Regulation of *N*-glycolylneuraminic acid biosynthesis in developing pig small intestine

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N-Glycolylneuraminic acid (Neu5Gc), an abundant sialic acid in animal glycoconjugates, is formed by the enzyme CMP-N-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase. The amount of Neu5Gc relative to other sialic acids is highly dependent on the species, tissue and developmental stage. Although the activity of the hydroxylase is a key factor in controlling Neu5Gc incorporation in adult animals, little is known about the regulation of hydroxylase expression and the role of this enzyme in determining changes in Neu5Gc during development. Using pig small intestine as a model system, the appearance of total sialic acid and the regulation of Neu5Gc biosynthesis during development were studied in various regions of this tissue. The amount of total sialic acid and Neu5Gc declined markedly in 2 weeks after birth. Although in subsequent developmental phases there were no positional differences in total sialic acid, a significant proximal-to-distal increase in Neu5Gc was detected. In all cases, a good correlation between the amount of Neu5Gc, the activity of the hydroxylase and the level of hydroxylase mRNA was observed. However, Western-blot analysis revealed considerable accumulation of less active enzyme in the *post partum* period, which persisted until adulthood. No evidence for cytosolic factors influencing the hydroxylase activity or for the formation of truncated enzyme was found, raising the possibility that other regulatory mechanisms are involved. The relevance of these results in the formation of Neu5Gc as a receptor for certain pig enteric pathogens is also discussed.

Key words: CMP-N-acetylneuraminic acid hydroxylase, cytochrome b_5, glycosylation, pathogen receptor, sialic acid.

INTRODUCTION

Development is frequently accompanied by tissue-specific changes in protein and lipid glycosylation. Among these, modifications in the type and linkage of sialic acid have been reported [1]. The sialic acids comprise a group of more than 40 acidic sugars, which are derived from several enzymic modifications of *N*-acetylneuraminic acid (Neu5Ac) [2,3]. They generally occupy terminal positions in the oligosaccharide chains of glycoconjugates where they are involved in mediating and regulating a variety of physiological cell–cell interactions and are also exploited by a number of pathogens as adhesion ligands [4]. *N*-glycolylneuraminic acid (Neu5Gc) is one of the most common sialic acid types and is formally derived by the addition of a hydroxy group to the N-acetyl moiety of Neu5Ac. Neu5Gc is abundant in the animal kingdom, although its expression in glycoconjugates varies widely, exhibiting considerable interspecies differences. Furthermore, within a certain species the appearance of Neu5Gc is tissue- and age-dependent [5] and may also be influenced by cell activation [6] and disease [7].

The biosynthesis of Neu5Gc occurs by the hydroxylation of CMP-Neu5Ac, giving rise to CMP-Neu5Gc which is utilized as a sialyl donor for glycoconjugate biosynthesis in the same way as CMP-Neu5Ac [8]. In mammals, CMP-Neu5Ac hydroxylase (EC 1.14.13.45) is a soluble, NAD(P)H-dependent mono-oxygenase, which requires the electron transport proteins cytochrome b_5 reductase and cytochrome b_5 for activity [9]. Studies on different adult pig tissues show that the hydroxylase activity of a particular organ or cell type is decisive in regulating the level of glycoconjugate sialylation with Neu5Gc [10,11]. However, little is known about the mechanisms underlying changes in the expression of this sialic acid during development. Developmental alterations in the appearance of total Neu5Gc have been observed in bovine and rat tissues [12,13] as well as in gangliosides isolated from the small intestine of pig [14,15] and rat [16]. Although evidence for ontogenic changes in hydroxylase activity was reported [13,16], these assays were performed under non-quantitative conditions owing to the omission of exogenous cytochrome b_5 and cytochrome b_5 reductase [17], prompting additional investigations. Since post-natal modifications in the sialic acid profile of pig and rodent small intestine have been found in several studies [18,19], the pig tissue was adopted in the present study as a model system for investigations on developmental changes in Neu5Gc biosynthesis.

Another motivating factor for this study is that in the pig intestinal tract, Neu5Gc-containing glycoconjugates play a crucial role in mediating infections by certain pathogens. For example, enterotoxigenic *Escherichia coli* with K99 fimbriae infect newborn piglets by binding to Neu5Gc in gangliosides such as Neu5Gcα2 → 3Galβ1 → 4Glcβ1 → 1′-ceramide [GM3(Neu5Gc)], *N*-glycolylsialoparagloboside and GM2(Neu5Gc) [20–22] attached to intestinal absorptive and mucus-secreting cells [23,24], causing a potentially lethal diarrhoea. Additionally, pig rotavirus, which similarly causes diarrhoea in young pigs, binds to GM3(Neu5Gc) [15]. Another pathogen, pig-transmissible gastroenteritis coronavirus, also recognizes glycoconjugates containing α2,3-bound Neu5Gc [25]. Enhanced levels of Neu5Gc-containing glycoconjugates were found in newborn pigs, which are particularly susceptible to these infections [15]. Studies on the regulation of Neu5Gc

Abbreviations used: DMB, 1,2-diamino-4,5-methylene dioxybenzene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Neu5Gc, *N*-glycolylneuraminic acid; GM3(Neu5Gc), Neu5Gcα2 → 3Galβ1 → 4Glcβ1 → 1′-ceramide; h.s.s., high-speed supernatant; Neu5Ac, *N*-acetylneuraminic acid.

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expression are, therefore, important for our understanding of the aetiology of these economically relevant pig diseases.

In this work, we have studied the regulation of Neu5Gc formation in different regions of pig small intestine during ontogenesis by analysis of CMP-Neu5Ac hydroxylase mRNA levels, immunodetection of the hydroxylase protein, assay of the CMP-Neu5Ac hydroxylase activity and quantification of Neu5Gc.

EXPERIMENTAL

Animals

Samples of small intestine were taken from foetal (day 100 of a 120 day gestation), newborn, suckled (2 weeks *post partum*), weaned (4 weeks *post-partum*) and adult (2-year-old female) large white × Landrace pigs from the Rowett herd. Foetal pigs were surgically exposed after 100 days gestation while under the influence of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) anaesthesia administered to the sow. Exposed foetuses were immediately killed by lethal injection of pentabarbitone. Newborn piglets were unsuckled and did not receive milk. Other animals were fasted for 4 h before intestinal tissue sampling. All animals were killed by a lethal injection of pentabarbitone, the intestinal tissues were immediately excised and any residual gut contents were removed carefully. The small intestine was divided into proximal and distal segments for foetal tissues and samples of duodenum, jejunum and ileum were taken from all other animals. For simplicity, the foetal proximal and distal regions were referred to as jejunum and ileum throughout this work. All tissues were frozen in liquid nitrogen and stored at −80 °C before analyses.

RNA analysis

PCR primers were used to amplify a 310 bp sequence corresponding to the N-terminus of the pig CMP-Neu5Ac hydroxylase [26] from adult pig intestine cDNA. The purified PCR products were cloned into pGEM-T Easy (Promega, Southampton, U.K.). DNA sequencing confirmed the correct amplification of the probe sequences. To obtain probes for Northern-blot analysis, the plasmids were digested with *Eco*RI (Promega). Double-stranded DNA probes of 310 bp were purified and used in Northern-blot analyses following standard methods. In summary, total RNA was extracted from flash-frozen jejunum tissues from newborn ($n = 3$), suckled ($n = 2$), weaned ($n = 2$) and adult pigs ($n = 2$) using the acid guanidinium thiocyanate–phenol chloroform method [27]. Extracted RNA was treated with RNase-free DNase (Promega) denatured and separated on a 1.2 % agarose gel containing 1.0 M formaldehyde (25 μg of total RNA/lane). The electrophoresed RNA was transferred on to Hybond N membranes (Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.) by overnight capillary blotting in 20 × SSC and hybridized for 1 h at 60 °C with the pig CMP-Neu5Ac hydroxylase probe that was ^{32}P-labelled using the High Prime Nucleic Acid Labelling system from Roche Diagnostics (Lewes, East Sussex, U.K.). The membranes were washed for 30 min at 60 °C in 0.1 × SSC/0.1 % SDS and hybridizing bands were visualized using autoradiography (Kodak Biomax MR film; Sigma). All blots were subsequently stripped and reprobed with a ^{32}P-labelled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The intensities of the bands were quantified using 'Quantity 1' image analysis software (Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.).

Assay for CMP-Neu5Ac hydroxylase activity

Tissues were homogenized with a Potter–Elvehjem homogenizer in ice-cold 50 mM Hepes/NaOH, pH 7.4 (0.5 g of wet mass tissue to 2.5 ml of buffer) and centrifuged using a Beckman TLA 45 rotor at 100000 ***g*** for 16 min at 4 °C to obtain high-speed supernatants (h.s.s.). Hydroxylase activity was measured using 15 μl of h.s.s. Enzyme tests were performed at 37 °C in the presence of 1 mM NADH, 0.5 mM $FeSO_4$, 12 μM CMP-Neu5Ac (Calbiochem, Bad Soden, Germany) containing 12.5 nCi CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac (Amersham, Braunschweig, Germany) and 33 μg of solubilized pig liver microsomal protein, in a final volume of 25 μl. After terminating the reaction with trichloroacetic acid, the released [^{14}C]sialic acids were quantitatively analysed by radio TLC [10]. All enzyme tests were performed in duplicate, using incubation times within the linear region of the reaction time course.

Western-blot analysis

Proteins in h.s.s. of the intestinal tissues were resolved by SDS/PAGE and blotted on a cellulose nitrate membrane as described previously [10]. Staining of the blots was performed using immuno-purified antibodies raised in rabbits against the CMP-Neu5Ac hydroxylase from pig submandibular glands (h-3) and a synthetic decapeptide, derived from the amino acid sequence of the same enzyme (p-1) [10]. Bound antibodies were detected with the BM chromogenic Western blotting kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Protein concentration

Protein concentrations were measured using the bicinchoninic acid protein assay reagent (Pierce, Sankt Augustin, Germany) with BSA as a standard.

Sialic acid analysis

Tissues were homogenized in 4 vols. (ml/g of wet mass) of ice-cold water with a Potter–Elvehjem homogenizer and further procedures were performed as described previously [10]. Briefly, total sialic acids were released from 0.3 ml of the homogenates by sequential acid hydrolyses in 2 M propionic acid followed by 0.1 M HCl and fractionated by chromatography on cation- and anion-exchange resins. After freeze-drying, the enriched sialic acid was resuspended in 1 ml of water. Aliquots (15 μl) of this solution were derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB) and analysed by reverse-phase HPLC with fluorimetric detection. To test for the presence of O-acetylated sialic acids, aliquots of the enriched sialic acid solution were subjected to saponification before derivatization with DMB. The HPLC column was calibrated using DMB derivatives of Neu5Ac, Neu5Gc and a mixture containing O-acetylated sialic acids purified from bovine submandibular gland mucin. The quantification of sialic acids was performed by integration of the chromatographic peaks for non-saponified samples, using a calibration curve obtained for the DMB derivative of Neu5Ac.

Statistical analysis

Three individual animals were studied at each developmental stage. Tissues from each animal were included in the sialic acid analysis and hydroxylase tests. Results were assessed by one-way

ANOVA using the Microcal Origin program (Microcal Software Inc., Northampton, PA, U.S.A.).

RESULTS

Expression of sialic acids

Representative HPLC analyses, showing temporal and spatial differences in the expression of sialic acids in pig small intestine, are presented in Figure 1. Two major peaks with the same retention times as the authentic Neu5Ac and Neu5Gc derivatives were detected in all samples. No difference between saponified and non-saponified samples was found (results not shown), proving the absence of O-acetylated sialic acids in all tissue regions and age groups. Developmental changes in the total amounts of sialic acids (expressed as μg of sialic acid/mg of protein) are shown in Figure 2. In the course of development from foetal to newborn pigs, a small decrease in the quantity of sialic acid occurred in the proximal small intestine, whereas no significant differences were observed in the distal region. However, during 2 weeks after birth, a 2–2.5-fold decrease in the sialic acid content in all parts of the small intestine was observed, which persisted until adulthood (Figures 1a and 2). In the foetal small intestine, no spatial dependence of the sialic acid quantity was detected, although in newborn animals, a significant gradient of sialic acids was evident, increasing from the duodenum to the ileum ($P = 0.04$). In later developmental phases, no obvious spatial gradient was found. Comparable temporal and spatial trends were apparent when the above results were expressed as μg of sialic acid/g of wet mass of tissue (results not shown), showing that the trends observed were not due to changes in protein content.

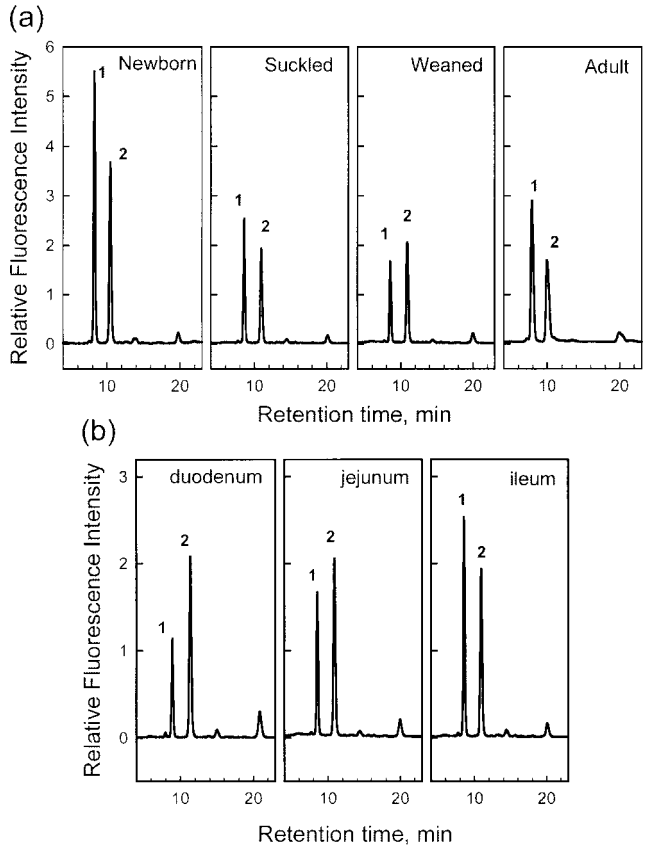

Figure 1 HPLC analysis of DMB-derivatized sialic acids isolated from pig small intestine

(**a**) Sialic acids at various stages of ileum development. (**b**) Sialic acids in various regions of the small intestine from suckled animals. In all cases, sialic acids corresponding to 1.1 mg of wet tissue were chromatographed. 1, Neu5Gc; 2, Neu5Ac. O-acetylated sialic acids have retention times ranging from 10.5 min (Neu5Gc7Ac) up to 23 min (Neu5,7,8,9Ac$_4$). The minor peak appearing at 20 min is an artifact of the derivatization reagent.

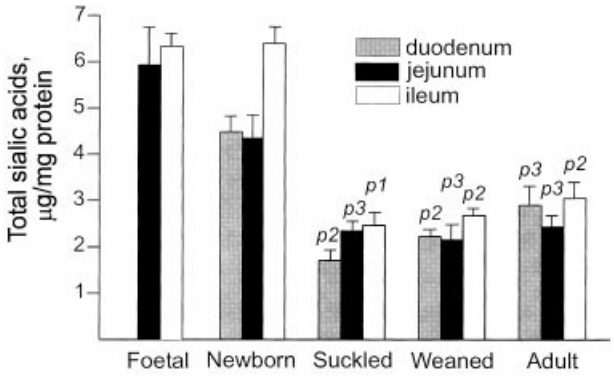

Figure 2 Total sialic acid levels in developing pig small intestine

Total amounts of sialic acid extracted from foetal (F), newborn (N), suckled (S), weaned (W) and adult (A) pigs are given relative to total protein in homogenates of the respective tissues. Values shown are means + S.E.M. ($n = 3$). The statistical analysis was performed by comparing total sialic acid in duodenum, jejunum and ileum from S, W, and A pigs with the respective tissues from N pigs. p1, $P < 0.001$; p2, $P < 0.01$; p3, $P < 0.05$. Proximal and distal regions of F small intestine are referred to as jejunum and ileum respectively.

Regarding Neu5Gc, little change in the content of this sialic acid was detected in proximal and distal regions of foetal and newborn small intestine (Figure 3a). However, in accordance with the results for total sialic acid, a significant, approx. 2.5-fold, decrease in the Neu5Gc content occurred in all regions in the second week after birth. The Neu5Gc level remained low during weaning and increased by approx. 50% in the adult, particularly in the duodenum and ileum. In foetal tissue, a slightly larger amount of Neu5Gc was detected in the distal relative to the proximal region. In newborn animals, a clear increase in Neu5Gc content proceeding from the duodenum to the ileum ($P = 0.005$) was visible. A similar gradient of Neu5Gc expression was also detected in the suckled ($P = 0.01$) and weaned ($P = 0.025$) groups (Figures 1b and 3a). Although a spatial Neu5Gc gradient was apparent in adult animals, this was not statistically significant ($P = 0.18$) (Figure 3a).

When expressed as a percentage (w/w) of total sialic acid, a clear proximal-to-distal gradient in Neu5Gc was observed in the small intestine at all stages of postnatal development (Table 1).

CMP-Neu5Ac hydroxylase activity

The specific activity of CMP-Neu5Ac hydroxylase in the various tissues investigated is presented in Figure 3(b). Comparing equivalent regions of foetal and newborn small intestine, no significant temporal alterations in the activity of the hydroxylase were discernable. However, birth was followed by a 2–8-fold decrease in activity, depending on the region of the small intestine. The enzyme activity increased slightly in the subsequent developmental phases. Although no spatial dependence of hydroxylase activity was evident in the intestines of foetal and newborn pigs, a clear increase in enzyme activity from duodenum to ileum was found in suckled pigs ($P = 0.0001$). A similar tendency was also observed in weaned ($P = 0.006$) and adult animals ($P = 0.03$).

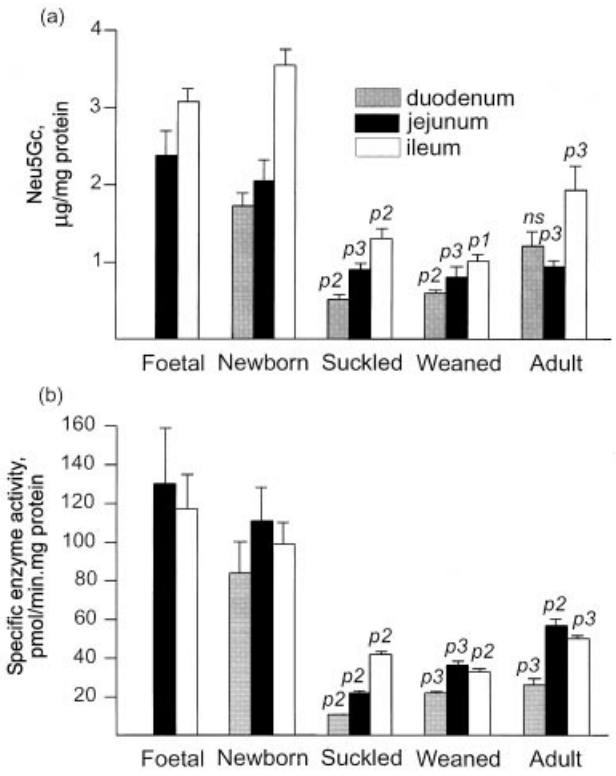

Figure 3 Biosynthesis of Neu5Gc in developing pig small intestine

Samples from F, N, S, W and A pigs were analysed for: (**a**) amount of Neu5Gc related to protein content in homogenate and (**b**) specific activity of CMP-Neu5Ac hydroxylase in the respective h.s.s. Values shown are means + S.E.M. ($n = 3$). Statistical analysis was performed by comparing the relevant parameters in duodenum, jejunum and ileum from S, W and A pigs with the respective tissues of N pigs. p1, $P < 0.001$; p2, $P < 0.01$; p3, $P < 0.05$; ns, no significant difference. Proximal and distal regions of F small intestine are referred to as jejunum and ileum respectively.

Table 1 Neu5Gc expressed as a percentage of total sialic acids in developing pig small intestine

Values shown are means + S.E.M. ($n = 3$). Statistical analysis comparing differences in duodenum and ileum percentage levels.

	Neu5Gc (% of total sialic acids)		
Developmental stage	Duodenum	Jejunum	Ileum
Foetal	–	41 ± 5	49 ± 3
Newborn	38 ± 4	47 ± 3	55.3 ± 0.5*
Suckled	30 ± 1	38.4 ± 0.6	53 ± 1†
Weaned	26 ± 2	37 ± 3	38 ± 2*
Adult	42 ± 7	40 ± 6	62 ± 6*

* $P < 0.01$.
† $P < 0.001$.

Western-blot analysis

A Western-blot analysis performed with h-3 revealed only small amounts of hydroxylase protein in all regions of foetal and newborn small intestine (Figure 4a). These results were confirmed by staining of representative samples with the anti-peptide antibody p-1 (Figure 4b). The presence of the enzyme in foetal and newborn tissues was clearly demonstrated by application of more protein to the gel (Figure 4d). In all regions of the small

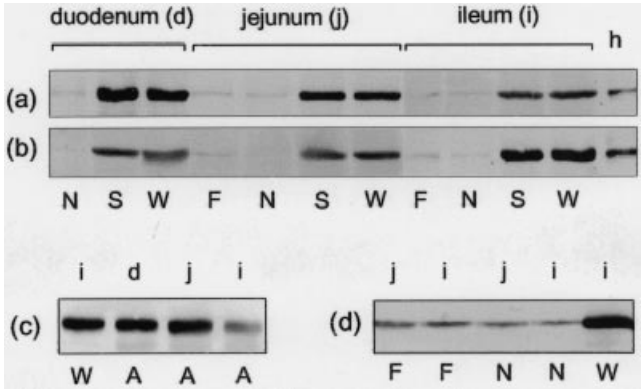

Figure 4 Western-blot analysis of CMP-Neu5Ac hydroxylase in h.s.s. of developing pig small intestine

(**a**) Protein (15 μg/lane) applied; immuno-detected with affinity-purified h-3. (**b**) Protein (30 μg/lane) applied; immuno-detected with affinity-purified antibody against p-1. (**c**) Protein (15 μg/lane) applied; immuno-detected with antibody h-3. (**d**) Protein (45 μg/lane) applied; immuno-detected with antibody h-3. Sections of small intestine: d, duodenum; j, jejunum; i, ileum. Animals: F, foetal; N, newborn; S, suckled; W, weaned; A, adult. A positive control, h, consisted of purified CMP-Neu5Ac hydroxylase from pig submandibular gland. Proximal and distal regions of F small intestine are referred to as jejunum and ileum respectively.

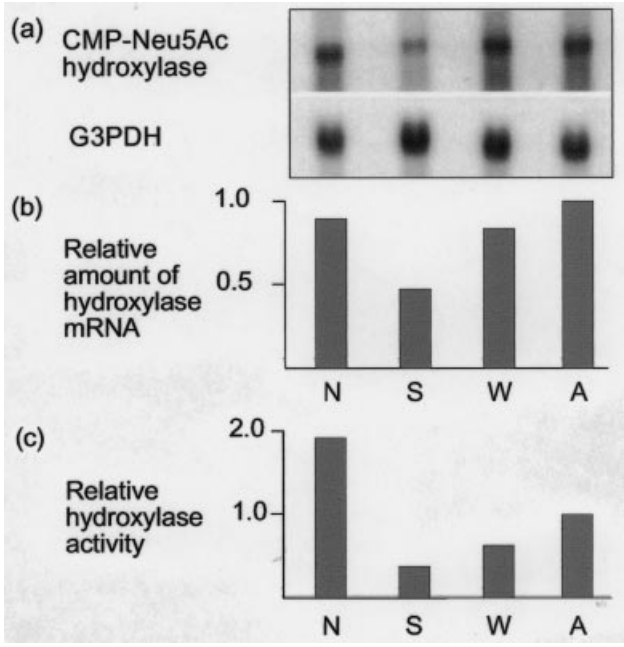

Figure 5 Northern-blot analysis of CMP-Neu5Ac hydroxylase mRNA in jejunum tissues of developing pig

(**a**) Total RNA was subjected to electrophoresis and probed with a radiolabelled cDNA, corresponding to the N-terminus of CMP-Neu5Ac hydroxylase, as described in the text. The same blot was subsequently stripped and reprobed with a radiolabelled cDNA for GAPDH. (**b**) The autoradiography film was scanned and the intensities of the CMP-Neu5Ac hydroxylase hybridizing bands are displayed after correcting for the respective GAPDH mRNA levels. (**c**) For comparison, the relative hydroxylase activity of jejunum from the various developmental phases is presented. Abbreviations for developmental phases are defined in Figure 4.

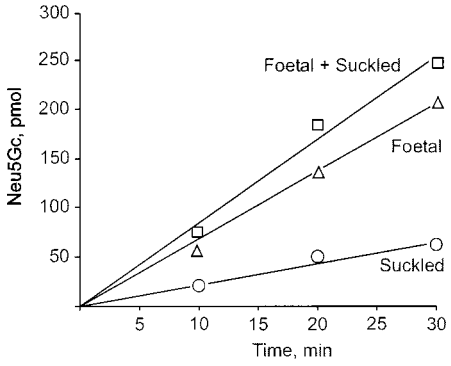

Figure 6 Assay of CMP-Neu5Ac hydroxylase activity in h.s.s. of pig intestine

The assays were performed in a total volume (25 μl) as described in the Experimental section. ○, 7.5 μl of h.s.s. of suckled pig jejunum. △, 7.5 μl of h.s.s. of foetal pig jejunum. □, 7.5 μl of h.s.s. of suckled pig jejunum +7.5 μl of h.s.s. of foetal pig jejunum.

intestine from suckled, weaned and adult animals, large and apparently constant quantities of hydroxylase protein were detected (Figures 4a and 4c). The immuno-detected hydroxylase bands in all samples exhibited the same electrophoretic mobility as the authentic enzyme from pig submandibular glands, corresponding to an apparent molecular mass of 65 kDa.

Northern-blot analysis

Investigations on the expression of mRNA for CMP-Neu5Ac hydroxylase were performed on jejunum tissues from newborn, suckled, weaned and adult pigs. A representative Northern blot is shown in Figure 5(a). When normalized against GAPDH mRNA, the hydroxylase mRNA level in the jejunum of newborn pigs decreased by approx. 50 % during suckling (Figure 5b). The abundance of hydroxylase mRNA subsequently increased during weaning and maturation to the adult animal.

Mixed enzyme assays

Since the above results suggest the existence of large amounts of less active protein at later stages of development, the possible presence of cytosolic inhibitors was investigated. The enzyme tests were performed with a mixture of h.s.s. of foetal (high hydroxylase activity, low levels of immuno-detected protein) and suckled (low hydroxylase activity, high levels of immuno-detected protein) tissues. Representative results from one of these experiments showed that the hydroxylase activity observed in mixed assays is essentially the sum of the individual activities over several time points (Figure 6).

DISCUSSION

Developmental changes in the expression of sialic acids in pig small intestine

Two main changes in the expression of sialic acids in pig small intestine were observed, namely (i) temporal alterations in their appearance during maturation and (ii) spatial as well as temporal differences in the content of Neu5Gc (Figures 1, 2 and 3a). In contrast with other pig tissues [28], no O-acetylated sialic acids were detected in the small intestine, in agreement with studies on gangliosides in this tissue [15].

Temporal changes

The general decrease in sialic acid occurring *post partum* (Figure 2) is consistent with the previously reported decline in the ganglioside content of pig small intestine during the first 2 weeks of life [14,15]. Indeed, higher levels of sialic acid in prenatal or initial postnatal stages of development may be a general phenomenon, as supported by observations on gangliosides of rat small intestine and other rat tissues [13,16] and total sialic acids in cows [12]. These changes may be associated with a postnatal switch from sialylation to fucosylation, as observed in rat and pig small intestine [29,30] and may also involve alterations in the activity of enzymes of sialic acid biosynthesis, activation and transfer [31–33].

With regard to sialic acid type, the considerable decrease in Neu5Gc, seen in all parts of the small intestine 2 weeks *post partum* (Figure 3a), correlates with the marked decline in the main ganglioside, GM3(Neu5Gc), observed in the distal small intestine of pigs during the same period [14]. The fact that bacteria do not possess Neu5Gc [5], together with studies on germ-free rats [34], suggest that our observations are only due to changes within the intestinal cells.

Spatial changes

Although only subtle site-specific differences in total sialic acid were generally observed (Figure 2), a clear proximal-to-distal gradient in Neu5Gc was detected at all stages of development (Figure 3a; Table 1). Interestingly, a similar positional gradient in Neu5Gc [35,36] was also observed in rat small intestine.

This gradient in Neu5Gc in the intestine may reflect site-specific variations in the cellular composition of epithelial and lymphoid tissues. The expression of Neu5Gc and CMP-Neu5Ac hydroxylase has previously been associated with mature T-lymphocyte populations in pigs [10]. The higher levels of Neu5Gc in the ileum at later stages of development may therefore partially result from the relatively large number of intra-epithelial and sub-mucosal lymphocytes in this region compared with the proximal intestine [37]. A histological analysis of Neu5Gc expression is evidently required to clarify this issue. The intestinal microflora may also influence the biosynthesis of glycoconjugates during the postnatal development of the small intestine [38]. Thus, commensal and pathogenic bacteria promote changes in the lymphoid tissue and modulate the formation and glycosylation of intestinal mucins [39,40]. Furthermore, parasitic nematode infections in rats result in reduced intestinal CMP-Neu5Ac hydroxylase mRNA expression and decreased levels of Neu5Gc in mucins [7]. Since the major changes in Neu5Gc content reported here occur during the colonization of the gut by normal enteric bacteria, microbial influences on glycosylation are possible and warrant further investigation.

Implications of the differential expression of Neu5Gc in pig small intestine for enteric infections

Neu5Gc in certain glycoconjugates can mediate several pig enteric bacterial and viral infections. These diseases are known to be most severe in neonatal pigs [41,42], thus correlating with the high level of Neu5Gc and hydroxylase activity reported here for animals of this age. Furthermore, the results on the spatial distribution of Neu5Gc are also relevant to the site of infection by these pathogens. For example, *E. coli* with K99 fimbriae were found to adhere preferentially to and colonize distal regions of pig small intestine [43,44]. Similarly, pig transmissible gastroenteritis virus was found to be most pathogenic in jejunum and ileum [45] and pig rotavirus initially infects the ileum [46]. Distal

regions of the pig small intestine therefore appear to be more prone to infection by pathogens, which require Neu5Gc for adhesion. Since the age and positional susceptibility for these enteric diseases correlate with the profile of Neu5Gc formation, CMP-Neu5Ac hydroxylase activity is a major factor determining the infection potential of these agents.

Regulation of Neu5Gc biosynthesis

The results presented in Figure 3 reveal a close relationship between the content of Neu5Gc and changes in the activity of CMP-Neu5Ac hydroxylase, suggesting that this enzyme is instrumental in controlling developmental alterations in Neu5Gc incorporation. Similar conclusions were reached on the basis of studies on various adult pig tissues [10] and pig lymphocyte subpopulations [11]. Developmental changes in the expression of GM3(Neu5Gc) in rat small intestine were also found to correspond to the activity of this enzyme [16].

The fact that the level of hydroxylase mRNA in developing pig jejunum correlated with variations in enzyme activity (Figure 5) indicates that the formation of Neu5Gc is regulated by transcription of the hydroxylase gene, a mechanism which is also operative in rodent tissues [7,47].

Despite the parallels between the levels of Neu5Gc, hydroxylase activity and hydroxylase mRNA observed here, quantification of enzyme protein by Western blotting revealed that the high activity in foetal and newborn small intestine was accompanied by small amounts of immunoreactive protein, whereas the low hydroxylase activity of suckled, weaned and adult tissue was associated with larger amounts of enzyme protein (Figures 3 and 4). In a preliminary report [48], it was suggested that cytosolic factors might regulate the hydroxylase activity. However, in mixed enzyme assays using tissue supernatants exhibiting high or low enzyme activity, no evidence for the presence of such substances was found (Figure 6). Furthermore, in the Western blots there was no evidence for an inactive, truncated form of the hydroxylase, observed previously in mouse liver [49].

These results suggest that significant amounts of presumably inactive hydroxylase protein accumulate in suckled, weaned and adult tissues. Interestingly, a similar phenomenon was also observed in the heart and lung of adult pigs [10]. The origin or function of this less active protein is unknown. To date, no post-translational modifications of the hydroxylase are known. The incorporation of a [2Fe–2S] cluster is probably an obligatory modification in the generation of active enzyme [50]. However, since little is known about the formation of such centres in cytosolic proteins [51], its possible role in influencing hydroxylase activity remains unclear. The observed accumulation of hydroxylase protein may reflect a decrease in its degradation, though as yet nothing is known about the mechanism of hydroxylase protein turnover. The correlation between the enzyme activity and the level of mRNA suggests that newly synthesized enzyme is detected in the activity test, and that differential degradation rates at various stages of development or in certain tissues [10] may contribute to the observed discrepancies between the amounts of hydroxylase protein and hydroxylase activity. Clearly, further research is required not only to elucidate the factors which govern the transcription of the hydroxylase gene, but also to study the holoenzyme assembly and turnover of the enzyme protein.

This work was supported by the Volkswagen-Stiftung, Hannover, Germany (grant no. I/71 754) and Sialic Acid Society Kiel, Germany. Research at the Rowett Research Institute is financed by the Scottish Executive Environment and Rural Affairs Department.

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Received 3 July 2002/13 November 2002; accepted 22 November 2002
Published as BJ Immediate Publication 22 November 2002, DOI 10.1042/BJ20021049