Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat

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Mеддингс, J. B., J. J. Аранд, S. J. Урбански, J. HARDIN, and D. G. Gall. Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G951–G957, 1999.—The BB rat spontaneously develops autoimmune diabetes. Feeding these animals a hydrolyzed casein diet significantly reduces the incidence of this disease, suggesting that a dietary antigen is involved in the pathogenesis of this disease. In other syndromes associated with luminal antigens, including celiac and Crohn's disease, increased intestinal permeability has been suggested to play an etiological role. Therefore, the objective of this study was to evaluate whether increased permeability was also present in BB rats before disease development. By measuring gastrointestinal permeability, in animals on a regular or hydrolyzed casein diet, we were able to demonstrate that increased gastric and small intestinal permeability appeared before the development of both insulitis and clinical diabetes. Although hydrolysis of dietary protein significantly reduced the incidence of diabetes, it did not alter the small intestinal permeability abnormality, suggesting that this is an early event. Increased permeability appears to have an early role in the genesis of several immunological diseases and may represent a common event in these diseases.

antigen delivery; Crohn's disease; celiac disease

There are several inflammatory diseases involving the gastrointestinal tract in which abnormal paracellular permeability defects appear to play an important role. In both celiac and Crohn's disease, an interaction between the host immune system and a luminal constituent is hypothesized as an initiating event. In the former, there is good evidence that the luminal constituent is a gluten fraction, whereas for the latter no clear candidate has been identified. However, both diseases share an important feature. Increased intestinal permeability appears to precede the development of both syndromes. In a dog model of celiac disease, increased permeability is clearly present before the onset of disease (8, 9) and a similar pattern has been reported in humans (2).

For Crohn's disease, the data are less clear. However, bypassing the epithelial barrier and injecting sterile extracts of luminal bacteria into the intestinal wall of the rat initiates a disease similar to Crohn's (24). In humans, several studies have now demonstrated that individuals at high risk of developing Crohn's disease contain a subgroup that either has increased baseline permeability (12, 13, 23) or an exaggerated increase in permeability in response to damaging agents (11). These data support the hypothesis that certain inflammatory diseases require several preexisting conditions. The first is a genetic susceptibility for the host immune system to recognize, and potentially misinterpret, an environmental antigen presented within the gastrointestinal tract. Second, the host must be exposed to the antigen or hapten. For instance, in the case of celiac disease in the Irish Setter model, preventing exposure to dietary gluten completely prevents disease expression. Finally, the antigen must be presented to the gastrointestinal mucosal immune system. In all cases, increased permeability appears to precede disease and suggests an abnormality in antigen delivery in the development of the clinical syndrome.

Other diseases share some of these features. In the BB rat, autoimmune diabetes develops spontaneously and is accompanied by evidence of insulitis and other autoimmune features. There are data in this model that dietary antigens may also play a role in disease initiation. Feeding these animals a hydrolyzed casein diet reduces the incidence and delays the onset of disease (20). A potential explanation for these findings would be the removal of a protein important in disease initiation.

Therefore, this study was designed to evaluate two questions. First, do BB rats have abnormal gastrointestinal permeability before disease development? Second, if abnormal permeability is present, does it depend on the presence of intact dietary protein? Hydrolysis of dietary protein might be beneficial in these animals, either due to removal of an antigen important for disease initiation or of a protein capable of inducing gastrointestinal damage.

METHODS

Study design. BB diabetes-prone (BBdp) and control, diabetes-resistant (BBc) rats were utilized in this study. Animals were weaned on either a protein-containing diet (control) or the hydrolyzed casein diet described previously (20). Four animal groups were therefore available as follows: BBc animals on either a control or hydrolyzed casein diet and BBdp on the same diets. Gastrointestinal permeability was measured noninvasively throughout the lifetime of each animal, and the incidence of diabetes in each group was determined.

Animals. Weanling (ages 21–28 days) BBdp (diabetes-prone) and BBc (non-diabetes-prone) rats were obtained from the Animal Resources Division of Health Canada. Animals were housed in Hepa-filtered Thoren units except for days when permeability was to be determined, on which they were switched to stainless steel metabolic cages. Serological testing was performed to monitor animals for mycoplasma. All animals had negative screening serological tests. Animal maintenance and experimental procedures were carried out...
in accordance with the guidelines of the Canadian Council on Animal Care.

Diet. Two diets were utilized in the study, a control standard rat chow (Laboratory Rodent Diet 5001; PMI Feeds, Richmond, IN) and an AIN 93G modified casein hydrolysate diet (BIO-SERV, Frenchtown, NJ). Animals were randomized into the following four groups: 1) BBc rats receiving the control diet, 2) BBc rats receiving the hydrolyzed casein diet, 3) BBdp rats receiving the control diet, and 4) BBdp rats receiving the hydrolyzed casein diet.

Definition of diabetes. Animals were weighed two times a week until day 70 after which they were weighed at weekly intervals. After day 60, rats were tested two times a week for glucose in their urine (Diatix; Miles Canada, Etobicoke, ON, Canada). Rats with a urine glucose value of $\geq 2+$ were fasted overnight, and blood glucose was measured the next morning with an Ames glucometer (Miles Laboratories, Rexdale, ON, Canada). Animals with a blood glucose $\geq 11.1$ mM were considered to be diabetic.

Intestinal permeability. Measurements were performed to evaluate gastrointestinal permeability in the stomach, small intestine, and colon. Sucrose is a large disaccharide that we have previously demonstrated to reliably determine gastro-duodenal damage (16). Because it is rapidly degraded in the small intestine, by sucrase-isomaltase, intact sucrose is only found high in the proximal gut. Increased permeation of this compound, in the absence of small intestinal damage, can be used to infer increased permeability in this location. Lactulose and mannitol have been used for many years to evaluate small intestinal permeability; however, they are destroyed in the colon and cannot provide information about damage to this organ. Sucralose is an artificial, trichlorinated analog of sucrose that is neither a substrate for sucrase-isomaltase nor colonic bacterial degradation (5). Therefore, increased permeation of this compound can be used to infer damage to either the small intestine or colon. Because the greatest contact time this probe has is with the colon, it preferentially reports colonic damage (5).

Each day, a stock solution was made containing 50 g sucrose, 4 g mannitol (BDH), 6 g lactulose (Technilab), and 12 ml of “Splenda” (25% wt/vol sucralose; McNeil Consumer Products, Guelph, ON, Canada) dissolved in distilled water to a volume of 100 ml. Enough solution was made so that each rat could be given 2 ml of the probe. Therefore, each rat received 1 g sucrose, 120 mg lactulose, 80 mg mannitol, and 60 mg sucralose.

Rats were placed in stainless steel metabolic cages with wire bottoms to separate feces from urine. Plastic tubes were mounted underneath a spout on the bottom of each cage to collect urine. As an additional filtering method, fine nylon mesh (300-µm perforations; Small Parts) was folded into a funnel shape and placed between the spout of each cage and the plastic tubes. Thymol, 100 µl of a 10% solution in isopropyl alcohol, was added to each tube to prevent bacterial degradation of urinary sugars. Control urine samples collected in this manner were spiked with known concentrations of the sugars and incubated at room temperature for up to 4 days. No loss of any sugar was apparent (data not shown).

Rats were denied access to water for 3 h, at which point they were allowed free access to water for the remainder of the experiment. Urine was collected for a total of 24 h, at which point the rats were returned to their normal cages. Urine volumes were measured, and the urine composition was analyzed by HPLC.

Permeability measurements were performed weekly at the initiation of the study and then monthly after 70 days of age. HPLC analysis. The methods to assay sucrose, lactulose, and mannitol have been reported previously (15). Briefly, cellobiose was added as an internal standard, and the urine was filtered through a 0.4-µm filter and diluted as necessary. Samples were deionized and then injected on a Dionex MA-1 ion exchange column. Sugars were eluted with NaOH at a flow rate of 0.4 ml/min with concentrations ranging from 400 to 600 mM. Peaks were detected using pulsed amperometric detection on a Dionex HPLC and quantitated as peak areas. Calibration was performed on a daily basis with authentic standards at multiple concentrations, and the experimental standards were diluted so that the areas of all peaks fell within the calibration range. Final data were reported as either fractional excretions (sucrose and sucralose) or as a ratio of fractional excretions (lactulose-mannitol). Fractional excretion is defined as the fraction of the gavaged dose recovered in the urine sample.

Sucralose was also assayed by HPLC. However, it cannot be detected under the conditions used for the other sugars. Separation was achieved using a Dionex I Ionpac NS1 column and acetonitrile-water as the eluent at a flow rate of 1 ml/min. An isocratic run was used beginning with acetonitrile in water increasing from 0 to 20% over the course of the run. Detection was performed with an electrochemical detector in a fashion identical to the other sugars. Because this only works at a high pH, postcolumn addition of 300 mM NaOH at a constant flow rate of 0.5 ml/min was used. For these assays, the internal standard used was phenyl-beta-D-thiogalactoside (Sigma Chemical) added to the initial urine sample at a concentration of 0.1 mg/ml. This compound was stable in urine for at least a week at room temperature and for at least 3 mo when frozen. Calibration and peak authentication were performed in a manner similar to that described.

Pancreatic histology. To define early disease onset, additional experiments were performed once the time course of altered permeability was apparent. The pancreas was removed by dissection, Bowen fixed, paraffin embedded, and routinely processed. Hematoxylin and eosin-stained slides were examined in a blinded fashion by an experienced gastrointestinal pathologist (Urbanski). Parameters measured included islet size and the presence of a lymphocytic infiltrate. BBdp or BBc animals maintained on either diet (n = 5/group) were killed shortly after the onset of increased gastrointestinal permeability (day 50), and the presence or absence of insulin was determined. In separate experiments, 75-day-old BBdp and BBc animals (n = 5/group) receiving the control diet were killed in a similar fashion. This time point was selected as being before the onset of clinical diabetes.

Statistical analysis. Data are expressed as means ± SE. Comparisons between groups were made using ANOVA with a Tukey test for post hoc comparisons. All calculations were performed using Systat (Bellevue, IL), and significance was assumed at P < 0.05.

RESULTS

BBc and BBdp rats thrived on both the control or hydrolyzed diet. Rates of weight gain are illustrated in Fig. 1. Figure 1A contains data for the BBc rats on either diet, whereas Fig. 1B illustrates the data obtained for the BBdp rats. No differences were apparent that could be attributable to the diet consumed. Furthermore, no significant differences were observed between the BBc and BBdp groups on either diet.

Diabetes was not observed in BBc rats consuming either diet. However, in the BBdp rats, maintained on
the control diet, diabetes became apparent by age 80 days in one of the 14 animals. By 100 days of age, 50% of this group had succumbed to the disease. In contrast, BBdp animals maintained on the hydrolyzed diet had a significantly lower incidence of diabetes; only 20% developed diabetes in the absence of intact dietary protein. These data are shown in Fig. 2 as disease-free survival.

Gastrointestinal permeability in these animals is illustrated in Figs. 3 and 4. In Figs. 3 and 4, A, B, and C refer to sucrose permeability, lactulose-mannitol ratio, and sucralose permeability, respectively. Figure 3 compares the results of BBdp and BBc rats maintained on the control diet. Gastric and small intestinal permeability was significantly increased in the BBdp rats as early as 50 days of age. The increase in gastric permeability was maintained to 100 days of age, but small intestinal permeability in the BBdp rat had normalized by this time point. The fractional excretion rate of sucralose, sensitive to colonic permeability, was not significantly increased in the BBdp rat at any time point, suggesting no increase in colonic permeability.

These significant differences in gastrointestinal permeability were also apparent in animals fed the hydrolyzed diet (Fig. 4). Once again, no differences were observed in colonic permeability between BBdp and BBc rats. Comparison of Fig. 3A and Fig. 4A shows that gastric permeability in the BBdp animals was higher in animals maintained on the control diet compared with animals maintained on the hydrolyzed casein diet. The hydrolyzed diet diminished, but did not abolish, the difference in gastric permeability between BBdp and BBc rats.

Examination of pancreatic histology was carried out in BBdp animals at day 50, shortly after the increase in gastrointestinal permeability, and day 75, immediately before the onset of clinical diabetes. None of the 50-day-old animals had any evidence of altered islet size or lymphocytic infiltration (data not shown). In contrast, pancreatic tissue from the 75-day-old BBdp animals maintained on a control diet all demonstrated prominent islet destruction by a lymphocytic infiltrate compared with BBc animals maintained on the same diet (Fig. 5). Clearly, the alterations in permeability illustrated in Figs. 3 and 4 occurred at a time when no evidence of diabetes was present either clinically or histologically.

DISCUSSION

It has long been recognized that the spontaneous diabetes seen in the BBdp rat is related to dietary constituents. Cereal-based diets prompt a high incidence of diabetes that can largely be prevented by feeding semipurified diets based on hydrolyzed casein (20). These data have been confirmed in the present study. A marked difference in diabetes incidence was
Fig. 3. Gastrointestinal permeability on the control diet. A, B, and C refer to sucrose permeability, the lactulose-to-mannitol ratio, and sucralse permeability, respectively. BBdp animals are represented by filled circles and a dotted line, whereas BBc animals are shown as open circles with a solid line. Significant increase in sucrose permeability and lactulose-to-mannitol ratio is seen between these groups after day 50. *P < 0.05 vs. BBc animals.

Fig. 4. Gastrointestinal permeability on the hydrolyzed diet. As in Fig. 3, it is clear that there is an increased gastric and small intestinal permeability in the BBdp animals (●) after day 50. ○, BBc animals. *P < 0.05 vs. BBc animals.
observed in the BBdp rat, depending on the diet received (Fig. 2).

Of even greater interest was the similarity between the diet dependence of diabetes in this model and other diseases. Celiac disease is clearly dependent on the exposure of genetically susceptible individuals to a well-recognized dietary component. With the elimination of gluten, the disease is well controlled in most patients. In Crohn's disease, it has also been postulated that disease activity is related to the presence of a luminal antigen. Diversion of the fecal stream clearly induces remission. Furthermore, animal models of Crohn's disease can be induced by exposure of the mucosal immune system to either luminal bacterial extracts or application of a chemical hapten [2,4,6-trinitrobenzenesulfonic acid (TNBS); see Refs. 17, 24].

A simple hypothesis can be generated regarding the etiology of these diseases. In patients with genetic susceptibility, exposure to an environmental antigen can trigger disease. Several things appear to be required for this to occur. Obviously, both the genetic susceptibility and antigen must be present. However, these do not appear to be sufficient in themselves. In animal models of Crohn's disease, the antigen must cross a damaged epithelial barrier. This can be achieved by destroying epithelial cells with ethanol to allow TNBS to penetrate to the mucosal immune system (17) or by the direct injection of bacterial extracts in the wall of the intestine (24). Simple abrogation of epithelial barrier function by the construction of a dominant negative E-cadherin chimeric mouse (10) also induces inflammation reminiscent of Crohn's disease. Thus it appears that abnormal presentation of antigen through a damaged epithelial barrier is important for disease development. This also appears to be the case in celiac disease. In the Irish setter dog model of celiac disease, abnormal small intestinal permeability is present before the animals have ever been exposed to gluten (8, 9).

The data in humans are more difficult to obtain. However, even after successful treatment of celiac disease, a persistent permeability defect exists (2). Patient populations at risk of developing Crohn's disease contain subgroups with either increased baseline permeability (12, 13, 23) or an exaggerated response to environmentally encountered agents that damage the epithelial barrier (11). These observations suggest that, in addition to genetic susceptibility and the presence of an environmental antigen, abnormal intestinal permeability is required for disease development. Whether this allows for abnormal antigen delivery or is associated in some other way with the etiology of these diseases is unclear at the present time.

It is in this regard that the BB model of autoimmune diabetes is of interest. Clearly, this disease is associated with genetic susceptibility and the presence of a luminal antigen, as altering luminal constituents can dramatically alter disease expression. If this syndrome is similar to the diseases mentioned in the preceding paragraphs, we would predict that altered gastrointestinal permeability should not only be associated with the development of the disease but should also occur before disease development. The experiments outlined in this manuscript were designed to test this hypothesis.

We utilized a series of orally administered probes that have been demonstrated to evaluate either gastric permeability (sucrose), small intestinal permeability (lactulose and mannitol), or colonic permeability (sucrose). Several observations are important. First, gastrointestinal permeability of the BBdp rat was not different from BBc animals at the time of weaning (21–28 days of age). However, by day 50, a marked increase in both gastric and small intestinal permeability was
apparent in these animals. Diabetes was not observed until almost a month later. Furthermore, histological evidence of pancreatic islet destruction was absent at the time of increased permeability but clearly present at a later time point (Fig. 5). Therefore, it appears clear that increased permeability occurred before either histological or overt manifestations of diabetes in these animals. There did appear to be an effect of diet on this process, but this was confined to the stomach. BBdp animals maintained on the hydrolyzed casein diet had lower sucrose fractional excretions than similar animals maintained on the control diet (Fig. 3A and 4A). The reason for this cannot be determined from these experiments, but it is recognized that dietary constituents, such as hot spices, may alter paracellular permeability (14). Whether similar effects on permeability could be induced by the control vs. the hydrolyzed casein diet cannot be concluded from these experiments, but even if true it does not appear to play a role in the small intestine. No diet-induced difference was apparent in small intestinal permeability in this study.

It could be argued that increased permeability is simply an early manifestation of diabetes, occurring even before lymphocytic destruction of the islets. However, in other animal models of diabetes, increased paracellular permeability does not appear to be present (7). Similarly, in humans, simple diabetes does not appear to alter gastrointestinal permeability, but some alterations have been reported with complications such as diabetic diarrhea (3). Furthermore, when BBdp rats received a hydrolyzed casein diet, a marked reduction in the incidence of diabetes was observed (Fig. 2). This was not associated with an improvement of the small intestinal permeability difference observed between the BBdp and BBc rats (Figs. 3 and 4). This suggests that diabetes itself did not induce the alterations in gastrointestinal permeability.

Colonic permeability was not different between BBdp and BBc rats; permeability alterations were restricted to the stomach and small intestine and maximal between 50 and 100 days of age. These observations support the general hypothesis. Increased gastrointestinal permeability appears to be associated with several diseases initiated by luminal antigens. Subtle differences exist between these models. In human populations at high risk of developing Crohn's disease, only small intestinal permeability is increased. No alterations in either baseline or nonsteroidal anti-inflammatory drug (NSAID)-evoked sucrose permeability have been reported. In animal models of celiac disease, gastric permeability has not been determined; however, in humans, active disease is associated with increased sucrose permeability (4, 21, 22). Whether this represents increased gastric permeability or is secondary to abnormal small intestinal permeability is a matter of debate (21). However, in this animal model of autoimmune diabetes, increased gastric permeability is clearly observed in association with increased small intestinal permeability (Figs. 3 and 4). Whether abnormal luminal antigen delivery occurs in the stomach or small intestine is impossible to determine from these data. It is also important to note when comparing these data with the human situation that measurements of small intestinal permeability differ in magnitude between these species.

In these experiments, we hypothesize that increased gastrointestinal permeability may allow increased delivery of a luminal compound, which is important in the genesis of disease, to the mucosal immune system. To obtain valid determinations of gastrointestinal permeability, we must use permeability markers that remain structurally intact in the gastrointestinal segment of interest under the conditions of the experiment. To achieve this, we have used a well-characterized system of small sugars as surrogate markers of permeability. However, this raises the important issue of whether permeation rates for these markers are representative of those observed for the compound involved in disease.

If we assume that the dietary constituent involved is a large protein, then this becomes a very difficult problem to elucidate in experimental models. As part of the digestive process, these large proteins are degraded in a series of steps by pancreatic enzymes. Therefore, the actual offending compound, although derived from a dietary protein, could be the original large molecule or any of the smaller degradation products, including small peptide haptons. Without knowledge of the actual compound that initiates disease, it is impossible to select a similar-sized permeability marker. We have assumed that alterations in permeability for the markers used in this study reflect alterations in permeability for the offending agent. This assumption appears tenable for several reasons. First, there are molecules of a size similar to disaccharides that induce immunological disease. An example of this would be f-Met-Leu-Phe, a formylated tripeptide that can initiate both inflammation and an alteration in immunologically relevant cell types (1). In other cases, a small luminal agent can act as a hapten and induce immunologically mediated inflammatory disease in the presence of abnormal permeability. This is clearly seen with the TNBS model of enterocolitis where the size of the disease-initiating agent, TNBS, is very similar to the disaccharides used to measure permeability (6, 17). However, the size of the protein compound involved in this model of type 1 diabetes might be much larger than that of the probes used to determine gastrointestinal permeability. This is true regardless of whether it is an intact dietary protein or a product of partial proteolysis that acts as a hapten. The question then arises whether gastrointestinal permeability, as determined by disaccharide probes, is a reasonable reflection of alterations that take place for larger molecules.

There is evidence to support this assumption in other models of disease. In patients with Crohn's disease, where there is a clear increase in permeability for small disaccharide molecules, there is a corresponding increase in permeability for larger molecules such as polysucrose (mol wt 15,000; see Ref. 18). Although the absolute permeabilities for these molecules differ tremendously, the relative increase observed in disease is similar. These findings are also apparent in experimen-
toral animal models of immunological disease. With immunological reactions in the intestinal wall, increased permeability is observed, and this is true whether the probe molecule is small, such as Cr-EDTA, or much larger, such as ovalbumin (mol wt 45,000; see Ref. 19). Therefore, the increased permeability for disaccharides, as observed in this study, is directly applicable to a smaller proinflammatory luminal compound or hapten or may represent an increase in permeability for a much larger molecule.

In conclusion, we have demonstrated that gastrointestinal permeability is increased in the BBdp rat before the development of autoimmune diabetes. There now appears to be a spectrum of diseases that are associated with intestinal presentation of environmental antigens in the context of abnormal gastrointestinal permeability. The type of disease developed by the host is probably dictated by the genetic background of the host and the antigen(s) presented to the mucosal immune system. However, abnormal gastrointestinal permeability appears to be a common denominator in these syndromes and may allow for abnormal delivery of luminal antigens to the mucosal immune system.

We express deep appreciation to Kim Tran for care and expertise in the HPLC analysis.

This work was funded by generous grants from Searle Canada, the Medical Research Council of Canada, and the Crohn’s and Colitis Foundation of Canada. J. B. Meddings is an Alberta Heritage Medical Scholar.

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Received 23 January 1998; accepted in final form 21 December 1998.

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