Expression and glycosylation of the filamentous brush border glycocalyx (FBBG) during rabbit enterocyte differentiation along the crypt-villus axis

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INTRODUCTION

Absorbing cells (enterocytes) and mucus-secreting cells (goblet cells) are the two main cell types present in the small intestinal epithelium. They originate from the same stem cells that proliferate near the bottom of the crypts, where the differentiation of each cell type begins (Cheng and Leblond, 1974a,b). The differentiation and maturation processes continue while the cells move up along the crypt-villus axis (Cheng and Leblond, 1974a,b,c; Wright and Irwin, 1982).

Goblet cells secrete the mucus which, thanks to the presence of gel-forming glycoproteins called mucins, forms a protective gel covering the epithelium. Enteroctyes synthesize an ‘enteric surface coat’, which was first described by Ito (1965, 1974) on the basis of electron microscopic data. We recently established that this structure, which has been called the ‘filamentous brush border glycocalyx’ (FBBG), is composed of one 400 kDa mucin-type glycoprotein, which is anchored to the membrane microdomain at the tip of the microvilli. A specific monoclonal antibody (3A4) against this membrane-bound mucin was produced and used to immunopurify it. It recognized a FBBG-specific glycosidic structure containing a terminal O-acetylated sialic acid (Maury et al., 1995).

Mucins are high molecular mass (>100 kDa) glycoproteins in which the glycosidic moiety amounts to 50-80% of the molecular mass (Strous and Dekker, 1992). The oligosaccharidic structures of mucins undergo changes during embryogenesis, cellular differentiation and cancer development, and therefore constitute appropriate antigenic determinants with which to monitor these phenomena using specific antibodies (Feizi and Childs, 1987; Feizi et al., 1984).

The glycosidic structures of highly glycosylated glycoproteins are very complex (Podolsky, 1985a,b; Roussel et al., 1988) and depend mainly on the glycosyltransferase pool of the producing cells (Paulson and Colley, 1989; Ichikawa et al., 1992). It is therefore to be expected that all the glycoproteins synthesized by the same cell will bear common glycosidic structures. This is indeed the case in rabbit enterocytes, as

SUMMARY

The filamentous brush border glycocalyx forming the ‘enteric surface coat’ of the intestinal epithelium is composed in rabbits of a 400 kDa mucin-type glycoprotein, which was purified using the 3A4 monoclonal antibody. This monoclonal antibody recognizes a filamentous brush border glycocalyx-specific glycosidic structure containing an O-acetylated sialic acid, which is absent from all the other glycoproteins in the epithelium, with the exception of certain goblet cell mucins. Here we establish that only 50% of the rabbits tested synthesized this glycocalyx structure. Upon immunolabeling surface epithelia and sections of jejunum from these rabbits, the carbohydrate epitope recognized by the 3A4 mAb was found to be present on the filamentous brush border glycocalyx of a variable number of enterocytes, which were patchily distributed over all the villi. This heterogeneous expression of 3A4 antigenicity, which was also observed in the crypts, suggests the existence of differences between the patterns of differentiation of enterocytes, which results in the expression of different pools of glycosyltransferases and/or acetyl transferases. In mature enterocytes, the 3A4 determinants were present only on the filamentous brush border glycocalyx, which is anchored solely to the membrane microdomain at the tip of brush border microvilli. However, expression of 3A4 antigenicity begins in the median third of crypts, in enterocytes with a short, thin brush border devoid of apical filamentous brush border glycocalyx. Here the 3A4 epitopes were present over the whole brush border membrane. A few cells higher up, the hyperpolarized expression of filamentous brush border glycocalyx, i.e. its segregation at the tip of the microvilli, began and both the apical filamentous brush border glycocalyx and the whole brush border membrane were labeled with the 3A4 mAb. The labeling of the lateral membrane of microvilli gradually decreased and then disappeared during the migration of the cells to the upper part of the crypts. The 3A4 structure was also detected in some of the granules of some rare goblet cell mucins in the vicinity of the most deeply located labeled enterocytes, whereas no labeled goblet cells were ever observed at higher cell locations.

Key words: enterocyte, differentiation, glycocalyx, mucin, brush border
regards the human blood group ABH type 1 and/or 2 glycosidic structures, which are present in almost all the plasma membrane glycoproteins (Feracci et al., 1982; Gorvel et al., 1982, 1987b). The same structures are also synthesized by goblet cells, and are consequently present on mucins (Zweibaum and Bouhou, 1973; Oriol and Dalix, 1977; Gorvel et al., 1987b). Rabbts termed A* express the A structures whereas A- rabbits express the H structures. In A+ rabbits the human blood group A type 3 (A3) determinant is present, but only in a few of these glycoproteins (Gorvel et al., 1987a,b). A specific monoclonal antibody (14A4) directed against this A3 structure was used to label the FBBG on jejunum sections of A+ rabbits and to distinguish it for the first time from unlabeled mucins stored in the goblet cells (Gorvel et al., 1987a).

We recently reported (Maury et al., 1995) that in both A+ and A- rabbits, the FBBG was the sole glycoconjugate in small intestinal mucosa that bears the glycosidic antigenic determinant recognized by the monoclonal antibody 3A4. O-acetylated sialic acid is an essential component of the 3A4 epitope. The 3A4 antibody was used here to study the expression of the 3A4 epitope during small intestinal epithelial cell differentiation along the crypt-villus axis.

MATERIALS AND METHODS

Materials
Biodyne B membrane was from Pall (Glen Cove NY 11542). Rabbit anti-mouse Igs and goat anti-mouse Igs coupled to either peroxidase or fluorescent were obtained from Cappel (Malvern PA, USA).

Monoclonal antibodies
Monoclonal antibody 3.3A, which was previously called Cl 3.3 (Gorvel et al., 1987a), was a gift from Dr Bara (IRSC, Villejuif). It specifically recognized human blood A glycosidic structures of types 1, 2, and 3 (Bara et al., 1988; Gane et al., 1987). The procedures used to produce and characterize 14A4 monoclonal antibody with a restricted specificity for the human blood group A type 3 (A3) glycosidic structure (Gane et al., 1987; Gorvel et al., 1987a,b) and of the anti-FBBG 3A4 monoclonal antibody (Maury et al., 1995) have been described in detail.

Partial purification of the FBBG from the brush border membrane
This technique has been described previously (Maury et al., 1995). Briefly, a crude brush border membrane preparation devoid of mucin contamination was prepared as described by Schmitz et al. (1973). After brush border membrane solubilization by Triton X-114 and phase separation (Pryde, 1986), the FBBG present in the aqueous phase was separated from any contaminants with a lower molecular mass by filtration through a Sepharose CL-2B column.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting
These techniques have been described previously (Feracci et al., 1986). Here a poorly crosslinked (monomer to crosslinker ratio of 200:1) 7.5% polyacrylamide gel and a 3.5% stacking gel were used. The proteins were transferred onto Biodyne B membrane instead of nitrocellulose. Either undiluted culture supernatants of 14A4 and 3A4 hybridomas or 3.3A ascites fluid (1:10^5 dilution) and goat anti-mouse Ig coupled to peroxidase (1:500 dilution) were used for the immunoblotting experiments.

Immunoprecipitation
A rat monoclonal antibody against the κ chain of mouse IgG, the 139 5211 mAb (Immunotech, Marseille, France), was coupled to Affi-Gel 10 (Bio-Rad, Hercules, California) as recommended by the manufacturer. The immunogel obtained was then incubated overnight with a large excess of 3A4 in culture supernatant (1 ml of supernatant with 60 μl of beads). The 3A4-beads obtained were used for the immunoprecipitation of the recognized antigen, as described previously (Massey et al., 1987).

Surface immunoperoxidase labeling
This technique was performed as described by Schmidt et al. (1985) with a few modifications. Segments (4 cm) of jejunum and ileum were cut out, opened longitudinally and pinned on a paraffin layer with the luminal surface uppermost. The preparations were then fixed for 1 hour in 4% formaldehyde solution in phosphate buffer, pH 7.3, containing 8.1 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl (PBS). To remove the mucus, the preparations were incubated for 2 hours in 20 mM dithiothreitol (DTT) added to 15 mM Tris-HCl buffer, pH 8.2, containing 0.1 M NaCl and 20% ethanol. The intestinal sheets were then washed in PBS (several times) and fixed again in the 4% formaldehyde solution for 1 hour. After being extensively washed with PBS, the preparations were incubated: (i) for 90 minutes in 0.1% sodium azide in PBS to block the endogenous peroxidase; (ii) for 30 minutes in PBS containing 10% bovine serum (PBS-BS); (iii) overnight with either culture supernatant of 3A4 hybridoma or 1:500 dilution of 3.3A ascites fluid in PBS-BS. After four washes with PBS-BS, the preparations were incubated for 5 hours with anti-mouse Iggs coupled to peroxidase (commercial solution diluted 1:200 in PBS-BS) and washed again with PBS-BS. The peroxidase was revealed using 1.3 mM diaminobenzidine (DAB) in PBS containing 0.01% H2O2. The fixation of the 3A4 and 3.3A antibodies on the cell surface was observed under a dissecting microscope as a brown reaction product. The villi were then dissected out, deposited on a glass slide, mounted in 50% glycerol, and examined with a Zeiss photomicroscope III.

Immunoelectron microscopy
Immunocolloidal gold labeling of Epon-embedded sections was performed as described previously (Bernadac et al., 1984; Chambraud et al., 1989). Briefly, immediately after the animal was killed, 1-2 mm thick pieces of jejunum were excised at a distance of about 1 metre from the pylorus. They were then fixed in 2.5% glutaraldehyde in PBS for 1 hour at 4°C. After being washed with PBS, they were post-fixed in 2% OsO4 in PBS for 1 hour at room temperature, and then dehydrated in a graded ethanol series and embedded in Epon 812. The post-embedding technique used was then performed as follows: thin sections (0.06 μm) on grids were treated with 1% H2O2 in water for 5 minutes, rinsed in water and then in PBS. In order to minimize any non-specific adsorption, the sections were floated on 1.5% BSA in PBS for 10 minutes and incubated first with 3A4 hybridoma culture supernatant for 90 minutes at room temperature. After washing, 3A4 was revealed using a double-sandwich technique with rabbit anti-mouse IgGs (dilution 1:1000; incubation 30 minutes) and then Protein A-gold (incubation 1 hour). Protein A-gold (15 nm) was prepared as previously described (Bernadac et al., 1984). After being washed with water, the sections were then lightly stained with uranyl acetate for 3 minutes and lead citrate for 2 minutes.

Immunofluorescence
The immunofluorescence technique was performed as described previously (Feracci et al., 1982), except that 0.25 μm sections of Epon-embedded rabbit jejunum were used instead of ultra-thin frozen sections, in which the FBBG was lost (Gorvel et al., 1987a). Briefly, the sections were deposited on a glass slide and incubated for 5 minutes in PBS containing 1.5% bovine serum albumin, and then for
15 minutes with either the 3A4 mAb in undiluted culture supernatant or the 3.3A mAb in ascites fluid diluted 1:1000 in PBS containing 1.5% BSA. After being washed with PBS, the sections were incubated for 30 minutes with fluorescein-labeled goat anti-mouse immunoglobulins diluted 1:100, washed, mounted in 50% glycerol, and examined with a Zeiss Photomicroscope III equipped with a III RS fluorescence epicondenser.

RESULTS

Immunolabeling of rabbit jejunum sections with the monoclonal antibody 3A4

Fig. 1 shows that along the whole villi and the upper part of the crypts, the 3A4 mAb specifically labeled the small intestinal surface coat. This coat is formed as shown in Fig. 2, by the fine filaments radiating from the tip of the brush border microvilli that we called ‘filamentous brush border glyocalyx’ (FBBG).

In the lower third of the crypts, the Paneth cells, goblet cells and poorly differentiated enterocytes with a few short, irregular microvilli were never labeled. In the median third of the crypts, labeling occurred in more differentiated enterocytes, such as cell 1 in Fig. 3A and C, which are characterized by the presence of numerous microvilli about 1-1.2 μm in length (as compared with 2.5 μm in mature enterocytes), forming a short brush border devoid of any apical FBBG. In these cells, the whole brush border membrane was labeled. Three to five cells higher up the crypts, the apical FBBG was present, and both the FBBG and the whole brush border membrane were labeled (cell 2 in Fig. 3A). The labeling of the lateral part of the brush border membrane disappeared during the migration of the cells to the upper part of the crypts. In the vicinity of the most deeply labeled crypt enterocytes, the secretory granules of some goblet cells were labeled (Fig. 3D). Above this restricted region, no goblet cells were ever labeled, as shown in Figs 1 and 3A.

Immunogold labeling using 3A4 mAb was performed on jejunum sections from four rabbits. Electron microscopy of villi present in these sections revealed that the FBBG of some enterocytes was devoid of any labeling (Fig. 4). Enterocytes with unlabeled FBBG were rare in the case of one rabbit (see Figs 1, 3 and 4), but constituted the majority of the enterocytes in the case of one other rabbit. In two other rabbits, the FBBG of about 50% of the enterocytes was unlabeled. In all four rabbits, heterogeneous expression of the 3A4 antigenicity occurred in the enterocytes of all the crypts examined. No crypts that were either unlabeled or uniformly labeled were ever detected.

Surface immunolabeling of rabbit small intestinal sheets with the 3A4 mAb

Investigation of the heterogeneous expression of 3A4 epitopes by the villus enterocytes was facilitated by performing surface labeling on the epithelium. As shown in Fig. 5, it was possible after most of the mucus has been eliminated, to label the villi with 3A4 mAb, except in some rabbits (see below). At low magnifications (Fig. 5) the labeling seemed to be uniform, whereas at high magnifications (Fig. 6) two types of labeling were observed: either all the enterocytes were labeled (Fig. 6A) or a patchy pattern of labeled and unlabeled enterocytes was detected (Fig. 6B). The proportion of labeled to unlabeled enterocytes on the various villi seemed to be uniform in any one rabbit, but was highly variable from one rabbit to another (compare Fig. 6A and B). Identical results were obtained for the jejunum and ileum.

When the labeling was performed using the 3.3 A mAb recognizing the human blood group A structures, the pattern of homogeneous labeling shown in Figs 5B and 6C was always observed in A+ rabbits.

Immunoblotting analysis of the expression of 3A4 and human blood group A antigenicities in rabbit small intestine

To test the presence of the 3A4 glycosidic structure in the FBBG of a large number of rabbits, high molecular mass soluble glycoproteins from 5 μg of mucosa obtained by scraping of the entire small intestine from each of 132 rabbits,
were separated by SDS-PAGE, transferred onto Biodyne B and immunostained with 3A4 mAb. In 50% of the rabbits, the 3A4 mAb specifically labeled the 400 kDa band corresponding to the FBBG (Maury et al., 1995), whereas in the other 50% no 3A4 antigenicity was expressed in the small intestinal epithelium. The same blots were then immunostained with 3.3A mAb to test the expression of the human blood group A antigenicity in the same rabbits. The results obtained showed that: 19% of the rabbits were 3A4+ and A+, 31% were 3A4+ and A−, 26% were 3A4− and A+, and 24% were 3A4− and A−. It was therefore concluded that no correlation seemed to exist between the expression of 3A4 and human blood group A glycosidic structures.

**Immunoprecipitation of FBBG from A+ rabbit with the 3A4 mAb**

The presence or absence of 3A4 epitopes in FBBG was also tested by carrying out immunoprecipitation with the 3A4 mAb of FBBG from A+ rabbits, in which the FBBG can also be specifically labeled by the 14A4 mAb which recognized the human blood group A type 3 structure (Gorvel et al., 1987a; Maury et al., 1995).

Complete immunoprecipitation of material recognized by the 3A4 mAb present in the FBBG partially purified from A+ rabbit brush border membrane vesicles was performed by successively incubating the material with three samples of 3A4 mAb beads. The bound and unbound material was then analysed using the immunoblotting technique with 3A4, 14A4 and 3.3A monoclonal antibodies. The data given in Fig. 7 show that, as expected, only the immunoprecipitated FBBG was revealed by these three antibodies. The results obtained with 14A4 mAb showed that FBBG devoid of 3A4 epitopes was present in the unbound material.

**DISCUSSION**

Techniques involving aggregation chimeras (Ponder et al., 1985; Schmidt et al., 1985), transgenic mice (Gordon, 1989; Sweetser et al., 1988) and somatic mutations (Winton et al., 1988), yielding mice composed of two well defined intestinal genotypes, have shown that in adult mice, the epithelium of one crypt is always composed of a single clone of cells that

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**Fig. 2.** Specific labeling obtained on the FBBG of a mature rabbit enterocyte after performing immunocolloidal gold labeling with 3A4 mAb on ultra-thin sections (600 Å) of Epon-embedded jejunum. Note the absence of any labeling on the lateral membrane of microvilli.

**Fig. 3.** Part of the median third of one crypt observed after performing immunocolloidal gold labeling with the 3A4 mAb on ultra-thin sections (600 Å) of Epon-embedded jejunum. In A, five cells can be easily delimited thanks to the presence of desmosomes, indicated by black arrowheads. Observations at higher magnification showed that the dark spot indicated by a white arrowhead was not a desmosome. The four enterocytes were numbered from 1 to 4, cell 1 being the lowest in the crypt. The appearance of the apical FBBG, forming the enteric surface coat, can be observed in cell 2. Note that it shows up only on one half of the microvilli of this cell. In B, magnification of part of cell 3. Note the labeling of both the FBBG and the lateral membrane of microvilli. In C, magnification of the part of cell 1 not shown in A. Note the absence of apical FBBG and the labeling of the whole membrane of short (1-1.2 μm) microvilli. In D, a goblet cell located in the same zone as the enterocyte shown in C, in which some of the mucus granules are labeled, whereas, as shown in A, the goblet cell located four cells higher up was unlabeled.
migrate in straight lines along the crypt-villus axis (Schmidt et al., 1985; Winton et al., 1988). The epithelium of one villus therefore originates from several crypts (Wright and Irwin, 1982). In these experimental systems, the epithelium covering one villus is formed of ribbons of cells expressing one of the two intestinal genetic markers introduced into the mice (for review see Gordon, 1989).

It was recently suggested that a heterogeneous differentiation of enterocytes originating from one crypt might occur, since different patchy patterns of expression were observed in the case of either lactase in hypolactasic adult humans (Maiuri et al., 1991), and adult rats and rabbits (Maiuri et al., 1992), or blood group A structure in A non-secretor humans (Maiuri et al., 1993). Here we provide another example of this heterogeneous pattern of expression in the case of enterocytes originating from the same crypt. This finding has to do with the synthesis of the FBBG-specific glycosidic 3A4 structure recognized by the 3A4 monoclonal antibody.

Electron microscopy observations carried out on Epon-embedded jejunum sections have shown that in the upper part of crypts and along the villi, all the enterocytes synthesize FBBG, a mucin-like glycoprotein (Maury et al., 1995) which forms the enteric surface coat first described by Ito (1965). Previous studies (Bernadac et al., 1984; Gorvel et al., 1987b) have shown the presence on FBBG of human blood group glycosidic structures of types A in A+ rabbits and H in A- rabbits. We recently established that the FBBG from both of these types of rabbit also bear a glycosidic epitope containing at least one O-acetylated sialic acid recognized by the 3A4 mAb (Maury et al., 1995). Immunoblotting analysis and immunolabeling experiments on jejunum epithelium from several rabbits showed however that the 3A4 structure was synthesized by enterocytes in only 50% of the rabbits tested.

Whereas the A antigenicity is always present on the FBBG of all the enterocytes in A+ rabbits, the 3A4 epitopes are present on the FBBG of either all or only a variable number of enterocytes in rabbits expressing the 3A4 antigenicity. In this last case, the 3A4 immunolabeled enterocytes have a patchy distribution over all the villi. Apparently, two programs of differentiation take place during differentiation in the crypts, where labeled and unlabeled immature enterocytes were observed. This leads to the expression of different glycosyltransferase(s) and/or O-acetyl transferase(s), which participate in the synthesis of the glycosidic moiety of the FBBG (Corfield et al., 1976; Paulson and Colley, 1989; Ichikawa et al., 1992).

In the vicinity of the less differentiated labeled enterocytes, the mucus granules of some goblet cells were also labeled, whereas no goblet cells were ever labeled above this level. In this restricted region of the crypts, the expression of enzymes involved in the synthesis of the 3A4 epitope might occur not

![Fig. 4. Brush border of three adjacent mature enterocytes after immunocolloidal gold labeling with the 3A4 mAb of ultra-thin section of Epon-embedded jejunum. Note the absence of any labeling of the FBBG synthesized by the central cell.](image-url)
only in enterocytes but also in goblet cells where a rapid change in the pool of enzymes involved in the mucin glycosylation might result in the disappearance of the 3A4 structure. The possibility cannot be excluded that the enterocytes which express no 3A4 antigenicity on their FBBG may, as in the case of the goblet cells, transiently synthesize the 3A4 antigenic determinant. Modifications (Corfield et al., 1976; Schauer et al., 1988) such as further acetylation, deacylation, glycosylation instead of acetylation, and/or cleavage of the O-acetylated sialic acid of the 3A4 epitope might destroy the 3A4 antigenicity. A further glycosylation of either the chain bearing this O-acetylated sialic acid or a neighbouring chain might also mask the 3A4 epitope.

The differentiation of the enterocytes along the crypt-villus axis has been investigated by studying the expression of various markers. Hydrolases such as aminopeptidase N (Gorvel et al., 1986; Norén et al., 1989) and sucrase-isomaltase (Hauri et al., 1980; Traber, 1990), which are integrated into the whole brush border membrane, have been used as markers of terminal differentiation although some monoclonal antibodies have also served to detect inactive sucrase-isomaltase and dipeptidylpeptidase IV in the microvilli of crypt enterocytes (Beaulieu et al., 1989; Gorvel et al., 1991). There also exist specific markers of crypt cells (Quaroni, 1985). Like some brush border membrane glycoproteins (Gorvel et al., 1986), brush border cytoskeleton proteins are expressed along the whole crypt-villus axis and, except for myosin I, are concentrated in the apical pole of even very poorly differentiated crypt cells (Fath et al., 1990; Heintzelman and Mooseker, 1990; Louvard et al., 1992). Myosin I is associated with the brush border cytoskeleton only in villus enterocytes (Heintzelman and Mooseker, 1990). From the bottom to the upper part of the crypts, however, the increase in the number and length of microvilli, which is correlated with the cytoskeleton organ-
Here the pattern of immunolabeling observed in the crypt enterocytes using 3A4 mAb showed the existence of three steps in the differentiation of the brush border membrane. The expression of 3A4 antigenicity began in the median third of the crypts. In these cells, 3A4 epitopes were present over the whole membrane of the short microvilli, and there was no FBBG concentrated at the tips. Three to five cells higher up, the apical FBBG was visible at the tips of the short microvilli of enterocytes which were in an intermediate state of differentiation characterized by the presence of 3A4 epitopes on the apical FBBG at microvillar tips as well as on the whole brush border membrane. The 3A4 antigenicity of the lateral membrane of the microvilli disappeared gradually during the migration of cells to the upper part of the crypts and no longer existed in mature enterocytes. If the 3A4 determinants were specifically borne by FBBG in immature cells, as in the mature enterocytes, their presence along the whole brush border membrane would suggest that these poorly differentiated enterocytes had not yet acquired their hyper-polarity, i.e. that the FBBG had not yet formed clusters at the tips of the microvilli. This phenomenon might be induced by a specific step in the assembly of submembrane cytoskeletal component(s). By conducting parallel studies on the two events it should be possible to test this hypothesis.

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