THE NEW ARMENIAN
MEDICAL JOURNAL
Vol. 2 (2008), N 4, 19 - 34
www.ysmu.am

IMMUNOHISTOCHEMISTRY OF MUCOSAL AND VASCULAR TISSUE BARRIERS OF PHARYNGEAL MUCOSA-ASSOCIATED LYMPHOID TISSUE IN CHRONIC RECURRENT TONSILLITIS

N. D. Vardazaryan, S. V. Hambardzumyan, K. A. Arsenyan*, A. G. Mkhitaryan, K. G. Gyulamiryan

Department of Pathological Anatomy, YSMU, Yerevan, Armenia

Abstract

To ascertain morphological manifestations of pharyngeal mucosa-associated lymphoid tissue (MALT) barriers, which prevent microbial invasion, surgically removed pharyngeal and palatine tonsils of patients with chronic tonsillitis were investigated by immunohistochemical and light-microscopic cytobacterioscopic methods. In preventive mechanisms against invasion of infection from the surface of tonsils, the decisive role is attributed to migration of granulocytes, which contain lysosomal cationic proteins with participation of CD_{31+} T-cells, phagocytosis of cationized microbes, as well as lymphocyte immunity. Intercellular interaction between CD_{4+} T-lymphocytes and macrophages on the surface of tonsils must be referred to as a process, which provides initial pre-immune physiological resistance against infection. The inflammation is accompanied by formation of intratissue persistent streptococcal foci and invasion of microbes into tonsillar microcirculation. The last barrier is endothelium of postcapillary high endothelium venules (PHEVs), which provides adhesion and inactivation of streptococci and their antigens.

The paper addresses undetermined barrier function of PHEVs to prevent penetration of microbes from tonsils into general circulation. The relative decrease of PHEVs number along with simultaneously expressed vascularization of interfollicular lymphoid tissue is the index of vascular barrier incompetence. The vascularization is formed against the background of gamma-aminobutyric acid (GABA), proline-rich polypeptide, cyclic 3',5'-adenosine monophosphate (cAMP) deficiency, as well as decreased activity of adenylate cyclase (AC) in the cells of lymphoid tissue. The simultaneous decrease in activity of NO- synthetase, prostaglandin-synthetase enzymes and AC, as well as decreased levels of cAMP and GABA in the endothelium of PHEV lead to decompensation of the vascular barrier.

Keywords: chronic tonsillitis, immunohistochemistry, mucosal and histohematic barrier, streptococci, postcapillary high endothelium venules.

Introduction

The tonsillar disease ranks high among various clinical-morphological manifestations of chronic local streptococcal infection [*Lindroos R., 2000; Bikova V., 2006*]. It is characterized by chronic tonsillitis and adenoiditis, as an inflammatory disease of lymphoid structures associated with pharyngeal mucosa. For non-specific protection against various pathogenic agents, which influence mucosal membranes, the tonsils participate in various humoral and cell-mediated immune reactions. As secondary lymphoid organs due to their localization, they are constantly under the influence of microbial flora realizing adaptive immunity through B- and T- lymphocytes [*Claeys S. et al., 2003; von Andrian U., Mempel T., 2003*], though in many cases turning into an infection focus. Many clinical-morphological, bacteriological, cytobacterioscopical, immunohistochemical and molecular-biological investigations have been carried out, but pathogenesis of the disease

Address for correspondence:
Yerevan State Medical University after M. Heratsi (YSMU)
2 Koryun Street, 0025, Yerevan, Armenia
Tel.: (+3741 0) 58 14 64
E-mail: arsenyan_kristina@yahoo.com

was not established [*Gaffney R., Cafferkey M., 1998; Vardazayan N. et al., 2006*]. The main etiological factor is group *A streptococcus*, although the influence of *Haemophilus influenzae, Staphylococcus aureus* and other microbes or viruses is not excluded [*Putto A., 1987; Lindroos R., 2000*].

It was established that in the development and persistence of chronic inflammation the main role is attributed to the persisting intratissue streptococcal foci in the epithelium, lymphoid tissue, in trabeculae, as well as in the walls and lumina of small vessels of tonsils [*Vardazayan N., 1981; Bernstein L. et al., 1999; Lindroos R., 2000*]. As a result of disturbance of local cellular and tissue processes, as well as immune and metabolic reactions [*Fujieda S. et al., 1999*], intratissue microbes present the main cause of chronic inflammation course, invasion of the causative agents into microcirculation and development of a decompensated form of the disease. These problems are not completely resolved until present. The specific role is attributed to pathogenetic mechanisms of barrier disturbance, metabolic changes in pharyngeal and tonsillar mucosa on the way of infection penetration.

Based on the aforementioned, our aim was to carry out complex immune-morphological and histochemical investigation on prostaglandin synthetase (PGS), adenylate cyclase (AC), NO- synthetase (NOS), gamma-aminobutyric acid (GABA), neuronal proline-rich polypeptide (PRP), cyclic 3',5'-adenosine monophosphate (cAMP) in vessels and tissues of tonsils for revealing the pathogenesis of chronic inflammation, succession of disturbance of antimicrobial tonsillar barriers from the surface of epithelial covering to deep tissue structures of tonsils.

Material and Methods

Surgically removed 105 pairs of palatine and 20 pharyngeal tonsils were investigated in children suffering from recurrent chronic tonsillitis and accepted to Otolaryngology Department of "Arabkir" hospital after preoperative conservative treatment.

Just before tonsillectomy, smears from the surface of pharyngeal mucosa and palatine tonsils were taken. The smears were stained by Jenner-Giemsa, lysosomal-cationic test was performed [*Pigarevksy V., 1983*] using alcohol solution of fast green and azure A, due to which lysosomal-cationic proteins (LCP) of neutrophil-granulocytes (lysosomes) were stained in bright-green. The surface and sections of tonsils were also investigated by scanning electron microscopy (SEM). After preliminary fixation and dehydration in ascending solutions of ethanol, specimens were dried up and then investigated by SEM Vega Tescan microscope.

The removed tonsils were divided into two parts. One part was fixed in 10% buffered neutral formalin solution and then in paraffin. Paraffin sections of 5-*mcm* thickness, were stained with hematoxylin and eosin, by Van Gieson, toluidine blue, and azan by Heidenhain, and, to reveal microbes, by Gram-Weigert's method. The second part was frozen under -20°C and 4-5 *mcm* thick sections were obtained. The following immune-histochemical procedures were used on paraffin and frozen sections: a) indirect immune fluorescence (IF) antibody technique, b) avidin-biotin peroxidase complex (ABC). For determination of CD_{31} antigen mouse monoclonal antihuman CD_{31} antibodies were used (Clone JC/70A, isotype: IgG1, kappa, Dako, Denmark) and diaminobenzidine as a chromogen. In control specimens bovine plasma albumin (kit "Zimed", USA)was used instead of primary antibodies.

To reveal CD_{34} antigen, Alkaline Phosphatase - Anti-alkaline Phosphatase (APAAP), the immunohistochemical method was used. The reaction was performed on the base of Pathology Institute of Göttingen Medical University (Germany), with the kind permission of Prof. Michael Amthor. After counterstaining in hematoxylin, the specimens were dried up and closed in mounting medium. Direct reaction of IF with *E. coli* lipopolysaccharide labeled with fluorescein isothiocyanate (FITC) (Serotype 055:B5, Sigma, USA), indirect reaction of IF with anti-cAMP serum (Sigma, USA), indirect reactions with rabbit anti-GABA monoclonal serum (Dia Sorin, USA), as well as with specific polyclonal serum against prolin-rich

polypeptide (PRP) were performed. The sections were rinsed out with physiological solution and anti-rabbit FITC labeled serum was used.

Adenylate cyclase (AC) was determined in frozen sections by the histochemical method [*Howell S. and Whitfield M., 1972*]. Non-fixed specimens were incubated in the medium for 30 *min* at +30°C. Per 100 *g* of incubation medium prepared on 80 *mm* of maleate tris-buffer the following was added: dextrose (8 *mg*), theoflavinum (36 *mg*), $MgSO_4$ (48 *mg*), ATP (28 *mg*). Prior to use, 159 *mg* $PbNO_3$ was added. After the reaction, specimens were rinsed out twice in distillated water, incubated in the solution of ammonium sulfide, and, after rinsing, fixed in 10% formalin. Control experiments were performed with incubation in the following media: a) without the substrate, b) adding $12.5x10^{-3}$ NaF. The sediment of black lead sulfide marked the localization of the enzyme. The presence of PGS was identified due to brown color of cells after specimen incubation at 35°C during 10-12 hours in medium containing arachidonic acid in presence of 3,3-diaminobenzydine. For suppression of non-specific coloration, KCN was added, which inhibits biosynthesis of PGS. The specificity of the reaction was assessed after estimation of results obtained in control experiment with the specimen incubation in medium without the substrate and inhibition of the enzyme by indometacin. As controls, sections from kidney were also used, the medullar part of which differs by high expressed levels of PGS.

To reveal NO-synthase (NOS) activity and localization, histochemical $NADPH^+$-diaphorase reaction was performed [*Scherer-Singler U. et al., 1983*], which is based on formation of dark-blue pigment formazan from nitro-blue tetrazolium under the influence of NOS and coenzyme $NADPH^+$. The intensity degree of different structures in the specimens was assessed by giving distinct number implication. In case of diffuse, intense coloration the result was assessed as *"3"*. Likewise, moderate, mild, and null staining was marked by *"2", "1",* and *"0"*, respectively. The enzyme activity was calculated by formula

$3\times a+2\times b+1\times c+0\times d/100$ units,

where *a, b, c,* and *d* are the number of similar type histological structures or cells with a certain degree of formazan coloration; *100* is the total number of counted cells. The examination of all slides was carried out by WILD microscope.

Counting of postcapillary venules in the interfollicular lymphoid tissue in each tonsil was carried out in 10 fields of vision under light microscope with objective 10; the number of postcapillary high endothelium venules (PHEVs) was counted as well. The results were represented, taking into account patients' age, duration of the disease and rate of tonsillitis exacerbation per year. Morphometric data underwent variation-statistical analysis, using computer based statistic program SPSS 13. The difference was considered statistically significant at $p<0.05$.

Results and Discussion

In cytobacterioscopic investigation of smears from the pharyngeal mucosa stained by Pigarevsky and Gram-Weigert, gram-positive and gram-negative microbes (streptococci), neutrophil granulocytes containing lysosomal-cationic proteins (LCP) and their destructed forms were constantly detected (Figure 1). Due to comparison of data with the morphology of surface epithelium of pharyngeal and palatine tonsils removed in case of tonsillectomy, it was revealed that along with leucocytes, macrophages, T- and B-lymphocytes, as non-specific antimicrobial factors of pharyngeal and tonsillar mucosa [*van Andrian U., Mempel T., 2003*], CD_{31} cells were also detected in the smears (Figure 2). The part of macrophages, those with bright-green cytoplasmic granular inclusions of LCP, cationized and non-cationized microorganisms are frequently surrounded by 3-4 contacting lymphocytes (Figure 3). By SEM investigation, the intercellular thin bridges of salients were found between macrophages and lymphocytes (Figure 4) that, to our opinion, is the morphological manifestation of cell interaction and local immune reactions to extracellular infection of tonsillar surface. The cellular elements are represented by macrophages and CD_{4+} T-lymphocytes, since it has been proved that fragmented antigens of phagocytosed and digested micro-

organisms in the cytoplasm of macrophages being complexed with molecules of MHC-II class are presented on the cellular surface and become recognized by CD_{4+} T-lymphocytes. Dendritic cells also have an important role participating in the process of initiation of primary immune response through antigen delivery to lymphoid tissue [*Howie A., 1980; Gorfien J. et al., 2001*]. Salients of these cells come into contact with microbes and small lymphocytes on the surface of epithelial cover. These data verify the opinion of J. Gorfien and co-authors on increasing amount of Langerhan's cells and macrophages delivering the antigen T to cells [*Gorfien J. et al., 2001*]. In this case, the chronic infection may have immunosuppressive effect on tonsillar tissue inhibiting maturation and activation of macrophages. Thus activated lymphocytes, which transfer information from the antigen-presenting macrophages to the tonsillar surface and crypts [*Liu Y., Banchereau J., 1996*], realize clone expression gaining effector immune function and memory [*Liu Y., Banchereau J., 1996; Hallman M. et al., 2001; Johansson-Lindbom B. et al., 2003; von Andrian U., Mempel T., 2003*]. This fact, as a morphological evidence of intercellular interaction of immune system cells on the tonsillar surface, allows to suggest that beginning from the surface of pharyngeal mucosa sowed by microbes with participation of CD_{31+} cells after migration of LCP+ granulocytes and their destruction, cationization of bacteria and phagocytosis, mechanisms of T-lymphocyte immunity with function of molecular recognition switch on. Intercellular interaction of macrophages with CD_{4+} T-lymphocytes outside the tissue on tonsillar surface should be regarded as manifestation of one of initial pre-immune physiological resistance mechanisms of an organism against infections. The role of LCP, which have regulating influence on the functional activity of macrophages, the activation of T-helpers' and suppressors' immune reactions is significant [*Mazing Yu., 1991*].

In bacterioscopic examination of sections stained by Gram-Weigert, the intratissue colonies of *streptococci* and their small foci as distinct cocci and chains are constantly revealed in tonsils of patients suffering from chronic recurrent tonsillitis. *Streptococci* are localized in the lumina of crypts, in the lymphoepithelial symbiosis (LES) zones, in the inter-follicular lymphoid tissue, sometimes in the follicles and in the connective tissue of trabecules. They are frequently detected in the walls and lumina of small vessels: that is the evidence of histohematic barrier disturbance and invasion of microbes into the vessels of tonsils (Figures 5, 6). Under such conditions the terminal chain of microcirculatory bed, the postcapillary high endothelium venules (PHEVs) serve as the last barrier. In the interfollicular lymphoid tissue of almost all investigated tonsils numerous PHEVs are detected with gram+ *cocci* fixed on apical and lateral surfaces of endothelial cells, and sometimes in their cytoplasm (Figure 7). Not infrequently, microbes are shrouded by thin and subtle fibrin film, while the proliferating endothelial cells with turgid cytoplasm, big nucleus, and nucleoli are frequently arranged in several rows, narrowing the vascular lumen (Figure 8). Thus, the chronic inflammatory process may arise and persist as a result of disturbance of local blood circulation, autoregulation mechanisms, protective barriers in conditions of permanent stimulation by microbes and their antigens.

Activation of PHEVs with adhesion of microbes by endothelial cells from the bloodstream of infected tonsils in chronic tonsillitis and adenoiditis allow to consider the role of PHEVs as a terminal barrier, which prevents spread of bacteria and their antigens from the vessels of infection focus to the circulation. Adhesion of microbes and their antigens by endothelial cells is, undoubtedly, a non-specific and universal process. To verify the aforementioned opinion on adhesive activity of the vascular endothelium towards microbial antigens, we performed the direct reaction of immunofluorescence with FITC labeled *E. coli* lipopolisaccharide (Serotype 055:B5, Sigma, USA) in frozen sections of tonsils. The results showed that endothelium of numerous vessels of interfollicular lymphoid tissue displays specific fluorescence (Figure 9), proving occurrence of fusion between the bacterial antigen and endothelium.

It is known that the main function of PHEVs of the mucosal lymphoid tissue is "homing" of lymphocytes from crypts into the bloodstream of tonsils [*Tohya K., Kimura M., 1996; Girard J. et al., 1999*].

Our data concerning adhesion of microbes and their antigens by endothelial cells of venules from the bloodstream of the infected tonsils, allow asserting that PHEVs are polyfunctional structures endowed with still uncertain specialized barrier function: in chronic inflammation they realize adhesion and neutralization of various microbes and their antigens in the microcirculatory bed from the focus of infection, preventing their entrance into the circulation. Due to K. Tudor and co-authors, in distinct cases the presence of *cocci* in the cytoplasm of endothelial cells is the evidence of phagocytosis, which is a typical feature for endotheliocytes [*Tudor K. et al., 1997*]. The mentioned processes are strictly associated with metabolism in the tonsillar tissue, especially with PHEVs, which are markedly altered in the presence of intratissue persisting infection. To this latter signify our data attained during the activity on examination of PGS, AC, and cAMP. The histochemical investigation of frozen and paraffin sections obtained from the palatine tonsils demonstrated that the main part of PHEV-endotheliocytes had expressed PGS and AC activity (Figure 10). The low enzymatic activity was noticed in palatine tonsils of patients with long duration of disease in the presence of expressed intratissue infection foci (Figures 11, 12). Changes to the activity of PGS are firstly attributed to prostaglandins and arachidonic acid, as well as to their ability to stimulate metabolism, chemotaxis, and phagocytosis of neutrophil leucocytes and macrophages. Probably, low activity of PGS, along with AC, is the cause of suppressed chemotaxis of phagocytes, as well as phagocytosis [*Shibata Y., 2005*]. In parallel sections due to indirect reaction of IF with anti-cAMP serum (Sigma, USA), marked changes of cAMP were revealed as well. In all tonsils endotheliocytes of PHEVs showed mild fluorescence. The intense luminescence of cAMP was seen in cells of diffuse lymphoid tissue as well as in interepithelial lymphocytes and macrophages of crypts. The decrease of cAMP in cells of lymphoid tissue and suppression of PGS and AC occurs mainly in tonsils of patients with recurrent tonsillitis, disease duration above 10 years, frequent recurrent angina within a year (Figures 13, 14). The decreased activity of enzymes was noticed in endotheliocytes of the main part of PHEVs. High activity of PGS and AC as well as intense fluorescence of cAMP in the cells of connective and lymphoid tissue was observed in case of small intratissue microbial foci. Moreover, numerous contacting salient forms are revealed in the cell population as a sign of processes of intercellular interaction (Figure 15). In cases of low activity of PGS and AC alongside with low content of cAMP, microbial foci are large and, in case of mildly expressed cellular reaction of tissues should be considered as morphological manifestation of areactivity, which promotes microbial invasion.

Data obtained after revelation of PRP in tissues are also of great interest. The neuroactive PRP, firstly educed by A. Galoyan from the neurosecretory granules of hypothalamic and pituitary cells [*Galoyan A., 1997*] and then in cells of lymphoid nodules and bone marrow [*Galoyan A. et al., 2004*] plays an important role in the immune and metabolic processes, as well as in the anti-infectious protective function of an organism. The indirect reaction of immunofluorescence with specific anti-PRP polyclonal serum in frozen sections of tonsils allowed to ascertain that PRP fluorescence is observed in cells of LES zone, focal accumulations in the diffuse lymphoid tissue, in granules of basophiles and especially in cells of mantle zone of lymphoid follicles (Figure 16). PRP was not observed in the endothelium of vessels. The severe decrease in luminescence intensity down to its disappearance was noticed in the tonsils of patients with long duration of the disease and high rate of recurring angina during a year. The decreased content of PRP in tonsil tissues is correlated with expressiveness of chronic inflammation, dimensions of intratissue

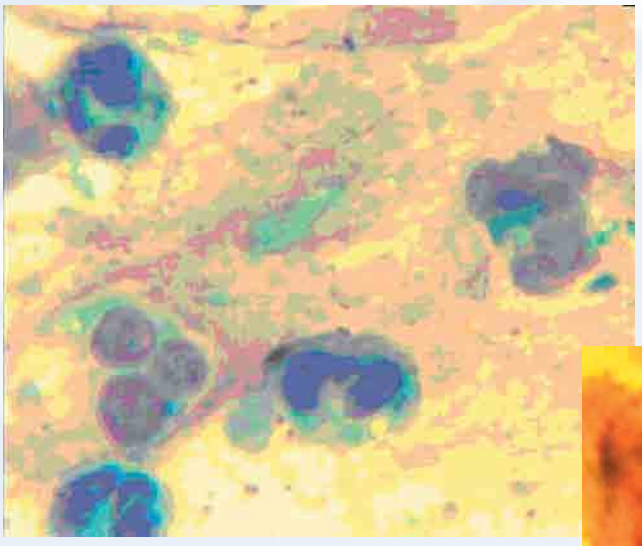


Figure 1. *Granulocytes with LCP and their destructed form. Smear from the surface of the palatine tonsils. B.E. Pigarevsky method. ×1000.*

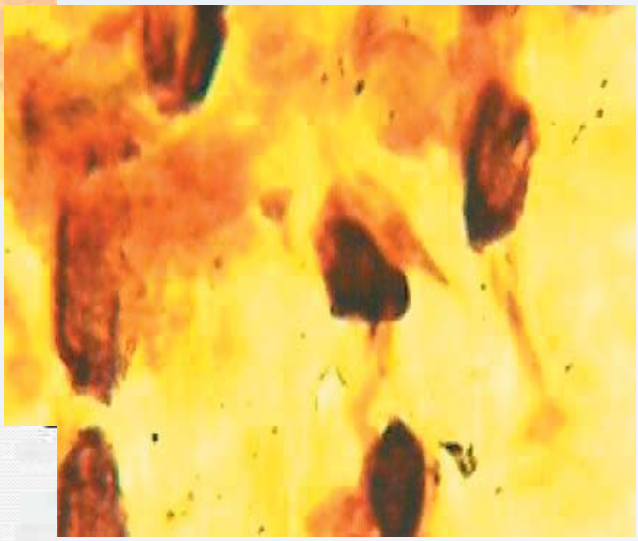


Figure 2. *CD_{31} antigen expressing cells from the surface of the palatine tonsils. ABC method. ×400.*

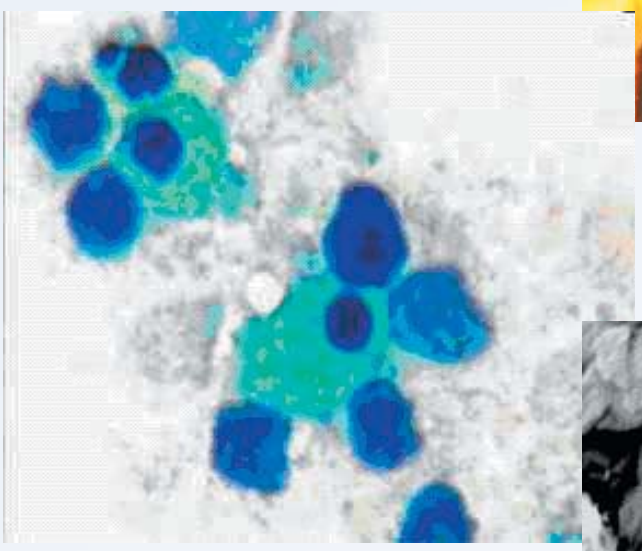


Figure 3. *Macrophage with the intracytoplasmic cationized and non-cationized bacteria, contacting with the lymphocytes. Smear from the surface of the palatine tonsils. B.E. Pigarevsky method. ×1000.*

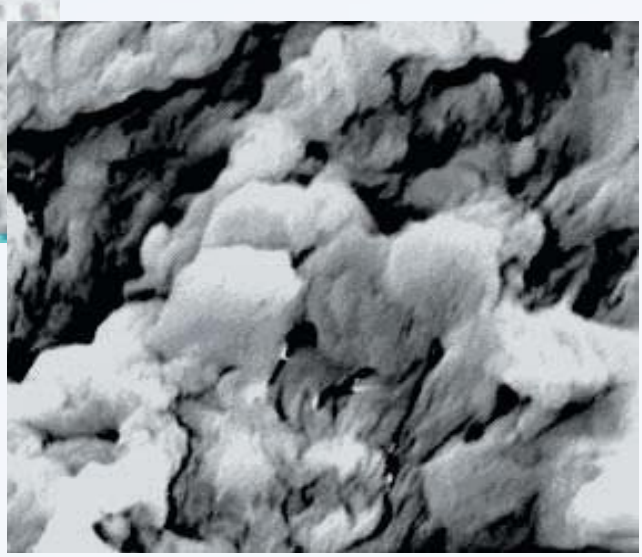


Figure 4. *Thin intercellular bridges between the cells from the surface of palatine tonsils. SEM ×5400.*

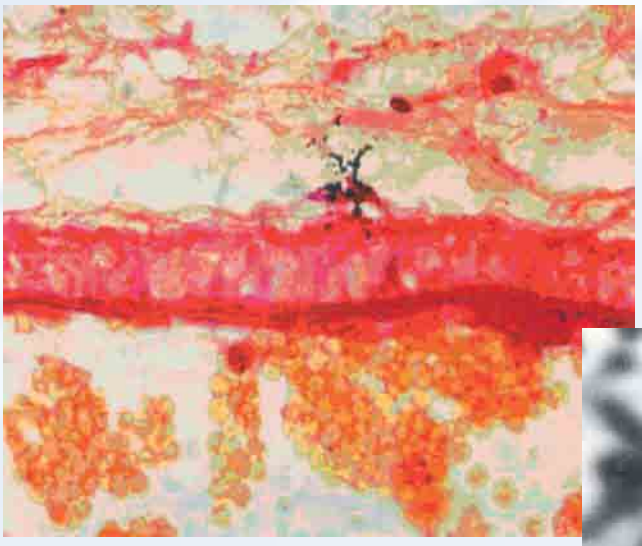
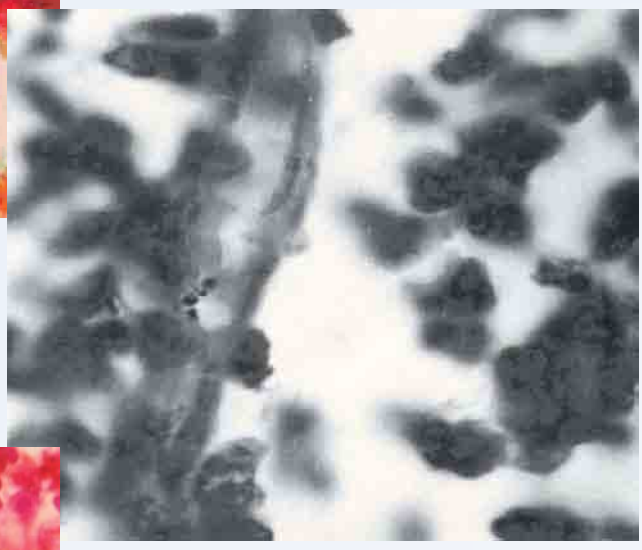


Figure 5. *Intratissue foci of streptococci with invasion into the vascular wall. Gram-Weigert's method. × 400*



Streptococci within the vascular wall. Gram-Weigert's method. × 1000. **Figure 6.**

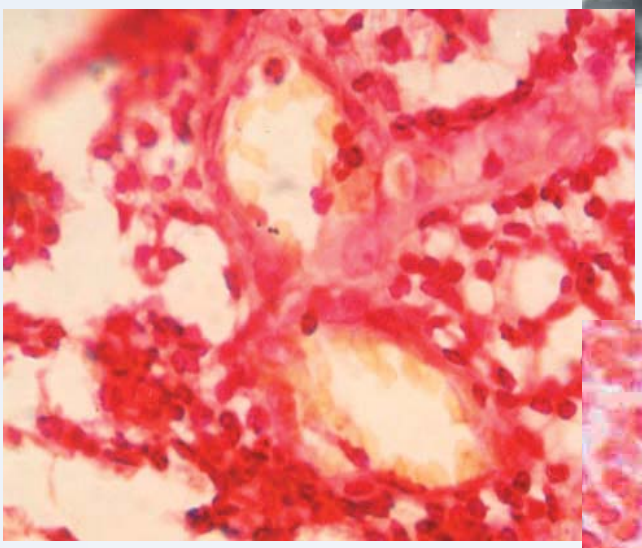
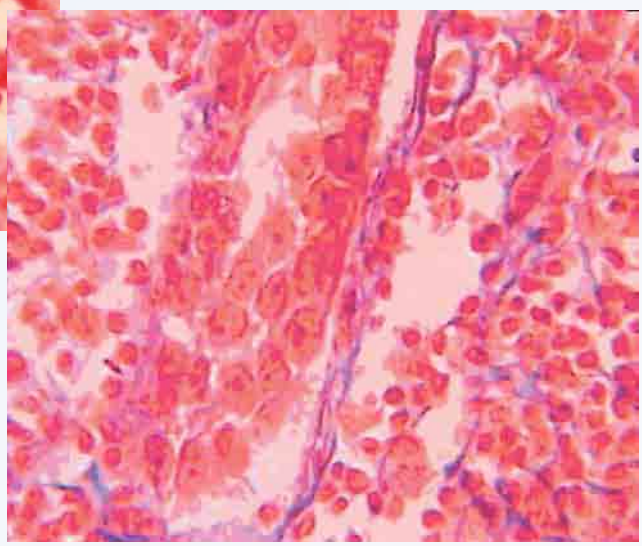


Figure 7. *Adhesion of Gram+ cocci on the surface of HEV endothelium. Gram-Weigert's method. × 1000*



Proliferation of endothelial cells of HEV, narrowing the vascular lumen. Heidenhain's azan method × 400. **Figure 8.**

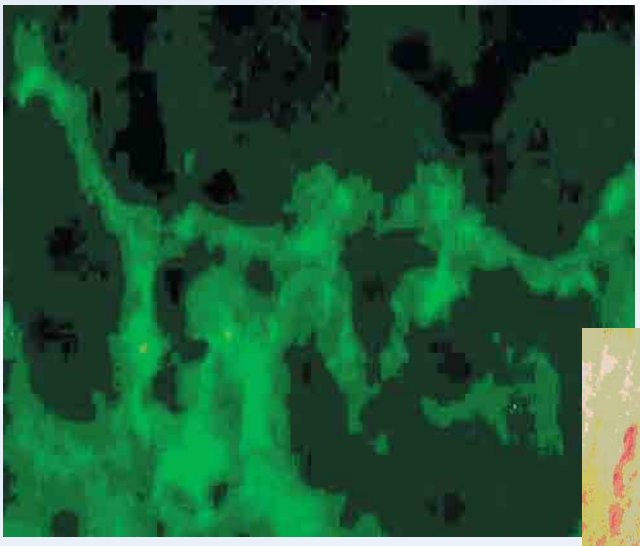


Figure 9. *Fluorescence of the endothelial cells of HEV. Direct immunofluorescence reaction with labeled FITC E. Coli lipopolysaccharide. × 400.*

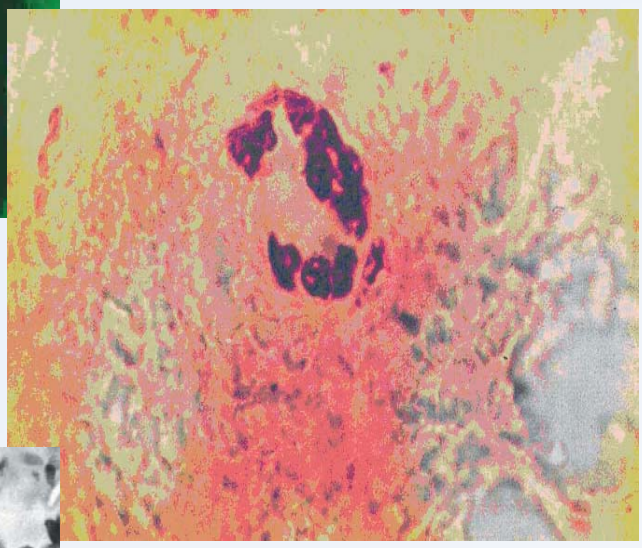


Figure 10. *PGS activity of the HEV endothelial cells within interfollicular lymphoid tissue of palatine tonsils. Histochemical reaction by S. Nimura and K. Ishida. × 400.*

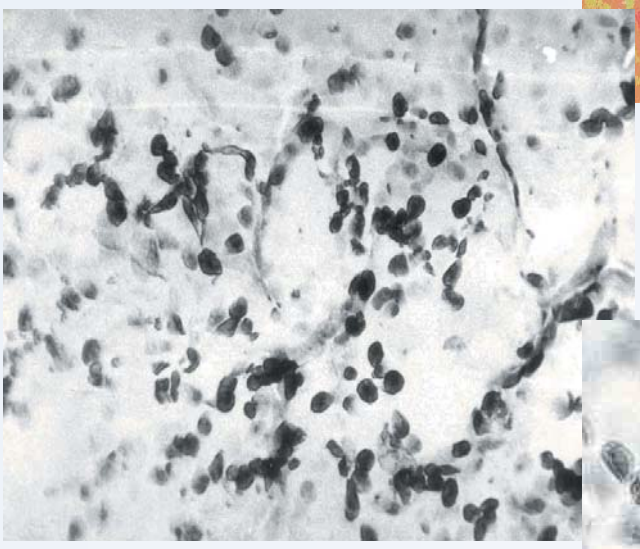


Figure 11. *High activity of AC within the interfollicular lymphoid tissue cells. Histochemical reaction by S.L. Bowell and M. Whitfield. × 400.*

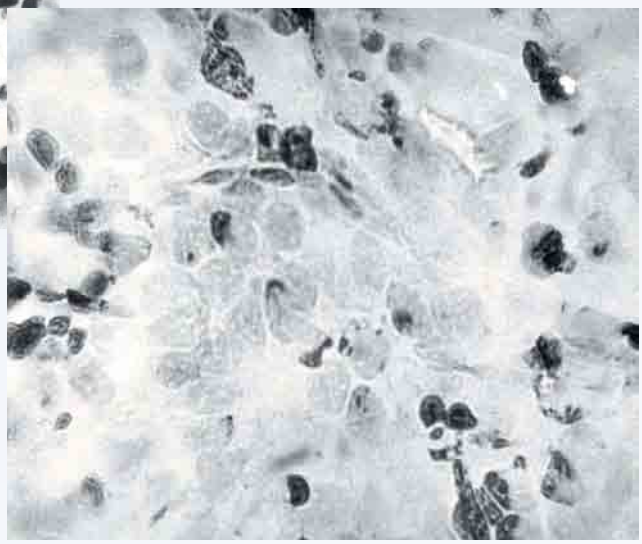


Figure 12. *Low activity of AC within the interfollicular lymphoid tissue cells. Histochemical reaction by S.L. Bowell and M. Whitfield. × 400.*

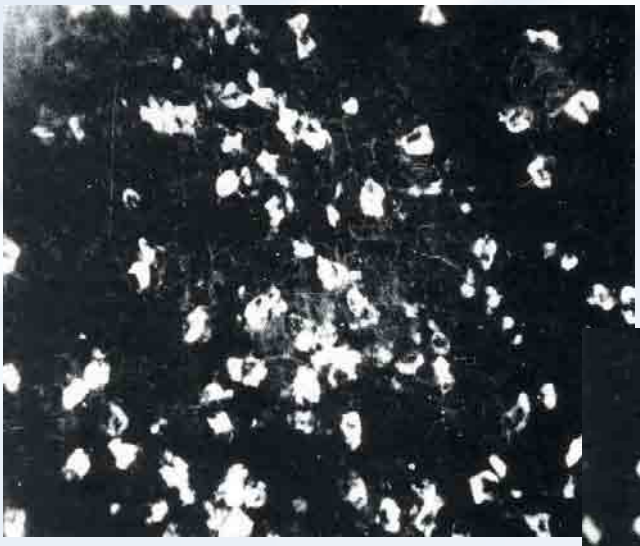


Figure 13. *High cellular cAMP of the diffuse interfollicular lymphoid tissue in case of small intratissue streptococcal foci. Indirect IF reaction. × 400.*

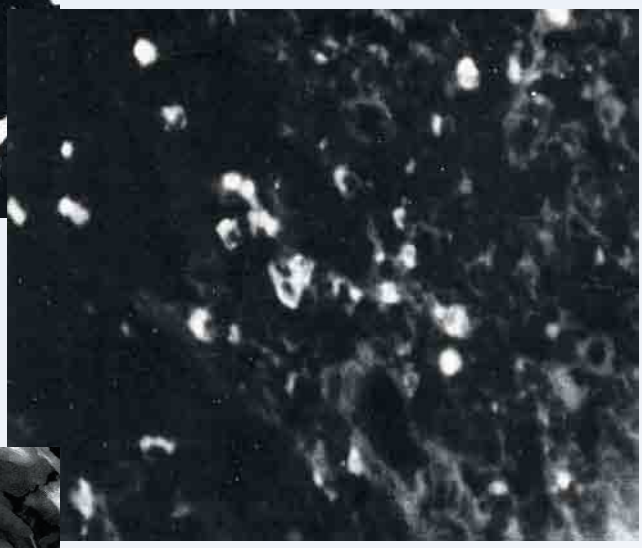


Figure 14. *Low cellular cAMP of the diffuse interfollicular lymphoid tissue in case of big intratissue streptococcal foci. Indirect IF reaction. × 400.*

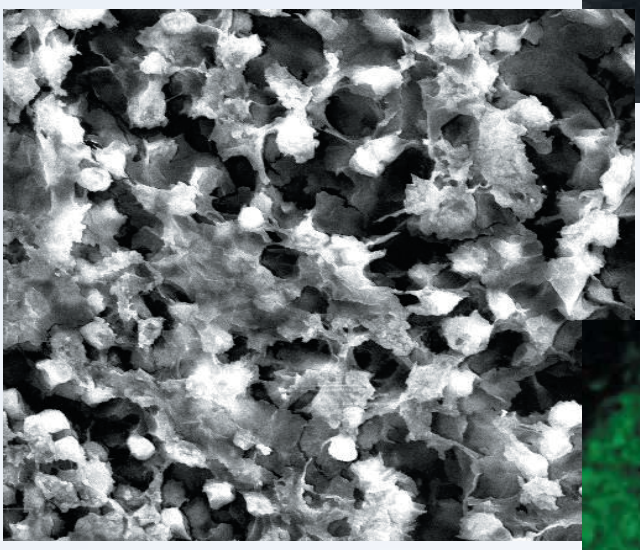


Figure 15. *Cells with cytoplasmatic processes in interfollicular lymphoid tissue. SEM × 2000.*

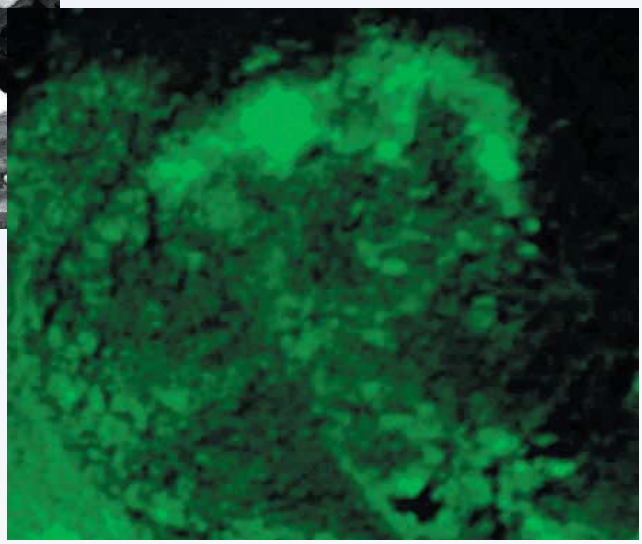


Figure 16. *PRP of the follicular mantle zone cells. Indirect IF reaction. × 100.*

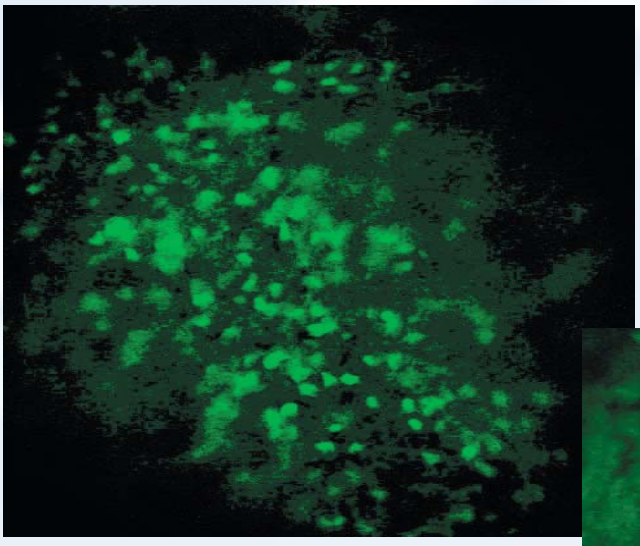
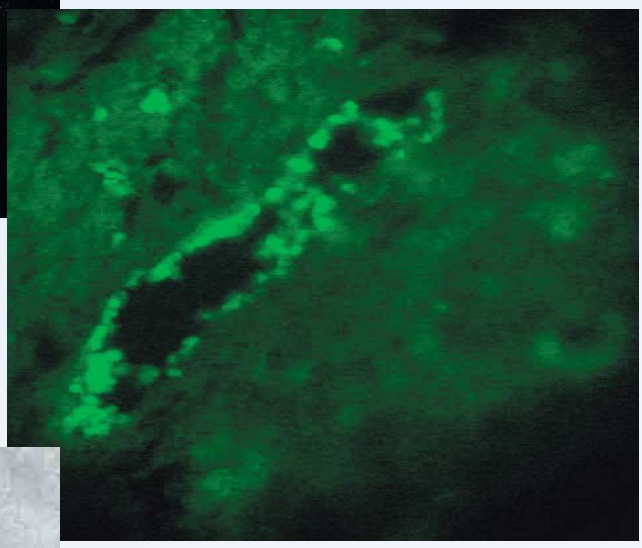


Figure 17. *GABA + cells of interfollicular lymphoid tissue. Indirect IF reaction. ×100.*



Fluorescence of GABA within the endothelial cells HEV of palatine tonsils. Indirect IF reaction. × 400.

Figure 18.

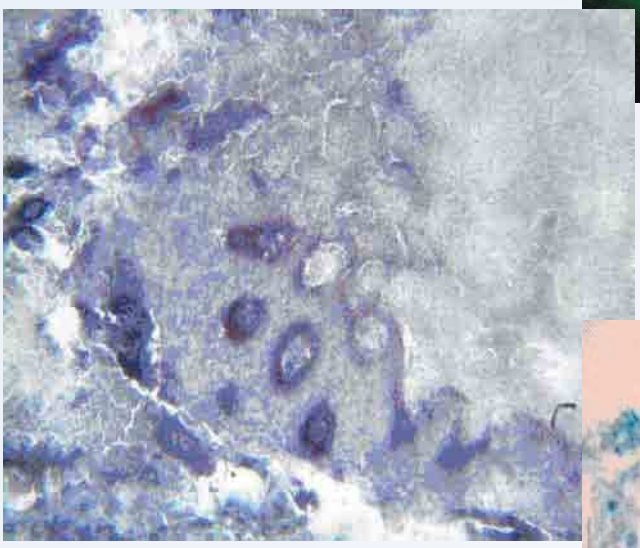
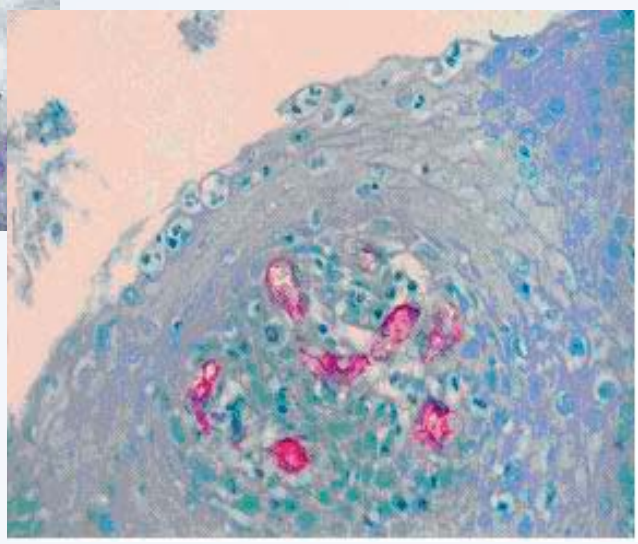


Figure 19. *NOS activity of the epithelium of the palatine tonsil crypts, around the vessels, and in the LES zone. NADPH+ diaphorase reaction. ×100.*



CD_{34} + vessels in non-reticulated epithelium of the tonsillar crypts with glomeruloid structures formation. APAAP immunohistochemical reaction. ×100.

Figure 20.

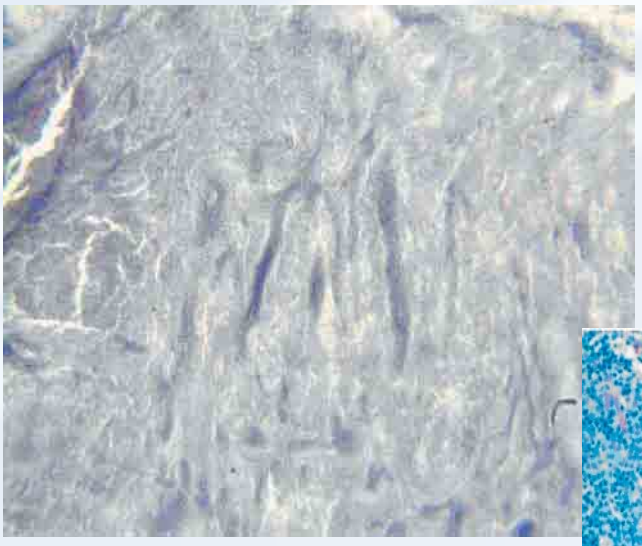


Figure 21. *Activity of the vascular NOS in subepithelial lymphoid tissue. NADPH+ diaphorase reaction. ×100.*

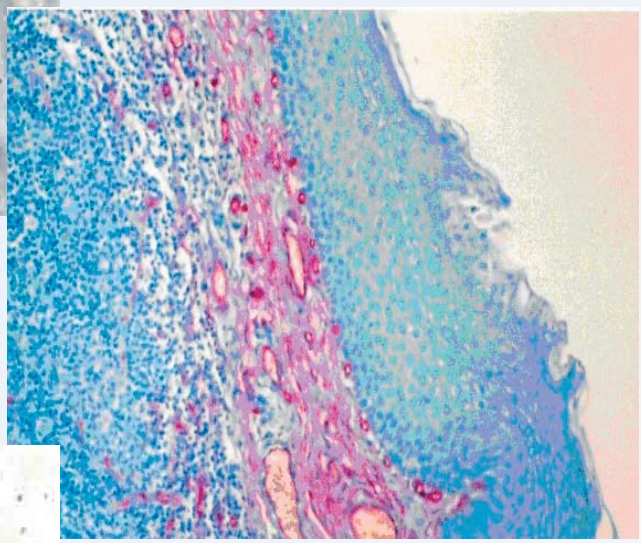
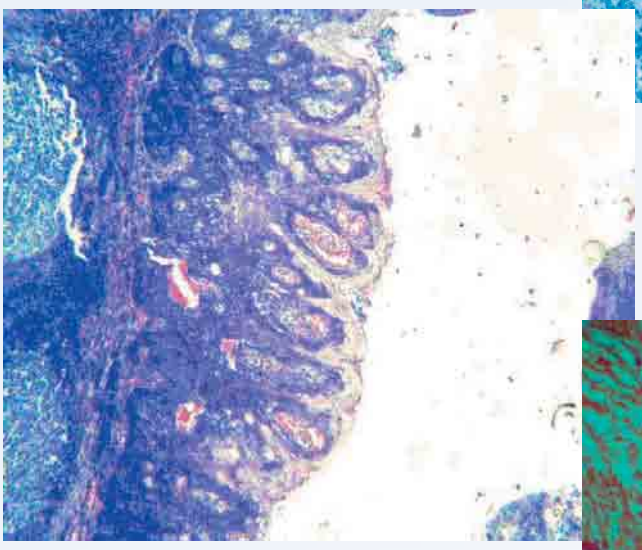
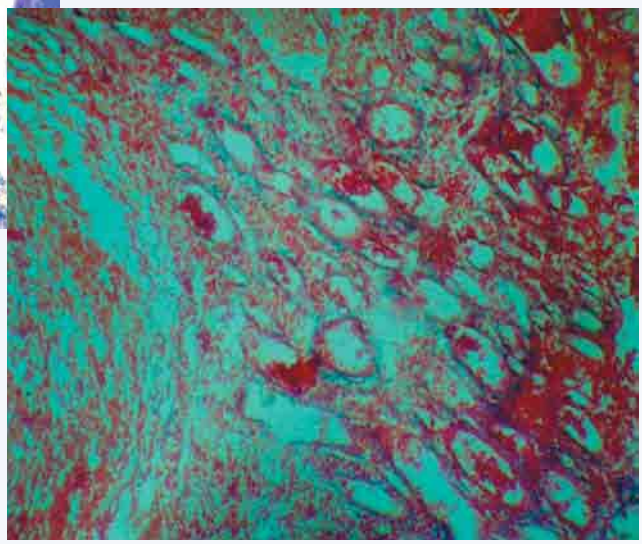


Figure 22. *Angiomatosis of the surface epithelium. Ienner-Gimza method. x 100*

Expressed angiomatosis (CD_{34} antigen) in subepithelial regions of the palatine tonsils in decompensated chronic tonsillitis. APAAP immunohistochemical reaction. ×100.

Figure 22.





Angiomatosis of the interfollicular lymphoid tissue of the pharyngeal tonsil. Heidenhain's azan method. x 100.

Figure 24.

streptococcal foci and presence of microbes in the walls and lumina of vessels. Our data allow suggesting that PRP has an important role in the local immune and protective reactions of tonsil tissue in the foci of chronic inflammation. The decreased level or absence of PRP in cells is coupled with activation of intratissue microbial foci, disturbance of barriers, and invasion of causative agents.

We also revealed that GABA, as a neuromediator, which has an essential role in the immune and protective reactions of an organism [*Hall N. et al., 1985; Devoino L. et al., 1992*], participates in metabolism and adhesive activity of PHEV endothelium. The immunohistochemical analysis of results obtained from sections of tonsils using indirect reaction of immunofluorescence with rabbit anti-GABA monoclonal serum, showed that along with intense luminescence of cells infiltrating cryptal epithelium, lymphocytes of interfollicular lymphoid tissue, small vessels, particularly PHEVs (Figures 17, 18) had especially high immunoreactivity to GABA. The considerable amount of GABA in the PHEVs in case of persisting streptococcal infection was observed alongside with depletion of PRP, and decreased levels of PGS and AC in the tonsillar lymphoid tissue. In tonsils of patients with low activity of PGS and AC enzymes, low content of cAMP and PRP in cells of lymphoid tissue, GABA-activity of PHEV endothelium was not expressed or was absent. In our opinion, these changes are the result of prolonged influence of infectious agent and functional incompetence of histohematic barrier at the site of infection entrance.

In mechanisms of chronic inflammation development, metabolic changes and invasion of microorganisms the role of NO and NO-synthase (NOS) is also of great importance in tissues and in the vascular bed of palatine tonsils, taking into account that NO is a unique vascular and neuronal mediator participating in both physiological and pathological processes. However, in pathological states dysregulation of its main producers takes place. Along with macrophages and other cells [*Moncada S. et al., 1991; Lowenstein C., Snyder S., 1992; Nathan C., 1992; Marletta M., 1993*], NO is formed also in the endothelium of vessels [*Furchgott R., Vanhoutte P., 1989; Ignarro L., 1990; Dawson V. et al., 1991; Moncada S. et al., 1991; Snyder S., Bredt D., 1992*]. The important isotypes of an enzyme are: (1) eNOS, which is mainly expressed in the endothelial cells, especially in inflammation; (2) inducible iNOS of macrophages, responsible for inactivation of infectious agents stimulated by pro-inflammatory cytokines and bacterial lypopolysaccharides [*Scherer-Singler U. et al, 1983*].

By NADPH-diaphorase reaction [*Scherer-Singler U. et al., 1983*] in cryostat sections of tonsils we managed to show that NOS-activity is displayed mainly by epithelial cells of crypt walls (Figure 19), especially at the parts of vessels and LES zones. Vascular loops displayed positive reaction to CD_{31} and CD_{34} of capillaries (Figure 20). This fact suggests that one of the factors, which prevent infection entrance from crypts into tonsillar tissue, is NO of epithelial origin. Its participation in the mechanisms of intercellular interaction of immunocytes in the LES zones cannot be excluded as well. In the microcirculatory bed the highest activity of NOS, along with stromal arterioles, is also observed in the vessels of sub-epithelial layer, i.e. in the regions of tonsils with intense processes of angiogenesis and sclerosis (Figure 21).

In the endothelium, the main part of PHEV NOS-activity was relatively low comprising 0.974±0.06 in children of younger age group, and 0.984±0.0041 in children of elder age group. Formazan formation and, thus, NOS-activity in the capillaries of follicles was not observed. Here arginase plays the potential role – as an arginine-dependent and NOS-regulating enzyme being a factor, which promotes chronic recurring infection development in tonsils [*Elgun S. et al., 2000*].

Taking into account the role of NO in the processes of vasodilatation, it may be suggested that its deficiency in the vessels, along with PHEV endothelium proliferation, and in some places with thrombi formation, is a factor, which prevents venous backflow of blood and holdup of microorganisms and their antigens within the

limits of an infectious focus. These data allow considering that low activity of NOS in the endothelium of PHEVs and absence of its activity in the capillaries of follicles in chronic inflammation is an index of decreased functional activity and reconstruction of the microcirculatory bed. It is known that NO is endowed with protective function and prevents apoptosis of lymphocytes, granulocytes, and endotheliocytes [*Liu L. and Stamler J., 1999*], may stimulate growth of endothelial cells [*Ziche M. et al., 1997*], as well as modulate angiogenesis [*Ying L., Hofseth J., 2007*]. Structural and metabolic changes in the vascular bed and barriers, low NOS activity of vascular endothelium of PHEV, as well as changes of PGS, AC, cAMP, and neuroactive substances like PRP and GABA are interconnected, being reflected on protective and immune processes in the cryptolymphones – main morpho-functional units of tonsils. On the whole, in most tonsils PGS, AC, vascular NOS-activity, as well as the content of cAMP, PRP, and GABA in the cells of lymphoid tissue are decreased. They are moderately increased in cases of recurrent tonsillitis, which is especially notable in cells infiltrating cryptal walls, diffuse lymphoid and perivascular tissue. The fact of adhesion of *streptococci* by endothelial cells from the infected blood of tonsils, allow asserting that PHEVs in the tonsils are polyfunctional structures endowed with specialized barrier function. Depending upon disease duration, the stimulation of angiogenesis in the interfollicular lymphoid tissue of tonsils is accompanied by increased amount of vessels (Figures 22-24), including PHEVs as well.

As obvious from the Table, in the tonsils of patients as compared with controls, the number of venules in a field of vision is increased almost 2.5 times. Statistically reliable increase in the average number of venules correlated with data on disease duration and angina rate during a year. The maximal index reaches up to 10.9±0.7 (control: 4.15±0.37). The percentage of PHEVs in the total amount of venules varies from 38% to 41.4% (control: 26.7%), which in the patients with long duration of the disease decreased up to 34.8% remaining higher compared with the control group. Taking into account that these data mainly refer tonsils removed in patients with clinically established recurrent chronic inflammation, the increase in average number of venules in the interfollicular lymphoid tissue and angiomatosis in persisting intratissue infection may be regarded as morphological manifestation of compensatory processes, which occurred as a result of hyperfunction of the vascular barrier.

Table.

The average number of venules including PHEVs in the interfollicular lymphoid tissue of palatine tonsils in patients with chronic recurrent tonsillitis (M±m, P<0.001).

	Control	Depending on patients' age		
		3-8 years	9-14 years	≥15 years
Venules	4.15±0.37	10.04±0.55	10.38±0.56	9.36±0.46
PHEV	1.11±0.18	3.8±0.28	4.3±0.33	3.83±0.2
		Depending on the disease duration		
		Up to 3 years	4-9 years	≥10 years
Venules	4.15±0.37	10.3±0.7	9.9±0.5	9.3±0.7
PHEV	1.11±0.18	4.0±0.3	4.1±0.4	2.9±0.7
		Depending on angina rate per year		
		2-3 times	≥4 times	
Venules	4.15±0.37	8.8±0.9	10.9±0.7	
PHEV	1.11±0.18	3.6±0.6	3.8±0.3	

In the period of decompensation at PHEV 34.8%, the average number of postcapillary venules (10.9±0.7) in tonsils of patients is the maximal index, which has no tendency to increase.

In summary, our findings demonstrate that LCP + neutrophil granulocytes participating in the mechanisms of intercellular interaction of CD_{4+} T-lymphocytes with macrophages containing phagosytosed cationized and non-cationized microorganisms are very important for mucosal barrier against infection on the surface of tonsils. Switching on the mechanisms of lymphocyte immunity with T-cells, the function of molecular recognition is a stage, which provides an initial preimmune physiological resistance of an organism to infection.

Under conditions of intratissue persisting microbial foci PHEVs take the role of terminal barrier as proliferating (polyfunctional) structures endowed also with the specialized barrier function detaining microorganisms and their antigens by endotheliocytes from the bloodstream of the infected tonsils, preventing their spread from the vessels of infection focus into the bloodstream. The compensatory increase in number of PHEVs with high activity and ability to fix and inactivate microbes takes place depending on the disease duration and sizes of intratissue microbial foci. Hence, the presence of large number of PHEVs in total amount of vessels in the tissue with moderately expressed angiomatosis, which has compensatory character, is an evidence of not completely lost barrier function against infection invasion. The low number of PHEVs against the background of expressed angiomatosis indicates both the functional incompetence and decompensation of vascular barrier. Its adequacy is ensured by levels of GABA, PGS, and AC in the endotheliocytes of venules and cAMP, PRP, AC, and NOS in the cells of lymphoid tissue, which are severely decreased in decompensation stage. Eventually, in case of complete decompensation of the vascular barrier, the average number of postcapillary venules is stabile, because the compensatory abilities of tonsils are lost.

References

1. *Bernstein I.M., Gorfien J., Brandtzaeg P.* 1999. The immunobiology of the tonsils and adenoids, p.1339-1362. In : L.Orga, J. Mestecky, M.E. Lamm, W. Strober, J. Bienenstock, and J.R. McGhee (eds.) Mucosal immunology, 2nd ed. Academic Press, New York, N.Y.
2. *Bikova V.P.* [Tonsils of Waldeyer's pharyngeal lymphoid ring in the system of mucosal immunity of upper airways] [published in Russian] In: Proceedings of the 2nd Congress of the Russian Society of Pathologists 2006, Vol. 1, p.98-101.
3. *Claeys S., de Belder T., Holtappels G., Gevaert P., Verhasselt B., van Cauwenberge P., Bachert C.* Human beta-defensins and toll-like receptors in the upper airway. Allergy 2003; 58: 748-753.
4. *Dawson V.L., Dawson T.M., London E.D., Bredt D.S., Snyder S.H.* Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc. Natl Acad. Sci. U S A. 1991; 88 (14): 6368–6371.
5. *Devoino L.V., Idova G.V., Beletskaya I.G.* Participation of a GABA-ergic system in the processes of neuroimmunomodulation. Int. J. Neurosci. 1992, 67: 4: 215-227.
6. *Elgun S., Guney K., Tuncer M., Gokhun I.H.* A potential role for arginase in recurrent infections and hypertrophy of tonsillar and adenoidal tissue. Eur. Arch. Otorhinolaryngol. 2000; 257(3):158-160.
7. *Fujieda S., Suzuki S., Sunaga H., Seki M., Mori S., Suzuki H., Funatsu I., Sato H.* Bacterial infection and production of immunoglobulin in the tonsil. Rinsho Byory, 1999; 47(8): 724-729.

8. *Furchgott R.F., Vanhoutte P.M.* Endothelium-derived relaxing and contracting factors. FASEB J. 1989; 3(9): 2007–2018.
9. *Gaffney R.J., Cafferkey M.T.* Bacteriology of normal and diseased tonsils assessed by fine-needle aspiration: Haemophilus influenzae and the pathogenesis of recurrent acute tonsillitis. Clin. Otolaryngol. Allied Sci. 1998; 23: 181-185.
10. *Galoyan A.A.* [Biochemistry of Novel Cardioactive Hormones and Immunomodulators of the Functional System Neurosecretory Hypothalamus-Endocrine Heart] [published in Russian] Nauka Publishers, Moscow. 1997.
11. *Galoyan A.A., Vardazaryan N.D., Melikyan N.V.* Immunohistochemistry of Hypothalamus Neurosecretory Cytokine-Proline-Rich Polypeptide (PRP) in Human Bone Marrow. Natural Sciences, 2004: 2(3): 3-5.
12. *Girard J.P., Baekkevold E.S., Yamanaka N., Haraldson G., Brandtzaeg P., Amarlic F.* Heterogeneity of endothelial cell: the specialized phenotype of human high endothelial venules characterized by suppression subtractive hybridization. Am. J. Pathol. 1999; 155(6): 2043-2055.
13. *Gorfien J.L., Noble B., Brodsky L.* Comparison of the microanatomical distributions of macrophages and dendritic cells in normal and diseased tonsils. Ann. Otol. Rhinol. Laryngol. 2001: 110 (2): 173-182.
14. *Hall N.R., Suria A., Goldstein A.L.* Elevated levels of gamma aminobutyric acid (GABA) in the thymus gland during the immune response. Lymphokine Res. 1985; 4(4): 339-341.
15. *Hallman M., Ramet M., Ezekowitz R.A.* Toll-like receptors as sensors of pathogens. Pediatr. Res. 2001; 50: 315-321.
16. *Hess K.L., Tudor K.S., Johnson J.D., Osati-Ashtiani F., Askew D.S., Cook-Mills J.M.* Human and murine high endothelial venule cells phagocytose apoptotic leukocytes. Exp. Cell. Res. 1997; 236 (2): 404-411.
17. *Howell S.L, Whitfield M.* Cytochemical localization of adenyl cyclase activity in rat islets of Langerhans. J. Histochem. Cytochem. 1972; 20: 873-879.
18. *Howie A.J.* Scanning and transmission electron microscopy on the epithelium of human palatine tonsils. J. Pathol. 1980; 130: 91-98.
19. *Ignarro L.J.* Biosynthesis and metabolism of endothelium-derived nitric oxide. Annual Rev. Pharmacol. Toxicol. 1990; 30: 535–560.
20. *Johansson-Lindbom B., Ingvarsson S., Borrebaeck C.A.* Germinal centers regulate human Th2 development. J. Immunol. 2003; 171: 1657-1666.
21. *Lindroos R.* Bacteriology of the tonsil core in recurrent tonsillitis and tonsillar hyperplasia - A short review. Acta Otolaryngol. Suppl. 2000; 543: 206-208.
22. *Liu L. and Stamler J.S.* NO: an inhibitor of cell death. Cell death and differentiation. 1999; 6(10): 937-942.
23. *Liu Y.J., Banchereau J.* The Paths and Molecular Controls of Peripheral B-cell Development. The Immunologist 1996; 4: 55-66. .
24. *Lowenstein C.J., Snyder S.H.* Nitric oxide, a novel biologic messenger. Cell 1992; 70(5): 705–707.
25. *Marletta M.A.* Nitric oxide synthase. Structure and mechanism. J. Biol. Chem. 1993; 268(17): 12231-12234.
26. *Mazing Yu. A.* [Neutrophil granulocytes and protective mechanisms of an organism] [published in Russian] Arch. pathol. 1991; 9: 70-73.
27. *Moncada S., Palmer R.M., Higgs E.A.* Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 1991; 43(2): 109–142.
28. *Nathan C.* Nitric oxide as a secretory product of mammalian cell. FASEB J. 1992; 6(12): 3051-3064.

29. *Pigarevsky V.E.* [Polymorphonuclear leukocyte and macrophage in inflammatory and hypersensitivity reactions] [published in Russian] Arch. pathol, 1983; 11: 14-22.

30. *Putto A.* Febrile exudative tonsillitis: viral or streptococcal? Pediatrics 1987; 80: 6-12.

31. *Scherer-Singler U., Vincent S.R., Kimura H., McGeer E.G.* Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. J. Neurosci. Methods. 1983; 9(3): 229–234.

32. *Shibata Y., Nishiyama A., Ohata H., Gabbard J., Myrvik Q.N., Henriksen R.A.* Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E2 byosynthesis between spleen and bone marrow macrophages. J. Leukocyte Biology 2005; 77: 544-551.

33. *Snyder S.H., Bredt D.S.* Biological role of nitric oxide. Scientific American 1992; 266: 68-77.

34. *Tohya K., Kimura M.* Preference of lymphocyte-homing to high endothelial venules in palatine tonsils of musk shrew (Suncus murinus). Acta Otolaryngol. Suppl. 1996; 523: 25-27.

35. *Vardazaryan N.D., Arsenyan K.A., Hambardzumyan S.V.* [Immunomorphological characteristics of endogenous active factors of vascular barrier of palatine tonsils in recurrent tonsillitis] [published in Russian] In: Proceedings of the 2nd Congress of the Russian Society of Pathologists 2006. Vol. 1. P.109-111.

36. *Vardazaryan N.D.*[Changes of palatine tonsils and intratissue microbial foci in chronic tonsillitis] [published in Russian] Arch. pathol., 1981; 5: 10-15.

37. *von Andrian U.H., Mempel T.R.* Homing and cellular traffic in lymph nodes. Nat. Rev. Immunol. 2003; 3(11): 867-878.

38. *Ying L., Hofseth L.J.* An emerging role of endothelial nitric oxide synthase in chronic inflammation and cancer. Inflammation and cancer. Cancer Res. 2007; 67: 1407-1410.

39. *Ziche M., Morbidelli, L. and Choudhuri R.* Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. J. Clin. Invest.1997; 99(11): 2625-2634.