ENZYMATIC STUDY OF WALDEYER’S RING LYMPHOID TISSUE: ACTIVITY OF ALKALINE AND ACID PHOSPHATASE IN PALATINAL TONSILS AND ADENOIDS IN CHILDREN WITH RECURRENT INFECTION OF THE RING

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Summary: Activity and kinetic properties of tissue nonspecific alkaline phosphatase and acid phosphatase were investigated in adenoids and tonsils of 62 children tonsiloadenoidectomised because of recurrent infection using p-nitrophenylphosphate as a substrate. Kinetic properties were calculated by the method of Levenberg-Marquardt. The mean value of tonsil TNAP activity was 3.525 U/mg of protein versus 7.280 U/mg of protein in adenoids (t = 5.928, df = 60, p < 0.01). ACP activity was also significantly lower in tonsils (10.844 U/mg of protein) than in adenoids (13.059 U/mg of protein) (t = 11.318, df = 60, p < 0.01). There were no influence of age and sex to both enzyme activities. TNAP activity was significantly higher in hypertrophic (4.132 U/mg of protein) than in atrophic (2.531 U/mg of protein) (t = 2.361, df = 20, p < 0.05). Tonsillar TNAP was more effective than adenoid TNAP (t = 11.769, df = 60, p < 0.01). Results suggest the possibility that recurrent infection influences the tonsils more than adenoids and age. Hypertrophy could be an adaptive mechanism of palatinal tonsils during the infection.

Key words: tonsils, adenoids, acid phosphatase, alkaline phosphatase

Introduction

Recurrent infection of the Waldeyer’s ring (RIW) is a form of chronic tonsillitis and adenoiditis. The term assumed that there are 3 to 5 episodes of acute infection a year. The mechanism of development recurrent tonsillitis has not been elucidated yet. It is estimated that lymphocytes from the diseased tonsils become refractory or tolerant to immune activation by certain pathogens, either bacterial or viral.

The enzymatic studies point to relation of enzymes such superoxide dismutase, nitric oxide synthase and arginase in chronic tonsillitis and tonsillar hypertrophy (1–3).

The tonsils and adenoids are lymphoid organs that are a part of mucosa-associated lymphoid tissue

(4). They consist of lymph follicles with B-lymphocytes dispersed in reticular fiber formation with presence of follicular dendritic cells (FDC), macrophages (M) and T lymphocytes (T) (5, 6). FDC and macrophages are known to be important in binding and processing the antigens to the immunocompetent T and B-lymphocytes (5, 7, 8). In contrast with the other mucosal lymphoid tissue macrophages are observed in palatinal tonsils (5). Enzyme characteristically detected in the lysosome compartment of M and FDC is acid phosphatase (8). The proliferation and differentiation of B cells are enzyme-mediated events. Enzyme tissue-nonspecific alkaline phosphatase (TNAP) as a glicoprotein is a part of cell membrane probably having the role of transmission the information between the different immunocompetent cells. TNAP is the enzyme principally expressed in B-lymphocytes, liver, bone and kidney. The expression of TNAP is in correlation with the stage of B cellular differentiation and proliferation (9, 10). If it is known that tonsils and adenoid are predominant B lymphocyte organs, it could be assumed that TNAP as a marker of B cells proliferation and activity.
Little is known about the activity and function of these enzymes in different parts of Waldeyer’s ring in children with chronic recurrent infection.

The aim of the study is to characterize the physicochemical and kinetic properties of tissue nonspecific alkaline phosphatase (TNAP) and acid phosphatase (ACP) in different part of Waldeyer’s ring, to establish the possible differences between the activity of these enzymes in palatinal tonsils and adenoids, to establish relation to sex and age, and to correlate the activity of TNAP in palatinal tonsils of children with RIW with and without hypertrophy.

**Material and Methods**

Palatinal tonsils and adenoids are obtained from 62 children, aged between 3–14. Most of them (29) were aged between 5–7 years, 20 were younger than 4, and 15 older than 8. There were 41 males and 21 females. The indication for tonsiloadenoidectomy was recurrent episodes of acute infection of Waldeyer’s ring 3–5 during a year. All of them were infection free a month prior the surgery and no antibiotic treatment was applied during that period. All the samples had undergone bacteriological, biochemical and histopathological examination.

According to the enlargement of the tonsils, the patients were divided in two groups. Patients with tonsillar asymmetry were excluded (11 patients). In the first group there were patients with a small tonsils which were either totally within the tonsillar fossa or minimum visible beyond the anterior tonsils pillous (6 patients), in the second group there were patients with enlarged tonsils occupying 50–70% of the oropharyngeal airway (45). These patients had no obstructive symptoms.

**Bacteriological examination.** Immediately after surgical procedure, the bacteriological investigation of tonsil core and adenoids on aerobic bacteria was undertaken.

**Histopathological investigation.** The paraffin sections of 22 palatinal tonsils were stained using hematoxylin-eosin technique. The histological criteria for tonsillar atrophy were the absence of lymph follicles or presence of one or two dark follicles without germative center (6 patients). Histological hypertrophied tonsils were assumed as tonsils with numerous lymph follicles with bright germative centers on the section (16 patients). The differences in TNAP activity between atrophic and hypertrophic tonsils were statistically tested using Student’s t test for small independent samples.

**Biochemical examination.** Immediately after the operation (max 30 min), the tissue was put on ice, their weight was measured and than homogenized for 60 s, using Polytron homogenizer at 4 °C, in 10 mmol/L Tris-HCl buffer pH 7.4 containing 50 mmol/L mannitol, 0.1 mmol/L MgCl2 and 0.02 mmol/L ZnCl2. Alternatively, the tissues were stored for 24 hours before homogenization at –20 °C. The homogenates of tonsils and adenoids were used for determination of alkaline and acid phosphatase activities

TNAP and ACP activity was estimated by the method of Japundžić (11).

Method of TNAP and ACP activity estimation is based on characteristics of both enzymes to hydrolyze p-nitrophenylphosphosphate (pNPP) in yellow colored p-nitrophenol with pick of absorption on 410 nm. The essential reagents are different. The essential reagenses for estimation of TNAP activity are 250 mmol/L carbonate bicarbonate buffer pH 10.6, 50 mmol/L MgCl2, 10 mmol/L ZnCl2, 10 mmol/L Dithiorthiol (DTT), 75 mmol/L paranitrophenilphosphat (pNP) and distilled water.

The mixture of 0.2 ml of 50 mmol/L carbonate bicarbonate buffer pH 10.6 with 0.1 ml of 5 mmol/L MgCl2, 0.1 ml of 1mmol/L DTT, 0.2 ml of 15 mmol/L pNP, 0.2 ml of 1 mmol/L ZnCl2 and 0.2 ml of distilled water was stored 5 min at 37 °C, than mixed with 0.1 ml of tissue homogenate and incubated 10 min at 37 °C. To stop the reaction 1 mL of 250 mmol/L NaOH was added. Absorbance of mixture was measured spectrophotometrically by Gilford method at 410 nm by means of a blank control lacking the enzyme (12). The essential reagents for ACP activity estimation consist of 500 mmol/L acetate buffer pH 4.6, 75 mmol/L pNP and distilled water. To determine ACP activity 0.2 mL 100 mmol/L of acetate buffer, 0.2 mL 15 mmol/L pNP and 0.5 mL of distilled water were mixed, stored 5 min at 37 °C, and than mixed with 0.1 mL of tissue sample and incubated for 10 min at 37 °C. The reaction was stopped with 250 mmol/L NaOH and concentration of p-nitrophenol was determined spectrophotometrically at 410 nm. TNAP and ACP activity was expressed in U/μg of protein.

**Protein concentration.** Total proteins were determined by the method of Bradford, using bovine serum albumin as a standard (12). The principle of the method is transformation of red color of Co-masie-Brilliant blue G-250 in blue variant after connection to protein with the absorbance pick at 595 nm. The intensity of blue color is directly proportional to the protein concentration from the standard curve.

**Kinetic properties.** Kinetic parameters (Michaelis-Menten constant-Km and Vmax) of TNAP and ACP for hydrolysis of pNPP as a substrate were determined in tonsillar and adenoid tissue of 62 tonsilloadenoidectomized children for chronic tonsillitis using increasing pNPP concentrations 1–40 mmol/L in incubating mixture on each enzyme optimal pH. Series of softest substrate solution were prepared using essential substrate solution of 200 mmol/L concentration. The method for initial V (Vi) estimation of TNAP and ACP mediated pNPP hydrolyze was the same as for estimation of both enzyme activities. The
reaction was carried out using different substrate concentrations. The results were shown in form of Michaelis-Menten hyperbole and its linear variant Lineweaver-Burk diagram (13). Fitted constants were calculated by non-linear regression estimation method of Levenberg-Marquardt (14, 15).

**pH optimum.** The effect of pH on TNAP activity was determined using 250 mmol/L carbonate/bicarbonate buffer in the 9.2–11.0 range, with pNPP as a substrate. For determination of pH for ACP, the activity was measured using 500 mmol acetate buffer in the 3.8–6.3 pH range, with pNPP as a substrate. The activity of TNAP and ACP was determined as described above but with different buffers concentrations representing different pH.

**Statistical analyses.** Standard errors, analysis of variance and Student’s t test for independent and paired samples were carried out using computer program SPSS.

**Results**

**TNAP and ACP activity in palatinal tonsils and adenoids from children suffering from recurrent infection of Waldeyer’s ring**

To determine the influence of recurrent infection on different parts of Waldeyer’s ring lymphoid tissue, the differences between TNAP and ACP activities in different parts of Waldeyer’s ring were examined. Both enzymes show higher activities in adenoids comparing to palatinal tonsils in the same patient (Table I). Mean value for TNAP activity in tonsils of 62 operated children was 3.525 U/mg of protein versus 7.206 U/mg of protein in adenoids. Paired Student’s t test shows high statistical significance (t = 5.928, df = 60, p < 0.01).

Mean value for ACP activity in tonsils was 10.844 U/mg of protein that was less than ACP activity in adenoids of the same children of 13.059 U/mg of protein. Student’s t test was highly significant (t = 11.318, df = 60, p < 0.01) (Table I).

**Influence of sex and age on TNAP and ACP activity in different parts of Waldeyer’s ring**

Table I represents specific activities of TNAP and ACP in palatinal tonsils and adenoids from females and males, respectively. Activities of both enzymes in either palatinal tonsils or adenoids are higher in females comparing to males, but the differences are not statistically significant.

Table II shows specific activities of TNAP and ACP in different parts of Waldeyer’s ring in children according to age. Although we have determined discrete decrease in both enzymes activity, in palatinal tonsils and adenoids, one way ANOVA shows no statistical significance.

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>TNAP activity (U/mg protein)</th>
<th>ACP activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Palatine tonsils</td>
<td>Adenoids</td>
</tr>
<tr>
<td>Male</td>
<td>41</td>
<td>3.397 ± 0.114</td>
<td>7.419 ± 0.163</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>3.908 ± 0.200</td>
<td>6.554 ± 0.490</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>3.525 ± 0.162</td>
<td>7.206 ± 0.334</td>
</tr>
</tbody>
</table>

TNAP – Tissue nonspecific alkaline phosphatase  
ACP – Acid phosphatase

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>TNAP activity (U/mg protein of proteins)</th>
<th>ACP activity (U/mg of proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenoids</td>
<td>Palatine tonsils</td>
</tr>
<tr>
<td>&lt; 2</td>
<td>7.789</td>
<td>4.167</td>
</tr>
<tr>
<td>2–4</td>
<td>7.268</td>
<td>3.769</td>
</tr>
<tr>
<td>5–7</td>
<td>7.067</td>
<td>3.589</td>
</tr>
<tr>
<td>&gt; 7</td>
<td>6.760</td>
<td>3.269</td>
</tr>
</tbody>
</table>

TNAP – Tissue nonspecific alkaline phosphatase  
ACP – Acid phosphatase
Physicochemical and kinetic properties of TNAP and ACP in palatinal tonsils and adenoids in children with recurrent infection of Waldeyer’s ring

The influence of pH on TNAP and ACP activity. pH optimum for TNAP from both palatinal tonsils and adenoids was determined using 250 mmol/L carbonate bicarbonate buffer with different pH values, ranging from 9.2–11.0.

TNAP activity was determined with different pH. The results showed higher TNAP activity for the enzyme in both tissues with pH value of 10.6 (Figure 1).

In order to determine pH optimum for ACP, activity of the enzyme was determined with different pH using acetate buffer. The maximum of ACP activity was estimated in both tissues with pH of 4.6 (Figure 2).

Kinetic properties. Maximal velocity of the reaction (Vmax) and Michaelis-Menten constant (Km) are kinetic properties specific for each enzyme and its substrate.

Vmax is velocity of enzyme-catalyzed reaction reached with that substrate concentration which saturated the whole enzyme (enzyme-substrate complex; ES complex). Km represents substrate concentration reaching the half of Vmax and affinity of the enzyme to the substrate. If Km is lower the enzyme affinity to substrate is higher.

TNAP and ACP activities in palatinal tonsils and adenoids were determined with different substrate (pNPP) concentrations in range 1–40 mmol/L at both enzyme pH optimum using the method described before.

The relation between enzyme catalyzed reaction initial velocity (Vi) and substrate concentration (S) in palatinal tonsils and adenoids is given in graphics look like hyperbola suggesting that both enzymes in both tissues behave according the rules of Michaelis-Menten kinetics. Transformed Michaelis-Menten hyperbola for TNAP in tonsils and adenoids in Line waever Burk diagram is shown in Figure 3.

Linewaever Burk diagram for ACP in tonsils and adenoids is shown in Figure 4.

Numeric values of kinetic parameters were counted using nonlinear regression method Levenberg Marquard by computed program SPSS.

Results showed that TNAP Km was higher in adenoids than in tonsils (Km adenoids 2.914 mmol/L pNPP versus Km tonsils 2.445 mmol/L pNPP) suggesting lower affinity of the enzyme for the substrate in adenoid tissue of the same children. Higher adenoid TNAP Km versus tonsillar TNAP Km was statistically significant (t = 11.769, df = 60, p < 0,01).

Tonsillar ACP Km was near adenoid ACP Km (adenoid ACP Km 0.950 mmol/L of pNPP versus tonsillar ACP Km of 0.965 mmol/L).

TNAP and ACP activity and histopatological findings

16 clinically hypertrophied tonsils and 6 clinically atrophic tonsils had been subjected to histopatological procedure using haemathoxilin-eosin technique. Hypertrophied tonsils showed increase of number of lymph follicles with large and bright germinate center. Atrophic tonsils show only one to two dark lymph follicles without enlargement of germinate center. Biochemical analysis showed lower level of TNAP activity in the tissue of atrophic tonsils, and the difference was of statistical significance. Mean value of TNAP specific activity in hypertrophic tonsils was 4.132 U/mg of protein versus in atrophic tonsils 2.531 U/mg of protein. Statistical analysis using Student’s t test for small samples showed the statistical significance (t = 2.361, df = 20, p < 0.05).

Figure 1 Influence of pH on (TNAP) activity in palatine tonsils and adenoids

Figure 2 Influence of pH on ACP activity in palatine tonsils and adenoids
**Discussion**

Palatinal tonsils and adenoids as a part of pharyngeal mucosal lymphoid system play the role of the first-line defence system of the body. They are composed of B-lymphocytes arranged in follicles, T-lymphocytes and macrophages located in the surrounding tissue. B-lymphocytes are responsible for synthesis of different immunoglobulins mostly IgG, especially with polyclonal activators such as different bacteria. The spectrum of bacteria in tonsillar tissue varies depending of the season and region (16, 17).

Our bacteriological analysis shows the same mixed flora in tonsils and adenoids of the same patient with predominant Haemophylus influenzae, Sterptococcus pneumoniae, Sterptococcus group A and Staphylococcus aureus.

Presence of TNAP is a characteristic of B-lymphocytes and might be presumed as a marker of B cell proliferation and differentiation (9).

This enzyme is mainly component of the cell membranes playing role in transmission the information between distinct immunocompetent cells. Results of the study show that TNAP and ACP activity is higher in adenoid tissue than in palatinal tonsils in all the investigated samples. Kinetic properties of the adenoid TNAP are different form those in palatinal tonsils. According to Km value the enzyme is more effective in tonsils than in adenoids. It could be that recurrent infection influences the adenoid immunoreactivity less than tonsillar one, although the same bacterial antigens are present in both tissues in one person.
Macrophages are important for ingestion, decomposition of bacteria and presentation of its antigens to T and B-lymphocytes. Similar role in processing the antigens have follicular dendritic cells (FDC). Presence of acid phosphatase is a characteristic of tissue macrophages and FDC. Higher activity of acid phosphatase in adenoids than in tonsils points to possibility that differences in this part of immune response exist between the two tissues.

The tonsillar and adenoid tissues undergo some changes according to age and infection. The mechanism of infectious influence is not yet clear. The assumption is that lymphocytes from diseased tonsils could be refractory or tolerant to these bacterial activators showing less degree of proliferation on stimulation with dominant bacterial antigens than healthy one (18, 19). The problem of investigator is the fact that healthy tonsils could be hardly defined. The term of chronic tonsillitis is clinically defined as the episodes of acute recurrent infection 3–5 a year or tonsillar hypertrophy. The mechanism of chronic tonsillitis is not yet clear. Nitric-oxide and its toxic metabolities are assumed as factors in chronic tonsillitis development (2). On the other hand enzyme arginase is in some relation with tonsillar hypertrophy (3).

The recurrent infections of palatinal tonsils lead to some histomorphological changes in term of tonsillar atrophy or hypertrophy. Some histomorphological changes could be noticed in tonsils with chronic infection. In this study the two types of histological changes were noticed. The first type represents the hypertrophied tonsils with large number of follicles with bright and active germinative centers. On the other side there are atrophic tonsils with only one or two dark follicles suggesting poor immunological activity. In our sample hypertrophic tonsils are present more often than atrophic (88% versus 12%).

Higher activity of TNAP is noticed in hypertrophic tonsils than in atrophic ones. If TNAP is assumed as a marker of B cell activation and proliferation, there is a positive correlation between the histomorphological signs of lymphoid activity and TNAP activity. The results suggest also that TNAP could be a marker of tonsillar immunological activity. Recurrent infection, producing a possible immunological tolerance to antigen stimulation, could lead to the tonsillar hypertrophy. Thus, hypertrophy could be an adaptive mechanism in term of improvement of immunological function destroyed by recurrent infection.

There is the influence of age to tonsillar changes. Some studies showed a correlation between the age and tonsillar B population (6). According to these changes number of Ig positive B cells decline with age. The area of activated follicles in human palatinal tonsils is largest between 4 and 10 years of age with the marked decline after the age of 20. The overall proportion is less affected by recurrent infection than by age (6). As the results of this study showed, there is no correlation between TNAP and ACP activity and sex and age.

The results suggest higher TNAP and ACP activity in adenoids than in tonsils in children with recurrent infection. TNAP is more effective in tonsils than in adenoids according to its kinetic properties. There is no correlation between enzymes activity and sex and age. Recurrent infection of the palatinal tonsils leads more often to the hypertrophy than atrophy of the tissue in children. According to the higher TNAP activity in hypertrophy tonsils, such a change could be an adaptive mechanism in preserving the immunological competence of the tonsil in contrast to the atrophic one.

ENZIMSKA STUDIJA LIMFNOG TKIVA WALDEYER-OVOG PRSTENA: AKTIVNOST ALKALNE I KISELKE FOSFATAZE U PALATALNIM TONZILAMA I ADENOIDE KOD DJECE SA REKURENTNIM INFEKCIJAMA PRSTENA

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Kratki sadržaj: Aktivnost i kinetičke osobine tkivno nespecifične alkalne fosfataze (TNAP) i kiselske fosfataze (ACP) ispitivane su u tkivu adenoida i tonsila 62 deteta operisana zbog ponavljanih infekcija Waldeyer-ovog prstena. Enzimskak aktivnost određivana je uz korišćenje p-nitrofenola kao supstrata, a kinetičke osobine metodom Levenberg-Marquardta. Srednja vrednost aktivnosti TNAP u tkivu tonsila iznosila je 3,525 U/mg proteina što je značajno niža nego aktivnost u adenoidnom tkivu (7,280 U/mg proteina). Srednja vrednost aktivnosti ACP u tkivu tonsila bila je statistički značajno niža nego u adenoidu (10,844 U/mg proteina u odnosu na 13,059 U/mg proteina) (f = 11,318, df = 60, p < 0,01). Pol i uzраст dece nisu bili od uticaja na enzimsku aktivnost. Aktivnost TNAP bila je značajno viša u hipertrofijnom (4,132 U/mg proteina) u odnosu na atrofičnom tonsilu (2,531 U/mg proteina) (f = 2,361, df = 20, p < 0,01). Efikasnost tonsilarnih TNAP bila je značajno viša od adenoidine (f = 11,769, df = 60, p < 0,01). Rezultati ukazuju na mogućnost da ponavljane infekcije imaju značajniji uticaj na tonsilarno nego na adenoidno tkivo, a da hipertrofija tonsila kod ovog tipa hroničnog tonsilitisa predstavlja adaptivni mehanizam u cilju očuvanja njene imunokompetentnosti.

Ključne reči: tonsil, adenoid, kiselka fosfataza, alkalna fosfataza
References


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