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Glucocorticoids and microbiota regulate ontogeny of intestinal fucosyltransferase *2* requisite for gut homeostasis

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At weaning, the intestinal mucosa surface glycans change from predominantly sialylated to fucosylated. Intestinal adaptation from milk to solid food is regulated by intrinsic and extrinsic factors. The contribution by glucocorticoid, an intrinsic factor, and colonization by microbiota, an extrinsic factor, was measured as the induction of α1,2/3-fucosyltransferase and sucrase-isomaltase (SI) activity and gene expression in conventionally raised, germ-free, and bacteria-depleted mice. In conventionally raised mice, cortisone acetate (CA) precociously accelerated SI gene expression up to 3 weeks and *fut2* to 4 weeks of age. In germ-free mice, CA treatment induces only SI expression but not fucosyltransferase. In post-weaning bacteria-deficient (germ-free and bacteria-depleted) mice, *fut2* expression remains at low suckling levels. In microbiota deficient mice, intestinal *fut2* (but not *fut1*, *fut4* or *fut7*) was induced only by adult microbiota, but not immature microbiota or CA. *Fut2* induction could also be restored by colonization by *Bacteroides fragilis*, but not by a *B. fragilis* mutant unable to utilize fucose. Restoration of *fut2* expression (by either microbiota or *B. fragilis*) in bacteria-depleted mice is necessary for recovery from dextran sulfate sodium-induced mucosal injury. Thus, glucocorticoids and microbes regulate distinct aspects of gut ontogeny: CA precociously accelerates SI expression and, only in colonized mice, *fut2* early expression. The adult microbiota is required for the *fut2* induction responsible for the highly fucosylated adult gut phenotype and is necessary for recovery from intestinal injury. *Fut2*-dependent recovery from inflammation may explain the high incidence of inflammatory disease (Crohn's and necrotizing enterocolitis) in populations with mutant *FUT2* polymorphic alleles.

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Introduction

Development of the mammalian gastrointestinal tract is divided into three major phases demarcated by birth and weaning: in utero development, the suckling period, and the juvenile phase. The transition from one phase to another is precisely coordinated and tightly regulated by intrinsic and extrinsic factors. The first major transition at birth prepares fetal gut for digestion and absorption of milk. The second transition at weaning prepares the gut for digestion and absorption of solid food (Henning 1987; Montgomery et al. 1999; Nanthakumar 2001). The first transition is often assessed by the expression of lactase, responsible for digesting the milk sugar lactose, a suckling phenotype marker (Henning and Nanthakumar 1994). The second transition is typically measured as the induction of disaccharidases, such as sucrase, isomaltase, maltase, etc., which are responsible for terminal digestion of carbohydrates of adult food. These markers are traditionally used to measure the onset of intestinal maturity (Henning and Nanthakumar 1994; Nanthakumar 2001). The maturational changes of these enzyme markers are regulated at the level of transcription by intrinsic and extrinsic mechanisms.

Critical intrinsic and extrinsic factors that regulate intestinal maturation are circulating glucocorticoids and gut microbiota, respectively. Using sucrase induction as an outcome, exogenous and endogenous circulating glucocorticoid has been shown to precociously induce intestinal maturation (Nanthakumar and Henning 1993). The in vivo importance of glucocorticoids was reaffirmed by the discovery of a developmental surge of circulating corticosterone in the rat and mouse 2 days prior to the initiation of sucrase induction (Martin and Henning 1984). Elimination of this endogenous surge by adrenalectomy confirms the importance of glucocorticoids for coordination of this developmental transition (Martin and Henning 1984; Nanthakumar and Henning 1993; Henning and Nanthakumar 1994). Both exogenous and endogenous glucocorticoids administered during the first week of post-natal life can also precociously induce the adult phenotype of rodent gut (Martin and Henning 1984; Nanthakumar and Henning 1993; Henning and Nanthakumar 1994). In addition to inducing digestive and absorptive capacity to adult food, glucocorticoids can also accelerate the proliferative and migratory capacity of the newly differentiated cells in the small intestine (Henning 1987;

Montgomery et al. 1999). The direct effect of glucocorticoids on the proliferative and maturational capacity of the intestine has been demonstrated in explant cultures (Simo et al. 1992). However, intestinal responsiveness to glucocorticoids is lost by the fourth post-natal week, after the intestine matures into the adult intestinal phenotype (Nanthakumar and Henning 1993); the mechanism responsible for the loss of glucocorticoid responsiveness is not known. Disaccharidases are widely used as tissue-specific markers to study the mechanism of steroid action on initiating the onset of the adult phenotype (Nanthakumar 2001; Nanthakumar et al. 2005). However, disaccharidase maturation is insensitive to luminal factors, including microflora (Henning 1987; Nanthakumar 2001). Developmentally dependent expression of glycosyltransferases can be influenced by the microbiome (Dai et al. 2000; Nanthakumar et al. 2003) and are also good markers of mucosal maturation. Thus, studying the interplay between steroids and luminal microbes in the ontogeny of gut maturation can be achieved by monitoring both a representative disaccharidase and glycosyltransferase.

The terminal ends of glycoconjugates in the suckling gut are predominantly sialic acid, whereas in the adult they are predominantly fucose (Biol et al. 1987; Dai et al. 2000). Therefore, the ratio of sialic acid to fucose in non-reducing terminal residues of glycoconjugates in the rodent gut epithelium reverses from high (sialic acid dominant) to low (fucose dominant) at the time of weaning (Biol et al. 1987; Dai et al. 2000, 2002; Nanthakumar et al. 2003). These changes in the composition of the terminal ends of glycans are due to decreasing levels of sialyltransferase activity with concurrent increases in the level of fucosyltransferase activity (Biol et al. 1987; Bry et al. 1996; Dai et al. 2000, 2002; Nanthakumar et al. 2003). The developmental decline in α2,3/6-sialyltransferase activity are observed in conventionally raised mice. However, in the absence of microbes in germ-free mice, these developmental changes of α2,3/6-sialyltransferase enzyme activities are not observed (Dai et al. 2002), and the suckling pattern of glycosylation persists (Dai et al. 2002; Nanthakumar et al. 2003). However, introduction of a mixture of adult fecal microbiota or *Bacteroides thetaiotaomicron*, one of its predominant species, can initiate fucosyltransferase expression to adult type within 2 weeks (Bry et al. 1996; Nanthakumar et al. 2003). Thus, differential glycosylation from the sialic acid predominant suckling phenotype to the fucose dominant adult phenotype of the mucosa defines the transition from pre- to post-weaning mouse intestine.

α1,2-Fucosyltransferase and sucrase activities were used as markers to delineate the role of extrinsic and intrinsic factors on this post-natal transition. The studies specifically address whether regulation of developmental α1,2-fucosyltransferase (FucT II) expression is independent of sucrase ontogeny, glucocorticoids regulate these processes, gut colonization influences this regulation, and post-weaning mucosal fucosylation influences homeostasis of adult gut. The ontogeny of α1,2-fucosyltransferase (GDP-Fuc: α1,2-fucosyltransferase) and sucrase-isomaltase (SI) gene expression was determined by measuring their enzyme activities, mRNA levels, and glycoconjugate expression in the mouse gut. These developmental studies were carried out using conventionally colonized mice, uncolonized germ-free and bacteria-depleted mice, and re-colonized mice. Recolonized mice were previously germ-free and bacteria-depleted mice, which had been inoculated with normal gut microbes freshly collected from age-matched colonized mice. The roles of intrinsic (glucocorticoid) and extrinsic (gut microbiota) factors were monitored in duodenum, jejunum, ileum and colon to determine their interactions on gut maturation pre- and post-weaning. Finally, the role of bacteria-induced fucosyltransferase activity on gut homeostasis was investigated. These experiments are designed to help understand the physiology of controlling gut development thought to underlie emerging inflammatory diseases whose risk factors include *FUT2* polymorphisms.

Results

In conventionally colonized mice, the developmental expression of α1,2/3-fucosyltransferase activity and of sucrase activity was measured in the intestine for the first 6 weeks of life. Week 2 represents the preweaning gut, and week 6 represents the mature gut. The α1,2/3-fucosyltransferase assay measured changes in α1,2- or 1,3-fucosyltransferase activity, simultaneously screening for changes in the expression of several fucosyltransferase genes. We devised the assay with phenyl-β-D-galactoside, a substrate that measures broad α1,2- and 1,3-fucosyltransfease activity, as we did not know *a priori* which might be affected. The fucosyltransferase activity increased in all regions of the intestine between the 2nd and 3rd weeks of life and reached the mature level found in the adults by the fourth week ($P < 0.001$; Figure 1). The sucrase activity followed a parallel increase in the maturing duodenum, jejunum, and ileum ($P < 0.001$); sucrase is not expressed in the colon. Although all four regions displayed maturation-dependent increases in fucosyltransferase activity, absolute activities were much lower in the proximal gut than in the distal gut. SI activity was highest in the jejunum and lowest in the duodenum and ileum. These systematic regional differences follow their function: the digestion of disaccharides occurs mostly in the jejunum, and this is where the disaccharidases are expressed most heavily. In contrast, if the fucosylated cell surface of the mucosa relates to their interaction with the microbiota (Nanthakumar et al. 2003), the increasing proximal-to-distal gradient follows that of the density of the microbiota. Both of these activities increase with the maturation of the gut at weaning. Note that fucosyltransferase activity is initially high in colon, even during the suckling period, and the colon is also heavily colonized at this time.

Mice were treated with CA on day 10; on day 14, fucosyltransferase and sucrase activities were measured to determine glucocorticoid effect on intestinal development. CA administration accelerated the maturation of intestinal fucosyltransferase and sucrase activities in all regions of the small and large intestine (Figure 2A).

The change in the α1,2/3-fucosyltransferase activity could be due to changes in the expression of any of several fucosyltransferase genes. To determine which of the α1,2- or α1,3-fucosyltransferase genes were driving this change, expression of *fut*1 and *fut*2 (the α1,2 gene family) and *fut*4 or *fut*7 (representing commonly expressed members of the α1,3 gene family) were measured as levels of their respective mRNAs. Under

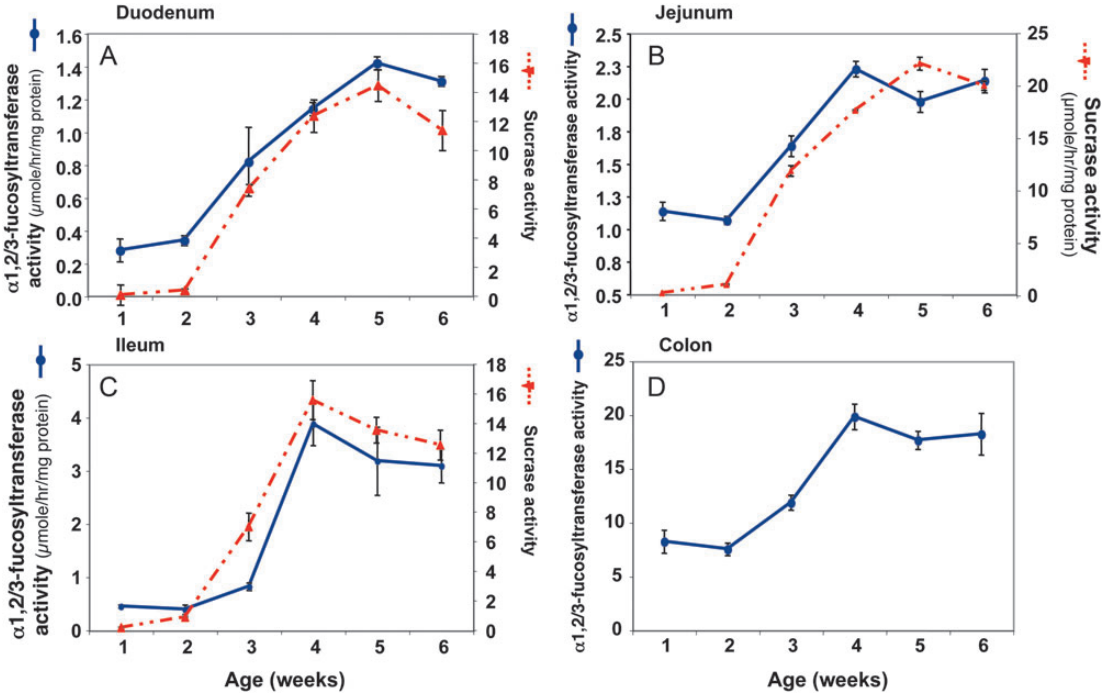

Fig. 1. Post-natal development of α1,2/3-fucosyltransferase activity (FucT —●—) (nmol/mg protein/h) and sucrase activity ···✝··· (μmol/mg protein/h) in the mouse (**A**) duodenum, (**B**) jejunum, (**C**) ileum and (**D**) colon. Six to eight litters of pups were used, and age denotes the post-natal week. Results are expressed as the mean ± SEM compiled from three samples. Tissues were pooled from six to eight mice per sample for 1- and 2-week-old mice and at three tissues per sample for 4- and 6-week-old mice.

all of our experimental conditions that resulted in induction of α1,2/3-fucosyltransferase activity, only expression of *fut*2 mRNA increased, and the magnitude of change was greater than that of the enzyme activity, consistent with the changes in the mixed fucosylation activity of the intestinal mucosa being driven primarily by altered transcription of the *fut*2 gene.

In Figure 2B, the increase in fucosyltransferase activity and sucrase activity in response to CA corresponded to an even more pronounced increase in *fut2* mRNA and SI mRNA, respectively. In contrast, these activities do not change in other organs such as the liver (not shown), indicating that cortisone induces precocious maturation specifically in the maturing intestine.

The duration of sensitivity to cortisone induction was measured for both fucosyltransferase and sucrase. Mice were treated on days 9, 13, 17, and 23 with CA or vehicle control, and the pups were sacrificed on postnatal days 14, 18, 22, and 28, respectively. Figure 3 shows data from the ileum, but all three regions of the small intestine displayed similar changes. The sensitivity of sucrase activity to glucocorticoid-mediated precocious induction was lost by day 17, whereas the sensitivity of *fut2* induction to CA persisted until day 22. Changes in enzyme activity were accompanied by parallel changes in *fut2* and SI mRNA expression, consistent with the cortisone effect on enzyme activity occurring at the level of transcription. The changes in fucosyltransferase activity coincided with even stronger parallel changes in *fut*2 mRNA expression, but no changes were evident in expression of *fut*1, *fut*4, or *fut*7. These data are consistent with the changes in intestinal fucosylation being specifically mediated by changes in *fut*2 gene expression. The changes in sensitivity for SI coincide with weaning and for *fut2* with the rapid change in the intestinal microflora that follows weaning.

The role of intestinal microbes *per se* on the ontogeny of intestinal *fut2* at weaning was investigated. To differentiate the effects of any programmed changes in the internal milieu at weaning from the concurrent changes in the external environment, diet, and colonization, post-natal development in conventionally colonized mice was compared with that of germ-free mice. Distinct from the intestinal increase in *fut2* during development observed in conventionally colonized mice (Figure 1), germ-free mice did not display age-related changes in fucosyltransferase activity through week 6, except for a modest increase in the colon (Figure 4). FucT II activity remained at the low levels typical of the immature mucosal phenotype, suggesting that luminal microbes might play an essential role in the normal induction of intestinal *fut2* gene expression. To directly test this hypothesis, germ-free mice were inoculated with microflora from conventionally colonized 6-week-old mice (ex-germ-free adult) and sacrificed 2 weeks after inoculation. Upon the introduction of luminal bacteria from aged-matched conventionally colonized mice, the levels of fucosyltransferase activity increased to that of conventionally colonized mice in the duodenum ($P < 0.01$), jejunum ($P < 0.01$), ileum ($P < 0.001$), and colon ($P < 0.001$) (Figure 4). In contrast, sucrase enzyme activities were similar in conventionally colonized, germ-free, and ex-germ-free mice in all regions of the small intestine, whether inoculated at 4 (Figure 4) or 6 (not

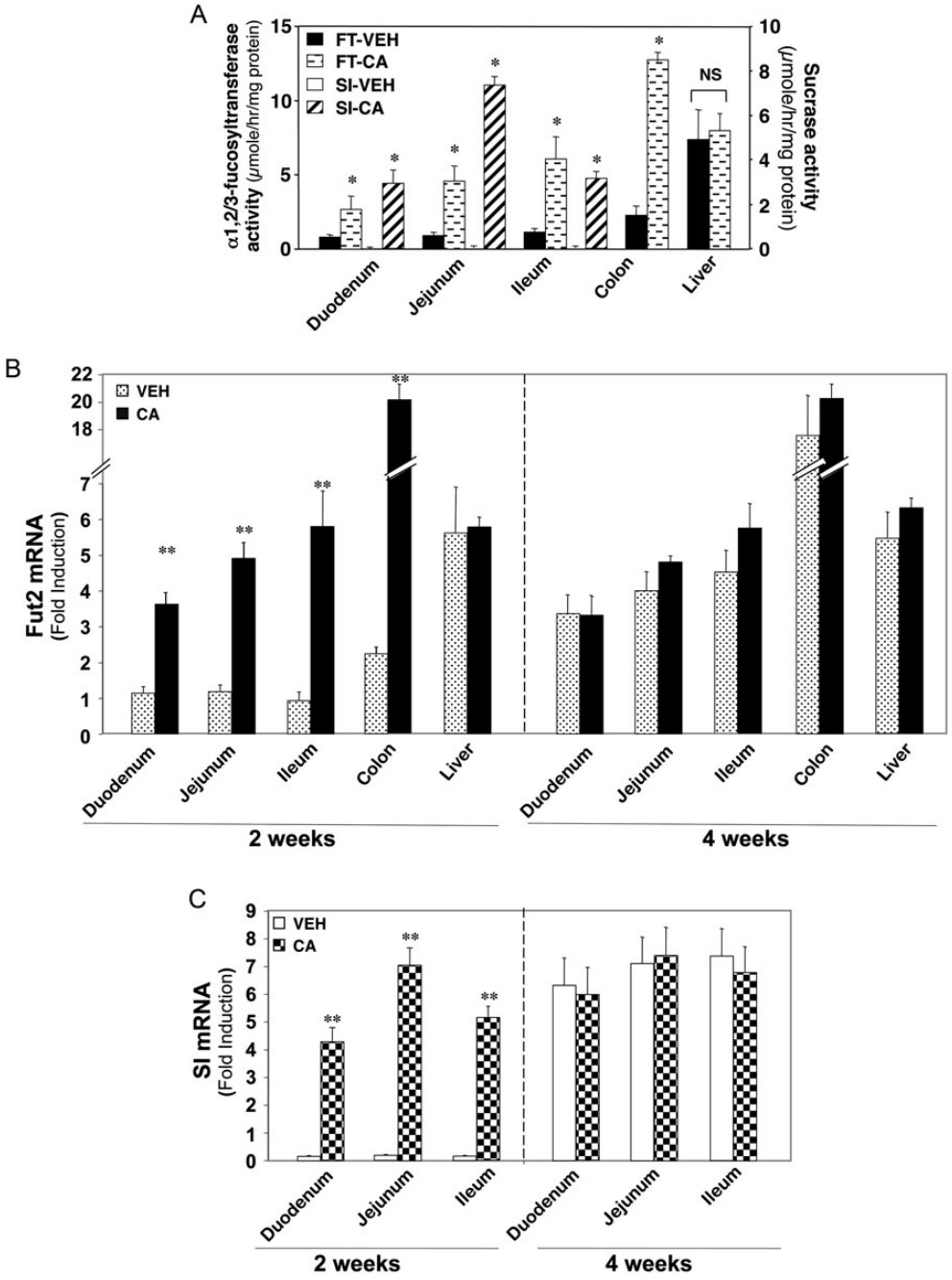

Fig. 2. Effect of CA on precocious maturation of (**A**) α1,2/3-fucosyltransferase activity (nmol/mg protein/h) and sucrase activity (μmol/mg protein/h) and (**B**) *fut2* mRNA and SI mRNA in the suckling mouse gastrointestinal tract. Four litters of pups were used; on post-natal day 10, half the pups in each litter were either treated with CA or vehicle (VEH; saline) and tissue were harvested and assayed on day 14. Each bar represents the mean ± SE of 3-4 samples. *$P < 0.05$ vs control (VEH) group.

shown) weeks of age. The next experiment was to determine if the microbiota of the suckling mouse is capable of inducing adult-type fucosylation. Bacteria-depleted mice were inoculated with the suckling microbiota isolated from a 7-day-old pup. Two weeks after inoculation, the microbiota of the adult mouse ($P < 0.001$), but not the microbiota of the suckling mouse ($P > 0.8$), was able to induce adult-type fucosylation in all segments of the small and large intestine (data not shown).

Confirming these results, levels of *fut2* mRNA (Figure 5) remained low in germ-free mice until inoculation with adult mouse microbiota, whereupon the *fut2* mRNA levels rose to those of conventionally colonized mice. In stark contrast, SI mRNA levels remained unchanged by colonization (Figure 5H). Thus, changes in intestinal FucT II activity are controlled by the induction of *fut2* mRNA by adult microbiota. No such changes were evident for the constitutive low levels of

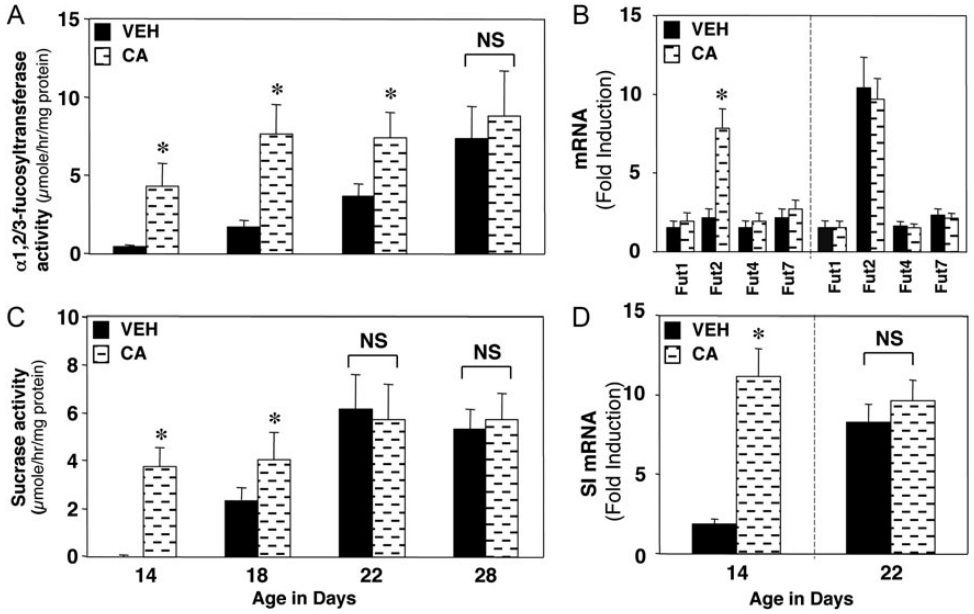

Fig. 3. Loss of glucocorticoid responsiveness of α1,2/3-fucosyltransferase activity (nmol/mg protein/h); and sucrase activity (μmol/mg protein/h); and *fut2* mRNA and SI mRNA in the developing mice ileum. Six litters of pups were used; on appropriate post-natal ages, pups were treated either with CA or vehicle (VEH; saline) and subsequently ileum was harvested and assayed. Each bar represents the mean ± SE of 3-4 samples. Each sample was pooled from six mice, *$P < 0.05$ vs control (VEH) group.

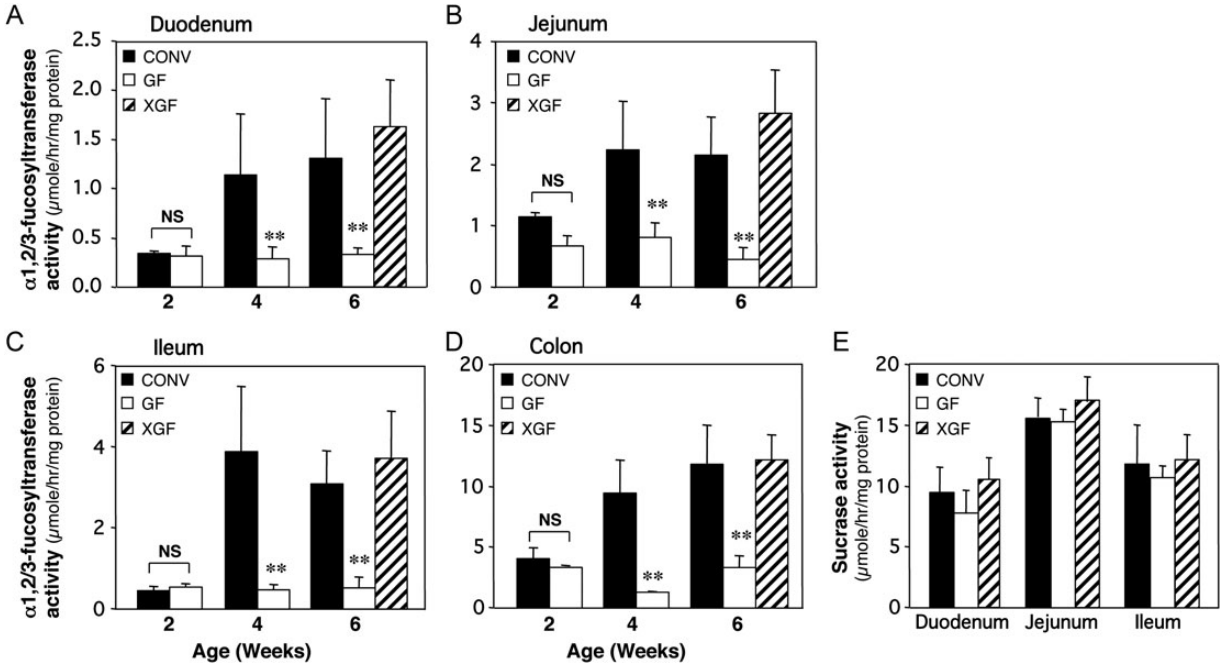

Fig. 4. Post-natal development of α1,2/3-fucosyltransferase activity (nmol/mg protein/h) in mouse (**A**) duodenum, (**B**) jejunum, (**C**) ileum and (**D**) colon of the germ-free (GF), conventional (CONV) and newly colonized germ-free (XGF) mice. Each bar represents the mean ± SE of three samples. Tissues were pooled from six to eight mice per sample for 1- and 2-week-old mice and at three tissues per sample for 4- and 6-week-old mice. **$P < 0.001$ vs CONV mice.

fut1 mRNA (Figure 5E), *fut4* mRNA (Figure 5F), or *fut7* mRNA (Figure 5G).

To determine the interaction between CA and colonization by microbiota in immature mice, the response of conventional mice to CA treatment was compared with that of germ-free mice (Figure 6). On day 10, conventional and germ-free mice were treated either with CA or vehicle control, and on day 14, the levels of SI and *fut2* were assessed in each region of the

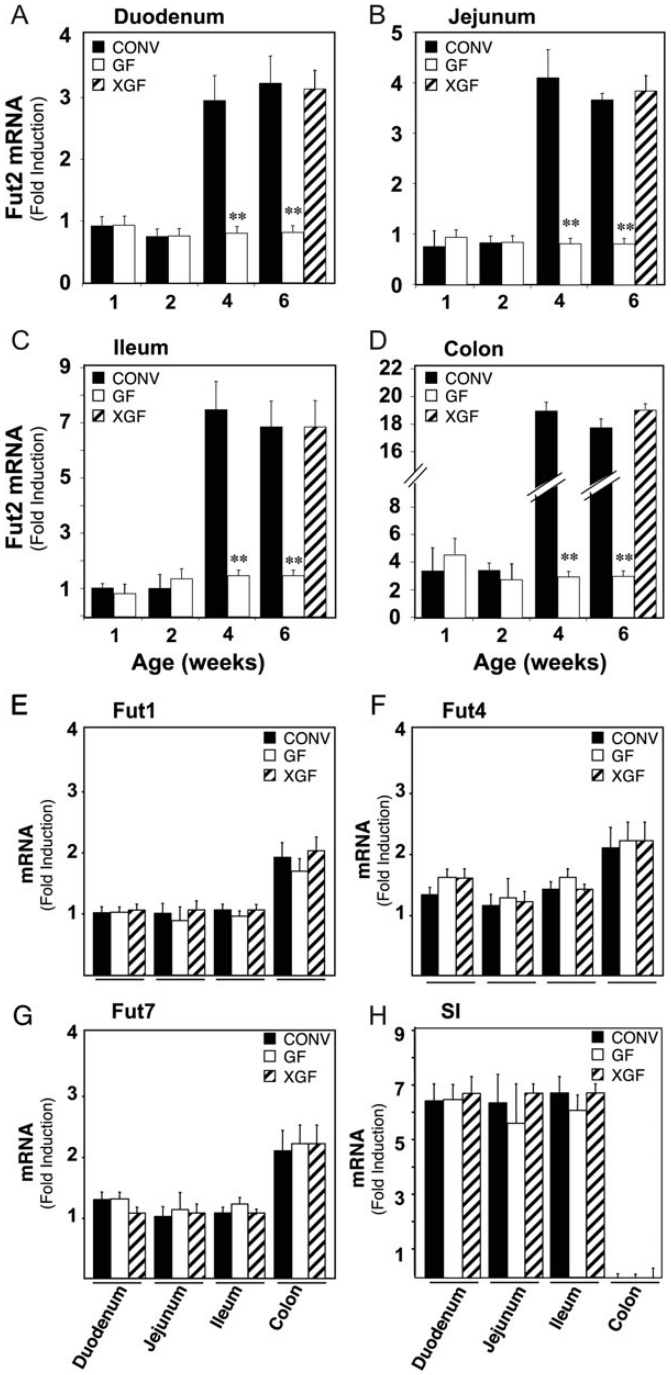

Fig. 5. Post-natal development of *fut2* mRNA in the mouse (**A**) duodenum, (**B**) jejunum, (**C**) ileum and (**D**) colon of germ-free (GF) and conventional (CONV) mice. Each bar represents the mean ± SE of three samples. Tissues were pooled from six to eight mice per sample for 1- and 2-week-old mice and at three tissues per sample for 4- and 6-week-old mice. $^{**}P < 0.001$ vs CONV mice.

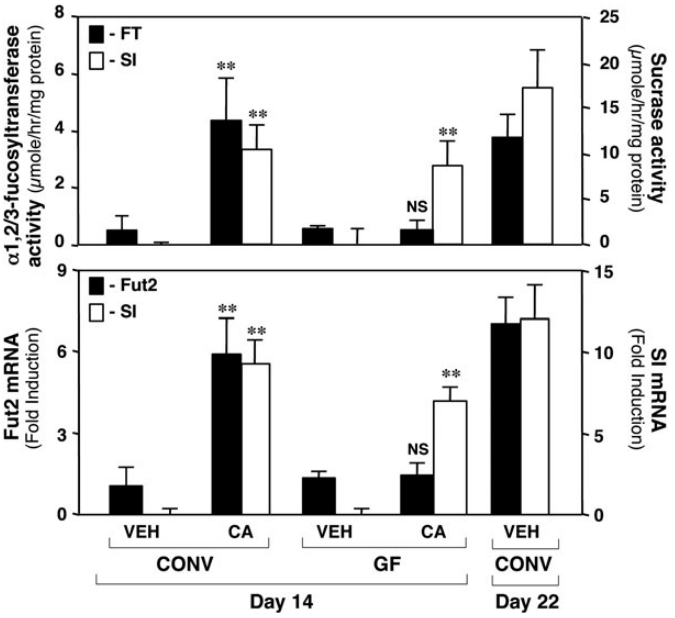

Fig. 6. Precocious induction of α1,2/3-fucosyltransferase activity (nmol/mg protein/h) and sucrase activity (μmol/mg protein/h) as well as the *fut2* mRNA and SI mRNA in mouse ileum of germ-free (GF) and conventional (CONV) mice treated with vehicle control or CA. Each bar represents the mean ± SE of three samples. Tissues were pooled from six to eight mice per sample for 1- and 2-week-old mice and at three tissues per sample for 4- and 6-week-old mice. $^{**}P < 0.001$ vs CONV mice.

intestine. As before, CA treatment precociously induced both sucrase and fucosyltransferase activities ($P < 0.001$), and their mRNA levels ($P < 0.01$), in conventional mice. However, only sucrase activity ($P < 0.001$) and mRNA ($P < 0.01$) were induced by CA in germ-free mice. The fucosyltransferase activity and *fut2* mRNA levels did not respond to CA and remained at the suckling level. This implies that signaling to the intestinal mucosa by the microbiota may be required for cortisone to induce precocious fucosylation of the gut mucosa.

Expression of α1,2-fucosylated-glycans was visualized in frozen sections by the α1,2-fucose-specific lectin, UEA-I, conjugated with Texas Red. Identical sections in which 100 mM L-fucose accompanied the Texas Red-UEA-1 during staining did not display any binding, indicating that the UEA-1 binding was specific to fucosylated moieties. Terminal α1,2-fucose-containing glycans were diminished in the colon of bacteria-depleted (Figure 7A) and germ-free (not shown) mice; however, reintroduction of the microbiota, or one of its major bacteria (*B. fragilis*), induced recovery of α1,2-fucosylated-glycans to the higher levels found in conventionally colonized mice. The changes in α1,2-fucosylated glycoconjugate expression were prominent on the cell surface, but also were apparent in the mucus of the goblet cells.

To determine whether the loss of the fucosylated phenotype of bacterially depleted mice was mediated by the loss of fucosyltransferase expression, fucosyltransferase activity and *fut2* mRNA levels were measured in these tissues. Adult levels of fucosyltransferase activity and *fut2* mRNA are lost with lack of bacterial colonization (BD; Figure 7B), but are restored upon recolonization (XBD); they are also restored by colonization with one of the dominant fucose utilizing bacteria of the adult microbiome, *B. fragilis*^{WT}, but not by the same bacterium with a mutation that deprives it of the ability to utilize fucose,

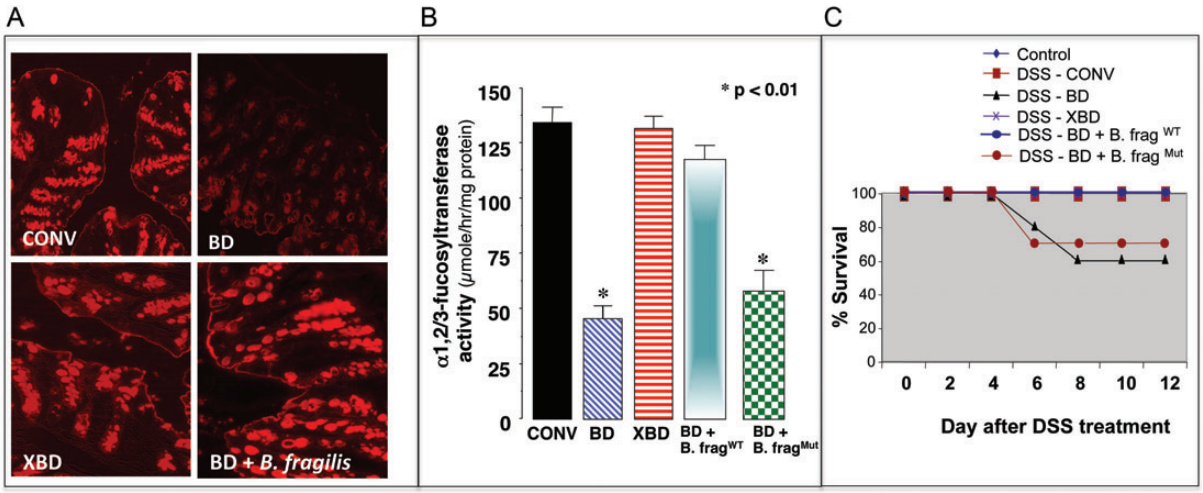

Fig. 7. (**A**) UEA-1 lectin staining of α1,2-fucosylted-conjugates in adult colonic tissue of conventional (CONV), bacteria depleted (BD) and ex-bacteria depleted (XBD) mice. The lack of fucosylation due to the lack of bacteria (BD) is reversed by recolonization (XBD) or by colonization by *B. fragilis*, a dominant bacteria of the microbiome. (**B**) Fucosyltransferase expression is lost with the loss of bacteria (BD), but is restored upon recolonization (XBD); fucosyltransferase expression is also restored by colonization with one of the dominant fucose utilizers of the adult microbiome, *B. fragilis*^{WT}, but not by the same bacterium with a mutation that deprives it of the ability to utilize fucose, *B. frag*^{Mut}. Thus, *B. fragilis* induces expression of the fucosylated phenotype (seen in panel A) through the induction of fucosyltransferase activity. (**C**) Recovery from DSS-induced mucosal injury occurs in mice that are colonized (CONV), recolonized (XBD) or colonized by wild-type *B. fragilis*, but not in mice that lack colonization (BD), or are colonized by the mutant *B. fragilis* (*B. frag*^{Mut}) and hence lack fucosylation. These data link the expression of the fucosylated phenotype with intestinal homeostasis, manifest as mucosal recovery from injury.

B. frag^{Mut}. Thus, *B. fragilis* induces the expression of the fucosylated phenotype through the induction of fucosyltransferase activity (Figure 7B).

The functional consequence of this immature, low fucosylation in bacteria-depleted mouse gut was investigated through the induction of mucosal injury by the chemical irritant, DSS. Recovery from DSS colonic inflammation was compared between conventionally colonized and bacteria-depleted mice. Conventionally colonized mice fully recover from the mucosal injury over 2 weeks, but in the absence of colonization (bacteria-depleted mice), recovery does not occur ($P < 0.01$). Recovery from DSS-induced mucosal injury occurs in mice that are colonized (CONV), recolonized (XBD), or colonized by wild-type *B. fragilis*. Recovery does not occur in mice that lack fucosylation due to a lack of colonization or are colonized by the mutant *B. fragilis* (*B. frag*^{Mut}; Figure 7C), which is unable to utilize fucose or induce mucosal fucosylation. These data link the expression of the fucosylated phenotype with intestinal homeostasis involved in mucosal recovery from injury. Recovery from the colonic damage induced by TNBS and OXA also required bacteria-dependent fucosylation (not shown). These results strongly suggest that fucosylation is dependent on colonization, and that proper *fut2*-dependent FucT II fucosylation of the intestinal mucosa is required to maintain tissue homeostasis and recovery from inflammatory chronic injury.

Discussion

Many intrinsic signals mediate and modulate the genetic program of development. Glucocorticoids are common intrinsic signals that mediate genetically programmed milestones of tissue development, including mammary gland development at puberty (Cunha and Hom 1996) and lactogenesis (Convey 1974), early lung maturation (Farrell 1977; Henning 1987), and post-natal development of the intestine (Henning 1987). Thus, the glucocorticoid surge that accompanies parturition (Farrell 1977; Henning 1987) mediates the rapid maturation of the lung needed for the immediate transition to breathing, the maturation of digestive function for the transition to obtaining nutrients directly from food (Henning 1987), preferably milk, and the modulation of the mucosal immune function (Rock 1999) that permits colonization without excess inflammation. Similarly, the glucocorticoid surge at weaning mediates intestinal adaptation to solid foods; it is modeled in the laboratory either as exogenous administration of steroids or as elicited surges in endogenous circulating glucocorticoid, and precociously induces the suckling to adult transition of the gut (Henning 1987; Henning and Nanthakumar 1994; Montgomery et al. 1999).

In the experiments herein, the initial definition of the mouse gut model involved measuring levels of mucosal fucosylation of the pre- and post-weaning small and large intestine, and defining which of the fucosyltransferase genes was responsible for any differences. All changes were compared with the changes in SI expression, a standard marker of maturation of developing intestine. As expected, glucocorticoids accelerated the maturation of gut, accompanied by precocious expression of both SI and fucosyltransferase activity. Of fucosyltransferase genes 1, 2, 4, and 7, only the gene responsible for fucosyltransferase II expression (*fut2*) was specifically induced by glucocorticoids (Figure 2). This accelerated expression by glucocorticoids could only occur in gut prior to weaning, consistent with glucocorticoids being mediators of specific maturational events in immature gut. In the mature post-weaning gut, the glucocorticoid had no further effect, as the milestones of

maturation had already been achieved in the course of normal development (Figure 3).

Likewise, in the germ-free animal, SI is only influenced by glucocorticoids before weaning (Figure 3); glucocorticoids after day 22 have no further effect. However, in the germ-free animal, fucosylation is not induced upon weaning, and the immature state of fucosyltransferase gene expression is maintained despite any surge of glucocorticoids at weaning. This is consistent with the lack of effect by exogenous glucocorticoids on fucosyltranferase expression observed in the conventionally colonized mouse after day 28 (Figure 3). These findings suggest a distinct type of developmental control of fucosyltransferase, and the difference between the germ-free and conventionally colonized mouse points to bacterial colonization as a prime candidate.

In the absence of a microbiota, the germ-free mouse gut retains its pre-weaning state of high sialyltransferase and low fucosyltransferase; with the introduction of the adult microbiota, the gut glycosylation rapidly shifts to high fucosyltransferase. This mimics the change at weaning for a normal conventionally colonized control. This is consistent with the primary control for the qualitative shift in gut glycosylation being signaling by the microbiota. Consistent with this explanation is that the adult microbiota, but not the distinctly different microbiota of the pre-weaning mouse, has the ability to induce fucosyltransferase activity in mature gut (Figure 4). Moreover, the colonization of germ-free mature gut with only one of the major mutualist symbionts of adult gut, *B. fragilis*, is sufficient to induce the adult pattern of fusosyltransferase expression resulting in the typical highly fucosylated mucosal surface (Figure 7). Moreover, the *B. fragilis* mutant unable to utilize fucose is unable to restore this fucosylation. Consistent with these data, SI is expressed only in the small intestine, the site of terminal carbohydrate digestion and absorption, whereas fucosyltransferase is expressed most heavily in the distal alimentary tract, the site of heaviest bacterial colonization. The developmental induction of *fut2* mRNA expression is accompanied by the induction of α2,3-sialyltransferase and β1,4 galactosyltransferase mRNA expression, but these are induced to a lesser extent and may represent the adaptation toward synthesizing the glycan scaffolds on the surface of the intestinal mucosa to which the fucose must be attached. In contrast to the α2,3 sialyltransferase, α2,6 sialyltransferase mRNA expression displayed a robust reciprocal decrease, consistent with the reduced sialylated phenotype in colonized mature intestinal mucosa.

Fucosylation of the intestinal mucosa is stimulated by the colonization of adult colon by adult microbiota. Mice that lack colonization, either by lack of inoculation by microbiota (germ-free mice) or by oral antibiotic treatment, lack the fucosylated mucosal phenotype. However, the colonization of germ-free mice and the recolonization of bacteria-depleted mice results in the expression of fucosylated glycans on the surface of the intestinal mucosa. This induction of fucosylation depends upon the activation of the ERK and JNK signal transduction pathways, which result in specific transcription of *fut2* mRNA, induction of fucosyltransferase activity, and the fucosylation of mucosal glycoconjugates (Meng et al. 2007). Inhibition of ERK and JNK activation in mucosa during colonization (germ-free) or recolonization (bacteria-depleted) prevents *fut2* mRNA induction and subsequent mucosal fucosylation. This suggests that constant communication between the microbiota and the epithelium is necessary for sustained expression of *fut2* mRNA transcription in the adult intestinal mucosa. *Fut1*, *fut4* and *fut7* did not display any such relationship with the microbiota, consistent with a highly specific relationship between the microbiota and *fut2*. Therefore, colonization by adult microbiota may be the primary regulator of mucosal fucosylation in the intestinal mucosa and, in turn, fucosylation of the mucosa may provide the niche for maintaining the stable microbial ecosystem in the adult gut. The interdependence of fucosyltransferase expression and the microbiota is consistent with the complex interkingdom communication typical of mutualist symbionts.

Mutualism includes both reciprocal communication and reciprocal benefit to both the microbiota and the host. Specific features of mutualism include the prokaryote microbe-inducing production of molecules in the eukaryote host thereby producing a niche that facilitates one side of the specific mutualistic relationship. The other side of the relationship is the benefit to the eukaryotic host. Experiments were designed to investigate the possibility that proper colonization would protect the host intestinal epithelium from damage or facilitate recovery from injury. Chemical irritants were used to cause mucosal injury under conditions that included or excluded the mutualist symbiotic relationship, and the consequence of colonization was measured. Normally colonized mice fully recover from the colitis caused by DSS or other chemical irritants (TNBS and OXA). In mice whose microbiome was greatly reduced, 40% did not recover and died. However, if the microbiota was replaced before treatment, recovery was complete (Figure 7C). It is noteworthy that *fut2* expression was restored even when the mice were recolonized with only one major bacterium of the normal adult microbiota (*B. fragilis*, Figure 7A and B), and this recolonization also supported recovery from mucosal injury. Recolonization with a mutant *B. fragilis* unable to utilize fucose did not induce *fut2* expression (Figure 7B), and was unable to support recovery from mucosal injury (Figure 7C). This suggests that bacteria-induced fucosylation is essential to cause a change in the internal state of the intestinal epithelial cell, rather than the microbiota *per se*, that is necessary for this recovery. The treatment with *B. fragilis* induced the same fucosylation through induction of *fut2* expression as recolonization with the entire microbiota. This implies that some microbes of the microbiota, especially predominant species like *B. fragilis*, might be the critical triggers of fucosylation during recolonization. Preliminary data indicate that monocolonization by any of several Gram-negative components of the adult microbiota can induce fucosylation, suggesting that *fut2* induction may be a response of uncolonized mature gut to Gram-negative prototypic molecules. The definition of the specific trigger and its ligand is ongoing and beyond the scope of this report.

These results strongly suggest that fucosylation is dependent on colonization and that proper fucosylation of the intestinal mucosa is required to maintain tissue homeostasis and recovery from inflammatory chronic injury. This conclusion is also consistent with previous observations in genetically reconstructed mice. FX mutant mice ($FX^{-/-}$) are unable to generate fucosylated glycans. They are born normal but shortly after weaning they die from colitis and diarrhea (Smith et al. 2002). Onset of

colitis in $FX^{-/-}$ mice can be prevented by maintaining them in germ-free conditions or by feeding them fucose. In light of the DSS data, the fucose can be seen as stimulating the colonization of their gut with fucose-dependent mutualists that can then protect the fucose-deficient mutant mice. Taken together, these data indicate a central and essential role of intestinal fucosylation induced by the gut microbiota for maintaining homeostasis in the host as well as mediating the mutualistic relationship between the adult gut microbiome and the adult gut.

Human populations contain a high frequency of a mutation in the *FUT2* gene that renders the gene product inactive. In most populations of European, Asian and African descent, ~20% are homozygous recessive for the *FUT2* allele and are known as non-secretors, because their exocrine secretions (milk, mucus, saliva etc.) lack the α1,2-fucosylated epitope; their intestinal mucosa also lacks this moiety. Thus, these individuals cannot respond to intestinal colonization with an induction of the gut α1,2-fucosylated phenotype. The colonization of non-secretors differs from that of secretors (data not shown), and non-secretors are predisposed to inflammatory bowel diseases such as necrotizing enterocolitis (Morrow et al. 2011) and Crohn's disease (McGovern et al. 2010). Thus, the data from our DSS colitis model, in conjunction with these clinical data, suggest that bacterially induced fucosylation can be of fundamental importance to human health. Conversely, polymorphisms in *FUT2* that result in low levels of fucosylation of secretions and intestinal mucosa are associated with dysbiosis of the gut microbiota (unpublished data). In clinical studies, dysbiosis of the human microbiota is implicated in various severe inflammatory conditions, such as necrotizing enterocolitis (Morrow et al. 2011), Crohn's disease (McGovern et al. 2010) and type 1 diabetes (Smyth et al. 2011; Morrow et al. 2013).

The negative consequences of the non-secretor phenotype raise the question of why this trait persists in the population. This can be considered a subset of why there is so much diversity in the human glycome. Many pathogens bind to specific glycoconjugate receptors. Humans are social beings. Hence, if an emerging human pathogen arises with high lethality, human populations would be at risk of extinction unless a portion of the population did not express the specific receptor for the lethal pathogen and hence could escape infection and ensure their survival. This could account for the extreme diversity of cell surface glycan expression in humans, and the survival of traits that otherwise have negative consequences.

The intestinal mucosa is predominantly sialylated before weaning and fucosylated after weaning. The ability of adult, but not suckling microbiota to stimulate fucosylation provides the basis for hypothesizing two distinct phases of mutualism. The adult fucose-centered mutualism is described above. It is interesting to speculate that the predominance of sialylation from birth to weaning might be supporting colonization by pioneering sialic acid-dependent species of our microbiota.

The maturation of the intestine, its colonization and its adaptation to various diets is increasingly recognized as being central to human health and wellbeing. Conversely, intestinal dysbiosis contributes to a variety of human diseases and chronic conditions in both infants and adults. Advances in metagenomic analysis now allow characterization of microbiome in normal and disease states, and distinct differences have been reported. The dearth of information on the major factors that direct early gut colonization by the microbiome, which include genetic variation of the host, and intrinsic and extrinsic signaling, have been limiting intervention strategies. Deeper knowledge of the interdependence and integration of factors that contribute to initiation and succession of a healthy microbiome and how dysbiosis leads to immunologic and other pathologies provides an attractive target for new rational therapies for diseases that are currently intractable.

Materials and methods

Reagents

Phenyl-β-D-galactoside, bovine serum albumin (BSA), 2-mercaptoethanol, dextran sulfate sodium (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS), oxazalone (OXA) and ultra-pure sucrose glucose assay kits were purchased from Sigma Chemical Co. (St Louis, MO). 10 mM GDP-[^{14}C] fucose was purchased from New England Nuclear Life Sciences (Boston, MA). Taqman reverse transcription kits and enzymes were purchased from Applied Biosystems (InVitrogen®, San Diego, CA). Biotinylated and fluorescein-conjugated *Ulex europaeus* agglutinin-1 (UEA-I) were from E-Y Laboratories (San Mateo, CA). Cortisone acetate (CA) was from Merck, Sharp & Dohme (West Point, PA). All other reagents were of analytical or molecular biology grade from Fisher Scientific (Fairlawn, NJ) or Sigma Chemical Co. Strains of *Bacteroides fragilis* were a gift from Dr. Laurie Comstock, Channing Lab, Brigham, and Women's Hospital, Boston, MA.

Animal studies

Developmental studies. Black Swiss mice were purchased from Taconic Farms (Germantown, NY) and C57/B6 mice from Jackson Labs (Bar Harbor, ME). Pups were from timed-pregnant mice, and the date of birth was designated day 0. Pups were housed with their dams through 21 post-natal days. Mice were fed mouse chow and water *ad libitum* and maintained under a 12-h light/dark cycle. Pups were treated with glucocorticoids on day 10 by subcutaneous injection of a single dose (5 mg/100 g body weight) of CA suspended in saline. Vehicle control pups were injected with the same volume of saline (0.9% NaCl) at the same time and maintained with their dam until sacrificed. All procedures relating to animals and their care conformed to the international guidelines "Principles of Laboratory Animals Care" and according to the approved animal protocol.

Mouse models. Germ-free mice were maintained in a germ-free environment until immediately before euthanasia at 6 weeks of age. Ex-germ-free mice were produced by removing germ-free mice from their germ-free environment at the ages of 4 or 6 weeks, inoculating them with a slurry of fresh fecal and cecal contents from age-matched conventional control mice (contents of one conventional mouse for inoculation of five germ-free mice) directly through both orogastric intubation and addition to their drinking water and keeping them in the same cage as conventional mice. The ex-germ-free mice were euthanized 2

weeks later along with the age-matched germ-free and conventional mice. All animals were euthanized between 12 p.m. and 3 p.m. to avoid circadian influences.

Three groups of 4-week-old mice were treated as follows. Bacteria-depleted and bacteria-repleted groups were fed an antibiotic cocktail in their drinking water for 2 weeks, whereas the conventional control group received untreated water. After 2 weeks, the bacteria-repleted group received cecal/colonic bacteria from age-matched conventionally reared mice in their drinking water, as described previously (Meng et al. 2007). At the designated age, mice were sacrificed and their colons were harvested for analysis.

Adult mice were depleted of luminal bacteria by consuming the antibiotic cocktail (100 µL antibiotic cocktail/mouse/day) added to their drinking water for 2 weeks, then commensal bacteria were introduced for 2 weeks whereupon the mice were euthanized and the fucosyltransferase activity and *fut2* mRNA levels of each colon were measured. The antibiotic cocktail consists of kanamycin (8 mg/mL), gentamicin (0.7 mg/mL), colistin (34,000 U/mL), metronidazole (4.3 mg/mL) and vancomycin (0.9 mg/mL). After the antibiotic cocktail was introduced, fresh fecal samples were collected from mice every day and assayed for the presence of bacteria in five different culture media, including both aerobic and anaerobic conditions, as described previously (Meng et al. 2007).

DSS treatment. Mice (6 weeks of age) received 3.5% (wt/vol) DSS (40,000 kDa; ICN Biochemicals Aurora, OH), ad libitum, in their drinking water for 5 days, then switched to regular drinking water. The amount of DSS water consumed per animal per diem was recorded and no differences in intake between strains were observed. Control mice received water only. For survival studies, mice were followed for 12 days post–DSS treatment. Mice were weighed every other day to determine changes in body weight, calculated as: % weight change = [(weight at day x – day 0/weight at day 0) × 100]. Animals were monitored clinically for rectal bleeding, diarrhea, and general signs of morbidity, including hunched posture and failure to groom. An essentially similar model was developed for 2.5% TNBS and OXA-induced colitis (Wirtz et al. 2007) to determine if phenomena observed with the DSS model were general to recovery from mucosal injury per se, irrespective of the cause. These three models gave essentially identical results, so we show the data from the most commonly used model, recovery from injury induced by DSS.

Enzyme assays

The entire small intestine and colon was removed and thoroughly flushed with ice-cold 0.9% NaCl. 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. Fucosyltransferase and sucrase enzyme activities were measured in samples of intestinal mucosa as described (Nanthakumar and Henning 1993; Nanthakumar et al. 2003).

Preparation of crude microvillus membrane preparation. A 10% mucosal homogenate was prepared from each segment of the intestine in 0.1 M Tris–HCl buffer (pH 7.4) and centrifuged at 1000 × *g* for 15 min to remove nuclei and cellular debris. The supernatant was then centrifuged at 105,000 × *g* for 1 h, resulting in a membrane fraction and soluble cell fluid. The resulting pellets were resuspended in homogenization buffer (0.1 M Tris–HCl, pH 7.4), frozen as aliquots at −80°C, and used for the fucosyltransferase assay.

Fucosyltransferase activity. α1,2/3-Fucosyltransferase enzyme activity was assessed using phenyl-β-D-galactoside as the acceptor (Nanthakumar et al. 2003). The reaction mixture for each assay contained, in a total volume of 0.1 mL, 25 mM phenyl-β-D-galactoside, 20 mM sodium phosphate buffer (pH 6.1), 10 mM fucose, 5 mM ATP, 20 mM $MgCl_2$, 50 mM NaCl, 0.5% Triton X-100, 10 nmol GDP-[^{14}C]fucose (0.1 µCi, spec. act. = 11 mCi/mmol; New England Nuclear) and homogenate containing 50–100 µg protein. GDP-fucose concentration was at saturation, and product formation was linear for 2 h of incubation for up to 100 µg of enzyme protein at 37°C. After 2 h, the reaction was terminated by addition of 100 µL of ethanol and dilution with 1 mL of 4°C H_2O followed by centrifugation at 15,000 × *g* for 5 min. The supernatant was applied to C-18 Bond Elute cartridges (500 mg) that had previously been washed with 6 mL of acetonitrile followed by 6 mL water. After application of the sample, the cartridges were washed with 5 mL of water to remove the radiolabeled precursor. The product, [14C]fucosylphenyl-β-D-galactoside, was eluted with 1.5 mL of 50% acetonitrile directly into scintillation vials. Five milliliters of scintillation cocktail (Ready Safe, Beckman, Fullerton, CA) was added to each vial, and radioactivity determined by scintillation counting of the clear solution. The specific activity is expressed as nmol [^{14}C] fucose incorporated/h/mg protein.

Sucrase activity. Frozen duodenum, jejunum, and ileum, whose lumen had been rinsed with sterile cold phosphate-buffered saline (PBS), were homogenized in 9 volumes of 0.154 M KCl and assayed in duplicate using sucrose as the substrate (Nanthakumar and Henning 1993). The glucose liberated by the enzyme reaction was quantified by a glucose-oxidase reaction kit (Sigma Chemical Co.). Protein concentration was determined by the BCA® protein assay kit (Pierce, Rockford, IL) using a standard curve generated by BSA. Sucrase activity is expressed as µmole glucose hydrolyzed/h/mg protein.

mRNA analysis

Total RNA was extracted from frozen tissues by homogenization in TRIZOL® reagent, and cDNA was synthesized using TaqMan Reverse Transcriptase (Applied Biosystems, Foster City, CA) and random hexamers. Real-time quantitative polymerase chain reaction (PCR) was performed using SYBR green Master mix. Fucosyltranferase-1 (*fut1*), fucosyltranferase-2 (*fut2*), fucosyltranferase-4 (*fut4*), fucosyltranferase-7 (*fut7*), SI and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were measured by quantitative reverse transcription-PCR (Nanthakumar et al. 2005). To calculate the fold increase or decrease of mRNA, the C_T of the target gene minus the C_T of the housekeeping gene (GAPDH) yields the corrected C_T. Change in the expression of the normalized target gene as a result of an experimental manipulation was expressed as $2^{-C_{Tc}}$ where $C_{Tc} = C_T$

samples – C_T controls. PCRs lacking cDNA, primers or reverse transcriptase were run as baseline controls.

UEA-I fluorescent staining

Tissue sections of 7 μm were used for UEA-I staining (E-Y Laboratories) to visualize α1,2-fucosylated glycoconjugates in fixed frozen tissue sections (Bry et al. 1996; Nanthakumar et al. 2003). One centimeter of tissue from each region of the intestine was fixed in 4% paraformaldehyde for 30 min and stored in ice-cold PBS containing 30% sucrose. The tissue block was embedded in optimal cutting temperature and sliced in a cryostat at −20°C. Frozen sections were blocked with PBS containing 2% BSA for 1 h, stained with 10 μg/mL Texas Red-UEA-I for 1 h, washed, mounted using Anti-Fade (Vector Laboratories, Burlingame, CA) and analyzed by fluorescent and/or confocal microscopy. Controls to ascertain fucose-specific binding under these conditions were identical sections in which 100 mM L-fucose was added at the same time as the Texas Red-UEA-1, and these displayed no binding.

Statistics

Results are expressed as the mean ± SEM. Effects of age and treatment on enzyme activities were analyzed by two-way analysis of variance, whereupon, if significant, post hoc tests for individual variables were performed by two-tailed unpaired *t*-tests. Differences with a *P*–value of <0.05 were considered significant.

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Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; CA, cortisone acetate; DSS, dextran sulfate sodium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GF, germ-free; OXA, oxazalone; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qRT, quantitative reverse transcription; SI, sucrase-isomaltase; TNBS, 2,4,6-trinitrobenzene sulfonic acid; UEA-I, Ulex *europaeus* agglutinin-1 FucT, fucosyltransferase.

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