Cell populations involved in the processes of local mucosal defense in extended partially edentulous and completely edentulous patients. Clinical and immunohistochemical study

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Abstract

Considering the fact that extended partial edentation and complete edentation have high incidence rates worldwide, the necessity for correct prosthetic treatment is very important. We performed a clinical study on 37 extended partially edentulous patients and completely edentulous patients, who were divided into three groups. We also performed a morphological study using classic techniques of histology and immunohistochemistry methods on sections obtained from oral mucosa fragments collected from these patients and processed by paraffin embedding technique. To identify the cell populations present in the inflammatory processes, we used the CD20, CD8, CD3 and CD68 markers. In the studied cases, we found the presence of changes that have interested both the epithelium and lamina propria. Epithelium showed in particular epithelial hyperplasia aspects, with orthokeratinization and parakeratinization areas and, in some sections, areas of ulceration. We found the inflammatory process present in the lamina propria to be chronic and it consists in particular of lymphocytes, plasma cells and macrophages. This process was differentiated in intensity from one case to another, but varied even within the same case, from one area to another. Inflammation was determined by the local microbial flora enhanced by the action of prosthetic appliances or by the prolonged edentulous state. We observed more intense changes in denture wearers patients. The inflammatory response indicates the reactivity of the edentulous mucosa in response to local aggression, the specific defense mechanism coexisting with the nonspecific defense mechanism, with predominance of cellular immune defense.

Keywords: edentulous patients, oral mucosa, immune cells, inflammatory processes.

5 Introduction

Extended partial edentation and complete edentation have high incidence rates worldwide and the necessity for correct prosthetic treatment for these patients is obvious. The prosthetic therapy consisting of mobile partial and complete dentures is largely used in daily dental practice [1]. The mucosal component of the potential denture bearing area is an important factor for a successful prosthetic treatment.

The state of extended partial edentation and, especially, complete edentation is the cause for morphological and functional alterations of the potential denture bearing area. Another contributing factor is represented by the fact that the oral mucosa is a dynamic structure influenced by systemic pathology and by associated local and general therapy.

The prosthetic treatment of edentulous patients that require mobile partial and complete dentures is an important problem for the dental practice, as it plays a major role for each patient’s quality of life [2–4]. The therapeutic purpose is to replace the missing teeth and bone with efficient dentures that respect the biological and socio-cultural imperatives and that are easily integrated by the patient [5]. Prosthetic rehabilitation is influenced not only by the techniques and by the dental materials used, but also by the quality of the mucosal substrate of the potential denture bearing area, all these factors contributing to an improved denture retention and stability.

The edentulous patient should be actively involved in the denture treatment and should be aware of the importance of acceptance of the prosthetic treatment’s result – the dentures. However, the existence of an individual intellectual and physical limit of cooperation has to be taken into consideration, as well as the presence of certain general illnesses such as Parkinson, senility, which causes oral mucosa dehydration, arthritis and deafness, that complicate the prosthetic therapy [6, 7].

The morphological study of the mucosal component of the potential denture bearing area of edentulous patients not only is important, but is also necessary in the process of elaborating the most favorable prosthetic treatment plan. The prosthetic treatment of edentulous patients should be the result of a comparative analysis of biofunctional advantages and disadvantages and it should be individualized for each patient [8].

The purpose of this study is to analyze the correlations between macroscopic and microscopic (histological and immunohistochemical) aspects of the oral mucosa of the potential denture bearing area in extended partially edentulous and completely edentulous patients.
Materials and Methods

We conducted, in parallel, a clinical study and a morphological study, using both classic techniques of histology and immunohistochemistry.

Clinical study

Patients involved in this study were selected during the period May 2014–September 2014 from the patients of the Prosthetic Dentistry Clinic of the Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania. The total number of patients was 37, both men and women aged 52 to 79 years. Twenty-two were extended partially edentulous patients and 15 were completely edentulous patients. We obtained the informed consent from all the patients included in this study.

The patients were divided into the following groups: the first group consisted of 15 extended partially edentulous patients and completely edentulous patients, without alterations of the oral mucosa of the potential denture bearing area; the second group was formed of eight extended partially edentulous and completely edentulous patients with alterations of the oral mucosa of the potential denture bearing area (such as traumatic injuries, congestive alterations of the oral mucosa, oral mucosal hyperplasia), who were never denture wearers before; the third group consisted of 14 extended partially edentulous and completely edentulous patients, denture wearers, with alterations of the oral mucosa of the denture bearing area (such as congestive alterations of the oral mucosa, oral mucosal hyperplasia, flabby ridge, denture stomatitis).

The patients were clinically examined. Fragments of oral mucosa were collected from these patients from different areas of the potential denture bearing area, during extractions that we performed in the cases where the clinical situation required them or by excision of oral mucosal hyperplasia, flabby ridge, denture stomatitis.

A morphological study, using both classic techniques of histological processing, was performed on 4 µm thick serial sections. These sections were processed in formalin for 48–72 hours and then processed by conventional histological techniques for paraffin embedding. The paraffin blocks were sectioned with the Microm HM325 microtome and 4 µm sections were obtained and stained with Hematoxylin–Eosin (HE), Goldner–Szekely (GS) trichrome, Van Gieson (VG) trichrome, Periodic Acid Schiff (PAS).

Immunohistochemical processing

After histological analysis, we selected sections from the initial three groups. These sections were processed for immunohistochemical study. Immunohistochemical analysis was performed on 4 µm thick serial sections, applied to slides treated with adhesive – poly-L-Lysine. The sections were dried for 12 hours at laboratory temperature, after which they were dewaxed in three successive baths of xylene (15 minutes for each bath). This was followed by rehydration through successive passage of the sections through four alcohol baths with decreasing concentrations (100% alcohol, 96% alcohol, 80% alcohol, 70% alcohol), for approximately 10 minutes per each bath. Finally, sections were passed through distilled water to remove any trace of alcohol. For the immunohistochemical study, the LSAB2 technique was used as a working method (Streptavidin–Biotin 2 Labeled System). The kit we used was manufactured by Dako, Redox, Romania. The result of these immunohistochemical reactions is to visualize the investigated antigens with 3,3’-diaminobenzidine (DAB) (Dako, Redox, Romania) by brown staining. Mayer’s Hematoxylin was used for counterstaining.

Negative controls were obtained by omitting the primary antibodies, and as external positive control were used normal oral mucosa specimens.

The antibodies used in this study are listed in Table 1.

Results

Through microscopic examination, we have highlighted different aspects depending on the existing clinical situation of the patient: partially edentulous patients and completely edentulous patients who were not wearers of removable dentures, with or without modification of the oral mucosa, and patients that were wearers of removable dentures and that also had obvious clinical oral mucosal modifications.

On all of the examined sections, we found changes that have interested both epithelium and lamina propria, but that vary in intensity depending on the presence and type of previous prosthetic therapy and on the clinical status of patients (associated systemic pathology). Structural changes were varied, both at the epithelia level and in the lamina propria and were due to local factors and individual factors. We found the most important modifications on sections from patients who had clinical macroscopic mucosal changes, as well as on sections from patients with a preexistent removable prosthetic therapy and on

Table 1 – Antibodies used in the immunohistochemical study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Code</th>
<th>Clone</th>
<th>Specificity</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD20</td>
<td>M0755</td>
<td>L26</td>
<td>B-lymphocytes</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:100</td>
<td>Dako</td>
</tr>
<tr>
<td>CD8</td>
<td>IS623</td>
<td>144B</td>
<td>T-lymphocytes</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:200</td>
<td>Dako</td>
</tr>
<tr>
<td>CD3</td>
<td>A0452</td>
<td>F7.2.38</td>
<td>T-lymphocytes</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:100</td>
<td>Dako</td>
</tr>
<tr>
<td>CD68</td>
<td>M0814</td>
<td>KP1</td>
<td>Macrophages</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:200</td>
<td>Dako</td>
</tr>
</tbody>
</table>

Images were taken using a Nikon Eclipse 55i Research microscope (Nikon, Apidrag, Munich) equipped with Plan Fluor objective, DS-Fil digital camera with 5 mega-pixel resolution, acquisition board, acquisition and Nikon NIS-Elements imaging analysis software.

Quantification of immunohistochemical expression was made using the criteria utilized by literature [9]: (-) negative staining; (+-) reduced staining; (+) weak staining, very focal, visible only at high magnification; (+++) focal staining of moderate intensity, visible to the average increase; (++++) intense positive staining clearly visible at low magnification.
sections from elderly patients who presented associated pathology (diabetes, cardiovascular disease). Thus, the mucosal morphology of the denture bearing area of edentulous patients is subjected to the action of a plurality of local factors (the presence or the absence of prosthetic restorations and their type) and of general factors (age, genetic factors, nutritional factors, systemic and local associated pathology).

**Histological aspects**

The microscopic examination has highlighted different aspects depending on the existing clinical situation of the patient: partially edentulous patients or completely edentulous patients, who had never been denture wearers, with or without modification of the oral mucosa and denture wearers’ patients with obvious clinical changes.

We found changes in all the patients, changes that have interested both epithelium and lamina propria, but that vary in intensity depending on the presence and on the type of previous prosthetic treatment. Structural changes varied, both at epithelial level and in the lamina propria and were due to local factors and individual factors. We encountered the most important changes on sections from patients who had clinical macroscopic mucosal alterations, as well as on sections from denture wearers’ patients and from elderly patients with a history of medical problems (diabetes, cardiovascular disease).

We frequently encountered hyperplasia at the level of the coverage epithelium with deep epithelial ridges (Figure 1, A–C). The epithelium presented intense keratinization areas (Figure 1, B and C) and discrete or even absent keratinization areas. The most present was the orthokeratinization process, while the parakeratinization process was rarely encountered. In some sections, keratinization areas were located only in the tip of the edentulous ridge mucosa.

We encountered acanthosis-like alterations, of different intensity with increase in epithelium thickness due to hyperplasia of the stratum spinosum cells (Figure 1A). On sections from some of the patients, the papillomatosis associated with orthokeratosis or parakeratosis and acanthosis was identified only in reduced areas. On sections from denture wearers’ patients and from elderly patients with a history of medical problems (diabetes, cardiovascular disease).

Histological aspects also included the presence of atrophic epithelium (Figure 1A), characterized by reducing or deleting the fibrillar component is disposed around areas with inflammatory infiltrate that they encircle and delineate (Figure 1E). Numerous blood vessels are present, especially near the inflammatory process area. The presence of numerous neoformation blood vessels was observed on sections obtained from patients who had old dentures. The vessels had sometimes parietal changes that have led to hematic extravasation and microhemorrhages. Sometimes, we identified the presence of polymorphonuclear neutrophils, which indicate the flare-up of the chronic inflammatory process.

**Immunohistochemical aspects**

On oral mucosa sections processed by paraffin-embedding technique, we identified the cell populations present in the inflammatory processes. We used the following markers: CD20, CD8, CD3, and CD68.

**Evaluation of CD20 immunoexpression**

We used anti-CD20 antibody to highlight specific B-lymphocytes. We identified relatively few lymphocytes in the inflammatory infiltrate present in the sections examined as compared to other cell types. Their distribution was uneven, as they were present mainly around blood vessels and around proliferated epithelial ridge (Figure 2, C–E). Localization of B-lymphocytes around blood vessels suggests that antigens present in the lamina propria, resulted from the external environment or from the damage resulting from its own structures, stimulate their passage from the blood capillaries. In most cases, B-lymphocytes had a diffuse location (Figure 2, A and B), sometimes they were grouped as small outbreaks, having a pseudofollicular aspect (Figure 2F).

On sections from patients with clinical ulcerative lesions, we encountered a greater number of B-lymphocytes, in the epithelium and in the connective tissue. This is due to discontinuities in the epithelium that favor the passage of antigens from the oral environment through the affected epithelial barrier.

**Evaluation of CD8 and CD3 immunoexpression**

In the areas with lymphocytic infiltration, we have shown the presence of T-lymphocytes using CD8 (a marker for cytotoxic T-lymphocytes) and CD3 (pan-T marker) immunomarkers. T-lymphocytes showed a subepithelial arrangement, particularly in lamina propria and a perivascular arrangement (Figure 3, B, C, E and F). In addition, lymphocytes have been identified among the bundles of collagen fibers (Figure 3A) and at intraepithelial level, in the cases that presented ulceration of the oral mucosa. Lymphocytic infiltrate was either diffuse or had a pseudofollicular aspect (Figure 3D). Cytotoxic T-lymphocytes were present in an increased number, especially in the ulcerated mucosal areas and perivascularly. T-lymphocytes were predominant in all sections examined, indicating a cellular immune defense process. Non-uniform distribution of T-lymphocytes is determined by the zonal presence of antigens.

**Evaluation of CD68 immunoexpression**

With the help of CD68 antibody, macrophages participating in the local defense process were evidenced. We noticed the different distribution of macrophages in the lamina propria. In some areas of the oral mucosa, we

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observed the presence of macrophages in the form of groups (Figure 4F). Sometimes, their arrangement was diffuse, adjacent to epithelial proliferations areas (Figure 4, A–C), in epithelium areas with erosion, among bundles of connective fibers, around blood vessels (Figure 4, D and E). The intensity of the inflammatory process, and thus macrophages’ density differs from one case to another, but there were regional differences within the same case, too. Macrophage infiltration intensity was higher in edentulous patients who were denture wearers. We also noticed clusters of macrophages in epithelial ulceration mucosal areas.

Figure 1 – (A) Epithelial hyperplasia with deep epithelial ridges, subepithelial inflammatory infiltrate and numerous blood capillaries. HE staining, ×200; (B) Papillomatosis, mild acanthosis, parakeratosis and the development of fibrillar collagen component in lamina propria. VG trichrome staining, ×200; (C) Epithelium with deep-branched epithelial ridges, with acanthosis and orthokeratinization areas; (D) Atrophic epithelium with inflammatory infiltrate in lamina propria. HE staining, ×200; (E) Subepithelial and perivascular lymphocytic inflammatory infiltrate. GS trichrome staining, ×200; (F) Perivascular inflammatory infiltrate, numerous fibroblasts and an intense collagen fibrillogenesis process. HE staining, ×200.
Figure 2 – Immunoreactivity for CD20: (A and B) Diffuse lymphocytic infiltrate with B-lymphocytes – CD20+ deeply in lamina propria, ×200; (C and D) Inflammatory infiltrate with rare B-cells – CD20+ adjacent to epithelial proliferations and one intraepithelial B-lymphocyte, ×200; (E) Diffuse infiltrate of B-lymphocytes – CD20+ adjacent to elongated epithelial ridges, ×100; (F) Pseudofollicular infiltrate with rare B-lymphocytes – CD20+ delimited by thick bands of collagen fibers, ×200.
Figure 3 – Immunoreactivity for CD8 and CD3: (A) Diffuse lymphocytic infiltrate with rare T-lymphocytes – CD8+ arranged among bundles of collagen fibers from lamina propria, ×200; (B) Rare T-lymphocytes – CD8+ diffusely arranged in the chorion of epithelial ridges, ×200; (C) Dense perivascular lymphocytic infiltrate with rare T-lymphocytes – CD8+ in lamina propria, ×200; (D) Pseudofollicular lymphocytic infiltrate through bundles of collagen fibers in lamina propria, with rare T-lymphocytes – CD8+, ×200; (E) Subepithelial cell infiltrate of T-lymphocytes – CD3+, ×100; (F) Abundant inflammatory infiltrate, T-lymphocytes, in an area presenting surface epithelial erosion – CD8+, ×100.
Discussion

Correct evaluation of edentulous patients’ oral mucosal substrate, denture wearers or not, requires not only a thorough clinical examination, but it especially requires a histological examination on the basis of morphological criteria which can lead, in conjunction with clinical data, to a diagnosis of certainty. Optimal therapeutic solution, including the impression taking method and the prosthetic treatment solution must be the result of a critical evaluation, which takes into consideration a tripod represented by: the general health condition of the patient (including associated pathologies), the state of edentulism and structural characteristics of the oral mucosa and its pathological alterations. The structural substrate at the prosthodontist’s disposal is the one that dictates the adequate prosthetic conduct in order to achieve functional prosthetic restorations and to ensure
adequate nutrition of the oral mucosa for as long as possible. The diagnosis and treatment strategies should be individualized.

Through this approach, the study that we conducted brought a double contribution for both basic research and for the clinical research, the two sides influencing each other. The basic research, centered on studying edentulous patients, mucosal substrate enables the assessment of etiopathogenic mechanisms involving the inflammatory process and especially involving a certain type of local defense: specific – immune-type defense, or non-specific – macrophage-type defense.

In the studied cases, we found the presence of changes that have interested both the epithelium and lamina propria. Epithelium showed in particular epithelial hyperplasia aspects, with orthokeratinization and parakeratinization areas. Very rarely, in the cases of two edentulous patients, epithelium presented dysplastic aspects, which indicate that a chronic aggression can induce major changes in the mucosa. Epithelium presented, in some sections, areas of ulceration. These issues were recorded in the cases of denture wearers’ edentulous patients. Epithelial changes result from actions of several local factors: the bacterial flora, vascularization disorders, mechanical factors [10].

The inflammatory process present in the lamina propria is chronic and consists in particular of lymphocytes, plasma cells and macrophages. This process was differentiated in intensity from one case to another, but varied even within the same case, from one area to another. Inflammation was determined by the local microbial flora enhanced by the action of prosthetic appliances or by the prolonged edentulous state.

The presence of lymphocytic inflammatory infiltrate shows the existence of a local specific (immune) defense process and of a non-specific (macrophages) defense process. Immunohistochemical study of cell populations involved in the inflammatory process of the oral mucosa of edentulous patients shows a significant reactivity of the edentulous territory under the action of various, both local and general, aggression factors [11–13]. Immune response seen in sections from all the studied cases was predominantly cellular, but also associated a humoral immune response. Intraepithelial presence of CD8-positive lymphocytes (cytotoxic) in the ulcers sections of oral mucosa, confirm their cytotoxic effect at this level.

Oral mucosal epithelium has immense importance in local defense and in alerting the immune system, as it is the first tissue structure originally facing with most microorganisms in the oral cavity. Interrelation between oral mucosal epithelium and immune system is essential because this is where is the difference between commensally flora and pathogenic flora is made and cellular and humoral mechanisms for maintaining local homeostasis are activated [14]. Initially, it was thought that the epithelium is only a passive barrier against invading pathogens, but relatively recent studies have shown that epithelial cells are capable of eliciting an immune response, thus playing an active role in microbial recognition. Therefore, the oral epithelium is able to secrete a variety of defense effector molecules [15] and to orchestrate an immune inflammatory response in order to activate immune cells in the lamina propria that destroy invading pathogens [16].

Immune responses to food antigens and commensally bacteria generally do not cause any inflammation and do not induce immune tolerance [17]. However, the oral mucosa is the seat of severe inflammatory or autoimmune diseases, such as periodontitis, Sjögren’s syndrome and oral lichen planus. In these cases, there is a more or less extensive destruction of the gingival barrier with gingival epithelial necrosis, including the basal layer, and massive infiltration of the lamina propria with lymphocytes, macrophages and polymorphonuclears [18, 19].

Neutrophils are essential cells in oral mucosal immune defense and in maintaining normal oral biofilm. They capture and destroy microbial agents by phagocytosis, the process by which microbes are internalized and digested in phagosomes. Some of neutrophils that reach the surface of the oral epithelium degranulate and release reactive bactericidal oxygen species (ROS) [20, 21].

In pathological situations, neutrophils are recruited to the site of invasion of pathogenic agents by a variety of potent chemoattractants such as interleukin 8 (IL8), the fragment C5a and other chemokines [22, 23]. In these places, they are intensifying phagocytosis and the release of ROS.

Cells of the immune defense system release, at lamina propria level, growth factors that induce fibroblast presence in large numbers. The higher number of fibrocytes encountered is a reactivation of these cells, that, through an increased fibrologenetic process conduct a reparative response, limiting the inflammatory process and restoring local homeostasis. Fibroblasts stimulation is determined by fibroblast growth factors secreted by bacteria or by the cells of the immune system, as shown also by literature.

Different types of cells that are present cooperate, influencing one another, in order to restore normal functionality. The activity of macrophages is enhanced due to lymphokines synthesized by T-lymphocytes that are present in large numbers in the inflammatory process.

Also, present on the examined sections, high number of capillaries, vasodilatation, stasis and microbleedings are the effect of chemical mediators that are released in the inflammatory areas or may be of mechanical nature – as in the case of denture wearers. Intense vascularization supports the specific and non-specific defense process, by increasing exchanges between blood and tissue. The presence of a large number of localized subepithelial capillaries is motivated by the increased needs of an epithelium in proliferation (acanthotic epithelium).

More intense changes were observed in patients who were denture wearers. It may be possible that mechanical forces that are beyond the means of local adaptation possibilities induce the inflammatory reactions that we observed in the lamina propria. Thus, inflammation is the response to local aggression.

Conclusions

Oral mucosal alterations interest, in different proportions, all the structural components: epithelium, basement membrane, lamina propria. These alterations differ from one patient to another and they are influenced by the
existence of previous prosthetic treatment and by the type of the previous prosthetic therapy. The results of the histological examination of the oral mucosa of edentulous patients corroborated with data from clinical examination are important both for basic research, helping to deepen the understanding of the local pathogenic mechanisms, as well as for clinical prosthetic practice, offering the possibility of individualized prosthetic treatment. The inflammatory response indicates the reactivity of the edentulous mucosa in response to local aggression, the specific defense mechanism coexisting with the non-specific defense mechanism, with predominance of cellular immune defense, in order to restore local homeostasis.

Conflict of interests

The authors declare that they have no conflict of interests.

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