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RESPONSE OF ORAL MUCOSA TO CONTACT WITH CLASS 4 TITANIUM

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Abstract

Although titanium dental implants are characterized by great biocompatibility, both electrochemical and galvanic corrosion may take place in the oral environment, even in the cases of full osseointegration of implants. The aim of the study was to evaluate processes occurring in the gingival mucous membrane collected from dental implants after a period of healing. In the gingival tissues in contact with implants fully integrated with the bone, infiltrations composed of subpopulations of T lymphocytes (CD45R0 and CD25) and Langerhans cells (S-100 positive) were found. The presence of immunologically competent cells in the infiltrations indicated that the titanium implant was recognized by the host's immune system. The lack of clinical symptoms of hypersensitivity may suggest local tolerance to a correctly healed intraosseous dental implant.

Key words: titanium implants, local tolerance, hypersensitivity.

REAKCJA BŁONY ŚLUZOWEJ JAMY USTNEJ NA KONTAKT Z TYTANEM KLASY IV

Abstrakt

Pomimo że tytanowe implanty zębowe charakteryzują się wysoką biokompatybilnością, to w środowisku jamy ustnej, nawet w przypadkach pełnej osteointegracji implantów, może dochodzić zarówno do elektrochemicznej, jak i galwanicznej korozji. Celem pracy była ocena procesów zachodzących w błonie śluzowej dziąsła pobranej znad implantów po okresie ich wgajania. W tkankach dziąsła kontaktujących się z w pełni zintegrowanymi z kością implantami wykazano nacieki złożone z subpopulacji limfocytów T (CD45R0 i CD25) oraz komórek Langerhansa (S-100 pozytywnych). Obecność komórek immunologicznie kompe-

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tentnych w naciekach świadczy o rozpoznaniu tytanowego wszczepu przez układ immunologiczny gospodarza. Brak klinicznych objawów nadwrażliwości może sugerować wytworzenie miejscowej tolerancji na prawidłowo wgojony śródkostny wszczep stomatologiczny.

Słowa kluczowe: implanty tytanowe, tolerancja miejscowa, nadwrażliwość.

INTRODUCTION

Although titanium is thought to be highly biocompatible, an increasing number of reports suggest its various local effects and even a negative influence on patients' general health as well as disruptions during a healing processes. (Thomas et al. 2006, Nawaz et al. 2007). *In vitro* and *in vivo* studies show that despite the passive activity of the external layer of oxides, both electrochemical and galvanic corrosion may occur in the environment of the oral cavity. Thus, titanium ions causing discolouration of gingiva, oedema, *gingivitis stomatitis*, skin rash, erythema as well as delayed healing may be released (Chaturvedi 2009, Koike et al. 2001). The low pH and the high concentration of fluorine ions damage the protective layer of oxides, leading to disorders in the osseointegration process and to changes in soft tissues (Chaturvedi 2009). Numerous macrophages with the cytoplasm containing titanium particles were found in the gingiva surrounding implants that were lost as a result of fracture or a loss of osseointegration. It is believed that the process of phagocytosis may stimulate macrophages to release inflammatory mediators, which lead to activation of osteoclasts and, in consequence, to bone resorption (Olmendo et al. 2003).

Sporadic cases of hypersensitivity in the oral cavity are explained by asmaller number of dendritic cells in the mucosa and lower permeability of mucous membrane in comparison with the skin (Bass et al. 1993, Schramm et al. 2000). Moreover, it is estimated that in order to cause a hypersensitive response in soft tissues, 5-12-fold greater exposure to allergens is necessary. On the other hand, the glycoproteins included in saliva form a protective barrier on the surface of a titanium implant that prevents the direct contact of the metal with the mucosa (Thomas 2000, Bass et al. 1993).

The processes observed in soft tissues have been diagnosed and documented on biopsy material almost exclusively in cases of failure of the implantological treatment (Gislason et al. 2004, Piatelli et al. 1998). There is no evidence demonstrating that in patients with full implant integration and without any pathological clinical symptoms, changes in the gingival mucosa could prove the release of titanium ions.

Aim of the study

The aim of the study has been to analyzed changes in soft tissues of the oral mucosa which may result from the contact with a titanium intraosseous implant during the period of osseointegration.

MATERIAL AND METHODS

The studies were conducted on clinical material collected from 15 patients, including 9 women and 6 men aged 40-69 (mean age 52). For this study, two-stage intraosseous screw implants: Osteoplant-Hex (PL), Neoss (USA), Ilerimplant (E), made of class 4 titanium were inserted (Table 1). In total, 43 implantations were carried out, including 25 procedures in the maxilla and 18 in the mandible. In the first stage of the implantation, control

Table 1

Description of research group

Number of patients	Sex F/M	Age	Place of implantation Maxilla/mandible	Number of implants	Implantation system
15	9/6	52	9/6	43	Osteoplant-Hex Neoss Illerimplant

material was collected from a patient before inserting the intraosseous part, during the preparation of the mucous lobe on the alveolar ridge, from the area of the planned implant bed. After 4-6 months of osseointegration, during the procedure of implant exposure, proper material for the study was collected from the same patient, including fragments of mucosa in direct contact with the titanium intraosseous implant during its healing period. In all the cases, clinical and radiological examination confirmed complete implant osseointegration. After fixing in Bouin's solution (picric acid + formaldehyde + acetic acid, 6 h, room temperature), fragments of the gingival mucosa were routinely dehydrated and immersed in paraffin. 5 μm thick cross-sections of the mucous membrane were placed on microscope slides. 5160 paraffin fragments placed on 1290 slides were prepared for morphological studies.

First, an analysis of the structure of the gingival mucosa was conducted so that which features changing independently of the conditions (before and after contact with an implant) or changing on consecutive cross-sections of a given fragment were excluded. In further examinations, the occurrence of cell clusters over a surface above 0.01 mm^2 was evaluated with an aid of the software programme MicroImage, v. 4.0 (Olympus, MS Windows 98)

coupled with the light microscope Olympus BX 50. Individual types of cells in the clusters were identified according to the expression of typical markers using the immunocytochemical technique ABC (Table 2).

Non-parametric Wilcoxon and Mann-Whitney tests were applied for statistical analysis. The correlation significance was checked with Student's *t*-test. The hypotheses were verified at $p < 0.05$. The statistical analysis of the results was performed with the use of Statistica v.6.1 (Statsoft, Inc.).

Table 2

Panel of antibodies (Ab) recognising surface or intracellular antigens (Ag) in gingival mucosa cells and commercial kits used in the immunocytochemical studies

Antibody (Ab)	Antigen (Ag)	Company	KIT	Labelled cell type
Rabbit N1573	S-100	Dako	Dako Cytomation LSAB2 System-HRP	Dendritic cells
Mice UCHL1	CD45RO	Novum Biologicals	Vectastain Elite ABC Kit	T lymphocytes
Mice 4C9	CD25	NovoCastra	Vectastain Elite ABC Kit	T lymphocytes
Mice HM47/A9	CD79a	NovoCastra	Vectastain Elite ABC Kit	B lymphocytes, plasmatic cells

RESULTS

In the mucosa from above the titanium implants, attention was drawn to the occurrence of cell infiltrations located in the subepithelial layer and in the neighbouring region of the epithelium (Figure 1). As shown in Figure 2, changes of this type were found in 11 patients, while in 6 patients they were observed only on single cross-sections. In 5 patients, the surface of the infiltrations was several times larger, and in 2 of these patients, cell infiltrations were found in the comparative material as well, and their surface increased statistically significantly after contact with the implant ($p < 0.05$).

The occurrence of the infiltrations did not depend on the type of implants or the number of implanted pillars in a given patient. In patients with more implants, infiltrations were observed next to all implants, although they differed in size. Detailed analysis of subsequent cross-sections of the mucosa collected above the implants allowed us to trace mononuclear cells which migrated from the subepithelial infiltrations to the epithelium, populating its successive layers. Within the epithelium, a significant number of mononuclear cells concentrated on a small area.

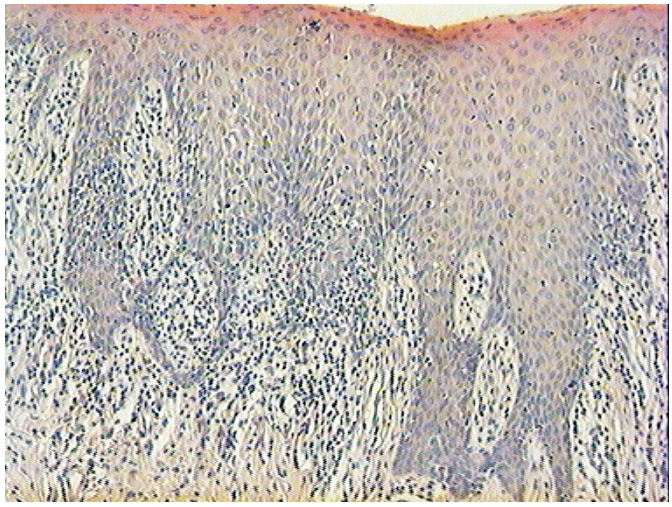

Fig. 1. Fragment of mucosa collected above an implant. Abundant infiltrations of mononuclear cells in the subepithelial layer infiltrating the epithelium visible. H+E. Magnification x 25

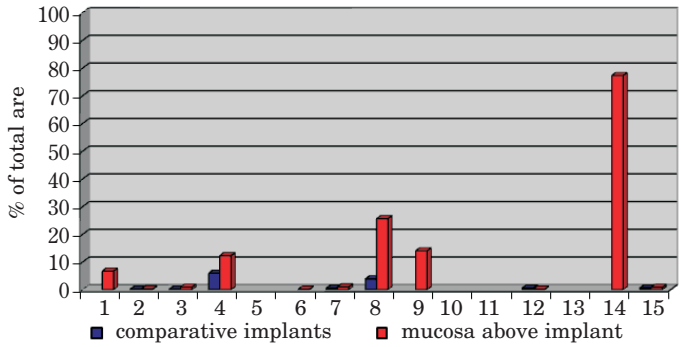

Fig. 2. Occurrence of cell infiltrations in individual patients (marked with consecutive numbers) and their area in the mucosa after contact with an implant and in comparative material

On all the cross-sections, a statistically significant (at $P < 0.05$) increase in the number of Langerhans cells was found in the epithelium of the mucosa covering the intraosseous implants, expressed by their great density 51 (± 30.9) per mm$_{.}$ in relation to the comparative material. The density of S-100 positive cells per unit of an epithelium area rose several times in most patients (Figure 3).

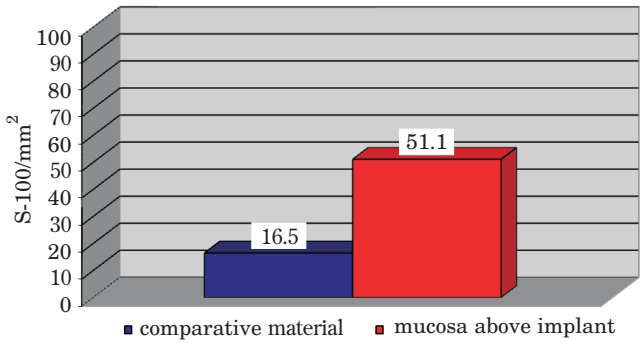

Fig. 3. Distribution of Langerhans cells density (S-100 positive) in mucosal epithelium after direct contact with an implant and in comparative material

In the material collected above implants, changes in the distribution of the investigated cells were also found within the epithelium, where many of the mononuclear cells creating characteristic clusters were S-100 positive. It should be emphasized that within these groupings, positive reaction cells made pairs with S-100 negative mononuclear cells of a lymphoid type. A statistically significant ($P < 0.05$) increase in S-100 positive cells both in the epithelium and in the subepithelial layer of the mucous membrane above the implant in relation to the comparative material collected from these patients is noteworthy. Furthermore, concentration of a considerable number of S-100 positive cells in the subepithelial zone in the infiltrations is a characteristic finding. However, no correlation between an increase in the number of S-100 cells and an area of the infiltrations was found.

The morphometric analysis demonstrated the distribution of cell types found in the subepithelial infiltrations. In all the analysed cases, most of the cells within the infiltrations were CD45-positive. In five cases evaluated in detail, the percentage of CD45RO cells increased significantly from 45.7% in the comparative material to 81.3% after contact with an implant (Figure 4). In the same patients, the average percentage of CD25-positive cells also increased from 21% to 42.9% (Figure 5). It was noticed that CD45RO- and CD25-positive cells were the most numerous group of cells in the infiltrations within the epithelium, beside S-100 positive cells. In the material collected above an implant, B lymphocytes and plasmatic cells were usually found among cells creating small clusters around vessels, and only single ones were dispersed in the connective tissue.

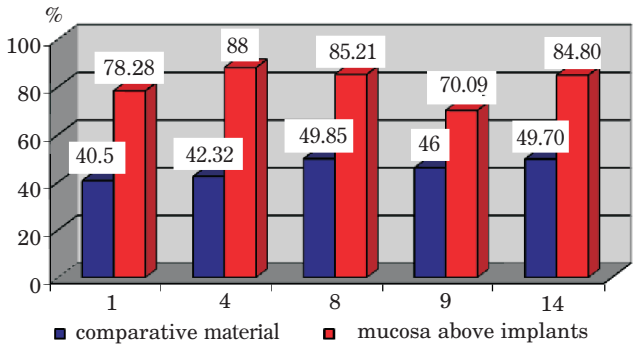

Fig. 4. Presence of CD45RO-positive T lymphocytes in cell clusters in the group of the patients with infiltrations in the mucosa above implants and in comparative material

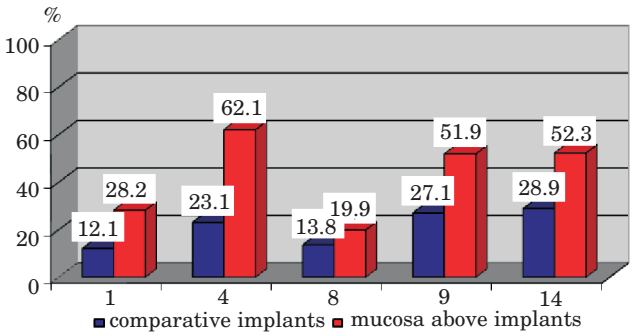

Fig. 5. Presence of CD25-positive T lymphocytes in cell clusters in the group of the patients with infiltrations in the material after contact with an implant and in comparative material

DISCUSSION

During the research, in most patients cell infiltrations located in the subepithelial region infiltrating the epithelium were found in the mucosa collected above implants. The infiltrations were tentatively defined as cell clusters covering an area above 0.01 mm¸ of a cross-section. In most cases, such cell infiltrations occurred on single cross-sections, which limited the possibility of further research by immunocytochemical methods. For this reason, detailed phenotypic analysis of cells in infiltrations was only possible in 5 cases in which the infiltrations appeared on consecutive cross-sections. In the other cases, B or T lymphocytes and Langerhans cells were present in infiltrations or in their direct vicinity.

On the basis of a semi-quantitative analysis, it was determined that CD45R0-positive T lymphocytes were the dominant cell population in the infiltrations. Moreover, in the same infiltrations, a considerable percentage of cells included a subpopulation of T lymphocytes with the CD25 expression, accompanied by Langerhans cells. It is noteworthy that together as the number of T lymphocytes in infiltrations in the subepithelial zone increased, so did the count of Langerhans cells, which may suggest that this is a "cumulative area" for cells migrating to the lamina propria of the gingival mucosa, from which these cells relocate further to the epithelium.

The direct contact of Langerhans cells with T lymphocytes observed within the epithelium indicates their interactions. Studies on dogs conducted by Pongnarisorn et al. pointed to the possible occurrence of cell infiltrations in gingival mucosa in cases of implants properly integrated with the bone, without any symptoms of inflammation. Similarly to the results presented in this study, some authors have demonstrated that T lymphocytes constituted the most numerous group of cells in the infiltrations. The occurrence of Langerhans cells was not taken into consideration in those studies (Pongnarison et al. 2007). In cases of inflammatory changes of *periimplantitis* type, B lymphocytes and plasmatic cells were considered to be the most abundant group (Gualini et al. 2003, Berglundh et al. 2004). As shown in the studies by Sanz et al., cell infiltrations constituted more than 65% of the total area of biopsy specimens in inflammatory processes occurring in the mucous membrane around titanium implants (Sanz et al.1991).

In recent years, attention has been drawn to the subpopulation of CD25+ immunoregulatory T lymphocytes which control the activity of T lymphocytes in response to organ-specific autoantigens (Itoh et al. 1999, Sun et al. 2006). As follows from the research on the phenomenon of tolerance by Cavani et al. (1998, 2000), the role of regulatory T lymphocytes consists in inhibiting the immunological response through secretion of Interleukin-10, which leads to a decrease in the extent of cell damage. T lymphocytes also have a direct influence on dendritic cells[1] . An increase in the number of CD25-positive T lymphocytes in the mucosa after contact with a titanium implant points to the well-known mechanism of these cells acting in the case of contact hypersensitivity to nickel. Nickel ions, similarly to ions of other metals such as chromium, cobalt, gold as well as titanium, are haptens which are capable of binding to different extracellular and intracellular proteins (Czarnobilska et al. 2007, Śpiewak et al. 2006). Research into the mechanism of reactions taking place locally shows that the protein + hapten complex is recognized by Langerhans cells, which transport it to neighbouring lymph nodes and present to CD4 and CD8 lymphocytes. These cells, then, migrate to the site of the hypersensitive reaction. CD8 T lymphocytes may locally exert cytotoxic influence on keratinocytes, inducing their apoptosis. When

[1]Cell family including Langerhans cells.

this happens, CD4 cells perform the role of regulatory cells, either stimulating or inhibiting the hypersensitive reactions (Büdinger et al. 2000, Saint-Mezard et al. 2004, Martin 2004).

Studies of the induction of tolerance to nickel drew attention to the role of CD4+CD25+ regulatory lymphocytes. In allergic patients, as well as in people without any symptoms of hypersensitivity, the presence of cells allergic to nickel was demonstrated. It was proven that clinical manifestation of hypersensitivity symptoms depended on the count of CD4+CD25+ cells and their activity (Cavani et al. 2003, Cavani 2005).

In vitro and *in vivo* studies show that despite the passive activity of the external layer of oxides, titanium ions are released as a result of electrochemical and galvanic corrosion in the oral cavity environment. Clinical manifestation of this phenomenon includes gingiva discolouration, oedema of soft tissues, *gingivitis*, *stomatitis*, skin rash, erythema, itch as well as delayed healing (Koike et al. 2007, Chaturvedi 2009). The presence of titanium ions was also revealed in soft tissues collected above implants correctly integrated with the bone, not accompanied by any clinical symptoms (Makuch et al. 2011).

Due to their characteristic image, the morphological changes occurring in the mucosa after contact with a titanium implant, observed in this study, point to an immunological process. The question arises whether these changes are a reflection of cell interactions inducing local tolerance in response to a titanium intraosseous implant. Such an assumption is undoubtedly supported by the increase in counts of Langerhans cells and T lymphocytes (CD45RO, CD25). Owing to a similar cell composition and the type of changes in the epithelial cells, the observed processes resemble a hypersensitive response to nickel. The lack of any clinical symptoms in patients subjected to implantation may be associated with a high increase in CD25-positive T lymphocytes in the mucosa collected above the implants. More detailed explanation of the processes occurring in soft tissues as a result of the contact with a titanium intraoseous implant requires further research on a larger group of patients.

CONCLUSIONS

1. In the healing process, cell infiltrations composed of a subpopulation of T lymphocytes and Langerhans cells appear in areas that are in contact with an implant.

2. The characteristic cell composition of the infiltrations within the gingival mucosa indicates that in areas of direct contact with an implant, processes of an immunological character take place.

3. The lack of clinical symptoms of hypersensitivity as well as the presence of immunologically competent cells may suggest that a titanium intraosseous implant is recognised by the immune system, with local tolerance being induced.

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