

## ORIGINAL PAPER

# Immunohistochemical aspects of the evaluation of the inflammatory answer of the dental pulp

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### Abstract

The inflammatory answer of the dental pulp in front of an antigen presents a series of particularities caused by its special anatomical conditions. The evaluation of the inflammatory answer in the evaluation tests of the biocompatibility of the dental materials is made by histological methods without defining precise quantitative criteria to measure the pulp reaction. In this study, we followed the reaction of the pulp tissue to five antibodies (CD20, CD45Ro, CD4, CD8, and CD68) in order to evaluate the inflammatory aspects of the dental pulp. We did not find positive answers for the CD20 protein, specific for B-lymphocytes, for the fragments of normal pulp tissue taken into study. Even if they are in small number among the pulp cells, the T-lymphocytes that express the protein CD45Ro may be also found in the normal dental pulp. Of the two subsets of T-lymphocytes, we found positive answers on the studied preparations only for the CD8 protein. For the CD68 protein, strongly expressed by the macrophages, we obtained positive results both for the inflamed pulp tissue, and for the normal dental pulp, yet in a very small amount. The use of immunohistochemical techniques, with well-defined markers of the pulp inflammation, can offer better results for a highly accurate evaluation of the inflammatory answer of the dental pulp.

**Keywords:** immunohistochemistry, dental pulp, evaluation tests.

### Introduction

The connective pulp tissue is surrounded by hard dental tissues, which realize a barrier against the injuries of the external environment. However, the bacteria elements may however penetrate into the pulp tissue, through the communication ways already existing between the dental pulp and the surrounding tissues (radicular apex, lateral radicular canals), or through obtained communication ways (carious lesions, iatrogenic lesions, dental fractures, etc.).

The special anatomical conditions of the dental pulp cause the appearance, in the condition of an antigen penetration, of an inflammatory answer with particular characteristics.

The ISO standards, by the ISO 10993 document, establish the evaluation of the pulpar response by the histological analysis of the pulp tissue taken from the experience animal after the extraction of the restored tooth with the researched material. Although there were proposed other methods for the evaluation of the biocompatibility of the dental materials, the histological methods remain the most frequently used methods. The use of the histological methods for the evaluation of

the inflammatory answer of the dental pulp both at primates and humans showed that they still are the most relevant [1].

Yet there are not definite precise quantitative criteria for measuring the pulpar reaction, these appreciations being left to the discretion of the investigators' subjectivity. Nowadays, the histological and anatomo-pathological studies have at hand a series of modern methods, which allow a higher accuracy in the diseases diagnosis and classification [2].

Among these modern methods, the immunohistochemical methods are to be distinguished, as they have the possibility to offer also quantitative analyses of the identified structures, and have also become an important part of the anatomo-pathological practice in the entire world [3].

In this study we used the immunohistochemical methods in order to evaluate the inflammatory aspects of the dental pulp.

### Material and Methods

For the realization of this study, we used 20 samples of pulp tissue divided into two groups.

The first study group was represented by ten samples of pulp tissue collected either directly from the pulp chamber following the pulpectomies made for therapeutic reasons, in six cases, either after the extraction of the teeth that presented also irreversible periodontal lesions, in the other four cases.

The second study group was represented by samples of pulp tissue collected from teeth without clinical symptoms of the pulp. The collecting was made following the pulpectomies made for prosthetic reasons in eight cases or after the teeth extraction, for two ectopic wisdom molars.

The pulpectomies were made under local anesthesia with 3% mepivacaine, without vasoconstrictor. The collecting of the pulp tissue subjected to the study was preceded by a less brutal opening of the pulp chamber with the aid of the micromotor and of spherical burs of adequate dimensions for the teeth on which the operation has been performed.

The proper collecting was made from the pulp chamber with the aid of well-sharpened dental excavators of adequate dimensions; they were used to cut off, systematically, the pulp tissue from the pulp chamber walls, together with the odontoblastic layer.

When the clinical situation imposed the tooth extraction, immediately after the tooth was pulled out, we made with the aid of a dental turbine, under water-cooling, two longitudinal ditches on opposite sides of the tooth. With a dental elevator applied in one of the two ditches, we separated the two fragments. The exposed pulp tissue exposed in this way was drawn with a dental excavator, together with the odontoblastic layer.

After the collecting, the pieces were passed in to a fixing solution of 10% neutral formalin, for 24 hours. Eventually, the pieces were treated following the classical histological technique for including into paraffin, technique that allowed us to slice serial 5  $\mu$ m-thick sections.

The sections were placed on histological blades treated with Poly-L-Lysine (Sigma). The immunohistochemical method used in this study was the Avidin Biotin Complex (ABC) three-stage indirect method [4].

In this study, we monitored the answer of the connective pulp tissue for five antibodies: CD20 (clone L26, DAKO), CD45Ro (clone UCHL1, DAKO), CD4 (clone 1F6, ZYMED), CD8 (clone SP16, NEOMARKERS), CD68 (clone KP1, DAKO).

## Results

For the identification of the B-lymphocytes on the pulp tissue samples, we used the anti-CD20 antibody. This one reacts with two non-covalently components bonded on the cytoplasmic side of the cell membrane in the majority of B-cells. It detects an epitope that survives tissue processing, and may be used for the formalin-fixed and paraffin-embedded tissues.

The antigen is expressed by most of the

B-lymphocytes present in peripheral blood and lymphoid tissue. Studies made with this antibody also showed that it does not stain erythrocytes, granulocytes, monocytes, platelets or T-cells from the normal tissues. In our study, we did not find B-lymphocytes on most of the fragments of the pulp tissue. From the whole group of normal pulp tissue taken into study, only on one sole tissue sample, completely isolated, we could identify a small group of cells with positive reaction placed in a vascular lumen and closely perivascular (Figure 1).

The anti-CD45Ro antibody was used for the identification of the T-lymphocytes from the dental pulp. It labels most thymocytes, a subpopulation of resting T-cells within both the CD4 and CD8 subsets, and mature activated T-cells. The antibody can be used for samples fixed in formalin, but it is not recommended for fixers that led to a pH decreasing below 3.1. Within the present study, we identified the presence in the normal dental pulp of the T-lymphocytes that express the CD45Ro protein. Although, they are in small number among the pulp cells, the T-lymphocytes are met especially along the blood vessels and the nerve fibers in the central area of the pulp. The presence of some inflammatory lesions on the level of the pulp determined a massive increase of the number of the T-positive lymphocytes for the CD45Ro protein (Figures 2–4).

The differentiation of the T-lymphocytes from the dental pulp in helper T-lymphocytes or suppressor T-lymphocytes was made with the aid of the anti CD4 and CD8 antibodies. CD4 is a transmembrane glycoprotein that works as a receptor for the molecules of the II<sup>nd</sup> class of major complex of histocompatibility. This antibody may be useful for the description of the inflammatory cells phenotype. CD8 is a molecule formed by two protein chains and expressed on the membrane surface of the suppressor T-lymphocytes, thymocytes and natural-killer lymphocytes. CD8 works like a co-receptor for the molecules of the I<sup>st</sup> class of major complex of histocompatibility.

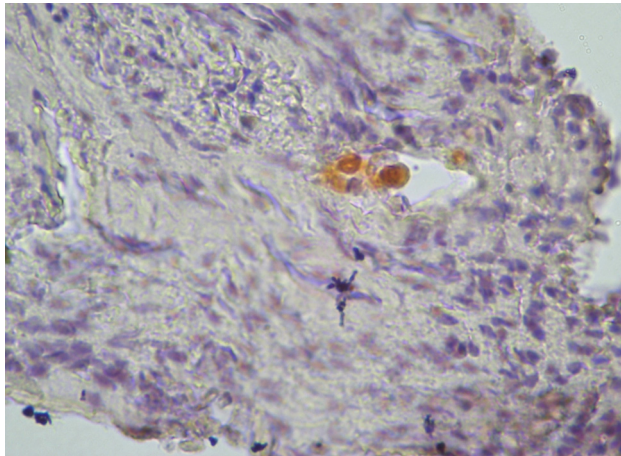
Of the two subsets of T-lymphocytes, on the studied preparations we found positive answers only for the CD8 protein. Actually, also the data in the literature mentioned below present the lymphocytes T-positive to the protein CD8 as a permanent resident of the dental pulp, while the presence of the T CD4-lymphocytes was much more reduced and cannot be generalized, depending on the type of the tooth (incisive, premolar or molar) and on the status of the dental pulp (Figure 5–7).

The anti-CD68 antibody was used to identify the macrophages from the samples taken into study. CD68 is a lysosomal protein expressed strongly in cytoplasmic granules, and weakly on the surface of macrophages, monocytes, neutrophils, basophils and natural-killer lymphocytes. Unlike many other CD-antigens, the CD68 is a molecule that is antigenically very heterogeneous, different antibodies for CD68 showing different cellular reactivity. On the studied preparations, we used the CD68 antibody and we obtained positive results both for the inflamed pulp

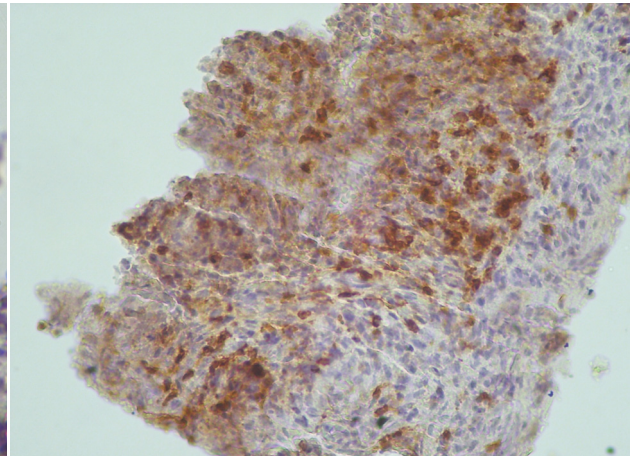
tissue, but also for the normal dental pulp. The number of the macrophages present in the normal pulp tissue was very small, located especially in the central area of the pulp perivascular (Figure 8).

However, the immunohistochemical marking allowed

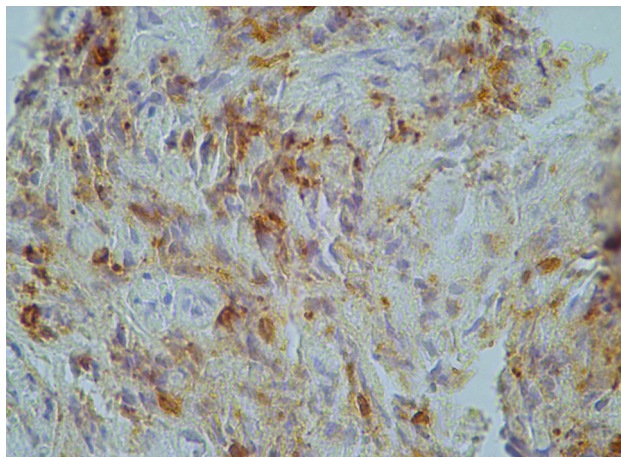
us to locate them and the observation of their form with stronger objectives. These cells appear in various shapes: round, oval, dendritic. In conditions of functional rest, they usually appear as elongated cells, located perivascularly.



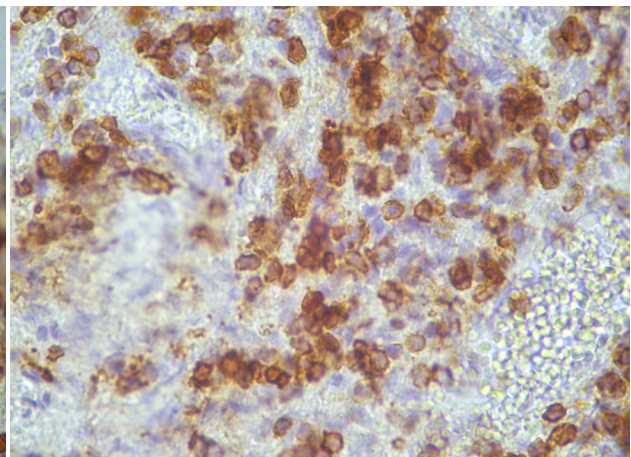
**Figure 1** – Image in which it can be observed the presence of a B-lymphocyte in the vascular lumen, but also other positive structures situated at the limit of the vascular wall ( $\times 400$ ).



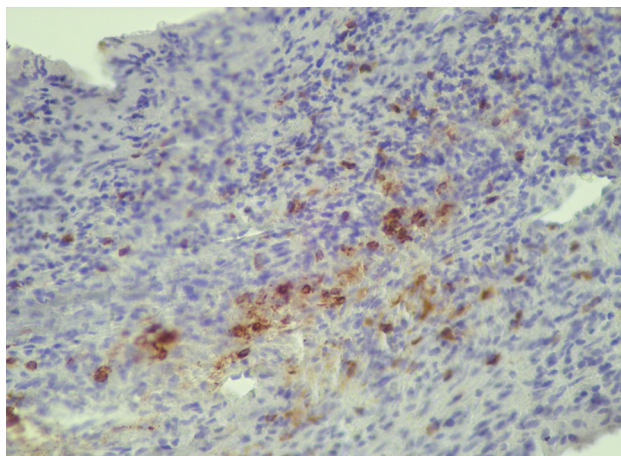
**Figure 2** – Inflamed pulp tissue in which it can be noticed the presence of a numerous population of T-lymphocytes ( $\times 100$ ).



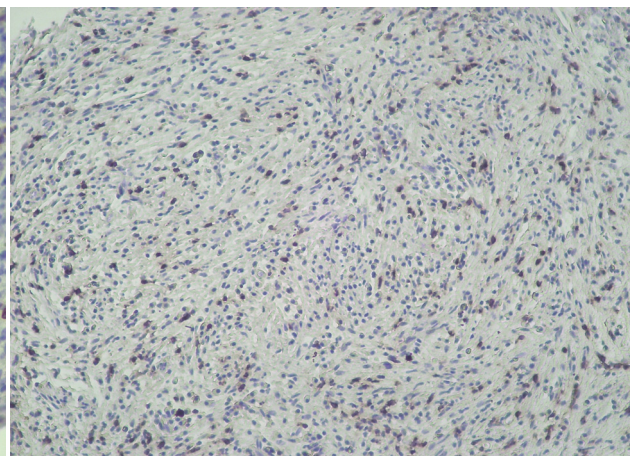
**Figure 3** – Normal pulp tissue with positive CD45Ro cells ( $\times 400$ ).



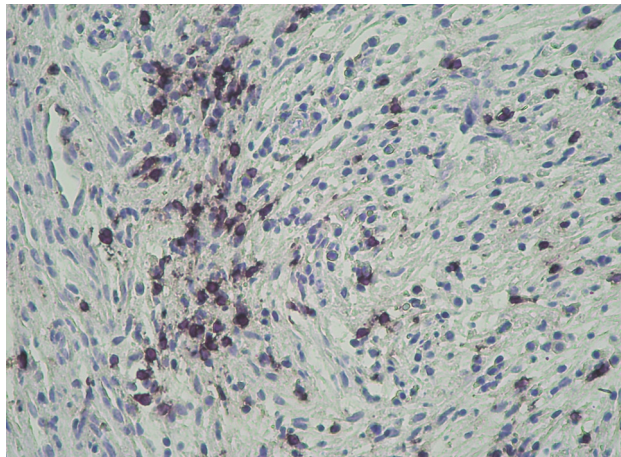
**Figure 4** – Numerous T-lymphocytes situated near a nerve fiber ( $\times 400$ ).



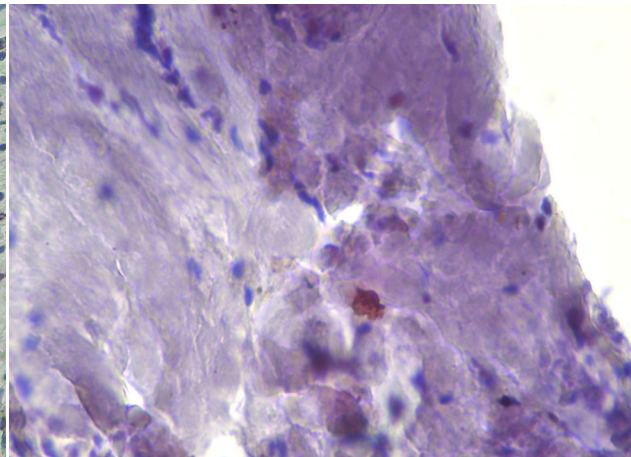
**Figure 5** – Positive reaction at CD8 protein for the dental pulp lymphocytes. It is noticed the more intense answer from the central area of the tissue ( $\times 200$ ).



**Figure 6** – Positive reaction for CD8 protein showing the presence and the distribution in an inflamed pulp tissue of the CD8 T-lymphocytes ( $\times 100$ ).



**Figure 7 – Detail of the previous image, showing a group of CD8 T-lymphocytes, located near a blood vessel ( $\times 200$ ).**



**Figure 8 – Positive reaction for CD68 on a normal dental pulp tissue sample ( $\times 400$ ).**

## Discussion

For a long time, the organizing way of the pulp immune system was not well known. Yet the researches in the last years brought a series of new elements that help us to understand the way the immune pulp system works.

The B-lymphocytes, unlike the T-lymphocytes, are unusually found in the normal dental pulp. Over the time, several studies showed the absence of the B-lymphocytes from the normal dental pulp with the aid of the immunohistochemistry techniques, using either antiserum against immunoglobulin [5], either monoclonal antibodies against B-lymphocytes [6]. Neither in the present study we did not find B-lymphocytes on the most of the fragments of the pulp tissue studied. However, some authors reported, occasionally, the presence of the B-lymphocytes in the human dental pulp [7].

In studies made on pulp tissue collected from rat molars, Okiji T *et al.* [8], found out the presence of a few plasmocytes at young animals, and the number of these cells had the tendency to grow with the age. In present, seems difficult to find a significant role for the B-lymphocytes in the normal dental pulp.

Physiologically, the T-lymphocytes are classified in:

- helper T-lymphocytes, which belong to CD4 subset; they activate the functions of other cells of the immune system (B-lymphocytes, T-lymphocytes or macrophages);
- suppressor T-lymphocytes, which belong to CD8 subset; they inhibit the functions of other B- or T-cells;
- cytolytic and cytotoxic lymphocytes, which belong to CD8 subset; they directly destroy the foreign cells after they have been stimulated [9].

The idea that the normal dental pulp is devoid of lymphocytes [10] is contradicted by the studies from the last years, the immunohistochemistry allowing the affirmation of the T-lymphocytes as normal and essential constituents of the dental pulp. Recently, there have been identified T-lymphocytes expressing the CD45RO protein in the human dental pulp [7]. In our study, we also identified the presence of the

T-lymphocytes that express the CD45RO protein, even in the normal dental pulp. The presence of inflammatory lesions on the level of the pulp determined a massive increase of the number of the T-positive lymphocytes for the CD45RO protein.

In 1987, Jontell M *et al.* showed for the first time, using monoclonal antibodies, the presence of the CD4 and CD8-lymphocytes in the normal dental pulp [11]. An unexpected finding was the ratio CD4/CD8 : 1/3.1 for molars and 1/1.6 for premolars, an inverted ratio of what might be expected from their occurrence in peripheral blood. In addition, on the studied preparations we found positive answers only for the CD8-protein, from the two subsets of T-lymphocytes. However, the same ratio as in the peripheral blood was found in chronic periapical lesions and in subepithelial infiltrates from different skin diseases [12].

However, the ratio between the two subsets of T-lymphocytes in pathological conditions or in peripheral blood is not necessarily to be the same as in normal tissue.

Hahn CL *et al.* found similar results. On human pulp tissue, and Okiji T *et al.* showed also by immunohistochemical methods the presence of T-lymphocytes CD4, CD5 and CD8 in the rat molars pulp [13, 14].

Cytometric analysis of cells from the human dental pulp has also demonstrated the presence of CD4 and CD8-cells, but there were detected more CD4 T-lymphocytes than CD8-lymphocytes [15].

The presence of the cells with macrophagic phenotype in the normal dental pulp was proven by many studies of optical and electronic microscopy. Yet, they are difficult to differentiate from the fibroblasts on the examination on the optical microscope [16, 17].

The studies of immunohistochemistry brought yet many clarifications on the presence of the cells of macrophagic type in the normal dental pulp or submitted to the action of various noxious external factors. As in other connective tissues, the macrophages from the dental pulp present a higher heterogeneity regarding the cytochemical markers expression.

On the studied preparations, we used the CD68-antibody and we obtained positive results both for the inflamed pulp tissue, but also for the normal dental pulp, even in this last case in a very small number.

Numerous recent studies showed that cells, which express a phenotype similar to that of the macrophages are distributed with a remarkable density in the dental pulp, at humans [18, 19], but also at rats [20]. These cells appear like the predominant cells in the dental pulp. These studies also showed that, beside histiocytes, in the dental pulp there are some cells with characteristics comparable with those of the dendritic cells. These cells present an intense dendritic profile, express strongly the II<sup>nd</sup> class molecules and there are distributed predominantly in the perivascular region of the central area of the pulp and in the periodontoblastic areas from the peripheral pulp [21].

In order to evaluate the pulp inflammation, within the E.U. there are valuable the standards imposed by *International Standards Organisation (ISO)*, which elaborated the ISO 10993 document, in which there are shown the standards for the evaluation of the medical materials and of the medical devices. This contains the "initial" and "secondary" tests to which the materials must be submitted.

The ISO standards, by the ISO 10993 document, establish the evaluation of the pulpar response by the histological analysis of the pulp tissue collected from the experience animal after the extraction of the restored tooth. Although there were proposed other methods of evaluation of the biocompatibility of the dental materials such as those which use the Doppler laser [22], the histological methods remain the most frequently used methods, but also the most relevant for evidencing the inflammatory response of the dental pulp. These standards divide the activity of the pulpar inflammatory cells into four categories (absent, easy, moderate, severe) according to a series of criteria. Yet there are not definite precise quantitative criteria for measuring the pulpar reaction, these appreciations being left to the discretion of the investigators' subjectivity.

The ISO standards do not use at the moment a set of modern methods of evaluation of the pulpar conjunctive tissue such as the immunohistochemical techniques or those of computerized morphometry, which could offer also quantitative analysis of the presence of the inflammatory cells in the dental pulp [23].

Also, in the evaluation of the biocompatibility of the dental materials it should be taken into account the initial status in which the pulpar conjunctive tissue is before applying the application of the material to be researched, an aspect less taken into account by the techniques used nowadays for the evaluation of the biocompatibility of the materials.

## ✉ Conclusions

The pulp tissue presents a well-organized immune system, which is adapted to the anatomical conditions of the dental pulp.

The B-lymphocytes were met only isolated in the normal pulp conjunctive tissue.

The T-lymphocytes, even if they are less numerous, represent important constituents of the normal dental pulp; they are situated especially along the blood vessels in the central area of the pulp.

Between the two subsets of T-lymphocytes, the T-suppressor lymphocytes represent constituents more frequently met and better represented numerically in the structure of the conjunctive tissue comparing to the T-helper lymphocytes.

The histiocytes and the macrophages are in different proportions according to the local pulp conditions; they present a large heterogeneity from point of view of the expression of the cytochemical markers.

The use of immunohistochemical techniques, with well-defined markers of the pulp inflammation, may offer superior results for the evaluation with higher accuracy of the inflammatory answer of the dental pulp.

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*Received: March 10<sup>th</sup>, 2009*

*Accepted: April 30<sup>th</sup>, 2009*