Changes in the content of neuropeptides in intestinal lymph nodes of pigs suffering from experimental *Brachyspira hyodysenteriae* infection

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ABSTRACT: The studies were performed in order to investigate the mutual interrelationship of the peripheral nervous system and particular types and sub-types of lymphocytes located in the intestinal lymph nodes of the pig. Using the ELISA method and the flow cytometry the tissue concentration of VIP, SP, GAL and SOM, as well as the number of lymphocytes containing antigens CD2, CD21, CD4, CD5, CD8 and TCRgamma/delta were determined. As compared to 4-months old pigs of the control group, in 4-months old pigs in which experimental enteritis was induced with *Brachyspira hyodysenteriae* infection, a statistically significant increase in SP and GAL concentration was shown in the lymph nodes. No statistically significant differences in the concentration of VIP and SOM were detected. As regards changes in the lymphocyte subpopulations of CD21+, CD4+/CD8+ and TCRgd+/CD8– (regarded as a subpopulation of NK cells) lymphocytes, the mean frequency of CD21+ changed from 50.05% in control pigs to 25.82% in animals suffering from dysentery, the number of CD4+/CD8+ lymphocytes changed from 6.98% to 18.97%, and at the same period, the subpopulations of TCRgd+/CD8– lymphocytes changed from 17.76% to 0.38%.

Keywords: pig; neuropeptides; lymphocytes; dysentery; intestinal lymph nodes

Numerous evidences suggest the existence of morphological and functional relations between the immune and nervous system.

Peripheral organs of the lymphatic system are innervated by autonomic and sensory neurons. Adrenergic nerve fibers reach lymph nodes together with blood vessels (Felten et al., 1984, 1985, 1992; Fink and Weihe, 1988; Weihe et al., 1991; Bellinger et al., 1992; Panuncio et al., 1999; Mignini et al., 2003). These fibers supply mainly regions of lymph nodes rich in lymphocytes T and are absent from regions rich in lymphocytes B (Felten et al., 1984). It was found that in nerve fibers also other biologically active substances are expressed, such as vasoactive intestinal polypeptide (VIP) (Fink and Weihe, 1988; Enzmann and Drossler, 1994; Martinez et al., 1996; Bellinger et al., 1997; Mignini et al., 2003), substance P (SP), calcitonin gene-related peptide (CGRP) (Fink and Weihe, 1988; Popper et al., 1988; Bellinger et al., 1990; Kurkowski et al., 1990; Weihe et al., 1991; Felten et al., 1992; Enzmann and Drossler, 1994) and neuropeptide NPY (Felten et al., 1985; Fink and Weihe, 1988; Weihe et al., 1991; Romano et al., 1994; Mignini et al., 2003). Among above mentioned lymph nodes of the man and different mammalian species (mouse, guinea pig, pig, cat, dog) a specific class of mesenteric lymph nodes exists (Popper et al., 1988; Bellinger et al., 1997; Rogausch et al., 2004). It was found that also other lymphatic tissues possess similar nerve

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supply. In the lymphatic tissue associated with the jejunum and ileum, catecholaminergic, cholinegic and peptidergic (SP-positive, VIP-positive) nerve fibres were found in the pig (Kulkarni-Narla et al., 1999) and sheep (Chiochcetti et al., 2008). Evidence was provided on the existence of tight morphological link between nerve terminals located in lymph nodes and other collections of lymphatic tissues and cells of the immune system such as lymphocytes or macrophages (Fink and Weihe, 1988; Felten et al., 1992; Romano et al., 1994). The next evidence on the existence of the association between the nerve and immune system are results of studies documenting the existence of receptors in cells of the immune system which are activated by neurotransmitters and/or neuromodulators released from nerve terminals: NPY (Wheway et al., 2005), SP, CGRP, VIP, SOM (Popper et al., 1988) and NA (Bellinger et al., 1992; Mignini et al., 2003).

It remained to be elucidated what is the role of nerve fibres localized in the lymphatic tissue. It is suggested that NPY has a significant effect on the functions of the lymphatic tissue (Wheway et al., 2005), that NA regulates the activity of cells of the immune system (Nance and Sanders, 2007), however (with some exceptions) the sympathetic nervous system is believed to exert an immunosupressive influence (del Rey and Besedovsky, 2008). Additionally, there is a possibility that SP and VIP are involved in the mechanism of sensitization of immune cells in the lymphatic tissue (Enzmann and Drossler, 1994), and VIP and PACAP specifically modulate the expression of different cytokines (Martinez et al., 1996). What more, evidences suggest that there is a strong psychoneurological influence on functions of the immune system (Weihe et al., 1991; Panuncio et al., 1999; Sloan et al., 2008). These findings open new questions on the role of biologically active substances being neurotransmitters or neuromodulators in pathological processes. In the end of XX century, it was proven that SP modulates inflammatory processes and lymphocyte proliferation (Felten et al., 1992), and the sympathetic as well as peptidergic innervation is involved in the processes of the development and progression of many autoimmune diseases (Bellinger et al., 1992), allergic disorders and inflammatory pain states (Weihe et al., 1991). Recently, very convincing evidences were provided that NPY and SP take part in the regulation of inflammatory states (Wheway et al., 2005; Straub et al., 2008) and that sympathetic nervous system takes part in the development and modulation of autoimmune lymphoprolipherative diseases (del Rey and Besedovsky, 2008). The optimal defense response of the host versus pathogens needs an efficient cross-talk between nervous and immune system (Bellinger et al., 2008). It was found that SOM and GAL present in the neurons of the enteric nervous system may influence the course of the experimental enteritis evoked by *Brachyspira hyodysenteriae* infection (Lakomy et al., 2005).

The literature data presented above showing the mutual relationship between the immune and nervous system in the course of diseases do not explain fully the mechanisms of this relation, but make a suitable basis for further studies aimed at solving this problem.

The present study investigated the relationship between tissue content of several neuropeptides (SP, VIP, SOM and GAL) and selected lymphocyte subpopulations expressing surface antigens CD2, CD21, CD4, CD5, CD8 and TCRgamma/delta in intestinal lymph nodes of pigs in which experimental enteritis was evoked by *Brachyspira hyodysenteriae* infection.

MATERIAL AND METHODS

The study was performed on 10 female pigs of the Large White Polish breed aged four months divided into two groups. Animals were purchased from the commercial fattening farm. Control group (n = 5) consisted of clinically healthy animals. Animals of the experimental group (n = 5) were kept in the animals room for one week. Next, they were infected per as with a *Brachyspira hyodysenteriae* bacterium cultured under anaerobic conditions on the culturing media (agar with sheep blood on Petri dish). Each animal received the content of two Petri dishes dispersed in phosphate buffer (PB; pH 7.4). First symptoms of the infection appeared in animals one week after the inoculation (partial anorexia, passage of soft feces and fever). One week later, the symptoms were easily visible (mucoid diarrhea with flecks of blood and mucus progressing to watery diarrhea). After several days, the feces were brown and contained flecks of fibrin and debris. Diarrheic pigs were dehydrated, profoundly weak, gaunt and emaciated. Animals at this stage were sacrificed.

All animals, control and experimental, were sacrificed according to the same procedure.

Animals were premedicated with propionylpromasine *i.m.* (0.4 mg/kg of body weight; Combelen,

Bayer, Germany), deeply anaesthetised with pentobarbital *i.v.* (25 mg/kg of body weight, Vetbutal, Biowet, Poland) and the abdominal cavity was opened. Ileocecal lymph nodes and part of the ileum adjacent to the colon were excised and placed on ice. The excised segment of the ileum was opened and the lymphatic plate was identified. The lymph node was cut by half with a scalpel. For neuropeptide assays, the samples of the lymph node and ileum with the lymphatic plate (ca. 200 mg) were excised, weighted, wrapped in Parafilm and alufoil and snap-frozen in liquid nitrogen until processed. For flow cytometry studies, the cut half of the lymph node was put flat on the cooled Petri dish with the surface of cut looking up and the stroma of the node was repeatedly chopped with scalpel. Then the tissue was placed in 1.5 ml of ice-cold PBS. The mucosa was scraped and chopped simultaneously. The scraped mucosa was placed in 1.5 ml of ice-cold PBS. The chopped tissues were shaken for 1 min in PBS, allowed to sediment for 2 min and the suspensions were removed. The "extraction" was repeated with 1.5 ml of ice-cold PBS. The pooled suspensions were filtered through polyester wool using 2 ml disposable syringes. Then the concentrations of lymphocytes were established in a haemocytometer.

For flow cytometry assays, the suspensions were prepared containing 10^6 lymphocytes in 50 µl of PBS. Fifty µl of suspension were placed in seven 2 ml Eppendorf tubes marked A-G. Appropriate antibodies (primary and secondary) were added to tubes and suspensions were incubated for 30 min at room temperature (RT). The data of primary antibodies used are shown in Table 1. The data of secondary antibodies used are shown in Table 2. The sequence of antibodies used is shown in Table 2. Two ml of PBS were added to each tube, mixed gently and centrifuged for 5 min at 1 200 rpm. The supernatant was removed and the sediment was dispersed with pipetting. To tubes A, B and C, 300 µl of 1% formaldehyde was added and the suspension was gently mixed and set aside. To tubes D-G, appropriate primary and secondary antibodies were added and tubes were incubated for 30 min at RT (Table 3). To all tubes, 2 ml of PBS was added, gently mixed and tubes were centrifuged for 5 min at 1 200 rmp. The supernatant was removed, the sediment was resuspended in the remaining supernatant and 300 µl of 1% formaldehyde was added and gently mixed. Tubes were analysed in a flow cytometer (Beckton Dickinson FACScalibur) and the data were analysed with Cell Quest (Beckton Dickinson).

For neuropeptide assays, samples were taken out of the liquid N_2 and homogenized with a homogenizer (UltraTurrax, Germany) in 0.5M acetic acid at 4°C and placed in boiling water bath for 10 min.

Table 1. Data of primary antibodies. All antibodies were mouse monoclonal antibodies purchased from VMRD Inc.

No.	Antigen	Antibody	Category No.
P1	CD2	IgG2a	MSA4
P2	CD4	IgG2b	74-12-4
P3	CD5	IgG1	PG114A
P4	CD8	IgG2a	76-2-11
P5	CD21	IgG1	BB6-11C9
P6	TCRγδ	IgG1	86D

Table 2. Data of secondary antibodies. Secondary antibodies and streptavidin conjugated with phycoerithrine (PE) were purchased from Pharmingen. All antibodies were anti-mouse antibodies

Symbol	Antigen or ligand	Marker	Category No.
S1	IgG1	PE	550083
S2	IgG2a	FITC	553390
S3	IgG2b	biotin	550333
S-PE	biotin	PE	554061

Table 3. Procedure used for staining CD antigens in lymphocyte suspensions

A	B	C	D	E	F	G
negative control	positive control	positive control	CD2/CD21	CD4/CD8	CD5/CD8	TCRγδ/CD8
lymphocyte suspension						
	1 µl S1	1 µl S2	1 µl P1 + 1 µl S2	1 µl P2 + 1 µl S3 + 1 ml S-PE	1 µl P3 + 1 µl S1	1 µl P6 + 1 µl S1
washing						
FA	FA	FA	1 µl P5 + 1 µl S1	1 µl P4 + 1 µl S3	1 µl P4 + 1 µl S3	1 µl P4 + 1 µl S3
END	END	END	washing	washing	washing	washing
			FA	FA	FA	FA
			END	END	END	END

After cooling on ice, the homogenates were centrifuged for 20 min at 10 000×g, the clear supernatant was collected and the pellet was re-extracted twice. The supernatants were pooled and lyophilized. The dried extracts were dissolved in 2 ml of water and stored frozen at –70°C. Tissue concentrations of VIP, SOM, SP and GAL were determined with ELISA tests using commercial kits (Peninsula Laboratories, USA) according to the manufacturer's instructions. Data of the ELISA kits are listed in Table 4. ELISA plates were read with a Dynex MRX (Dynex Technologies, USA) immunoplate reader equipped with a 450 nm filter. Ten-point standard curve was prepared and absorbancies were converted to peptide concentrations. The results were re-calculated for 1 g of fresh tissue.

Table 4. Enzyme immunoassay kits used (Peninsula Laboratories Inc.)

Substance	Code	Lot No.
VIP	S-1183 (EIAH-7161)	016535
GAL	S-1210 (EIAH-7100)	016537
SOM	S-1179 (EIAH-8001)	016538
SP	S-1180 (EIAH-7451)	016536

RESULTS

Results obtained with an ELISA kits (Figure 1) indicate that the content of GAL in lymph nodes of the experimental group raised 2-fold in comparison to control animals (Figure 1) and this difference was statistically highly significant. The level of SP in lymph nodes of experimental animals has risen also almost 2-fold and also this difference was statistically significant. No statistically significant differences were found between experimental and control animals as regards VIP and SOM.

Cytometric study

In the ileocecal lymph node, statistically significant differences regarded the number of CD21+, mCD4+/CD8+and TCRgd+/CD8– (regarded as a subpopulation of NK cells) lymphocytes (Figure 2). The mean frequency of CD21+ changed from 50.05% in control animals to 25.82% in animals suffering from dysentery. The number of CD4+/CD8+ lymphocytes changed from 6.98% to 18.97%. At the same time, the subpopulations of TCRgd+/CD8– lymphocytes changed from 17.76% to 0.38 %.

DISCUSSION

As it was found recently, socially inhibited individuals show increased vulnerability to viral infections and in the some time, the activity of the sympathetic nervous system is increased. Lymph nodes from Low Sociable animals showed much higher density of adrenergic innervation than lymph nodes from High Sociable animals (Sloan et al., 2008). This phenomenon may be explained by the fact of the existence of beta-adrenergic receptors in the cells of the immune system present, among others, in lymph nodes (Bellinger et al., 1992). Additionally, it was found that activation of the sympathetic system restrains the activity of cells associated with the innate immunity, and may affect (stimulate, or inhibit) the cells associated with the

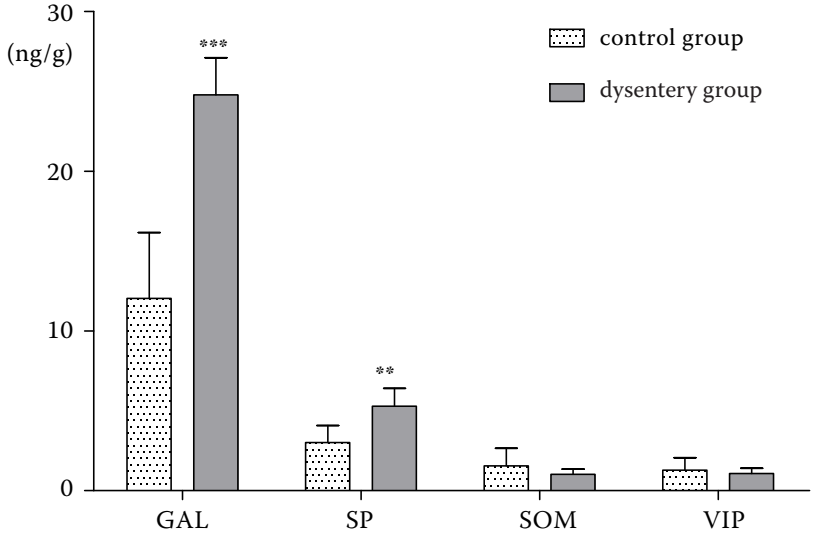

Figure 1. Tissue concentration of neuropeptides in intestine lyph nodes

acquired immunity response. It was documented that that the cells associated with innate immunity possess both alpha- and beta-adrenergic receptors. It is assumed that with these receptors, noradrenaline may regulate the level of the activity of immune system cells (Nance and Sanders, 2007).

As results from to-date studies on the porcine lymph nodes, also intestinal ones, they are supplied with adrenergic nerve fibers which contain also NPY and VIP, as well as with sensory nerve terminals which express SP and CGRP (Fink and Weihe, 1988). It may be stated that neuropeptides investigated in this paper (SP and VIP) are located in nerve fibers supplying the studied lymph nodes, and other considered in this paper neuropeptides (SOM and GAL) show similar localization.

It was found without doubts that in the course of some intestinal disorders, changes in tissue concentrations of some neuropeptides in intestinal wall may be detected. It was shown that, for example, the content of VIP decreases in Crohn's disease (Eysselein and Nast, 1991).

The unanimous opinion exist that VIP is the neuropeptide present in and released from both nerve terminals and cells of the immune system (especially lymphocytes T_{h2}). Moreover, cells of the immune system show the presence of VIP receptors. VIP exerts an inflammatory effect on the cells involved in both innate and acquired immunity and the mechanism of this action relies on the inhibition of the production and release of pro-inflammatory cytokines and chemokines (Delgado, 2004). Numerous studies showed that T helper cells of type 1 (T_{h1}) derived cytokine profile predominates at the induction and acute phase of the inflammatory disease, whereas T helper cells of type 2 (T_{h2})

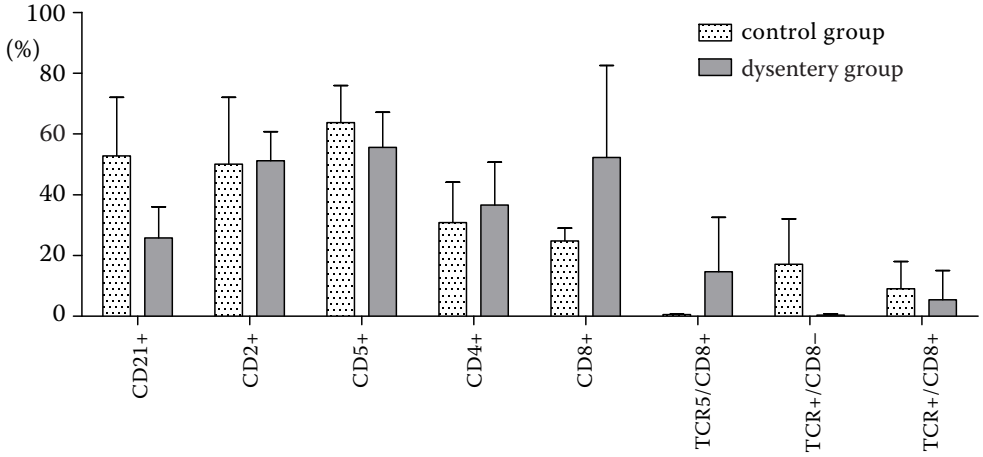

Figure 2. Sub-populations of lymphocytes in intestine lymph nodes

mediated response is associated with the remission phase of the inflammatory disease, what suggests a pathogenic role of T_{h1}-derived cytokines (Leceta et al., 2004). VIP preferentially induces differentiation toward a T_{h2} response following antigen stimulation.

Results of the present paper indicate that differences in the concentration of VIP in the porcine lymph nodes between control and experimental group are not statistically significant. Hence, these results do not confirm a substantial involvement of VIP in the modulation of the inflammation of intestines evoked by *B. hyodysenteriae* infection.

Another neuropeptide localized in the neurons supplying the intestinal lymph nodes is SP, which is localized in the sensory fibers and intrinsic enteric neurons of the gut. It seems conceivable that tachykinin receptor antagonists which mediate the action of SP can be employed as spasmolytic, antidiarrhoeal, antiinflammatory and antinociceptive drugs (Holzer, 1998). Results of other studies (Straub et al., 2008) indicating that the substance P has a pro-inflammatory effect in Crohn's disease, in which an increase in the number of SP-positive nerve fibers is observed, accompanied by a decrease in the number of sympathetic nerve fibers. A decrease in number of sympathetic nerve fibers having anti-inflammatory effect enables an increase in the number of SP-positive ones. An increase in the density of SP-positive fibers is observed also in ulcerative colitis in humans (Watanabe et al., 1997). Also other authors consider SP as a pro-inflammatory peptide (Qian et al., 2001).

In our studies, a statistically significant increase in SP tissue concentration was found in lymph nodes of the experimental pigs in comparison to the control animals. These results indicate the indisputable influence of this peptide on the modulation of the enteritis. This was the first time when it was shown that the inflammation of the intestinal wall induces changes in SP concentration also in the mesenteric lymph nodes which are probably supplied by the primary afferent sensory fibers, postganglionic sympathetic nerve fibers and the fibers from the enteric nervous system. In the light of the results of the mentioned studies and our results, it seems plausible that the tachykinin antagonists may be a new therapeutic tool in the treatment of enteric inflammations (Evangelista, 2001).

The tissue content of SOM in the mesenteric lymph nodes found in the present paper was remarkably lower in the experimental group in comparison to the control group, however, this difference was statistically insignificant. The studies on the participation or involvement of SOM in pathological processes in intestines are very scarce. In 1994, Reubi and co-workers (Reubi et al., 1994) showed that this neuropeptide play an active role in the pathophysiology of inflammatory states of the gut in humans (Crohn's disease, ulcerative colitis) in which they found an increase in the density of intramural receptors for SOM. In previous studies (Lakomy et. al., 2005), a significant increase in SOM content was shown in the ileum of pigs infected with *B. hyodysenteriae* in comparison to control animals. The results of the present study as well as the results of two studies cited above do not give the definite foundation for drawing conclusions, however, they show necessity of undertaking further studies to explain what role, if any, SOM plays in pathological processes, not only in guts.

Galanin belongs to the group of substances regulating the contractility of intestines, may modulate the transepithelial ion transport and the intestinal epithelial cells express Gal1 receptors (Benya et al., 1998). In the same paper, it was found that the expression of this receptor increases in the human colon undergoing inflammatory changes. The authors mentioned point to galanin as a next substance responsible for modulating intestinal inflammation. In the recent studies, where the experimental enteritis was evoked by *B. hyodysenteriae* inoculation it was found that galanin receptor concentration rose not only in the colonic wall, but also in the wall of the stomach and in the inferior mesenteric ganglion (IMG), coeliac-superior mesenteric ganglion (CSMG) and the dorsal root ganglia (DRG) (Lakomy et al., 2005). It suggests that the enteritis influences also peptide concentration in comparatively distant autonomic ganglia being the partial source of the intestinal innervation. A significant increase in galanin tissue concentration in the studied lymph nodes of the experimental animals in comparison to control ones shows that this peptide may be one of the substances most important for complex modulation of inflammatory states.

Statistically significant differences in the lymphocyte subpopulations were found only in subpopulation of CD21+ (lymphocytes B), CD4+/CD8+ (differentiating and maturing lymphocytes T), and TCRgamma/delta+/CD8– lymphocytes similar in their functions to "natural killers" (NK-like lymphocytes) (Murphy et al., 2007). However,

surprising phenomena occurred in case of CD21+ and TCRgamma/delta/CD8– lymphocytes. Their number dropped very significantly in animals sick of dysentery. The number of B lymphocytes dropped by half (from ca. 50% to ca. 25%). Taking into consideration the fact that these lymphocytes produce antibodies necessary for fighting infection, so significant drop in their number is quite astonishing. Similar phenomenon occurred in case of NK-like lymphocytes. Their number dropped dramatically from ca 18% to less than 1%. The same time, the number of mature T lymphocytes (cytotoxic and helper) did not rise significantly. The only rise was found in the sub-class of CD4+/CD8+ cells which are in the process of differentiation and did not make decision about their final phenotype. It is quite tempting to speculate that the surprising changes in lymphocyte subpopulations reflect some kind of the immune system collapse when it is unable to fight the infection efficiently. This is somehow backed by the data on the neuropeptide concentrations, where changes were found only in case of GAL and SP. GAL in the pig is exprimed by nerve cells, also of the enteric nervous system, in response to noxious stimuli, including inflammation. However, GAL doesn't influence proliferation of lymphocytes, what more, it can have anti-proliferative activity by inducing apoptosis of differentiating lymphatic cells (Trejter et al., 2002). SP is heavily involved in the development of inflammation by triggering a neurogenic mechanism and even some bacterial alimentary infections use this path in the development of enteritis (Mantyh et al., 1996, 2000). On the other hand, no rise in the concentration of SOM and VIP was found in the lymph nodes. These neuropeptides are regarded to have a strong anti-inflammatory factors (Delgado et al., 2002; Paran and Paran, 2003) and VIP has a strong lymphocyte stimulating activity, what can in part explain its anti-inflammatory activity. The rise in GAL concentration and a lack of VIP increase can partly explain the drop in the number of B and NK-like lymphocytes. The animals were sacrificed when the experimental enteritis was well developed and their clinical state was very serious. It may be that these animals would die of dysenteria if they were not euthanasized and used for our studies. It is possible that the changes in neuropeptide tissue concentrations and lymphocyte subpopulations reflect the state of the terminal illness associated with an immune system collapse leading to death of infected organisms.

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