

## BASIC RESEARCH IN OTORHINOLARYNGOLOGY

# Quantification of cells expressing markers of proliferation and apoptosis in chronic tonsillitis

## Quantificazione delle cellule che esprimono i marcatori di proliferazione e apoptosi nelle tonsilliti croniche

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## SUMMARY

During chronic tonsillitis, the relationship between proliferation and apoptosis of lymphocytes in tonsillar follicles can be disturbed, which gives rise to attenuation of tonsil immunocompetence and diminishing its contribution in systemic immunity. In this study, we have quantified the cells expressing the markers of proliferation and apoptosis in the follicles of the palatine tonsil. Six tonsils from patients aged 10-29 years with hypertrophic tonsillitis and five tonsils from patients aged 18-22 years with recurrent tonsillitis were studied. The sections of paraffin blocks of tonsillar tissue were stained by the immunohistochemical LSAB/HRP method with the utilisation of antibodies for: Ki-67 antigen-cell marker of proliferation; Bcl-2 and survivin anti-apoptotic factors and Fas/CD95, caspase-3 and Bax pro-apoptotic factors. The size of lymphoid follicles, i.e. mean follicle area and number of lymphoid follicle immunopositive cells per mm<sup>2</sup> of a slice area, i.e. numerical areal density were determined by the quantitative image analysis. The localisation of Ki-67, Bcl-2, survivin, Fas/CD95, caspase-3 and Bax- immunopositive cells inside the palatine tonsil was similar in both types of tonsillitis. The number of Ki-67 immunopositive cells was significantly ( $p < 0.01$ ) larger in the tonsils with hypertrophic tonsillitis ( $14681.4 \pm 1460.5$ ) in comparison to those with recurrent tonsillitis ( $12491.4 \pm 2321.6$ ), although the number of survivin and caspase-3 immunopositive cells was significantly ( $p < 0.05$ ) larger in recurrent tonsillitis (survivin,  $406.9 \pm 98.4$ ; caspase-3,  $350.4 \pm 119.4$ ) when compared to those with hypertrophic tonsillitis (survivin,  $117.4 \pm 14.5$ ; caspase-3,  $210 \pm 24$ ). Our results show that the rate of the proliferation and apoptosis of follicular lymphocytes is different in various types of tonsillitis. This suggests that the immunological potential of the palatine tonsil varies in patients with hypertrophic and recurrent tonsillitis, which in practice poses a dilemma over the choice of conservative or surgical treatment.

KEY WORDS: Human palatine tonsil • Lymphoid follicle • Cell proliferation • Apoptosis • Quantification

## RIASSUNTO

*Durante una tonsillite cronica, il rapporto tra proliferazione e apoptosi dei linfociti nei follicoli tonsillari può essere alterato: ciò spiega l'attenuazione dell'immunocompetenza tonsillare e la riduzione del suo contributo all'immunità sistemica. In questo studio abbiamo quantificato le cellule che esprimono i marker di proliferazione e apoptosi nei follicoli delle tonsille palatine. Sono state studiate sei tonsille da pazienti di età compresa tra 10 e 29 anni con tonsillite ipertrofica e cinque tonsille da pazienti di età compresa tra 18 e 22 anni con tonsillite ricorrente. Le sezioni di tessuto tonsillare incluso in paraffina sono state colorate con il metodo immunoistochimico di LSAB/HRP attraverso l'applicazione di anticorpi per: l'antigene ki-67, marcatore cellulare di proliferazione; Bcl-2 e survivina, fattori antiapoptotici; Fas/CD95, caspasi 3 e Bax, fattori proapoptotici. La dimensione dei follicoli linfatici, ossia l'area del follicolo e il numero delle cellule immunopositive del follicolo per mm<sup>2</sup>, ossia la densità numerica dell'area, sono state determinate attraverso una analisi quantitativa dell'immagine. La localizzazione delle cellule immunopositive a ki-67, Bcl-2, survivina, Fas/CD95, caspasi 3 e Bax all'interno della tonsilla palatina è stata simile nei due tipi di tonsillite. Il numero delle cellule immunopositive per Ki-67 è stato significativamente ( $p < 0,01$ ) maggiore nelle tonsille con tonsillite ipertrofica ( $14681,4 \pm 1460,5$ ) rispetto a quelle con tonsillite ricorrente ( $12491,4 \pm 2321,6$ ), sebbene il numero di cellule immunopositive per survivina e caspasi 3 fosse significativamente ( $p < 0,05$ ) maggiore nelle tonsilliti ricorrenti (survivina,  $406,9 \pm 98,4$ ; caspasi-3,  $350,4 \pm 119,4$ ) rispetto alle tonsille con tonsilliti ipertrofiche (survivina,  $117,4 \pm 14,5$ ; caspasi-3,  $210 \pm 24$ ). I nostri risultati mostrano che il tasso di proliferazione e apoptosi dei linfociti follicolari è diverso nei vari tipi di tonsillite. Questo indica che il potenziale immunologico della tonsilla palatina varia nei pazienti con tonsillite ipertrofica rispetto a quelli con tonsillite ricorrente: ciò, nella pratica, pone un dilemma in merito alla scelta del trattamento conservativo o chirurgico.*

PAROLE CHIAVE: Tonsilla palatina • follicolo linfatico • proliferazione cellulare • Apoptosi • Quantificazione

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## Introduction

Human palatine tonsil is an organ of a local (mucous) and systemic immune response<sup>1-4</sup>. Due to the specific location in the oropharynx and permanent antigen stimu-

lation, the human tonsil is subject to inflammatory processes that frequently turn chronic. According to clinical parameters and morphological substrate, Surjan et al.<sup>5</sup> have defined two types of chronic tonsillitis in adult pa-

tients: hypertrophic tonsillitis (HT) and recurrent tonsillitis (RT).

The lymphoid follicles (B-dependent zone) of the human tonsil contain two morphologically and functionally different parts: germinal centre (GC) and mantle zone (MZ). The mantle zone is populated by B memory lymphocytes and GC contains centroblasts and centrocytes, representing the spot where proliferation, differentiation and clonal selection of antigen-stimulated B lymphocytes takes place<sup>6</sup>. Differentiation of stimulated B lymphocytes into B memory cells and plasma cells is precisely regulated by the cellular interaction among follicular B cells, CD4<sup>+</sup> (Th2) cells and follicular dendritic cells (FDC)<sup>7,8</sup>. Apoptosis represents one of the control mechanisms in the process of clonal selection of stimulated B lymphocytes, which protects the organism from created "low-grade" B cells<sup>9,10</sup>. In particular, the follicular B cells that did not receive the signals for positive selection leave their cell cycle, undergo apoptosis<sup>11-13</sup> and are phagocytosed by follicular macrophages<sup>14</sup>.

In order to determine the role of the human palatine tonsil in the humoral immune response, Surjan et al.<sup>5</sup> and Korsrud and Brandtzaeg<sup>15</sup> were the first who quantified the immunoglobulin (Ig)-producing cells in human palatine tonsils with HT and RT, and found some differences related to the type of tonsillitis. Over the last two decades, a specific microenvironment of lymphoid follicles was defined by immunohistochemistry as the place of production of cell-stimulating cytokines<sup>16-18</sup>, presence of a variety of FDC subtypes<sup>19</sup>, expression of Fas receptors and Fas ligands (FasL)<sup>9,20</sup> and caspase-3<sup>21</sup>; however, there is a small number of published works<sup>22,23</sup> which have quantified the above mentioned factors using morphometric methods.

In recent years, there has been a growing interest in survivin, which is described as a member of the inhibitor of apoptosis protein family and an indicator of cell survival<sup>24</sup>. Its expression has been observed in the cell nucleus, which confirms its role, primarily in the control of cell division<sup>25,26</sup>.

In order to determine if there is a difference in the immune activity of the lymphoid follicles in chronic tonsillitis, we quantified the cells expressing biological markers of proliferation and apoptosis in the lymphoid follicles of human tonsils with HT and RT.

## Materials and methods

Palatine tonsils were obtained at the ENT Clinic of Clinical Centre Niš from patients who had undergone elective tonsillectomy due to chronic tonsillitis, and were obtained from 5 patients aged 18-22 years (mean 20.3 years) with RT and 6 patients aged 10-29 years (mean 20.8 years) with HT. In their case histories, patients reported recurrent episodes of tonsillitis (more than four times a year for more than 1-2 years), which, in patients with HT were commonly associated with dysphagia due to enlarged tonsils.

After removal, only one tonsil from each patient was taken; all tonsils were routinely processed from paraffin blocks for light microscope study. Histopathological analysis was performed on 3-5 µm thick sections that had been routinely stained with H&E.

### Immunohistochemistry

The immunohistochemical LSAB/HRP method was applied with antibodies for: (1) Ki-67 antigen (mouse monoclonal antibody, N1633, ready to use, DAKO, Denmark) for proliferative activity; (2) Bcl-2 protein (mouse monoclonal antibodies, M0887, DAKO, Denmark, dilution 1:100) and survivin (mouse monoclonal antibodies, M3624, DAKO, Denmark, dilution 1:100) for anti-apoptotic activity; and (3) Fas/CD95 receptor (rabbit polyclonal antibody, ab2437, Abcam, UK, dilution 1:10); caspase-3 (mouse monoclonal antibody, ab2171, Abcam, UK, dilution 1:200) and Bax protein (rabbit polyclonal antibody, A3533, DAKO, Denmark, dilution 1:1000) for apoptotic activity.

Immunohistochemistry was performed on 3-5 µm thick paraffin sections using 45-minute heat-induced epitope retrieval in 0.01 M citrate buffer at pH 6.0, followed by overnight incubation with the primary antibody at +4°C°. For visualisation, we used the LSAB2 system HRP (K0673, DAKO, Denmark). After staining with DAB, slides were counterstained with Mayer's haematoxylin, dehydrated through a graded ethanol series and mounted with Canada balsam.

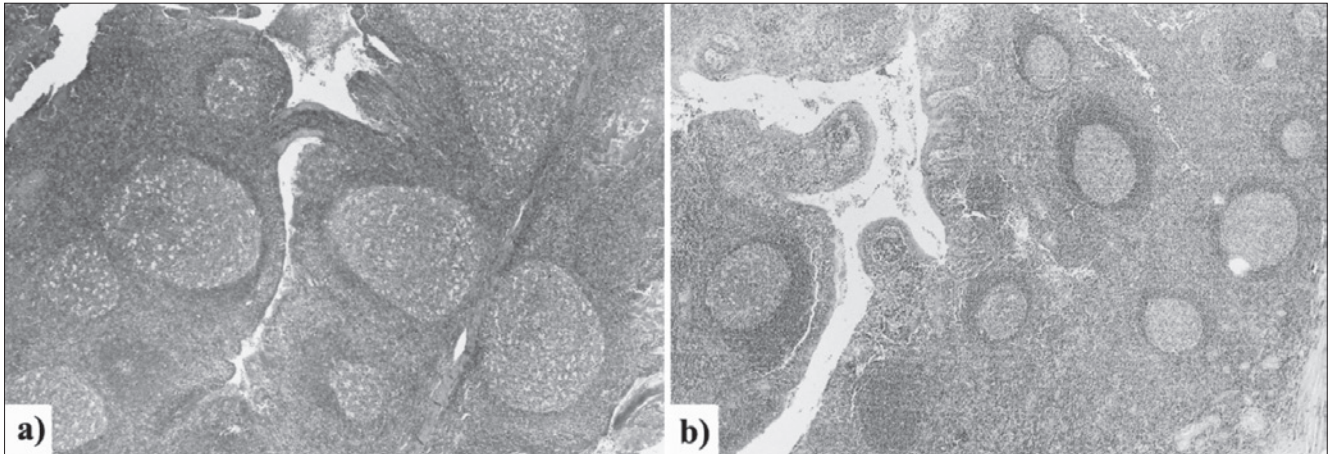
### Quantitative image analysis

Lymphoid follicles area and numerical areal density ( $N_A$ ) of follicular immunopositive cells were determined by digital image analysis using Image J software (National Institutes of health, Maryland, USA, <http://imagej.nih.gov/ij/>). The images were obtained on Leica DMR light microscope equipped with a digital camera (Leica Micro-Systems, Reuil-Malmaison, France). The objective × 4 was used for determination of the follicle area as well as germinal centre area, while × 40 was applied to determine the numerical areal density of follicular immunopositive cells, i.e. the average number of cells per mm<sup>2</sup> of tonsillar tissue.

For measuring lymphoid follicle surfaces, we examined all lymphoid follicles on three sections of each tonsil; the distance between the slices was 30 µm. For quantitative analysis of follicular immunopositive cells, 20 fields per slice (simple random sampling) were examined. Statistical analysis of the results was performed using the Mann-Whitney rank sum test.

## Results

The lymphoid follicles in tonsils with HT contained hyperplastic GC and thinned MZ (Fig. 1a), while the lymphoid follicles in the tonsils with RT were small and the space



**Fig. 1.** Light micrograph of the palatine tonsil with: a) hypertrophic tonsillitis and b) recurrent tonsillitis. The pictures show the lymphoid follicles in which the pale-stained germinal centres and dark-stained mantle zones are visible. H&E  $\times 100$ .

between them, the interfollicular region, was dominant (Fig. 1b). The mean follicle area in HT ( $0.38 \pm 0.17 \text{ mm}^2$ ) was significantly ( $p < 0.05$ ) increased compared with RT ( $0.28 \pm 0.12 \text{ mm}^2$ ). The measurement of the GC area showed significant ( $p < 0.01$ ) differences between HT ( $0.26 \pm 0.13 \text{ mm}^2$ ) and RT ( $0.17 \pm 0.09 \text{ mm}^2$ ).

#### *Ki-67 expression in lymphoid follicles*

Follicular Ki-67-immunopositive cells (Fig. 2a) have similar localisation in both types of tonsillitis. The majority of Ki-67-immunopositive cells can be spotted in the dark zone of GC (Fig. 2b). They are also present to a lesser extent in the light zone of GC and in MZ. Numerical areal density ( $N_A$ ) of Ki-67-immunopositive cells (Table I) was greater in tonsils with HT compared with RT, with a statistically significant ( $p < 0.01$ ) difference for the dark zone of GC and MZ of lymphoid follicles.

#### *Bcl-2 and survivin expression in lymphoid follicles*

In both types of tonsillitis, Bcl-2-expressing cells were found primarily in MZ of lymphoid follicles (Fig. 2c, d). By quantitative analysis, we obtained a larger number of Bcl-2-expressing cells in RT, in comparison with HT, without statistical significance (Table I).

The expression of survivin (Fig. 2e) had typical nuclear localisation and the nuclei of the survivin immunopositive cells in GC displayed mitotic figures with strong delineation of chromosomes (Fig. 2f). By quantification of survivin-expressing cells, we obtained significantly higher values for GC of the lymphoid follicles in tonsils with RT in comparison with HT (Table I).

#### *Fas/CD95, caspase-3 and Bax expression in lymphoid follicles*

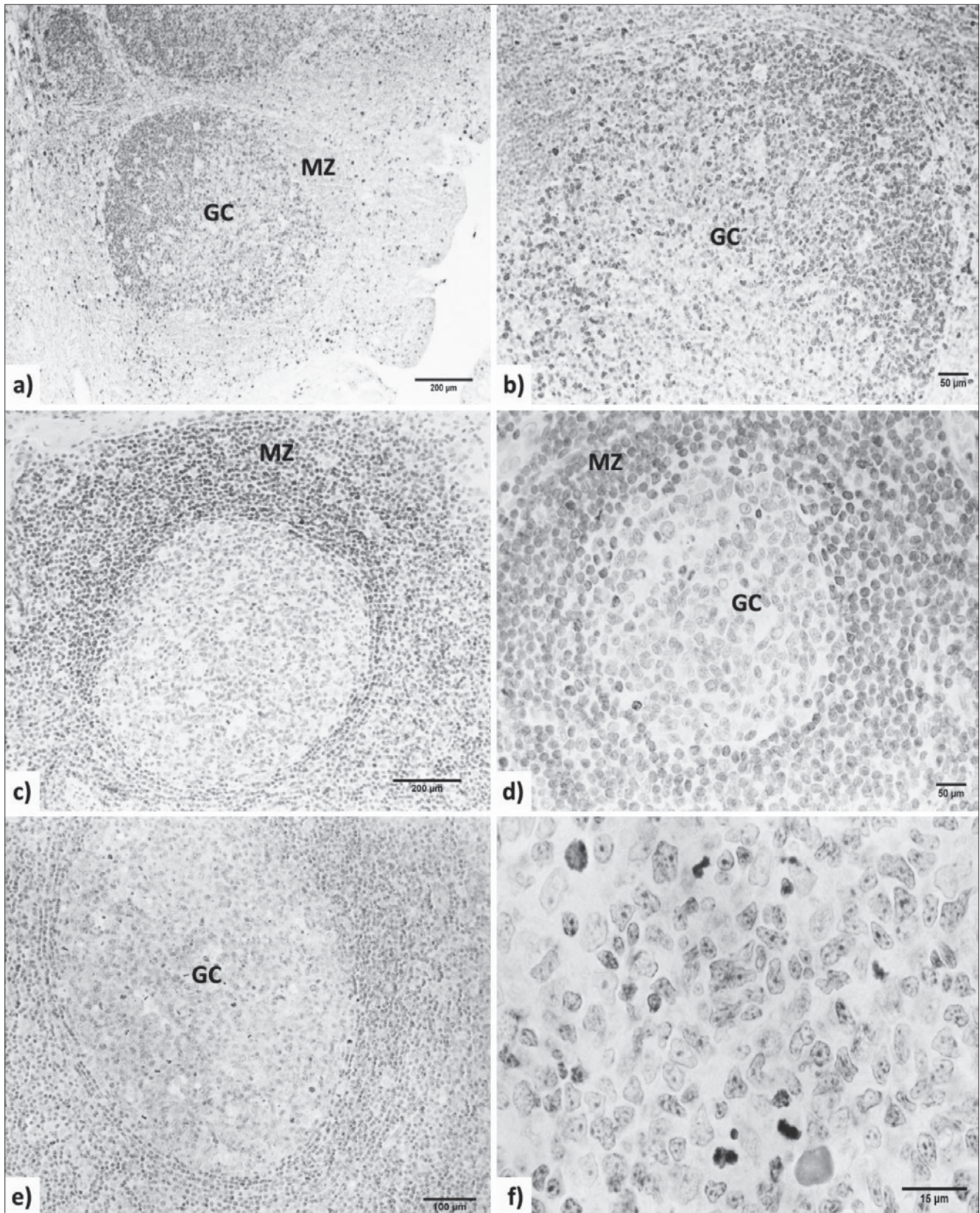
Fas/CD95 is expressed, mostly, by the cells of GC and smaller cells of MZ (Fig. 3a, b). In both types of tonsillitis

**Table I.** Number of lymphoid follicle cells per  $\text{mm}^2$  of tonsillar slice area ( $N_A$ ) in hypertrophic tonsillitis (HT) and recurrent tonsillitis (RT).

Antigen	Localisation	Numerical areal density ( $N_A$ )	
		HT (n = 6)	RT (n = 5)
Ki-67	Germinal centre dark zone	14,681.4 $\pm$ 1460.5	12,491.4 $\pm$ 2321.6**
	Germinal centre light zone	8014.4 $\pm$ 1404.7	7844.2 $\pm$ 1360.6
	Mantle zone	1406.9 $\pm$ 393.1	1001 $\pm$ 540.7**
Bcl-2	Germinal centre	Absent	Absent
	Mantle zone	10,856.4 $\pm$ 1171.3	13,253.7 $\pm$ 2226
Survivin	Germinal centre	117.4 $\pm$ 14.5	406.9 $\pm$ 98.4*
	Mantle zone	44.7 $\pm$ 9.1	50.2 $\pm$ 13.6
Fas/CD95	Germinal centre	291.1 $\pm$ 73.5	285.6 $\pm$ 94.7
	Mantle zone	85.9 $\pm$ 31.5	116 $\pm$ 21.9
Caspase-3	Germinal centre	210 $\pm$ 24	350.4 $\pm$ 119.4*
	Mantle zone	60.5 $\pm$ 25	257.8 $\pm$ 29.6*
Bax	Germinal centre	Scattered	Scattered
	Mantle zone	Absent	Absent

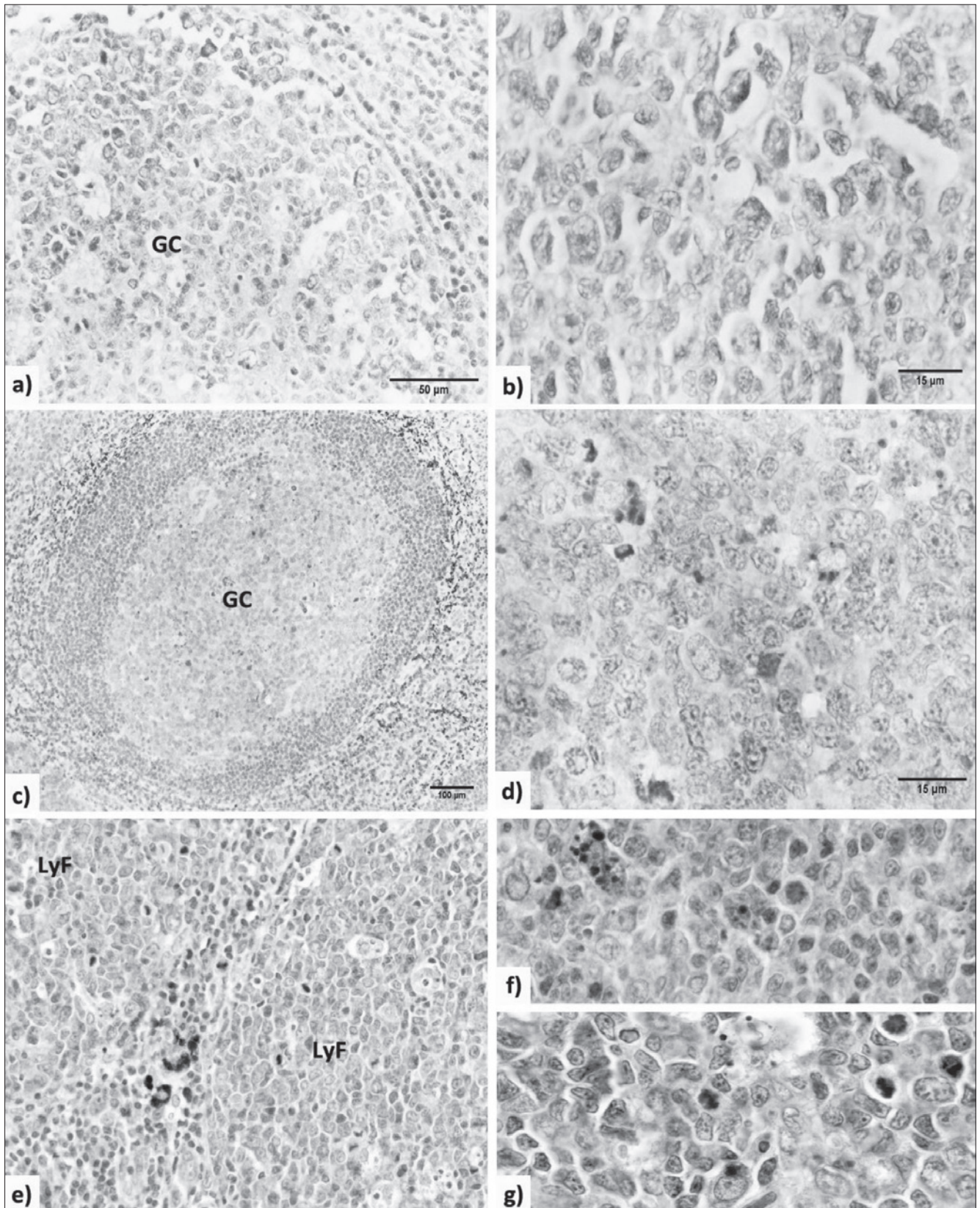
\* $p < 0.05$  vs HT; \*\* $p < 0.01$  vs HT.





**Fig. 2.** Immunohistochemistry of proliferative and anti-apoptotic activity in tonsillar lymphoid follicles. LSAB/HRP method: a) Expression of Ki-67 in germinal centre (GC) and mantle zone (MZ) of lymphoid follicle ( $\times 200$ ); b) Detail from the previous picture: Ki-67-immunopositive cells are the most numerous in the dark zone of germinal centre (GC) ( $\times 400$ ); c) Bcl-2 protein is expressed by the cells of the follicular mantle zone (MZ), ( $\times 200$ ); d) Absence of Bcl-2 expression in the germinal centre (GC) and strong expression in the mantle zone (MZ) is noticed ( $\times 400$ ); e) Survivin-expressing cells are present in the germinal centre (GC) of the lymphoid follicle ( $\times 100$ ); f) Survivin expression in a non-dividing cell (upper left corner) as well as the survivin-expressing cells with strongly stained mitotic figures (low central) are noticed ( $\times 400$ ).





**Fig. 3.** Immunohistochemistry of apoptotic activity in tonsillar lymphoid follicles. LSAB/HRP method: a) A part of the lymphoid follicle germinal centre (GC) with numerous Fas/CD95- expressing cells ( $\times 400$ ); b) Detail from the previous picture ( $\times 1000$ ); c) Individual caspase-3- expressing cells in the germinal centre (GC), ( $\times 100$ ); d) Detail from the previous picture: cytoplasm of the cell which morphologically corresponds to the follicular macrophage (upper left part), contains caspase-3 immunopositivity ( $\times 1000$ ); e) Strong Bax immunopositivity can be seen between the two lymphoid follicles (LyF), and weak immunopositivity in germinal centre cells ( $\times 400$ ); f) Germinal centre cells (upper lower part) show granular Bax immunopositivity, possibly phagocytosed apoptotic bodies ( $\times 630$ ); g) In the lower part of the picture a cell with strong Bax immunopositivity can be seen ( $\times 1000$ ).

there was the presence of a larger number of Fas/CD95-expressing cells in GC, comparing to MZ, but without any statistical difference (Table I).

In both types of tonsillitis, caspase-3 is detected in GC and MZ (Fig. 3c, d). The larger number of cells that express caspase-3 was seen in RT in comparison with HT (Table I).

Clear expression of Bax protein was observed in a few cells in the GC and on the border between the GC and MZ (Fig. 3e-g), with no differences in relation to the type of tonsillitis. Due to the small number of Bax-immunopositive cells, quantitative analysis was not performed.

## Discussion

In this study, we have quantified the cells expressing the cell proliferation and cell death markers in the lymphoid follicles of the human palatine tonsil in two different entities of chronic tonsillitis: HT and RT.

The ultimate role of the GC of the lymphoid follicle is to differentiate B memory lymphocytes which express Ig-receptors with high affinity for binding to an antigen, and to differentiate into plasma cells secreting Ig with high affinity for a specific antigen<sup>36</sup>. Some B cells do not allow antigen-specific B-cell responses, and as aberrant cells activate pro-apoptotic factors and die by apoptosis<sup>7 12 13</sup>.

The cell marker of proliferation, Ki-67 protein<sup>27</sup>, was the most strongly expressed in the dark zone of GC. A larger number of follicular Ki-67-immunopositive cells in HT clearly displays higher proliferative activity in the lymphoid follicles of tonsils with HT, compared to those with RT. Based on the data according to which FDC precursors are predominantly localised in the dark zone and highly differentiated FDC subtypes in the light zone of GC<sup>2</sup>, and considering the localisation of Ki-67-immunopositive cells in our samples, we can assume that the highest proliferative activity of B lymphocytes in GC co-localises with precursors of FDC, whereas the lowest proliferative activity can be observed in the region corresponding to the localisation of differentiated FDC subsets. A fewer number of Ki-67-immunopositive cells in apical region of the light zone of GC is expected, because this part of GC is the place where selection of B cells occurs exclusively, with the help of corresponding subsets of FDC<sup>19</sup>. In our study, the higher GC proliferative activity in HT correlated with the GC surface values, which is in agreement with the results of Zhang et al.<sup>28</sup> who determined the size of the lymphoid follicles in HT and RT by the similar morphometric method.

The apoptosis inhibitor Bcl-2, together with pro-apoptotic factor Bax protein, controls transport through the pores on the external mitochondrial membrane by preventing the exit of cytochrome c from mitochondria and apoptosis initiation<sup>29 30</sup>. Kondo and Yoshio<sup>13</sup> recently reported that GC-cells do not display the expression of Bcl-2, whereas

cells in the MZ express a high level of Bcl-2. In our study, strong expression of Bcl-2 in MZ-cells, along with the absence of Bax expression, confirms that B memory cells in the MZ are highly differentiated cells that underwent a process of clonal selection and are protected from apoptosis by expression of Bcl-2 protein.

Studies of apoptosis in lymphoid organs have shown strong expression of Fas and FasL<sup>9 12</sup>. Apart from that, the Fas/FasL signal is one of the best studied and described mechanisms of apoptosis, which is characteristic of various cell types including B lymphocytes in lymphoid follicles<sup>6 10 21 30</sup>. By comparing the number of the cells expressing Fas/CD95 with the number of Bax-expressing cells in GC, we noticed domination of expression Fas/CD95, which shows that apoptosis in GC cells is initiated primarily by the extrinsic pathway of activation<sup>24</sup>.

We demonstrated that tonsillar follicle cells express caspase-3, as described by Lukeš et al.<sup>21</sup>. It is necessary to point out that we found expression of caspase-3 in the cytoplasm of numerous cells that are morphologically related to follicular macrophages, which confirms the role of such cells in phagocytosis of apoptotic cells<sup>14</sup> and supports the fact that proteolytic activity of caspase-3 takes place rapidly<sup>24</sup>. Hence, it cannot always and in due time be noted in dying cells on histological preparations. The presence of caspase-3 expression and absence of Bcl-2 expression in GC are indicators of two diametrically opposed processes taking place in GC, namely cell death and cell survival.

Unlike previous studies that mostly relied on morphological identification and description of the localisation of Fas- and caspase-3-expressing cells, we morphometrically quantified these cells and found a significantly larger number of caspase-3-immunopositive cells in RT than in HT. Lopez-Gonzales et al.<sup>11</sup> previously pointed out that apoptotic parameters decreased in the tonsils with HT compared to those with RT. With respect to their data, as a verification of the validity of our results, more prominent apoptosis in the tonsils with RT can be explained by the greater production of inflammatory cytokines and their effect on immunological processes inside GC<sup>17</sup>.

A significantly smaller number of survivin-expressing cells in GC in HT suggests that its expression is independent of proliferative activity, yet it may be related to the expression of pro-apoptotic factors, i.e. with apoptosis. Our hypothesis is supported by the fact that intracytoplasmic localisation of survivin is associated with apoptosis inhibition through the degradation of caspase-3, caspase-7 and caspase-9<sup>31 32</sup>. Considering our findings that both greater apoptotic activity and stronger expression of survivin were observed in lymphoid follicles of tonsils with RT, it is assumed that survivin is expressed in those cells which have already received some death signal and which attempt to prevent dying by stopping the cell cycle.

In this study, we have shown that proliferative activity of



cells in GC of lymphoid follicles is dominant in tonsils with HT in comparison to those with RT, and that, contrary to this, the cells in GC die by apoptosis at a higher rate in tonsils with RT than in those with HT. Moreover, the similar expression of Fas receptors in both types of tonsillitis confirms that activation of Fas receptors is the main signal for the initiation of apoptosis in the cells of GCs.

## Conclusions

The relationship between the intensity of proliferative and apoptotic activities in tonsils with HT and RT is a good indicator of tonsil immunological potential, and can thus serve to better understand the immunological status of patients with various types of chronic tonsillitis. However, the knowledge of biological processes going on in the palatine tonsil is not yet sufficient to guide final decisions on how to best treat chronic tonsillitis, conservatively or surgically.

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