Anti-glycosyl antibodies in lipid rafts of the enterocyte brush border: a possible host defense against pathogens


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The pig small intestinal brush border is a glycoprotein- and glycolipid-rich membrane that functions as a digestive/absorptive surface for dietary nutrients as well as a permeability barrier for pathogens. The present work was performed to identify carbohydrate-binding (lectinlike) proteins associated with the brush border. Chromatography on lactose-agarose was used to isolate these proteins, and their localization was studied biochemically and by immunofluorescence microscopy and immunogold electron microscopy. IgG and IgM were the two major proteins isolated, indicating that naturally occurring anti-glycosyl antibodies are among the major lectinlike proteins in the gut. IgG and IgM as well as IgA were localized to the enterocyte brush border, and a brief lactose wash partially released all three immunoglobulins from the membrane, indicating that anti-glycosyl antibodies constitute a major part of the immunoglobulins at the luminal surface of the gut. The antibodies were associated with lipid rafts at the brush border, and they frequently (52%) colocalized with the raft marker galectin 4. A lactose wash increased the susceptibility of this membrane toward labeling with lectin peanut agglutin (PNA) and cholera toxin B, suggesting that anti-glycosyl antibodies compete with other carbohydrate-binding proteins at the brush border. In particular, we wanted to identify novel lectinlike proteins that either contribute to the stability of these specialized membrane microdomains or use them as platforms for the final digestion of nutrients but unfortunately may also be exploited by pathogens as attachment sites during infection.

In addition to its absorptive functions, the small intestinal epithelium constitutes a protective barrier separating the apical aspect of the mucosa from the underlying tissue as well as being a site of intense immunological activity. The transcytotic route taken by IgA passes through an endosomal, lipid raft-containing subapical compartment, indicating the involvement of membrane microdomains in the sorting process.

In the present work, we further explored the role of lipid rafts as stable glycoconjugate-enriched platforms in the enterocyte brush border. In particular, we wanted to identify novel lectinlike proteins that either contribute to the stability of these specialized membrane microdomains or use them as platforms to fulfill their function. Immunoglobulins were the main type of lactosyl-binding mucosal proteins thus identified. The major part of these immunoglobulins was released from the brush border by a lactose wash, indicating that they belong to a common group of anti-glycosyl antibodies that has previously been isolated from antisera of animals immuninized with nonviable streptococcal cells. Anti-glycosyl antibodies are defined as antibodies induced in the host by a glycosyl antigen and combine with a specific carbohydrate moiety of that antigen. This group of antibodies comprises anti-galactose antibodies, which combine with terminal galactose, as well as anti-lactose antibodies combining with terminal lactose residues of the same antigens. Interestingly, the release of antibodies from the brush border by the lactose wash was paralleled by an increased sensitivity of this membrane toward labeling with lectin peanut agglutin (PNA) and cholera toxin B (CTB). This relationship indicates that anti-glycosyl antibodies in the gut lumen may act as protectors of the brush border against pathogens.
MATERIALS AND METHODS

Materials. Lactosylagarose was obtained from Sigma-Aldrich (Copenhagen, Denmark). Horseradish peroxidase-conjugated swine antibodies to rabbit immunoglobulins, goat anti-rabbit immunoglobulins, and rabbit antibodies to swine immunoglobulins, IgA, IgM, IgG, and IgD were from DAKO (Glostrup, Denmark). A monoclonal mouse antibody to actin was from Santa Cruz Biotechnology (Santa Cruz, CA), and a rabbit antibody to intestinal alkaline phosphatase was from Biogenesis (Poole, UK). Alexa Fluor 594-conjugated CTB subunit from Vibrio cholerae and a lectin PNA-Alexa Fluor 488 conjugate were from Molecular Probes (Copenhagen, Denmark).

Pig small intestines were kindly given by Letty Klarskov and Mette Olesen (Department of Experimental Medicine, The Panum Institute, Copenhagen, Denmark).

Isolation of mucosal lactosyl-binding proteins. Portions of washed and frozen pig small intestines (about 60 g) were thawed, and the mucosa was scraped off and homogenized in 80 ml of 25 mM HEPES-HCl and 150 mM NaCl (pH 7.1) containing 0.1 μg/ml leupeptin and aprotinin. The homogenate was centrifuged at 48,000 g for 1 h, and the supernatant was filtered and passed through a column packed with 3 ml lactosyl agarose at 4.5 ml/h. After the column was washed with 35 ml of 25 mM HEPES-HCl and 150 mM NaCl (pH 7.1), lactosyl-binding proteins were eluted with the above buffer containing 100 mM lactose. The eluate was dialyzed exhaustively against distilled H2O, centrifuged at 48,000 g for 30 min, and dried by evaporation in a SpeedVac centrifuge. The resulting pellet of lactosyl-binding proteins was stored at −20°C.

For purification of the 50-kDa lactosyl-binding protein, the pellet of lactosyl-binding proteins was dissolved in 150 μl of 50 mM sodium phosphate and 150 mM NaCl (pH 7.0), filtrated, and pumped through a Superose 12 PC 3.2/30 column mounted in a SMART System (Pharmacia Biotechnology; Uppsala, Sweden) at a rate of 40 μl/min. Eluate fractions of 50 μl were collected, and those containing the purified 50-kDa protein were pooled and stored at −20°C until further use.

Preparation of antibodies to 50-kDa lactosyl-binding protein. Rabbits were immunized intracutaneously at 2-wk intervals with the purified 50-kDa lactosyl-binding protein (about 10 μg/injection) mixed with an equal volume of Freund’s incomplete adjuvant. The rabbits were bled a week after the fourth injection, and booster injections were given at 6-wk intervals, followed by new bleedings. Finally, the immunoglobulin fraction was isolated from the antisera by chromatography on a column of protein A-Sepharose as earlier described (50). In immunoblotting, this antibody specifically recognized a broad band of 50 kDa in mucosal fractions. A commercially available antibody to IgG exhibited the same labeling.

SDS-PAGE and immunoblotting. SDS-PAGE in 10% polyacrylamide gels was performed as described (32). After electrophoresis and electrotransfer of proteins onto Immobilon membranes, immunoblotting was performed with antibodies to total immunoglobulins or specifically to IgA, IgG, and IgM. Blots were developed by an electrochemiluminescence detection reagent kit according to the protocol supplied by the manufacturer (Amersham Biosciences). After the immunoblotting, total protein was visualized by staining with Coomassie brilliant blue R250 (0.2% dissolved in methanol-H2O-acetic acid (50:43:7) for 1 min), followed by destaining in the same solvent for 30 min.

MALDI-TOF analysis. Eluates from the lactosyl agarose chromatography were subjected to SDS/PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane. The 50- and 70-kDa bands, visualized by staining with Coomassie brilliant blue, were carefully excised and submitted to commercial MALDI-TOF analysis (Alphalyse; Odense, Denmark). For the 50-kDa band, four peptides were identified, matching the amino acid sequences 23–38, 147–159, 341–352, and 402–412 of the immunoglobulin-α chain of Sus scrofa. For the 70-kDa band, three peptides were identified, matching the amino acid sequences 49–62, 145–152, and 242–252 of the immunoglobulin-μ chain of Sus scrofa. In addition, two peptides were identified in the 70-kDa band, matching the amino acid sequences 20–35 and 187–200 of the immunoglobulin-δ heavy chain of Sus scrofa.

Preparation of microvillar membranes. Microvillar membrane vesicles were prepared from small intestinal mucosa by the divalent cation precipitation method (7). Briefly, mucosal scrapings were homogenized in 2 mM Tris-HCl and 50 mM mannitol (pH 7.1) containing 0.1 μg/ml aprotinin and leupeptin using a manually operated Potter-Elvehjem homogenizer. The homogenate was cleared by centrifugation at 500 g for 10 min, and MgCl2 was added to a final concentration of 10 mM. After incubation on ice for 15 min, the preparation was centrifuged at 1,500 g for 10 min to pellet intracellular and basolateral membranes. Finally, the supernatant was centrifuged at 48,000 g for 1 h to obtain a pellet of microvillar membrane vesicles.

Lipid raft analysis. Microvillar membranes were resuspended in 1 ml HEPES-HCl and 150 mM NaCl (pH 7.1) and extracted with 1% Triton X-100 for 10 min on ice. Detergent-resistant membranes (lipid rafts) were prepared as described (10) with the modification that the extract was placed in a 60% sucrose cushion with a 50–25% sucrose gradient layered on top. The extract was centrifuged in an SW40 Ti rotor (Beckman Instruments; Palo Alto, CA) for 20–22 h at 35,000 rpm (γmax = 217,000), as described previously (13).

Fluorescence microscopy. Mucosal explants of about 1 cm² were excised from the small intestine, taken 1–2 m from the pylorus. The explants were gently washed at 4°C for 10 min in MEM medium in the absence or presence of 10 mM lactose and then fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2) for 2 h at 4°C. After being rinsed in PB, mucosal explants were frozen in precooled 2-methylbutane and mounted on a precooled cryostat table. Sections (7 μm thick) were cut in a Leitz cryostat at −20°C, collected on glass slides, and labeled with the appropriate primary antibody followed by the secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulins). Control experiments were performed by omission of the primary antibody. For lectin PNA and cholera toxin binding studies, sections were incubated for 2 h with Alexa 488-conjugated lectin PNA (0.25 μg/ml) and Alexa 594-conjugated CTB (5 μg/ml), respectively.

All sections were finally mounted in antifade mounting medium and examined in a Leica DM 4000B microscope equipped with a Leica DC 300FX camera.

Electron microscopy. Pieces of the pig small intestine were fixed in 2% paraformaldehyde-0.1% glutaraldehyde in PB at 4°C. After being rinsed in PB, tissue pieces were immersed in 2.3 M sucrose, mounted on top of a metal pin, and frozen in liquid nitrogen. Ultracyrosectioning and immunogold double labeling of IgG and galectin 4 were performed as previously described (26, 27). Briefly, ultracyrosections of ~85–100 nm were cut in a RMC MT 6000-XL ultracytomicrotome, collected with a sucrose droplet, and attached to formvar-coated 90-mesh nickel grids. Goldparticles of 7 and 13 nm were prepared according to Slot and Geuze (52) and conjugated to goat anti-rabbit immunoglobulins as previously described (27). Control experiments were performed by omission of both the first and second primary antibody and by omission of only the second primary antibody.

The ultracyrosections were finally examined in a Zeiss EM 900 electron microscope equipped with a Mega View photography system. For a morphometric analysis of colocalization of IgG and galectin 4 in the microvillar membrane, 500 gold particles representing IgG were examined for colocalization with gold particles representing galectin 4. Particles <10–15 nm apart from another were scored as “colocalized.”
RESULTS

Identification of small intestinal lactosyl-binding proteins. In a screen for small intestinal mucosal lactosyl-binding proteins, two bands of 50 and 70 kDa, respectively, were by far the major components isolated by lactosyl agarose chromatography (Fig. 1). Both bands reacted strongly in immunoblotting with an anti-immunoglobulin antibody, and, by MALDI-TOF analysis of the excised bands, they were identified as the heavy chains of IgG (50-kDa band) and IgM and IgD (70-kDa band), respectively.

Immunolocalization of IgG, IgA, and IgM. Figure 2 shows the distribution of IgG in freshly obtained pig small intestinal mucosa as visualized by labeling with the rabbit antibody prepared to the purified 50-kDa polypeptide identified as the IgG heavy chain. The strongest labeling was seen in the subepithelial lamina propria, where numerous lymphocytes were present. In addition, the antibody distinctly but more weakly labeled the brush border as well as the basolateral lining of the enterocytes in both crypts and villi. The brush border labeling was patchy, in particular in the villus region, probably reflecting that IgG is only a peripherally associated protein bound to the membrane by relatively weak interactions. A commercially available antibody to IgG showed a similar labeling pattern (data not shown). As shown in Fig. 2, a brief wash with medium containing 10 mM lactose removed the major part of the labeling associated with the brush border, whereas the basolateral surface of enterocytes and the stong labeling of the lamina propria was unaffected by the lactose treatment. In controls, lacking a primary antibody, only weak autofluorescence over the lamina propria was observed (Fig. 2).
IgA is known to be the major immunoglobulin synthesized and secreted by lymphocytes of the small intestine, and, by receptor-mediated basolateral to apical transcytosis through the enterocyte, dimeric IgA is subsequently released into the lumen of the gut (31, 39, 45). As seen for IgG, labeling of IgA was most intense in the lamina propria. In the epithelium, labeling was strongest over the brush border both in the crypt and villus regions, but basolateral and intracellular staining of the enterocytes was also detectable (Fig. 2). After a wash with lactose, the brush border labeling was greatly diminished relative to the staining of the lamina propria and the basolateral/intracellular parts of enterocytes.

For IgM, epithelial labeling was observed in the crypt region (Fig. 2), whereas labeling was absent or very weak in the villus region (data not shown). In enterocytes, this polymeric immunoglobulin was mainly localized to the brush border region of the enterocytes, where the labeling appeared patchy as for IgG, but IgM was also seen intracellularly and at the basolateral surface. As with IgG and IgA, the lactose wash effectively removed most of the brush border labeling for IgM relative to the staining of the lamina propria and the intracellular/basolateral parts of the enterocytes.

Taken together, these results indicate that a fraction of mucosal IgG and IgM, like IgA, is localized at the enterocyte brush border. In addition, the lactose susceptibility of the brush border labeling agrees well with the finding that immunoglobulins were the major proteins isolated from intestinal mucosa by lactosylagarose chromatography (Fig. 1), and it implies that at least a fraction of the immunoglobulins in the brush border belongs to the class of anti-glycosyl antibodies previously isolated from serum (9, 36, 43).

Finally, labeling for IgD distinctly stained lymphocytes of the villus stroma, but epithelial cells were only diffusely labeled by this antibody (data not shown).

**Anti-glycosyl antibodies in the microvillar membrane fraction.** Right-side-out microvillar membrane vesicles of high purity (7) were prepared by the divalent cation precipitation method to confirm the presence of anti-glycosyl antibodies in the enterocyte brush border. Figure 3A shows that by immunoblotting, IgG and IgM as well as IgA are all present in the microvillar membrane fraction, in agreement with the labeling of the brush border seen by immunofluorescence microscopy. In addition, a brief washing of the microvillar vesicles in the presence of 10 mM lactose markedly reduced the amounts of antibodies associated with the membranes, implying that at least a fraction of microvillar IgG and IgM as well as IgA can be characterized as anti-glycosyl antibodies. In contrast, association of the major microvillar protein actin (42 kDa) with the microvillar vesicles was unaffected by the lactose wash, indicating that microvillar proteins are not released nonspecifically by this brief treatment.

IgD, in contrast to IgG, IgM, and IgA, was not readily detectable by immunoblotting in the microvillar fraction (data not shown). As stated above, immunofluorescence microscopy likewise failed to detect this immunoglobulin at the enterocyte brush border.

To further confirm the presence of immunoglobulins in the microvillar fraction, Fig. 3B shows that they were isolated from a microvillar membrane Triton X-100 extract by protein A-Sepharose. Furthermore, binding to protein A-Sepharose of all three classes of immunoglobulins was unaffected by the presence of 100 mM lactose, indicating that the carbohydrate does not affect the interaction between protein A and the Fc region of immunoglobulin heavy chains.

Figure 4 shows a lipid raft analysis of microvillar membranes by Triton X-100 extraction at low temperature followed by sucrose gradient centrifugation. The intestinal raft markers galectin 4 (15) and glycosylphosphatidylinositol-anchored alkaline phosphatase (57) were predominantly seen in the buoyant fractions of the gradient, as previously shown (8, 25). Likewise, a major proportion of both the 50-kDa band of IgG and the 70-kDa band of IgM was present in the buoyant
fractions, indicating that the immunoglobulins are associated with lipid raft membranes. However, the distribution of IgM and IgG only partially overlapped, with the latter being present in fractions of the lowest density. This heterogeneity might be related to the observation that IgM was mainly confined to the brush border of enterocytes in the crypts, whereas IgG was localized at the surface of enterocytes along the entire crypt-villus axis (Fig. 2). Alternatively, IgM and IgG may also have differing affinities for lipid raft components that reside in rafts with different densities.

Taken together, the subcellular analyses shown above confirm the presence of anti-glycosyl antibodies of all three classes of immunoglobulins in the enterocyte brush border. The predominant raft localization of IgG and IgM is in agreement with a membrane association mediated at least in part by galactosyl-conjugated components (proteins or lipids) of the brush border.

Coclustering of anti-glycosyl antibodies with galectin 4 in the brush border. Figure 5 shows the localization by double immunogold labeling electron microscopy of IgG and the lipid raft marker galectin 4 in the enterocyte brush border. Both proteins were present along the entire length of the microvilli, and the labeling was most frequently seen as small clusters of particles, although single particles were also detectable. In addition, colocalization of IgG (13-nm particles) and galectin 4 (7-nm particles) was frequently observed; a morphometric analysis indicated that 52% of the IgG labeling was colocalized with galectin 4 (260 of 500 gold particles). This strong colocalizing most likely reflects that anti-glycosyl antibodies and galectin 4 share common binding sites at the brush border and supports the notion that IgG is predominantly associated with lipid rafts.

Anti-glycosyl antibody protection of the brush border against pathogens. Figure 6 shows the labeling of small intestinal mucosa with a fluorescent lectin PNA conjugate. Like a number of pathogens, this lectin specifically recognizes terminal galactosyl residues of glycoconjugates (34), and it predominantly labeled the enterocyte brush border surface (as well as the goblet cells) of the epithelium with only weak labeling of the basolateral and intracellular parts of enterocytes and the lamina propria. A brief wash with 10 mM lactose markedly increased the staining intensity over the brush border without increasing the weak basolateral/intracellular labeling seen over enterocytes and the lamina propria (Fig. 6). This result shows that the brush border is indeed the major galactosyl conjugate-containing membrane in the mucosa. In addition, it suggests that lactose-mediated release of anti-glycosyl antibodies increases the number of available binding sites for lectin PNA at the brush border.

Ganglioside GM1, the receptor for CTB (12, 29), contains a terminal galactosyl residue, and Fig. 6 shows that fluorescent
CTB labeled the enterocyte, including the brush border, as previously reported (23). In addition, a strong labeling was seen in the underlying lamina propria. As observed with lectin PNA, a lactose wash resulted in a more intense labeling of the brush border relative to the intracellular staining of enterocytes and the lamina propria (Fig. 6). This result thus parallels that obtained with lectin PNA in suggesting that the presence of anti-glycosyl antibodies protects the brush border against toxin binding.

**DISCUSSION**

The starting point of the present investigation was the observation that IgG and IgM were the major lactose-binding proteins isolated from pig small intestinal mucosa. This finding agrees well with previous works showing that anti-glycosyl antibodies are naturally occurring in humans and have been purified from serum by lactoseagarose chromatography (9, 43). They are known to comprise about 1% of total serum IgG and IgM in almost all human individuals (5, 22). The binding constants of anti-glycosyl antibodies have been reported to vary considerably, and the relatively higher affinity of the IgG antibodies arises from their specific interaction with both hexosides of lactose in contrast to IgM antibodies, which do not engage the nonterminal hexoside as effectively (36). The novel observation that a considerable fraction of these antibodies are targeted to lipid raft microdomains of the enterocyte brush border is most likely explained by the high local concentration of ligands in this membrane, including lactosyl- and galactosylcerebrosides (11).

With regard to the targeting of anti-glycosyl antibodies to the brush border, IgA and IgM are both ligands for the polymeric immunoglobulin receptor, which enables the massive transcytosis of IgA, and to a lesser extent IgM, across the epithelium (31, 39, 45). In addition, the transport of IgA through the enterocyte has been previously shown to occur through the glycolipid raft-containing apical endosomal compartment (26). At the brush border, the fraction of IgA and IgM representing anti-glycosyl antibodies may then tether to their lipid raft-associated ligands, preventing a true secretion to the gut lumen. In contrast, IgG is not a ligand for the polymeric immunoglobulin receptor but is instead recognized by Fc receptors, such as FcRn, which in the neonate is responsible for transcytosis of maternal IgG from colostrum in the apical to basolateral direction (45). However, this receptor, which is expressed in adult human but not rodent intestinal epithelial cells, is capable of transcytosis in both directions (17), and it might be active in transporting porcine IgG from the basolateral surface to the brush border of the enterocyte. Alternatively, IgG might reach the brush border by a process of transsudation through the interstitial space between disintegrating enterocytes, which has previously been observed to be the site where albumin and other serum proteins leak into the gut lumen of the rat intestine (2).

Antibodies acting in the gut lumen, of which the vast majority are IgA produced locally by lymphocytes in the lamina propria, are generally thought mainly to recognize commensal bacteria of the intestinal flora as well as pathogens constantly challenging the intestinal epithelium (18, 35). By binding to the surface of luminal microorganisms, antibodies interfere with their mobility and ability to penetrate the epithelial lining (37). In addition to this direct type of defense against pathogens and toxins, the results obtained in the present work indicate the existence of another, indirect defense strategy mediated by anti-glycosyl antibodies: as ligands competing with pathogens for the receptors at the brush border. As shown by the labeling with lectin PNA, this surface, with its high density of glycolipid/galectin 4-based lipid raft microdomains containing some of the major, heavily glycosylated digestive enzymes, is the most prominently glycoconjugated membrane in the gut. The preference of brush border-localized IgG and IgM for the lipid raft fractions supports the notion that these naturally occurring antibodies may function as guardians of the pathogen-susceptible lipid raft microdomains. Here, aminopeptidase N is known to act as receptor for the transmissible gastroenteritis virus (16, 24). In rabbits, sucrase-isomaltase contains a functional receptor in its carbohydrate domain for...
Clostridium difficile toxin A (44), and, in the pig, it is capable of binding CTB (23). In addition to proteins, glycolipids, including galactosylceramide and asialo-GM₁, have been shown to be present in lipid rafts from the brush border (8), and a wide range of pathogens, including viruses, bacteria, fungi, and parasites, use glycolipids as cellular binding sites (46, 53). For instance, the main natural receptor for the exotoxin B subunit of Vibrio cholera is ganglioside GM₁ (12, 29), and it has been shown recently that CTB associates with lipid rafts of the enterocyte brush border before its internalization by a clathrin-dependent mechanism (23). The marked increase in labeling observed both with lectin PNA and CTB after a lactose wash suggests that release of antibodies from the brush border indeed renders the surface more susceptible to other types of galactosyl-binding proteins.

As described above, the ability of naturally occurring anti-glycosyl antibodies to protect the epithelial lining of the gut by shielding its membrane glycoconjugates from pathogens and toxins depends on the presence of receptors with terminal galactosyl residues. In this context, it is well known that bacterial microflora are able to modulate certain epithelial characteristics of the mucosal surface (55), including the cell glycosylation pattern of the host (21). Of particular interest, it has been shown that a nonpathogenic microflora component, Bacteroides thetaiotaomicron, was able to increase specifically the galactosylation activity in the epithelial cell line HT29-MTX, whereas other glycosylation steps remained unaffected (20). The interaction between the bacterium and intestinal cells was mediated by an unknown bacterial heat-labile soluble factor of low molecular weight. Because galactosylated glycoconjugates mediate attachment of a number of pathogens, including Listeria monocytogenes (40), this so-called bacterial-host cross-talk was suggested as a mechanism to confer stability to the microflora-gut ecosystem (20). We propose that anti-glycosyl antibodies, by acting as guardians of the brush border lipid rafts as suggested above, likewise might be a part of an ongoing cross-talk between indigenous bacteria and the host.

The observation that IgG was shown to be present in the enterocyte brush border and to be released by lactate indicates that also this immunoglobulin class may serve an important role in the immune defense of the gut. In support of this notion, it has previously been observed that protection of small intestinal epithelial cells from rotavirus infection does not require IgA (42). Thus infected IgA knockout mice were able to clear the virus as effectively as normal control mice, and the compensatory mechanism was high levels of antirotavirus serum IgG and IgM.

In conclusion, many types of proteins are known to be associated with lipid rafts, including glycosylphosphatidylinositol-linked membrane proteins, transmembrane cell surface receptors, signal transducers, and membrane transporters as well as various structural proteins (3). In the present work, we identified immunoglobulins as a major novel class of lipid raft-associated proteins in the small intestine. Their proposed protective function at the enterocyte brush border underlines the notion that the microvillar lipid rafts, unlike those of other cell types, are designed as stable microdomains that require active maintenance in the harsh environment of the gut (8, 14, 25).

REFERENCES


