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Developmental Changes of Blood Group A-Active Glycosphingolipids with Type 1 and Type 2 Chains in Rat Small Intestine

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Blood group A-active glycosphingolipids of the small intestine, A-6 and A-12, which have been characterized previously in the adult rat [Breimer ME, Hansson GC, Karlsson K-A, Leffler H (1982) J Biol Chem 257:906-12], were found to appear during postnatal development, using immunostaining on thin layer chromatograms with two monoclonal anti-A antibodies, A005 and A581. In this system, A005 was found to be specific for the A determinant based on the type 2 chain, while A581 reacted mainly with the A determinant based on the type 1 chain and only weakly with the A determinant based on the type 2 chain. A-6 Type 1 was detected first at 18 days after birth. Its concentration increased markedly during the fourth week. A-6 Type 2 was detected, at a very low level, in neonates. Its concentration increased between days 15 and 20 and then decreased almost to the neonate level by 28 days. Dodecaglycosylceramide A-12 followed the same pattern of reactivity as A-6 type 1 with A581, and remained strongly reactive with A005 after 20 days. Linear A-6 and branched A-12 appeared simultaneously. Antibodies directed against blood group H determinants based on the type 1 or type 2 chains did not detect any H structure which might have appeared as a precursor of either A-6 or A-12 at the early stages of postnatal development.

The small intestine is known to be rich in fucolipids with blood group ABH antigenic activities [1]. Its glycolipid composition displays species [2] and individual specificities [3].

Abbreviations: A-6, A-12, H-5, H-10 etc - the glycolipids are abbreviated by giving blood group activity, and number of sugars (see also Fig. 1); G_{M3}, G_{M3}-ganglioside, II^{3}NeuAc-LcCer; PBS, phosphate-buffered saline.

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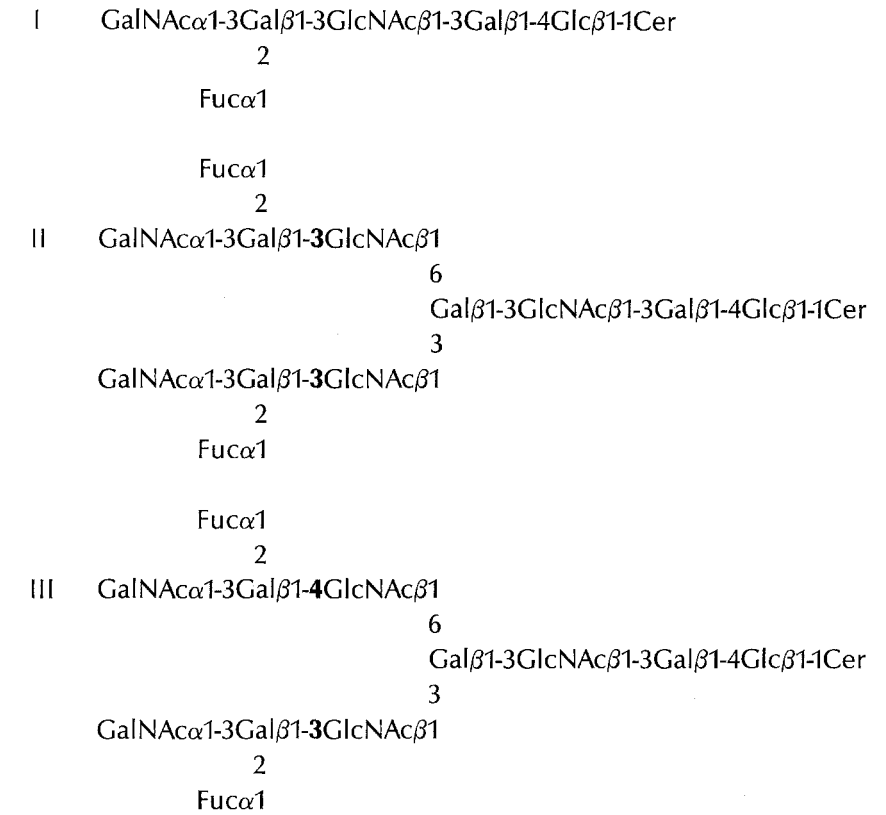

Figure 1. Blood group A-active glycolipids of the small intestine of the adult rat.
I, A-6 based on the type 1 chain (Galβ1-3GlcNAc) [9]. II, A-12 with only type 1 chains. III, A-12 with type 1 chains at two sites and type 2 chain (Galβ1-4GlcNAc) on the (1-6)-linked antenna [10]. In the rat species expressing A-glycolipids, an H-active pentaglycosylceramide, H-5, is found with the same structure as A-6, but lacking the terminal *N*-acetylgalactosaminyl residue [9]. In the rat species not expressing blood group A glycolipids, two decaglycosylceramides, H-10, are present with the same structure as A-12 but lacking the terminal *N*-acetylgalactosaminyl residues [8].

Two rat strains have been found, in which the small intestine expresses blood group H-active glycolipids [4, 5], while in another strain it expresses both A- and H-active glycolipids [4].

The glycolipid composition of the small intestine of the rat undergoes changes during the course of development [6, 7]. Previous work has shown that a branched blood group H-active decaglycosylceramide (H-10), characterized in the small intestine of the adult rats which express only blood group H-active glycolipids [4, 8], is not detected during the ontogenic development before 21-22 days in Sprague-Dawley rats, when using chemical visualization on thin layer chromatograms [5]. In the rat strain which expresses both A- and H-active glycolipids, the adult small intestine does not express H-10 [9], but expresses a longer chain glycolipid A-12, which is a branched dodecaglycosylceramide present in two forms [9, 10], and a shorter linear structure A-6 [9] (Fig. 1).

The blood group A determinant is synthesized by addition of an *N*-acetylgalactosamine moiety to an H determinant [11, 12], and cellular differentiation may be accompanied by gradual elongation of the carbohydrate chains [13]. We therefore investigated in the small intestine of this latter strain (A- and H-expressing) the postnatal development pattern of glycolipids with blood group antigenic activity, looking for a chronology of expression of A-6 and A-12, and their precursors H-5 and H-10, by immunostaining on thin layer chromatograms with monoclonal antibodies. We report here that A-6 and A-12 could be detected earlier than 21 days, which was the threshold of the chemical detection, that the linear (A-6) and branched (A-12) glycolipids appeared simultaneously, and that their precursors H-5 and H-10 could not be detected before the appearance of A-6 and A-12. Furthermore, the characterization of the epitope specificity of two monoclonal anti-A antibodies against A-6 according to the type of internal carbohydrate chain (type 1 or type 2) (Fig. 1) allowed us to demonstrate that a shift between the two types of chains occurred in A-6 after the third week of life.

Experimental Procedures

Animals

The rats used in this study were hooded rats (black and white) of the strain used for the characterization of A-active glycosphingolipids [9, 10]. Day 1 was the day after birth. The small intestines were flushed with saline after excision. In one series of experiments, small intestines of 4-5 rats of the same age were pooled at birth, or after 1, 2, 3 and 4 weeks. In all other experiments, rats of a given litter were taken individually at one day intervals during the third week of life.

Lipid Extraction and Neutral Glycosphingolipid Purification

Tissue samples obtained from pooled small intestines were first lyophilized and submitted to extraction in two steps in a Soxhlet apparatus [14]. Individual small intestines were cut into small pieces and extracted with 10 ml of methanol at 70°C for 30 min. The mixture was centrifuged. The supernatant was collected and the tissue was re-extracted three times with 10 ml of chloroform/methanol, 1/2 by vol, at 70°C for 30 min [15].

The neutral glycosphingolipids were purified from the lipid extract according to a published procedure [14, 15]. In short, after mild alkaline degradation and dialysis, the total glycolipid fraction was obtained by chromatography on a silicic acid column (Mallinckrodt, St. Louis, MO, USA). The total glycolipid fraction was separated into neutral and acid glycolipids by chromatography on DEAE-cellulose (DE-23, Whatman Ltd, Maidstone, UK), acetate form. The neutral glycolipids were purified, after acetylation, by chromatography on a column of Iatrobeads 6RS-8060 (60 μm particles; Iatron Laboratories Inc., Tokyo, Japan). After deacetylation of the neutral glycolipid mixture, the final step of purification was done by chromatography on a column of DEAE-cellulose, acetate form.

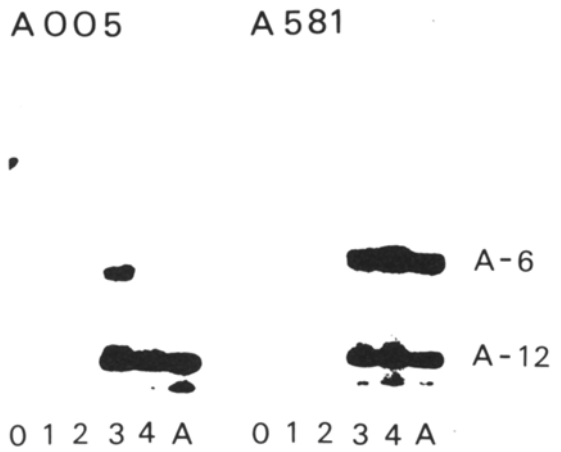

Figure 2. Autoradiogram after TLC and immunostaining by monoclonal anti-A antibodies A005 and A581 of the neutral glycolipids of rat small intestine at different ages during the postnatal development. The same amount of purified neutral glycolipid mixtures (1.5 nmol) was applied to the plate at each age. Numbers refer to the approximate age of rats in weeks. The actual ages were : 0, 1 day; 1, 7 days; 2, 12 days; 3, 22 days; 4, 28 days; A, adult.

Immunostaining of Glycolipids after TLC

The immunostaining of glycolipids was performed according to a modified procedure [16] of Magnani *et al.* [17]. Glycolipids were analyzed on aluminium-backed silica gel 60 sheets (HPTLC, Merck, Darmstadt, W. Germany) in chloroform/methanol/water, 60/35/8 by vol. After drying, the plates were dipped in a solution of 0.3% polyisobutylmethacrylate (Plexigum P28, Röhm GmbH, Darmstadt, W. Germany) in hexane/diethylether, 1/1 by vol. After drying, the plates were sprayed with 137 mM NaCl in 20 mM sodium potassium phosphate buffer pH 7.3 (PBS) containing 2% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO, USA) and 0.1% NaN_3, and soaked in this solution for 2 h. The plates were then overlaid with the appropriate antibody 1:100 diluted in the above buffer solution. Four monoclonal antibodies were used in this study: anti-A A581 (Dakopatts, Glostrup, Denmark), anti-A A005 (BioCarb, Lund, Sweden), anti-H A583 (Dakopatts), and anti-Le^b NS-10-17 [18]. The plates were incubated for 2 h at room temperature and washed five times in PBS. The solution of rabbit anti-mouse immunoglobulins (Dakopatts) was radiolabeled with $Na^{125}I$ (The Radiochemical Centre, Amersham, UK) using Iodogen [19] (Pierce Chemical Co., Rockford, IL, USA), diluted to about 10^6 cpm/ml, and applied over the plates. After 2 h of incubation, the plates were washed six times in PBS, dried and exposed to XAR-5 film (Eastman-Kodak, Rochester, NY, USA) for 20-60 h at 20°C. The autoradiograms were scanned with a Shimadzu CS-910 TLC scanner with wavelength at 550 nm. The signals were recorded on a Shimadzu C-RIB integrator. For quantitative analysis, each series of glycolipid samples was analyzed in duplicate with appropriate A-6 references on a thin layer plate. The plate was cut into two parts. The immunological reactions with both anti-A antibodies were done simultaneously. They were followed by overlay with the same solution of ^{125}I-labeled anti-immunoglobulin. The plates were exposed simultaneously to X-ray film.

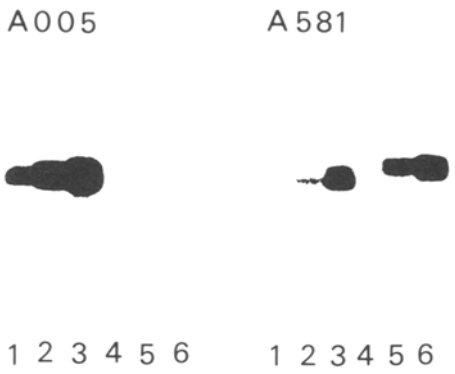

Figure 3. Autoradiogram after TLC and immunostaining of two anti-blood group A antibodies (A005 and A581) against A-active hexaglycosylceramides with type 1 (A-6 type 1) and type 2 (A-6 type 2) chains.
The amount of glycolipid applied to the TLC plate was determined after measurement of the sphingosine content of stock solutions of the purified glycolipids. TLC of A-6 type 2 from dog small intestine; 1, 6 pmol; 2, 60 pmol; 3, 600 pmol: and A-6 type 1 from human meconium; 4, 7 pmol; 5, 70 pmol; 6, 700 pmol.

Chemical Assays

The quantities of total neutral glycolipid mixtures prepared from each tissue samples were expressed as their sphingosine content which was determined by the method of Naoi *et al.* [20] as described in [21].

Results

Blood group A-active glycolipids A-6 and A-12 (Fig. 1), as well as blood group H-active glycolipid H-5, from the small intestine of adult black and white rats have been previously characterized [9, 10]. Rats of the same stock were used for the present developmental study.

In a first approach, the neutral glycolipids were purified from the small intestine of rats taken at one week intervals from birth to four weeks after birth, and at adult age. The presence of blood group A-active glycolipids was detected by TLC-immunostaining with two monoclonal anti-A antibodies, A005 and A581 (Fig. 2). When 1.5 nmol of neutral glycolipid mixture were chromatographed, both antibodies reacted at the level of the six-sugar (A-6) and the twelve-sugar (A-12) glycolipids at 22 days. However, A005 did not detect A-6 at 28 days, while A581 did. The difference of reactivity of A005 and A581 with A-6 was supposed to originate in the type of sugar sequence (type 1 or type 2 chain) on which the blood group A determinant was based.

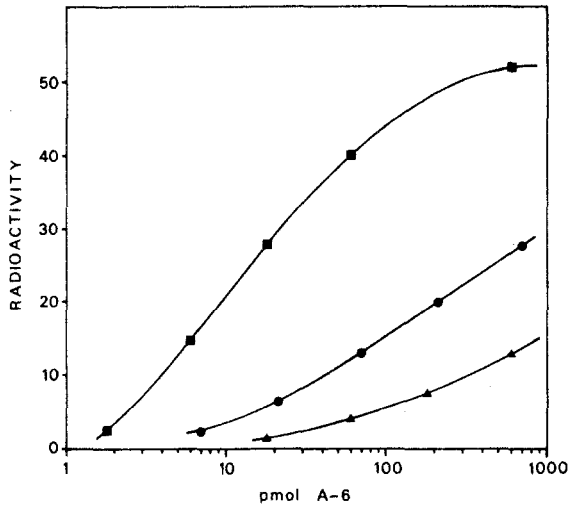

Figure 4. Binding of monoclonal anti-A antibodies A005 and A581 to A-6 type 1 and A-6 type 2 on a thin layer chromatogram. Different amounts of A-6 type 1 (●) or A-6 type 2 (■, ▲) were chromatographed on TLC plates. Chromatograms were overlaid first with either A005 (■) or A581 (●, ▲), and then with the same solution of ^{125}I-labeled anti-mouse immunoglobulins. They were exposed simultaneously to X-ray film for 60 h. After development of the film, the spot intensities were measured on a densitometric scanner. The amount of radioactivity retained on each spot was expressed in arbitrary units of integration.

Specificity of Anti-A Antibodies

In order to verify the above hypothesis, the epitope specificity of both antibodies was tested on thin layer chromatograms of two known blood group A-active hexaglycosylceramides: A-6 with type 1 chain (A-6 type 1) from human meconium [22, 23] and A-6 with type 2 chain (A-6 type 2) from dog small intestine [24] (Fig. 3). Antibody A005 bound to A-6 type 2 and gave a strong reaction with 7 pmol of antigen (detection limit 1 pmol). However, it did not react with A-6 type 1, even with the largest quantity tested (700 pmol). Thus, within this range of antigen concentration and with this method of detection, A005 was regarded as an antibody specific for the A determinant based on the type 2 chain. A581 bound to both A-6 type 1 and A-6 type 2. However, its reactivity was higher with the A determinant based on type 1 (detection limit 3-4 pmol) than with the one based on type 2 chain (detection limit 12 pmol), although its affinity for A-6 type 1 was lower than the affinity of A005 for A-6 type 2 (Fig. 4).

These results supported the hypothesis concerning a change in the internal carbohdyrate chain of A-6 at three weeks (Fig. 2). It is likely that two A-6 glycolipids were present in this age: then the A-6 type 2 faded away, and the A-6 type 1 increased in concentration during the fourth week of life.

Figure 5. Immunodetection of the appearance of A-6 and A-12 during the postnatal development of rat small intestine with anti-blood group A antibodies A005 and A581.
Neutral glycolipid mixtures (3.5 nmol) from the small intestine of individual rats between 14 and 21 days and from pooled small intestines at 22 and 28 days were analyzed by TLC. Chromatograms were overlaid with A005, type 2 chain-specific anti-A antibody; and A581, type 1 chain-specific anti-A antibody (under the present experimental conditions). The actual threshold of appearance was one day earlier than can be seen on this picture.

Developmental Changes of A-6 and A-12 during the Third Week

The subsequent study was focused on the expression of A-active glycolipids during the third week, taking one rat of a litter per day. A-6 and A-12 were immunologically detectable before 22 days with both anti-A antibodies (Fig. 5).

The onset of detection depended on the quantity of glycolipid used. When 3.5 nmol of glycolipid mixture were chromatographed, A-6 and A-12 were detected from 16 days on with A005 and two days later with A581. A-6 and A-12 could be detected already at birth with A005, but not with A581, when 16 nmol of glycolipids were chromatographed (not shown). For reference, A-6 and A-12 began to be chemically detectable at 21-22 days when 35 nmol of glycolipid mixture were chromatographed (Fig. 6).

The calibration curve of the reactivity of the two antibodies against the A determinant based either on type 1 or type 2 chain (Fig. 4) made possible the quantitative analysis of the appearance of A-6 in the small intestine during development (Fig. 7). From birth to six days, A-6 type 2 was present at the level of 0.1 pmol/nmol glycolipids. The concentration was 0.3 pmol/nmol glycolipids between 12 and 15 days, and then rose about tenfold

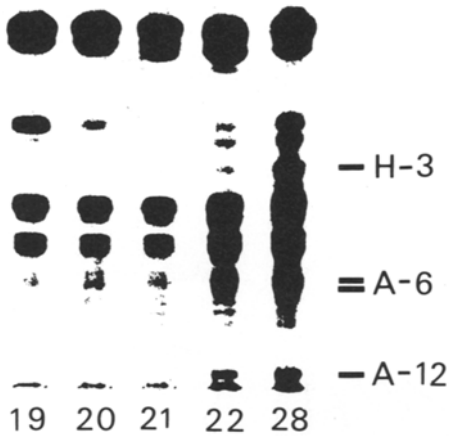

Figure 6. TLC and chemical visualization of neutral glycosphingolipids of rat small intestine at different ages during postnatal development.
Neutral glycosphingolipids (35 nmol) were analyzed by TLC in chloroform/methanol/water, 60/35/8 by vol. They were visualized with 1-naphthol/sulfuric [25]. The numbers refer to the age of the rats in days. The position of the major immunoreactive glycolipids is indicated in the right margin.

during the third week, before decreasing to the low adult level during the fourth week (0.4 pmol/nmol glycolipids).

Under our experimental conditions, A581 reacted only with A-6 type 1, as the quantity of A-6 type 2 (measured with A005) in the aliquots of glycolipids used for the assays was always below the detection limit with this antibody. The threshold of detection of A-6 type 1 (around 1 pmol/nmol glycolipids) was found at 18 days. Its concentration increased sevenfold by 22 days and seventeenfold by the end of the fourth week.

A-12 appeared at the same time as A-6 with both monoclonal anti-A antibodies and increased in quantity in a similar way until 21 days. Thereafter, the reactivity of A-12 with A581 continued increasing in parallel with that of A-6. Its reactivity with A005 increased also after 21 days, whereas that of A-6 disappeared (Fig. 5). This finding is consistent with the fact that A-6 type 1 is the only form of A-6 present in the adult small intestine (Fig. 1, structure I) [9], while 40% of the A-12 structures have a blood group A-type 2 determinant on the 6-linked antenna (Fig. 1, structure III) [10]. The heterogeneity of A-12 and the possible presence of structures different from the adult ones in the early time of appearance prevented us from quantitating the appearance of A-12 more precisely.

An A-active glycolipid, which migrated slightly above A-12 upon TLC and reacted strongly with A005 (A-X, Fig. 5), was present between 19 and 22 days but not in the adult. Its structure was not characterized, nor was the structure of the A-reactive material present at the origin.

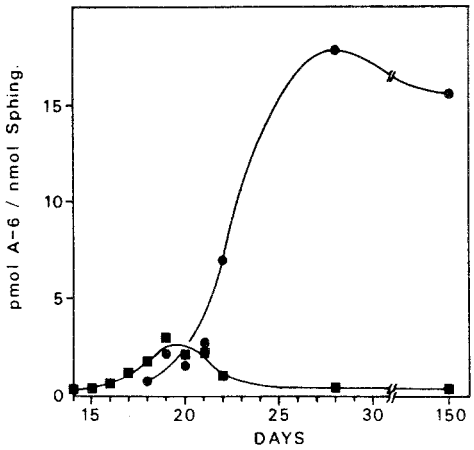

Figure 7. Concentration changes of A-6 type 1 and A-6 type 2 during the postnatal development of the rat small intestine.
Concentrations of A-6 type 1 detected with A581 (●) and A-6 type 2 detected with A005 (■) were expressed as pmol of A-6 per nmol of sphingosine in the total glycolipid extracts of the whole small intestine. One rat of a litter was taken at each time point between 14 and 28 days. Results were obtained from autoradiogram scannings of one representative set, out of three, of TLC-immunostainings processed simultaneously.

Presence of H-Active Glycolipids

The developmental regulation of the expression of blood group A-active glycolipids in the small intestine of black and white rats prompted us to investigate the blood group H-active glycolipids in order to see whether precursor structures of A-6 and A-12 such as H-5 [9] or H-10 [8] could be detected before A glycolipids were expressed. The anti-H antibody A583 reacted only with H-3 (fucosyllactosylceramide), which has been characterized in the small intestine of the adult black and white rat [26] (Fig. 8). The presence of H-3 was detected already at birth. A583 reacts with H determinants based on the type 2 chain, but not with H determinants based on the type 1 chain (Hansson GC, Strömberg N, unpublished results). The possible presence of the latter type was investigated with the anti-Le^b antibody NS-10-17 which cross-reacts with blood group H determinants based on the type 1 chain [18]. Antibody NS-10-17 gave the same pattern of reactivity with H-3 as A583 (Fig. 8). In addition, H-5 type 1 appeared as a faint band at 22 days and a stronger one at 28 days. Therefore, H-5 type 1, which is a known component of the small intestine of black and white rats [9] (Fig. 1, legend) appeared in small quantities at the same time or later than A-6 type 1. A structure such as H-10, which is not found in the small intestine of the adult black and white rat [9], was never detected with either antibody, even when intermediate time points between 12 and 22 days were tested on chromatograms similar to those of Fig. 5.

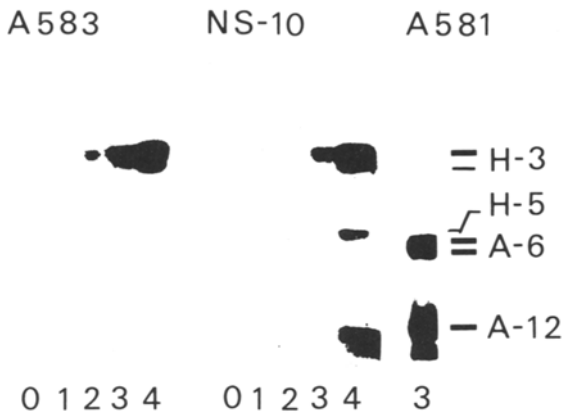

Figure 8. Autoradiogram after TLC and immunostaining of neutral glycolipids of rat small intestine with anti-H antibodies during the postnatal development. The numbers refer to the approximate age of rats in weeks as in Fig. 2. Quantity of neutral glycolipids per lane: 8 nmol. Immunostaining with anti-H antibody A583, and anti-Le^b NS-10-17 [18]. For reference: sample of neutral glycolipids from the small intestines of 22 day old rats run on the same plate and overlaid with anti-A antibody A581.

One can assume that NS-10-17 and A583 had the same reactivity toward H-active glycolipids as anti-A antibody A581, as they detected H-10 from 18 days on in another rat strain (see legend of Fig. 1) that does not express A-active glycolipids (results not shown). It was concluded that H-5 and H-10 were not present in the small intestine of black and white rats at a stage of development preceding immediately the appearance of A-6 and A-12.

Discussion

Two monoclonal anti-blood group A antibodies were used for the present study. It was shown that they had distinct specificities, depending on the carbohydrate sequence on which the A determinant was based. A005 was shown to be specific for the A determinant based on the type 2 chain. The second antibody, A581, had a less narrow specificity, as it was able to react with the A determinant based on both the type 1 and type 2 chain. However, its reactivity with the A-type 2 determinant was lower than its reactivity with the A-type 1 determinant. It was possible, in the present system, to use such an antigen concentration such that the A-type 2 determinant could not react with A581. Thus, A581 could be considered in most cases of this study as specific of the A-type 1 determinant.

The immunological detection of blood group A-active glycolipids gave evidence that the concentration of these glycolipids changed during the postnatal development of the small intestine. It allowed a better appraisal of the changes occurring around 21 days than the chemical visualization of all the constituent glycolipids on thin layer

chromatograms. It increased the specificity and lowered the limit of detection. Chemical means puts the threshold of detection of A-6 and A-12 around 21-22 days (Fig. 6). This result is similar to that found in rats not expressing A-active glycolipids, but expressing a branched decaglycosylceramide (H-10), which begins to be detected chemically at the same period of development [5]. However, A-6 and A-12 could be detected before 21 days by immunostaining on thin layer chromatograms. The rapid rise in concentration occurring after the third week might explain why both glycolipids appeared at a chemically detectable level from 21 days onwards, but not before.

A slight decrease of the A-6 type 1 concentration was observed between 28 and 60 days. This finding might be explained by the increasing contribution of the mesenchyme to the whole intestine after four weeks, as it is known from previous studies that the mesenchyme lacks blood group A and H-active glycolipids [15].

A blood group A-active glycolipid, slightly less polar than A-12, was detected with A005 around 21 days. It is absent from the adult small intestine, and has therefore escaped direct structural characterization. This glycolipid did not react with anti-H antibodies. Thus, it was not a branched structure with an H determinant on one antenna and an A determinant on the other, such as that found by Slomiany and Slomiany in hog stomach [27]. It might be a linear structure similar to the one detected in human umbilical cord erythrocytes, but not in adult human erythrocytes, by Fukuda and Levery [28].

The use of two monoclonal anti-A antibodies of distinct specificities allowed us to demonstrate that not only the concentration of A-6 and A-12 changed, but also the structure of their carbohydrate chain. Before 20 days, A-6 type 2 was more abundant than A-6 type 1, which became the major form of A-6 glycolipid after 21 days. The disappearance of the reactivity of A005 with A-6 and its persistence with A-12 suggest that distinct β(1-4)galactosyltransferases were involved in the synthesis of lactoneotetraosylceramide which is the backbone of A-6 type 2, in 20-day-old rats, and in the synthesis of the type 2 chain of the 6-linked antenna of A-12 in the adult (Fig. 1), [9, 10]. These findings indicate that additional structures of A-12 might be present during the third week of postnatal development, but are not subsequently found in the adult (Fig. 1). Glycolipid A-12 might be based also on lactoneotetraosylceramide and one or both antennae might bear A-type 2 determinants. The unknown reactivity of the anti-A antibodies with the many likely structures of A-12 prevented its precise quantitation during this period of development.

It has been found that, during cellular differentiation, carbohydrate chains can be elongated by one unit at a time [13], or that a straight chain can be replaced by a branched chain structure [29, 30]. In the present study, no such modifications of the carbohydrate structures were observed. Linear A-6 and branched A-12 were present simultaneously. No immediate precursor H-active glycolipids could be detected before the A-active glycolipids were expressed. One can conclude that precursor glycolipids did not accumulate at any time of development and that they were efficiently converted to A-active glycolipids as soon as synthesized.

In conclusion, this work showed that changes in the structure of the carbohydrate moiety of A-active glycolipids could be observed during the postnatal development of the small intestine. They occurred at the same time as the changes previously found in the structure of the ceramide moiety of glucosylceramide and hematoside G_{M3} (ap-

pearance of 2-hydroxylated fatty acids), and in the structure of the sialic acid of G_{M3}-ganglioside (appearance of *N*-glycolylneuraminic acid) [6, 7]. How these structural modifications, which affect the carbohydrate as well as the lipid part of glycosphingolipids, play a role in the tremendous functional changes that occur in the rat small intestine around 21 days [31] is a question that remains to be elucidated.

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