Antibodies in the small intestine: mucosal synthesis and deposition of anti-glycosyl IgA, IgM, and IgG in the enterocyte brush border

Gert H. Hansen, Lise-Lotte Niels-Christiansen, Lissi Immerdal, and E. Michael Danielsen

Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

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Antibodies in the small intestine: mucosal synthesis and deposition of anti-glycosyl IgA, IgM, and IgG in the enterocyte brush border. Am J Physiol Gastrointest Liver Physiol 291: G82–G90, 2006. First published March 24, 2006; doi:10.1152/ajpgi.00021.2006.—Synthesis and deposition of immunoglobulins in the brush border was studied in organ-cultured pig small intestinal mucosal explants. Surprisingly, comparable amounts of IgM and IgA were synthesized during a 6-h pulse, and also newly made IgG was detected in media and explants, including the microvillar fraction. For IgA and IgM, this subcellular distribution is consistent with basolateral-to-apical transcytosis, mediated by the polymeric immunoglobulin receptor. IgG is a ligand for the Fc receptor FcRn, and β2-microglobulin, the light chain of FcRn, are associated with lipid rafts. Interestingly, a lactose-agarose (LA) chromatography (23). These antibodies are hereby delivered to the mucus layer of the gut to perform immune exclusion and clearance of antigens (7).

“Anti-glycosyl” antibodies are defined as antibodies induced in the host by a glycosyl antigen (39). Experimentally, they were initially characterized as antibodies from antisera of rabbits immunized with a vaccine of nonviable cells of Strep-tococcus faecalis that contains an antigenic diheteroglycan of glucose and galactose, but similar types of antibodies also occur naturally in high levels in the sera of humans (19). With the aim to characterize lectin-like proteins in the small intestinal mucosa, we recently identified anti-glycosyl antibodies of IgG and IgM classes of immunoglobulins as the main proteins isolated by lactose-agarose (LA) chromatography (23). These antibodies were present in the enterocyte brush border, where they were associated with lipid rafts. Interestingly, a lactose wash that released the antibodies from the brush border increased the binding of other types of glycosyl-binding proteins like lectin PNA and cholera toxin B chain, suggesting that anti-glycosyl antibodies, by acting as competitors to pathogens, play a protective role as “guardians” of lipid rafts in the enterocyte brush border (23). In the present work, we studied the biosynthesis and brush border deposition of anti-glycosyl antibodies in organ cultured mucosal explants of the pig small intestine. Surprisingly, IgM was synthesized in amounts comparable with IgA and was preferentially retained in the brush border. Newly synthesized IgG was also detected in the brush border and seemed to reach this membrane by basolateral-to-apical transcytosis, possibly transported by the FcRn receptor. The synthesis and protective functions of these enzymes caused by proteolytic/lipolytic cleavage of their membrane anchors in the harsh working environment of the gut (11).

Unfortunately, a large number of pathogens, including bacteria, viruses, fungi, parasites, and toxins, specifically recognize and “hijack” raft components in their initial contact with a target cell (16, 34, 44, 46, 52). In the small intestine and at other luminal surfaces, the host response toward pathogens relies upon antibodies to provide a first line of defense, and mainly secretory IgA and, to some extent secretory IgM, produced by mucosal plasma cells in the lamina propria, accomplish this function (7). These cells are believed to be derived largely from B cells initially activated in the gut-associated lymphoid tissue. By a well-known process of transcytosis, the secretory antibodies, bound to their cognate polymeric immunoglobulin receptor, traverse the enterocytes from the basolateral surface to the apical brush border (31, 38, 43). The antibodies are hereby delivered to the mucus layer of the gut to perform immune exclusion and clearance of antigens (7).
brush border deposition of anti-glycosyl antibodies of IgA, IgM, and IgG classes therefore appear to represent a significant contribution to the overall mucosal immune defense of the gut.

**MATERIALS AND METHODS**

Materials. LA was obtained from Sigma-Aldrich (Copenhagen, Denmark), and protein A-Sepharose (PAS) CL-4B and protein G-Sepharose (PGS) 4 fast flow were obtained from Amersham Biosciences (Uppsala, Sweden). Antibodies to human IgG, IgA, IgM, β2-microglobulin, horseradish peroxidase-conjugated albumin, and horseradish peroxidase-conjugated secondary antibodies were from DakoCytomation (Glostrup, Denmark). An antibody to the Na+/K+-ATPase α-subunit was from Affinity Bioreagents (Golden, CO), and antibodies to pig IgG and aminopeptidase N were obtained as described previously (22, 23). Protein G-Alexa 488 conjugate and Alexa 488/594-conjugated secondary antibodies were from Invitrogen (Copenhagen, Denmark). [35S]methionine (specific radioactivity >1,000 Ci/mmol) was purchased from Perkin-Elmer.

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Organ culture. Mucosal explants of 20–100 mg wet weight were excised from freshly obtained pig small intestines taken 1–2 m from the pylorus. Explants were placed villus side up on metal grids in culture dishes and cultured at 37°C in 1 ml MEM medium for periods up to 6 h as previously described (12). The medium was changed for every 2 h of culture, and, in some experiments, explants were radioactively labeled with [35S]methionine (0.2 mCi/ml) (9). Corresponding explants and media were collected and analyzed in parallel. In other experiments, a protein G-Alexa 488 conjugate was added to the culture medium (10 μl/ml) at 4°C, and explants were incubated for 30 min at this temperature. The explants were then briefly washed in fresh medium and incubated for additional 30 min at 37°C. After culture, explants were either rapidly frozen at −20°C for subsequent subcellular fractionation or immersed in fixative for microscopy.

Subcellular fractionation. Mucosal tissue was fractionated by the divalent cation precipitation technique (5). Briefly, the mucosa was homogenized in 2 mM Tris–HCl and 50 mM mannitol (pH 7.1) containing 10 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 15 min on ice, the preparation was centrifuged at 1,500 g for 10 min to pellet the intracellular and basolateral membranes. The supernatant was centrifuged at 48,000 g for 1 h to obtain a pellet of microvillar membranes and a supernatant of soluble proteins. For solubilization, Mg2+-precipitated and microvillar membranes were resuspended in 1.0 ml of 25 mM HEPES–HCl and 150 mM NaCl (pH 7.1) and extracted by the addition of 1% Triton X-100. After 10 min at room temperature, membrane extracts were cleared by centrifugation at 20,000 g for 20 min.

Isolation of immunoglobulins. IgG, IgA, and IgM were isolated from mucosal detergent-solubilized subcellular fractions and culture media by subsequent affinity purification steps using PGS first to isolate IgG, followed by PAS to isolate IgA and IgM. One to two milliliters of medium or explant subcellular fraction was incubated with 10 μl (settled volume) PGS for 1 h at room temperature on a rocking table. After incubation, the supernatant was collected for subsequent incubation with PAS, and PGS was washed twice in 1 ml of 25 mM HEPAS–HCl and 150 mM NaCl (pH 7.1) before the IgG was eluted by incubation in 200 μl of 25 mM glycine (pH 2.0) for 15 min. The pH of the eluate was then adjusted to 7–8 by the addition of about 2 μl of 3 M Tris–HCl (pH 8.8). The supernatant from the PGS incubation was incubated with 10 μl PAS (settled volume) for 1 h at room temperature. The PAS was washed, and IgA and IgM were eluted as described above for the PGS step.

In some experiments, the pH-adjusted eluates of isolated IgG and IgA-IgM were mixed with 20 μl (settled volume) LA and incubated for 1 h on a rocking table. After incubation, the LA was washed once with 1 ml HEPES–HCl and 150 mM NaCl (pH 7.1). The supernatant from the LA incubation was mixed with an equal volume of acetone, cooled on ice, and centrifuged at 20,000 g for 20 min to obtain a pellet of protein.

SDS-PAGE and immunoblot analysis. SDS-PAGE in 10% or 15% gels was performed according to Laemmli (32). After the electrophoresis and transfer of proteins onto Immobilon membranes, immunoblot analysis was performed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The blots were developed with an electrochemoluminescence detection kit according to the protocol supplied by the manufacturer (Amersham Biosciences). After the immunoblot analysis, total protein was visualized by staining with Coomassie brilliant blue.

Radioactively labeled proteins were visualized by a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Fluorescence microscopy. Sections of the pig small intestinal mucosa and mucosal explants cultured in the presence of protein G conjugated to Alexa 488 were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2) for 2 h at 4°C and embedded in paraffin. Rehydrated paraffin sections were labeled with primary antibodies to β2-microglobulin, aminopeptidase N (22), or IgG, followed by fluorescent secondary antibodies. Sections were mounted in antifade mounting medium (Dako) and finally examined with a Leica DM 4000 B microscope equipped with a Leica DC 300 FX camera.

Immunogold electron microscopy. Pieces of the pig small intestine were fixed in 4% paraformaldehyde in PB for 2 h at 4°C. After being rinsed in PB, pieces were immersed in 2.3 M sucrose, mounted on top of a pin, and frozen in liquid nitrogen. Ultracryosectioning and immunogold double labeling of β2-microglobulin and IgG were performed as previously described (21). Briefly, ultracryosections were cut in a RMC MT6000-XL ultracytomicrotome and collected on 90 mesh nickel grids. Goldparticles of 7 and 13 nm were prepared according to Ref. 50 and conjugated to goat anti-rabbit immunoglobulins as previously described (24). As a control for immunogold double labeling, the second primary antibody was omitted. Ultracryosections were finally examined with a Zeiss EM 900 electron microscope equipped with a Mega View II camera system.

**RESULTS**

Isolation of IgG, IgA, and IgM from mucosal fractions. Figure 1 shows a subcellular fractionation of small intestinal mucosal tissue by the divalent cation precipitation method (5), yielding fractions of soluble proteins (marker, albumin), Mg2+-precipitated membranes (marker, Na+–K+–ATPase), and microvillar membranes (marker, aminopeptidase N). As evidenced by the distribution of the three markers, little or no cross-contamination of the microvillar fraction with soluble or basolateral/intracellular proteins occurred.

Figure 2 shows the affinity isolation of antibodies from the soluble, Mg2+-precipitated, and microvillar fractions. Antibodies were present in all three subcellular fractions, including the microvillar fraction, which only contains 5–10% of total mucosal protein. In particular, IgM appeared to be relatively abundant in the microvillar fraction. IgG bound equally well to PAS and PGS, whereas both IgA and IgM were efficiently isolated by PAS but bound only poorly (IgA) or not at all (IgM) to PGS. In the experiments with radioactively labeled mucosal explants described below, the three immunoglobulin classes were therefore selectively isolated from the culture media and subcellular fractions by subsequent incubations, first with PGS.
to purify the ~50-kDa band of the IgG γ-chain followed by incubation with PAS to purify the ~50-kDa band of the IgA α-chain and the ~70-kDa band of the IgM μ-chain.

**Antibody synthesis in mucosal explants.** We (21) have previously studied mucosal immunoglobulin secretion in organ culture by radioactive pulse-chase labeling and observed that after a 30-min pulse, newly synthesized IgA first appeared in the culture medium after 30–60 min by a temperature-dependent process. This long lagtime most likely reflects the time required for synthesis in the plasma cells of the lamina propria, secretion into the intercellular space, receptor-mediated uptake into enterocytes, transcytosis, and finally secretion to the medium from the brush border. In addition, the lagtime and temperature dependency showed that release of IgA to the medium did not occur through leakages in the epithelium. In the experiment shown in Fig. 3B, the immunoglobulins isolated from media and explants by PGS and PAS were further subjected to affinity purification by LA chromatography. For all three classes of immunoglobulins, a significant fraction of those newly synthesized bound to LA, implying that they can be characterized as anti-glycosyl antibodies (39).

**Subcellular distribution of antibodies.** To analyze in further detail the mucosal localization of the newly synthesized immunoglobulins, the labeled explants were fractionated by the divalent cation precipitation technique shown in Fig. 1. Figure 4 shows that labeled IgG (PGS fractions) as well as IgA and IgM (PAS fractions) were present in all three subcellular fractions. For all three immunoglobulins, the major part was present in the soluble and Mg2+-precipitated fractions, probably reflecting their abundant presence in plasma cells in the lamina propria and at the basolateral surface and intracellular compartments of enterocytes. However, smaller amounts of newly synthesized IgG, IgA, and, in particular, IgM were also present in the microvillar fraction. From the results shown in

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**Figure 1.** Subcellular fractionation of the small intestinal mucosa. Mucosal tissue was homogenized and fractionated into soluble (Sol), Mg2+-precipitated (Mg), and microvillar (Mic) fractions as described in MATERIALS AND METHODS. The fractions were subjected to SDS-PAGE in a 10% gel followed by electrotansfer to Immobilon membranes and immunoblot analysis for albumin (67 kDa), the Na+-K+-ATPase α-chain (100 kDa), and aminopeptidase N (ApN; the 160-kDa mature form and 140-kDa “high mannose”-glycosylated transient form). Finally, total protein was visualized by staining with Coomassie brilliant blue. Notice that unlike the mature 160-kDa form, the transient 140-kDa form of ApN from the endoplasmic reticulum is only present in the Mg2+-precipitated fraction. Molecular mass values are indicated by arrows.

**Figure 2.** Affinity isolation of immunoglobulins from mucosal subcellular fractions. Soluble, Mg2+-precipitated, and microvillar fractions were prepared from the small intestinal mucosa and extracted with Triton X-100. One microliter of each fraction was incubated with 10 μl of either protein A-Sepharose (PAS) or protein G-Sepharose (PGS) for 1 h at room temperature. After samples were washed twice in 1 ml HEPES-HCl and 150 mM NaCl (pH 7.1), the PAS and PGS fractions were subjected to SDS-PAGE, followed by immunoblot analysis for IgG, IgA, and IgM. Finally, total protein was visualized by staining with Coomassie brilliant blue. Molecular mass values are indicated by arrows.
In conclusion, the labeling experiments show that not only IgA but also IgM and IgG are synthesized by the small intestinal mucosa. Although all three immunoglobulin classes were released to the culture medium, substantial amounts of newly synthesized IgA, IgM, and IgG remained associated with the mucosal explants, and a fraction hereof was detected in the microvillar fraction, indicating a deposition in the enterocyte brush border. Importantly, the high specific radioactivity of IgG in the explant fractions relative to the IgG released into the medium (Fig. 3A) implies that at least a fraction of this immunoglobulin after synthesis and secretion from plasma cells in the lamina propria is taken up at the basolateral surfaces of the enterocytes and transported through the enterocyte by a process of transcytosis, followed by release/deposition at the luminal surface.

**Colocalization of IgG and β2-microglobulin in enterocytes.** FeRn is the cognate receptor for IgG in the small intestine and is a heterodimer consisting of an α-chain and β2-microglobulin (47, 48). As shown in Fig. 5, intense labeling for β2-microglobulin was seen at the lateral surfaces of enterocytes. In addition, punctate labeling was observed intracellularly, particularly over the subapical region. Weaker labeling was seen over the apical brush border. By immunogold double-labeling electron microscopy, β2-microglobulin and IgG frequently colooclustered in a subapical endosomal compartment and in the basolateral plasma membrane, suggesting the presence of receptor-ligand complexes (Fig. 6).

By subcellular fractionation, the 12-kDa band of β2-microglobulin was present in both Mg2+-precipitated and microvillar fractions (Fig. 7), in agreement with its distribution seen by immunofluorescence and immunogold microscopy. Further-
more, it was copurified with IgG by PGS from both these subcellular fractions, indicating that at least a fraction of IgG in the enterocytes is associated with FcRn.

**Apical endocytosis of IgG.** To study the endocytic trafficking of IgG associated with the enterocyte brush border, mucosal explants in organ culture were incubated with a protein G-Alexa 488 conjugate. After 30 min at 4°C, the lumenal surface of both crypts and villi was distinctly labeled (data not shown). However, after 30 min at 37°C, a similar labeling pattern was observed (Fig. 8). Thus protein G remained associated with the lumenal brush border surface, and it was not internalized to intracellular compartments positive for IgG or transcytosed to the intercellular space along the basolateral membranes. Therefore, assuming that Alexa 488-conjugated protein G reflects apical IgG trafficking, this experiment showed that IgG present in the subapical endosomal compartments of enterocytes (Fig. 6) is unlikely to have originated from apical uptake of the antibody. Interestingly, however, single cells along the epithelium were occasionally observed that had taken up the fluorescent probe (Fig. 9). Such cells often appeared irregular in shape, indicating that they are undergoing apoptosis and in the process of being extruded from the epithelium.

**DISCUSSION**

Circulating anti-glycosyl antibodies evoked by commensal bacteria of the gut that recognize antigens bearing terminal galactose or lactose residues have been known for a long time (33, 39). The present work was carried out with the aim to decide whether anti-glycosyl antibodies primarily of the IgM and IgG classes, recently shown also to be present at the enterocyte brush border (23), are derived solely from the plasma or, alternatively, may be locally synthesized by plasma cells in the lamina propria. Mucosal organ culture experiments clearly showed that not only IgA but also IgM and IgG are synthesized by plasma cells in the lamina propria. In fact, the relative levels of IgM and IgG synthesis were surprisingly high. Compared with the corresponding relative immunoglobulin class production reported for the human small intestine (4), IgM synthesis in the present work was more than twofold higher and that of IgG, which was fourfold higher. The discrepancy may be attributed to species variation, but the observed high levels of IgM and IgG production in the pig small intestine underscores the considerable contribution of these antibody classes in the overall immune defense of the gut.

![Fig. 6. Co-clustering of IgG and β2-microglobulin in the enterocyte. Double immunogold electron microscopy for IgG (7-nm particles) and β2-microglobulin (13-nm gold particles) in ultracyosections of enterocytes is shown. A: labeling of β2-microglobulin was seen over the microvillar membrane (MM) and in a subapical compartment (*), whereas mitochondria (Mi) were devoid of any labeling. B: higher magnification of the subapical compartment in A showing frequent co-clustering of small and large gold particles (arrows). C: a large endosome with co-clustering (arrows) of IgG and β2-microglobulin along the inner periphery. D: co-clustering of IgG and β2-microglobulin along the basolateral plasma membrane (BM). Bars = 0.2 μm in A and 0.1 μm in B–D.](http://ajpgi.physiology.org/):
Anti-glycosyl antibodies of the IgM class have previously been shown to have a lower affinity toward their glycosyl epitopes compared with IgG (33), but IgM nevertheless seemed to be the antibody class that binds most efficiently to the brush border, as judged both by the relative steady-state levels and by the relative amounts of newly synthesized proteins in the microvillar fraction (Figs. 3 and 4). A likely explanation for this could be that a pentameric immunoglobulin with multiple binding sites is the best suited to adhere to the lipid raft platforms described above that are densely populated with ligands. However, LA-binding immunoglobulins of all three classes were isolated from the media as well as from explants, suggesting they have only a modest affinity toward the brush border. Most likely, the protective anti-glycosyl antibody “coating” of the lipid raft microdomains of the brush border is a dynamic equilibrium where the steady state is achieved by a constitutive synthesis and deposition balanced by a constant release to the gut lumen.

For IgA and IgM, locally synthesized and secreted by mucosal plasma cells, the route and mechanism whereby they reach the gut lumen is well-known (7, 31, 38, 43). By virtue of their association with the J chain, the polymeric immunoglobulin receptor specifically recognizes these antibodies at the enterocyte basolateral surface and transports them through a series of endosomal compartments to the apical surface. During transcytosis, the receptor undergoes proteolytic cleavage, and the cleaved extracellular domain, known as secretory component, is released to the lumen together with its cargo. Thus the transcytotic transport of IgA and IgM is strictly unidirectional in the basolateral to apical direction, ensuring an efficient delivery of secretory immunoglobulins to the gut lumen. In contrast, FcRn, the cognate receptor for IgG, is able in principle to function bidirectionally (3, 15, 17, 36). Initially, this major histocompatibility complex class I-related receptor was shown to provide immune protection to neonate rodents by intestinal uptake of maternal immunoglobulins from colostrum and milk, followed by apical-to-basolateral transcytosis and release into the circulation (41, 42). In primates, this maternal transfer of IgG mainly takes place prenatally across the placental syncytiotrophoblast, likewise by transcytosis in the apical-to-basolateral direction (17, 18). However, in humans and nonhuman primates as well as in rodents, FcRn is also expressed in maturity in intestinal and bronchial epithelial cells (15, 28, 51). Its transcytotic function relies upon its pH-dependent binding of IgG, where the ligand binds at slightly acidic pH, as may be found in endosomes, but less so at neutral pH. This reversibility with regard to ligand binding enables FcRn to establish and maintain a steady-state distribution of IgG across the epithelium. In consequence hereof, it has been reported to play a role in immune surveillance by promoting both lumenal secretion of IgG as well as a reuptake of antigen-antibody complexes for recognition by the dendritic cells of the immune system (55). However, the findings of the present work imply that in the mature pig small intestine, IgG made locally by plasma cells of the lamina propria, like IgA and IgM, is transported predominantly in the basolateral-to-apical direction. Thus the observed inability of enterocytes to internalize Alexa 488-conjugated protein G argues against apical endocytosis being a major trafficking route for IgG. This conclusion is also supported by the observation that the specific radioactivity of IgG from the explants was higher than that of IgG collected...
from the medium. Nevertheless, we cannot exclude the possibility that small amounts of luminal IgG may reenter the epithelium by this pathway. However, an alternative route of internalization could be via apoptotic cells of the rapidly renewable mucosal epithelium. This view is supported by the occasionally observed epithelial cells that took up protein G from the culture medium. Although relatively few in number, this might be a physiologically relevant pathway for returning IgG-antigen complexes back across the intestinal barrier into the lamina propria for processing by dendritic cells and presentation to T cells.

GRANTS

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Fig. 8. Protein G binds to the enterocyte brush border but is not internalized. Mucosal explants were cultured in the presence of a PG-Alexa 488 conjugate (PG) as described in MATERIALS AND METHODS. After 30 min at 37°C, protein G-Alexa 488 conjugate was seen along the enterocyte brush border (arrows), as evidenced by its colocalization with the microvillar membrane enzyme ApN. However, no uptake into enterocytes (E) was observed, and no colocalization with IgG in the subapical compartment or along the basolateral membranes was seen. LP, lamina propria. Bars = 10 μm.

Fig. 9. Protein G entry into apoptotic cells. Although protein G-Alexa 488 bound to the brush border surface was not generally internalized by enterocytes (E), occasionally a single epithelial cell was seen that had taken up the fluorescent probe (arrows). By incident light microscopy, such cells appeared irregular, indicating that they may be apoptotic cells in the process of being extruded from the epithelium. Bars = 10 μm.
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