Neurotrophins and neurotransmitters in human palatine tonsils: An immunohistochemical and RT-PCR analysis

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Received February 1, 2006; Accepted March 27, 2006

Abstract. Lymphoid organs are supplied by many nerve endings associated with different kinds of cells and macrophages. The role of these neuromediators on the release of locally active molecules is still unknown. Here we focused our attention on the expression of some neurotrophins (NTs), their high- and low-affinity receptors and several neurotransmitters in human palatine tonsils. Light and electron microscopy immunohistochemistry showed that human tonsillar samples were positive for all analyzed neurotrophins (NGF, BDNF and NT-3) and their high-affinity receptors (TrkA, TrkB and TrkC, respectively). All of these molecules were strongly expressed in macrophages whereas, in some patients, a weaker specific staining of lymphocytes and blood vessels was also found. The low-affinity receptor for NGF (p75) was always absent in the analysed samples. RT-PCR confirmed the occurrence of specific transcripts for NTs and their high-affinity receptors as well as the absence of mRNA for p75 protein. Also, specific immunoreactivity for neurotransmitters SP, VIP, CGRP, ChAT and nNOS was mainly expressed by macrophagic cells. These results suggest the presence of an extensive network of innervation in the human palatine tonsils which may play a role in the regulation of some immune functions as well as in the modulation of a possible functional scenario of interactions among different immune cellular subtypes.

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Key words: human tonsil, immunohistochemistry, neurotrophins, nNOS, SP, VIP, CGRP, cholinacetyltransferase, electron microscopy

Introduction

The nervous and the immune systems are functionally related and human mucosae lymphoid tissue are considerably innervated (1). Mucosae-associated lymphoid tissue, particularly the tonsils, are in contact with the external environment and are known to contain numerous lymphocytes and macrophages which defend the host against pathogens. As a part of the immune system, the tonsils are supposed to interact with the nervous system by an extensive network, involving many signaling molecules. Regarding this connection, information is still scarce about neurotrophin expression and innervation of the human tonsils. Neurotrophins (NTs), also known as neurotrophic factors, constitute a family of dimeric proteins working as polypeptidic growth factors, which include nerve growth factor (NGF), brain derived growth factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6, the last being apparently specific for fish (2). Biological actions of NTs are mediated by the binding with two families of membrane receptors, the high-affinity tyrosine kinase (Trk) and the low-affinity p75 (p75NT receptor) receptors (3,4). The Trk family includes TrkA, TrkB and TrkC receptors, whereas the p75NT receptor belongs to the trans-membrane molecules serving as receptor for tumor necrosis factor and cytokines (4). TrkA is specifically activated by NGF, whereas TrkB and TrkC are primarily receptors for BDNF and NT-3 respectively (5,6). NTs are involved in vertebrate neuronal cell development, differentiation, survival and functional activities (2,7). NTs are also involved in the modulation of adult central nervous system functions and organization, as well as in the vegetative innervation of several organs (5,7-12). Moreover, detailed studies have revealed significant actions of neurotrophins in a wide variety of tissues outside the nervous system, especially in the immune system (13-17). In

particular, it is supposed that immune tissue is capable of concentrating NGF, which in turn may modulate the level of innervation by the sympathetic nervous system (18-21).

In order to gain information about this matter, we studied the innervation and the expression of NTs and their receptors in different cellular subtypes (lymphocytes, macrophages and epithelial cells) of human palatine tonsils to investigate a possible relationship between functional and physio-pathological mechanisms.

Materials and methods

Clinical material. Human tonsil tissue was surgically removed from seven patients and subjected to immunohisto-chemical and RT-PCR analysis. Experiments were performed in compliance with the Italian laws and guidelines concerning the informed consent of the patients.

Immunohistochemical analysis by light microscopy. The following molecules were investigated: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), tyrosine kinase A (TrkA), tyrosine kinase B (TrkB), tyrosine kinase C (TrkC), protein 75 (p75), substance P (SP), vasointestinal peptide (VIP), calcitonin gene-related peptide (CGRP), choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS).

For light microscope immunohistochemical analysis, small fragments of tonsil tissue were washed in PBS, fixed in 10% formalin and embedded in paraffin according to a standard procedure. Serial 10-μm thick sections were cut using a rotatory microtome, mounted on gelatin-coated slides and processed for immunohistochemistry. To study the immunolocalization of neurotrophins and their receptors, the antibodies we used were: rabbit anti-NGF polyclonal antibody (Santa Cruz, CA, USA), which displayed less than 1% cross-reactivity against recombinant human NT-3, NT-4 and BDNF; rabbit anti-BDNF polyclonal antibody (Santa Cruz), which recognized the amino-terminus of mouse BDNF and did not cross-react with NT-3 or NGF; rabbit anti-NT-3 polyclonal antibody (Santa Cruz), which was raised against the amino-terminus of mouse NT-3 and did not cross-react with NGF or BDNF; rabbit anti-TrkA polyclonal antibody (Santa Cruz) which recognized an epitope correspond-ing to aminoacids 763 to 777, mapping adjacent to the carboxy terminus of human TrkA p140 and was not cross reactive with TrkB or TrkC; rabbit anti-TrkB polyclonal antibody (Santa Cruz), which recognized an epitope corresponding to aminoacids 794 to 808 of mouse TrkB p145 and was not cross- reactive with TrkA or TrkC; rabbit anti-TrkC polyclonal antibody (Santa Cruz), which recognized an epitope corresponding to aminoacids 798 to 812 of porcine TrkC p140 and was not cross-reactive with TrkA or TrkB; and goat polyclonal antibody to human NGF receptor p75 (Santa Cruz), which recognized the amino acid sequence mapping the carboxy terminus of the NGF receptor p75 precursor of human origin and was not cross-reactive with other growth factor receptors. For analysis of neuro-transmitters, the following antibodies were used: rabbit anti-SP polyclonal antibody (Chemicon International, CA, USA); rabbit anti-VIP polyclonal antibody (Chemicon International); mouse anti-CGRP monoclonal antibody (Chemicon International); goat anti-ChAT polyclonal antibody (Chemicon International); and rabbit anti-nNOS polyclonal antibody (Chemicon International). The immunohistochemical recognition of macrophages was performed by a mouse anti-human CD68 monoclonal antibody (DakoCytomation, Denmark) whereas the immunohisto-chemical recognition of lymphocytes was performed by a mouse anti-CD38 monoclonal antibody (Santa Cruz). Incubation with primary antibodies was performed overnight at 4˚C at a final concentration of 2-5 μg/ml. Optimal antisera dilutions and incubation times were assessed in a series of preliminary experiments. After exposure to the primary antibodies, slides were rinsed twice in phosphate buffer and incubated (1 h and 30 min at room temperature) with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (final dilution 1:100). The secondary antibody-HRP linked against rabbit immuno-globulins was purchased from Boehringer (Boehringer Mannheim GmbH, Mannheim, Germany), while secondary antibodies-HRP linked against mouse and goat immuno-globulins were from Sigma (Sigma Chemicals Co, St Louis, MO, USA). After a further wash with phosphate buffer, slides were treated with 0.05% 3,3-diaminobenzidine and 0.1% H_2O_2. Finally, sections were counterstained with Mayer's hematoxylin and observed by using a light microscope. To block endogenous peroxidase activity, slides were pretreated with 3% H_2O_2, whereas the non-specific binding of immunoglobulins was prevented by adding 3% fetal calf serum to the incubation medium. Negative control experiments were performed: by omitting the primary antibody; by substituting the primary antibody with an equivalent amount of non-specific immunoglobulins; by pre-incubating the primary antibody with the specific blocking peptide (antigen/antibody = 5 according to manufacturer's instructions). In preliminary experiments, immunohisto-chemistry was performed also on frozen sections of human tonsil tissue. We found no differences in the intensity or distribution of immunostaining using the two types of sections, but we preferred paraffin-embedded material because microanatomical details were better preserved. The intensity of immune reaction was assessed microdensitometrically by an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected via a TV camera to the microscope. The system was calibrated taking as zero the background obtained in sections exposed to non-immune serum. Ten 100 μm^2 areas were delineated in each section by a measuring diaphragm. Quantitative data of the intensity of the immune staining were analyzed statistically by analysis of variance (ANOVA) followed by Duncan's multiple range test as a post hoc test.

Immunolabeling by electron microscopy. Tonsillar samples were cut into small pieces (1-2 mm^3), fixed with 4% paraformaldehyde plus 0.1% glutaraldehyde in PBS, pH 7.4, for 2 h at 4˚C, washed in PBS and then infiltrated with 2.3 M sucrose in PBS for 3 h at 4˚C, frozen in liquid nitrogen, and cryosectioned following the method by Tokuyasu (22). Ultrathin cryosections, obtained by using a Leica Ultracut UCT device (Leica Microsystem, Wien, Austria), were collected using sucrose and methylcellulose and incubated overnight at 4˚C with the specific primary antibodies and

Table I. Results of the immunohistochemical analysis performed on the tonsil tissue of 7 patients for NTs, NT receptors and neurotransmitters. ++, strong immunoreactivity; +, moderate immunoreactivity; ±, weak immunoreactivity; -, absence of immunoreactivity.

	Expressing Cells	Pat 1	Pat 2	Pat 3	Pat 4	Pat 5	Pat 6	Pat 7
NGF	Macrophages	++	++	+	++	+	++	++
	Lymphocytes	+	+	±	+	+	±	+
BDNF	Macrophages	+	++	+	+	++	+	+
	Lymphocytes	+	+	+	±	+	+	±
NT3	Macrophages	++	++	++	+	++	++	+
	Lymphocytes	±	±	-	±	±	±	-
	Blood vessels	+	+	+	+	+	+	+
TrkA	Macrophages	++	++	++	+	++	+	++
	Lymphocytes	+	+	±	±	+	±	±
	Blood vessels	±	+	±	±	+	±	±
TrkB	Macrophages	++	++	+	+	++	++	++
	Lymphocytes	++	+++	++	++	+++	+++	+++
	Blood vessels	+	+	+	+	+	+	+
TrkC	Macrophages	++	+	++	++	++	+	++
	Lymphocytes	-	±	±	±	±	±	-
p75		-	-	-	-	-	-	-
SP	Macrophages	++	++	++	++	++	++	++
	Lymphocytes	±	±	±	-	±	±	±
VIP	Macrophages	++	++	++	+	++	++	++
	Lymphocytes	±	+	+	±	+	±	+
	Blood vessels	+	+	+	+	+	+	+
CGRP	Macrophages	++	++	++	+++	++	++	+++
	Lymphocytes	±	-	-	-	±	-	-
ChAT	Macrophages	-	±	±	+	+	±	±
	Lymphocytes	++	++	+	++	++	+	++
nNOS	Macrophages	+	±	+	±	+	±	+
	Lymphocytes	+	+	+	+	+	+	+
	Blood vessels	+	±	+	±	+	+	+

then with goat anti-mouse or anti-rabbit immunoglobulins 5- and 10-nm gold conjugated (Sigma-Aldrich, Milan, Italy). Control samples were obtained by omitting the incubation with the specific primary antibody. Finally, ultrathin cryosections were stained with a 2% methylcellulose and 0.4% uranyl acetate solution. Samples were examined with a Philips 208 transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

RT-PCR. Total RNA was isolated from human tonsil tissue by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA was synthesized from 1 μg total RNA in a final reaction volume of 20 μl. Briefly, a mixture of total RNA, oligo (dT), dNTP mix and DEPC-treated distilled water was pre-incubated for 5 min at 65˚C; then SuperScript III reverse transcriptase (200U), RNase ribonuclease inhibitor, DTT and buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) were added to the mixture and incubation was continued for 45 min at 50˚C. Finally, Superscript III was inactivated by heating for 15 min at 70˚C. All reagents were from Invitrogen. Of the resulting cDNA, 3-5 μl were amplified by polymerase chain reaction (PCR). Each PCR tube contained the following reagents: 0.2 μM of both sense and antisense primers, 3 to 5 μl template cDNA, 0.2 mM 4-dNTP mix (Invitrogen), 2.5 U Platinum TaqDNA polymerase (Invitrogen) and 1X reaction buffer (Invitrogen). $MgCl_2$ was added at a final concentration of 1 mM for BDNF, NT-3, TrkA and TrkB and at a final concentration of 1.5 mM for NGF, TrkC and p75. The final volume was 50 μl. The PCR primers used for amplifying neurothrophins and their receptors (M-Medical, Florence, Italy) were as follows: for NGF, forward TCATCATCCCATCCCATCTT, reverse CTTGACAAAGGTGTGAGTCG; for BDNF, forward AGCCTCCTCTGCTCTTTCTGCTGGA, reverse CTTTT

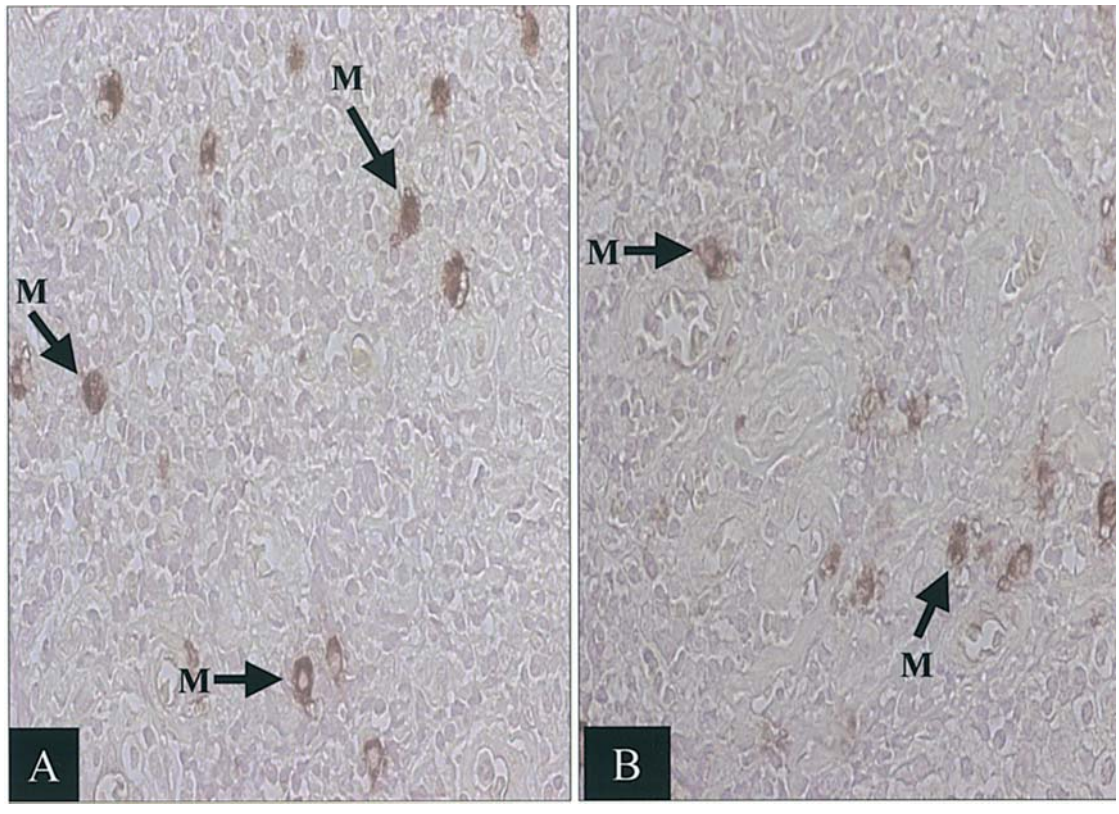

Figure 1. Human tonsil tissue: patient no. 5. Micrographs of NGF (A) and Trk-A (B) immunostaining. Strong immunoreactivity for NGF and its specific receptor, TrkA, is observed in macrophagic cells (M) whereas lymphocytes and blood vessel endothelium are weakly stained.

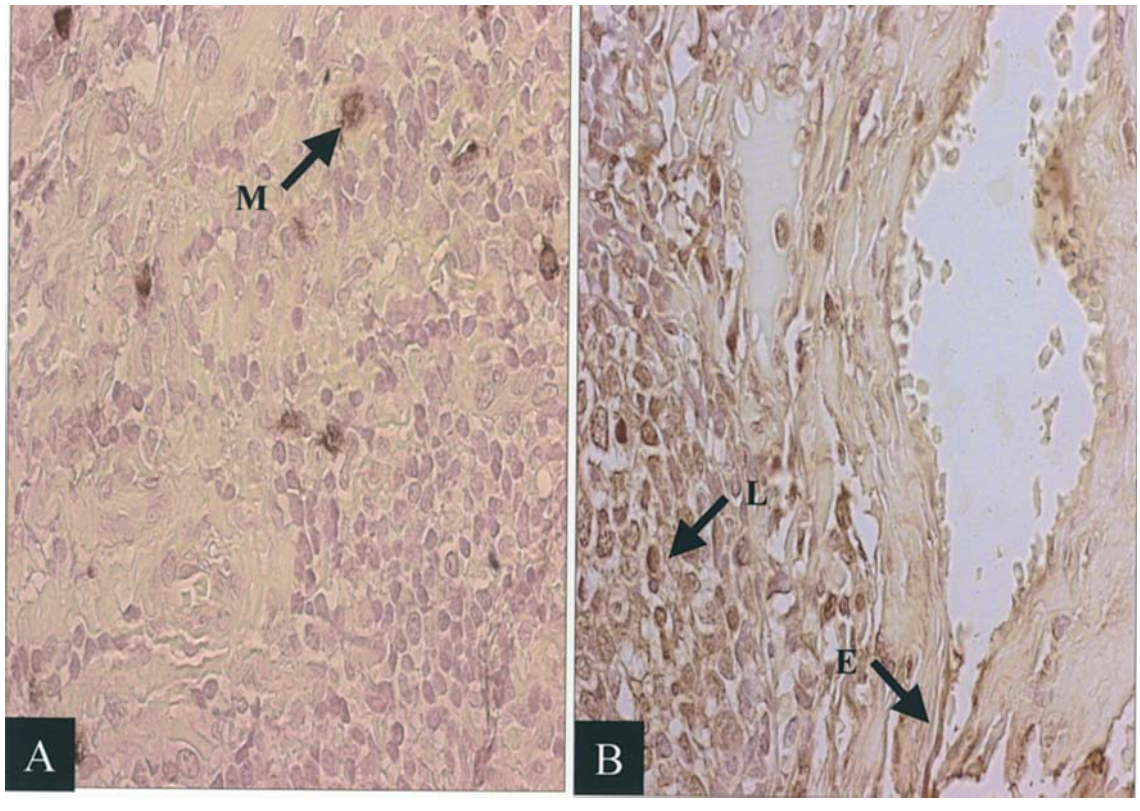

Figure 2. Human tonsil tissue: patient no. 1. Micrographs of BDNF (A) and TrkB (C) immunostaining. Strong immunoreactivity for BDNF is associated to macrophages (M) whereas (B) its specific receptor, TrkB, is strongly expressed by lymphocytes (L). Weak immunostaining for TrkB is also observed in macrophages and blood vessel endothelium (E).

GTCTATGCCCCTGCAGCCTT; for NT-3, forward TTTCTCGCTTATCTCCGTGGCATCC, reverse GGCAGG GTGCTCTGGTAATTTTCCT; for TrkA, forward TCTT CACTGAGTTCCTGGAG, reverse TTCTCCACCGG GTCTCCAGA; for TrkB, forward AAGACCCTGAAGG ATGCCAG, reverse AGTAGTCAGTGCTGTACACG; for TrkC, forward GGAAAGGTCTTCCTGGCCGAGTGC, reverse GCTTTCCATAGGTGAAGATCTCCC; for p75, forward TGGACAGCGTGACGTTCTCC, reverse GATCT CCTCGCACTCGGCGT. The specificity of the primers was verified by searching the NCBI database for every possible homology to cDNAs of unrelated known proteins. The PCR reaction consisted of incubation for 2 min at 94˚C followed by 30-35 cycles of incubation at 94˚C for 30 sec, 56˚C (for NGF, TrkA and TrkB) or 62˚C (for BDNF, NT-3, p75 and TrkC) for 30 sec and 72˚C for 1 min. PCR products were separated by agarose gel electrophoresis (Submarine agarose gel unit; Hoefer, San Francisco, CA, USA) and visualized using a digital gel documentation system (GelDoc 2000 System/QuantityOne software; Bio-Rad Laboratories, Hercules, CA, USA).

Results

Light microscopy immunohistochemistry. Sections of tonsil samples exposed to primary/secondary antibodies developed a dark-brown (intense), yellow-brown (slight) or no immunostaining. Immunoreactivity was specific, since no immunostaining was obtained in control sections incubated with each primary antibody adsorbed with the specific peptide or with pre-immune serum (not shown). Immunolabeling was located in the macrophages, lymphocytes and blood vessels of tonsil tissue (Table I).

Strong immunoreactivity for NGF was observed in macrophage cells and moderate immunoreactivity was found in lymphocytes of all patients (Fig. 1A). Immunoreactivity for BDNF was well expressed in macrophage cells of all patients; lymphocytes showed a moderate staining (Fig. 2A). Strong immunoreactivity for NT-3 was observed in the macrophages of all patients and slight immunoreactivity was detected in the lymphocytes of five out seven analyzed patients; moderate immunoreactivity was present in the blood vessels (Fig. 3A). Strong immunoreactivity for TrkA receptor was detected in the macrophages and weak reaction was evident in the lymphocytes and blood vessels of all patients (Fig. 1B). Appreciable immunoreactivity for TrkB receptor was detected in the macrophages while strong expression was evident in the lymphocytes of all patients; blood vessels showed weak immunoreactivity (Fig. 2B). Strong expression of TrkC was relevated in macrophages of all patients and weak immunoreactivity was detected in the lymphocytes of five out of seven analyzed patients (Fig. 3B). No expression of p75 was relevated in anyone of the seven analyzed patients (not shown). Strong immunoreactivity for SP was detected in the macrophages and weak positivity was observed in the lymphocytes of all analyzed patients (Fig. 4A). Immunoreactivity for VIP was strong in macrophages, weak in lymphocytes and moderate in blood vessels of all patients (Fig. 4B). Strong immunoreactivity for CGRP was evident in the macrophages of all patients; lymphocytes were only weakly labelled in two out of seven analyzed patients (Fig. 4C). Weak expression for ChAT was detected in the macrophages while strong immunoreactivity was detected in the lymphocytes of all analyzed patients (Fig. 4D). Weak immunoreactivity for nNOS was detected in the macrophages while expression was moderate in lymphocytes and blood vessels of all analyzed patients (Fig. 5).

The results obtained are summarized in Table I. It can be observed that macrophages stained for all of the markers analyzed with the exception of p75. On the contrary, lymphoid cells and vascular endothelial cells showed a variable expression of the different markers, although, in general, not as strong as macrophages.

ImmunoTEM. The observation by transmission electron microscopy of cryosectioned human tonsillar samples revealed a relatively good morphological preservation. Fig. 6C showed a portion of a control unlabeled cryosection in which a macrophagic cell with indented nucleus, a fibroblastic cell with elongated nucleus and, adjacent to a cross-sectioned bundle of collagene, a capillary vessel surrounded by endothelial cells were visible.

When the same sections were labelled with anti-NGF antibody, an intense positivity was revealed in macrophages (Fig. 6A). Fig. 6A shows numerous gold particles randomly distributed inside the cytoplasm whereas the nucleus appears to be completely negative. Macrophages were positive also for BDNF (not shown) and NT-3 (not shown) and the receptors, TrkA (Fig. 6B), TrkB (not shown) and TrkC (not shown). Lymphocytes were stained with all analyzed markers, but they were particularly reactive with anti-TrkB antibody (Fig. 7B). In TrkB-labelled samples, numerous clusters of gold particles were observed inside the cytoplasmic matrix and associated to plasma membrane (Fig. 7B). Labelling of the tonsillar samples with anti-CD38 mAb demonstrated that the observed cells were indeed lymphocytes since they showed numerous CD38 molecules on the outer side of the plasma membrane (Fig. 7C).

RT-PCR. RT-PCR analysis revealed the occurrence in tonsil tissue of specific transcripts for neurotrophins (NGF, BDNF, NT-3) and their high-affinity receptors (TrkA, TrkB, TrkC) (Fig. 8). Furthermore, in line with our immunohistochemical results, mRNA for p75 was undetectable (Fig. 8).

Discussion

Neurotrophins (NTs) are neurotrophic signaling polypeptides which play physiological roles in the development, maintenance and regeneration of the sympathetic and sensory nervous system (3,7). Moreover, NGF induces differentiation and decreases growth rate in a variety of neoplastic cells from neurogenic and non-neurogenic origin (9,23-26). The present study describes the localization in human tonsil tissue of some neurotransmitters (SP, VIP, CGRP, ChAT, and nNOS), some NTs, such as NGF, BDNF, NT-3, and their high- and low-affinity receptors (TrkA, TrkB, TrkC and p75), that usually mediate the neurotrophic actions in the central nervous system (6,27).

The possible action of neurotrophins on lymphocytes was first reported by Dean *et al* (28), who observed that NGF

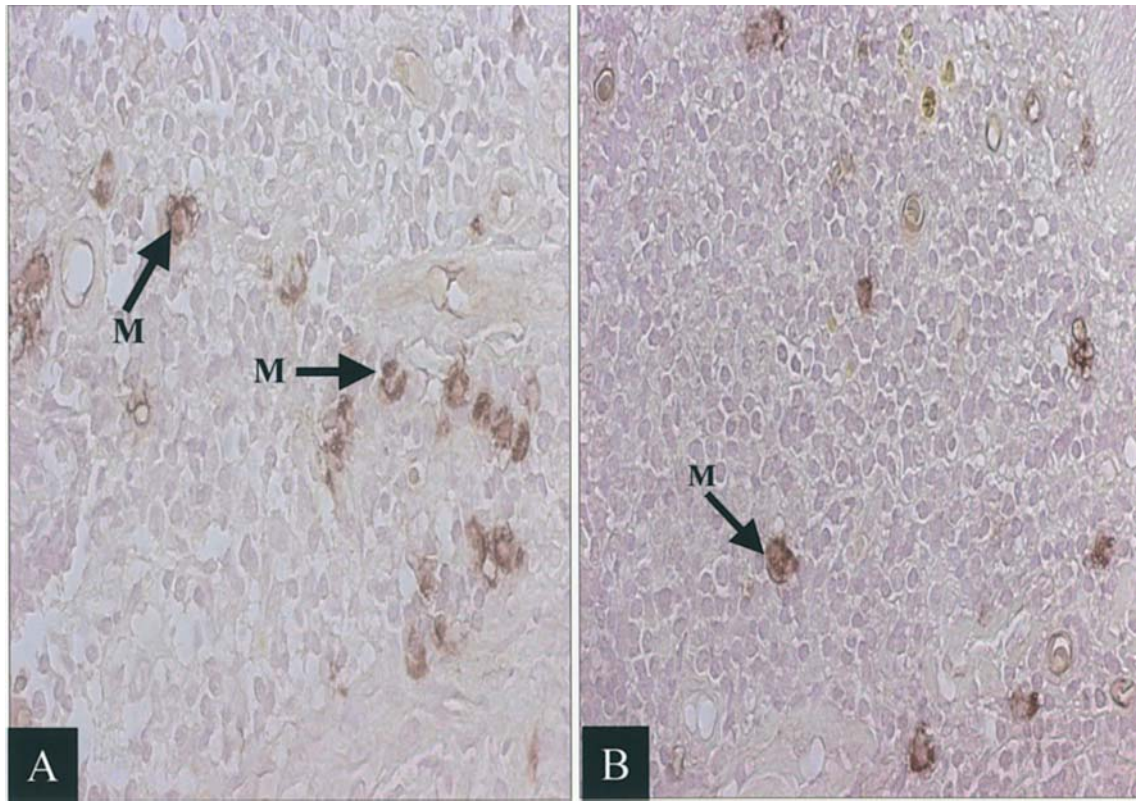

Figure 3. Human tonsil tissue: patient no. 2. Micrographs of NT-3 (A) and Trk-C (B) immunostaining. Strong immunoreactivity for NT-3 and its specific receptor TrkC is evident in macrophages (M). Lymphocytes are slightly stained only in some patients.

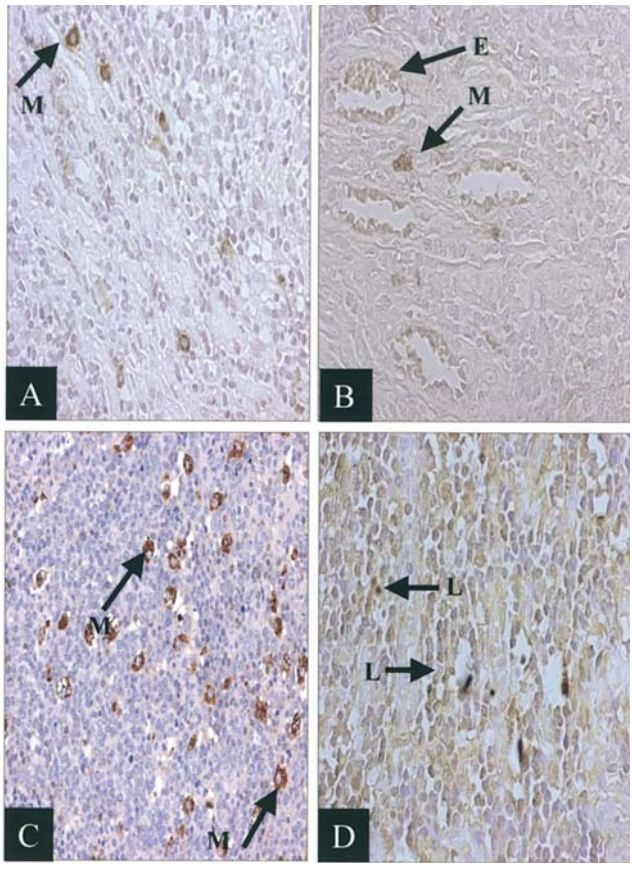

Figure 4. Human tonsil tissue: patient no. 2. Micrographs of SP (A), VIP (B), CGRP (C) and ChAT (D). Strong immunoreactivity for SP, VIP and CGRP is associated to macrophages (M), whereas ChAT immunostaining is strong in lymphocytes (L).

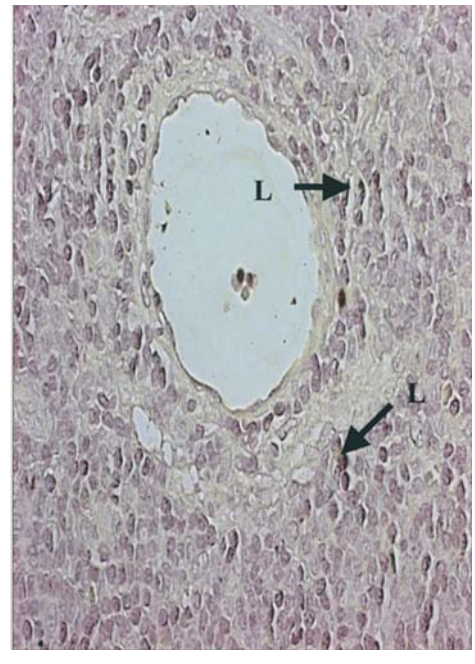

Figure 5. Human tonsil tissue: patient no. 3. Micrographs of nNOS immunostaining. Moderate immunoreactivity is evident in lymphocytes (L) and blood vessel endothelium. Macrophages are slightly stained.

increased the blastogenic response of mouse spleen cells. This observation suggested that lymphocytes (and perhaps other immunocompetent cells) expressed neurotrophin receptors. Further observations proved that these cells synthesize and release neurotrophins, leading to the hypothesis that there might be an autocrine and paracrine action of NTs

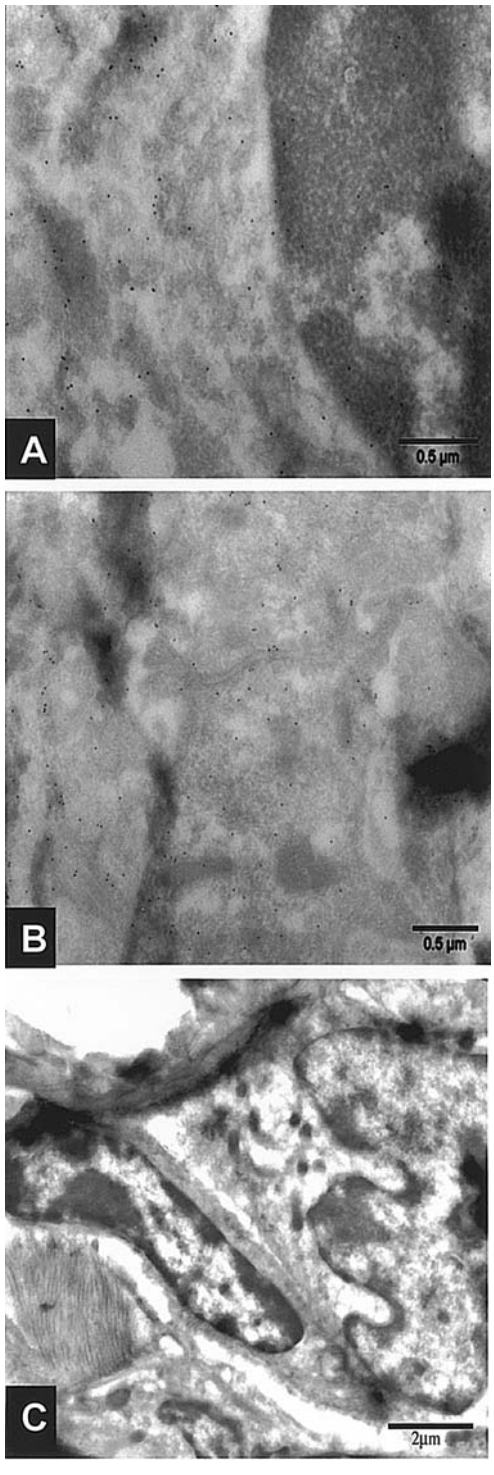

Figure 6. Transmission electron microscopy cryosections of human tonsil tissue. A-Macrophagic cell labelled with anti-NGF antibody. Numerous gold particles are randomly distributed inside the cytoplasm. B-Macrophagic cell labelled with anti-TrkA antibody. Clusters of gold particles are visible in the cytoplasm of both cells. C-Negative control cryosection showing that morphology of the different cell types appears to be well preserved.

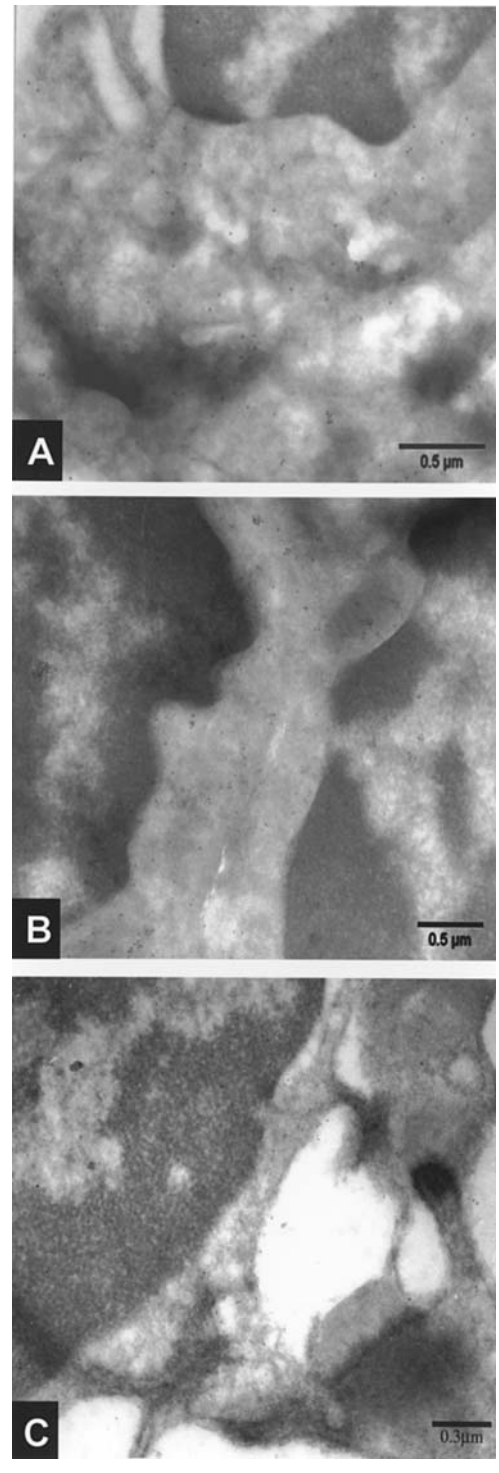

Figure 7. Transmission electron microscopy cryosections of human tonsil tissue. A-Lymphocyte labelled with anti-BDNF antibody. Numerous gold particles are evident inside the cytoplasm whereas the nucleus is completely negative. B- Lymphocyte labelled with anti-TrkB antibody. Numerour gold particles are observed inside the cytoplasmatic matrix and plasma membrane. C-Lymphocyte labelled with anti-CD38 antibody. The presence of numerous CD38 molecules on the cell surface demonstrates the lymphocytic nature of these cells.

on the same cells (29). Moreover, the most important and interesting result concerning the expression of both NTs and their receptors in lymphocytes is represented by the fact that this possibility depends on the cell activation (30,31). In fact, expression of both NGF and TrkA is induced by mitogen activation in $CD4^+$ T cells (32). Moreover, both $CD4^+$ and $CD8^+$ T cells produce NGF, which is increased after antigenic stimulation in the Th2 subset (33). Furthermore other studies (34,35) confirmed that unstimulated human $CD4^+$ Th1 and Th2 cells (but not Th0 cells) express both NGF and TrkA, and Th1 cells express full-length TrkB and low levels of TrkC. $CD4^+$ and $CD8^+$ T cells transcribe also BDNF mRNA and produce bioactive BDNF, NT-3 and

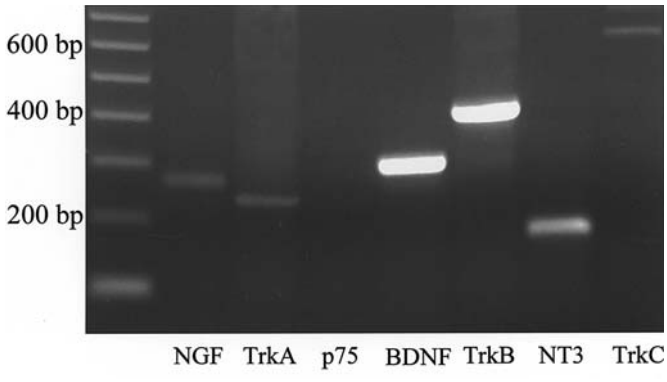

Figure 8. RT-PCR analysis of neurotrophins and related receptors in human tonsil tissue. All analysed NTs and high-affinity receptors are expressed in human palatine tonsils whereas mRNA for low-affinity receptor p75 is absent.

NT4/5 (30,31,36). Otherwise, the expression of p75 NT by T cells remains controversial (32,37). The expression of TrkA (38-40) and p75NT (41) in B cells has been reported. According to Schenone *et al* (42), B cells do not express mRNA for either p75NT or TrkA and, moreover, BDNF activates TrkB receptors on B cells. The occurrence of TrkB and TrkC on B cells has been previously confirmed (35,40). Furthermore, it has been shown that B cells produce NGF and NT-3 (33,35,39). In human palatine tonsils, p75 is exclusively present in follicular dendritic cells and in periarteriolar macrophages (43-46). Concerning TrkA, dendritic cells and interdigitated reticular cells have been found in cryptic tonsilar epithelium (44-46). Analogous results have been observed in bovine lymphoid organs (47,48).

In the present study, a moderate to strong immunoreactivity for neurotransmitters, NTs and NT receptors was observed in tonsil tissue; in particular, NTs and their own high-affinity receptors were strongly expressed in macrophages and, to a lesser extent, in lymphocytes. Only immunoreactivity for TrkB was evident also in lymphocytes. The whole tissue does not show any expression of p75 in any of the analyzed patients and this is in line with the majority of reports available in the literature (32,37). Specific immunoreactivity for NTs and NT receptors was also demonstrated within different layers of large, medium and small sized arteries and veins, according to a recent study of our group (49) performed in other mucosae-associated lymphoid tissues. This evidence supports the role of these growth factors in the modulation of the immune functions in the mucosae-associated lymphoid tissues (1,7,8,50-55). Electron microscopy immunolabelling confirmed the localization of NTs and their receptors, mainly in macrophages, as well as the strong expression of the TrkB receptor by lymphocytes. In line with our immunohistochemical analysis, RT-PCR experiments demonstrated the occurence of specific transcripts for NTs and their high-affinity receptors as well as the absence of the low-affinity NGF receptor, p75.

In the present study, the evidence of a moderate expression of NGF within the germinative centre is consistent with previous studies demonstrating that NGF was constitutively produced by B cells (39). Regarding this connection, NGF may be considered an autocrine survival factor for memory B lymphocytes, even though our study revealed it to be well expressed in macrophages. This last finding is in line with previous observations (35,36,56-58) indicating that NGF, BDNF and NT-4/5 may be expressed by macrophages. Moreover, a recent very interesting observation (59) has demonstrated that both NGF and BDNF influence the cytokine expression pattern in peripheral blood mononuclear cells, as well as in antigen-specific T cells, modulating the production of interleukin-4, TGF-ß, TNF-α and γ-interferon. It is already well known (13,60) that NGF has an inflammatory role and its increase is directly related to inflammation, allergies and diseases of the immune system. NGF levels are also increased in asthma and in other allergic diseases (34,61-64). Moreover, systemic NGF administration increases histamine-induced bronchial hyperreactivity, probably via an intervention of tachykinins released by macrophages or mast cells (65). An altered regulation of NT production or NT receptor expression may be responsible for the control of the growth, differentiation and apoptosis of some kinds of non-neuronal tumours (29,66), such as pancreatic ductal adenocarcinoma (67), melanoma (68), prostate cancer (69) and lung cancer (70).

In the present study, ChAT has been found to be expressed in the lymphocytes, but also in macrophages. SP, VIP and CGRP were well-expressed in macrophages, and a weak immunoreaction was detected in lymphatic cells and blood vessels. nNOS was only slightly expressed in all analyzed structures.

In conclusion, our data collectively suggest the existence of an extensive network of innervation in human tonsil lymphoid tissue, which may play a role in the regulation of some immune functions and in the modulation of complex interactions among different immune cellular subtypes in physiological as well as inflammatory conditions. Our data also corroborate previous studies suggesting that neurotransmitters and neurotrophins may mediate functional signals in lymphoid aggregates (5,7,8-12), thus confirming an important role of growth factors in the complex regulation of immune functions (29). Regarding this connection, owing to their widespread expression in immune organs and immunocompetent cells, NTs are candidates for a prominent role in the regulation of immune and neuroimmune interactions.

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