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Characterization of Autonomic Nerve Markers and Lymphocyte Subsets in the Ileal Peyer's Patch of Pigs Infected Experimentally with *Brachyspira hyodysenteriae*

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Summary

The aim of the present study was to investigate potential interrelationships between immune and neural elements of Peyer's patches in normal pigs ($n = 8$) and in pigs infected experimentally with *Brachyspira hyodysenteriae* and suffering from swine dysentery ($n = 8$). Assessment of tissue concentration of neuropeptides by enzyme linked immunosorbent assay revealed increased levels of galanin (GAL) and substance P (SP) in samples from the infected animals. In contrast, concentrations of vasoactive intestinal polypeptide (VIP) and somatostatin (SOM) were similar in both groups. Immunohistochemistry demonstrated reactivity of nerve fibres with antibodies specific for dopamine β hydroxylase, vesicular acetylcholine transporter, SOM, GAL, VIP and SP in the interfollicular region and peripheral areas of the Peyer's patch lymphoid follicles. In the dysenteric pigs, the GAL-positive nerve fibres were more numerous and more intensely labelled than those in the normal animals. Flow cytometry revealed a decreased percentage of $CD21^+$ lymphocytes and lymphocytes expressing T-cell receptor (TCR)-γ, with or without CD8 ($TCR\text{-}\gamma^+CD8^-$ and $TCR\text{-}\gamma^+CD8^+$), in the dysenteric pigs as compared with the normal animals. Percentages of other lymphocyte subsets ($CD2^+$, $CD4^+$, $CD5^+$, $CD8^+$, $CD5^-CD8^+$) were comparable between the groups. Immunohistochemical investigations generally correlated with results obtained by flow cytometry related to lymphocyte subpopulations. Swine dysentery can therefore affect neuroimmunomodulatory processes in the ileal Peyer's patch, in addition to the large intestine. GAL and SP may play a specific role in this neuroimmune cross-talk.

Keywords: autonomic nerve markers; ileal Peyer's patch; lymphocyte subsets; pig; swine dysentery

Introduction

There is a growing body of evidence suggesting the existence of functional interconnections between the immune and nervous systems, although the data available are frequently incomplete and not always focussed on their relevance for different physiological and pathological states (Mignini *et al*., 2003; Nance and Sanders, 2007). Primary (i.e. bone marrow and thymus) and secondary (i.e. spleen and lymph nodes) lymphoid organs are supplied with an efferent autonomic and afferent sensory innervation. Lymph nodes of various somatic and visceral regions are supplied with nerve fibres that have been shown by immunohistochemistry (IHC) to express noradrenaline, acetylcholine, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP) and substance P (SP). These neurotransmitter substances can alter immune responses as immune cells may express specific receptors able to bind them. Alternatively, changes in immune function

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can modify the distribution of nerves and expression of neural receptors in lymphoid organs (Mignini *et al*., 2003; Rosas-Ballina and Tracey, 2009).

Recent studies have revealed profound changes in the number of enteric neurons expressing some neuropeptides and the vesicular acetylcholine transporter (VAChT; marker of cholinergic nerve structures) supplying the stomach and small and large intestine in pigs affected by swine dysentery and adenomatosis (Sienkiewicz *et al*., 2006; Kaleczyc *et al*., 2007; Pidsudko *et al*., 2008). The tissue concentration of several neuropeptides is also altered in such infectious diseases (Lakomy *et al*., 2005). There is some evidence that such adaptations occur not only to help enteric neurons to survive under pathological conditions, but also to help the inflamed part of the gastrointestinal tract to recover (Ekblad and Bauer, 2004; Vasina *et al*., 2006).

The gut-associated lymphoid tissues include Peyer's patches, mesenteric lymph nodes and a large number of lymphoid cells distributed in the epithelium and lamina propria of the intestinal mucosa. Immunohistochemical studies have characterized subpopulations of lymphocytes that express diverging cell surface molecules according to the lymphoid tissue being considered (LeBien and Tedder, 2008; Gerner *et al*., 2009). Porcine lymphocytes have been well defined. There are marked changes in the intestinal immune system of the pig after weaning, with alterations in the numbers of $CD4^+$, $CD8^+$, double positive $CD4^+CD8^+$, $CD21^+$, T-cell receptor (TCR)-γ^+, $SWC3^+$ and SLA-DQ^+ cells (Solano-Aguilar *et al*., 2001). Significant differences have been also found in porcine circulating and intestinal lymphocyte subpopulations before and after experimentally induced swine dysentery or following parenteral immunization of pigs with a pepsin-digested *Serpulina hyodysenteriae* bacterin (Waters *et al*., 1999a,b; Jonasson *et al*., 2004; Hontecillas *et al*., 2005). However, no information is available on such changes within Peyer's patches. Although swine dysentery is commonly believed to affect the stomach and large intestine, recent studies have revealed profound changes in the chemical coding of enteric neurons not only in these parts of the gastrointestinal tract, but also in the ileum of dysenteric pigs. Porcine jejunal and ileal Peyer's patches are known to possess well developed, specifically arranged adrenergic, cholinergic and peptidergic innervation (Kulkarni-Narla *et al*., 1999; Vulchanova *et al*., 2007) and morphological associations between the nerve fibres and immune cells have been described (Vulchanova *et al*., 2007).

The aim of the present study was to investigate potential interrelationships between some immune and neural elements of the ileal Peyer's patch in normal pigs and in pigs suffering from swine dysentery.

Materials and Methods

Animals and Experimental Procedure

The study was carried out on 16 female pigs (aged 4 months and of approximate body weight 40 kg) of the large white Polish breed obtained from a commercial fattening farm in Purda, Poland. The animals were housed and treated in accordance with the rules of the local Ethics Commission (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education).

The pigs were assigned into two groups. Group 1 ($n = 8$) consisted of healthy animals, which served as controls. Group 2 ($n = 8$) comprised healthy pigs, which were inoculated intragastrically with a mixture of two strains of *Brachyspira hyodysenteriae* (the reference B78 strain and the wild 'Siarczyna' strain isolated from the faeces of dysenteric pigs). Both strains were cultured in the Department of Swine Diseases, National Veterinary Research Institute, Pulawy, Poland as described by Kaleczyc *et al*. (2007). The control pigs were given phosphate buffer instead of the inoculum.

Ten to 14 days post-infection (dpi), classical signs of the disease were observed in the infected animals. The pigs were killed 4–7 days after the first clinical signs appeared (14–18 dpi). At this time the infected pigs had a gaunt appearance with sunken eyes and were weakened and dehydrated due to haemorrhagic diarrhoea. The pigs were pretreated with azaperone (Stresnil; Janssen Animal Health BVBA, Belgium; 2 mg/kg body weight) by intramuscular injection and 30 min later were given thiopental sodium (thiopental; Biochemie, GmbH, Austria; 20–30 mg/kg body weight). Three animals from each group were then perfused transcardially with 1 litre of pre-perfusion solution containing 0.9% sodium chloride (Chemia, Gliwice, Poland), 2.5% polyvinylpyrrolidone (Sigma, Deisenhofen, Germany), 0.5 procaine hydrochloride (Polfa, Warsaw, Poland) and 20,000 IU of heparin (Heparinum; Polfa, Warsaw, Poland; added *ex tempore*), followed by 4 l of 4% ice-cold buffered paraformaldehyde (pH 7.4). Tissue samples were then collected from the ileum and Peyer's patch. Tissues were post-fixed by immersion in the same fixative for 20 min, rinsed with phosphate buffer (pH 7.4) and transferred to and stored in 18% buffered sucrose solution (pH 7.4) until processing.

Tissue samples for flow cytometric assessment of lymphocyte subsets and determination of tissue