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The immunohistochemical localization of the glycosphingolipid asialo-GM1 in the intestine of weaned piglets

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ABSTRACT

The duodenum, jejunum, ileum, cecum and colon of three male hybrid piglets, 4 weeks old just after weaning, were investigated for the immunohistochemical localization of the asialoganglioside, GM1 (asialo-GM1). The study revealed various degrees of labelling for this acid glycosphingolipid in neural, epithelial and blood elements in all the gut segments. The immunolabelled neural structures, represented by ganglionic perikarya and nerve fibers, were distributed throughout the intestinal wall and showed quantitative variations in the various regions. In contrast the numerical evaluation of labelled epithelial cells was encountered only in the terminal jejunum and along the entire ileum, cecum and large intestine. In addition, a heterogenous population of immunolabelled leukocytes was spread randomly in the lamina propria and submucosa of the entire intestine and did not show any apparent quantitative fluctuations between the different parts. The observations regarding the typical distribution patterns of the asialoganglioside GM1 in ganglionic perikarya and epithelial cells of weaned piglets are discussed in relation to their possible functional significance in the intestine and other mammalian organs.

Introduction

The asialoganglioside GM1 (asialo-GM1), a glycosphingolipid of about 88,000 Da, was originally isolated from the bovine brain by gel filtration chromatography and SDS-PAGE electrophoresis (US Patent 5.275.939). Since its production in 1994 until now, this glycosphingolipid, which contains in its molecular structure neuraminic acid, has been firmly associated with the outer lipidic plasmalemma of neurons and also with the plasmalemma of epithelial cells. In malignant tumors, in comparison with corresponding healthy tissue, there is a different and much more abundant expression of asialo-GM1. It has been proposed that asialo-GM1 may play an active role in the abnormal "behavior" of malignant cells, and this has led to the adoption of several anti-tumoral therapies (Livingston et al., 1989; Dumontet and Portoukalian, 1991).

Although the role of gangliosides still remains doubtful, several biochemical and immunological studies have led to the proposal of their involvement as "receptors" for specific viruses (Karupiah and Blanden, 1990; Suzuki et al., 1992), or bacterial toxins (Saiman and Prince, 1993; MacKenzie et al., 1997). Other activities ascribed to the acid glycosphingolipids include their involvement in the modulation of mitosis in thymocytes (Spiegel et al., 1985), in the proliferation of circulating T lymphocytes (Welte et al., 1987) and also in other cellular proliferations (Kim et al., 2008), including the solid tumors (Berra et al., 1997; Kawashima et al., 2003). In addition, it has been shown that they may possibly play a role as cellular markers of tumors (Fredman, 1989; Miyake et al., 1990), in particular for melanoma and neuroblastoma (Dumontet and Portoukalian, 1991; Ruan and Lloyd, 1992). It has also been suggested that they may be associated with depression of the immune response of the host organism against the development of these neuroectodermal tumors. In this way the self-maintenance and tumoral development are facilitated (Dumontet and Portoukalian, 1991). There is also some experimental evidence indicating the existence of an inverse correlation between the expression of diverse gangliosides in the tissues of a host animal and their immunogenicity (Livingston et al., 1989; Dumontet and Portoukalian, 1991). This correlation has resulted in the development of passive immunotherapies (with monoclonal antibodies against the diverse asialogangliosides) and active immunotherapies (inoculations with gangliosides); however, little is known about how they are effective or their modes of mechanism and such treatment is still regarded as controversial and unproven (Kawashima et al., 1990; Reisfeld, 1992; Grayson and Ladisch, 1992).

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Considering the above history and in view of the difficulties in highlighting an antigen of glycolipidic nature such as the asialo-GM1, we considered it useful to undertake the present immunohistological study on asialo-GM1 in the absence of any information regarding its localization along the porcine intestinal wall. It has been claimed that the asialogangliosides GM1 and GM2 located in the pharyngeal and middle ear epithelial cells may serve as adhesion molecules for pathogenic bacteria (Ahmed et al., 2002) and it was considered highly possible that our study could reveal some details on their mechanisms of action.

Our study has demonstrated for the first time the immunolocalization of asialo-GM1 in the intestine of piglets and these observations might explain the multiple histological alterations of a toxic nature reported even after the controlled short-time ingestion of aflatoxin B1 (Trandaburu et al., 2003) and fumonisin B1 (Trandaburu et al., 2004).

Material and methods

Animals

The study was performed according to the French National Guidelines for the care and use of animals for research purposes. A total of three healthy male (Dalan X France) hybrid piglets 4 weeks old, were used in the study. They were purchased from a local commercial source at 3 weeks of age, just after weaning, and were further acclimatized for 1 week in the animal care facilities of the INRA Laboratory of Pharmacology and Toxicology at Toulouse, France. During acclimatization the animals had free access to water and were fed with a commercial starter diet as previously described (Bouhet et al., 2006).

Tissue preparation

The piglets were killed by electrical stunning and equal 1-cm-long fragments from the duodenum, jejunum, ileum, cecum and large intestine were processed for immunohistochemical investigations. The tissues were immersion fixed in Bouin's solution for 60 h, dehydrated in increasing concentrations of ethanol, cleared with toluene and embedded in paraffin wax. A series of 6-μm-thick sections were cut from each fragment, spaced out at 2 mm intervals, using a rotary microtome (Reichert, Austria) and were mounted on poly-L-lysine (Sigma-Aldrich, St. Louis, Mo, USA) or gelatin-coated glass slides.

Primary antibody

The primary antibody, monoclonal mouse IgM anti-asialo GM1 (clone AG-1), was raised against gangliotetraosilceramide ($GgOse_4Cer$) and not against any other glycosphingolipids (GSLs) (Watari et al., 1987), and was purchased from Seikagaku Corporation (Tokyo, Japan).

Immunohistochemical protocol

Sections were deparaffinized, rehydrated and immunolabelled for the binding of $GgOse_4Cer$ using a streptavidin–biotin immunostaining kit (K9011P, lot no.: 30547j, American Qualex Manufacturers, San Clemente, CA, USA). Briefly, the procedure included three consecutive incubations of the sections at room temperature with (1) the primary antibody diluted 1:100 overnight, (2) biotinylated goat anti-mouse IgM properly diluted for 30 min and then (3) streptavidin–biotin–horseradish peroxidase complex also properly diluted for 20 min. A solution of 10 mM phosphate buffered saline (PBS, pH 7.4) was used as diluent for the three main steps of the procedure and as rinsing solution between these steps. The same buffer was used also for the dilution of goat blocking serum, in which the sections were incubated for 20 min. The sections were finally dehydrated through an ascending series of ethanol, cleared with xylene and mounted in Entellan (E. Merck, Darmstadt, Germany).

The specificity of the immunohistochemical procedure was confirmed by incubation of sections with non-immune goat serum (blocking serum) instead of the primary antibody.

Semi-quantitative evaluations

Transverse sections through each processed gut fragment were examined and at least seven of them, separated from each other by approximately 2 mm, were photographed using a Carl Zeiss (Jena, Germany) microscope with Azopan IS-100 monochrome film. Images, representing adjacent and overlapping intestinal sectors, were taken at the same magnification (3.2×25) and arranged to construct "photomaps" of the entire cross-section. The average numbers of immunolabelled ganglionic perikarya and also of the nerve fibers from the reconstructed "photomaps" were considered to be those of marked neural structures. The occurrence and distribution of both ganglionic perikarya and nerve fibers along the intestine of the three piglets under study were classified in one of the following categories: (very numerous, ++++), >30; (numerous, +++), 15–29; (moderate, ++), 5–14; (a few, +), 1–4.

Results

The asialo-GM1 immunolabelled structures encountered along the gut wall of the piglets were derived from various embryological origins. The densely immunostained ganglionic perikarya and their extensions, were detected in both the superficial (Auerbach) and the deep (Meissner) myenteric plexuses and occurred with uneven distribution along the entire length of the intestine. The labelled epithelial cells, of endodermal origin, were only found in the distal portion of jejunum and along the entire ileum, cecum and large intestine.

With regard to the immune labelled cells of mesodermal origin, most of them were represented by various categories of leukocytes localized in the lamina propria and in the submucosa. The number of these labelled leukocytes did not appear to exhibit significant quantitative changes along the gut segments. In the submucosa of the ileum in all the piglets, the developing lymphatic nodules and their diffuse infiltration with labelled lymphocytes possibly signal the chronic inflammatory reaction of the animals after birth.

Ganglionic perikarya and nerve fibers

The labelled neural structures encountered along the gut wall showed not only semi-quantitative variations (Table 1), but also intracellular peculiarities of asialo-GM1 distribution within the two distinct categories of ganglionic perikarya. Thus, the ganglioside labelled in most of the cases exclusively the neuroplasm of both superficial (Auerbach) and deep (Meissner) ganglionic perikarya (Fig. 1a, b), in far fewer cases, especially in the ones revealing single perikarya in submucosa, the immunolabelling covered the entire neurons, including their nuclei (Fig. 1c).

Unlike the ganglionic perikarya, their asialo-GM1 positive extensions detected in the superficial myenteric plexus, were

Table 1
The semi-quantitative distribution of immunolabelling for asialo-GM1 in ganglionic perikarya and their extensions along the intestinal wall of piglets.

Immunoreactive neural elements	Duodenum	Jejunum	Ileum	Large intestine
Superficial myenteric nerve plexus				
Ganglionic perikarya	++	+–++	+–++	+
Nerve fibers and terminals	++–+++	+++	++–+++	++
Deep myenteric nerve plexus				
Ganglionic perikarya	+++	++–+++	++–+++	+–++
Nerve fibers and terminals	++++	++++	+++–++++	+++

Symbols (number of immunostained neural structures/gut cross-section): ++++, >30; +++, 15–29; ++, 5–14; +, 1–4.

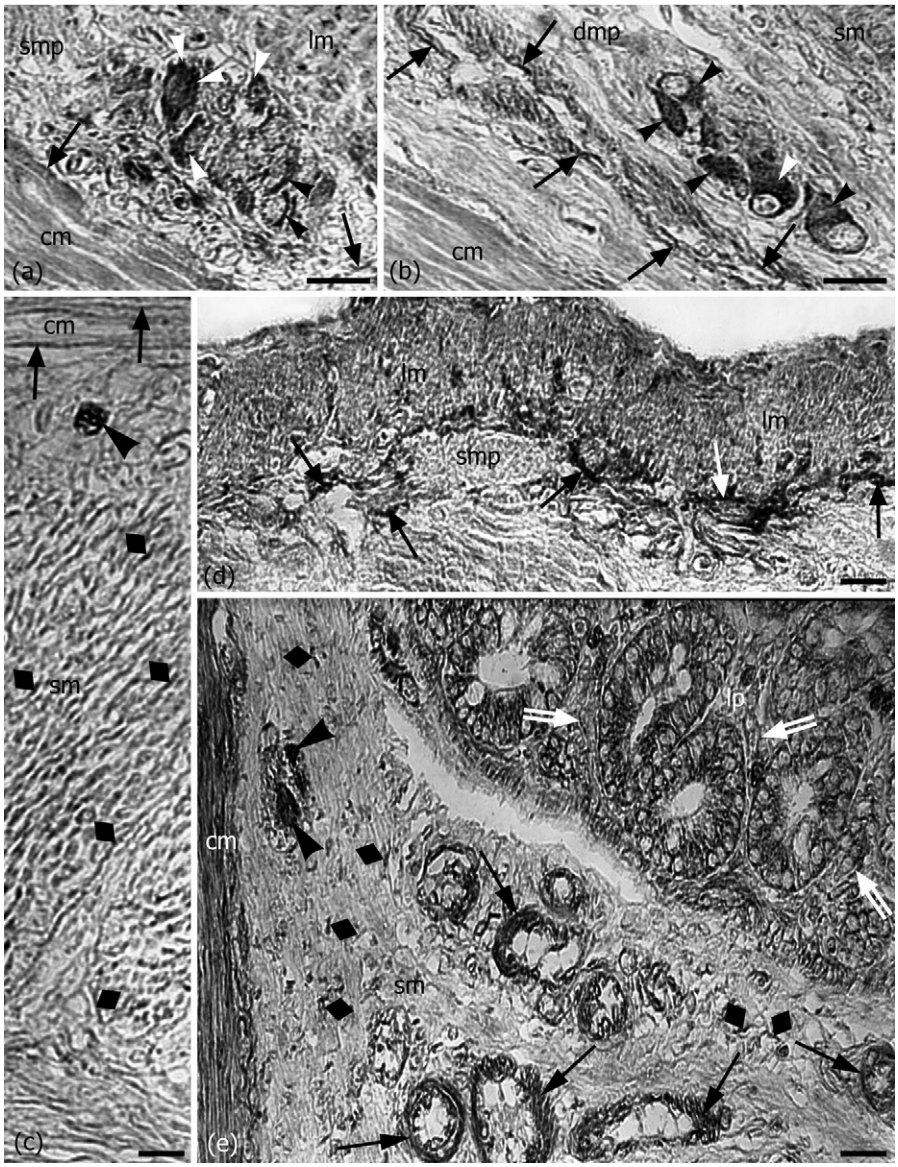

Fig. 1. Neural elements immunopositive for asialo-GM1 detected in the (a) duodenum (b) jejunum (c, e) ileum and (d) the ascending colon of the weaned piglets. The neuroplasm (small arrowheads) of both (a) superficial and (b) deep ganglionic perikarya appears strongly immunostained. In a few cases, immunolabelling of (c) single or (e) grouped perikarya in the submucosa (sm), stains entirely the profiles of the neurons (arrowheads). Both longitudinal (lm) and circular (cm) muscle layers of the gut wall, but also the superficial (smp) and deep (dmp) myenteric nervous plexuses display a few, strongly immunostained nerve fibers (small arrows). In the submucosa varicose, fine nerve profiles (small rhombs) and also thicker nerve profiles surrounding the blood and lymphatic vessels (arrows) can be seen. Scale bars (a–e) represent 20 μm.

relatively scarce, but strongly immunostained (Fig. 1a). In contrast, there were abundant free nervous profiles from the submucosal plexus, mostly short or varicose (Fig. 1b–d), but also of the thick ones delineating the lymphatic and blood vessels, which always appeared densely immunolabelled (Fig. 1e).

The results of our semi-quantitative evaluations of the ganglionic perikarya and their nerve fibers recorded along all the intestinal segments are presented in Table 1. They showed a general decreasing trend of both categories of myenteric neurons (superficial and deep), in spite of a relative uniform distribution of

their extensions, particularly of those that were much more abundant in the submucosal plexus.

Finally, the presence of less dense immunostained nerve profiles in the submucosa and in the lamina propria and also their parallel arrangement with the long axes of villi should be recorded (as illustrated in Fig. 2a–c, e).

Epithelial cells

The epithelial cells immunolabelled for asialo-GM1 from the distal portion of jejunum and along the entire ileum, large intestine and even the cecum of weaned piglets, exhibited a common intracellular distribution of this ganglioside. Thus, apart from the completely non-reactive mucous cells, in these intestinal segments the asialo-GM1 labelling always strongly marked the basolateral membrane of epithelial cells (Fig. 2a–c, e). The cytoplasm of these cells was also stained for asialo-GM1, although only with a slight staining (Fig. 2a–e). From these observations, it was difficult to quantify, even on a semi-quantitative basis, the labelling of epithelial cells along the investigated gut segments.

Blood cells

A relative rich and very heterogeneous population of labelled blood cells, spread fairly randomly in the submucosa, lamina propria and also in the axes of villi of the investigated intestinal segments could be observed. Although their morphological affiliation was not determined, it is highly probable that this heterogeneous population of asialo-GM1-positive cells would include segmented neutrophils and eosinophils, small and large lymphocytes and very numerous monocytes (Fig. 3a–d). It is important to emphasize that the membrane markers of blood cells are not identical among the rodents, pigs and humans. As already described, the population of asialo-GM1 immunoreactive leukocytes did not seem to show quantitative fluctuations along the intestinal fragments under study.

Discussion

As far as we are aware, this study is the first to report asialo-GM1 immunolabelling of neural, epithelial and blood elements in the intestinal wall of disease-free piglets (absence of mycotoxins).

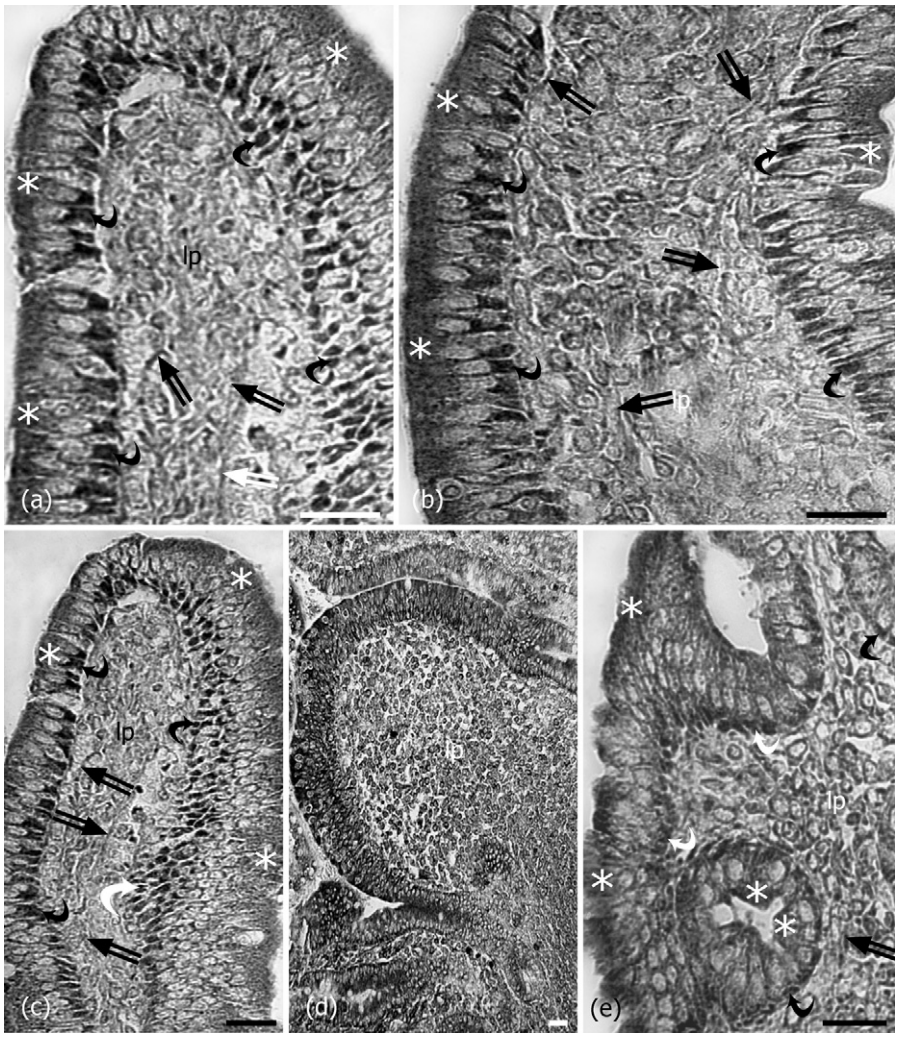

Fig. 2. Micrographs showing the distribution of immunolocalization for asialo GM1 within the epithelial cells from the distal region of the following: (a) jejunum (b) ileum (c) ascending colon (d) descending colon and (e) cecum of weaned piglets. In micrographs (a–c, e), the membrane of microvilli (small asterisks) and the basolateral membrane (small arrows with incurved tail) of epithelial cells show densely immunolabelling. Note also the slender and somewhat less immunostained nerve profiles in the lamina propria (lp) running in parallel with the long axes of villi (double small arrows). Scale bars (a–e) represent 20 μm.

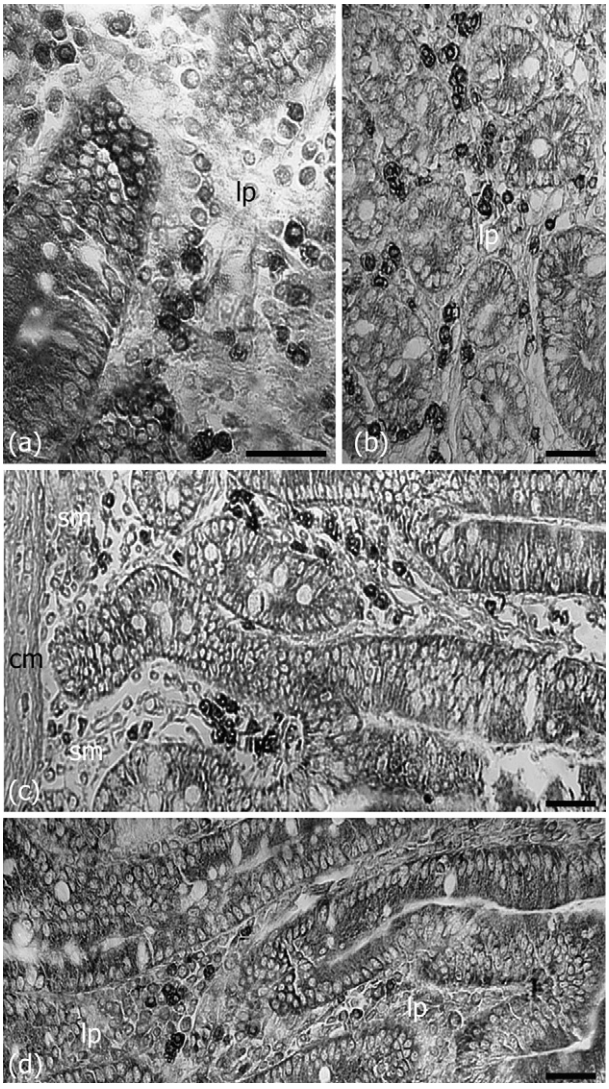

Fig. 3. Micrographs of asialo-GM1 labelled blood cells (a–d) and their distribution in the lamina propria (lp) and submucosa of the duodenum (d), jejunum (c) ileum (a) and large intestine (b) of weaned piglets. Scale bars (a–d) represent 20 μm.

With regard to mammals and mammalian organs, only the small intestine of mouse (Umesaki, 1984) and adult rat liver (Enzan et al., 1991) have been studied for the immunohistochemical localization of this glycosphingolipid. The detection of asialo-GM1 described in this paper illustrates the immunoreactive structures for this ganglioside in the intestinal wall, thus contributing, even indirectly, to a more detailed knowledge of the molecular mechanisms in normal, non-pathological conditions (Weiser et al., 1978; Umesaki et al., 1982). Infections induced by micro-organisms (Suzuki et al., 1992; MacKenzie et al., 1997; Bouhet and Oswald, 2005) in the gut have been studied. The lack of information on the immunohistochemical localization of this glycosphingolipid in the gut or in any other organs of birds and poikilotherm vertebrates, and also the methodological difficulties in revealing an antigen of glycolipidic nature such as asialo-GM1, considerably increases the interest in such a research approach.

The presence of labelled ganglionic perikarya and their extensions, such as those detected in this study, is not an isolated case among mammals. There are several reports which have shown the association of anti-asialo-GM1 antibodies with the plasmalemma of neurons of the central and peripheral nervous system (O'Hanlon et al., 1996) in autoimmune neuropathies (Willison, 2002) and in various lysosomal storage diseases (Prieur et al., 1991; Murnane et al., 1991). Among the biochemical significance of the above specific association there is a correlation with ubiquitin 1 expression in human neuroblastoma cells and rat cortical neurons (Liu et al., 2006), with the distribution of immunoreactive epitopes in the human peripheral nervous system (O'Hanlon et al., 1998), with the prevention of guanethidine-induced sympathectomy in athymic rats (Thygesen et al., 1992) and also with those neurologic maladies provoked by anti-GM1 antibodies (Gregson et al., 1991; Weng et al., 1992).

The epithelial cells spread from the distal portion of jejunum up to the terminal end of the large intestine and also along the cecum represent another category of asialo-GM1 immunolabelled elements. The immunostaining pattern of these cells, which includes the basolateral membrane and to some extent the membrane of microvilli, generally appeared similar to that described previously in mice (Suzuki and Yamakawa, 1981; Umesaki, 1984). Although at present the physiological significance of the labelling of epithelial cells has still not been clarified, some authors consider that the asialoganglioside may take part in the cell–cell or cell–basal lamina interaction or may be receptors for the colonization factors of the gut with pathogenic or non-pathogenic micro-organisms (Leffler and Eden, 1981). Yamada et al. (1981) have postulated that glycolipids, such as gangliosides, may be receptors for fibronectin, whereas recently Yoneshige et al. (2009) have ascribed an important role to the interrelation between the developmental changes in the composition of glycolipids and synchronized expression of nutrient transporters in the mouse small intestine during suckling-to-weanling transition.

Finally, with regard to the occurrence of a large variety of labelled blood cells in the intestine of weaned piglets, there is considerable biochemical information confirming the presence of asialogangliosides, not only along the gut wall (Alberti et al., 1985; Umesaki et al., 1995; Kawamoto et al., 1996), but also in other rodent (Enzan et al., 1991; Yanagi et al., 2003; Moore et al., 2008) or human (Leffler and Eden, 1981) organs. Concerning pigs, an update on porcine T lymphocytes and NK cells has recently been published (Gerner et al., 2009). According to Enzan et al. (1991), almost all the monocytes, segmented neutrophils and eosinophils, small or large lymphocytes in the sinusoids and the mast cells in Glisson's sheath in the adult rat liver were positive for asialo-GM1. Alberti et al. (1985) emphasized the morphological differences between the large granular lymphocytes obtained from murine blood and intestinal epithelium, Umesaki et al. (1995) noted the role of indigenous intestinal bacteria in activating intraepithelial mouse lymphocytes. Nohara et al. (1994) proposed the role of glycosphingolipids of rat T cells expressing predominantly asialo-GM1 in the immune response.

The above data, and also that regarding the rapid killing of murine lymph node T cells by intestinal intraepithelial lymphocytes (Kawamoto et al., 1996) and the differential regulation of GM1 and asialo-GM1 expression by T cells and natural killer cells in respiratory viral infections (Moore et al., 2008) have contributed to a better understanding of the diversity of blood elements and their expression mechanisms on their surface of asialo-GM1 or of other gangliosides. Now that we have determined a baseline level concerning the immunolocalization of asialo-GM1 in the porcine gut, the next step would be to determine the extent in which this immunolocalization could be modulated, especially by food contaminants. In this regard, we have already demonstrated that Fumonisin B1 alters the glycolipids of porcine jejunal segment (Loiseau et al., 2007). As a result, it seems important to delve further into the detailed biochemical changes occurring in the intestine as they might have consequences in terms of susceptibility to infection by mycotoxins (Oswald et al., 2003; Bouhet and Oswald, 2005).

In conclusion, our findings on the occurrence and distribution of asialoganglioside GM1 along the intestine of weaned piglets enhance our knowledge of the spectrum in which this glycosphingolipid was detected immunohistochemically in specific organs of mammalian species. In addition, it should be emphasized the necessity of promoting such investigations, not only on the gut but also on other organs of birds and poikilotherm vertebrates in order to understand the phylogenetic changes of this antigen of glycolipidic nature in the vertebrates.

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