Developmental regulation of sialic acid modifications in rat and human colon

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ABSTRACT

Using high-pressure liquid chromatography (HPLC) and gas-liquid chromatography/mass spectrometry (GLC/MS), we have confirmed the existence of several sialic acid modifications in the adult rat and human colon. The major *O*-acetylated sialic acid in both species is 9-*O*-acetyl-*N*-acetylneuraminic acid; *N*-glycolylneuraminic acid is a major component of the adult rat colon. Both of these major modifications were found to be developmentally regulated during the perinatal period in the rat. The *N*-glycolyl modification is present prenatally and disappears rapidly in the postnatal period. It reappears in the preweanling period, reaching levels at weaning comparable to those found prenatally. In contrast, the 9-*O*-acetyl modification is very low prenatally, and undergoes a marked increase shortly after birth in both the rat and human colon. The difference in the kinetics of appearance of the two modifications suggests that they are independently regulated. Regulation of these modifications seems to be influenced by exposure to bacterial by-products or environmental stimuli. The *N*-glycolyl modification in the rat colon reappeared at weaning, a time of major change in enteral colonic substances. Spontaneously aborted human fetuses, including three with intrauterine infection at 27, 33, and 35 wk of gestation, showed adult levels of *O*-acetylation in colonic tissue. Also, although *O*-acetylation in freshly isolated colon tumor specimens was only somewhat lower than that in the adult normal colon, all established colon cancer cell lines studied showed minimal *O*-acetylation. — MUCHMORE, E. A.; VARKI, N. M.; FUKUDA, M.; VARKI, A. Developmental regulation of sialic acid modifications in rat and human colon. *FASEB J.* 1: 229–235; 1987.

Key Words: sialic acids • colonic mucosa • O-acetylation • developmental regulation

THE SIALIC ACIDS ARE A FAMILY OF *N*- and *O*-substituted derivatives of neuraminic acid, a nine-carbon polyhydroxaminoketoacid sugar (1, 2). For animals, they are almost always found in α-glycosidic linkage as the outermost sugar on cell membrane glycoproteins and glycolipids. *N*-Acetylneuraminic acid (Neu5Ac)[2] can be *O*-substituted at the 4, 7, 8, and 9 positions, and the *N*-acetyl group can be converted into an *N*-glycolyl group, giving rise to a diverse group of compounds that appear to be highly tissue- and species-specific in their distribution (2). Precise biological roles have not yet been assigned to these modifications. However, they have been shown to dramatically alter several biological properties of the parent sialic acid molecule, including cleavage by sialidases (3–5), recognition by antibodies (6–8), binding of viruses (9), modulation of the alternate pathway of complement activation (10), and bacterial capsular antigenicity (11).

Previous studies have suggested that the sialic acids of normal human, rat, and rabbit colonic mucosa are *O*-acetylated, and that Neu5Ac can be converted to *N*-glycolylneuraminic acid (Neu5Gc) in the rat colon (2, 12–15). Histochemical techniques suggested that *O*-acetylation may be markedly diminished or absent in human colonic tumors (12–15).

We have recently described improved biochemical techniques for the accurate study of sialic acid modifications (16, 17). In this report, we have applied these techniques to analyze developmental changes in the sialic acids of human and rat colon. In addition, we have analyzed human colonic adenocarcinoma samples to reexamine the previously reported changes.

MATERIALS AND METHODS

Chemicals

Chemically synthesized Neu5Ac was purchased from Kantoishi Pharmaceutical Company (Tokyo, Japan).

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[2]Abbreviations: The sialic acids are designated by combinations of Neu (neuraminic acid), Ac (acetyl), and Gc (glycolyl). For example, *N*-acetyl-9-mono-*O*-acetylneuraminic acid is written as $Neu5,9Ac_2$ (after R. Schauer and others; ref 2). Other abbreviations: GLC/MS, gas-liquid chromatography/mass spectrometry; PFA, periodate/formaldehyde/acetylacetone method; HPLC, high-pressure liquid chromatography.

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Neu5Gc was purchased from Sigma Chemical Co. (St. Louis, MO). Other sialic acid standards were prepared as previously described (5, 16). Dowex 50 AG1-×2 (100–200 mesh, hydrogen form) and Dowex 3-×4A (100–200 mesh, chloride form) were purchased from Bio-Rad (Richmond, CA). Dowex 3-×4A was converted to the formate form. Diazomethane in ether was kindly provided by Dr. Alan Hoffman (UCSD School of Medicine). All other chemicals were of reagent grade and were purchased from commercial sources.

Cell lines

All cell lines were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in culture in RPMI 1640 with 10% fetal calf serum supplemented with 2 mM sodium pyruvate, grown in large amounts, harvested, washed in phosphate-buffered saline, and stored frozen at −20°C until further work-up. The cell lines studied were ATCC HTB 38 (HT-29); ATCC CCL 231 (SW48); ATCC CTL 228 (SW 480); and ATCC CCL 225 (HCT 15). All were originally established from primary colonic adenocarcinomas (18).

Tissue samples

Samples were collected on ice and the enteral contents were removed by lavage. For human colon samples, the mucosa was stripped from the serosa by blunt dissection. Adult Sprague-Dawley rat colons were analyzed without the stripping procedure to maintain comparability with the fetal rat samples, which could not be stripped. Human colon tumor nodules were dissected free of underlying tissue and normal mucosa. All samples were placed in ice-cold 10 mM sodium formate buffer, pH 5.5, and gently homogenized by using a Brinkmann Polytron Unit (Model No. 10/35) at speed 5 for 30–45 s. Low-molecular-weight materials were removed by dialysis against 200 volumes of 10 mM formate buffer pH 5.5 containing 10 ppm of butylated hydroxytoluene to retard lipid peroxidation (10).

Release and purification of sialic acids

This was carried out as previously described (16, 17), with some significant modifications. The dialyzed samples were adjusted to 2 M acetic acid and heated for 4.5 h at 80°C to obtain maximal release of bound sialic acids with minimal loss of *O*-acetylation. The samples were chilled, debris was removed by centrifugation at 100,000 × *g* for 30 min, and the supernatants were lyophilized. The acid-released low-molecular-weight material was collected by dialyzing twice against a 10-fold volume of 10 mM formate buffer, pH 5.5. The pooled dialysates were then passed over a 1-ml Dowex 50 (H^+ form) column and washed through the column with an additional 6 ml of water. The pooled washings were adjusted (if necessary) to pH > 3 with 100 mM sodium formate buffer, pH 5.5, and immediately passed over a 1-ml Dowex AG3 × 4A (formate form) column equilibrated in 10 mM sodium formate buffer, pH 5.5. The columns were washed with 10 ml 0.01 M formic acid and the sialic acids were eluted with 10 ml of 1 M formic acid, and evaporated to dryness. The samples were resuspended in 1 ml of 10 mM acetic acid (which had previously been decationized by passage over Dowex 50), and subjected to three extractions with 5 volumes each of diethyl ether. Residual ether was removed under a stream of nitrogen. The purified samples were evaporated to dryness and stored at −20°C in a dessicator. All evaporations were performed with a Buchler Shaker-Evaporator apparatus with a 35°C water bath.

High-pressure liquid chromatography (HPLC)

HPLC was carried out by a modification of our previously described method (17, 19). A Varian SP oligonucleotide column (4 × 300 mm) was eluted in the isocratic mode with 66% acetonitrile, 18% water, and 16% 0.25 M NaH_2PO_4 at 1 ml/min, and the effluent was monitored at 200 nm.

Gas-liquid chromatography/mass spectrometry (GLC/MS)

GLC/MS was carried out on selected samples (2, 20, 21). Purified sialic acids were dissolved in methanol, converted to methyl esters with diazomethane in ether, and then taken to dryness. The methyl esters were then trimethylsilylated with a mixture of trimethylchlorosilane, hexamethylsilane, and pyridine (1:2:10) at room temperature for 45 min. The resulting methyl esters/trimethylsilyl ethers of the sialic acids were taken to dryness again and extracted into *n*-hexane. Aliquots were fractionated on a capillary column (0.25 mm × 30 m) of 5% DB-5 on a Finnigan 4510 gas chromatography/mass spectrometer. The column temperature was increased at 20°C/min increments from 50 to 230°C, held isothermic for 20 min, and then increased at 4°C/min increments from 230 to 250°C. Mass spectra at 40 eV were analyzed on an Incos 200 series data system (ion source temperature 120–130°C; accelerating voltage 13 kV; trap current 320 A).

Measurement of sialic acid side-chain *O*-acetylation

Purified samples were studied by HPLC (see above) and by the periodate-formaldehyde-acetylacetone (PFA) method (22). Each sample was studied with or without de-*O*-acetylation in 0.1 *N* NaOH at 37°C for 30 min. The increase in the peak area of the parent sialic acids (in the case of HPLC) or the increase in yield of formaldehyde (in the case of the PFA method) was used to measure the amount of side-chain *O*-acetylation.

RESULTS

Optimization of conditions for the release and purification of colonic sialic acids

We and others have previously pointed out the difficulties in the accurate study of sialic acid modifications and the necessity of optimizing methodology for each tissue that is studied (5, 16, 17). For colonic tissue, we found that it was necessary to remove low-molecular-weight contaminants by dialysis before release of bound sialic acids. Release by neuraminidases could not be carried out because of the potential for bacterial overgrowth during enzyme incubations. However, we found that a 4.5-h hydrolysis in 2 M acetic acid at 80°C gave maximal release of sialic acids, with minimal loss of *O*-acetylation (data not shown). After release, we used our previously described method of purification (16), with the exception that the ether extraction step was carried out last. This was found to maximize removal of lipid impurities that interfered with HPLC analysis.

Direct biochemical analysis of the sialic acids of adult rat and human colon

Previous studies have carried out indirect histochemical or incomplete biochemical analyses of the sialic acids of rat and human colon (12–15). Sialic acids were purified from adult rat and human colon and were analyzed by HPLC and GLC/MS. HPLC analysis showed the presence of a variety of sialic acids, some of which are alkali-labile, suggesting the presence of *O*-acetyl esters. Direct demonstration of the structure of the major sialic acids was then carried out by GLC/MS. Figure 1*A* shows an example of the total mass profile of adult rat colon sialic acids. Figure 1*B* shows the individual mass fragmentation patterns of the three major sialic acids in this sample. These were identified by comparison with published spectra of the same derivatives (2). Neu5Gc was a major component in the rat but not the human colon. The single major *O*-acetylated sialic acid in both species is Neu5,9Ac_2 (9-*O*-acetyl-*N*-acetylneuraminic acid).

Developmental regulation of Neu5Gc in the rat colon

Pre- and postnatal rat samples were studied by using HPLC (after base treatment to eliminate *O*-acetyl esters). As shown in Fig. 2, Neu5Gc is present in prenatal rat specimens. Similar levels are seen in samples collected within hours of birth (data not shown). However, by the first postnatal day, the level of Neu5Gc drops markedly. Subsequently, low levels appear by day 8, but full reexpression is noted only at the beginning of the weanling period. The percentage of Neu5Gc to total sialic acid in the prenatal colon is 40%. In samples from postnatal days 1, 4, 8, 16, and 21, the values are 5, <1, <1, 12, and 33%, respectively.

Optimization of conditions for analytical de-*O*-acetylation of purified colonic sialic acids

We have previously shown that the study of *O*-acetyl esters on the sialic acids is fraught with difficulties. The problems include variable release by neuraminidases and acid hydrolysis, and the destruction and migration of *O*-acetyl esters during release and purification (5, 16, 18). However, the methodology that we have developed does allow the accurate release and purification of 9-mono-*O*-acetylated sialic acids. 7-Mono-*O*-acetylated and 8-mono-*O*-acetylated sialic acids undergo rapid migration to the 9 position (2, 16). The less abundant side-chain di- and tri-*O*-acetylated sialic acids are derived from the 9-*O*-acetylated species (2). We therefore conclude that the major 9-*O*-acetylated sialic acids in the purified samples are representative of the overall *O*-acetylation state in the original tissue.

The level of 9-*O*-acetylation in the purified sialic acids could be measured either by HPLC or by the PFA method (23). The latter method makes use of the fact that mild periodate treatment causes release of a single mole of formaldehyde per mole of sialic acid, and that such release is blocked by the presence of an *O*-acetyl group at the 9 or 8 position. Because *O*-acetyl groups at the 8 position rapidly migrate to the 9 position (2, 16), this assay is an accurate and specific way to measure the amount of the major 9-*O*-acetylated species in the purified samples from colonic tissues. We found this assay to be somewhat more reproducible and reliable than the HPLC method, which was occasionally affected by baseline waver. Therefore, all quantitative *O*-acetylation results presented below are based on the PFA assay. However, all samples were also monitored by HPLC analysis to confirm purity and general composition.

Developmental regulation of *O*-acetylation in the rat colon

The total amount of sialic acid in rat colonic tissue ranged from 19 to 46 nmol/mg of protein and was unrelated to the developmental state of the individual samples (data not shown). As shown in Fig. 3, *O*-acetylation of sialic acids is essentially absent in the prenatal rat colon, but develops rapidly within 1–2 days after birth, and subsequently remains high for about 3 wk. In the weanling period (19–21 days) and beyond, a remarkable degree of individual variation in levels of *O*-acetylation develops. This variation has been confirmed by repeated analysis of the same specimens.

Developmental regulation of *O*-acetylation in human colon

Similar overall finding were obtained with regard to *O*-acetylation in samples of human colon. As shown in Fig. 4, electively aborted fetal samples (20–23 wk of gestation) had the lowest levels of *O*-acetylation, and a gradual increase was seen in the postnatal period. Although the number of samples is relatively small, the

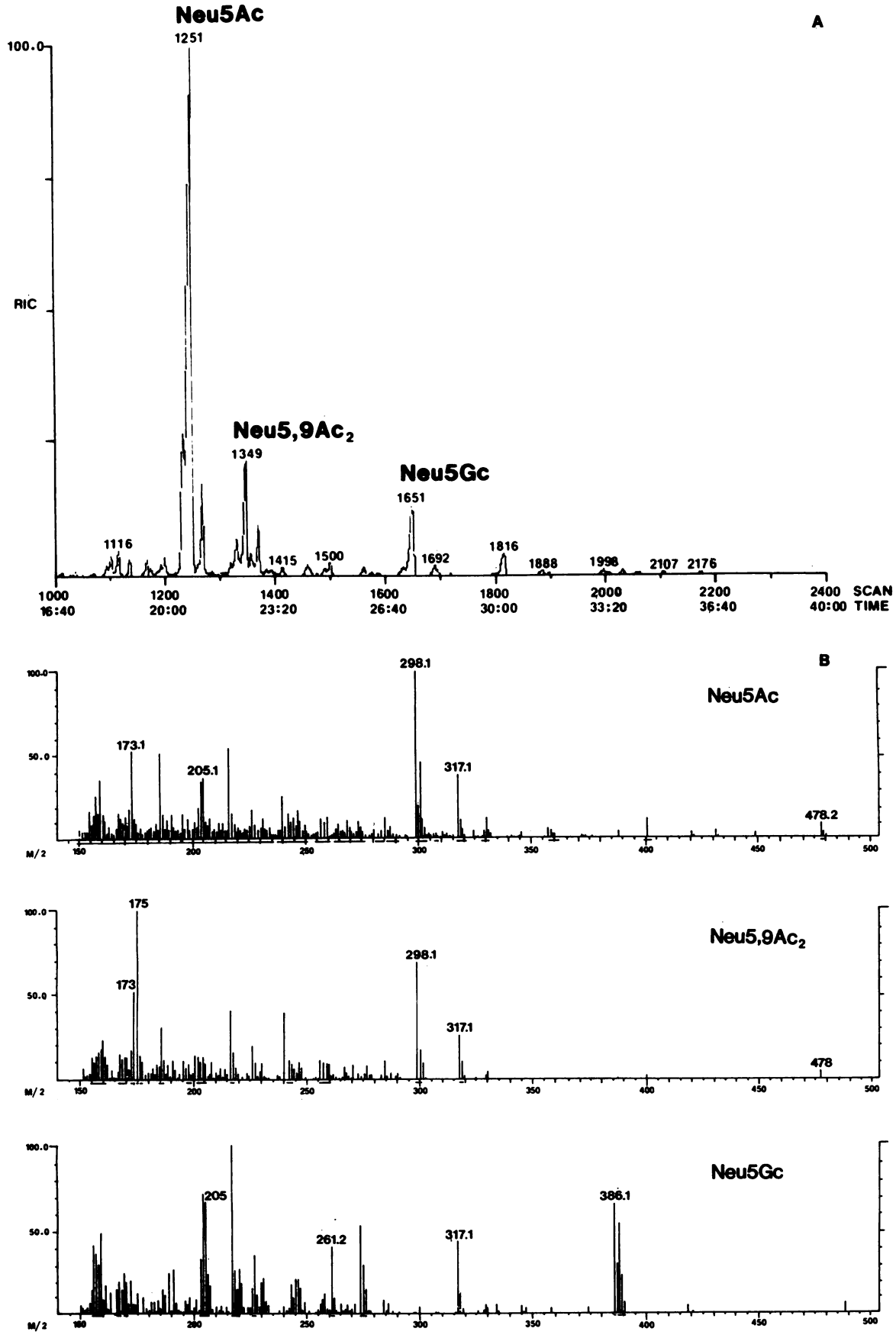

Figure 1. Mass spectrometric analysis of purified sialic acids from adult rat colon. The purified sialic acids were converted to methyl esters/trimethylsilyl ethers and studied by capillary GLC/MS as described under Materials and Methods. The total mass spectrum profile is shown (*A*) along with representative spectra of the major sialic acids (*B*). The typical mass fragments of each sialic acid are indicated.

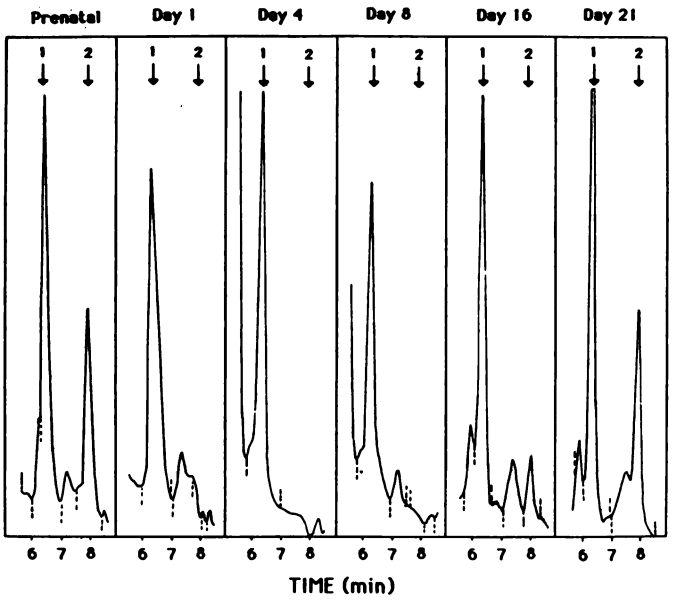

Figure 2. HPLC analysis of purified sialic acids. Purified sialic acids from pre- and postnatal rat colon samples were de-*O*-acetylated and studied by HPLC as described under Materials and Methods. The region of the HPLC profiles containing Neu5Ac (peak 1) and Neu5Gc (peak 2) is shown in each case. The peak between 1 and 2 is an unknown.

general trend was the same as that seen with the rat model. As with the adult rat, human adult colonic samples showed considerable variation in levels of *O*-acetylation (Fig. 4). There was no clear association between the level of *O*-acetylation and the presence of an adjacent colorectal cancer in the same specimen.

Colonic carcinoma is not always associated with decreased *O*-acetylation

Previous indirect histochemical studies have suggested that a marked decrease in *O*-acetylation occurs in colonic adenocarcinomas (13–15). Eleven colon carcinomas and four colon tumor cell lines were analyzed for the level of *O*-acetylation. As shown in Fig. 4, the fresh colon tumor samples demonstrated variable levels of *O*-acetylation but only tended to be somewhat lower than those of normal adult colon. However, in striking contrast, all four colon tumor cell lines showed no detectable *O*-acetylation.

Elevated levels of *O*-acetylation in spontaneously aborted fetuses

Spontaneously aborted fetuses had significantly higher levels of *O*-acetylation (see Fig. 4). One of these samples was from a 27-wk fetus in which many organs had grown a pure culture of *Peptostreptococcus anaerobicus*. Another grew *Listeria monocytogenes* and the third had histological evidence of bacterial infection. The fourth had evidence of hydrops fetalis of uncertain origin, but no definite evidence of bacterial infection.

DISCUSSION

More than 25 different kinds of sialic acids have been described (1, 2). Most if not all of this diversity arises from various modifications and substitutions of the parent molecule, Neu5Ac (2). The appreciation of the extent of this complexity is recent and has spurred the search for its biological significance. Many clues have emerged (1, 6, 8, 10, 11) but no definitive biological roles have been established for these modifications.

There have been many other detailed and careful studies of the structure and distribution of colonic mucosal glycoconjugates in both normal and diseased states (8, 14, 23–28). However, except for the histochemical studies previously mentioned (12–15), none have considered *O*-acetylation of sialic acids. We have confirmed the presence of side-chain *O*-acetylated sialic acids in adult rat and human colon by direct biochemical analysis.

In this study we demonstrate the developmental regulation of expression of 9-*O*-acetylated sialic acids in the Sprague-Dawley rat colon. *O*-Acetylation is absent prenatally, and a marked increase occurs postnatally, sometimes within hours of birth. Our data also demonstrate clear developmental regulation of conversion of Neu5Ac to Neu5Gc in the rat colon. It is of particular note that the changes in the expression of this modification are quite different from that of *O*-acetylation. This implies that the two modifications are differentially regulated and are not the consequence of a coordinated single response to environmental stimuli and/or bacterial colonization. The *N*-glycolyl modification is not seen in the normal adult human colon, but it has recently been reported to occur in very small amounts in malignant human colon samples (8).

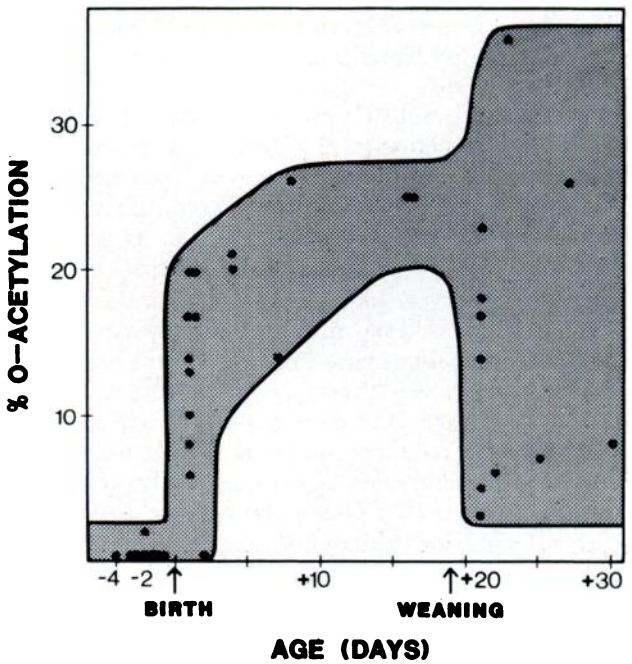

Figure 3. *O*-Acetylation of rat colonic sialic acids. The percentage of *O*-acetylation in purified sialic acids from colonic samples obtained at various times before and after birth was determined by the PFA method as described under Materials and Methods.

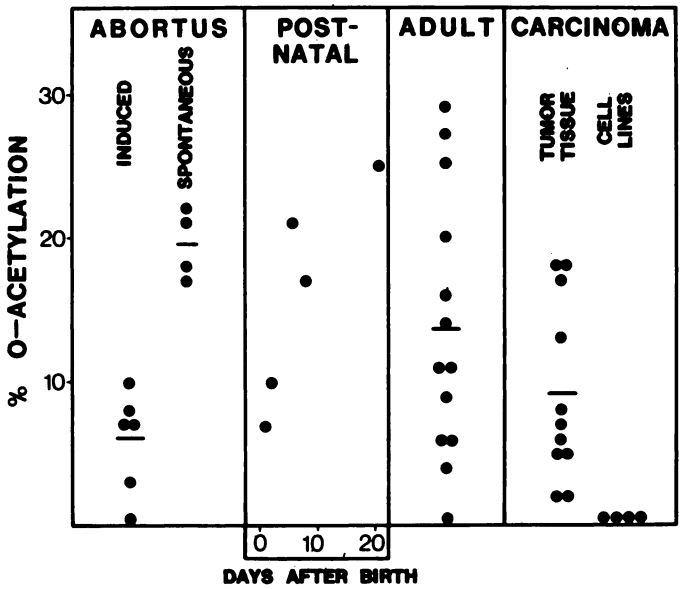

Figure 4. *O*-Acetylation of sialic acids in human colon samples. The various groups of human colonic samples indicated were studied for *O*-acetylation by the PFA method as described under Materials and Methods.

In the human colon, the same trend is seen for *O*-acetylation. Electively aborted fetal samples showed low levels of *O*-acetylation, and an increase is seen in the postnatal period. However, because the elective abortions were performed by prostaglandin infusion and hypertonic urea injection, they do not represent strictly normal specimens.

Bouhours and Bouhours have previously showed that the *N*-glycolyl modification on hematoside is developmentally regulated in the postnatal rat small intestine (29); however, they did not examine prenatal samples. Furthermore, *O*-acetyl groups would have been removed by the alkaline hydrolysis step used in the purification of the ganglioside.

One obvious event that could be involved in the postnatal changes is the exposure to environmental organisms and their by-products. However, because bacterial neuraminidases are present in the colon postnatally, it could be argued that the increase in *O*-acetylation results from a selective cleavage of the non-*O*-acetylated molecules. However, inasmuch as the total amount of sialic acid (expressed per milligram of protein) does not change significantly during this period, this explanation is improbable. It was therefore of interest to find that high levels of *O*-acetylation were present in human fetal colon samples from spontaneous abortuses, most of which had clear evidence of intrauterine bacterial infection. This implies that *O*-acetylation could be induced by intrauterine infection and/or stress as early as the 27th wk of gestation. Thus, the induction could be related to environmental stimuli rather than the process of birth itself.

The levels of *O*-acetylation in the adult human and rat colon are extremely variable. In the laboratory rat the onset of this variability coincides with the period of weaning. One possible explanation for these findings is that the level of *O*-acetylation is in a dynamic state that is determined by an ongoing response to multiple environmental stimuli such as the composition of bacterial flora, the presence of food contents, and environmental stress.

We found that the level of *O*-acetylation in human colonic adenocarcinomas is highly variable, and is only slightly lower than that in the normal adult colon. The difference is not as striking as that described in previous histochemical work, which concluded that levels were markedly decreased in tumors (12–15). There are two potential explanations for this discrepancy. First, it is well known that *O*-acetyl groups are exceedingly labile and could have been destroyed at some point in the collection, storage, and processing of the tumor samples. Second, the sensitivity of the histochemical methods used may have been inadequate for the detection of the somewhat lower levels of *O*-acetylation. In striking contrast, four established human colon tumor cell lines had no detectable *O*-acetylation. This could be the result of selection of specific tumor subpopulations in tissue culture. A more intriguing possibility is that continued exposure to certain environmental stimuli may be necessary to sustain expression of *O*-acetylation, and that these are lost in the sterile, uniform conditions of tissue culture.

The biochemical level of regulation of *O*-acetylation of sialic acids is currently unknown. We and others have previously described many activities, including *O*-acetyltransferases, acetyl-CoA transporters, and *O*-acetylesterases, that could be involved in determining the exact level of *O*-acetylation in a given tissue (2, 19, 30, 31). Further studies of the activities in colon samples will be necessary to define the mechanisms of the postnatal changes. The distribution of *O*-acetylation between the different colonic cell types and among the different macromolecules of each cell and the topology of *O*-acetylation also require further investigation. With the availability of more specific probes for *O*-acetylated sialic acids (32–35), we are currently examining these questions. FJ

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