ORIGINAL RESEARCH

For the Assessment of Intestinal Permeability, Size Matters

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ABSTRACT

The purpose of this review is to demonstrate that an intestine leaky to small molecules can be impermeable to large antigenic molecules. The author proposes that the permeability of the epithelium to very small sugar molecules such as lactulose/mannitol—used for the past 50 years to gauge intestinal permeability—does not necessarily correlate with epithelial permeability to macromolecules. This article begins with the history and science behind the use of small sugars to measure permeability, a method developed in 1899. The lactulose/mannitol test may give useful information regarding the overall condition of the digestive tract; however, the author suggests that the test is not indicative of the transport of macromolecules such as bacterial toxins and food antigens, which have the capacity to damage the structure of the intestinal barrier and/or challenge the immune system. This article describes the various mechanisms and physiological transport pathways through which increased antigen uptake may result in immunological reactions to food antigens and bacterial lipopolysaccharides, resulting in the pathogenesis of disease. Finally, the article presents evidence indicating that increased intestinal, antigenic permeability plays a key role in the development of various inflammatory and autoimmune disorders. Therefore, more knowledge about the epithelium's permeability to large molecules undoubtedly contributes not only to early detection but also to secondary prevention of many inflammatory autoimmune, neuroimmune, and neurodegenerative disorders.
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The understanding of the science behind gut permeability is said to have started in 1899 with Hober, who studied the absorption rates of several sugars and salts and found that dogs absorbed galactose slightly faster than glucose.[1] In 1900, Hedon published a study comparing the rates of absorption of glucose, fructose, galactose, and arabinose in a loop of rabbit's gut.[2] Hewitt later compared the rates of absorption of dilute solutions of glucose, fructose, and galactose from loops of gut.[3] The results were inconclusive in rabbits, but Hewitt observed that cats absorbed glucose much more rapidly than fructose, with galactose registering at an intermediate rate. Killing the intestinal mucosa with infusions of hot liquids or sodium fluoride resulted in equal absorption rates for all of the sugars. None of these methods were applicable, however, to humans. For this reason, McCance and Madders designed a method by which it was possible to compare the absorption rates of rhamnose, arabinose, and xylose.[4] In humans, these three sugars are all excreted readily by the kidney and are destroyed in the tissues comparatively slowly. The researchers compared the rate and amount of each sugar excreted (a) when injected intravenously and (b) when taken orally. They concluded that:

1. Arabinose, rhamnose, and xylose are readily excreted when intravenously injected in humans. Their curves of excretion are of identical shape and may all be superimposable.
2. The relative rates of absorption of arabinose, rhamnose, and xylose are the same in rats and humans. If the rate of absorption of rhamnose = 1, then those of arabinose and xylose are 2.33 and 3.6, respectively.
3. All of these sugars are absorbed at the same location high in the small intestine. Little or no absorption occurs further down the intestine.

4. In a normal person, the absorption of these sugars proceeds rapidly and linearly for 1.5 hours. After that point, absorption almost ceases, even when a large excess still remains in the intestine.
5. It is reasonable to assume that the relative rates of absorption of glucose, galactose, and fructose found in rats also hold true in humans; galactose is absorbed slightly faster than glucose, and glucose is absorbed twice as fast as fructose.

In the 1970s, the introduction of nonmetabolizable oligosaccharides as test substances made it possible to develop feasible methods for assessing intestinal barrier function.[5] The intestinal permeability test consists of the oral administration of sugars and the subsequent measurement of these substances in the urine; it is a noninvasive method that has been used to assess the integrity of the epithelial barrier to small sugar molecules. Some evidence supports the role of gut-barrier dysfunction as a primary disease mechanism in intestinal disorders.[6] As a result, the intestinal permeability test is used in both clinical practice and research.[7]

Two different sugars, mannitol—a monosaccharide with a molecular weight (MW) of 182 Da and a molecular radius of less than or equal to 0.4 nm—and lactulose—a disaccharide with an MW of 342 Da and a molecular radius of 0.42 nm, have been used in this noninvasive, functional, intestinal-permeability test. The different sizes (MWs) of these molecules allow the evaluation of the relative importance of the two separate entrance routes that are postulated. Molecules up to the size of monosaccharides, such as mannitol, are believed to use the transcellular route, and disaccharides or larger molecules are believed to be transported through the paracellular route across the intestinal wall, as shown in Figure 1.[8,9]

This sugar permeability test has been used in the determination of health and disease, including celiac disease (CD) and Crohn's disease.[10-12] In the case of Crohn's disease, Blomquist et al and Bjarnason et al all suggested that a defect in the intestinal barrier function might be an etiological factor in the pathogenesis of the disease.[12,13] Many factors, however, can influence the uptake of these sugars by epithelial cells, including (1) GI motility; (2) the body's distribution of the tracers; (3) the use of medications such as nonsteroidal anti-inflammatory drugs like methotrexate; (4) smoking; (5) the use of alcohol; (6) variations in gastric emptying; (7) intestinal transit time and surface area; (8) mucosal blood flow; and (9) renal clearance. These factors can change the permeability of the epithelial cells, and hence, cause the tests to yield false-positive results.[13-16]

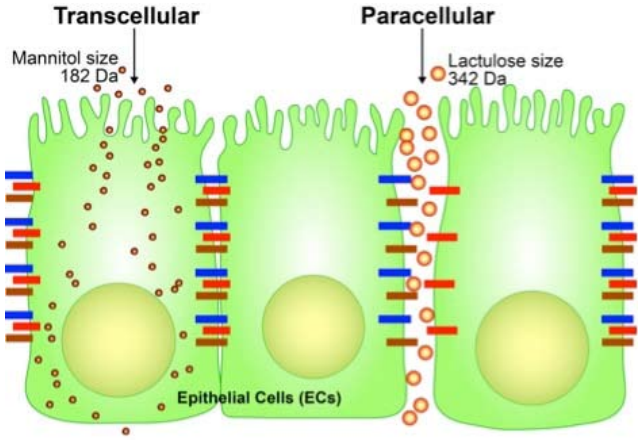
Furthermore, normal ranges vary from laboratory to laboratory and country to country, with the ranges being higher in most tropical areas because of eating habits and the presence of tropical enteropathy.[17] In addition, intestinal permeability to very small molecules (182-342 Da) is not necessarily related to structural damage in the tight-junction barrier that permits increased penetration of large molecules. Only molecules 5000 Da or larger can challenge the immunological system of the bowel, resulting in a T-cell response and the production of cytokines and antibodies.[16] The alteration of the gut barrier to antigenic molecules leads to the absorption of endotoxin and lipopolysaccharides from the lumen. Both of these substances potently stimulate acute-phase reactions and the secretion of IL-1β, IL-6, and tumor necrosis factor (TNF), which have been shown to be important mediators of inflammation in many GI disorders.[7,18]

For this reason, many attempts have been made to measure intestinal permeability to large, 12 000- to 15 000-Da polysugars in humans. The size of these sugars is similar to that of many food proteins, suggesting that these sugars may be suitable markers for intestinal permeability to macromolecules such as bacterial toxins and food antigens.[10,19]

This conclusion is based on the fact that the human gastrointestinal tract allows a certain degree of physiological absorption of undegraded, macromolecular dietary antigen, whether free or antibody bound. This antigen uptake is influenced by the permeability of the gut and the local and systemic immune responses.[20] Husby et al studied the passage of dietary antigens into the blood of children with celiac disease and other children who were suspected of celiac disease but exhibited normal jejunal biopsies (silent celiac).[20]

For 7 hours after a test meal, researchers investigated the uptake of egg ovalbumin (OA) and beta-lactoglobulin (BLG) from cow's milk into the blood of five children with confirmed celiac disease, both when on a gluten-free diet and after gluten challenge, and the blood of five children suspected of silent celiac disease with normal jejunal mucosas. An ELISA detected OA in three of the five confirmed celiac children (maximal concentrations 8-178 ng/mL in the serum) and in all of the five children suspected of silent celiac disease (maximal concentrations 4-91 ng/mL in the

Figure 1. The Transcellular and Paracellular Pathways Permit the Transfer of Differently Sized Molecules



serum). BLG was detected in three of the five confirmed celiac children (maximal concentrations 0.6-6 ng/mL in the serum) and in two of the five children suspected of silent celiac disease (maximal concentrations 0.5 and 50 ng/mL in the serum).

HPLC fractionation in combination with ELISA detected OA and BLG in the serum of all the confirmed celiac children and suspected silent celiac children. The serum concentrations of OA and BLG were increased after gluten challenge in four of the five confirmed celiac children, indicating increased macromolecular passage through the gut mucosa in untreated celiac disease.[20]

It was concluded that challenge with gluten and the subsequent development of villous atrophy in celiac children leads to the increased uptake of macromolecular dietary antigens. Therefore, intestinal permeability should be measured against molecules that are representative of dietary antigens and bacterial toxins and not against very small sugar molecules.

Menard discussed the issue of gut permeability to large macromolecules in a very elegant 2010 review, "Multiple Facets of Intestinal Permeability and Epithelial Handling of Dietary Antigens."[21] Menard found that the intestinal epithelium is not fully impermeable to macromolecules. In the steady state, the transepithelial passage of small amounts of food-derived antigens and microorganisms contributes to the induction of a homeostatic immune response that is dominated by immune tolerance to dietary antigens and the local production of secretory immunoglobulin A (SIgA), thus preventing pathogenic and commensal microbes from entering internal compartments.

Obviously, no universal marker can provide a definitive answer on the capacity of the intestinal mucosa to sense the intestinal content and deliver antigens or bacteria to the underlying immune system. It is important to keep in mind that beyond the controversies of paracellular versus transcellular permeability, one important feature in intestinal disease is the failure of the intestinal barrier to contain the macromolecular luminal content, a phenomenon likely to exacerbate unwanted immune responses.

Whether, and the degree to which, the entrance of antigenic macromolecules across the gut epithelium initiates and/or perpetuates chronic inflammation remains a matter of debate, as do the respective contributions of paracellular and transcellular permeability. Thus, the experimental studies that use small inert molecules to assess intestinal permeability in vivo do not necessarily correlate with the uptake of larger dietary antigens.

MACROMOLECULES AND INERT SUGARS DO NOT CORRELATE

Currently, the gold standard for measuring intestinal permeability to small molecules is the lactulose/mannitol test. Although it is a useful test in clinical studies, providing information on the overall condition of the digestive tract (villous atrophy, inflammation), it does not indicate the transport of macromolecules such as food antigens and bacterial lipopolysaccharides (LPS). The transport of large intestinal molecules does not correlate with intestinal electrical resistance or the lactulose/mannitol permeability test.

Indeed, studies have shown the lack of correlation between the permeation of inert sugars and macromolecules. In neonatal pigs, intestinal closure to β-lactoglobulin (molecular weight 18 000 Da), a major allergen in cow's milk, occurs within 6 days of birth. The permeation of lactulose, a marker of paracellular permeability, persists, however, throughout the suckling period. This finding means that the body develops tight-junction structures such as occludin/zonulin, claudin, and JAM family proteins between paracellular spaces and prevents the movement of large antigen molecules into the submucosa within 6 days of birth.[22,23] These spaces, however, are not tight enough to prevent the permeation of very small molecules such as lactulose, which continues to move throughout the first 6 to 12 months of life.[23,24] This result indicates that inert soluble markers do not represent macromolecular absorption and do not reflect antigen handling by the gut.[23]

In addition, the lack of direct correlation between lactulose/mannitol IPT and the absorption of beta-lactoglobulin can also be observed in children with rotavirus diarrhea.[25] Finally, in a mouse model of a celiac-like disease, mice challenged with gluten exhibited increased fluxes of horseradish peroxidase (HRP, a molecular tracer) in the absence of increased ionic conductance, whereas the addition of indomethacin to gluten promoted an increase in ionic conductance (paracellular pathway) and a further increase in HRP transcytosis. Thus, one should remember that electrical resistance (or its reverse, ionic conductance) is mainly related to the permeation of ions, and at best, small molecules but not always food-type antigens.[25,26] Thus, studies that use very small, inert molecules to assess intestinal permeability in vivo do not necessarily correlate with the uptake of larger dietary antigens and bacterial toxins.[21]

It is accepted that intestinal permeability is a generic term related to the absorption of variously sized molecules ranging from small, inert solutes (mannitol) to large macromolecules. In intestinal diseases, the increased permeability of large molecules (food antigens, microbial fragments) can have a detrimental effect by facilitating or magnifying inappropriate immune responses. Whether the transport pathway is paracellular or transcellular, it is mandatory to use appropriate probes (proteins, bacteria) to delineate which materials can cross the epithelial barrier. In this regard, small inert markers cannot mimic large molecules because of the size selectivity of tight junctions.

MICROBIAL TRANSLOCATION AND IMMUNE ACTIVATION

A recent study showed that compromised gastrointestinal integrity in pigtail macaques (PTMs) was associated

with microbial translocation (increased levels of LPS in the submucosa), immune activation, and IL-17 production by T_H17 cells.[27]

The study of nonhuman primates is essential in understanding how and to what extent dysfunction and damage to the mucosal immune system can affect systemic immune activation in vivo. Infecting Asian rhesus macaques (RMs; *Macaca mulatta*) with pathogenic simian immunodeficiency virus (SIV) is the most widely studied nonhuman primate model for the pathogenesis of the human immunodeficiency virus (HIV) to date.

A comparison of the pigtail and rhesus macaques is interesting in that PTMs typically progress to AIDS more rapidly than RMs. After infection with SIVsmE543, the majority of PTMs progress to AIDS within 6 months of infection, as opposed to approximately 2 years for RMs. The rapid disease progression observed in PTMs is most likely not associated with viral inoculation but is instead due to host factors. Interestingly, uninfected PTMs in captivity have an increased incidence of diarrhea and GI diseases, and older animals frequently present with systemic amyloidosis. Indeed, a 5-year study of uninfected monkeys revealed that the majority of them had at least two bouts of diarrhea requiring treatment. Therefore, these animals are more susceptible to death resulting from intestinal permeability and the deposition of LPS in the mucosal tissue.[28,29]

To determine the mechanisms underlying the permeability of the GI tract and consequent microbial translocation, the GI tract tissues were stained with antibodies against the tight-junction protein claudin-3 to measure the continuity or observed damage of the structural barrier of the gut epithelia.[27] Significant damage to the tight-junction proteins was observed both through immunohistochemical studies and through calculations that measured the breach/intact ratio by comparing the length of the tight epithelial barrier that was not stained for claudin to the length of the colon barrier that was stained for claudin. In comparison to controls, which exhibited a breach/intact ratio of 0.017, monkeys with diarrhea showed a breach/intact ratio of 0.303; hence, a putative mechanism for the increased diarrhea, intestinal permeability, and microbial translocation in these monkeys may be associated with increased pre-existing damage to the structural barrier of the GI tract.

To determine whether these breaches in the epithelial tight junctions correlated with the increase in microbial translocation, the researchers studied the colon sections with an antibody against the LPS core antigen to directly measure the bacterial products present within the lamina propria (LP). They found that the monkeys with diarrhea had increased levels of LPS in the LP of the colon compared to the controls. Using quantitative image analysis, they determined the percent of the colonic LP area that contained LPS and found that, on average, LPS accounted for 13.00% of the LP area in PTM monkeys, whereas only 0.274% of the LP was occupied by LPS in the RMs (controls). These data strongly suggest that the mechanism underlying the increased microbial translocation involves structural damage to the gut epithelium in monkeys with diarrhea.

Furthermore, the authors demonstrated a significant positive correlation between damaged tight-junction proteins and the level of LPS in the tissue and blood.[27] In addition, a positive correlation was found between the extent of LPS staining in the colon and mesenteric lymph nodes. Both the degree of damage to the tight junctions and the level of LPS staining in the colon and lymph nodes correlated with the level of LPS in the plasma. The level of LPS in the plasma of pigtail macaques with diarrhea averaged 45.3 pg/mL, a level that was much higher than that observed in the control monkeys, which averaged 19.2 ± 13 pg/mL.[27] The researchers hypothesized that the rapid disease progression observed in the PTMs after SIV infection may in part be due to pre-existing conditions that cause the dysfunction of and damage to the mucosal immune system and lead to increased microbial translocation and consequent immune activation.

Based on these findings, it can be concluded that an assessment of intestinal permeability to large antigenic molecules can use the bacterial toxins that first play a significant role in damaging tight-junction and structural proteins (occludin/zonulin) and actomyosin and then open the paracellular pathway, thus facilitating the entry of tight-junction proteins, actomyosin, and bacterial LPS into the submucosa, the regional lymph nodes, and the circulation. This entry of tight-junction proteins and bacterial LPS into the circulation can challenge the immune system, resulting in the production of significantly elevated levels of occludin/zonulin-, actomyosin- and LPS-specific IgG, IgM and IgA in the blood.

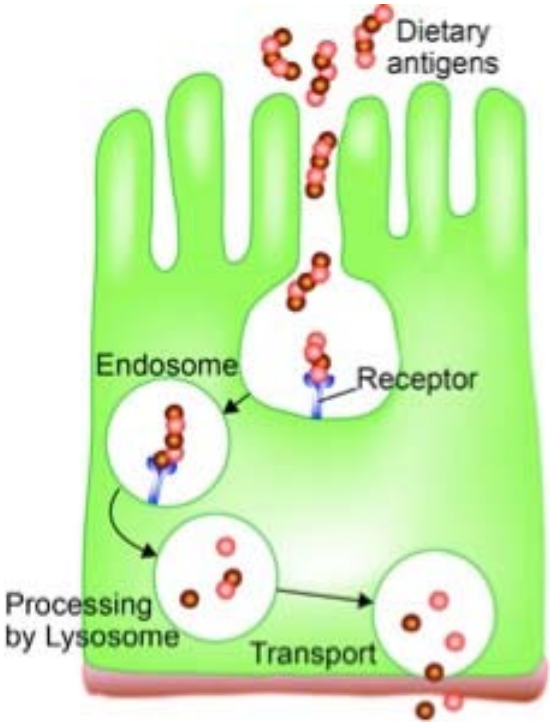
MECHANISM RESPONSIBLE FOR UPTAKE OF IMMUNOGENIC MOLECULES

The uptake of immunogenic molecules (antigens) from the lumen plays a significant role in the pathogenesis of gastrointestinal disease. The excessive uptake of these antigens, in addition to a breakdown in immunological tolerance or the suppression of immune responsiveness, can induce immunological activity both within the intestine and beyond. This activity seems to be a prerequisite for disease development.[30] For example, serum immunoglobulins to the food antigens beta-lactoglobulin, wheat, and maize have been found in inflammatory bowel disease (IBD). It is probable that protein macromolecules permeate in increased amounts, causing systemic immune responsiveness.[31-33]

Because an increase in the uptake of antigens is involved in this immunological reaction to food antigens and bacterial toxins, an understanding of the physiology of this uptake is central to an appreciation of the pathogenesis of disease.[30] This antigen uptake process is divided into physiological transport and pathological transport.

The physiological-transport pathways include (1)

Figure 2. Transcellular Transport Pathways



Under steady-state conditions, epithelial cells sample molecules with molecular weights greater than 600 Da (such as food antigens and peptides) by endocytosis at the apical membrane and transcytosis toward the lamina propria. During transcytosis, the full-length peptides or proteins are partly degraded in acidic and lysosomal compartments and released in the form of amino acids (total degradation) or breakdown products (partial degradation) at the basolateral pole of the enterocytes.

ligand-receptor uptake, (2) antibody uptake, and (3) microfold or M cell transport.

Pathological transport can be antigen-nonspecific or antigen-specific. Antigen-nonspecific transport occurs through transcellular (intracellular) or paracellular pathways when the tight junction becomes more permeable or damaged by environmental factors. This excessive uptake of antigens may occur as a result of an immature gut, postenteritis, allergic enteropathy, gut dysbiosis, and other environmental factors that activate inflammatory cascades.[34-36]

Antigen-specific transport via the transcellular or paracellular pathways can induce a specific disease. For example, gliadin has been linked to celiac disease; casein and beta-lactoglobulin cause allergic gastroenteropathies; beta-glucan from baker's yeast has been implicated in Crohn's disease and bacterial antigens can cause inflammatory bowel disease and other autoimmune disorders.[30]

From all the above information, the authors conclude that increased antigen uptake in the intestine precedes the onset of many immunologically mediated, gastrointestinal diseases.

LIGAND-RECEPTOR TRANSPORT OF ANTIGENS

Macromolecules cross intestinal epithelial cells in two ways of which we can be certain. They can be shuttled through absorptive cells using specific receptors—in which case, only those macromolecules that bind to a receptor will pass—or they can pass through specialized epithelial cells (ie, the M cells previously mentioned).

Macromolecules are transferred by a mechanism that is altogether different from those that transport nutrients such as glucose and amino acids. Nutrient molecules enter the intestinal-cell cytoplasm at the apical membrane and exit through the basolateral membrane. Macromolecules, however, transverse the cell in membrane-bound compartments that invaginate from the apical membrane. The first step in this process is attachment of macromolecules to receptors on the apical surface of enterocytes, where they are endocytosed and processed by lysosomal enzymes, which degrade the antigen and transport it to the basolateral pole.[37] This process of transcellular transport is shown in Figure 2.

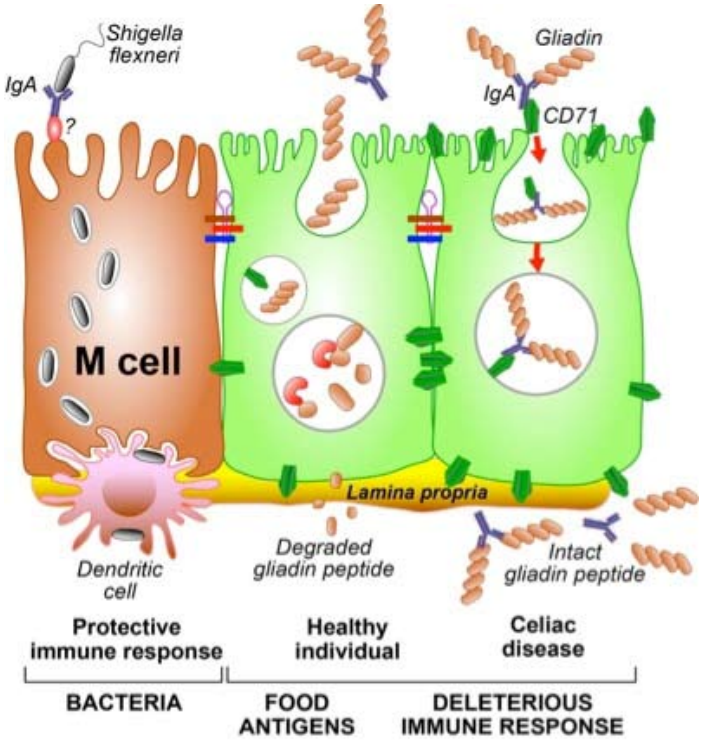
ANTIBODY-MEDIATED UPTAKE OF ANTIGENS

Humoral immunity as mediated by secretory IgA (SIgA) and IgG plays an important role as a first line of defense against microorganisms in mucosal tissues. SIgA is transported from tissue spaces and across epithelial cells into the lumen through an active, unidirectional process involving the polymeric Ig receptor pIgR. IgG can also be detected within the intestinal lumen of the adult human; in certain tissue locations, it may reach levels approximating those observed for SIgA.

The passive administration of neutralizing IgGs has also been reported to prevent mucosal transmission of human immunodeficiency virus in rhesus macaques or neonatal macaques. Together, these observations support the concept that the presence of IgG along the mucosal surfaces can serve an important role in mucosal protection.[38-40] The mechanism(s) by which IgG accesses the lumen and biological functions once in place, however, remain to be defined.

Evidence has recently indicated a role for the neonatal Fc receptor (FcRn) in these processes. Interestingly, FcRn has been functionally linked not only to the passive acquisition of immunity in neonatal rodents through the transport of maternal IgG but also to IgG-mediated immune surveillance. This finding is based upon the indirect morphological observation that FcRn is also capable of transporting antigen-antibody complexes across the intestinal epithelium from the lumen during neonatal rodent life. From this finding, one can hypothesize that a major function of FcRn in adult human life is to transport IgG into the apical region of the epithelium for the retrieval of antigens so that FcRn can recycle these complexes for transport back into the

Figure 3. Immunoglobulin A-mediated Retrotransport of Luminal Food Antigens



lamina propria. Such a pathway could contribute significantly to the regulation of immune responses by providing a mechanism for luminal antigen(s) to gain access to professional antigen-presenting cells such as dendritic cells (DCs). These cells are known to be present at this location and are capable of interacting with regulatory T cells to induce immunological tolerance.[41]

ANTIBODY-MEDIATED TRANSPORT OF ANTIGENS

The most representative Ig isotype at the mucosal surface is IgA. The basal-to-apical secretion of dimeric IgA, in the form of SIgA, through the polymeric Ig receptor is a common receptor-mediated IgA transport mechanism in the intestines. SIgA retains potentially noxious antigens in the intestinal lumen, thus performing a vital role in intestinal immunity. While restricting the passage of exogenous antigens into the intestinal mucosa seems to be the main function of SIgAs, apical-to-basal retrotransport, can occur, with either deleterious or beneficial effects on the intestinal mucosa.[42]

In animal models, *Shigella flexneri* alone or as an SIgA immune complex (ICs) was administered into ligated intestinal loops containing Peyer's Patches (PP); this process allowed ICs, but not free bacteria, to enter the PP and be captured by DCs, thereby contributing to the induction of protective immunity and preserving the integrity of the intestinal barrier.

In some pathological situations, the abnormal retrotransport of SIgA ICs can allow bacterial or food antigens to enter the intestinal mucosa, with various outcomes. Indeed, SIgA can mediate the intestinal entry of SIgA/*S flexneri* ICs through M cells and interactions with dendritic cells, inducing an inflammatory response aimed at improving bacterial clearance and the restoration of intestinal homeostasis. In healthy individuals, undigested gliadin peptides are taken up by nonspecific endocytosis in enterocytes and entirely degraded/detoxified during transepithelial transport. In celiac disease, however, the ectopic expression of the transferrin receptor CD71 (also known as the IgA receptor) at the apical membrane of epithelial cells favors the retrotransport of IgA ICs and inappropriate immune responses. SIgA allows the protected transcytosis of gliadin peptides. Because of the constant flow of gluten in the gut, this process is likely to trigger exacerbated adaptive and immune responses and precipitate mucosal lesions. This IgA-mediated transport of antigens is shown in Figure 3.

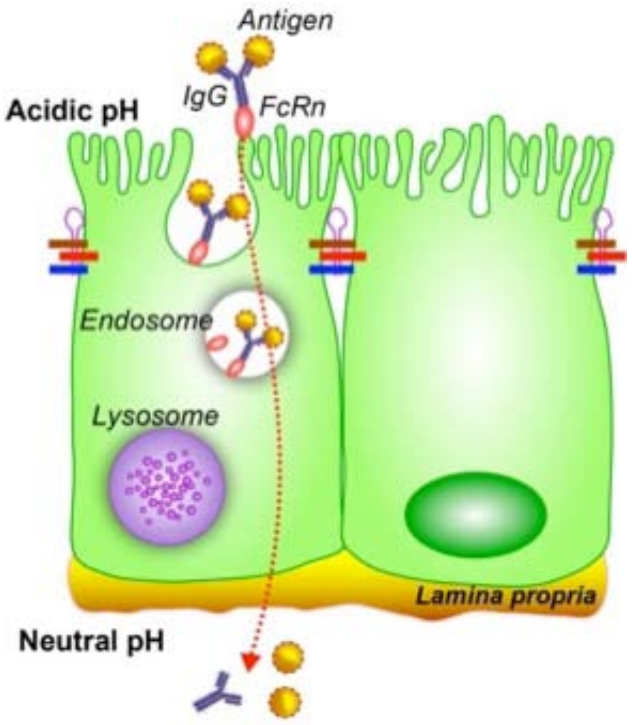
Whereas the retrotransport of SIgA/bacterial ICs aids in the development of immune responses to clear pathogenic microbes, this retrotransport might turn deleterious to the host when food antigens are concerned. This deleterious effect occurs in CD, an enteropathy induced by the abnormal activation of T cells by gluten-derived gliadin peptides. In CD, gliadin peptides are transported intact across the intestine by IgA/gliadin ICs.[21]

Studies suggest that dietary antigens, including gluten peptides, are complexed to antigen-specific, intraluminal SIgA. The gliadin peptides now complexed with secretory IgA bind to the IgA receptor, which then transports and protects them from lysosomal degradation through a specific transcytosis pathway.[43-45] This IgA receptor has been recently identified as CD71.[46]

Normally, CD71 is only expressed on the basolateral enterocyte membrane in the normal intestine and in patients on a gluten-free diet. This receptor efficiently binds polymeric and secretory IgA but not monomeric IgA. In contrast, in active CD, CD71 expression is greatly increased and CD71 is found at the apical enterocyte membrane, where it colocalizes with IgA. The gliadin peptides that complex with SIgA can then bind to CD71, which mediates their protected endocytosis and translocation from the intestinal lumen into the lamina propria.[44]

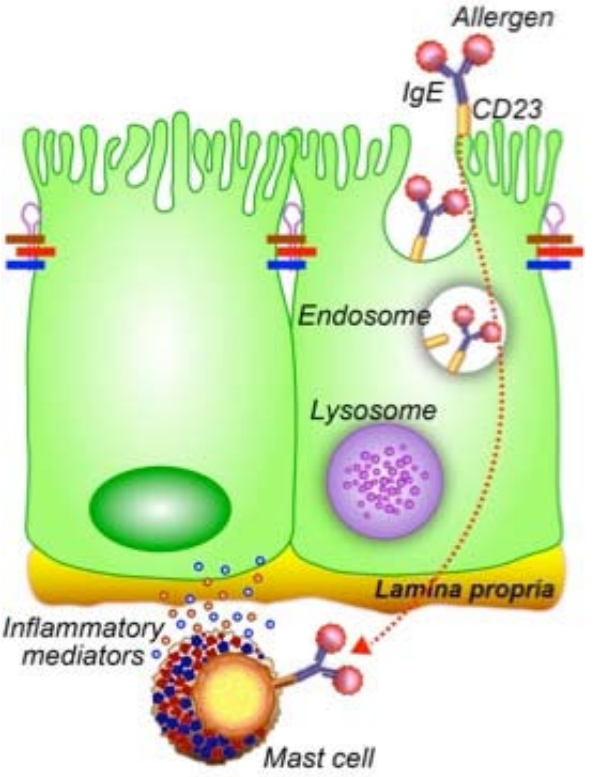
In healthy individuals, gliadin peptides are taken up nonspecifically by enterocytes and degraded by lysosomal proteases during fluid-phase transcytosis. Very few toxic peptides are delivered into the intestinal lamina propria. In patients with active CD, the abnormal expression of CD71 at the apical pole of enterocytes allows the receptor-mediated uptake of SIgA-gliadin peptide complexes and their protected transport toward the lamina propria and, thus, the local immune system. The exact domain of the SIgA

Figure 4. The Immunoglobulin G-mediated Transport of Antigens



Although IgGs are not classical secretory antibodies, their presence in the intestinal lumen suggests a protective role. An IgG-antigen immune complex is shown binding to the neonatal Fc receptor (FcRn) on intestinal epithelial cells in the acidic environment close to the apical membrane. The FcRn mediates transcytosis of the IC, allowing the protected transport and release of the IC on the basal side of the enterocyte, where the neutral environment induces the dissociation of the IC from the receptor.

Figure 5. Immunoglobulin E-mediated Allergen Transport



The low-affinity IgE receptor CD23 is abnormally overexpressed in intestinal epithelial cells in allergic humans and murine models of allergy. The overexpression of CD23 at the apical side of enterocytes can drive the transport of intact IgE/allergen ICs from the intestinal lumen to the lamina propria, triggering mast-cell degranulation and an allergic inflammatory cascade.

molecule involved in CD71 binding is not known. Blocking gliadin peptide entry into the intestinal mucosa might serve as the basis for a novel therapeutic strategy in CD. The CD71-mediated transport of IgA food complexes is also shown in Figure 3.

IgA-mediated gliadin transport is involved in the overstimulation of the local immune system. While the IgA-mediated transport of pathogenic bacteria might be beneficial for improving bacterial clearance and restoring intestinal homeostasis, the application of the same mechanism to a normally nonpathogenic antigen such as gliadin may cause the effects to be deleterious rather than protective.[47] Additionally, the presence of large aggregates of gliadin-specific IgA in duodenal secretions, the lamina propria, and the serum of celiac patients could provide a danger signal that promotes the rupture of oral tolerance and/or triggers tissue damage. The damaging effects of IgA-complex deposition in tissues have been exemplified in IgA nephropathy.[48]

THE IGG-MEDIATED TRANSPORT OF ANTIGENS

It is now accepted that gastrointestinal secretions contain significant amounts of IgG. IgG-mediated intestinal transport primarily seems to be implicated in protective immunity. The role of intestinal FcRn was initially reported in suckling rats that receive passive immunity from their mother through the intestinal absorption of IgG from maternal milk. The polarized absorption of IgG is explained by the binding properties of IgG to FcRn at the acidic pH (less than 6.5) recorded close to the apical membrane of the intestinal epithelial cell. The dissociation of IgG from FcRn at neutral pH leads to the release of IgG on the basolateral side of the epithelium. In contrast, while the human neonatal intestine is not a major site for the transfer of passive immunity, FcRn can be found at the apical pole of enterocytes in the fetal and adult intestine, even though the relevance of such expression has not been clearly established.[49,50] The Fc ligand valency influences the intracellular processing of IgG during transcytosis (protection versus degrada-

tion). The Fc fragment displays two binding sites for FcRn, and the presence of both binding sites is required for efficient transcytosis and the protection of IgG from catabolism.[51,52] The functional role of FcRn in the transfer of IgG ICs has been characterized using polarized, epithelial cell lines and transgenic mice. The cells transfected with hFcRn did not degrade OVA during the apical-to-basal transport of IgG/OVA ICs, and OVA-specific $CD4^+$ T cells were activated after IC transport.[41]

Moreover, in vivo studies investigating transgenic mice expressing hFcRn and β_2 microglobulin, showed the FcRn-mediated transcytosis of IgG ICs and their efficient presentation to OVA-specific, helper T lymphocytes by $CD11c^+$ DCs. While the outcome of this immune response in vivo is not known, it has been reported that IgG ICs might induce immune suppression.[53] In addition to food antigens, bacteria can also be transported as IgG ICs through FcRn, a feature likely to have a role in the defense against intestinal pathogens. These findings thus underline a potential role of FcRn in the maintenance of intestinal homeostasis. The IgG-mediated transport of antigens is shown in Figure 4.

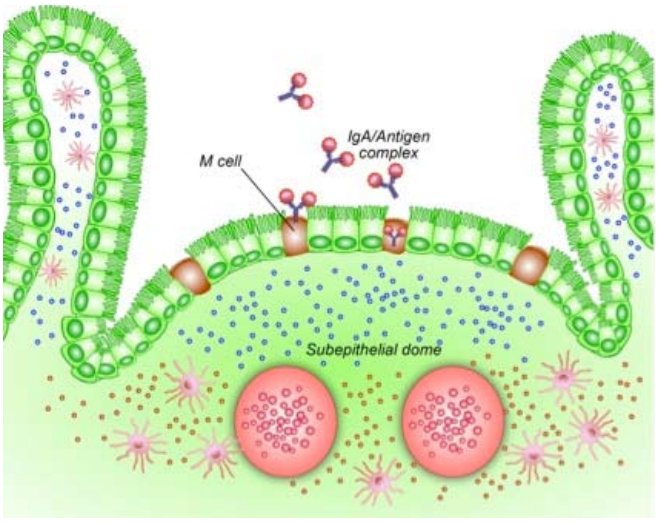
IGE-MEDIATED TRANSPORT OF ANTIGENS OR ALLERGENS

The human, low-affinity IgE receptor (Fc-epsilon-RII, CD23) can mediate the transport of IgE ICs in food allergies (Figure 5). CD23 is primarily expressed on hematopoietic cells but is also observed on the apical and basal surfaces of IECs in patients with gastrointestinal diseases such as autoimmune enteropathy, cow's milk protein enteropathy, CD, ulcerative colitis, and immune activation. High levels of T_H2 cytokines, which are involved in allergic disease, enhance expression of the IgE receptor.[54]

Although it can be found in lavage fluids from parasitic infection and in food allergy patients, IgE is not considered a secreted Ig. Rodent models of allergy have unraveled the roles of epithelial CD23 and IgE ICs in the mucosal entry of food allergens. Sensitizing rats to horseradish peroxidase (HRP) led to the increased uptake of HRP into IECs and faster transcellular transport in these rats compared with naïve control rats. This enhanced transport has been shown to involve an IgE-dependent, receptor-mediated process. Immune sensitization enhanced CD23 expression on the IECs and allowed the IgE/allergen complexes to bypass epithelial lysosomal degradation, resulting in the penetration of a large concentration of intact allergens into the mucosa.[54-56] Intra-epithelial lymphocytes express high levels of the CD23 receptor, which is involved in the apical-to-basal transport of IgE or IgE ICs.

It is likely that the IgE ICs delivered to the lamina propria after epithelial transport can degranulate mast cells, underlining the ability of the IC to activate local cells. This mechanism could be involved in the rapid onset of intestinal symptoms in IgE-dependent food allergy.

Figure 6. M-cell Mediated Allergen Transport



IgA/allergen complexes adhere to the brush border on the apical membranes of M-cells and are transported into the intraepithelial pocket. This can induce a secretory immune response and the production of antigen-specific IgA in the secretions.

TRANSPORT OF ANTIGENS BY M CELLS

The passage of intact macromolecules across the gut is at odds with the role of the gut as a macromolecular barrier. For macromolecules to cross the gut in a controlled manner, specialized epithelial cells have evolved to control the passage of antigens and larger particles through the intestinal epithelium. These cells are called microfold or M cells.

M cells are highly specialized epithelial cells that are joined to their neighbors by tight junctions that restrict the paracellular pathway. Several important immunological and pathophysiological functions, such as the capture of antigens from the gut lumen and their transport to lymphocytes and macrophages, have been recognized for M cells.

The transepithelial, vesicular transport activity of M cells, however, provides functional openings in the epithelial barrier. M cell membranes are equipped with a thick, brush border to promote the sampling of foreign materials from the lumen through the adherence and uptake of food antigens, microbiota, and mucosal pathogens. Because of this unique structure, the SIgA that has been transported into the lumen selectively adheres to the M cells' apical membranes. Furthermore, IgA/antigen complexes also adhere to the M cells' membranes and are transported into the intraepithelial pocket. This uptake of specific IgA/antigen complexes by M cells can induce a secretory immune response and the production of antigen-specific IgA in the secretions.[57,58] The IgA/antigen interaction may promote the uptake of IgA-opsonized, commensal microorganisms, thereby promoting the production of anticommensal, IgA immune responses that could control the luminal microflora, clear microorganisms from the mucosa, and prevent

bacterial invasion. The M cell mediated transport of IgA/allergen complexes is shown in Figure 6.

The microorganisms, macromolecules, and particles taken up at the M cell's apical surface are internalized into endosomal tubules and vesicles and large multivesicular bodies that lie between the apical membrane and the intraepithelial pocket. These macromolecules and particles can be released at the pocket membrane within 10 to 15 minutes. The antigens and pathogens are then captured by immature dendritic cells in the subepithelial region that lies in close proximity to the organized T cell and B cell zones where antigen presentation may occur. These cellular interactions with antigens and pathogens are likely to be important determinants of the mucosal immune response against these same antigens and pathogens; they may also facilitate the dissemination of pathogenic factors that exploit the M cell transport pathway.[59,60]

This pathway, however, provides rapid entry into the mucosa and consequently has a vital role in the pathogenesis of certain bacterial and viral diseases. The risk of pathogen invasion at these sites is high because the intestinal M cells are constantly exposed to the lumen of the gut and are relatively accessible to pathogens.

The uptake of microorganisms by M cells may also play a key role in the maintenance of the normal bacterial flora in the intestine. M cells can transport noninvasive, commensal bacteria into Peyer's Patches, a process that may be crucial in regulating endogenous microbial populations in the lumen or eliminating and inactivating bacteria that have crossed the mucosal epithelium.[58] In neonates, the uptake of nonpathogenic bacteria may be vital for the maturation of the mucosal immune system and for the development of tolerance to food antigens.[61] The excessive internalization of antigens by M cells, the subsequent capture of these antigens by dendritic cells, and the initiation of the immune response against them, however, can cause the overproduction of IgA antibodies, the formation of immune complexes, the initiation of the inflammatory response, and the breakdown of immunological tolerance to various food antigens. The specialized antigen-transporting feature of the M cell is only one of several mechanisms employed by the epithelia of all mucosal surfaces to provide samples of the external environment to the immune system. Normally presenting a selective barrier against invaders, the epithelia use different strategies for this sampling. In addition to the M cell pathway, we have shown how some epithelia also allow the transepithelial traffic of professional, antigen-presenting DCs.

THE ROLE OF THE INTESTINAL BARRIER FUNCTION IN INFLAMMATION AND AUTOIMMUNITY

Over the past decades, accumulating evidence has indicated that increased intestinal-barrier permeability to large molecules plays a key role in the development of various inflammatory and autoimmune disorders, including Parkinson's disease (PD).[62-64] Therefore, insight into the function and loss of gut-barrier integrity is vital in improving researchers' knowledge of the etiology and pathophysiology of diseases and transferring it into clinical practice. Being able to assess the level of intestinal, epithelial-cell damage and the enhanced permeability of large macromolecules undoubtedly contributes not only to early detection but also to the secondary prevention of many inflammatory autoimmune, neuroautoimmune, and neurodegenerative disorders.[62-64]

It is well accepted that gene-environmental triggers and their interaction play a significant role in the production of autoantibodies against various tissue antigens and the development of autoimmune diseases.[65-67] In fact, scientists have often observed that less than 10% of the subjects with a genetic susceptibility to autoimmunity progress to clinical disease in their lifetime.[68-71] This suggests that exposure to environmental factors such as toxic chemicals, infection, and dietary proteins is involved in the development of autoimmune disease.[67-72] In addition to the gene-environment interaction, however, GI dysfunction and the trafficking of macromolecules to the submucosa and into the circulation are additional factors in autoimmunity.[63] This situation occurs because the intercellular tight junctions of the intestinal epithelial barrier control the equilibrium between tolerance and immunity to nonself antigens that originate from dietary proteins and infectious agents.[63,68] Thus, when the zonulin/occludin pathway is deregulated in genetically susceptible individuals, intestinal and extraintestinal inflammatory and autoimmune disorders can occur.[63] In these cases, the intestinal tight junctions allow the passage of macromolecules from the intestine to the submucosa, and the regional lymph nodes stimulate the immune system to mount cellular and humoral immune responses against various tissues or organs.[63] This theory is echoed and strengthened by different studies, lending support to the understanding of the role that the gut-associated lymphoid tissue (GALT) plays in the excessive increase in intestinal permeability during development of autoimmune diseases, such as celiac disease, type 1 diabetes, rheumatoid arthritis and multiple sclerosis.[63,68,73-83]

This finding was verified by measuring the zonulin levels in the sera of patients with autoimmune diseases. Elevated serum zonulin was detected in 70% of the subjects at a time point of 3.5 plus or minus 0.9 years before the onset of the disease.[68] In addition to an increase in the permeability of the blood-brain barrier (BBB) of multiple sclerosis patients, a subgroup of these patients demonstrated increased intestinal permeability.[68,84,85] To lend further support to the detection of intestinal permeability abnormalities in MS patients, the serum zonulin levels were measured in different patient subgroups.[68] Approximately 30% of the patients with either relapsing-remitting MS (RRMS) or secondary-progressive MS (SPMS) showed elevated serum-zonulin levels that were 2.0-fold higher than the serum-zonulin levels in healthy controls. Interestingly, this percentage was similar in MS patients who had

Table 1. Why Size Matters

Lactulose/Mannitol Test	Antigenic Intestinal Permeability
1. The test assesses the permeability to small molecules in the upper region of the small intestine.[4,7-9]	1. The test assesses not only the entire length of the small intestine but the large intestine as well.[10,19]
2. The test measures the permeability of small sugar molecules 342 Da in size.[8,9]	2. The test measures permeability to large molecules (ie, 10 000 Da or larger).[10,19,20]
3. Small sugars the size of lactulose are not antigenic, and therefore, do not challenge the immune system.[16]	3. The 10 000 Da large molecules are antigenic and challenge the system.[16,20,21]
4. The intestinal permeability to small sugar molecules does not necessarily correlate with the uptake of much larger dietary antigens and bacterial toxins.[16,21-23,25]	4. Intestinal permeability to large molecules does correlate with digestion-resistant fragments of food antigens and bacterial endotoxins.[30-33]
5. The interaction between small molecules and the immune system cannot lead to immunologically mediated damage.[16,21]	5. Interaction between macromolecules and the immune system could lead to immunologically mediated damage.[30-33]
6. Measuring permeability to small sugar molecules does not correlate with gut dysbiosis, endotoxin release, microbial translocation, and activation of the mucosal immune system.[16,21]	6. Measuring permeability to large molecules such as LPS does correlate with gut dysbiosis, microbial translocation, and immune activation.[27-29]
7. Epithelial cells permeable to small sugar molecules will not be permeable to large molecules; hence, more false positive results.[13-16]	7. The epithelial cells that are permeable to large molecules will be permeable to small molecules as well; hence, no false positive results.[16,21,23,25]
8. Permeability to small sugar molecules does not reflect damage to the structure of tight junctions.[16]	8. Large-molecule permeability indicates damage to the structure of tight junctions.[48]
9. Due to a repair mechanism, small openings in tight junctions can be repaired within hours, which means more false negative results.[24]	9. Large openings in tight junctions (which are associated with structural damage to tight-junction proteins) cannot be repaired within hours and do not lead to false negative results.[21]
10. Lactulose/mannitol is inconvenient. It entails the oral administration of a tracer and the collection of urine hours later.[5-7]	10. Measuring permeability to large molecules is more convenient. It requires neither a tracer nor urine collection.[21,27]
11. Lactulose/mannitol can be affected by GI motility, the distribution of the tracer, variations in gastric emptying, renal clearance, the use of medication, smoking, and alcohol consumption, leading to even more false positive results.[13-16]	11. Permeability to large molecules is not affected by GI motility, the distribution of the used tracer, variations in gastric emptying, renal clearance, the use of medication, or smoking and alcohol consumption, thus producing fewer false positives.[13-16]
12. The passage of small inert materials is not an indication of a breakdown in immunological tolerance, which is the root cause of allergies and autoimmunity.[21]	12. Permeability to large antigenic molecules and the immune response against them is an indication of a breakdown in immunological tolerance, which is the root cause of allergies and autoimmunity.[21]

increased intestinal permeability. These findings further support the pivotal role that increased intestinal and BBB permeability plays in the development of severe autoimmune disorders.

As with autoimmune disorders, the pathology of PD is believed to be associated with an interaction between genes and susceptibility to environmental factors.[86] The GI tract and its large number of neuronal cells serve as the largest interface between the environment and neural tissue, but it can also serve as a major site of oxidative stress.[87] The close proximity of this extensive neuronal network to microbiota permits the creation of a proinflammatory environment and an increase in oxidative stress in the enteric nervous system (ENS) due to gut dysbiosis and the release of bacterial toxins. This situation may result in the formation of the neuronal inclusions called *Lewy bodies*,[64,88] which consist of aggregated and phosphorylated alpha-synuclein.[89,90] The discovery of these abnormal protein aggregates in the intestinal enteric nerves led to the hypothesis that the GI tract might present the first evidence of PD as a response to pathogens or environmental toxins.[64] These findings further support the concept that the ENS may be the route by which a toxin or pathogen initiates the production of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, thereby affecting the permeability of the BBB and initiating neuroinflammation and its progression into PD over a period of many years.[91-93]

A different line of evidence indicates that an endotoxin-induced increase in intestinal permeability also triggers neuroinflammation in PD. For example, the administration of LPS either directly into the CNS or systematically can induce the selective loss of dopaminergic (DA) neurons in the *substantia nigra* and the development of PD in an animal model.[94-96] Thus, PD patients appear to have an intestinal epithelium that is hyperpermeable to bacterial toxins, which can induce oxidative stress and the misfolding of alpha-synuclein. This situation may lead to very important biological consequences and even the initial injury of the ENS, which is followed by the induction of neuroinflammatory events, enhanced BBB permeability and the development of PD in genetically susceptible individuals.[64,97,98]

CONCLUSION

The lactulose/mannitol test has long been held to be the gold standard for determining the permeability of the intestinal epithelium. The information presented in this article, however, calls for a reassessment as to what may actually be the best methodology for determining intestinal barrier function. Table 1 provides an easy-to-view, side-by-side comparison of the information the authors have reviewed, which can be summarized as follows: The permeability of the epithelium to small sugar molecules does not necessarily correlate with its permeability to larger macromolecules. A misconception may exist that a system sensitive enough to detect and measure the permeability of small sugar molecules makes the measurement of larger molecules superfluous; however, this statement is simply not true.

The table details how small sugars are not antigenic, do not challenge the immune system, do not lead to immunologically mediated damage, do not correlate with the conditions of barrier dysfunction, do not indicate real damage to tight junctions, and in fact, are not an indication of a breakdown in immunological tolerance, thus leading to false negative or false positive results. In comparison, large molecules are antigenic and challenge the immune system, can lead to immunologically mediated damage, correlate with intestinal barrier dysfunction, indicate real damage to the structure of tight junctions, and indicate a breakdown in immunological tolerance without false negative or positive results. All of the transport pathways for the different ligands and antibodies that have been detailed in this review are associated with large antigenic molecules, not small sugar molecules such as lactulose and mannitol.

The loss of the intestinal barrier to antigenic molecules that occurs secondary to the upregulation of occludin/zonulin and environmentally induced inflammation is largely responsible for the switch from tolerance to an immune response against nonself antigens that cross the intestinal barriers. This continuous stimulation of the immune system by nonself antigens and activation of the inflammatory cascade and/or cross-reaction with various self-antigens appears necessary to perpetuate the autoimmune and neurodegenerative processes. Therefore, a hyperpermeable intestinal epithelium may first injure the ENS and then induce neuroinflammatory events, increase BBB permeability and ultimately promote the development of neuroautoimmunity and neurodegenerative disorders.[62-99]

Despite significant progress in the field of mucosal immunology during the past 2 decades, much still remains to be learned regarding everything that happens to transported antigens and the factors that influence the nature and magnitude of the resulting immune responses[99]; however, it is clear that size does matter.

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