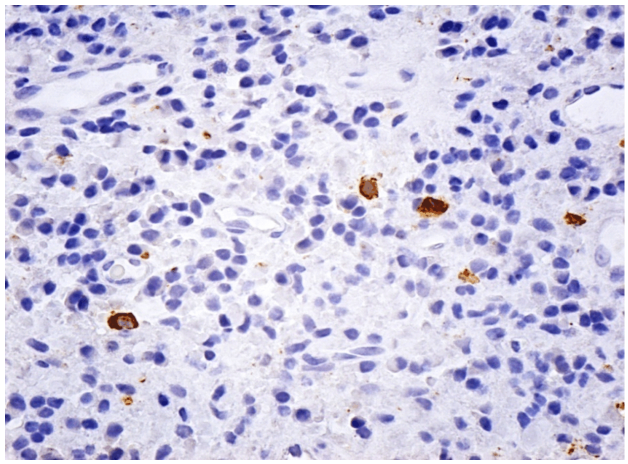

Figure 10 – ***Chronic periodontopathy with rare CD68-positive cells (macrophages) disposed in the superficial chorion (LSAB technique, ×200).***

Discussion

The association of diabetes mellitus and periodontal diseases (such as gingivitis and periodontosis) has received the most attention in the last 20 years in stomatological medical practice, the periodontal disease being considered a major complication, which determines an unfavorable evolution of diabetes mellitus. On its turn, it seems that diabetes favors the occurrence of periodontopathy by an inflammatory exaggerate response to the microflora within oral cavity.

Various studies have shown that periodontopathy in patients with diabetes mellitus may be associated with a worse metabolic control of diabetes mellitus and with a bigger number of diabetic chronic complications [4, 6]. Other studies have suggested that the appropriate treatment of periodontal disease in patients with diabetes mellitus may be beneficial to reduce diabetic complications [7, 9].

In our study, we have tried to evaluate the inflammatory reaction in patients with periodontopathy and diabetes mellitus. We have shown that the immune reaction of periodontium in patients with diabetes mellitus is characterized by the presence of a chronic inflammatory infiltrate, abundant, mainly formed of lymphocytes, plasma cells and macrophages. Graves DT *et al.* (2006) [10], when reviewing the pathogenesis of periodontal disease in patients with diabetes mellitus, concluded that, besides a strong inflammatory response, the cell apoptosis may contribute to the occurrence of periodontopathy. Consequently, the strong inflammation associated with an intense cell apoptosis may lead to the destruction of periodontal tissues.

Other authors [11] have shown that patients with diabetes mellitus had a particular immune response, characterized by the increase of some interleukins (IL-6 and IL-1β) which, together with alpha factor of tumor necrosis (TNF-α) and prostaglandin E2 (PGE2), could act by stimulating the periodontal inflammatory response. Other studies confirmed that patients with diabetes mellitus and periodontopathies have an increased production of inflammatory mediators in gingival tissues, as compared to non-diabetic patients. These mediators may contribute to the pathogenesis of periodontal diseases and the alteration of healing process [12, 13].

Determination in blood of some inflammatory mediators, such as TNF-α and IL-6 in diabetic patients also had increased concentrations when have been associated with chronic periodontopathies [14–16].

We consider that the periodontal inflammatory process has been triggered by the penetration of microbial microflora from the oral cavity at the level of periodontium, because of the particular conditions created by the excess of glucose or the products resulted from its incomplete metabolization. The exacerbation of microbial microflora in the oral cavity, associated with a defective local hygiene, may determine the necrosis of the covering epithelium, especially the epithelium in the gingivodental canal, which opens the path of pathogen agents to penetrate in lamina propria and the occurrence of the inflammatory process.

Ohlrich EJ *et al.* (2010) [17] have shown that the immune response against periodontal pathogen agents may be altered in diabetes, fact which could lead to the exaggerate multiplication of certain microbial species. In the oral cavity, by molecular techniques, have been identified more than 700 bacterial species or phenotypes [18]. Out of these, *Porphyromonas gingivalis* is one of the most incriminated bacteria in etiopathogeny of periodontal disease. This bacteria was identified 37.5% in subgingival plaque and 32.5% in the cells of buccal epithelium in health patients and 69.23% in gingival plaque, respectively 46.15% in the cells of buccal mucosa in patients with periodontitis [19].

The immunohistochemical study allowed us to notice the fact that the most numerous cells in the inflammatory infiltrate have been T-lymphocytes. These cells carry out several functions within the immune response: lyses the cells that express no-self molecules on their surface, regulates the immune response, mediates the reactions of delayed hypersensitivity, synthesizes the most part of lymphokines, stimulates the differentiation of B-lymphocytes towards plasma cells, etc. Inflammatory cytokines such as IL-1, IL-6 and TNF-α also play an important role in carbohydrate and lipid metabolism and for this reason has been suggested the fact that the inflamed periodontium could act as a source of endocrine type of inflammation mediators and could determine the consecutive increase of resistance to insulin [20].

In respect of the reaction of monocyte-macrophage system cells, in patients with chronic periodontopathy and diabetes mellitus, we have found that, in general, the number of this type of cells has been reduced, as compared to that of lymphocytes, and the arrangement has been totally non homogenous. According to other authors [17], monocytes-macrophages suffer alterations in diabetic patients, exacerbating the progression and the severity of periodontal disease. Salvi GE *et al.* (1997) [21] have substantiated that the monocytes in diabetic patients have a production of TNF-α from 24 up to 32 times bigger, as compared to non-diabetic control subjects. The same group of researchers has also emphasized the increased concentrations of IL-1β and PGE2 in monocyte cultures in type 1 diabetic patients as compared to non-diabetic patients with the same stage of periodontal disease.

We consider that macrophages have more complex roles in the process of tissue recovery. Their reaction depends on the coordination with the other cells of the immune system and with the connective cells in the periodontal tissue. Their main function is to phagocytize the cell rests following the microbial aggression.

Conclusions

Chronic periodontopathies in patients with diabetes mellitus have been characterized by the presence in the periodontal connective tissue of an abundant chronic inflammatory infiltrate, formed of lymphocytes, plasma cells and macrophages. Out of the cells of the immune system, the most numerous have been T-lymphocytes.

References

[1] Löe H, *Periodontal disease. The sixth complication of diabetes mellitus*, Diab Care, 1993, 16(1):329–334.

[2] Lalla E, Lamster IB, Feit M, Huang L, Spessot A, Qu W, Kislinger T, Lu Y, Stern DM, Schmidt AM, *Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice*, J Clin Invest, 2000, 105(8):1117–1124.

[3] Pontes Andersen CC, Flyvbjerg A, Buschard K, Holmstrup P, *Relationship between periodontitis and diabetes: lessons from rodent studies*, J Periodontol, 2007, 78(7):1264–1275.

[4] Taylor GW, Borgnakke WS, *Periodontal disease: associations with diabetes, glycemic control and complications*, Oral Dis, 2008, 14(3):191–203.

[5] Mealey BL, Oates TW; American Academy of Periodontology, *Diabetes mellitus and periodontal diseases*, J Periodontol, 2006, 77(8):1289–1303.

[6] Thorstensson H, Kuylenstierna J, Hugoson A, *Medical status and complications in relation to periodontal disease experience in insulin-dependent diabetics*, J Clin Periodontol, 1996, 23(3 Pt 1):194–202.

[7] Arrieta-Blanco JJ, Bartolomé-Villar B, Jiménez-Martinez E, Saavedra-Vallejo P, Arrieta-Blanco FJ, *Bucco-dental problems in patients with diabetes mellitus (I): index of plaque and dental caries*, Med Oral, 2003, 8(2):97–109.

[8] Mansour AA, Abd-Al-Sada N, *Periodontal disease among diabetics in Iraq*, MedGenMed, 2005, 7(3):2.

[9] Nelson RG, *Periodontal disease and diabetes*, Oral Dis, 2008, 14(3):204–205.

[10] Graves DT, Liu R, Alikhani M, Al-Mashat H, Trackman PC, *Diabetes-enhanced inflammation and apoptosis – impact on periodontal pathology*, J Dent Res, 2006, 85(1):15–21.

[11] Sims TJ, Lernmark A, Smith T, Page RC, Persson GR, *Treatment outcome for IDDM patients in relation to glutamic acid decarboxylase autoantibodies and serum IgG to periodontal pathogens*, J Clin Periodontol, 2001, 28(6):550–557.

[12] Nishimura F, Takahashi K, Kurihara M, Takashiba S, Murayama Y, *Periodontal disease as a complication of diabetes mellitus*, Ann Periodontol, 1998, 3(1):20–29.

[13] Stewart JE, Wager KA, Friedlander AH, Zadeh HH, *The effect of periodontal treatment on glycemic control in patients with type 2 diabetes mellitus*, J Clin Periodontol, 2001, 28(4):306–310.

[14] D'Aiuto F, Parkar M, Andreou G, Suvan J, Brett PM, Ready D, Tonetti MS, *Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers*, J Dent Res, 2004, 83(2):156–160.

[15] Havemose-Poulsen A, Sørensen LK, Stoltze K, Bendtzen K, Holmstrup P, *Cytokine profiles in peripheral blood and whole blood cell cultures associated with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis*, J Periodontol, 2005, 76(12):2276–2285.

[16] Engebretson S, Chertog R, Nichols A, Hey-Hadavi J, Celenti R, Grbic J, *Plasma levels of tumour necrosis factor-alpha in patients with chronic periodontitis and type 2 diabetes*, J Clin Periodontol, 2007, 34(1):18–24.

[17] Ohlrich EJ, Cullinan MP, Leichter JW, *Diabetes, periodontitis, and the subgingival microbiota*, J Oral Microbiol, 2010, 2:1–8.

[18] Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE, *Defining the normal bacterial flora of the oral cavity*, J Clin Microbiol, 2005, 43(11):5721–5732.

[19] Pan CL, Pan YP, Lin L, Zhao J, Zhang DM, *Detection of Porphyromonas gingivalis in buccal epithelial cells and subgingival plaque*, Hua Xi Kou Qiang Yi Xue Za Zhi, 2005, 23(5):377–379.

[20] Preshaw PM, Foster N, Taylor JJ, *Cross-susceptibility between periodontal disease and type 2 diabetes mellitus: an immunobiological perspective*, Periodontol 2000, 2007, 45:138–157.

[21] Salvi GE, Collins JG, Yalda B, Arnold RR, Lang NP, Offenbacher S, *Monocytic TNF alpha secretion patterns in IDDM patients with periodontal diseases*, J Clin Periodontol, 1997, 24(1):8–16.

Corresponding author

Adrian Camen, University Assistant, PhD, Department of Oral and Maxillo-Facial Surgery, University of Medicine and Pharmacy of Craiova, 2–4 Petru Rareş Street, 200349 Craiova, Romania; Phone +40251–522 458, Fax +40251–593 077, e-mail: adycamen@yahoo.com

Received: November 25th, 2011

Accepted: February 27th, 2012