Glucagon-like peptide-2-enhanced barrier function reduces pathophysiology in a model of food allergy

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Penetration of the gut epithelial barrier by intact luminal antigen is necessary for immunologically mediated pathophysiology in the context of food allergy. We investigated if glucagon-like peptide-2 (GLP-2) could affect immediate hypersensitivity and late-phase allergic inflammation in a murine model. Mice were sensitized to horseradish peroxidase (HRP); studies were conducted 14 days later. Mice were treated with 5 μg GLP-2 subcutaneously 4 h before antigen challenge. For immediate hypersensitivity, jejunal segments in Ussing chambers were challenged by luminal HRP antigen. GLP-2 treatment reduced the uptake of HRP and the antigen-induced secretory response after luminal challenge. GLP-2 appears to reduce macromolecular uptake independent of the CD23-mediated enhanced antigen uptake pathway. For the late phase, mice were gavaged with antigen, and 48 h later the function and histology of the jejunum were examined. GLP-2 prevented the usual prolonged permeability defect and reduced the number of inflammatory cells in the mucosa. Our studies demonstrate that a single treatment of sensitized mice with GLP diminishes both immediate and late-phase hypersensitivity reactions characteristic of food allergy by inhibiting transepithelial uptake of antigen.

food allergy; transcellular and paracellular permeability; epithelial barrier function

Glucagon-like peptide-2 (GLP-2) is a 33-amino acid peptide secreted by the enteroendocrine L cells of the intestinal epithelium (16). The biological activities of GLP-2 include augmented growth of the gut mucosa [by both increased crypt cell proliferation and decreased enterocyte apoptosis (20)], stimulation of nutrient absorption (7), and enhanced barrier function (2). Such changes may account for the beneficial effects of GLP-2 in models of massive small bowel resection (18), total parenteral nutrition (8), and intestinal inflammation (6, 11). Specifically, improved barrier function may prevent the uptake of luminal antigens, bacterial products, and other proinflammatory material into the mucosa where they can provoke immune/inflammatory reactions, although this hypothesis has not yet been tested precisely. However, our previous study (2) in mice demonstrated that GLP-2 was able to reduce penetration of the epithelium by both small and large molecules; this effect was documented within 4 h after a single subcutaneous injection.

Food allergy is a condition in which ingested food antigens rapidly provoke gastrointestinal symptoms in sensitized individuals (in humans or rodent models; reviewed in Ref. 9). Food allergic reactions are caused by antigen cross-linking of IgE bound to mucosal mast cells in the subepithelial compartment. Released mast cell mediators then act on cell receptors to induce intestinal anaphylaxis involving ion secretion, the driving force for water secretion leading to diarrhea, and increased paracellular permeability. Theoretically, the epithelial barrier should prevent uptake of luminal macromolecules since the paracellular pathway is not available to molecules >500 Da. Although some antigenic proteins are endocytosed into enterocytes and transported across the epithelium via the transcellular pathway, most proteins are degraded by intracellular enzymes, thus eliminating their antigenic properties (22). However, increased intestinal permeability has been documented in vivo in patients with food allergy (4, 19), as well as in biopsy specimens (12) and in animal models of food hypersensitivity (3, 10, 17, 21, 24). It is clear that in sensitized individuals intact antigen does penetrate the epithelium to induce both immediate hypersensitivity and late-phase allergic reactions.

We previously identified that a unique mechanism accounts for enhanced transepithelial antigen transport in allergic rodents. We provided evidence that enhanced transcellular transport (increased uptake of luminal antigen into enterocyte endosomes and rapid transcytosis across the cell) is mediated by increased expression of CD23/FcεRII, the low-affinity IgE receptor, on enterocytes (23, 25). Subsequent to antigen activation of mast cells in the subepithelial compartment, mast cell mediators act on epithelial receptors to open the paracellular pathway (3, 23), enabling non-specific transepithelial passage of luminal material, including the antigen itself. The immediate hypersensitivity reaction [shown by a rise in the short-circuit current (I_sc), indicating secretion of charged ions] is
followed several hours later by another phase of pathophysiology and infiltration of inflammatory cells in the mucosa (24).

Although we had shown that short-term treatment of normal mice with GLP-2 could improve their intestinal epithelial barrier function (2), it was not clear if GLP-2 would be effective in sensitized mice where barrier function was compromised in such a specific manner. Therefore, the aim of this study was to examine the potential benefit of GLP-2 in a mouse model of food allergy, including effects on transepithelial antigen transport, the immediate hypersensitivity reaction (examined within minutes after antigen challenge), and the late-phase reaction (examined 48 h after antigen challenge in vivo).

MATERIALS AND METHODS

Animals

Experiments were approved by the Animal Care Committee at McMaster University. Male BALB/c mice (n = 36; 9–12 wk old; Harlan Sprague-Dawley, Indianapolis, IN) were housed in cages equipped with filter hoods. After 1 wk of acclimatization, mice were sensitized to horseradish peroxidase (HRP type II; Sigma-Aldrich Canada, Oakville, ON, Canada) by intraperitoneal injection of HRP with pertussis toxin, as described previously (15). Naive mice were sham sensitized by saline injection. On day 14, mice received a single subcutaneous injection of 5 μg of a synthetic protease-resistant analog of GLP-2, human [Gly7]-GLP-2 (a gift from Allelix Biopharmaceuticals, Mississauga, ON) in sterile PBS. Control mice received only subcutaneous PBS. Mice were assigned to either the immediate hypersensitivity group or the late-phase hypersensitivity group.

Immediate Hypersensitivity Reaction

At 4 h after in vivo GLP-2 treatment, mice were killed by cervical dislocation. The jejunum was excised beginning 5 cm distal to the ligament of Treitz and was placed in warmed oxygenated Krebs buffer. The immediate hypersensitivity reaction was evaluated by measuring ion secretion and mucus secretion from antigen challenge of tissues in Ussing chambers (3).

Ussing chambers. A 12-cm segment of small intestine was opened along the mesenteric border and cut into four flat sheets. Full-thickness segments (devoid of Peyer’s patches) were mounted in modified Ussing chambers and bathed with oxygenated Krebs buffer. The immediate hypersensitivity reaction was evaluated by measuring ion secretion and mucus secretion from antigen challenge of tissues in Ussing chambers (3).

Ion transport studies. Unless otherwise indicated, tissues were challenged by addition of HRP antigen (10−5 M) to the luminal buffer. In some experiments, for comparison, HRP was added to the serosal buffer. The ion secretory response to HRP was calculated as the total charge transfer during the 5-min period after antigen challenge, measured as the area under the Isc curve (expressed as C/cm2). The effect of GLP-2 on this response was compared with the response to antigen challenge in tissues from vehicle-treated sensitized mice. To establish if the immediate Isc change observed after exposure to HRP was an antigen-specific phenomenon associated with the hypersensitivity reaction, jejunal tissues from mice sensitized to HRP were exposed to ovalbumin (OVA) on either the mucosal or the serosal side in the Ussing chamber. In addition, tissues from mice sensitized to OVA were exposed to HRP in the Ussing chamber to verify that the sensitizing antigen was required to evoke the secretory response.

Flux of HRP. The flux of HRP across the tissue from the luminal to serosal compartment was determined as previously described (3). Briefly, HRP was added to the luminal buffer at a concentration of 10−5 M. Duplicate samples of serosal buffer (500 μl) were obtained at 30-min intervals and were replaced with Krebs buffer. HRP activity was measured by assaying enzyme activity using a modified Worthington method (13). Briefly, 180 μl sample was added to 800 μl of phosphate buffer containing 0.003% H2O2 and 80 μg/ml o-dianisidine (Sigma). Enzyme activity was determined from the rate of increase in optical density at 460 nm during a 1.5-min period. The flux was calculated and expressed as picomoles per square centimeter per hour.

HRP-containing endosomes in enterocytes. After exposure of the intestine in Ussing chambers to luminal HRP for 2 min, the tissue segment was removed and fixed in 2% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer for 2 h at room temperature, transferred to sodium cacodylate buffer, and stored at 4°C overnight. The tissue was washed three times in 0.05 mol/l Tris buffer and then incubated for 30 min in 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Canada) in 10 ml of 0.05 mol/l Tris buffer and 0.01% hydrogen peroxide. The tissue was subsequently processed for electron microscopy, and photomicrographs were prepared of well-oriented epithelial cells. The total area occupied by HRP product-containing endosomes located within a 300-μm2 window (in the apical region of the enterocyte above the nucleus) was measured in coded photomicrographs by one of the investigators (Yang) using a computer-supported image analysis system. A total of six windows for each of six mice per group were analyzed (averages per mouse obtained before calculating group means; n = 6).

Role of epithelial CD23. To determine if the inhibitory effects of GLP-2 involved the transepithelial CD23 pathway, an anti-CD23 antibody was employed. The rat anti-mouse monoclonal antibody was derived from hybridoma cells and isolated as described (25). The anti-CD23 antibody (0.05 mg/ml) was added to the luminal buffer 30 min before challenge with HRP antigen (added to the luminal buffer). Tissues were exposed to luminal HRP for 2 min. Endosomal uptake and HRP flux were measured as described above. Results were compared with those obtained using an isotype control antibody. (The same protocol was followed as that which was shown to inhibit transepithelial antigen transport in sensitized mice as reported in our previous study (25).)

Late-Phase Reaction

At 4 h after GLP-2 treatment, mice were challenged orally (ig) by gavage with 0.1 ml sensitizing antigen (5 × 10−6 M or vehicle). Mice were killed 48 h later. The jejunum was excised beginning 5 cm distal to the ligament of Treitz and placed in warmed oxygenated Krebs buffer for Ussing chamber studies of ion transport and permeability. In addition, segments of jejunum distal to those used in Ussing chambers were fixed

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and stained for light microscopic analysis of inflammatory infiltrate.

**Flux of $^{51}$Cr-EDTA.** The inert probe $^{51}$Cr-labeled EDTA (6 µCi/ml; Radiopharmacy, McMaster-Chedoke Hospital, Hamilton, ON, Canada) was added to the luminal buffer of the Ussing chambers. Four 50-µl samples were taken from the serosal buffer at 30-min intervals after equilibrium (30–120 min), and duplicate 50-µl samples were obtained from the luminal buffer at the beginning and end of the experiment. The radioactivity of $^{51}$Cr-EDTA was measured in a gamma counter. The flux was calculated and expressed as picomoles per square centimeter per hour.

**Light microscopy.** Tissues fixed in 4% formaldehyde were processed and stained with hematoxylin and eosin. The number of eosinophils and mononuclear cells was counted in 10 random fields (magnification, ×200) for each mouse (80 mice/group). Cell numbers were expressed per square millimeter of mucosa. All sections were coded to avoid observer bias.

**Experimental Design**

In the first part of the study, the effect of GLP-2 treatment on the immediate hypersensitivity reaction was examined by studying tissues in Ussing chambers. Because we had previously shown enhanced antigen uptake in enterocyte endosomes as a characteristic feature in sensitized rodents, here we measured the total area of HRP-containing endosomes in tissues from both control and sensitized mice treated or not treated with GLP-2. HRP was used both as a probe and the specific antigen in mice since it can be visualized in tissues to observe its pathway of penetration, and the intact molecule can be quantified by an enzymatic activity assay. The immediate hypersensitivity reaction was indicated by the change in $I_{sc}$ (area under the curve) within 15 min as well as the overall flux of HRP across the tissues during a 2-h period.

In the second part of the study, sensitized mice were treated with GLP-2 4 h before in vivo antigen challenge by gavage. The late-phase reaction was examined 48 h later when pathophysiology was assessed in Ussing chambers and tissue morphology was evaluated by light microscopy. The inert probe $^{51}$Cr-EDTA was used to measure paracellular permeability.

**Statistical Analysis**

Results were analyzed by ANOVA with post hoc comparisons using Newman–Keul’s test or Student’s t-test where appropriate. Results for each group are expressed as means ± SE. A P value of <0.05 was accepted as significant.

**RESULTS**

**Immediate Hypersensitivity Response**

GLP-2 attenuated the ion secretory response to antigen challenge. In response to antigen, sensitized intestine shows a rapid rise in $I_{sc}$ due to luminal directed chloride secretion resulting from the action of mast cell mediators on the epithelium (10). When antigen is added to the serosal side of the tissue, the $I_{sc}$ increases within 1 min, whereas when antigen is added to the luminal side, $I_{sc}$ changes occur within 5 min. The lag represents the time needed for antigen to penetrate the epithelial barrier and activate mast cells. In these experiments, the $I_{sc}$ response to luminal antigen challenge was attenuated significantly ($P < 0.001$) by GLP-2 treatment compared with nontreated sensitized mice (by ~80%; 224.3 ± 26.7 vs. 1,106.2 ± 95.4 C/cm², n = 6; Fig. 1). To assess whether this reduced secretory response was the result of reduced epithelial penetration by antigen or an effect on mast cells or the secretory apparatus, we challenged tissues with serosal antigen. In contrast to luminal challenge, GLP-2 did not alter the secretory response to serosal antigen challenge. No $I_{sc}$ changes were documented in jejunal segments from mice sensitized to HRP and exposed to OVA (a bystander antigen). Furthermore, jejunal segments from mice sensitized to OVA did not respond to luminal or serosal HRP.

**GLP-2 decreased endosomal uptake of HRP antigen.** Electron microscopy was used to examine the uptake of HRP antigen in the epithelium before mast cell activation, 2 min after antigen challenge. We observed that HRP was present within enterocyte endosomes but not in the paracellular regions, indicating that the movement of HRP was restricted to an endocytic pathway across the epithelium (Fig. 2). In tissues from sensitized mice (Fig. 2B), the HRP product-filled endosomes appeared larger and more numerous than those in control mice (Fig. 2A). GLP-2 treatment resulted in a significant decrease in the area of HRP product-containing endosomes (Fig. 2C). Computerized image analysis (Fig. 3A) indicated that the total area of epithelial HRP-containing endosomes in a 300-µm² apical window was enhanced significantly in sensitized mice compared with naive mice and reduced by GLP-2 in both sensitized and nonsensitized mice.

**GLP-2 inhibited enhanced transepithelial antigen transport.** GLP-2 treatment significantly reduced the transport of HRP across intestinal tissue from naive mice (from 13.17 ± 2.09 to 3.24 ± 0.61 pmol·cm⁻²·h⁻¹; P < 0.001, n = 6; Fig. 3B). The flux of HRP across tissue from sensitized mice (38.07 ± 4.88 pmol·cm⁻²·h⁻¹)
was increased significantly compared with values in naive control mice. This enhanced antigen uptake was attenuated dramatically by the treatment of sensitized animals with GLP-2 (10.04 ± 1.56 pmol·cm⁻²·h⁻¹; P < 0.001, n = 6). GLP-2 significantly reduced the flux to 27% of that observed in sensitized untreated mice, a value not different from that in naive control mice.

To examine if GLP-2 reduced permeability by acting via the CD23 pathway for enhanced antigen transport (25), we exposed intestinal segments to monoclonal CD23 antibody. Compared with sensitized tissues treated with isotype control antibody, tissues treated with anti-CD23 antibody showed a significantly reduced endosomal uptake of HRP in enterocytes (Fig. 3A). When tissues from GLP-2-treated sensitized mice were treated with anti-CD23, there was a similar decrease in HRP uptake. However, for the overall flux of HRP (Fig. 3B), GLP-2-treated mice showed a significant reduction in transepithelial HRP transport beyond that induced by the anti-CD23 antibody. Taken together, these findings suggest that GLP-2 did not influence CD23-mediated antigen uptake in endosomes but did inhibit transcytosis of antigenic macromolecules.

Late-Phase Reaction

GLP-2 prevented the prolonged permeability defect. Sensitized mice showed a persistent jejunal permeability defect 48 h after oral antigen challenge compared with sham-challenged mice. This was indicated by a significant threefold increase in ⁵¹Cr-EDTA flux (Fig. 5). In contrast, in mice treated with GLP-2, the flux of ⁵¹Cr-EDTA was decreased significantly, being restored to normal values.

GLP-2 reduced intestinal allergic inflammation. The numbers of eosinophils and mononuclear cells were
increased in tissues from sensitized mice compared with naive controls (Fig. 6, A and B). Tissues from mice that had been orally challenged 48 h before death showed a further increase in numbers of eosinophils and mononuclear cells. The number of eosinophils in tissues from GLP-2-treated mice was significantly lower compared with both sensitized controls and sensitized challenged mice, whereas the number of mononuclear cells was significantly less than those found in tissues from sensitized challenged mice.

**DISCUSSION**

Luminal antigens play a role in the pathogenesis of several gastrointestinal disorders, including food allergy, celiac disease, cow’s milk enteropathy, and possibly inflammatory bowel disease (reviewed in Ref. 4). A common feature of such conditions is the presence of reactive immune cells in the mucosa. Therefore, the epithelium plays a critical role as a barrier to restrict access of luminal material to the subepithelial tissue, thus controlling the immune/inflammatory response. Many endogenous and exogenous substances can disrupt the epithelial barrier to increase permeability, whereas relatively few agents are known to enhance barrier function. GLP-2 is one factor that appears to act specifically in the gut to increase mucosal growth by an action (direct or indirect) on the epithelium. In addition, we have recently identified that GLP-2 also has epithelial barrier-enhancing properties that occur as rapidly as 4 h after a single injection in mice, well before any morphological changes in the mucosa (2).

Here we examined the ability of GLP-2 to affect the pathophysiology of immediate and late-phase hypersensitivity reactions that have been identified as occurring within defined times after exposure of sensitized animals/tissues to luminal antigen. In addition, in sensitized rodents, transepithelial antigen transport has been shown to be receptor mediated, enhanced, and accelerated (3, 25). Our study showed that treatment of sensitized mice with GLP-2 increased barrier function to significantly reduce antigen uptake and, by this mechanism, limited the secretory response in the immediate hypersensitivity phase. Moreover, GLP-2 treatment inhibited the long-lasting permeability defect and allergic inflammation characteristic of the late-phase reaction.

Food allergy is one type of luminal antigen-driven disorder that has been extensively investigated experimentally for >30 yr, and the pathophysiology and sequence of events are well described (reviewed in Ref. 9). In experimental models of food allergy, intestinal anaphylaxis occurs in two stages after oral antigen challenge of sensitized rodents. The rapid reaction occurring within minutes is known as the immediate
hypersensitivity reaction. Its features include stimulated epithelial chloride ion secretion, which provides the driving force for water secretion leading to diarrhea, followed by a generalized increase in epithelial permeability (9). The late phase occurs hours to days later and involves both pathophysiology and an influx of inflammatory cells, which may contribute to the long-lasting response (24). Recently, a novel receptor was identified on epithelial cells of sensitized rodents that accelerates antigen delivery in the lamina propria (23). The studies showed that the initial uptake of luminal antigen in sensitized mice/rats is mediated by IgE antibody binding to its low-affinity receptor, CD23, on epithelial cells. CD23-mediated transepithelial antigen transport occurs via endosomes where the antigen is protected from degradation, resulting in an increased rate and quantity of intact protein crossing the basolateral membrane. The current studies were undertaken to determine if the barrier-enhancing ability of GLP-2 would be sufficient to limit receptor-mediated antigen uptake and alter the subsequent functional and morphological abnormalities. Separate experiments were designed to investigate the effect of GLP-2 on either the immediate or the late-phase allergic hypersensitivity reaction.

Immediate hypersensitivity can be studied effectively by mounting sensitized tissues in the Ussing chamber. Luminal antigen challenge evokes an increase in I_sc resulting from directed chloride secretion (10). In this study, we confirmed that the secretory response of sensitized tissues was specific for the sensitizing antigen. We showed that GLP-2 treatment of mice 4 h before the experiment attenuated the secretory response to luminal antigen by ~80%. This reduced response to antigen could either be a result of reduced epithelial permeation of antigen to limit access to mast cells in the lamina propria or an effect of GLP-2 on immune cell activation. Electron microscopy revealed that GLP-2 caused a decreased uptake of antigen in enterocyte endosomes and a reduction in transepithelial flux. In addition, GLP-2 treatment did not affect the ability of sensitized tissue to respond to serosally applied antigen, where passage across the epithelium was not a factor. Therefore, we propose that GLP-2 treatment before antigen exposure attenuated the immediate hypersensitivity reaction by enhancing epithelial barrier function.

To examine the possibility that GLP-2 was affecting the CD23 pathway for enhanced antigen transport, we blocked this pathway using an anti-CD23 antibody. This antibody has previously been shown to compete with IgE for its low-affinity receptor, and we previously demonstrated that it inhibits CD23-mediated transepithelial antigen transport (25). In this study, anti-CD23 treatment dramatically reduced (by ~85%) the uptake of HRP antigen in enterocyte endosomes in sensitized mice. There was no further reduction in tissues from GLP-2-treated mice. Anti-CD23 also decreased the flux of antigen across the tissues, but only by ~30%. This lesser degree of inhibition is likely because of the fact that the flux is measured over a 2-h period during which time mast cells become activated and mast cell mediators increase the permeability of the paracellular pathway. GLP-2 did significantly reduce the HRP antigen flux beyond that produced by anti-CD23 antibody. In addition, GLP-2 inhibited HRP flux across tissues from nonsensitized mice. Taken together, these results suggest that GLP-2 does not affect the CD23- and IgE-mediated flux beyond that produced by anti-CD23 antibody. Therefore, our results imply that the mechanism by which GLP-2 reduced macromolecular permeability is separate from the CD23 pathway.

The late-phase response has previously been characterized in the rodent intestine by a prolonged permeability defect after in vivo antigen challenge as well as recruitment of inflammatory cells to the mucosa (24). The time of the most severe intestinal abnormalities corresponded to the time of the greatest inflammatory infiltrate. Previously, we reported that, 4 h after GLP-2...
treatment of naive mice, the flux of $^{51}$Cr-EDTA was reduced (2), and, here, we have shown that GLP-2 treatment before oral antigen challenge prevented the permeability defect 48 h after antigen exposure. The finding that the flux of the paracellular probe, $^{51}$Cr-EDTA, was reduced to control values suggests that GLP-2 prevented the increased leakiness of the tight junctions, either directly or more likely by reducing the initial transcellular antigen uptake so no hypersensitivity (immediate or late) occurred. The paracellular pathway has been shown to allow even large macromolecules, including the sensitizing antigen, to permeate after the immediate hypersensitivity reaction. In addition, this study documented that the inflammatory infiltrate appeared 15 min after GLP-2 stimulation followed by maximal crypt cell c-fos induction at 90 min. In addition, GLP-2-mediated crypt cell proliferation was inhibited after local blockade of neuronal function. This suggests an indirect pathway of action for GLP-2 on epithelial cells, mediated by neuronally released mediators. It is conceivable that GLP-2-enhanced barrier function is mediated by neurons since neural regulation of macromolecular transport has previously been reported in intestinal epithelium (14).

In summary, we have shown that treatment of sensitized mice with a single injection of GLP-2 can reduce the pathophysiology of the immediate and late-phase hypersensitivity reactions by enhancing epithelial barrier function. This enhanced barrier function translates to a decreased uptake of luminal antigen and a consequent reduction of the secretory response to antigen challenge. The long-term effects of antigen exposure have shown that GLP-2 pretreatment can prevent the prolonged permeability defect and the influx of inflammatory cells in the mucosa. This study supports the role for GLP-2 as a therapeutic option for disease characterized by impaired barrier function and identifies the usefulness of GLP-2 in preventing the inappropriate/exaggerated inflammatory response in a model of food allergy.

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