Regulation of intestinal ontogeny: effect of glucocorticoids and luminal microbes on galactosyltransferase and trehalase induction in mice

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Intestinal maturation can be influenced by intrinsic factors (glucocorticoid hormones) and by extrinsic factors (resident microflora); their relative roles in ontogeny of mouse intestinal trehalase expression, a marker of general gut development, and of β1,4-galactosyltransferase (βGT), a marker of glycosyltransferase development, were investigated. In conventional (CONV) mice, βGT and trehalase gene expression rapidly increased to adult levels by the fourth postnatal week. In germ-free (GF) mice, βGT expression remained at initial low levels and was rapidly induced on reintroduction of luminal microbes of the adult gut but not of microbes characteristic of the suckling gut. Similar developmental patterns were observed for colonic galactosyl β1,4-linked glycoconjugates, products of βGT activity. These results indicate an essential role for microbes in the ontogeny of βGT. In both CONV and GF mice, cortisone acetate (CA) precociously accelerated the ontogeny of βGT and trehalase until maturation of the gut occurred (day 22). In the mature gut of CONV mice, both βGT and trehalase are elevated and insensitive to CA; in GF mature mice, the expression of βGT remains low, whereas the expression of trehalase was at mature levels, regardless of CA treatment. These changes in enzyme activity were accompanied by parallel changes in mRNA, implying transcriptional regulation. Thus both microbes and cortisone regulate gut ontogeny, but only suckling gut responds to CA, an intrinsic factor, whereas adult gut βGT expression remains sensitive to microflora, an extrinsic factor. However, induction of the adult pattern of glycosyltransferase expression in mature gut requires colonization by microflora typical of adult gut, suggesting an essential role for intestinal colonization in the ontogeny of normal intestinal mucosal cell surface glycoconjugate receptors.

Key words: germ-free mice/hormonal regulation/microflora/postnatal development

Introduction

Postnatal development of the mouse gut can be divided into three phases: suckling, weaning, and adulthood. The transition from the suckling to the adult phenotype occurs during the third postnatal week, coinciding with the dietary change from milk to solid food (Buller et al., 1990; Henning, 1987) and is accompanied by characteristic changes of digestive enzymes. Lactose and sucrose are characteristic of milk and adult food, respectively; hence the enzymes digesting these disaccharides, lactase and sucrase, have been used to measure the onset of postnatal gut maturation (Henning, 1987). Maturational changes reflected by these enzyme markers are regulated at the level of transcription by multiple mechanisms. However, trehalase, which digests the more ubiquitous Glc α1,6 Glc from branched dietary glucans, is intrinsic to and potentially a superior marker of enterocyte development.

Intestinal maturation can be regulated by three major factors: an intrinsic timing mechanism, circulating hormones, and extrinsic factors, such as luminal microbes. An intrinsic timing mechanism coordinates the onset of the adult phenotype (Biol et al., 1987; Dai and Walker, 1999; Henning, 1987; Hooper et al., 1998). In the case of sucrase induction, however, glucocorticoids accelerate the ontogeny, leading to a rapid transition to the adult phenotype. The in vivo importance of glucocorticoids was reaffirmed by the discovery of a developmental surge of the circulating level of corticosterone in the rat and mouse 2 days prior to the initiation of gut ontogeny (Martin and Henning, 1984; Nanthakumar and Henning, 1993). In addition, both exogenous and premature induction of endogenous glucocorticoids can precociously induce an adult phenotype of rodent gut during the first 2 weeks postpartum. During this induction period, glucocorticoids stimulate proliferative cells to initiate differentiation into mature villus epithelium (Nanthakumar and Henning, 1993; Henning, 1987). The kinetics of this effect in vitro and in explant cultures suggests that steroids may affect differentiated epithelium indirectly through gut mesenchyme (Simo et al., 1992). This sensitivity to glucocorticoids is lost after the third postnatal week with the onset of an adult intestinal phenotype (Nanthakumar and Henning, 1993) by a mechanism that is not completely understood. Sucrase and lactase have been used as tissue-specific markers to elucidate the effect of steroids and their interplay with intrinsic timing mechanisms that lead to the onset of the adult phenotype. However, they are insensitive to luminal factors, such as changing microflora (Reddy and Wostmann, 1966). Thus disaccharidases alone are not useful as markers for studying the interplay between steroids...
and luminal microbes in establishing the adult phenotype in the gut.

Gut microflora may also influence intestinal ontogeny. In all mammals, rapid colonization of gut microflora begins at birth (Berg, 1996; Dai and Walker, 1999), followed by an equally rapid transition to a new complex microbial ecosystem at weaning (Berg, 1996; Falk et al., 1998). Adult human microflora usually includes more than 500 species of bacteria. This complex and dynamic community of commensal (and symbiotic) microflora forms a stable niche by adhering to the surface of the gut lumen (Berg, 1996; Falk et al., 1998; Karlsson, 1995). This adherence is mediated through binding to glycoconjugates on the brush border membrane (Dai and Walker, 1999; Midtvedt et al., 1987; Pang et al., 1987).

For example, the ratio of sialic acid to fucose in the non-reducing terminal residues of surface carbohydrates of the rodent intestinal epithelium reverses from a predominance of sialic acid to fucose after weaning (Dai et al., 2002); this has been attributed to a decrease in intestinal sialyltransferase activity coincident with a reciprocal increase in fucosyltransferase activity (Bry et al., 1996; Dai et al., 2002; Hooper et al., 2001).

The developmental induction of α1,2-fucosyltransferase and ontogenic decline of α2,3/6-sialyltransferase observed in conventional mice are not observed in germ-free (GF) mice. However, on inoculation of GF mice with luminal bacteria from adult mice, even after maturity, these two enzymes achieve adult patterns of expression within 2 weeks (Dai et al., 2002; Hooper et al., 2001; Nanthakumar et al., 2003). The level of expression of these two glycosyltransferases in adults is primarily regulated by microbial colonization (Bry et al., 1996; Dai et al., 2002). Bacteroides thetaiotaomicron is a commensal bacterium that colonizes the gut lumen by adhering to glycoconjugates with terminal α1,2-linked fucose; when adult GF mice are colonized by this bacterium alone, the expression of α1,2-fucosyltransferase is induced to normal mature levels (Bry et al., 1996; Hooper et al., 2001). Thus microflora may be an important extrinsic factor controlling the ontogeny of glycosyltransferases, leading to the reciprocal changes in the surface glycoconjugate content during the suckling to weaning transition. However, the interaction between extrinsic and intrinsic regulators of the developing gut could not be investigated without using markers specific to each. β1,4-Galactosyltransferase (βGT), because it synthesizes part of the glycan core rather than the more variable nonreducing terminus, is potentially a marker for developmental changes in general glycan expression. Mice whose gene for βGT had been inactivated displayed phenotypes that included early death at the third postnatal week, coinciding with the loss of sucrase induction in the intestine (Asano et al., 1997; Lu et al., 1997). Therefore, we investigated whether βGT and trehalase were suitable intestinal markers for comparing the regulatory control of extrinsic factors (luminal microbes) and intrinsic regulators (glucocorticoids). These two markers could be used to study the relative contributions of extrinsic and intrinsic factors in regulating the transition from the suckling to the adult intestinal phenotype.

In this study, experiments were designed to address five major issues. (1) To determine whether expression of βGT is a marker of maturation distinct from that of trehalase, their ontogeny was compared in conventional (CONV) mice. (2) To determine the influence of glucocorticoids on ontogeny of intestinal βGT. βGT expression was compared in steroid-treated versus untreated CONV mice. (3) To determine the role of microbes in this process, the ontogeny of these two enzymes was compared between CONV and GF mice. (4) To determine influence of glucocorticoid regulation on βGT and trehalase in the absence of microbes, ontogeny of these two markers was compared between GF and CONV mice. (5) To determine whether the loss of glucocorticoid responsiveness is dependent on microbial colonization, both βGT and trehalase gene expression were measured in developing GF mice.

To answer these questions, the ontogeny of βGT (UDP-Gal: GlcNAc βGal β1,4-galactosyltransferase, EC 2.4.1.38) and trehalase (EC 3.2.1.28) expression was determined by measuring their enzyme activities, mRNA induction, and glycoconjugate expression in the mouse duodenum, jejunum, ileum, and colon during the first 4 weeks of postnatal life. These experiments were performed in GF, CONV, and GF mice conventionalized (XGF) by inoculating GF mice with normal intestinal flora obtained from age-matched CONV mice.

Results

The developmental expression of βGT was compared with developmental expression of trehalase as a marker of gut maturation over the first 6 weeks of life in CONV mice. In duodenum, jejunum, ileum, and colon, the expression of βGT increased between the second and third weeks of life and achieved adult levels by the fourth week (overall analysis of variance [ANOVA] p < 0.001). As the mice matured, large regional differences in the level of βGT activity were apparent, with the lowest activity in the duodenum and jejunum and the highest activity in the colon (p < 0.001). The expression of trehalase essentially followed a similar increase in the maturing duodenum, jejunum, and ileum (overall ANOVA p < 0.001). Trehalase, a disaccharidase, is not expressed in colon. As the mice matured, large regional differences in the level of trehalase activity were apparent, with the lowest activity in the ileum and the highest activity in the duodenum (p < 0.001) (Figure 1).

Intestinal ontogeny can be regulated by glucocorticoids. The influence of cortisone acetate (CA) on ontogeny of intestinal βGT and trehalase was studied. Mice were treated with CA on day 10; βGT and trehalase activities were measured on day 14 (Figure 2). Cortisone treatment caused precocious induction of βGT in all areas of the gut (p < 0.01) (Figure 2A); in like manner CA induced trehalase activity in all areas of the small intestine (p < 0.001) (Figure 2B). During this period of development, CA seems to specifically affect the gut, as evident by the lack of CA response in liver. Thus the ability of CA to accelerate development of the immature gut was reflected by changes in...
both of these enzyme markers, implying that CA is inducing a general maturation of the gut.

Next, the duration of cortisone sensitivity during postnatal development was determined. Each of these marker enzyme activities was measured in the gut 5 days after CA treatment from the second to fourth week of life. All three regions of the small intestine displayed similar changes; the data from the ileum is shown in Figure 3. The βGT of the immature intestine was responsive to CA until day 18 (Figure 3A), and trehalase was responsive until day 14 (Figure 3C). These changes in enzyme activity were accompanied by parallel changes in mRNA expression (Figure 3B and 3D), consistent with the cortisone effect on enzyme activity occurring at the level of transcription. This change in sensitivity occurs during a period of rapid turnover of intestinal microflora.

Therefore the role of microbes on the ontogeny of intestinal βGT was investigated in GF and CONV mice during postnatal development. In CONV mice, βGT was fully induced during development in all regions of the gut (ANOVA: \( p < 0.001 \)), as shown in Figure 1. However, in GF mice, there were no significant (ANOVA) age-related changes from week 1 to week 6 of age in the duodenum (2.0 ± 0.2 to 2.8 ± 0.2 nmol/mg protein/h), jejunum (2.1 ± 0.2 to 3.2 ± 0.4), or ileum (3.0 ± 0.4 to 4.4 ± 0.6) and only a modest increase in βGT activity in the colon (3.1 ± 0.3 to 5.9 ± 0.4; \( p < 0.05 \)). This lack of normal induction in GF mice suggests that luminal microbes play an important role in the ontogeny of intestinal βGT.

To test whether indigenous microflora affect the development of intestinal βGT, GF mice were inoculated with microflora from suckling mice (XGF-suckling) or CONV 6-week-old mice (XGF-adult). The XGF mice were sacrificed 2 weeks after the introduction of microflora. In the absence of microflora, the levels of βGT activity (Figure 4A) remained low, which is typical of immature suckling mice regardless of actual age. On the introduction of luminal bacteria from aged-matched CONV mice (data

![Fig. 1. Developmental expression of βGT activity and of trehalase activity in the mouse gastrointestinal tract: (A) duodenum, (B) jejunum, (C) ileum, and (D) colon. Six to eight litters of pups were used, and age denotes the postnatal age. Trehalase is not expressed in colon. Results are expressed as mean ± SEM from three to four samples. Each sample of mice under 2 weeks old was a pool of six to eight pups; each sample of older mice was a pool of three.](https://academic.oup.com/glycob/article-abstract/15/3/221/574687)
from inoculation at 6 weeks shown), the levels of βGT activity increased to that of CONV mice in the duodenum ($p < 0.01$), jejenum ($p < 0.01$), ileum ($p < 0.005$), and colon ($p < 0.001$) (Figure 4A). In contrast, the introduction of gut microflora from the suckling mice had no effect on βGT activity. Unlike βGT, the levels of trehalase enzyme activity (Figure 4C) of CONV, GF, and XGF mice showed no significant difference in any region of the small intestine whether inoculated at 4 weeks or 6 weeks of age. The mRNA levels for βGT and trehalase displayed parallel changes during these treatments: Levels of βGT mRNA (Figure 4B) increased on inoculation of microbes from the adults, whereas trehalase mRNA levels were unchanged (Figure 4D), suggesting that changes in intestinal βGT activity (but not trehalase) is controlled by microflora that is unique to the suckling mice and these effects are manifested at the level of transcription.

The consequence of these changes of βGT activity on the expression of cell surface glycans in these intestinal regions was tested. Cell surface galactose-containing glycans were visualized in frozen sections of mouse colon by use of the galactose-specific lectin, RCA-I (Venkatesh and Lambert, 1997), conjugated with fluorescein isothiocyanate (FITC) (Figure 5). In GF mice, galactose-containing glycans were undetectable in the colon; reintroduction of microbes induced expression of galactoglycans to the higher levels found in CONV mice.

Thus there appear to be two important variables regulating developmental changes in the gut: the intrinsic genetic program and the change in microflora. In CONV suckling mice, the ability of CA to induce gut maturation is lost by day 18 (as shown in Figure 3); however, this period of loss of responsiveness coincides with the rapid conversion from the suckling to adult microflora, confounding these two variables. We use trehalase as a developmental marker of the intrinsic genetic program and βGT as a developmental marker for changes regulated by microflora. By measuring changes in expression of these enzymes induced by CA in the developing GF mice, these two control mechanisms can be studied independently.

GF mice were treated with CA on days 9 and 17 and sacrificed 5 days thereafter. When these GF pups were treated with CA on day 9, trehalase activity was induced from low levels to elevated adult levels by day 14 ($p < 0.01$). However, when these GF pups were treated with CA on day 17, there was no change in trehalase activity. Note that the trehalase activity had already reached adult levels by this time and probably could not be further induced by CA (Figure 6C). Induction of trehalase activities was accompanied by induction of mRNA levels (Figure 6D), suggesting that these changes are regulated at the level of transcription. In GF mice, βGT activity and mRNA were also low on day 9 and were induced by CA treatment to adult levels by day 14 ($p < 0.01$) (Figure 6A and 6B). However, following treatment with CA on day 17, the βGT expression remains at low suckling levels. Thus in the absence of microflora, trehalase activities follow the normal developmental pattern, but in the absence of microflora βGT activity remains at immature levels, irrespective of CA treatment, suggesting that the developmental induction of βGT is primarily regulated by colonization by adult microflora. This observation suggests that the loss of glucocorticoid responsiveness is an intrinsic property of intestinal ontogeny and is independent of any enzyme induction by microflora. Thus early postnatal development is primarily controlled by intrinsic mechanisms and is sensitive to steroids, while after weaning, some enzymes, such as glycosyltransferases, are modulated primarily by extrinsic factors (microbial colonization).

**Discussion**

This is the first report that intestinal βGT activity is induced during weaning, increasing from low levels in the immature gut to elevated levels that characterize the adult intestine. These changes in enzyme activity are accompanied by...
parallel changes in the level of βGT mRNA, suggesting transcriptional control of the increase in expression of this gene during gut maturation. βGT activity proved to be a useful marker for maturity of intestinal glycoconjugate expression.

Traditionally, disaccharidases have been used as developmental markers for investigating intestinal maturation (Henning, 1987; Mircheff et al., 1985; Nanthakumar and Henning, 1993). The phenotype of the suckling gut is characterized by high lactase and low trehalase and sucrase activities (Buller et al., 1990; Nanthakumar and Henning, 1993, 1995; Oesterreicher et al., 1998). During the weaning period, both trehalase and sucrase rapidly increase to adult levels in the mouse intestine while lactase decreases. Glycosyltransferase activities also change rapidly during this period, with α2,3/6-sialyltransferase activity changing from an initial high activity to the lower activity of the adult gut, whereas fucosyltransferase activity increases from an initially low activity to the elevated activity of adult gut (Dai et al., 2002; Nanthakumar et al., 2003). In this study, we found that both trehalase and βGT increase with the maturation of the gut, but trehalase, like sucrase, is controlled more by an intrinsic mechanism of gut ontogeny. In contrast, βGT, like α1,2-fucosyltransferase (Bry et al., 1996; Hooper et al., 2001), is controlled more by extrinsic factors. Therefore, trehalase and βGT are good markers for comparing the relative contributions of intrinsic and extrinsic factors in gut ontogeny.

In mature mice, neither glucocorticoids nor luminal microbes caused increases in levels of trehalase and sucrase

![Fig. 3. Effect of cortisone acetate (CA) on (A) βGT activity and (B) mRNA levels and (C) trehalase activity and (D) mRNA in the mouse ileum during postnatal development. Six litters of pups were used; on postnatal days 9, 13, 17, and 23, half the pups in each litter were treated with CA and the other half were treated with vehicle (VEH; saline). The pups were sacrificed after 5 days and assayed. Each bar represents the mean ± SE of three to four samples. Each sample was a pool of six mice, *p < 0.05 versus control (VEH) group.](https://academic.oup.com/glycob/article-abstract/15/3/221/574687)
activities (Midtvedt et al., 1987; Nanthakumar and Henning, 1993). However, because these marker enzymes are already at maximal levels by the fourth postnatal week, the lack of glucocorticoid responsiveness could be due to true lack of intrinsic tissue responsiveness at this age or simply due to the use of markers that have already reached maximum activity. In GF mice, the continued low expression of βGT levels into adulthood provides an opportunity to determine whether the lack of intestinal responsiveness to steroids is truly a characteristic feature of mature gut.

Figure 6 shows that GF mouse intestine after day 18 of postnatal life is unresponsive to steroids, as measured by the lack of change in the immature levels of βGT. In a similar manner, 4-week-old GF mice also failed to respond to steroids (data not shown). Thus the loss of steroid responsiveness during the third postnatal week is an independent intrinsic feature of mouse gut development and not affected by extrinsic factors. This may provide guidance in the timing of the prophylactic use of glucocorticoids in premature infants to prevent necrotizing enterocolitis (NEC), a destructive inflammatory disease of the immature gut.

NEC is a devastating gastrointestinal tract disease of premature infants whose etiology is unclear. When premature infants are treated with glucocorticoids to prevent respiratory distress syndrome (Jobe, 2004; Kattner et al., 1992; MacKendrick and Caplan, 1993; Neu, 1996), a coincidental decrease in NEC is observed. The degree of prevention is much greater with prenatal than with postnatal treatment (Bauer et al., 1984). NEC is typically associated with an underdeveloped gut (Furlano and Walker, 1998;
Kliegman et al., 1993). Thus exogenous glucocorticoids may act to accelerate intestinal maturation to afford protection against inflammation. However, not all studies on the use of glucocorticoids resulted in a decrease of NEC incidence (Jobe, 2004; Kosloske, 1994) because the intestine is sensitive to glucocorticoids for a restricted period during development (Nanthakumar and Henning, 1993, 1995). The loss of tissue responsiveness to steroids observed herein is consistent with a greater reduction in the risk of NEC with prenatal steroid exposure than with neonatal steroid treatment; however, NEC is a multifactorial disease whose etiology includes an inappropriate response to bacterial colonization after the introduction of enteral feeding (Furlano and Walker, 1998; Kattner et al., 1992; Kliegman et al., 1993; MacKendrick and Caplan, 1993; Neu, 1996). Therefore the interrelationship between steroid-induced intestinal maturation and bacterial colonization may be an important element in our understanding the pathophysiology of this inflammatory disease of the immature gut.

Bacteria colonize the luminal surface of the gut through binding to specific glycoconjugates (Dai and Walker, 1999; Karlsson, 1995). The surface of the intestinal epithelium has abundant glycoproteins and glycolipids intrinsic to the apical brush border membrane, as well as secreted glycoproteins on the apical surface (such as digestive fluids and mucus) and on the basolateral surface (such as extracellular matrix components and signaling molecules) (Dudeja et al., 1988; Mircheff et al., 1985; Morita et al., 1986; Mulivor et al., 1978; Robbe et al., 2003; Roth et al., 1985, 1986; Srivastava et al., 1987). Many of the terminal glycans of these glycoconjugates serve as receptors for pathogenic microbes in the gut lumen. For example, galactose residues of O-linked glycoconjugates play an essential role in the early stages of Entamoeba histolytica attachment and invasion (Hughes et al., 2003) and in enteroaggregative Escherichia coli adhesion (Grover et al., 2001). A more detailed knowledge of the galactose-terminal glycoconjugate structures and their functions in gut and the role of βGT in generating these receptors is needed to better understand the pathophysiology of galactose-binding organisms. Specifically, regional specific expression of galactose-containing glycans and their temporal changes in expression during development helps us understand the regional affinity and temporal susceptibility of gut to infection during development.

Changes in expression of glycosyltransferases can result in an alteration in the composition of the terminal moieties of glycoconjugates that control important developmental processes (Biol et al., 1987; Kotani et al., 2001; Lu et al., 1997). The importance of the βGT is apparent by the disruption of normal development in its absence (Kido et al., 1999; Kotani et al., 1999; Love et al., 2001). The targeted disruption (knock-out) of the mouse βGT gene is semi-lethal during the weaning period. However, the cause
of this lethality is not well understood (Asano et al., 1997; Lu et al., 1997). Curiously, in these mutant mice, the expression of an adult phenotype in the gut is apparent by the end of the first postnatal week (Asano et al., 1997). These studies suggest that βGT plays a critical role in epithelial differentiation in developing mouse intestine and may be necessary for handling the microbial colonization in the gut lumen associated with weaning (Asano et al., 1997).

Although endocrine insufficiency of the anterior pituitary gland has been postulated as a mechanism in the lethal phenotype of these mutant mice (Lu et al., 1997), alteration in the gut physiology due to lack of βGT during the weaning period may also contribute.

In CONV mice, the timing of the changes in intestinal βGT coincides with a shift from microflora characteristic of suckling animals to that of adults. In this study we have attempted to begin addressing the question of whether changes in mucosal glycoconjugate expression select for the nature of colonizing microflora or whether a change in colonizing microflora induces changes in intestinal glycoconjugate expression (microbial-epithelial cross-talk).

CONV suckling mice have a different microbial ecosystem than that of adult mice. When the adult GF mice were inoculated with microbes from immature animals, βGT expression was not induced, but when inoculation occurred with adult microflora βGT expression was induced to the levels comparable to CONV adult mice (Figure 4). These data imply that the elevated levels of intestinal βGT that characterize the adult gut are dependent on colonization by specific bacteria at the time of weaning that are intrinsic...
constituents of the commensal flora in the adult intestine. This change in βGT can be used as a marker for changes in activity of many glycosyltransferases that are induced during the general shift in microflora at weaning (Biol et al., 1987; Lenoir et al., 2000; Srivastava et al., 1987), which in turn cause a modification in the types of glycoconjugates expressed in gut during development. For example, the activity of N-acetylagalactosyltransferase also increases during postnatal development of the mouse gut (Dall’Olio et al., 1990). One would expect the change in expressed glycoconjugates in the intestinal mucosa to influence the type of bacteria colonizing the gut. It is possible, however, that one subset of specific colonizing bacteria induced these shifts in glycoconjugate receptors through specific alteration in gene expression, which in turn select for those and other bacteria with this glycoconjugate as an adhesion factor to become part of the stable adult microflora (Midtvedt et al., 1987; Umesaki et al., 1995). These phenomena may represent a mutually beneficial cross-talk during intestinal ontogeny, that is, a reciprocal communication between colonizing bacteria and the gut in which bacteria influence the ontogeny of the gut, which in turn causes expression of glycoconjugates that help select and stabilize the colonizing microflora of the adult gut.

This study distinguishes between intrinsic and extrinsic regulation of intestinal ontogeny in mice using two markers of development: (e.g., trehalase as a marker for the general development of gut and βGT as a marker of maturation of glycosylation subsystems in gut development). Glucocorticoids may modify the intrinsic genetic program of the developing intestine before the onset of weaning, as exemplified by disaccharidase expression, whereas microbial colonization appears to be a major extrinsic modifier of development from weaning to adulthood, as exemplified by glycosyltransferase expression. Extrinsic factors, circulating glucocorticoids, and the intrinsic genetic program of a tissue work synergistically and are responsible for the rapid development of the gut that occurs during the third postnatal week, thus accommodating the digestive function of the nutrients as well as accompanying changes in the resident microbial ecosystem and development of the mucosal defense. These mechanisms may underlie reciprocal control between resident bacteria and the intestinal mucosa (cross-talk) in which bacteria induce the production of glycoconjugates to which they bind, reinforcing their stable colonization of the gut to the mutual benefit of the human host and the microflora. We hypothesize that premature infants are unable to participate in such symbiosis and that their inappropriate colonization may lead to the onset of imbalance leading to diseases (e.g., NEC). In addition, the use of GF mice provides an approach to the understanding of the role of changes in intestinal glycoconjugates in elucidating the pathobiology of enteric infections.

Materials and methods

Reagents

Agalacto-mucin, bovine serum albumin, 2-mercaptoethanol, phenylmethylsulfonylfluoride, and ultra-pure sucrose were purchased from Sigma Chemical (St. Louis, MO). UDP [14C]galactose (0.1 μCi, specific activity = 1.8 mCi/mmol) was purchased from New England Nuclear Life Sciences (Boston, MA). TaqMan reverse transcription reagents and TaqMan Gold real time polymerase chain reaction (RT-PCR) kits were purchased from Perkin-Elmer (San Ramone, CA). Streptavidin–horseradish peroxidase conjugate was purchased from Amersham Life Sciences (Piscataway, NJ). Biotinylated Ricinus communis-I agglutinin (RCA-I) and FITC-conjugated RCA-IA were purchased from E-Y Laboratories (San Mateo, CA). Control glycoprotein transferrin was purchased from Roche Diagnostics (Indianapolis, IN). Cortisone acetate was from Merck, Sharp & Dohme (West Point, PA). All other reagents were of analytical or molecular biology grade from Fisher Scientific (Fairlawn, NJ) and Sigma.

Experimental animals

Black Swiss mice were purchased from Taconic Farms (Germantown, NY) as young adults (4–6 weeks) or as pregnant dams at 16–18 days of gestation. Their pups’ date of birth was designated as day 0. Pups were housed with their dams through 21 postnatal days under conventional conditions, whereupon they were weaned to mouse chow and water ad libitum in an animal room with a 12-h light/dark cycle and access.

Cortisone treatment

Each litter of suckling mice was divided randomly into two groups. At 10 days of age, one group was injected subcutaneously with a single dose (5 mg/100 g body weight) of cortisone acetate suspended in saline. The other group (controls) was injected with the same volume of normal saline (0.9% NaCl). The animals were maintained with their dams until they were sacrificed at 14 days of age. Cortisone-treated adult animals were injected on day 28 and sacrificed on 28, 32, 35, and 42 postnatal days.

GF animals and conventionalization

GF mice of the same strain were purchased from the same vendor at the desired ages. All GF animals were removed from the GF environment immediately before being sacrificed by cervical dislocation. XGF mice were produced by removing GF mice from their GF environment at the age of 4 weeks, inoculating them with intestinal contents from age-matched CONV mice as previously described (Dai et al., 2002; Midtvedt et al., 1987), and keeping them in the same cage as CONV mice. They, along with age-matched GF and CONV mice, were sacrificed 2 weeks later by cervical dislocation. All animals were sacrificed between 12 noon to 3 PM to avoid circadian influences.

Preparation of enzyme fractions

The entire small intestine and colon were removed and thoroughly flushed with ice-cold 0.9% NaCl. The small intestine was divided into duodenum, jejunum, and ileum as follows: The small intestine from the stomach to the ligament of Treitz was defined as duodenum, and the proximal and distal halves of the remaining small intestine were defined as jejunum and ileum, respectively. The intestine was placed on a glass plate maintained at 4°C and cut open;
the mucosa was harvested by scraping with a microscope glass slide. All subsequent procedures were performed at 4°C. A 10% mucosal homogenate in 0.1 M Tris–HCl buffer (pH 7.4) was centrifuged at 1,000 × g for 15 min to remove nuclei and cellular debris. The supernatant was then centrifuged at 105,000 × g for 1 h in a Beckman L6-65 ultracentrifuge with 50.3 Ti rotor, leading to sedimentation of a microsomal fraction and to a soluble cell fluid. The enzyme activities were determined on the microsomal fraction. The resulting pellets were resuspended in the same buffer used for homogenization, aliquoted, frozen, and stored at −80°C, or used immediately for the enzyme assay.

Protein determination

Protein was determined using a BCA protein assay (Pierce, Rockford, IL) modified for use in 96-well microtiter plates according to the manufacturer’s protocol. To each protein sample of 50 µl, 200 µl of working reagent was added, followed by incubation at 37°C for 30 min. Absorbance at 560 nm was measured on a microtiter plate reader (BT 2000 Microkinetics Reader Spectrophotometer, Fisher Biotech, Pittsburgh, PA). The concentration of each protein sample was calculated using a standard curve produced with bovine serum albumin.

GT assay

The βGT (UDP-Gal: GlcNAc βGal βGT) is the primary enzyme responsible for transferring terminal galactose from UDP-galactose to terminal N-acetylglycosamine of complex N-glycans. Asialo-agalactofetuin was the exogenous acceptor for the assay of intestinal βGT (Lenoir et al., 2000; Ozaki et al., 1989). The reaction mixture (total volume 0.1 ml) contained 0.4 mg asialo-agalactofetuin, 5 mM ATP, 100 mM cacodylate acetate (pH 6.3), 20 mM MgCl₂, 0.5% Triton X-100, an appropriate amount of enzyme solution (50–100 µg protein), and UDP-[14C]galactose (29.9 Ci/mmol, New England Nuclear) diluted with nonradioactive UDP-galactose (Sigma) to attain a concentration of 0.6 mM, 0.5 µCi. Incubation was carried out at 37°C for 1 h. The radioactive product formed was collected on Gelman GN-4 nitrocellulose filters and measured in an LKB scintillation beta counter (Dai and Walker, 1999). Both UDP-galactose and the exogenous protein acceptor were present at saturating concentrations, and product formation was linear for 1 h of incubation time and up to 150 µg enzyme protein under these conditions. The activity of βGT was stable in storage at −20°C for a year.

RNA preparation

Tissues were frozen in liquid nitrogen and stored at −80°C. The frozen tissue was homogenized, and total RNA was extracted using the TRIZOL reagent according to the manufacturer’s protocol (Invitrogen, San Diego, CA). Total RNA was then dissolved in 50 µl of RNase-free water, and concentration was determined by measuring absorbance at 260/280 nm.

cDNA synthesis

RNA (1 µg) was used to generate cDNA using TaqMan Reverse Transcription Reagents according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Briefly, random hexamers were used to prime RNA samples for reverse transcription using MultiScribe Reverse Transcriptase.

qRT-PCR

Real-time quantitative PCR (qRT-PCR) was performed in an ABI 7700 sequence detection system using the TaqMan Cytokine Gene Expression Plate I (Applied Biosystems). The plate consists of a MicroAmp Optical 96-well reaction plate arranged into six columns for each mRNA in triplicate. Each column is made up of identical wells containing TaqMan primers and probes for βGT or trehalase mRNA with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. PCR primers and probes for βGT, trehalase and GAPDH were made as described previously (Dai et al., 2002; Lu et al., 1997). The TaqMan probe was labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5′ end and a fluorescent dye quencher, TAMRA (6-carboxy-tetramethyl-rhodamine), at the 3′ end. PCR primers and a TaqMan probe for GAPDH were obtained from Perkin-Elmer.

Detection of PCR products was measured by two dye layers to detect the presence of target and control sequences. The FAM dye layer yields the results for quantitation of target mRNA, and the VIC dye layer yields the results for quantification of the GAPDH RNA endogenous control. Reaction mixtures for the qRT-PCR had a final volume of 50 µl containing 5 µl of cDNA and 25 µl of the master mix. Amplification conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The endpoint used in RT-PCR quantification, C_T, is defined as the PCR cycle number that crosses the signal threshold. C_T values range from 0 to 40, with the latter number assumed to represent no product formation. Quantification of gene expression was performed using the comparative C_T method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to the housekeeping gene. To calculate the fold change (increase or decrease), the C_T of the housekeeping gene GAPDH was subtracted from the C_T of the target gene to yield the net C_T(C_Tn). Change in expression of the normalized target gene as a result of an experimental manipulation was expressed as 2−C_Tn where C_Tn = C_T samples − C_T controls. PCR reactions lacking either cDNA, primers, or reverse transcriptase were run as controls.

Lectin-fluorescent staining with RCA-I

Analyses of β1,4 galactosyl glycoconjugates was performed on frozen tissue sections using FITC-conjugated RCA-I agglutinin. One centimeter of tissue from each region was fixed for 2 h in 4% paraformaldehyde at 4°C, washed in ice-cold phosphate buffered saline (PBS) containing 30% sucrose overnight at 4°C, and embedded in OCT. Frozen sections (6–7 µm thick) were blocked with PBS containing 2% bovine serum albumin, then stained with FITC-RCA-I for 1 h (10 µg/ml). Sections were then washed three times in cold PBS, mounted using Anti-Fade (Vector Laboratories, Burlingame, CA), and analyzed by fluorescent and/or confocal microscopy.
Statistical treatment of results

Results are expressed as the mean ± SE. Effects of age and treatment on enzyme activities were analyzed by two-way ANOVA. After overall significance was confirmed, post hoc tests for individual variables were performed by a two-tailed unpaired t-test. Differences with a p value < 0.05 were considered significant.

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Abbreviations

ANOVA, analysis of variance; βGT, β1,4-galactosyltransferase; CA, cortisone acetate; CMP, cytosine monophosphate; CONV, conventional; FITC, fluorescein isothiocyanate; GALT, gut-associated lymphoid tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GF, germ-free; NEC, necrotizing enterocolitis; PBS, phosphate buffered saline; RCA-I, Ricinus communis-I agglutinin; RT-PCR, real time polymerase chain reaction; XGF, germ-free conventionalized.

References


