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Diagnostic Value of Histological and Microbiological Screening in Etiopathogenesis of Recurrent and Hypertrophic Tonsillitis

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Abstract: *Interest in the mechanisms and causes of recurrent tonsillitis is considerable. CD4+CD25+ T-lymphocytes have an important role in the maintenance of immunological tolerance. The aim of our research was to compare the diagnostic value of palatine tonsils histological and microbiological screening in patients with hypertrophic and recurrent tonsillitis. 14 patients with hypertrophic and 10 patients with chronic tonsillitis undergoing tonsillectomy were enrolled in the study. Rapid diagnosis of adenovirus, parainfluenza, influenza A and B, and respiratory syncytial virus infection was made before tonsillectomy by viral antigen detection using the immunofluorescence procedure from tonsils. Herpes simplex and cytomegaloviruses DNA were detected by the polymerase chain reaction. Samples for bacteriological studies were collected using a cotton swab. Immunohistochemical methods were used to evaluate S-100 and TGF-beta1 expression. The obtained results showed that patients with recurrent tonsillitis had less S-100 and TGF-beta1 positive cells in parafollicular regions compared to patients with hypertrophic tonsillitis. In both groups, tonsils were colonized predominantly by gram-positive microorganisms and adenovirus (36% of cases). However, in patients with recurrent tonsillitis, associations of gram-positive, gram-negative bacteria and viruses (40% of cases) were observed. To conclude, recurrent tonsillitis is characterized by the breakdown of the immunological tolerance to oral microflora.*

Keywords: *Immunological tolerance, Tonsillitis, TGF-β1.*

Introduction

The oral mucosa are bombarded immediately after birth by a large variety of microorganisms as well as by protein antigens from the environment, the latter particularly in formula-fed infants, and the mucosal surface to be protected is enormous, probably almost 200 times that of the skin [4]. During the evolution over millions of years, the mucosal immune system has generated two arms of adaptive defence to handle these challenges: antigen exclusion performed by secretory IgA (SIgA) and secretory IgM (SIgM) antibodies to modulate or inhibit the colonization of microorganisms, and dampen the penetration of potentially dangerous soluble luminal agents and suppressive mechanisms to avoid local and peripheral overreaction (hypersensitivity) against innocuous substances bombarding the mucosal surfaces [2, 3]. The latter arm is referred to as "immunological tolerance". Similar

downregulatory mechanisms apparently operate against antigens from the commensal microbial flora [9].

The palatine tonsils represent inductive sites for oral mucosal immune responses. The tonsil structure includes B-cell follicles, intervening T-cell areas, and a variety of antigen-presenting cell subsets with different lymphatics supplying antigens for immunological stimulation.

The induction of immunological tolerance is an important mechanism for maintaining homeostasis in the immune system and for regulating the fates of lymphocytes following encounters with self-end foreign antigens. There are natural (CD4+CD25+) and inducible (CD4+CD25-) populations of T-regulatory (Treg) cells, which play an important role in suppressing responses to self-antigens, and controlling the immunity to pathogens. Treg cells can be expanded by stimulation with antigen loaded mature dendritic cells [8, 14, 26].

Recurrent tonsillitis typically caused by the Streptococcus species occurs in children aged 5-16 [1, 6]. In turn to recurrent tonsillitis, tonsillar hypertrophy (hypertrophic tonsillitis) itself is not a disease, but only a result of increased immunologic activity. It is not necessary to be due to inflammation or tonsillitis [17]. Those hypertrophic tonsils with acute upper airway obstruction are usually associated with acute infection but not with the chronic type. Chronic tonsillar hypertrophy can be asymptomatic and may not lead to any problem. However, a marked increase in the size of tonsil accounts for up to 80% of obstructive sleep apnoea (OSA) in children. In the severe form, OSA can lead to cor pulmonale, pulmonary hypertension, pneumonia, chronic hypercapnia or hypoxia and eventually right heart failure [18].

The pathogenesis of recurrent tonsillitis is largely unknown. In recent years, a new category of CD25+ regulatory lymphocytes has been identified [13, 26]. These cells are accumulated at tissue sites of antigen invasion where they exert site-localized immune suppression producing IL-10 and transforming growth factor-β1, TGF-*β1* [8]. In turn, mast cells appear to be key players in the initiation of inflammation, but dendritic cells enhance the antigen specific T-cell activation [16, 25]. There are certainly different initial factors, but the mechanisms that lead to the recurrent tonsillitis are not clear. Possibly, in the natural evolution of tonsillitis, two phases may be distinguished: 1) phase of immune tolerance and 2) phase of fault-tolerance. In the first phase, immature dendritic cells present antigen in the tolerogenic rather than immunogenic fashion. As a result, Treg develops making immunocompetent cells tolerant to constant exposure of resident microflora. In the second phase, increased secretion of sex hormones may stimulate the maturation of dendritic cells and inhibit the ability of these cells to perform their normal “tolerance inducing” role [30].

The aim of our study was to compare the diagnostic value of histological and microbiological screening to elucidate the etiology and pathogenesis of recurrent and hypertrophic tonsillitis. For this purpose, we investigated the microanatomical distribution, quantity and morphology of TGF-*β1* positive cells, dendritic cells and mast cells, and the microbiological tonsil’s status of patients with recurrent and hypertrophic tonsillitis.

Patients and methods

The study was approved by the local ethical committee, and it conformed to the Helsinki Declaration. 24 patients undergoing tonsillectomy were enrolled in the study. They were subdivided into two groups according to the clinical criteria of tonsillitis [18]. 14 patients

(aged from 4 to 12 years) had recurrent tonsillitis, and 10 patients (aged from 4 to 12 years) had tonsils hypertrophy with no history of recurrent tonsillitis.

Histological and immunohistochemical studies

The palatine tonsils were sampled and marked appropriately, fixed in 10% neutral buffered formalin, processed and embedded routinely. The paraffin-embedded tissue was cut in a 4 μm thick section and stained with Mayer's hematoxylin and eosin. For immunohistochemistry, the formalin-fixed paraffin embedded tissue was cut in 4-μm thick sections on Histobond electrostatic slides (*Menzel-Glasser*, Germany). Antigen retrieval was achieved by treatment in a domestic microwave for 30 min in citrate buffer pH = 6.0. The endogenous peroxidase activity was blocked by 0.5% H_2O_2. Primary antibodies against the following antigens were used: CD25 (1:50), TGF-β (1:100), mast cells tryptase (1:200) and S100 (1:1000). *LSAB+* (labelled streptavidin and biotin from *Dako Cytomation*) was used for visualization of the bound with the primary antibodies. Briefly, slides were incubated in a humidity chamber for 30 min each with biotinylated secondary antibody and streptavidine with preceding, interventing and subsequent rinses in isotonic buffer (pH = 7.6), 2*5 min. 3'3-diaminobenzidine-tetrahydrochloride (DAB) was applied as chromogen (7 min). Sections were counterstained in hematoxylin (1 min). Positive and negative controls reacted appropriately. Slides were analysed using *Image ProPlus* software *Leica 4000B*. Positive immunostained cells were counted in ten fields (*400), and their total number was recorded. The results were expressed as mean values ± SEM. The *Mann-Whitney U* test was employed for two-group comparison. All calculations were performed with *GraphPadPrism* 3 version software. Any P value < 0.05 was considered significant.

Microbiological studies

The samples for bacteriological and virological studies were collected before tonsillectomy by a cotton swab. Identification of different microorganisms species was performed routinely. The rapid diagnosis of adenovirus and respiratory syncitial virus was made by the indirect immunofluorescence procedure. The Herpes simplex and CMV virus DNA was detected by PCR.

Results

Histological studies

Our results showed that there was no significant difference in the mean follicle numbers per counting area between two groups. However, the mean follicle area in hypertrophic tonsillitis was significantly larger than in recurrent tonsillitis (0.275 ± 0.013 and 0.1583 ± 0.016, p = 0.002).

Dense infiltrates of S-100 positive dendritic cells were noted in the surface and cryptal epithelium, and in parafollicular regions. Patients with hypertrophic tonsillitis had more S-100 positive cells in parafollicular regions compared to recurrent tonsillitis (136 ± 12 *vs* 55 ± 6 cell, p = 0.002). It was shown that patients with hypertrophic tonsillitis had more mast cells in parafollicular regions compared to the case of recurrent tonsillitis (38 ± 6 *vs* 28 ± 5 cell, p = 0.04).

TGF-β1 positive cells were identified in parafollicular regions and cryptal epithelium. We observed that patients with recurrent tonsillitis had less TGF-*β1* positive cells in parafollicular regions compared to hypertrophic tonsillitis (28 ± 7 *vs* 13 ± 5 cell, p = 0.02).

Microbiological studies

A total of 57 microorganisms species (41 bacterial and 16 viral species) were isolated from palatine tonsils. Table 1 shows the incidence of isolated microorganisms species in tonsillitis patients. In both groups, tonsils were predominantly colonized by gram-positive microorganisms (*S. aureus*, *Streptococcus spp.*) and adenovirus (42% of cases). However, in patients with recurrent tonsillitis, in 42% of cases, associations of gram-positive, gram-negative bacteria (predominantly *K. pneumonia*) and viruses (adenovirus) were observed. Furthermore, patients with recurrent tonsillitis had increased tonsils *C. albicans* colonization as compared to hypertrophic tonsilllits (20% *vs* 5% of cases).

Table 1. Isolated microorganisms species from palatine tonsils of recurrent and hypertrophic tonsillitis patients

Microorganisms species	**Number of patients**
Streptococcus spp.	11
S. aureus	9
S. aureus and *S. epidermidis*	4
S. epidermidis and *Streptococcus spp.*	5
E. coli	2
K. pneumoniae	3
C. albicans	5
C. albicans and *S. aureus*	4
C. albicans and *Streptococcus spp.*	3
Streptococcus spp. and gram-negative bacteria	3
S. aureus and gram-negative bacteria	4
S. aureus, *Streptococcus spp.* and gram-negative bacteria	3
S. aureus, *Streptococcus spp.* and *C. albicans*	4
S. epidermidis and gram-negative bacteria	2
Adenovirus	10
RSV	2
HSV	3
CMV	1
Adenovirus and *S. aureus*	5
Adenovirus and *Streptococcus spp.*	6
Adenovirus and gram-negative bacteria	2
Adenovirus, *S. aureus* and *C. albicans*	4
RSV and *S. aureus*	1
RSV and *Streptococcus spp.*	1
HSV and *S. aureus*	2
HSV and *Streptococcus spp.*	2
HSV and gram-negative bacteria	1

Discussion

The palatine tonsils participate in a variety of functions involving innate, cellular and humoral immunity both at the local and systemic levels. In spite of the immunological nature of the tonsils, some microorganisms have acquired adaptations that allow them to circumvent the tonsillar immune defences and utilize the tonsils as the entry, replication and colonization. However, the pathogenic mechanism of recurrent and hypertrophic tonsillitis has not yet been completely elucidated [4, 5, 19].

The present findings indicate that the mean follicle area in hypertrophic tonsillitis is significantly larger than in recurrent tonsillitis. Furthermore, the increased mast cells infiltration in the parafollicular region in hypertrophic tonsillitis was observed. Activated mast cells generate many cytokines among this IL-4, leucotriene B_4. It is well known that the IgE sensitization of mast cells may in various ways drive the mucosal response to the chronic inflammatory reaction [3].

Mast cells play an important role in defending an individual against bacterial infection. Mast cells possess a variety of surface receptors and may be activated by inflammatory mediators, IgE, IgG, light chains, complement fragments, proteases, hormones, neuropeptides, and microbial products. Following activation, they produce a plethora of pro-inflammatory mediators and participate in inflammatory reactions in many organs. Furthermore, it has been shown that mast cells have the capacity to modulate the host's innate immune response to gram-negative bacteria by their ability to phagocytose bacteria, process and present bacterial antigens to T cells, and recruit the phagocytic help through the release of physiological amounts of pro-inflammatory mediators [15].

We believed that the increase in the number of mast cells is beneficial as it serves to clear the infecting agent.

We observed that patients with recurrent tonsillitis had less dendritic cells in parafollicular regions compared to hypertrophic tonsillitis that, in our opinion, is associated with an impaired response to foreign antigens.

Dendritic cells (DCs) are a heterogeneous group of cells that display differences in anatomic localization, cell surface phenotype, and function, originating from CD34 bone marrow stem cells. Productive immune responses occur when DCs that have taken up an antigen are activated and present optimal levels of MHC/peptide complexes in the context of accessory molecules. In the normal host if self-antigens are presented, no T cells should be available to respond because of central tolerance induction [28]. However, if T cells recognize only low levels of MHC/peptide, have a low affinity for their cognate ligand, or receive no costimulation from DCs, they become anergic or undergo apoptosis. For example, immature DCs treated with IL-10 fail to mature and, as a result, induce anergy in responder T cells [20, 24]. Once generated, anergic T cells can suppress the development of an immune response by directly suppressing the expression of MHC class II, CD80, and CD86 on DCs in culture [12, 21, 27].

Our research data showed that patients with recurrent tonsillitis had less TGF-beta1 positive cells in parafollicular regions compared to patients with recurrent tonsillitis.

TGF-beta1 plays an important role in the induction of immunological unresponsiveness (tolerance) to antigens [29]. It has been shown that IL-10 and TGF-*βI* deficient mice develop inflammatory bowel diseases, which are characterized by breakdown in an analogous state of tolerance to commensal bacteria [10, 23].

TGF-*β* has multiple immunosuppressive effects at the cellular level and has been described as inhibiting type 1 and type 2 cells, B cells, CD8+ T cells, macrophages and natural killer cells. TGF-β blocks cell-cycle progression and may have a direct effect on the expression of the gene encoding IL-2. Importantly, it can suppress the expression of IL-12 and IL-2 receptors, as well as downregulate the MHC class II expression on macrophages (specifically by antagonising TNF-*α* and IFN-*γ*) [22].

Our results showed that, both in recurrent and hypertrophic tonsillitis, tonsils were predominantly colonized by gram-positive bacteria. However, in patients with recurrent tonsillitis, gram-negative flora, viral-bacterial and fungal associations were observed.

Our microbiological data are consistent with other research data available in the literature, suggesting that *Streptococci* and *Staphylococci* are still common etiological agents in tonsillitis [1, 17, 7].

However, in contrast, we have observed that the etiopathogenesis of recurrent tonsillitis is predominantly multietiological. This includes bacteria, viruses and fungi (*C. albicans*).

We observed that patients with recurrent tonsillitis had four-fold increase of tonsils' fungal colonization compared to the case of hypertrophic tonsillitis. The increased incidence of tonsils colonization by *C. albicans* can be explained by an increased use of antimicrobial therapy [11]. It is increasingly recognized that antimicrobiol therapy in recurrent tonsillitis is often ineffective and recurrences are observed. In our opinion, this could be explained by the fact that, in recurrent tonsillitis, viral-bacterial and viral-bacterial-fungal associations frequently take place. Furthermore, isolated microorganims species frequently prohibit the antimicrobial resistance to widely used antimicrobials.

Our study supports the hypothesis that recurrent tonsillitis is characterized by the breakdown of immunological tolerance to oral microflora. On the other hand, it is possible that, in tonsils of patients with hypertrophic tonsillitis, immunological tolerance is maintained, and the immune response is not impaired.

To conclude, in recurrent tonsillitis, dendritic cells probably lose their ability to prime Treg cells effectively. At the same time, in the presence of bacterial, viral and fungal biofilms, antigen presentation by dendritic cells favours the induction of inflammatory response. This is the main reason for frequent spontaneous exacerbation of recurrent tonsillitis. It seems that, if a child suffers from recurrent tonsillitis, it is an immunodeficient condition, which co-presents with impaired immunological tolerance.

Conclusions

Patients with recurrent tonsillitis have less dendritic cells, TGF-beta1 positive and mast cells in palatine tonsils compared to patients with hypertrophic tonsillitis.

In patients with hypertrophic tonsillitis, the tonsils are predominantly colonized by gram-positive bacteria. However, in recurrent tonsillitis, bacterial-viral and fungal associations are observed.

The data of the present paper suggest that the upper aerodigestive tract microflora-specific Treg lymphocytes function may be compromized relatively early in tonsillar inflammation.

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