(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 November 2009 (19.11.2009)

PCT

(10) International Publication Number
WO 2009/139887 A2

(51) International Patent Classification:
A61K 48/00 (2006.01)

(21) International Application Number:
PCT/US2009/002997

(22) International Filing Date:
13 May 2009 (13.05.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/053,949 16 May 2008 (16.05.2008) US

(71) Applicant *(for all designated States except US)*: **THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY** [US/US]; 1705 El Camino Real, Palo Alto, CA 94306-1106 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BETHUNE, Michael, Thomas** [US/US]; 119 Quillen Court, Apt. 603, Stanford, CA 94305 (US). **KHOSLA, Chaitan** [US/US]; 740 La Para Avenue, Palo Alto, CA 94306 (US).

(74) Agent: **SHERWOOD, Pamela, J.**; Bozicevic, Field & Francis LLP, 1900 University Avenue, Suite 200, East Palo Alto, CA 94303 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2009/139887 A2

(54) **Title**: NON-INFLAMMATORY GLUTEN PEPTIDE ANALOGUES AS BIOMARKERS FOR CELIAC SPRUE

(57) **Abstract**: The diagnosis of an enteropathic disease or the response of a patient with an enteropathic disease to therapy, in a clinical trial setting or in a long-term disease management setting, is assessed by detecting the ability of the patient to metabolize an orally administered gluten peptide analog. The peptide metabolism may be monitored in a variety of ways. Conveniently, the appearance of a metabolite of the peptide is detected in a patient sample over a period of time following oral administration, e.g. in urine, plasma, breath, saliva, etc. The gluten peptide analog is optionally labeled, e.g. with an isotopic, fluorescent, etc. label.

NON-INFLAMMATORY GLUTEN PEPTIDE ANALOGUES AS BIOMARKERS FOR CELIAC SPRUE

GOVERNMENT RIGHTS

[01] This invention was made with Government support under contract DK063158 awarded by the National Institutes of Health. The Government has certain rights in this invention.

[02] In 1953, it was first recognized that ingestion of gluten, a common dietary protein present in wheat, barley and rye causes disease in sensitive individuals. Gluten is a complex mixture of glutamine- and proline-rich glutenin and gliadin molecules, which is thought to be responsible for disease induction. Ingestion of such proteins by sensitive individuals produces flattening of the normally luxurious, rug-like, epithelial lining of the small intestine known to be responsible for efficient and extensive terminal digestion of peptides and other nutrients.

[03] Clinical symptoms of celiac sprue include fatigue, chronic diarrhea, malabsorption of nutrients, weight loss, abdominal distension, anemia, as well as a substantially enhanced risk for the development of osteoporosis and intestinal malignancies (lymphoma and carcinoma). The disease has an incidence of approximately 1 in 200 in most populations. Although no non-dietary therapy has been approved thus far for the treatment of celiac sprue, several efforts are under way to develop oral enzyme therapies (hereafter referred to as "glutenases") that accelerate the digestion, detoxification and assimilation of proteolytically resistant, immunotoxic gluten peptides in the celiac patient's gastrointestinal tract. Other types of drugs are also being considered for treatment of celiac sprue.

[04] A related disease is dermatitis herpetiformis, which is a chronic eruption characterized by clusters of intensely pruritic vesicles, papules, and urticaria-like lesions. IgA deposits occur in almost all normal-appearing and perilesional skin. Asymptomatic gluten-sensitive enteropathy is found in 75 to 90% of patients and in some of their relatives. Onset is usually gradual. Itching and burning are severe, and scratching often obscures the primary lesions with eczematization of nearby skin, leading to an erroneous diagnosis of eczema. Strict adherence to a gluten-free diet for prolonged periods may control the disease in some patients, obviating or reducing the requirement for drug therapy. Dapsone, sulfapyridine and colchicines are sometimes prescribed for relief of itching, although the underlying disease is unaffected by these drugs. Given the close relationship between celiac sprue and dermatitis herpetiformis pathogenesis, the above-mentioned therapies are also expected to be useful for the treatment of dermatitis herpetiformis.

[05] The environmental trigger of celiac sprue is dietary gluten from common food grains such as wheat, rye, and barley. Duodenal digestion of ingested gluten releases proteolytically resistant, immunotoxic peptide fragments, such as the immunodominant 33-mer from α-gliadin (Shan et al. (2002) Science 297: 2275-2279). These peptides traverse the mucosal epithelium by unknown mechanisms and are deamidated at specific glutamine residues by an endogenous enzyme, tissue transglutaminase (tTG) (Molberg et al. (1998) Nat Med 4: 713-717; Arentz-Hansen et al. (2000) J Exp Med 191: 603-612). Deamidated peptides bind with high affinity to the primary genetic determinant of celiac sprue, human leukocyte antigen (HLA) DQ2 (Quarsten et al. (1999) Eur J Immunol 29: 2506-2514; Kim et al. (2004) Proc Natl Acad Sci USA 101: 4175-4179), a class II major histocompatibility complex (MHC) molecule found in >90% of diagnosed celiac sprue cases (Spurkland et al. (1997) Tissue Antigens 49: 29-3), and the remaining cases are associated with HLA DQ8 (Karell et al. (2003) Hum Immunol 64: 469-477). Upon encountering DQ2-gluten complexes on the surface of antigen presenting cells (APC), gluten-specific, DQ2-restricted $CD4^+$ T cells are activated to induce a Th1 response comprising the secretion of pro-inflammatory cytokines, such as IFN-γ, and the recruitment of $CD8^+$ intraepithelial lymphocytes, ultimately causing mucosal damage (Jabri et al. (2005) Immunol Rev 206: 219-231). Additionally, $CD4^+$ T cells give help to a humoral immune response comprising production of both gluten-specific antibodies and TG2-specific autoantibodies (Sollid LM (2002) Nat Rev Immunol 2: 647-655).

[06] In many affected individuals, this molecular pathogenesis is manifested symptomatically as nutrient malabsorption, wasting, and/or chronic diarrhea, and chronic inflammation caused by recurrent exposure to gluten is associated with the increased incidence of T cell lymphoma of the small intestine (Green and Jabri (2006) Annu Rev Med 57: 207-221). Inflammation, antibody production, and clinical symptoms are gluten-dependent, such that strict adherence to a gluten-free diet causes remission, while reintroduction of dietary gluten causes relapse. However, a gluten-free diet is extremely difficult to maintain due to the ubiquity of gluten in human foods (Pietzak (2005) Gastroenterology 128: S135-141). Consequently, non-dietary therapies could substantially improve the health and quality of life of celiac sprue patients.

[07] Several recent studies have highlighted the potential of orally administered gluten-specific proteases (i.e., glutenases) for treating celiac sprue (reviewed in Stepniak and Koning (2006) Trends Biotechnol 24: 433-434; Cerf-Bensussan et al. (2007) Gut 56: 157-160). Since the proteolytic resistance of gluten peptides stems from their high proline (~15%) and glutamine (~35%) content, efforts are primarily focused on enzymes capable of cleaving proximal to these residues.

[08] Bacterial prolyl endopeptidases (PEP) detoxify immunodominant gluten *in vitro* (Stepniak et al. (2006) Am J Physiol Gastrointest Liver Physiol 291: G621-629), and are especially effective when complemented with a barley endoprotease, EP-B2, that preferentially cleaves gluten C-terminal to glutamine residues (Siegel et al. (2006) Chem Biol 13: 649-658; Gass et al. (2007) Gastroenterology 133: 472-480). Importantly, the cleavage sites for EP-B2 in the immunodominant peptide, 33-mer, are coincident with those glutamine residues that are selectively deamidated by tTG (Bethune et al (2006) Chem Biol 13: 637-647), suggesting EP-B2-catalyzed cleavage of gluten may interfere specifically with this critical step in disease progression. The therapeutic promise of EP-B2 is further underscored by its digestion of gluten *in vivo* (see Gass et al. (2006) J Pharmacol Exp Ther 318: 1178-1186) and by its ability to protect a gluten-sensitive rhesus macaque against gluten-induced clinical relapse when dosed orally (Bethune et al. (2008) PLoS ONE 3: e1857).

[09] To bring therapeutic glutenases to bear on the human condition of celiac sprue, however, safe and effective means of assessing enzyme efficacy in human celiac patients are needed. A similar need also exists for therapies that have alternative modes of action. For example, AT-1001 (Alba Therapeutics Corp.) is an investigational drug for celiac disease that is thought to reverse tight junction dysfunction in celiac patients, thereby preventing gluten transport across the epithelial layer.

[10] There is an urgent need for the development of sensitive, specific and non-invasive biomarkers for assessing drug efficacy in the treatment of patients with enteropathic diseases such as celiac sprue. The ideal biomarker would not only facilitate clinical trials of drug candidates, but would also find utility in disease management of patients who are prescribed such medications. Current diagnostic methods for celiac sprue, such as ELISA-based methods in which either anti-gliadin or anti-tTG antibodies in the patient's serum are detected or T cell methods in which cell proliferation or γ-IFN secretion is measured upon stimulation with gliadin, are unsuitable for this purpose. Antibody tests are unsuitable because patients must be exposed to relatively high doses of gluten over extended durations before they seroconvert. T cell proliferation assays are more sensitive, but they require invasive procedures (e.g. withdrawal of a small intestinal biopsy or relatively large quantities of blood to harvest adequate numbers of peripheral blood mononuclear cells) and are deemed to be too expensive for routine use. The present invention addresses this emerging but unmet medical need.

SUMMARY OF THE INVENTION

[11] Methods are provided for diagnosis and clinical monitoring of enteropathic disease, which diseases include, without limitation, celiac sprue, Crohn's disease and irritable bowel

syndrome, and inflammation associated with intestinal infection, e.g. infection with rotavirus, giardia, enteroaggregative *E. coli*, *Cryptosporidium*, and the like. In some embodiments the methods are utilized for monitoring ongoing therapeutic regimens for such enteropathic diseases. In other embodiments, the methods of the invention are used in determining the efficacy of a therapy for treatment of an enteropathic disease, either at an individual level, or in the analysis of a group of patients, *e.g.* in a clinical trial format. Such embodiments typically involve the comparison of two or more time points for a patient or group of patients. The patient status is expected to differ between the two time points as the result of administration of a therapeutic agent, therapeutic regimen, or challenge with a disease-inducing agent to a patient undergoing treatment.

[12] The efficacy of therapy in a patient with an enteropathic disease is assessed by detecting the ability of the patient to metabolize an orally administered gluten peptide analog, where the term "metabolize" refers to proteolytic cleavage of gluten peptides and peptide analogs; or refers to the ability of the gut to maintain normal permeability. Such gluten analog peptides are substantially similar to native gluten peptides in susceptibility to proteolytic cleavage; but are substantially decreased in pathogenic properties of the native gluten peptide, *i.e.* are not presented by the DQ MHC protein; and are not a substrate for deamidation by tissue transglutaminase. As a result, these peptides are not presented to gluten-specific T cells and are consequently non-inflammatory. Such analog peptides can be safely administered at a higher dose than native gluten proteins or peptides. Gluten analog peptide compositions are also a feature of the invention.

[13] The patient metabolism of the peptide may be monitored in a variety of ways. The peptide analog may be labeled, *e.g.* with an isotopic, fluorescent, *etc.* label, and the appearance of labeled amino acids that result from metabolism of the peptide is detected in a patient sample over a period of time following oral administration, *e.g.* in urine, plasma, breath, saliva, *etc.* Alternatively, the level of labeled or unlabeled peptide itself (or a partially proteolyzed metabolite) in a bodily fluid may be determined by various methods, including immunoassays. The determination of the presence of the peptide is used in determining peptide cleavage, or for assessing the extent of leakiness of the epithelial barrier of the small intestine in the context of celiac sprue, or other inflammatory bowel diseases where mucosal leakiness is elevated.

[14] In other embodiments, the methods of the invention are used to diagnose celiac sprue. The enhanced permeability of the epithelial barrier in patients with active disease is correlated to more established measures of diagnosis such as tTG auto-antibodies and upper intestinal villus atrophy.

[15] In one embodiment, the sequence of a 33-residue immunotoxic gluten peptide derived from α2-gliadin (see Genbank accession number CAB76964) is modified so that

three key glutamine (Q) residues that are selectively deamidated by TG2 are substituted with non-substrate amino acids, *e.g.* by asparagine (N) or histidine (H). Labeled or unlabeled biomarker peptides can safely be administered orally to celiac sprue patients in conjunction with a drug or placebo.

[16] Various formats may be used in the pharmacokinetic analysis. In some embodiments, a patient sample is obtained prior to treatment, as a control, and compared to samples from the same patient following treatment. In other embodiments, the CYP3A function is assessed over long periods of time to monitor patient status.

BRIEF DESCRIPTION OF THE DRAWINGS

[17] Figure 1. Synthetic gluten and biomarker peptide sequences. Sequences are shown for the native 33-mer (designated QQQ-33-mer) derived from ⟨2-gliadin, the synthetically deamidated 33-mer (EEE-33-mer), the biomarkers NNN-33-mer and HHH-33-mer, and a non-inflammatory control peptide of unrelated sequence derived from myoglobin. Bonds that are scissile to EP-B2-mediated cleavage are designated by arrowheads. Glutamine residues that are selectively deamidated by TG2 or synthetically replaced in the biomarker peptides are in bold. Leucine residues that are isotope-labeled for *in vivo* experiments are underlined.

[18] Figure 2. The 33-mer gluten peptide and gluten peptide-based biomarkers are similarly resistant to cleavage by pepsin and susceptible to cleavage by the glutenase EP-B2. **(a-f)** Reverse-phase HPLC traces for **(a,d)** QQQ-33-mer, **(b,e)** NNN-33-mer, and **(c,f)** HHH-33-mer (300 μM) after simulated gastric digestion for specified durations with either **(a-c)** pepsin alone or **(d-f)** pepsin and 120 μg/ml EP-B2. The TAME internal standard (T), intact peptide (peak 5), minimally processed peptide lacking only the N-terminal LQ (peak 4), and other major digestion products are indicated for each peptide trace overlay. **(g-j)** Integrated area-under-the-curve analysis for intact peptides **(g)** QQQ-33-mer, **(h)** NNN-33-mer, **(i)** HHH-33-mer, and **(j)** myoglobin peptide showing dose- and time-dependency of EP-B2-mediated digestion. Each peptide (300 μM) was digested *in vitro* with pepsin supplemented with specified concentrations of EP-B2, and reaction products were analyzed by HPLC. The area-under-the-curve for each intact peptide peak (and, where applicable, the minimally-processed –LQ peptide peak) was calculated and normalized to that for the internal standard. Values are expressed as the % of intact peptide remaining after a given digestion duration relative to the initial peak area. **(k)** LC-MS identification of major digestion products from simulated gastric digests with pepsin ± EP-B2. HPLC peak number corresponds to the peak numbers in panels (a-f). Data shown are representative of 3 independent experiments.

[19] Figure 3. The 33-mer gluten peptide and gluten peptide-based biomarkers are similarly resistant to cleavage by pancreatic enzymes and susceptible to cleavage by prolyl endopeptidase. **(a)** Integrated area-under-the-curve analysis for intact QQQ-33-mer, NNN-33-mer, and HHH-33-mer after simulated intestinal digestion ± supplementation with prolyl endopeptidase from *Flavobacterium meningosepticum* (FM PEP). Following treatment with pepsin, each peptide (300 μM) was digested *in vitro* with pancreatic proteases (trypsin, chymotrypsin, elastase, carboxypeptidase A (TCEC)) and rat intestinal brush border membrane (BBM) ± 1.2 U/ml FM PEP. Reaction products were analyzed by reverse-phase HPLC. The area-under-the-curve for each intact peptide peak was calculated and normalized to that for the internal standard. Values are expressed as the % of intact peptide remaining after a given duration relative to the initial peak area. **(b)** Cleavage map derived from LC-MS/MS analysis of major digestion products following simulated gastrointestinal digests. Blue arrowheads designate major cleavage sites resulting from EP-B2 supplementation. Red arrowheads designate major cleavage sites resulting from FM PEP supplementation. Underlined, numbered sequences designate major products of digestion with EP-B2. **(c-e)** HPLC traces for **(c)** QQQ-33-mer, **(d)** NNN-33-mer, and **(e)** HHH-33-mer (300 μM) after simulated gastric digestion with pepsin + 120 μg/ml EP-B2 for 60 min followed by treatment with TCEC + BBM ± 1.2 U/ml FM PEP for 10 or 60 min. T, TAME internal standard. HPLC peak numbers corresponds to the numbered sequences in panel (b). Data shown are representative of 2 independent experiments.

[20] Figure 4. Biomarkers are non-inflammatory in the context of celiac sprue. **(a)** Specific activity of transglutaminase 2 (TG2; 5 μM) in the presence of 100 μM gluten peptide QQQ-33-mer, biomarkers NNN-33-mer or HHH-33-mer, control myoglobin peptide, or no peptide. Means ± s.d. for triplicate assays are shown. Data are representative of 3 independent experiments. Statistical comparisons were performed with respect to samples containing QQQ-33-mer. *** $p < 0.001$. **(b)** Ratio of DQ2-bound to unbound fluorescein-conjugated (f-) peptides following incubation of thrombin-cleaved DQ2-(I-gliadin peptide complexes (9.4 μM) with 0.185 μM f-QQQ-33-mer, f-EEE-33-mer, f-NNN-33-mer, -f-HHH-33-mer, or f-myoglobin peptide in a citrate-PBS buffer, pH 5.5 for 45 h at 37 °C. Means ± s.d. for triplicate assays are shown. Data are representative of 3 independent experiments. Statistical comparisons were performed with respect to samples containing f-EEE-33-mer. ** $p < 0.01$.

[21] Figure 5. Biomarker transepithelial transport parallels gluten peptide transport *in vitro* and *in vivo*. **(a)** Experimental design for *in vitro* studies. Transwell supports bearing mature T84 epithelial cell monolayers were preincubated with media alone or with 600 U/ml IFN-γ in the basolateral chamber. After 48 h, 20 μM Cy5-labeled QQQ-33-mer, NNN-33-mer, or

HHH-33-mer was added to the apical chamber, and samples from the apical and basolateral chambers were sampled over time to determine the stability and apical-to-basolateral flux of each intact peptide. **(b)** Apical-to-basolateral flux of each Cy5-labeled peptide under basal (0 U/ml IFN-γ) and simulated inflammatory (600 U/ml IFN-γ) conditions. Means ± s.d. for triplicate assays are shown. Data are representative of 2 similar experiments. Statistical comparisons were performed with respect to QQQ-33-mer; no significant differences were observed. **(c-e)** Reverse-phase HPLC traces from LC-MS analysis of samples taken from the apical and basolateral chambers at 0 and 10 h. Elution of **(c)** Cy5-QQQ-33-mer, **(d)** Cy5-NNN-33-mer, and **(e)** Cy5-HHH-33-mer were monitored by their absorbance at 640 nm. Intact Cy5-peptides elute as the major peak between 9-10 min. The peak eluting in each trace at 8 min. is Cy5-LQ, signifying limited processing of the N-terminus by T84 cells. **(f)** Area-under-the-curve analysis quantifying the data from panels (c-e).

DETAILED DESCRIPTION

[22] Enteropathic inflammatory disease is clinically monitored by measuring the metabolism and digestion kinetics of gluten peptide analogs. In preferred embodiments such substances are orally administered as a solution, capsule, enteric formulation, *etc.*

[23] As used herein, the term "therapeutic drug" or "therapeutic regimen" refers to an agent used in the treatment or prevention of a disease or condition, particularly an enteropathic condition for the purposes of the present invention. Of interest are clinical trials using such therapies, and monitoring of patients undergoing such therapy.

[24] In some embodiments, the therapy involves treatment of celiac sprue patients with glutenase. In other embodiments, the therapy involves treatment of celiac sprue patients with a permeabilibity modifying agent. Assessment of treatment may utilize a gluten challenge. In some embodiments, 1-14 days of a moderate dose (at least about 1 g/day, at least about 5 g/day, at least about 10 g/day, or more) of oral gluten is utilized for this for this purpose. Patients may be control patients that have not been treated, or patients subject to a clinical regimen of interest, e.g. dietary restriction of gluten, treatment with permeability modifier, treatment with glutenase, and the like.

[25] A "patient," as used herein, describes an organism, including mammals, from which samples are collected in accordance with the present invention. Mammalian species that benefit from the disclosed systems and methods for therapeutic drug monitoring include, and are not limited to, apes, chimpanzees, orangutans, humans, monkeys; and

domesticated animals (e.g., pets) such as dogs, cats, mice, rats, guinea pigs, and hamsters.

[26] The term "pharmacokinetics," refers to the mathematical characterization of interactions between normal physiological processes and a therapeutic drug over time (i.e., body effect on drug). Certain physiological processes (absorption, distribution, metabolism, and elimination) will affect the ability of a drug to provide a desired therapeutic effect in a patient. Knowledge of a drug's pharmacokinetics aids in interpreting drug blood stream concentration and is useful in determining pharmacologically effective drug dosages

[27] The term "patient sample" or "sample" as used herein refers to a sample from an animal, most preferably a human, seeking diagnosis or treatment of a disease, *e.g.* an enteropathic disease. Samples of the present invention include, without limitation, urine, saliva, breath, and blood, including derivatives of blood, *e.g.* plasma, serum, *etc.*

[28] *Sample analysis.* Patient samples are analyzed to determine the metabolism of a gluten peptide analog, usually an orally administered gluten peptide analog. Sample may be quantitatively analyzed for the presence of the substrate and/or its metabolites by any suitable assay, which are well-known in the art. Methods of analysis include liquid chromatography-mass spectroscopy (see Kanazawa *et al.* (2004) J. Chromatography 1031:213-218, Gorski *et al., supra.*); HPLC; ion-monitoring gas chromatography/mass spectroscopy (see Paine *et al., supra.*); gas chromatography; semiconductive gas sensors; immunoassays; mass spectrometers (including proton transfer reaction mass spectrometry), infrared (IR) or ultraviolet (UV) or visible or fluorescence spectrophotometers (i.e., non-dispersive infrared spectrometer); binding assays involving aptamers or engineered proteins *etc.*

[29] In some embodiments, the biological sample is patient urine. The concentration of the peptide and its metabolites, *i.e.* amino acids, dipeptides, *etc.* can be monitored in a 6-hour urine collection.

[30] Conditions of interest for monitoring methods of the present invention include a variety of enteropathic conditions, particularly inflammatory chronic conditions. In some embodiments of the invention, a patient is diagnosed as having an enteropathic condition, for which treatment is contemplated. The patient may be initially tested for activity prior to treatment, in order to establish a baseline level of activity. Alternatively, the patient may be released from a treatment regimen for a period of time sufficient to induce an enteropathic state, in which state the patient is tested for activity in order to establish a baseline level of activity. Enteropathic conditions of interest include, without limitation, celiac sprue,

herpetiformis dermatitis, irritable bowel syndrome (IBS); Crohn's Disease; and inflammation associated with intestinal infection.

[31] *Celiac sprue* is an immunologically mediated disease in genetically susceptible individuals caused by intolerance to gluten, resulting in mucosal inflammation, which causes malabsorption. Symptoms usually include diarrhea and abdominal discomfort. Onset is generally in childhood but may occur later. No typical presentation exists. Some patients are asymptomatic or only have signs of nutritional deficiency. Others have significant GI symptoms.

[32] Celiac sprue can present in infancy and childhood after introduction of cereals into the diet. The child has failure to thrive, apathy, anorexia, pallor, generalized hypotonia, abdominal distention, and muscle wasting. Stools are soft, bulky, clay-colored, and offensive. Older children may present with anemia or failure to grow normally. In adults, lassitude, weakness, and anorexia are most common. Mild and intermittent diarrhea is sometimes the presenting symptom. Steatorrhea ranges from mild to severe (7 to 50 g fat/day). Some patients have weight loss, rarely enough to become underweight. Anemia, glossitis, angular stomatitis, and aphthous ulcers are usually seen in these patients. Manifestations of vitamin D and Ca deficiencies (eg, osteomalacia, osteopenia, osteoporosis) are common. Both men and women may have reduced fertility.

[33] The diagnosis is suspected clinically and by laboratory abnormalities suggestive of malabsorption. Family incidence is a valuable clue. Celiac sprue should be strongly considered in a patient with iron deficiency without obvious GI bleeding. Confirmation usually involves a small-bowel biopsy from the second portion of the duodenum. Findings include lack or shortening of villi (villous atrophy), increased intraepithelial cells, and crypt hyperplasia. Because biopsy results may be non-specific, serologic markers can aid diagnosis. Anti-gliadin antibody (AGA) and anti-endomysial antibody (EMA, an antibody against an intestinal connective tissue protein) in combination have a positive and negative predictive value of nearly 100%. These markers can also be used to screen populations with high prevalence of celiac sprue, including 1st- degree relatives of affected patients and patients with diseases that occur at a greater frequency in association with celiac sprue. If either test is positive, the patient may have a diagnostic small-bowel biopsy performed. If both are negative, celiac sprue is unlikely. Other laboratory abnormalities often occur and may be sought. These include anemia (iron-deficiency anemia in children and folate-deficiency anemia in adults); low albumin, Ca, K, and Na; and elevated alkaline phosphatase and PT. Malabsorption tests are sometimes performed, although they are not specific for celiac sprue. If performed, common findings include steatorrhea of 10 to 40 g/day and abnormal D- xylose and (in severe ileal disease) Schilling tests.

[34] Conventional treatment is a gluten-free diet (avoiding foods containing wheat, rye, or barley). Gluten is so widely used that a patient needs a detailed list of foods to avoid. Patients are encouraged to consult a dietitian and join a celiac support group. The response to a gluten-free diet is usually rapid, and symptoms resolve in 1 to 2 months. Ingesting even small amounts of food containing gluten may prevent remission or induce disease.

[35] Complications include refractory sprue, collagenous sprue, and the development of intestinal lymphomas. Intestinal lymphomas affect 6 to 8% of patients with celiac sprue, usually presenting in the patient's 50s. The incidence of other GI malignancies (eg, carcinoma of the esophagus or oropharynx, small-bowel adenocarcinoma) increases. Adherence to a gluten-free diet can significantly reduce the risk of malignancy.

[36] *Dermatitis herpetiformis* is a chronic eruption characterized by clusters of intensely pruritic vesicles, papules, and urticaria-like lesions. The cause is autoimmune. Diagnosis is by skin biopsy with direct immunofluorescence testing. Treatment is usually with dapsone or sulfapyridine.

[37] This disease usually presents in patients 30 to 40 yr old and is rare in blacks and East Asians. It is an autoimmune disease. Celiac sprue is present in 75 to 90% of dermatitis herpetiformis patients and in some of their relatives, but it is asymptomatic in most cases. The incidence of thyroid disease is also increased. Iodides may exacerbate the disease, even when symptoms are well controlled. The term “herpetiformis” refers to the clustered appearance of the lesions rather than a relationship to herpesvirus.

[38] Patients may have skin biopsy of a lesion and adjacent normal-appearing skin. IgA deposition in the dermal papillary tips is usually present and important for diagnosis. Patients should be evaluated for celiac sprue.

[39] Strict adherence to a gluten-free diet for prolonged periods (eg, 6 to 12 mo) controls the disease in some patients, obviating or reducing the need for drug therapy. When drugs are needed, dapsone may provide symptomatic improvement. It is started at 50 mg po once/day, increased to bid or tid (or a once/day dose of 100 mg); this usually dramatically relieves symptoms, including itching, within 1 to 3 days; if so, that dose is continued. If no improvement occurs, the dose can be increased every week, up to 100 mg qid. Most patients can be maintained on 50 to 150 mg/day, and some require as little as 25 mg/wk. Although less effective, sulfapyridine may be used as an alternative for those who cannot tolerate dapsone. Initial oral dosage is 500 mg bid, increasing by 1 g/day q 1 to 2 wk until disease is controlled. Maintenance dosage varies from 500 mg twice/wk to 1000 mg once/day. Colchicine is another treatment option. Treatment continues until lesions resolve.

[40] *Gluten peptide analog.* Gluten peptide analogs of the present invention have properties of native gluten peptides, non-limiting examples of which are provided in Table 1, but are modified to decrease the pathogenic properties of stable binding to MHC DQ proteins, and of acting as a substrate for tissue transglutaminase.

[41] Native gluten peptides suitable for modification are at least about 14 amino acids in length, and not more than about 40 amino acids in length, and may be obtained by partial proteolytic digestion of a grain gluten or gliaden polypeptide, *e.g.* wheat, barley, oats, *etc.* as is known in the art, for example as set forth in Shan *et al.* (2005) J. Proteome Res. 4:1732-1741 (herein specifically incorporated by reference). Such oligopeptides have the properties of being (i) resistant to digestion with mammalian pancreatic and gastric proteases, *e.g.* as described in detail in U.S. Patent no. 7,303,871, and in Shan *et al.* (2002), *supra.* (each herein specifically incorporated by reference); (ii) are substrates for deamidation by tissue transglutaminase, *e.g.* as described in Bethune *et al.* (2006) supra. (herein specifically incorporated by reference); and (iii) are presented by human MHC DQ, e.g. as described by Quarsten *et al.* (1999), *supra.*, and Kim *et al.* (2004), *supra.*, (each herein specifically incorporated by reference).

Table 1

Native Gluten Peptides

Sequence identifier	Sequence
SEQ ID NO:1	QPFPQPQLPYPQPQLPYPQPQLPYPQPQP
SEQ ID NO:2	PFPQPQLPYPQPQLPYPQPQLPYPQPQP
SEQ ID NO:3	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF
SEQ ID NO:4	QPFPQPQLPYPQPQPFRPQ
SEQ ID NO:5	PFPQPQLPYLQPQPFRPQQPYPQPQP
SEQ ID NO:6	QPFPQPQLPYPQPQPFRPQQ
SEQ ID NO:7	PLFQLVQGQGIIQPQQPAQLEVIRSLVLG
SEQ ID NO:8	QVPQPQQPQQPFLQPQQPFPQQPQQPFPQTQQPQQPFPQQP
SEQ ID NO:9	FLQPQQPFPQQPQQPFPQTQQPQQPFPQQP
SEQ ID NO:10	PQPQQPQQPFLQPQQPFPQQPQQP
SEQ ID NO:11	PQQPQQPFLQPQQPFPQQPQQP
SEQ ID NO:12	PFLQPQQPFPQQPQQPFP
SEQ ID NO:13	LQPQQPFPQQPQQPFPQ
SEQ ID NO:14	QQSEQIIPQQLQQPFPLQPQQPFPQQPQQPFP
SEQ ID NO:15	QPFPLQPQQPFPQQPQQPFPQPQQPIPVQ
SEQ ID NO:16	QPFPLQPQQPFPQQPQQPFPQPQQPIP
SEQ ID NO:17	PQQPQQPFPQTQQPQQPFPQQPQQPFPQTQQPQQPFPQQP
SEQ ID NO:18	TQQPQQPFPQQPQQPFPQTQQPQQPFPQQPQQPFPQ
SEQ ID NO:19	TQQPQQPFPQQPQQPFPQTQ
SEQ ID NO:20	FPQTQQPQQPFPQQPQQPFP
SEQ ID NO:21	TQQPQQPFPQQPQQPFPQ
SEQ ID NO:22	TQQPQQPFPQQPQQPFP
SEQ ID NO:23	PQQLFPELQQPIPQQPQQPFPLQPQQPFPQQPQQPFPQQP

SEQ ID NO:24	FPELQQPIPQQPQQPFPLQPQQPFPQQPQQP
SEQ ID NO:25	PQQPFPQQPQQPVPQQSQQPFPQTQQPQQ
SEQ ID NO:26	QPQQPTPIQPQQPFPQQPQQPQQPFP
SEQ ID NO:27	QPFPQQSQQPFPQQPQQS
SEQ ID NO:28	QQSQQPFPQQPQQS
SEQ ID NO:29	PQQPQQPFPQQPQQP
SEQ ID NO:30	QPQQPFPQQPQ
SEQ ID NO:31	PRQPYPQQPQQP
SEQ ID NO:32	SQQQQPPFSQQQPPFSQQQQPV
SEQ ID NO:33	SQQQPPFSQQQQPV
SEQ ID NO:34	SQQQLPPFSQQQPPFSQQQQPV
SEQ ID NO:35	PPFSQQQQPVLPQQPPFSQQQQQQQQQPPFSQQQQPV
SEQ ID NO:36	VLPQQPPFSQQQQPVLPPQQSPFPQ
SEQ ID NO:37	FSQQQLPPFSQQLPPFSQQQQQVLPQQPPFSQQQQPV
SEQ ID NO:38	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF
SEQ ID NO:78	FLQPQQPFPQQPQQPYPQQPQQPFPQ

[42] The amino acid sequence of a native gluten peptide is modified from the native peptide, *e.g.* a peptide selected from SEQ ID NO:1-SEQ ID NO:38 to reduce the pathogenicity without substantially changing the metabolic profile of the peptide. Analog peptides of the invention are typically altered from a native peptide in the replacement of at least one, at least two, at least three, and not more than about five amino acid residues.

[43] The analog peptides are similarly resistant to gastrointestinal proteases, as compared to native gluten peptides, *e.g.* SEQ ID NO:38. Under test conditions, *e.g.* as set forth in the Examples, as set forth in U.S. Patent no. 7,303,871, etc., an analog peptide of interest has at least about 75% of the resistance of the native gluten peptide, at least about 85% of the resistance, at least about 95% of the resistance.

[44] Analog peptides of interest have significantly decreased activity as a tissue transglutaminase (tTG) substrate. Under test conditions, *e.g.* as set forth in the Examples, as set forth in Bethune *et al.* (2006) *supra., etc.*, an analog peptides of interest has less than about 10% of tTG substrate activity; less than about 5% of tTG substrate activity; less than about 1% of tTG substrate activity.

[45] Analog peptides of interest have low affinity for DQ2, thereby minimizing presentation to T cells, which also correlates with having poor immunostimulatory capacity toward gluten-specific T cells derived from celiac patient intestinal biopsies. This minimizes the risk of inducing inflammation upon oral administration of the biomarker. Under test conditions, *e.g.* as set forth in the Examples, as set forth in Quarsten *et al.* (1999), *supra.*, and Kim *et al.* (2004), *supra. etc.*, an analog peptides of interest has less than about 10% of T cell stimulatory or DQ binding affinity activity; less than about 5% of T cell stimulatory or DQ binding affinity activity; less than about 1% of T cell stimulatory or DQ binding affinity activity.

[46] In some embodiments of the invention, the criteria set forth above are accomplished by substituting one, two or three glutamine residues of a native gluten peptide, *e.g.* a peptide set forth in SEQ ID NO:1-SEQ ID NO:38, with amino acids other than glutamine and other than a negatively charged amino acid. In some embodiments, the substituting amino acid is a histidine, an asparagine, or a combination thereof.

[47] Glutamine residues susceptible to deamidation by tTGase can be experimentally determined, as described above and as known in the art. In some embodiments, the deamidated glutamine is the underlined residue in the motif PQPQLPY. In other embodiments, the deamidated glutamine is present in the motif QXP, where Q is a glutamine targeted by tTG and X is an amino acid intervening between Q and P. In such motifs, the deamidated glutamine is replaced by X, where X is an amino acid other than glutamine.

[48] Examples of analog peptides, without limitation, are provided in Table 2, where one or more X residues in a given peptide are an amino acid other than glutamine, preferably a neutral or positively charged amino acid, and which may be histidine or asparagine. One of skill in the art will understand that all X residues need not be substituted, and some may remain as a glutamine, provided that such glutamine does not result in a pathogenic peptide as described above.

Table 2

Analog Gluten Peptides

Sequence identifier	Sequence
SEQ ID NO:39	QPFPQPXLPYPQPXLPYPQPXLPYPQPQP
SEQ ID NO:40	PFPQPXLPYPQPXLPYPQPXLPYPQPQP
SEQ ID NO:41	LQLQPFPQPXLPYPQPXLPYPQPXLPYPQPQPF
SEQ ID NO:42	QPFPQPXLPYPQPQPFRPQ
SEQ ID NO:43	PFPQPXLPYLQPQPFRPXQPYPQPQP
SEQ ID NO:44	QPFPQPXLPYPQPQPFRPQQ
SEQ ID NO:45	PLFXLVQGQGIIQPXQPAXLEVIRSLVLG
SEQ ID NO:46	QVPXPXQPXQPFLQPXQPFPXQPXQPFPQTXQPQQPFPXQP
SEQ ID NO:47	FLQPQXPFPQXPQXPFPXTQQPQXPFPQQP
SEQ ID NO:48	PQPQQPQXPFLXPQQPFPQQPQQP
SEQ ID NO:49	PQQPXQPFLQPXQPFPXQPQQP
SEQ ID NO:50	PFLXPXQPFPXQPXQPFP
SEQ ID NO:51	LQPXQPFPXQPXQPFPQ
SEQ ID NO:52	QQSEQIIPQQLXQPFPLQPXQPFPXQPXQPFP
SEQ ID NO:53	QPFPLQPXQPFPXQPXQPFPQPXQPIPVQ
SEQ ID NO:54	QPFPLXPXQPFPXQPXQPFPQPQQPIP
SEQ ID NO:55	PXQPXQPFPXTXQPXQPFPXQPXQPFPXTXQPXQPFPXQP
SEQ ID NO:56	TXQPXQPFPXQPXQPFPXTXQPXQPFPXQPXQPFPQ
SEQ ID NO:57	TXQPXQPFPXQPXQPFPQTQ
SEQ ID NO:58	FPXTXQPXQPFPXQPXQPFP

SEQ ID NO:59	TXQPXQPFPXQPXQPFPQ
SEQ ID NO:60	TXQPXQPFPXQPXQPFP
SEQ ID NO:61	PQQLFPELXQPIPXQPXQPFPLQPXQPFPXQPXQPFPXQP
SEQ ID NO:62	FPELXQPIPXQPXQPFPLQPXQPFPXQPXQP
SEQ ID NO:63	PXQPFPXQPXQPVPQQSXQPFPQTXQPQQ
SEQ ID NO:64	QPXQPTPIQPXQPFPXQPXQPXQPFP
SEQ ID NO:65	QPFPQQSXQPFPXQPQQS
SEQ ID NO:66	QQSXQPFPXQPQQS
SEQ ID NO:67	PXQPXQPFPXQPXQP
SEQ ID NO:68	QPXQPFPXQPQ
SEQ ID NO:69	PRQPYPXQPXQP
SEQ ID NO:70	SQQXQPPFSQXQPPFSQQXQPV
SEQ ID NO:71	SQXQPPFSQQXQPV
SEQ ID NO:72	SQQQLPPFSQXQPPFSQQXQPV
SEQ ID NO:73	PPFSQQXQPVLPXQPPFSQQQQQQQXQPPFSQQXQPV
SEQ ID NO:74	VLPXQPPFSQQXQPVLPPQQSPFPQ
SEQ ID NO:75	FSQQQLPPFSQQLPPFSQQQQQVLPXQPPFSQQXQPV
SEQ ID NO:76	LQLQPFPQPXLPYPQPXLPYPQPXLPYPQPQPF
SEQ ID NO:79	FLQPXQPFPXQPXQPYPXQPXQPFPQ

[49] In some embodiments, the analog peptide consists or comprises of the sequence set forth in SEQ ID NO:76, where each X is histidine, asparagine, or a combination thereof, i.e. one histidine substitution and two asparagine substitutions; three histidine substitutions; three asparagine substitutions; and the like. In one embodiment of the invention, the peptide is LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77)

[50] Analog peptides optionally comprise one, two or more labeled amino acids, where the label provides for specific detection of the peptide and its metabolites. Preferred labels are non-radioactive, biogenic moieties, *e.g.* ^{13}C, ^{2}H, *etc.* Other labels of interest include radiography moieties (e.g. heavy metals and radiation emitting moieties), positron emitting moieties, magnetic resonance contrast moieties, and optically visible moieties, e.g., fluorescent or visible-spectrum dyes, quantum dots, visible particles, FRET pairs; *etc.* Among the most commonly used positron-emitting nuclides in PET are included ^{11}C, ^{13}N, ^{15}O, ^{18}F, and ^{124}I. Isotopes that decay by electron capture and/or γ emission are used in SPECT, and include ^{125}I, ^{128}I, ^{123}I and ^{99m}Tc. Optically visible moieties include fluorescent dyes, or visible-spectrum dyes, visible particles, and other visible labeling moieties. Fluorescent dyes such as ALEXA dyes, fluorescein, coumarin, rhodamine, bodipy Texas red, and cyanine dyes, are useful when sufficient excitation energy can be provided to the site to be inspected visually. Acceptable dyes include FDA-approved food dyes and colors, which are non-toxic, although pharmaceutically acceptable dyes which have been approved for internal administration are preferred.

Methods of the Invention

[51] The ability of an individual to metabolize a gluten peptide analog via an intestinal route is analyzed by administering an oral dose of a gluten peptide analog to an individual suffering from an enteropathic disorder, and quantitating the presence of the gluten peptide analog and/or its metabolite(s) in at least one patient sample.

[52] In some embodiments, the method comprises identifying a patient as having an enteropathic disorder, *e.g.* by criteria described above for specific disease conditions; administering an oral dose of a gluten peptide analog to an individual identified as having an enteropathic disorder, and quantitating the presence of the gluten peptide analog and/or its metabolite(s) in at least one patient sample.

[53] Patient samples include a variety of bodily fluids in which the gluten peptide analog and/or metabolites will be present, *e.g.* blood and derivatives thereof, urine, saliva, breath, *etc.* The samples will be taken prior to administration of the peptide, and at suitable time points following administration, e.g. at 15 minutes, 30 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 4 hours, 6 hours, etc., following administration.

[54] In some preferred embodiments, the methods of the invention are used in determining the efficacy of a therapy for treatment of an enteropathic disease, either at an individual level, or in the analysis of a group of patients, e.g. in a clinical trial format. Such embodiments typically involve the comparison of two time points for a patient or group of patients. The patient status is expected to differ between the two time points as the result of a therapeutic agent, therapeutic regimen, or disease challenge to a patient undergoing treatment.

[55] Examples of formats for such embodiments may include, without limitation, testing peptide metabolism at two or more time points, where a first time point is a diagnosed but untreated patient; and a second or additional time point(s) is a patient treated with a candidate therapeutic agent or regimen. An additional time point may include a patient treated with a candidate therapeutic agent or regimen, and challenged for the disease, particularly for celiac sprue and/or dermatitis herpetiformis, which may be challenged with administration of gluten.

[56] In another format, a first time point is a diagnosed patient in disease remission, *e.g.* as ascertained by current clinical criteria, as a result of a candidate therapeutic agent or regimen. A second or additional time point(s) is a patient treated with a candidate therapeutic agent or regimen, and challenged with a disease-inducing agent, particularly for celiac sprue and/or dermatitis herpetiformis, which may be challenged with administration of gluten.

[57] In such clinical trial formats, each set of time points may correspond to a single patient, to a patient group, e.g. a cohort group, or to a mixture of individual and group data.

Additional control data may also be included in such clinical trial formats, e.g. a placebo group, a disease-free group, and the like, as are known in the art. Formats of interest include crossover studies, randomized, double-blind, placebo-controlled, parallel group trial is also capable of testing drug efficacy, and the like. See, for example, Clinical Trials: A Methodologic Perspective Second Edition, S. Piantadosi, Wiley-Interscience; 2005, ISBN-13: 978-0471727811; and Design and Analysis of Clinical Trials: Concepts and Methodologies, S. Chow and J. Liu, Wiley-Interscience; 2003; ISBN-13: 978-0471249856, each herein specifically incorporated by reference.

[58] Specific clinical trials of interest include analysis of therapeutic agents for the treatment of celiac sprue and/or dermatitis herpetiformis, where a patient is identified as having celiac sprue by conventional clinical indicia. For example, in celiac sprue a daily dose of 5-10 g gluten (equivalent to 2-3 slices of bread) for two weeks can induce malabsorption, as measured by a 72-hour quantitative fecal fat collection or a D-xylose urinary test (Pyle, 2005), providing for a means to challenge the efficacy of a treatment.

[59] In one embodiment, a blinded crossover clinical trial format is utilized. A patient alternates for a set period of time, *e.g.* one week, two weeks, three weeks, or from around about 7-14 days, or around about 10 days, between a test drug and placebo, with a 4-8 week washout period. The patient is challenged with gluten during both alternating time periods with around about 1 g gluten, about 5 g. gluten, about 10 g. gluten, or more, usually not more than about 25 g gluten daily. Subjects are tested with a gluten peptide analog, as described above, at the beginning and end of each alternating time period. The duration of gluten challenge may be about 1, about 3, about 5, about 7, about 10 days, about 14 days, and the like. By decreasing the duration of the gluten challenge or the magnitude of the daily gluten dose, adverse symptoms can be minimized.

[60] In another embodiment a randomized, double-blind, placebo-controlled, parallel group trial is used to test drug efficacy. In one embodiment, individuals identified as having celiac sprue, who are on a gluten-free diet, undergo three sequential treatment periods, each of 1-14 day durations. Subjects will be assessed with the gluten peptide analog at entry and at the end of each treatment period. During the entire study, subjects will consume regular gluten-free meals plus drug or placebo as indicated. During the first treatment period (run-in), all subjects will receive placebo. During the second treatment period, the subjects will be randomized into drug or placebo groups. During the third treatment period, subjects will remain on the same (drug or placebo) treatment as in the second period. In addition, all subjects will receive 1-5 g gluten with each meal. Drugs that are effective will show a statistically lower frequency of relapse in the treatment arm versus placebo arm of the study.

[61] In all such methods, the gluten peptide analog is administered at a dose that is sufficient to monitor the metabolism over time, which will vary with the specific peptide that is selected. The dose may be at least about 1 mg, at least about 5 mg at least about 10 mg, at least about 25 mg, at least about 100 mg, at least about 500 mg, and not more than about 1 g.

[62] The peptide may be administered in any conventional formulation, e.g. solution, suspension, tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents. An alternative formulation is an aqueous solution containing 5 g lactulose, 2 g mannitol and 22.3 g glucose as an osmotic filler. The lactulose-mannitol cocktail in this solution facilitates an independent assessment of intestinal permeability.

[63] In one embodiment of the invention, the oral formulations comprise enteric coatings, so that the active agent is delivered to the intestinal tract. Such formulations are created by coating a solid dosage form with a film of a polymer that is insoluble in acid environments, and soluble in basic environments. Exemplary films are cellulose acetate phthalate, polyvinyl acetate phthalate, hydroxypropyl methylcellulose phthalate and hydroxypropyl methylcellulose acetate succinate, methacrylate copolymers, and cellulose acetate phthalate. Other enteric formulations comprise engineered polymer microspheres made of biologically erodable polymers, which display strong adhesive interactions with gastrointestinal mucus and cellular linings and can traverse both the mucosal absorptive epithelium and the follicle-associated epithelium covering the lymphoid tissue of Peyer's patches. The polymers maintain contact with intestinal epithelium for extended periods of time and actually penetrate it, through and between cells. See, for example, Mathiowitz et al. (1997) Nature 386 (6623): 410-414. Drug delivery systems can also utilize a core of superporous hydrogels (SPH) and SPH composite (SPHC), as described by Dorkoosh et al. (2001) J Control Release 71(3):307-18.

[64] The presence of the peptide may be determined by an affinity assay. For example an antibody that specifically binds to the peptide may be used in a quantitative or semi-quantitative assay. Such antibodies are known in the art, *e.g.* see Morón et al. (2008) PLoS ONE 3(5): e2294. For such assays it is not necessary to label the peptide.

[65] Other assays may utilize a labeled peptide, where the presence of the label is determined, e.g. by a chromatographic separation of peptide and free amino acids, by detection of cleavage through a peptide labeled with a FRET pair, and the like.

DATABASES OF METABOLIC ANALYSES

[66] Also provided are databases of metabolic analyses. Such databases will typically comprise analysis profiles of various individuals following a clinical protocol of interest *etc.*, where such profiles are further described below.

[67] The profiles and databases thereof may be provided in a variety of media to facilitate their use. "Media" refers to a manufacture that contains the expression profile information of the present invention. The databases of the present invention can be recorded on computer readable media, *e.g.* any medium that can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. One of skill in the art can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising a recording of the present database information. "Recorded" refers to a process for storing information on computer readable medium, using any such methods as known in the art. Any convenient data storage structure may be chosen, based on the means used to access the stored information. A variety of data processor programs and formats can be used for storage, *e.g.* word processing text file, database format, *etc.*

[68] As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used to analyze the information of the present invention. The minimum hardware of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled artisan can readily appreciate that any one of the currently available computer-based system are suitable for use in the present invention. The data storage means may comprise any manufacture comprising a recording of the present information as described above, or a memory access means that can access such a manufacture.

[69] A variety of structural formats for the input and output means can be used to input and output the information in the computer-based systems of the present invention. Such presentation provides a skilled artisan with a ranking of similarities and identifies the degree of similarity contained in the test expression profile.

REAGENTS AND KITS

[70] Also provided are reagents and kits thereof for practicing one or more of the above-described methods. The subject reagents and kits thereof may vary greatly. Reagents of interest include reagents specifically designed for use in production of the above described analysis. Kits may include a gluten peptide analog, reagents for analysis of the peptide and/or metabolites, and such containers as are required for sample collection.

[71] The kits may further include a software package for statistical analysis of one or more phenotypes. In addition to the above components, the subject kits will further include instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, *e.g.*, a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, *etc.* Yet another means would be a computer readable medium, *e.g.*, diskette, CD, *etc.*, on which the information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access the information at a removed site. Any convenient means may be present in the kits.

[72] This invention also provides rapid commercial test methods and devices that use at least one gluten analog peptide of the invention. In such methods, detection may utilize, for example, standard immunochromatographic technology with visible colorimetric readout. A positive result for the increased presence of at least one gluten analog peptide in a biological fluid, *e.g.* blood, urine, saliva, etc., from the patient being tested, relative to a normal control or normal reference sample, indicates active disease, i.e. increased intestinal permeability and/or lack of gluten peptide metabolism.

[73] Such assay devices may contain a single test membrane, or two or more test membranes. The test membranes may be present within a cassette, each receiving fluid from a single application, such as through an aperture in a test cassette equipped with means such as tubules for distributing said fluid to each test membrane. This invention also provides kits which comprise one or more test membrane strips comprising binding partners for the antigens used in the assay method. Assay devices may include puncture or other physical means known to the art, e.g. finger prick devices.

[74] Preferred binding partners for gluten analog peptides are antibodies specific thereto. They can be polyclonal or monoclonal antibodies. A first antibody may bind to the gluten analog peptide of the invention to form a complex, and a second antibody may bind to the complex. Either the first or second antibody may be labeled, and either the first or second antibody may be immobilized on a substrate such as a test membrane for ease of detection.

[75] This invention further provides an assay system comprising: (1) a cassette; (2) a test membrane housed within the cassette; (3) first antibodies specific to one or more gluten analog peptide(s), said first antibodies being capable of binding to the corresponding peptides to form first antibody-antigen complexes; (4) binding partners specific to such complex; and (5) labels attached to said first antibodies or said binding partners. The binding partners capable of binding to the complexes may be second antibodies specific to said gluten analog peptide; or they may be second antibodies specific to the first antibodies.

The binding partners may also be detection antigens capable of binding specifically to each of the first antibodies.

[76] The binding partners for the complex, or the first antibodies, may be immobilized on a substrate such as a test membrane. The first antibodies or the binding partners for the complex may be labeled. Preferably, the labels used are detectably different for detecting each antigen. Immobilized antibodies or binding partners for the complexes may be laid down on the substrate in different patterns.

[77] The assay system may be in the form of a cassette comprising all needed antibodies and antigens, or may be in the form of a kit which includes necessary antigens and/or antibodies or other reagents as separate reagents. In some embodiments, the kit includes a cassette comprising all needed antibodies; and a predetermined dose of gluten analog peptide, which may be in a form suitable for ingestion.

[78] A preferred cassette comprises a sample aperture for introducing sample fluid into the assay, preferably with a sample pad positioned beneath the sample aperture. The cassette also comprises a substrate such as a test membrane for immobilizing antibodies and/or antigens. A filter may be positioned downstream from the sample aperture. The cassette also preferably comprises a test window positioned above the point on the test membrane wherein labeled first antibodies, labeled second antibodies, or labeled detection antigens are immobilized. The human eye or a detection device may be used to view test results through the test window.

[79] The cassette may also comprise control peptides. These may or may not be immobilized on the test membrane and may or may not be labeled. The cassette also comprise binding partners for the control antigens. The binding partners may be labeled and may be immobilized on the test membrane. Preferably, the cassette also comprises a control window positioned above the test membrane at the point where the control antigens or their binding partners are immobilized, so that results can be viewed through the control window by the human eye or a detection device.

[80] While the methods of this invention can be carried out using an immunological assay device as described above, other testing methods known in the art for measuring antigens and gluten analog peptide of the invention levels, either directly or indirectly, such as western blot, sandwich blot, ELISA, dot blot, slot blot, Northern blot, PCR, and antibody precipitation, are also useful in the methods of this invention.

[81] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of the invention or to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure

accuracy with respect to numbers used (e.g., amounts, temperature, and the like), but some experimental errors and deviations may be present. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

EXAMPLE 1

[82] Our strategy for designing a biomarker for disease management and clinical drug development was inspired by the physical, chemical and biological properties of immunotoxic gluten peptides such as the 33-mer from α-gliadin. Specifically, we sought to engineer a gluten peptide analogue that mimics the 33-mer with respect to some criteria but can be differentiated from the natural product with respect to others. Like the 33-mer, the biomarker must be resistant to gastrointestinal proteases, so that it is not rapidly cleared from the stomach or intestinal lumen. Also like the 33-mer, it must be efficiently proteolyzed by therapeutic glutenases, so that its amino acid metabolites are rapidly assimilated into the bloodstream in a glutenase dose dependent manner. And finally, like the 33-mer, it must be able to penetrate across the intestinal epithelium. However, unlike 33-mer, the biomarker must neither be recognized by human TG2 nor HLA-DQ2. Consequently, it must be unable to stimulate disease-specific T cells so as to elicit an inflammatory response. We present two examples of biomarkers that meet these criteria, and demonstrate their utility in animal models.

[83] Design of gluten peptide-based biomarkers. In order to engineer a biomarker with the desired properties, we replaced the reactive Gln residues in the 33-mer (QQQ-33-mer) with either Asn (NNN-33-mer) or His (HHH-33-mer) residues (Figure 1). The rationale for the Q→N analogue was that, within the scope of naturally occurring (i.e. dietary) amino acids, this conservative change would minimally perturb recognition by the EP-B2 glutenase34 (Figure 1, arrowheads). The rationale for the Q→H analogue was that, in the acidic environment of the post-prandial stomach and the upper small intestine, these substitutions were expected to position positive charges at sites where TG2-mediated introduction of negative charge increases peptide affinity for HLA-DQ25,6. Importantly, neither Asn nor His is a preferred residue for cleavage by gastrointestinal proteases, so these biomarkers were expected to be as proteolytically resistant in the gastrointestinal lumen as the natural 33-mer peptide.

[84] As controls for our study, we also synthesized a peptide derived from myoglobin with moderate proteolytic resistance but unrelated sequence, as well as EEE-33-mer, the synthetically deamidated analogue of the 33-mer (Figure 1). The structures and purity of all peptides were confirmed by liquid chromatography-assisted mass spectrometry (LC-MS).

[85] Biomarkers are resistant to gastrointestinal digestion but susceptible to therapeutic glutenases. To evaluate the resistance of the 33-mer and the derivative biomarkers to gastrointestinal proteolysis, we first performed simulated gastric digests at 37 °C, pH 4.5, using commercially available pepsin, the major protease present in the stomach. The extent of digestion at multiple time-points was determined by analytical high performance liquid chromatography (HPLC) and LC-MS. As previously observed, the 33-mer was not cleaved by pepsin under simulated gastric conditions (Figure 2A). Similarly, both biomarkers were completely resistant to pepsin digestion over the course of 60 min (Figure 2B,C). To test whether the experimental glutenase EP-B2 could accelerate digestion of these biomarkers, we performed identical digests with the addition of proEP-B2, the acid-activated proenzyme form of EP-B2. The addition of proEP-B2 rapidly degraded the 33-mer (Figure 2D), as well as the derivative biomarkers (Figure 2E,F) in a time- and dose-dependent manner (Figure 2G,H,I). By contrast, pepsin alone catalyzed nearly complete cleavage of the myoglobin control peptide within 10 min (Figure 2J). The major products of biomarker digestion identified by LC-MS were similar to those produced by 33-mer cleavage (Figure 2K), indicating that the substitutions in these 33-mer analogues did not substantially alter the sites of susceptibility to EP-B2-mediated digestion.

[86] We next performed simulated duodenal digests to determine whether the biomarkers are resistant to pancreatic proteases (trypsin, chymotrypsin, elastase, and carboxypeptidase A (collectively, TCEC)), as well as to the exopeptidases contained in the intestinal brush border membrane (BBM). Following a 60 minute gastric digest containing either pepsin alone or pepsin and EP-B2, reactions were adjusted to pH 6.0 and commercial TCEC was added with BBM purified from rat intestine. Intestinal digests were carried out at 37 °C for 60 minutes, over which period several time points were taken and analyzed as above. Both biomarkers were highly resistant to high concentrations of intestinal proteases, with ~80% of intact NNN-33-mer remaining after 60 minutes, similar to 33-mer, and ~60% of intact HHH-33-mer remaining (Figure 3A). Supplementation of these digests with the proline-specific glutenase *Flavobacterium meningosepticum* (FM) PEP resulted in complete digestion of the 33-mer, as previously reported, and also of both biomarkers within 10 min (Figure 3A). Tandem mass spectrometry of biomarker digests revealed complementary EP-B2 and FM PEP cleavage patterns (Figure 3B), demonstrating these biomarkers can be used to assess efficacy of combination enzyme therapies. Indeed, addition of FM PEP enabled further digestion of fragments remaining after treatment of the 33-mer and biomarkers with EP-B2 (Figure 3C,D,E).

[87] *Biomarkers are non-inflammatory in the context of celiac sprue.* For biomarkers to be administered safely to celiac sprue patients, they must not be deamidated by TG2, bind HLA DQ2, or stimulate a strong immune response by pre-existing gluten-specific T cells. To

determine the capacity for gluten peptide-based biomarkers to elicit an inflammatory T cell response in celiac sprue patients, these characteristics were tested *in vitro* and compared to the immunodominant 33-mer gluten peptide. The extent to which each biomarker is deamidated by TG2 was determined by a spectrophotometric assay in which the ammonium ion released by TG2-catalyzed substrate deamidation is coupled to glutamate dehydrogenase-catalyzed oxidation of NADH36. Consistent with previous results, the 33-mer was readily deamidated by TG2 (Figure 4A).

[88] By contrast, TG2 activity in the presence of NNN-33-mer and HHH-33-mer was significantly reduced, 33.1- fold and 25.0-fold, respectively, relative to that in the presence of native 33-mer. Activity in the presence of the biomarkers was slightly higher than that detected in the absence of a peptide substrate, though this difference was not significant for HHH-33-mer (p = 0.04 for NNN-33- mer). The control myoglobin peptide, which lacks glutamine residues entirely (Figure 1), elicited no activity from TG2, suggesting the residual activity elicited by the biomarkers might be attributable to deamidation of glutamines at positions other than the preferred sites that were synthetically altered. The affinity of each biomarker for HLA-DQ2 was determined using a peptide exchange assay in which fluorescein-labeled peptides were incubated with soluble HLA-DQ2 molecules at pH 5.5, 37 °C to simulate the endocytic environment. After 45 h, DQ2-bound and free fluorescein-labeled peptides were separated by high-performance size exclusion chromatography (HPSEC) and the ratio of their peak heights determined by fluorometry. Consistent with previous results, >80% of synthetically deamidated 33-mer (EEE-33-mer) bound HLA-DQ2, a 9.9-fold increase over the native peptide and a 15.6-fold increase over the NNN-33-mer biomarker (Figure 4B). Neither HHH-33-mer nor the myoglobin control peptide exhibited any detectable binding to HLA-DQ2. The immunostimulatory capacity of each biomarker was measured via T cell proliferation assays employing gluten-specific T cells derived from celiac patient intestinal biopsies. The low immunostimulatory capacity of the biomarkers toward any of the cell lines and clones precluded response saturation and EC50 determination. However, it is apparent that the immunostimulatory capacity of NNN-33-mer was reduced ~1000-fold relative to TG2-treated 33-mer. The HHH-33-mer biomarker was even less immunogenic, eliciting minimal or no response at micromolar concentrations. Treatment of the biomarkers with TG2 did not increase their immunostimulatory capacity.

[89] *Biomarker transepithelial transport parallels gluten peptide transport under basal and inflammatory conditions.* In addition to reporting on glutenase activity *in vivo*, noninflammatory gluten peptide-based biomarkers are useful tools for understanding the factors and mechanisms that modulate intestinal permeability of immunogenic dietary peptides, as well as for practical applications related to the diagnosis of celiac sprue and its treatment with modulators of epithelial permeability. To be used in such applications, these

biomarkers must be similar to inflammatory gluten peptides in terms of their transport and transepithelial stability across healthy and inflamed mucosa. The T84 epithelial cell line was used to model the intestinal epithelium because its responsiveness to IFN-γ, the major inflammatory cytokine present in celiac lesions, has been extensively studied. Additionally, the effect of IFN-γ on the intact transport of the 33-mer and other gluten peptides across T84 epithelial monolayers has recently been described. To simulate transport under healthy and inflammatory states, media alone or media containing IFN-γ was incubated for 48 hours on the basolateral side of T84 epithelial cells cultured on transwell supports, and the apical-to-basolateral flux of Cy5-labeled 33-mer and biomarkers was measured thereafter. The flux of Cy5-33-mer and both Cy5-labeled biomarker peptides was ~6 pmol/cm2/h under basal conditions. Following exposure of T84 monolayers to IFN-γ, the flux of all 3 peptides was increased ~10-fold. No significant difference in flux was observed between 33-mer and either biomarker under basal or simulated inflammatory conditions. Some processing of the 33-mer may occur upon its transport across the intestinal epithelium. Therefore, to evaluate the stability of apical and translocated Cy5-labeled biomarkers, the apical and basolateral media were analyzed by LC-MS immediately after peptide addition to the apical chamber and 10 h thereafter. The absorbance at 640 nm was monitored during chromatographic separation. Both Cy5-labeled biomarkers, as well as Cy5-labeled 33- mer, eluted between 9-10 min as a single major peak. A smaller peak, eluting at ~8 min in all samples, was identified as Cy5-LQ, indicating that some processing of the peptides' N-terminus occurred in the presence of epithelial cells. Nonetheless, after 10 h, no other breakdown products were identifiable by mass spectrometry, and >95% of each intact peptide remained in the apical chamber of control cells. Intact Cy5-peptides were present at somewhat lower levels (>80% of initial) after 10 h in the apical chambers of those cells preincubated with IFN-γ. This was due to increased N-terminal processing, evidenced by the more apparent Cy5-LQ peak present in these samples, as well as to IFN-γ-induced enhancement of apical-to-basolateral flux. All three Cy5-labeled peptides remained intact during transport. After 10 h, 0.2-0.3% and 2-3% of the initial 20 μM apical peptide was observed intact on the basolateral side of cells preincubated with media alone or with IFN-γ, respectively. Thus, both biomarkers were highly stable in the presence of epithelial cells, and were translocated intact to a similar extent as the 33-mer under basal and simulated inflammatory conditions. To examine biomarker transport under basal and inflammatory conditions *in vivo*, catheterized rats were administered 20 mg [^{13}C3]-HHH-33-mer biomarker by oral gavage following 2 days of daily intravenous treatment with vehicle or IFN-γ. The level of intact peptide in peripheral plasma 60 min after peptide administration was determined by 3Q LCMS/ MS. As predicted by the lack of a serological response to ingested gluten in rodents,

biomarker was detected in the plasma of only 1 of 8 control rats administered oral biomarker. Pretreatment with IFN-γ did not elicit a general increase in biomarker transport in the test animal group relative to controls, as only 1 of 4 IFN-γ-treated animals exhibited plasma biomarker. However, the level of plasma biomarker in this IFN-γ-treated animal (101.7 nM) was ~10-fold higher than that in the control animal exhibiting detectable plasma biomarker (9.5 nM), a difference similar to that caused by IFN-γ *in vitro*. Additionally, 33-mer dosed together with [^{13}C3]-HHH-33-mer was detected in the plasma of both animals exhibiting plasma biomarker, but not in other animals, suggesting this inflammatory gluten peptide and its non-inflammatory counterpart are transported in parallel *in vivo*.

[90] A recent report demonstrated that the 33-mer is absorbed intact across the gut epithelium of an enteropathic gluten-sensitive rhesus macaque, but not across that of a healthy control. To determine if similar absorption of a non-inflammatory gluten peptide-based biomarker occurs, a gluten-sensitive macaque with chronic diarrhea and elevated plasma anti-gliadin antibodies was administered 100 mg [^{13}C3]-HHH-33-mer intragastrically. Peripheral blood samples were collected at hourly intervals and analyzed for biomarker content by 3Q LC-MS/MS. The labeled biomarker was clearly detected (2.3 ± 0.1 nM) in peripheral blood 60 min after administration, similar to the extent and rate of absorption reported for the 33-mer gluten peptide. In a repeat experiment, intragastrically administered labeled biomarker was again detected in the peripheral blood of this gluten-sensitive macaque (2.2 ± 0.5 nM), but was not detected in samples from two identically dosed healthy controls.

[91] Celiac sprue affects up to 1% of many human populations, but despite the wide prevalence and serious manifestations of the disease, the only treatment available remains a life-long gluten-free diet. Compliance with this burdensome dietary treatment is poor, and recurrent exposure to gluten causes chronic inflammation, increased morbidity, and more serious health effects over time. Moreover, in asymptomatic celiac sprue patients, disease management is especially difficult, as invasive histological evaluations are the only reliable way to assess response to a gluten-free diet. Finally, the development of non-dietary treatment alternatives to the gluten-free diet requires a long-term gluten challenge in celiac patients, which is inherently problematic. Therefore, novel tools for monitoring compliance with the gluten-free diet, and safely evaluating non-dietary treatments *in vivo* are needed.

[92] Here we used the disease-causing agent in celiac sprue as a template for designing noninflammatory analogs as tools for fundamental and translational research. Celiac sprue is uniquely suited for this biomarker strategy because we know the environmental trigger and have structural information about its binding mode to the primary genetic determinant

for the disease. Additionally, gluten peptides are extraordinarily stable in the relevant physiological compartment, making them ideal scaffolds for drug and biomarker design.

[93] Research into the molecular basis for celiac sprue has elucidated many of the properties of gluten peptides that allow them to persist through gastrointestinal proteolysis, access the gut-associated lymphoid tissue, and interact with the key players in disease pathogenesis: TG2, DQ2, and gluten-specific T cells. Guided by these findings, our goal was to abrogate those properties that contribute to the inflammatory response to gluten while retaining those properties that render biomarker metabolism and transport relevant to disease. By altering key residues in a gluten peptide scaffold, we developed peptide biomarkers that mimic gluten peptides in their resistance to gastrointestinal proteases and susceptibility to therapeutic glutenases, but that are neither substrates for TG2 nor ligands for DQ2. As a result, these peptides are not presented to gluten-specific T cells and are consequently noninflammatory.

[94] In the present study, the 33-mer from α2-gliadin was used as a scaffold for biomarker design because its metabolism in the presence and absence of glutenases has been extensively characterized and because its intact transepithelial translocation has been demonstrated. Other examples of disease-relevant gluten peptide sequences that are suitable for biomarker design include the 26-mer from γ5-gliadin and the p31-49 peptide that stimulates an innate immune response in mucosal biopsies from celiac sprue patients.

[95] Oral protease therapy is one of the more promising non-dietary treatments being developed for celiac sprue, but few studies have been conducted *in vivo*. Celiac patients administered an undefined enzyme mixture from animal digestive extracts showed modest improvement in a clinical trial. More recently, clinical efficacy of oral EP-B2 was demonstrated in a gluten sensitive rhesus macaque. These studies relied on histological, clinical, and serological readouts, complex parameters which require weeks to register a response and which are indirect measures of glutenase-mediated gluten detoxification. By contrast, in the present study, the metabolism of [^{13}C3]-HHH-33-mer in rats dosed with EP-B2 provided an immediate and direct readout for glutenase activity *in vivo*. This effect was observed by mass spectrometric analysis of gastric contents. Studies will also focus on optimizing non-invasive readouts for biomarker metabolism, such as the measurement of isotope-labeled (or alternatively labeled) amino acid metabolite concentrations in bodily fluids such as serum or urine. Alternatively, a stable isotope breath test (e.g. for $^{13}CO_2$) may be adapted to the detection of biomarker metabolites.

[96] In animal studies, we observed intact biomarker absorption across the intestinal epithelium of a subset of tested rats as well as an enteropathic gluten-sensitive macaque. Whereas the results of our rodent studies support the hypothesis that proteolytically stable gluten oligopeptides can be transported intact across the gut epithelium, they also highlight

the need for a considerably larger study to quantify inherent permeability differences between individual animals and to explore the role of IFN-γ in enhancing intestinal permeability. In the glutensensitive macaque, the extent and kinetics of biomarker transport during active disease were similar to those reported for the 33-mer itself in a previous experiment.

[97] Biomarker transport was not observed in healthy controls, also consistent with the 33-mer experiment. These data demonstrate that the level of the intact peptide in blood (or urine) can be used as a disease-relevant biomarker for intestinal barrier function. Such a metric would facilitate the evaluation of drugs intended to modulate intestinal permeability. One such drug candidate, AT-1001, has shown evidence of decreasing paracellular permeability in a diabetes-prone rat model and in preliminary clinical trials. By analogy to the use of neutralizing antibodies against TNF-α to treat Crohn's disease, anti-IFN-γ antibodies may represent another candidate for reducing gut permeability and inflammation in celiac sprue.

[98] Lastly, strict adherence to a gluten-free diet reduces intestinal permeability in a majority of celiac patients and this reduction precedes measurable improvements in histology. Therefore, biomarkers are a useful clinical tool for monitoring adherence to a gluten-free diet as well. Notwithstanding the effect of a gluten-free diet on intestinal permeability, epithelial uptake of gluten remains altered in treated celiac patients with respect to healthy controls. This is likely related to the 7.6-fold higher levels of IFN-γ present in treated patients relative to healthy controls. In our experiments, both biomarkers were translocated intact across epithelial monolayers to a 10-fold greater extent following preincubation of the cells with IFN-γ. This suggests these peptides may be used as a screening tool for celiac sprue, en route to a diagnosis, as well as for other inflammatory bowel diseases in which intestinal IFN-γ levels and mucosal leakiness of antigenic peptides and proteins are elevated.

Materials and Methods

[99] *Materials.* Peptide synthesis reagents were purchased from Chem-Impex (Wood Dale, IL), Peptides International (Louisville, KY), Anaspec (San Jose, CA) and Novabiochem (San Diego, CA). Cy5-NHS ester was purchased from Amersham Biosciences (Piscataway, NJ). Isotopelabeled amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA). Recombinant IFN-γ was purchased from Peprotech, Inc. (Rocky Hill, NJ). Cell culture media, antibiotics, human serum, 5'(and 6') carboxyfluorescein, and fluorescently labeled dextrans were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville,

GA). Gluten flour was purchased from Bob's Red Mill (Milwaukie, OR). Thrombin was purchased from Novagen (Madison, WI). Protease inhibitor cocktail set 1 was purchased from Calbiochem (San Diego, CA). Pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, vancomycin, and Nα-p-tosyl-L-arginine methyl ester hydrochloride (TAME) were purchased from Sigma-Aldrich (St. Louis, MO). Brush border membranes were purified from rat intestine as previously described and stored at -80°C until use. The recombinant proenzyme precursor of barley endoprotease EP-B234, *Flavobacterium meningosepticum* PEP44 and human TG236 were expressed in *E. coli* and purified as previously described. Recombinant soluble DQ2 heterodimer-αI gliadin peptide fusion molecules were prepared and purified in insect cells as previously described.

[100] *Animals*. Twelve 8 week old male Wistar rats weighing between 250 and 300 grams (Charles River Laboratories, Wilmington, MA) were singly housed in standard polycarbonate shoebox cages measuring 10.5" x 19" x 8" h with wire bar lids and micro-isolator tops (Allentown, Inc., Allentown, NJ). Eight 8 week old male Wistar rats weighing between 250 and 300 grams (Charles River Laboratories) with indwelling jugular vein catheters were housed similarly. Rats were allowed access to rodent chow #5010 (Purina, Richmond, IN) and water *ad libitum* prior to the onset of the studies. The room was maintained on a 12:12-hr light:dark cycle. The ambient temperature remained between 64 and 72 °F with a relative humidity of 30-70%. All experimental procedures were approved by the Animal Care and Use Committee of the Tulane National Primate Research Center (Covington, LA).

[101] Gluten-sensitive juvenile macaque FH45 (4.5 kg, male) was selected from a population of rhesus macaques exhibiting clinical diarrhea, intestinal villous blunting, and elevated AGA on a gluten-containing diet. Healthy controls HI48 (2.65 kg, male) and HK31 (2.70 kg, male) were selected from a population that exhibited no clinical or serological responses to gluten intake. Throughout the study, animals consumed 4% of their respective body weights daily of monkey chow #5K63 (Purina) containing 20% (by weight) crude protein including oats and gluten sources such as ground wheat. The animals were housed under biosafety level two conditions in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care. Investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council.

[102] *Peptide synthesis, labeling, and purification.* Peptides were synthesized using Boc/HBTU chemistry on solid-phase as previously described. To prepare isotope-labeled peptides for the study of biomarker metabolism *in vivo*, [1-^{13}C]-leucine was incorporated at positions 11, 18, and 25 in the HHH-33-mer biomarker (SEQ ID NO:77) (LQLQPF(PQPHLPY)$_3$PQPQPF) and [5,5,5-D3]- leucine was incorporated at position 8 in

the myoglobin peptide ((SEQ ID NO:80) KGHHEAELKPL; underlined). For DQ2 binding assays, peptides were labeled at their amino terminus on solid-phase with carboxyfluorescein, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), and 1-hydroxy-7-azabenzotriazole (HOAt) in 1:1:1 ratio and 10% (v/v) diisopropylethylamine in dimethylformamide/methanol (2:1) as solvent. Following cleavage from the resin, peptides were purified over a reverse-phase C18 column by HPLC using a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid, lyophilized, and stored at -20°C. For transepithelial translocation assays, purified peptides were labeled at their amino terminus with Cy5-NHS ester in DMSO according to the manufacturer's instructions, repurified by HPLC, lyophilized, and stored at -20°C. The correct mass of all peptides was confirmed by LC-MS. Prior to use, peptides were resuspended in 50 mM sodium phosphate, pH 7.0 supplemented with 0.02% NaN_3. The concentrations of unlabeled and Cy5-labeled peptides were determined at pH 7.0 by spectrophotometric measurement of A280 (ε280 = 3840 $M^{-1}cm^{-1}$) and A652 (ε652 = 250,000 $M^{-1}cm^{-1}$), respectively. Due to the absence of aromatic residues in the myoglobin peptide, the concentration of the unlabeled myoglobin peptide was determined by spectrophotometric measurement of A205 (ε205 = 27 (mg/ml)-1^{cm-1}) as previously described. The concentrations of fluorescein-labeled peptides were determined at pH 9.0 by spectrophotometric measurement of A495 (ε495 = 80,200 $M^{-1}cm^{-1}$). Working stocks were stored at 4°C and their integrity confirmed periodically by RP-HPLC.

[103] *Cell culture.* T84 epithelial cells from the American Type Culture Collection (Manassas, VA) were grown in T84 media (Dulbecco's Modified Eagle Medium:Ham's F12 (1:1) supplemented with antibiotics (penicillin/streptomycin) and 5% (v/v) fetal bovine serum). Media was changed every alternate day, and the cells were split once a week. DQ2 homozygous antigen-presenting cells (CD114, an Epstein Barr virus-transformed B lymphoblastoid cell line) were grown in APC media (RPMI supplemented with antibiotics and 5% (v/v) fetal bovine serum). Every other day, CD114 were split to 0.4 x 106 cells/ml. Gluten-specific, DQ2-restricted T cell lines (TCL 432.1.4, TCL 421.1.4, TCL 446.1.3) and T cell clones (TCC 436.5.3 (DQ2-α-II specific), TCC 430.1.142 (DQ2-α-I and DQ2-α-III specific) were isolated from celiac patient intestinal biopsies and expanded as previously described. T cell proliferation assays were performed in T cell media (RPMI 1640 supplemented with antibiotics and 10% (v/v) human serum). All cells were grown and assayed at 37°C with 5% atmospheric CO_2.

[104] *Simulated gastrointestinal digests with glutenase supplementation.* In preparation for HPLC analysis, all buffers and reagents were filtered (0.2 μm) prior to use in digests. To simulate gastric digestion, peptides (300 μM) were incubated at 37°C in a 10 mM sodium acetate buffer, pH 4.5 with 1:10 (w/w) pepsin (120 μg/ml) supplemented with 0, 6, 12, 24,

48, or 120 μg/ml of recombinant proEP-B2 glutenase. Samples were collected at 0, 10, 30, 45, and 60 min timepoints. Samples were heat-deactivated at 95°C for 5 min, diluted 1:5 in HPLC solvent A (95% H_2O, 5% acetonitrile, 0.1% trifluoroacetic acid) supplemented with an internal standard (TAME), and analyzed by reverse-phase HPLC. Samples (50 μl) were separated over a C18 column (Grace Vydac, Hesperia, CA) using a water-acetonitrile gradient in the presence of 0.1% TFA. The absorbance at 215 nm was monitored. The area-under-the-curve for each intact peptide was calculated and normalized to the area under the curve for the internal standard, TAME. Due to the rapid EP-B2-catalyzed removal of the N-terminal LQ from 33-mer and biomarkers (Figure 1 and 2), this minimally-processed product was included as intact peptide in area-under-curve analyses. Following simulated gastric digestion of each peptide (300 μM) with pepsin ± 120 μg/ml proEP-B2, digests were adjusted to pH 6.0 with sodium phosphate buffer (50 mM, final concentration) and commercially available pancreatic proteases trypsin (30 μg/ml), chymotrypsin (30 μg/ml), elastase (6 μg/ml), and carboxypeptidase A (6 μg/ml), as well as 27 μg/ml rat intestinal brush border membrane, were added. Recombinant prolyl endopeptidase from *Flavobacterium meningosepticum* (FM PEP) was supplemented at 1.2 U/ml when added. Simulated duodenal digests were performed at 37°C. Samples were collected at 0, 10, 30, and 60 min and processed for HPLC as described. The area-under-the-curve for each intact peptide (together with the minimally processed -LQ product) was calculated and normalized to the internal standard. To identify digestion products, select samples were analyzed by LC-MS. Samples (50 μl) processed for HPLC as described were injected on a reverse-phase C18 HPLC system (Waters Corporation, Milford, MA) coupled to a UV/Vis detector and a ZQ single quadrupole mass spectrometer with an electrospray ionization source. Samples were eluted with a wateracetonitrile gradient in the presence of 0.1% formic acid. Absorbance at 214/254 nm and total ion current were monitored, and spectra corresponding to major absorbance peaks were examined.

[105] *Transglutaminase deamidation assay.* Coupled spectrophotometric assays for TG2 activity in the presence of each peptide were performed as previously described36. Briefly, each peptide (100 μM) was added to a 200 mM MOPS, pH 7.2 buffer containing 5 mM CaCl2, 1 mM Na4EDTA, 10 mM α-ketoglutarate, and 250 μM NADH. Glutamate dehydrogenase was added to a final concentration of 0.036 U/μl, and this incomplete reaction mixture was incubated at room temperature for 10 min to stabilize the initial absorbance at 340 nm. Finally, 5 μM TG2 was added and A340 was monitored. The specific activity of the enzyme in the presence of each peptide was calculated from the rate of NADH consumption (ε340 = 6220 M-1cm-1).

[106] *HLA-DQ2 binding assay.* Peptide exchange assays for determining the equilibrium occupancy of each peptide on HLA-DQ2 were modified from previously described methods. Briefly, recombinant DQ2-α1-gliadin peptide fusions (35 μM) were treated with 0.15 U/μl thrombin in phosphate-buffered saline (PBS), pH 7.4 supplemented with 0.02% (w/v) NaN_3 for 2 h at 4°C, after which protease inhibitor cocktail was added. Thrombin-cleaved DQ2-α1-gliadin peptide complexes (9.4 μM) were incubated with 0.185 μM fluorescein-conjugated peptides in a citrate- PBS buffer, pH 5.5 (24 mM sodium citrate, 55 mM Na_2HPO_4, 75 mM NaCl, 0.02% (w/v) NaN_3) for 45 h at 37 °C. To quantify the equilibrium occupancy of each fluorescent peptide on DQ2, binding reactions were diluted 1:5 in PBS, pH 7.4 supplemented with 0.02% (w/v) NaN_3 and 12.5 μl was injected on an HPSEC system coupled to a fluorescence detector (Shimadzu, Columbia, MD). DQ2-bound and unbound fluorescent peptides were separated using a BioSep 3000 size exclusion column (Phenomenex, Torrance, CA) with a flow rate of 1 ml/min PBS, pH 7.4 supplemented with 0.02% (w/v) NaN_3. The detector was set to monitor excitation at 495 nm and emission at 520 nm. The bound/free ratio for each peptide was calculated by dividing the measured peak height for bound peptide by that for the free peptide.

[107] *T cell proliferation assay.* The T cell proliferation assay was modified from previously described methods, as follows. Briefly, the 33-mer, NNN-33-mer, and HHH-33-mer peptide stocks (250 μM) were deamidated by treatment with 100 μg/ml TG2 in 100 mM Tris, pH 7.4 for 2 hours at 37 °C in the presence of 2 mM $CaCl_2$. Antigen-presenting cells (CD114, 60,000 cells/well) were irradiated (80 Gy) and incubated overnight at 37°C in U-bottom, 96-well plates with various concentrations of native or TG2-deamidated peptides in T cell media. As positive and negative controls, 2 μM synthetically deamidated EEE-33-mer or no peptide was added to antigen-presenting cells. The next day, three T cell lines and two T cell clones isolated from intestinal biopsies of HLA-DQ2+ celiac patients were thawed and added (40,000 cells/well) to triplicate wells containing peptide-loaded antigen-presenting cells. Cells were co-incubated 48 hours to allow T cells to proliferate in response to DQ2-peptide complex stimulation. After 48 hours, 1 μCi [^{3}H]-thymidine was added to each well and cells were incubated an additional 16 hours. Finally, DNA was collected on a filter mat using a cell harvester and counts-per-minute (cpm) resulting from incorporated [3H]-thymidine were measured with a liquid scintillation counter.

[108] *Biomarker and glutenase dosing to rats.* Procedures for administration of gluten and therapeutics to rats were modified from those previously described. The study comprised 3 days, including fasting, acclimation to the gluten test meal, and glutenase/biomarker dosing. Adult rats (n = 4 for each of 3 groups) were fed a commercially available rat chow until Day 1 of the study. Rats were then fasted for 12 h, while drinking water remained freely available

throughout the study. On Day 2, all 3 animal groups were administered a freshly prepared gluten-sugar test meal (a dough ball composed of 1 g gluten, 0.6 g white sugar, 0.6 g brown sugar, 0.35 g croscarmellose sodium, 2.45 ml water) to acclimate them to eating this meal following a fast. After 60 min, the test meal was removed and animals were once again fasted for 24 h. On Day 3, animals were administered a freshly prepared gluten-sugar test meal supplemented with 0, 10, or 40 mg proEP-B2 (for Groups 1, 2, or 3, respectively), 19.7 mg [^{13}C3]-HHH-33-mer, 3.3 mg [D3]-myoglobin peptide, and 10 mg vancomycin. Vancomycin was added as a non-absorbable dosing internal standard, as previously described. Animals consumed the test meal completely within 60 min, and animals were euthanized 30 min thereafter (i.e. 90 min after meal administration). The gastric, duodenal, jejunal, and ileal contents were collected immediately and stored at -80 °C as previously described. Peripheral plasma samples were collected via cardiac puncture at the time of euthanasia and stored at -80 °C.

[109] *Reverse-phase HPLC analysis of rat gastrointestinal contents.* Gastric samples (100 mg) were thawed on ice and suspended in 190 µl 0.01 M HCl and 10 µl 10 mM leupeptin (an inhibitor of EP-B2, to prevent digestion of material *ex vivo*). Suspensions were incubated 10 min at 37 °C, pH 2.5, and then 50 mM sodium phosphate, pH 6.0 and sodium hydroxide were added to increase the pH of the suspensions to above 6.0. Trypsin (0.375 mg/ml) and chymotrypsin (0.375 mg/ml) were added to maximize dissolution of gluten, and reactions were incubated 30 min at 37 °C. Samples were heat-deactivated for 5 min at 95 °C, supplemented with ethanol to a final concentration of 70% (v/v), and centrifuged for 10 min at 16,100 x g. Syringe-filtered (0.45 µm) supernatants (100 µl) were diluted 1:5 in 95% H2O, 5% acetonitrile, 0.1% trifluoroacetic acid supplemented with an internal standard (TAME), and analyzed by reverse-phase HPLC. Samples (50 µl) were separated over a C18 column (Grace Vydac, Hesperia, CA) using a water acetonitrile gradient in the presence of 0.1% TFA. The absorbance at 215 nm was monitored. Intestinal flushes were thawed on ice and centrifuged for 10 min at 4 °C, 3100 x g. Supernatants were heat-deactivated and processed for HPLC as described for gastric contents.

[110] *Competitive ELISA on rat gastric contents.* Gastric samples (100 mg) were thawed on ice, suspended in 950 µl 70% (v/v) ethanol and 50 µl 10 mM leupeptin, incubated 10 min at 37 °C, and then centrifuged for 10 min at 16,100 x g. Syringe-filtered (0.45 µm) supernatants were tested for the gluten sequence QPQLPY using a monoclonal antibody-based competitive ELISA modified from previous methods. Briefly, equal volumes of coating solution (5 µg/ml gliadin (Sigma) in 20 mM PBS, pH 7.2) and 20 mM sodium bicarbonate, pH 9.6 were added to 96-well microtiter plates (Nunc Maxisorp), and incubated 1 h at 37 °C and overnight at 4 °C. The next day, gliadin-coated plates were washed twice with washing

buffer (PBS, pH 7.2 containing 0.05% Tween-20) then blocked with blocking buffer (5% (w/v) nonfat milk in PBS, pH 7.2) for 2 h at room temperature. Synthetic 33-mer standard (10^{-5} – 10 μg/ml) or gastric supernatants were serially diluted in assay buffer (3% (w/v) bovine serum albumin in PBS, pH 7.2). An equal volume of G12-HRP monoclonal antibody-horseradish peroxidase conjugate (Biomedal, Seville, Spain) diluted 1:10,000 in assay buffer was added to each standard or sample dilution. Mixtures were incubated with gentle agitation for 2 h at room temperature, and then added to plate wells in triplicate. After 30 min incubation at room temperature, wells were washed 5 times with washing buffer, and TMB liquid substrate solution (Sigma) was added to wells. The reaction was stopped after a 30 min dark incubation by addition of an equal volume of 1 M sulfuric acid and the absorbance at 450 nm was measured. Origin 6.0 (OriginLab, Northampton, MA) was used to fit the 33-mer standard curve to the sigmoidal model: A450 = A450min + (A_{450}max - A_{450}min)/[1 + (x/IC_{50})n] where x is the peptide concentration, IC_{50} is the 33-mer concentration at which competition is half-maximal, and n is the Hill slope. The concentration of peptides containing the sequence QPQLPY in gastric samples was determined by comparison to the linear portion of the 33-mer standard curve.

[111] *3Q LC-MS/MS analysis of intact peptides and biomarker metabolites.* The amounts of gluten-derived 33-mer, [^{13}C3]-HHH-33-mer, and [D3]-myoglobin peptide present in rat gastric contents and plasma were determined using a Micromass Quattro Premier triple quadrupole LC MS system. Gastric samples (100 mg) were thawed on ice and suspended in 950 μl 0.01 M HCl and 50 μl 10 mM leupeptin. Suspensions were adjusted to pH 6.0 and pancreatic proteases were added as described above to release the 33-mer from gluten present in the samples. After 30 min, samples were heat-deactivated for 5 min at 95 °C and centrifuged for 10 min at 16,100 x g. Prior to 3Q LC-MS/MS analysis, syringe-filtered (0.45 μm) gastric supernatants, or plasma samples, were depleted of larger proteins by addition of acetonitrile. Samples were mixed with an equal volume of cold acetonitrile containing 0.1% formic acid and 200 nM NNN-33-mer as an internal standard. Samples were vortexed, incubated for 2 h at 4 °C, and centrifuged for 10 min at 4 °C, 16,100 x g. Supernatants were mixed with an equal volume of 0.1% formic acid in water to dilute the acetonitrile concentration to 25%, and used directly for intact peptide analysis or, for plasma samples only, processed for metabolite analysis as below. Mass spectrometry analysis of intact peptides was performed as previously described30 with the following modifications. Samples were injected in triplicate (80 μl each) and eluted with a water-acetonitrile gradient in the presence of 0.1% formic acid. For 33-mer detection, positive ion SRM mode was used for monitoring the transitions of ions at *m/z* 978.84+ → 263.2+ (30V cone voltage, 27eV collision energy) for the quantification assay and *m/z* 1304.73+ →263.2+ (40V cone

voltage, 50eV collision energy) as a confirmatory transition. For [^{13}C3]-HHH-33-mer detection, the transition monitored was *m/z* 986.754+ →263.2+ (45V cone voltage, 32eV collision energy) for the quantification assay. For [D3]-myoglobin peptide detection, the transitions monitored were *m/z* 632.72+ →229.2+ (35V cone voltage, 25eV collision energy.) for the quantification assay and *m/z* 632.72+ →129.2+ (35V cone voltage, 33eV collision energy) as a confirmatory transition. For NNN-33-mer internal standard, the transitions monitored were *m/z* 968.64+ →263.4+ (32V cone voltage, 32eV collision energy) for the quantification assay and *m/z* 968.64+ →226.0+ (40V cone voltage, 50eV collision energy) as a confirmatory transition. Levels of 33-mer, isotope-labeled biomarker, and isotope-labeled myoglobin control peptide in each sample were determined by comparison of the area under their transition peaks to the area under the NNN-33-mer internal standard transition peak and to a calibration curve corresponding to each peptide. Mass spectrometry analysis of biomarker metabolites was performed on plasma samples processed as above. Protein-depleted samples (30 μl) were dried and resuspended in 300 μl 20 mM ammonium acetate, pH 5.0 containing 100 nM [D10]-leucine as an internal standard. Samples were injected in triplicate (20 μl each) and eluted from an Atlantis T3 column (3 μm, 2.1x100mm, Waters) with a 20 mM ammonium acetate, pH 5.0-acetonitrile gradient. For leucine quantification, the transitions monitored were *m/z* 132.2+ →86.2+ (15V cone voltage, 10eV collision energy). For [^{13}C]-leucine quantification, the transitions monitored were *m/z* 133.2+ →86.2+ (15V cone voltage, 10eV collision energy). For [D3]-leucine quantification, the transitions monitored were *m/z* 135.2+ →89.2+ (15V cone voltage, 10eV collision energy.). For [D10]-leucine internal standard quantification, the transitions monitored were *m/z* 142.2+ →96.2+ (15V cone voltage, 10eV collision energy.)

[112] *Peptide translocation assays.* Peptide translocation assays were performed as previously described. Briefly, cultured T84 cells were seeded on rat tail collagen-coated polycarbonate transwell permeable supports (5 μm pore size, 6.5 mm diameter; Corning Life Sciences, Lowell, MA) at 5 x 104 cells/well and the media was exchanged every other day for 2 weeks while the cells grew to confluence and formed tight junctions. Following maturation, cell monolayers were preincubated for 48 h with basolateral media containing either 0 or 600 U/ml recombinant IFN-γ. After preincubation, the translocation assay was performed by replacing media in both the apical and basolateral chambers with serum-free T84 media (1:1::Dulbecco's Modified Eagle Medium:Ham's F12 media supplemented with antibiotics), and adding 2 μM dextran (3,000 mol. wt.)-Alexa Fluor-488, 2 μM dextran (70,000 mol. wt.)-Texas Red, and 20 μM Cy5-labeled peptide to the apical chamber. Labeled dextrans were added to confirm monolayer integrity and size-selective transport41. Samples of the apical and basolateral media were taken at the 0 h time-point, and

basolateral samples were taken every hour over a 4 h experiment. Fluorescence in collected samples was measured in 96-well format on a Flexstation II 384 (Molecular Devices, Sunnyvale, CA), monitoring three channels (excitation 490 nm, emission 525 nm for Alexa Fluor-488; excitation 585 nm, emission 620 nm for Texas Red; excitation 640 nm, emission 675 nm for Cy5). The slope of basolateral fluorescence units versus time (from 1-4 h) was calibrated to the initial apical fluorescence and divided by the permeable support area (0.33 cm^2) to yield the transepithelial flux (pmol/cm^2/h).

[113] *Chromatographic and mass spectrometric analysis of translocated gluten peptides.* During the peptide translocation assay described above, additional samples from the apical and basolateral chambers were collected at 0 and 10 h for analysis of peptide stability and intact translocation. Samples (50 μL) were analyzed by LC-MS as described for digests, except absorbance at 640 nm was monitored. Spectra corresponding to A_{640} peaks were examined. Samples were also analyzed by HPSEC with fluorescence detection as described for DQ2- peptide binding analysis, except the detector was set to monitor excitation at 647 nm and emission at 665 nm.

[114] *Biomarker dosing in rats following IFN-gtreatment.* Adult catheterized rats (n = 4 for each of 2 groups) were administered a daily dose of vehicle (PBS) or 108 U/m2 IFN-γ intravenously via catheter for 2 days. On Day 3, 48 hours after the initial dose of vehicle or IFN-γ, all animals were administered 0.5 ml water containing 20 mg 33-mer and 20 mg [13C3]-HHH-33-mer via oral gavage. Animals were euthanized 60 min thereafter and peripheral plasma samples were collected, stored, and tested via 3Q LC-MS/MS for intact peptides as described above.

[115] *Biomarker inoculation in gluten-sensitive and healthy rhesus macaques.* Prior to the study, gluten-sensitive macaque FH45 was administered a gluten-containing diet and exhibited elevated anti-gliadin antibodies, intestinal villous blunting, and clinical symptoms indicative of gluten sensitivity. On the day of the study, a dose of 100 mg of isotopically labeled biomarker ([^{13}C3]-HHH-33-mer) dissolved in 10 ml of Gatorade was administered directly into the fasted stomach of FH45 by intragastric tube. A 0.5 ml sample of EDTA-blood was collected from an ear vein at 0, 60, 120, 180, and 240 min following biomarker inoculation. The experiment was repeated four months later with FH45 and two healthy control macaques, HI48 and HK31, on a gluten-containing diet. In this second experiment, 50 mg of isotopically labeled biomarker was intragastrically administered to each animal. Animals were sedated and anesthetized prior to peptide inoculation and blood collections. Plasma samples were analyzed for intact biomarker by 3Q LC-MS/MS as described above.

[116] *Statistics.* Statistical comparisons were conducted using a two-tailed Student's t-test assuming unequal variances. A statistical probability of $p < 0.05$ was considered significant.

Example 2

Method of Determining Treatment Efficacy (I)

[117] In conjunction with a clinical trial for determining efficacy of a study drug ALV003 in mitigating the effects of gluten ingestion in patients with celiac disease. Adult patients are selected for inclusion in the study based on biopsy-proven CD in past 5 year. Subjects are given the study drug at one of four different dose levels, and following a meal containing a small amount (1 gram) of gluten.

[118] In combination with the gluten, the subject is given a dose of unlabeled peptide consisting of the amino acid sequence LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77), where the dose may be 1; 10; 100 or 500 mg of peptide.

[119] At four hours after ingestion, a urine sample is obtained from the subject, which sample is contacted with a dipstick comprising a dye antibody that selectively binds to the peptide; and an immobilized second antibody that selectively binds to the peptide or the peptide antibody complex. Upon contact, the presence of the peptide is determined by localization of the dye label at the position where the second antibody is immobilized, which may for convenience be a shape of a "+", or other easily recognized symbol.

[120] In an alternative determination, a sample of blood is tested for the presence of peptide one hour after ingestion.

[121] The presence of the peptide in urine is indicative that intestinal permeability is undesirably high, suggesting that the peptide (and, by analogy, dietary gluten) is inadequately metabolized by ALV003 in the gastrointestinal lumen.

Example 3

Method of Determining Treatment Efficacy (II)

[122] In conjunction with a clinical trial for determining efficacy of the study drug CCX282-B (an orally active inhibitor of chemokine receptor CCR9) in mitigating the effects of gluten ingestion in patients with celiac disease. Alternative efficacy determination is made by evaluation of the effect of CCX282-B compared to placebo on the villous height/crypt depth ratio of small intestinal biopsy specimens taken from subjects with celiac disease, before and after gluten exposure. Adult patients are treated with 250mg capsule, twice daily, 13 weeks of the study drug.

[123] At selected time periods, the subject is given a dose of unlabeled peptide consisting of the amino acid sequence LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77), where the dose may be 1; 10; 100 or 500 mg of peptide.

[124] At four hours after ingestion, a urine sample is obtained from the subject, which sample is contacted with a dipstick comprising a dye antibody that selectively binds to the peptide; and an immobilized second antibody that selectively binds to the peptide or the

peptide antibody complex. Upon contact, the presence of the peptide is determined by localization of the dye label at the position where the second antibody is immobilized, which may for convenience be a shape of a "+", or other easily recognized symbol.

[125] The presence of the peptide in urine is indicative that the intestinal permeability is undesirably high, suggesting that an inflammatory response to gluten persists in the patient.

Example 4.

Method of Determining Treatment Efficacy (III)

[126] In conjunction with a clinical trial for determining efficacy of the study drug AT-1001 (Larazotide acetate). AT-1001 is an orally administered octapeptide zonulin receptor antagonist that appears to exert its inhibitory effect on gliadin-induced tight junction disassembly by blocking putative zonulin receptors on the luminal surface of the small intestine. Pretreatment with the peptide fails to inhibit gliadin induced zonulin release, while administration of zonulin analogues or gliadin in the presence of AT-1001 fail to significantly affect intestinal permeability, confirming the effect of the molecule is specific to the zonulin receptor. in mitigating the effects of gluten ingestion in patients with celiac disease.

[127] Adult celiac patients are given capsules of AT-1001 at doses of 1, 4, and 8 mg of drug, with 900 mg of gluten.

[128] At selected time periods, the subject is also given a dose of unlabeled peptide consisting of the amino acid sequence LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77), where the dose may be 1; 10; 100 or 500 mg of peptide.

[129] At four hours after ingestion, a urine sample is obtained from the subject, which sample is contacted with a dipstick comprising a dye antibody that selectively binds to the peptide; and an immobilized second antibody that selectively binds to the peptide or the peptide antibody complex. Upon contact, the presence of the peptide is determined by localization of the dye label at the position where the second antibody is immobilized, which may for convenience be a shape of a "+", or other easily recognized symbol.

[130] The presence of the peptide in urine is indicative that the intestinal permeability is undesirably high.

Example 5.

Method of Determining Treatment Efficacy (IV)

[131] In conjunction with a clinical trial for determining efficacy of the study drug Nexvax2. NexVax2 is a peptide-based vaccine to treat or prevent celiac disease. The vaccine will include the gluten peptides most commonly recognized by T cells in people with celiac disease.

[132] Adult celiac patients are given Nexvax2 at the following doses:

9 micrograms Nexvax2 weekly intra-dermal injection, 3 week duration

30 micrograms Nexvax2 weekly intra-dermal injection, 3 week duration

90 micrograms Nexvax2, weekly intra-dermal injection, 3 week duration

300 micrograms Nexvax2, weekly intra-dermal injection, 3 week duration

900 micrograms Nexvax2, weekly intra-dermal injection, 3 week duration

[133] At selected time periods, the subject is also given a dose of unlabeled peptide consisting of the amino acid sequence LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77), where the dose may be 1; 10; 100 or 500 mg of peptide.

[134] At four hours after ingestion, a urine sample is obtained from the subject, which sample is contacted with a dipstick comprising a dye antibody that selectively binds to the peptide; and an immobilized second antibody that selectively binds to the peptide or the peptide antibody complex. Upon contact, the presence of the peptide is determined by localization of the dye label at the position where the second antibody is immobilized, which may for convenience be a shape of a "+", or other easily recognized symbol.

[135] The presence of the peptide in urine is indicative that the intestinal permeability is undesirably high, presumably due to ongoing T cell inflammation by dietary gluten.

Example 6

Method of ongoing patient monitoring

[136] In conjunction with treatment modality for celiac sprue, the subject is given a dose of unlabeled peptide consisting of the amino acid sequence LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77), where the dose may be 1; 10; 100 or 500 mg of peptide to be ingested with normal meals.

[137] At four hours after ingestion, a urine sample is obtained from the subject, which sample is contacted with a dipstick comprising a dye antibody that selectively binds to the peptide; and an immobilized second antibody that selectively binds to the peptide or the peptide antibody complex. Upon contact, the presence of the peptide is determined by localization of the dye label at the position where the second antibody is immobilized, which may for convenience be a shape of a "+", or other easily recognized symbol.

[138] The presence of the peptide in urine is indicative that the peptide is not metabolized, and is indicative that intestinal permeability is undesirably high, and is indicative that the patient's diet and/or therapeutic regimen should be adjusted accordingly.

Example 7

Method of ongoing patient monitoring

[139] In conjunction with treatment modality for celiac sprue, the subject is given a dose of labeled peptide consisting of the amino acid sequence

LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF (HHH-33mer, SEQ ID NO:77), where a highly fluorescent label is conjugated to the amino terminus. The dose may be 1; 10; 100 or 500 mg of peptide to be ingested with normal meals. In an alternative embodiment the peptide is conjugated with a fluorescent label and a quencher, such that the fluorescence is quenched in the intact peptide, and is unquenched in a proteolytically cleaved peptide.

[140] At four hours after ingestion, a urine sample is obtained from the subject. Where the peptide is labeled with a fluorescent dye and quencher pair, the sample is analyzed for the presence of fluorescence. Fluorescence is indicative that the peptide is cleaved and is indicative that the treatment is appropriately metabolizing gluten.

[141] Where the peptide is labeled with only a fluorescent dye, the sample is separated by chromatography to determine whether the label is associated with free amino acids or with peptide.

[142] The presence of the label at a position corresponding to the peptide in urine is indicative that the peptide is not metabolized, and is indicative that intestinal permeability is undesirably high, and is indicative that the therapeutic regimen should be adjusted accordingly.

[143] These and other diagnostic methods of the invention can be practiced using the methods provided by the invention.

[144] All publications, patents, and patent applications cited in this specification are herein incorporated by reference as if each individual publication, patent, or patent application were specifically and individually indicated to be incorporated by reference.

[145] The present invention has been described in terms of particular embodiments found or proposed by the inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. Moreover, due to biological functional equivalency considerations, changes can be made in methods, structures, and compounds without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for assessing the efficacy of a therapeutic agent or therapeutic regimen in the treatment of an individual with an enteropathic disorder, the method comprising:

identifying an individual as having an enteropathic disorder;

administering an oral dose of a gluten peptide analog to the individual identified as having an enteropathic disorder, and

quantitating the presence of the gluten peptide analog and/or its metabolite(s) in at least one patient sample;

wherein the extent of metabolism of the gluten peptide analog to its metabolite is indicative of the efficacy of the therapeutic agent or regimen.

2. The method of Claim 1, wherein the enteropathic disorder is selected from celiac sprue, dermatitis herpetiformis, Crohn's disease and irritable bowel syndrome.

3. The method of Claim 2, wherein the individual is a human.

4. The method of Claim 1, wherein the metabolism is proteolytic digestion.

5. The method of Claim 1, wherein the metabolism is gut permeability.

6. The method of Claim 1, wherein the patient sample is selected from blood, saliva, urine and breath.

7. The method of Claim 1, wherein the steps of administering a gluten peptide analog and quantitating the presence of the gluten peptide analog and/or its metabolite(s) in at least one patient sample are performed at two or more time points, where the disease status of the individual is expected to differ between the time points as the result of administering a therapeutic agent, therapeutic regimen, or disease challenge to the individual.

8. The method of Claim 7, wherein the individual is one of a group of individuals in a clinical trial.

9. The method of Claim 8, wherein the clinical trial is a crossover trial.

10. The method of Claim 8, wherein the clinical trial is a double blinded parallel trial.

11. An oligopeptide analog of a native gluten peptide, wherein the peptide is from 14 to 40 amino acids in length, and is modified from a native gluten peptide in the replacement of at least one, at least two two, at least three, and not more than about five amino acid residues, wherein the analog peptide has substantially similar resistance to gastrointestinal proteases of a native gluten peptide, but is has decreased activity as a substrate for tissue transglutaminase, and has decreased binding affinity to human MHC DQ protein.

12. The oligopeptide analog of Claim 1, wherein the native gluten peptide is selected from SEQ ID NO:1 to SEQ ID NO:38.

13. The oligopeptide of Claim 12, wherein from one to three glutamine residues of a native gluten peptide are substituted with an amino acid other than glutamine and other than a positively charged amino acid.

14. The oligopeptide of Claim 13, wherein said one to three glutamine residues are substituted with histidine, asparagine, or a combination thereof.

15. A method of diagnosing an enteropathic disorder, the method comprising:

administering an oral dose of a gluten peptide analog to the individual suspected of having an enteropathic disorder, and

quantitating the presence of the gluten peptide analog and/or its metabolite(s) in at least one patient sample;

wherein the extent of metabolism of the gluten peptide analog to its metabolite is indicative of the presence of the enteropathic disorder.

16. The method of Claim 15, wherein the enteropathic disorder is selected from celiac sprue, dermatitis herpetiformis, Crohn's disease and irritable bowel syndrome.

17. The method of Claim 16, wherein the individual is a human.

18. The method of Claim 17, wherein the metabolism is proteolytic digestion.

19. The method of Claim 17, wherein the metabolism is gut permeability.

20. A kit for monitoring treatment of an enteropathic disorder, the kit comprising an analog peptide according to Claim 14, and a container for sample collection.

21. The kit according to Claim 20, further comprising a therapeutic agent for treatment of said enteropathic disorder.

QQQ–33–mer:	LQLQPF(PQPQLPY)$_3$ PQPQPF (native 33-mer)
EEE–33–mer:	LQLQPF(PQPELPY)$_3$ PQPQPF (deamidated 33-mer)
NNN–33–mer:	LQLQPF(PQPNLPY)$_3$ PQPQPF (biomarker)
HHH–33–mer:	LQLQPF(PQPHLPY)$_3$ PQPQPF (biomarker)
Myoglobin peptide:	KGHHEAELKPL (control)

FIG. 1

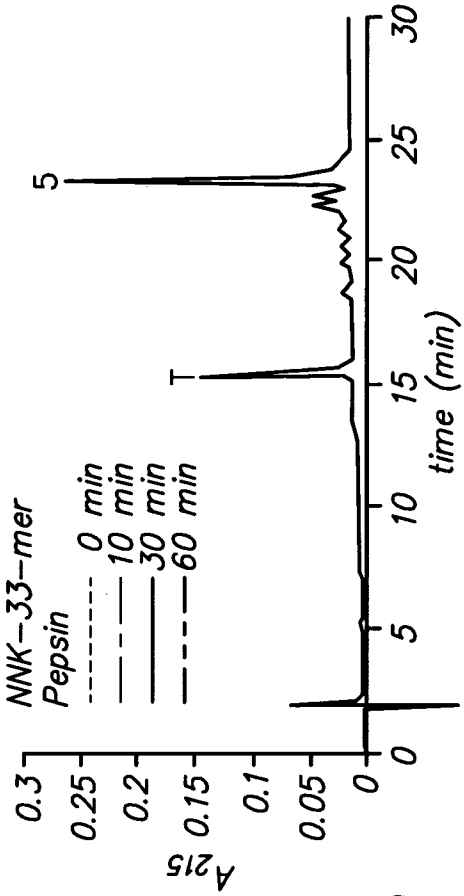


FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

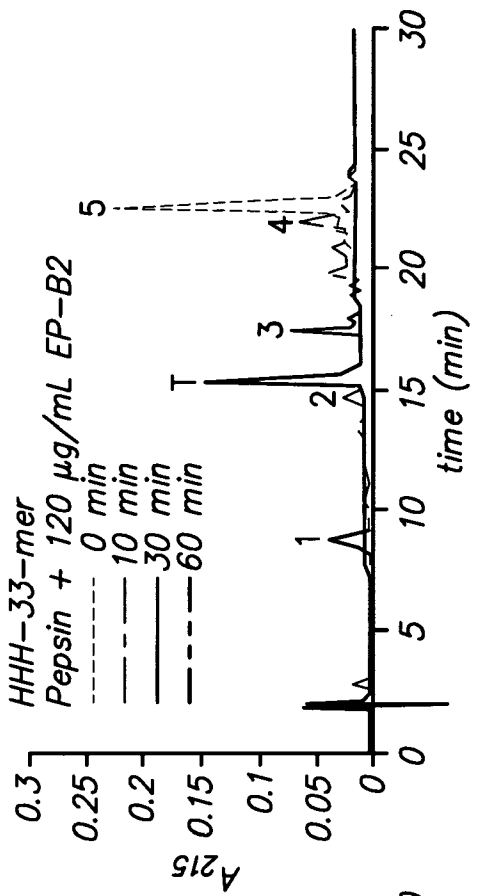


FIG. 2F

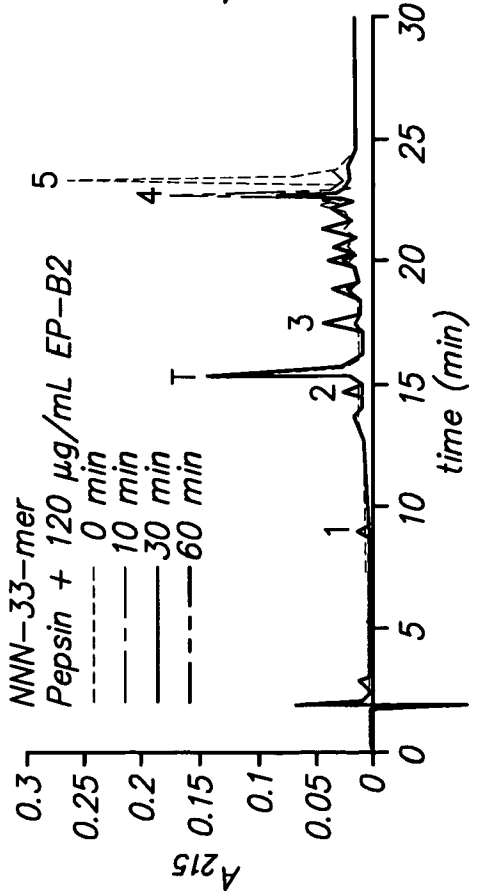


FIG. 2E

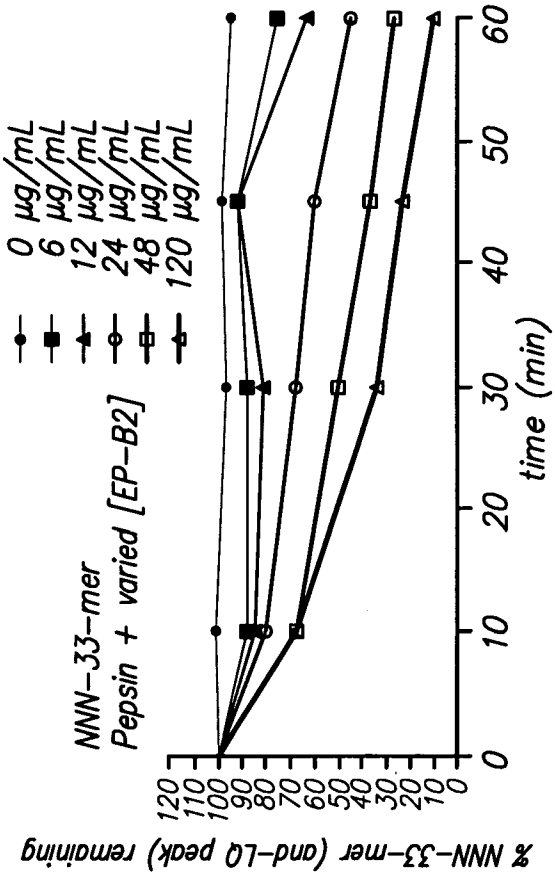


FIG. 2H

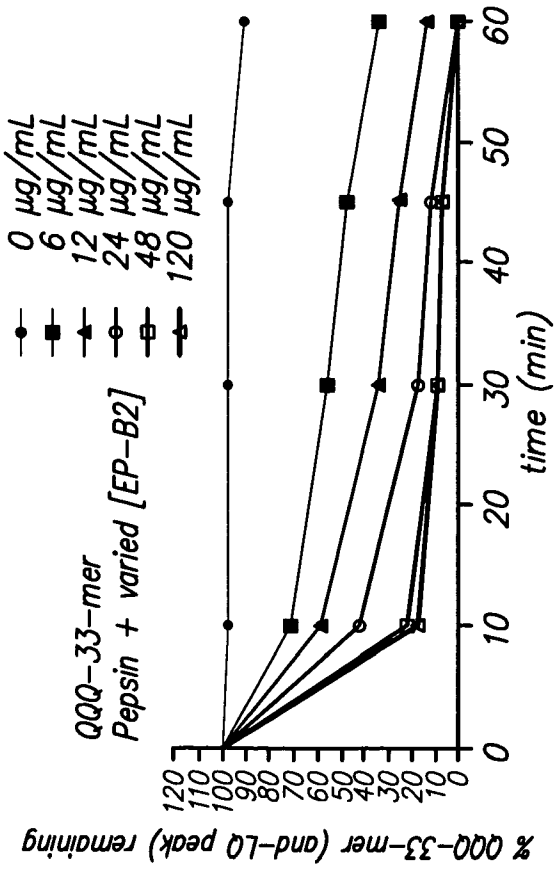


FIG. 2G

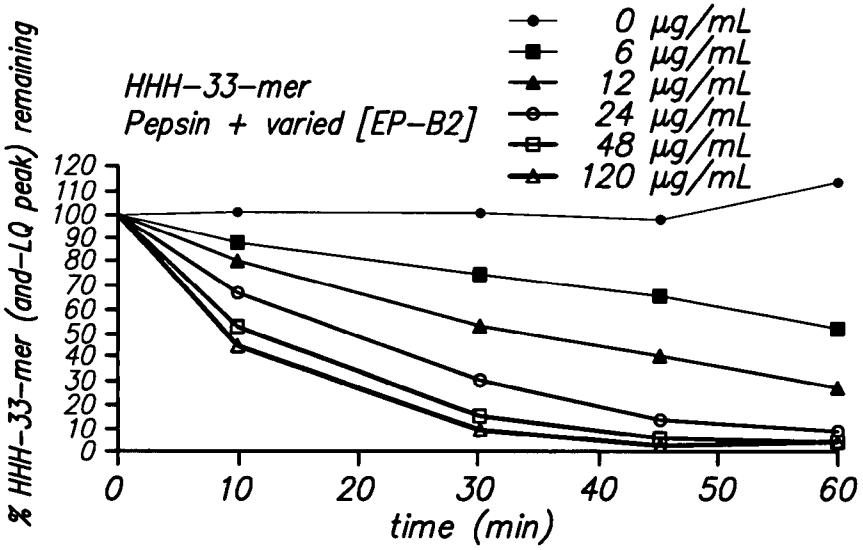


FIG. 2I

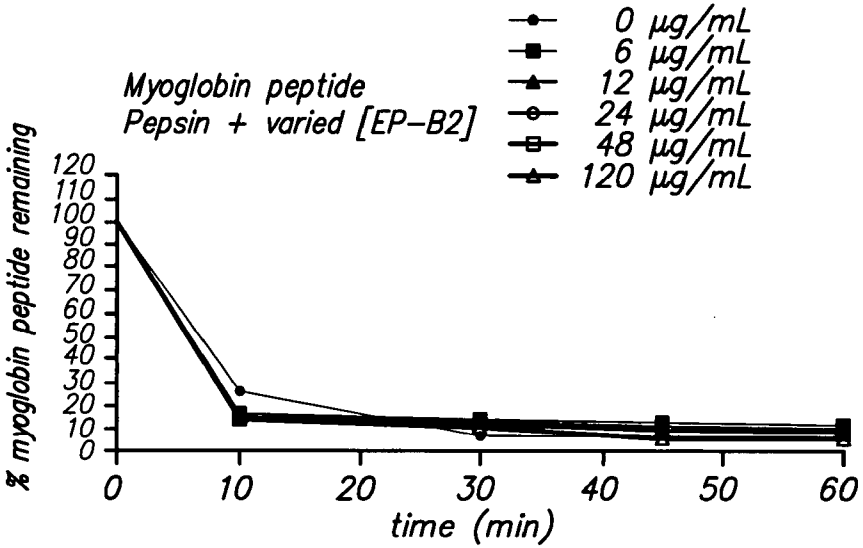


FIG. 2J

HPLC peak A	QQQ–33–mer MW (daltons)	QQQ–33–mer Peptide sequence	NNN–33–mer MW (daltons)	NNN–33–mer Peptide sequence	HHH–33–mer MW (daltons)	HHH–33–mer Peptide sequence
1	842	LPYPQPQ	828	LPYPQPN	851	LPYPQPH
2	954	LQPFPQPQ	940	LQPFPQPN	983	LQPFPQPH
3	1088	LPYPQPQPF	1088	LPYPQPQPF	1088	LPYPQPQPF
4	3670	LQPF(PQPQLPY)$_2$PQPQPF	3626	LQPF(PQPNLPY)$_2$PQPQPF	3687	LQPF(PQPHLPY)$_2$PQPQPF
5	3911	LQLQPF(PQPQLPY)$_2$PQPQPF	3859	LQLQPF(PQPNLPY)$_2$PQPQPF	3938	LQLQPF(PQPHLPY)$_2$PQPQPF

T TAME internal standard

FIG. 2K

FIG. 3A

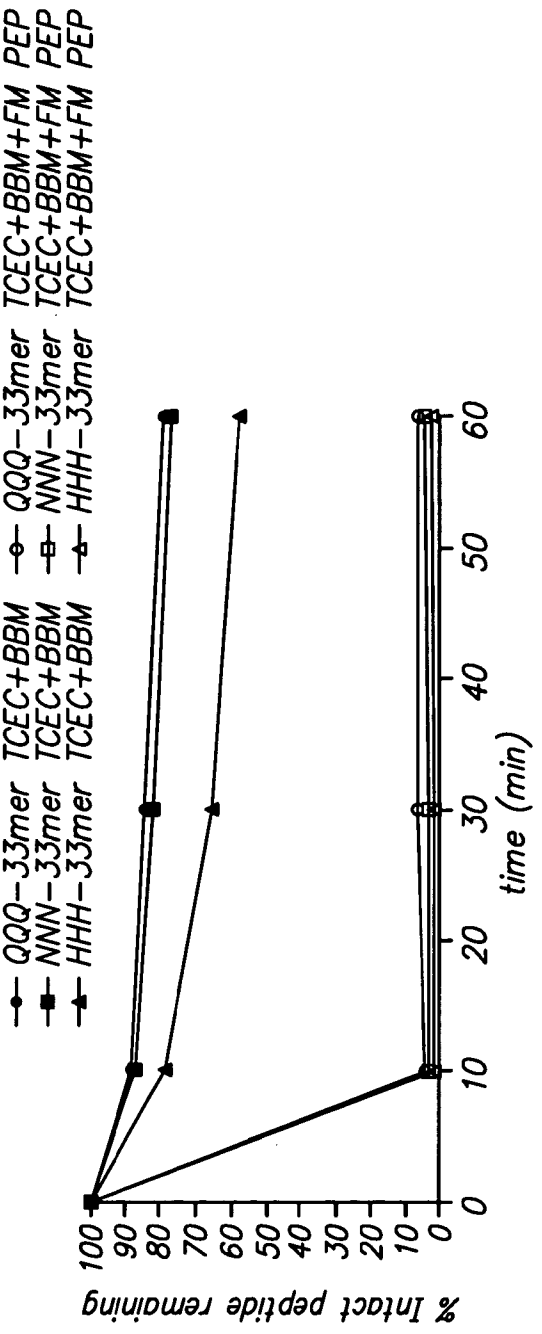


FIG. 3B

▲ Cleavage site from Pepsin + EP–B2, 60 min –> TCEC, 10min
▵ Cleavage site from Pepsin, 60 min –> TCEC + FM PEP, 10min

QQQ–33–mer: LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF

NNN–33–mer: LQLQPFPQPNLPYPQPNLPYPQPNLPYPQPQPF

HHH–33–mer: LQLQPFPQPHLPYPQPHLPYPQPHLPYPQPQPF

2 1 1 3

FIG. 3C

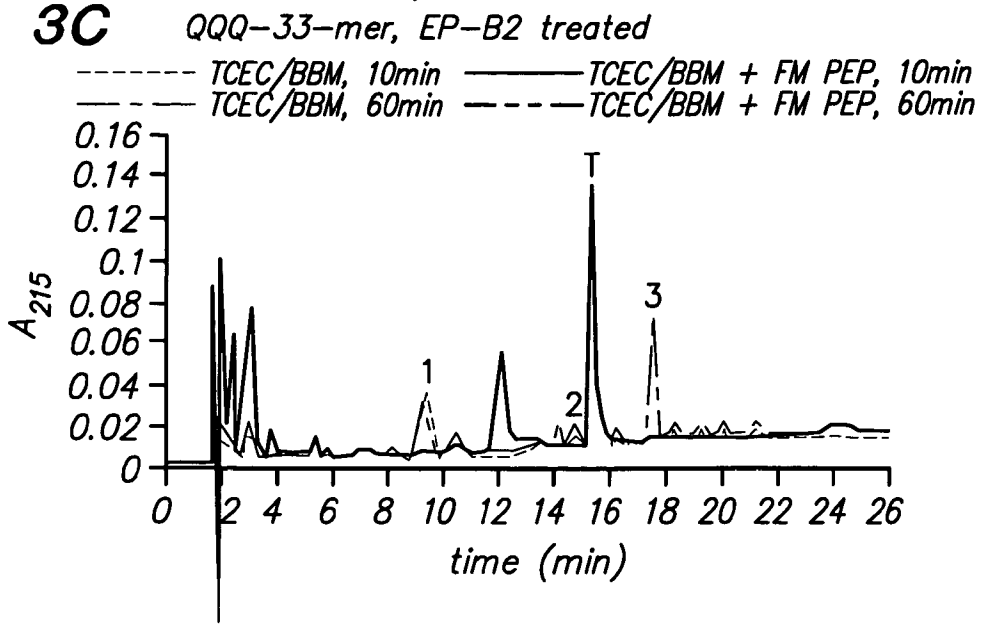


FIG. 3D

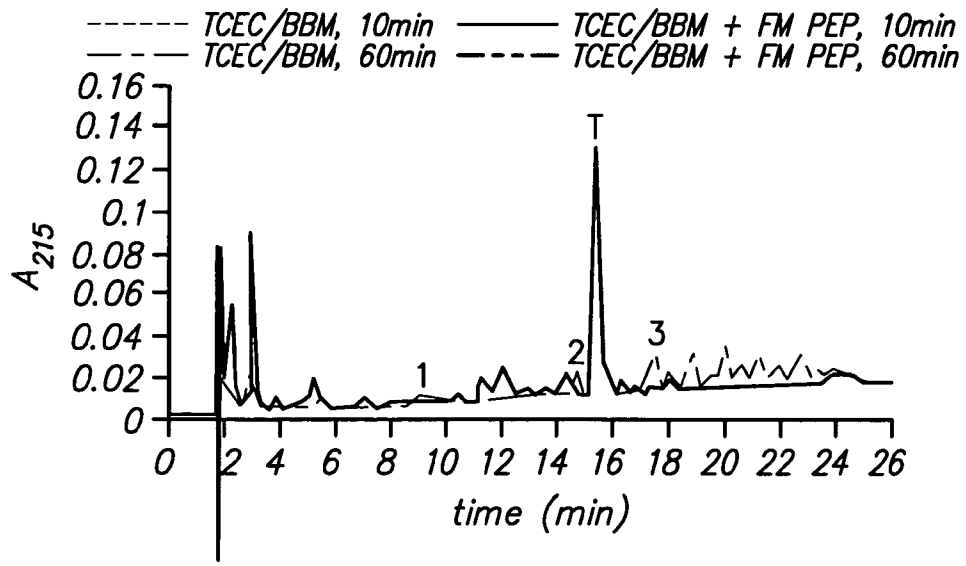
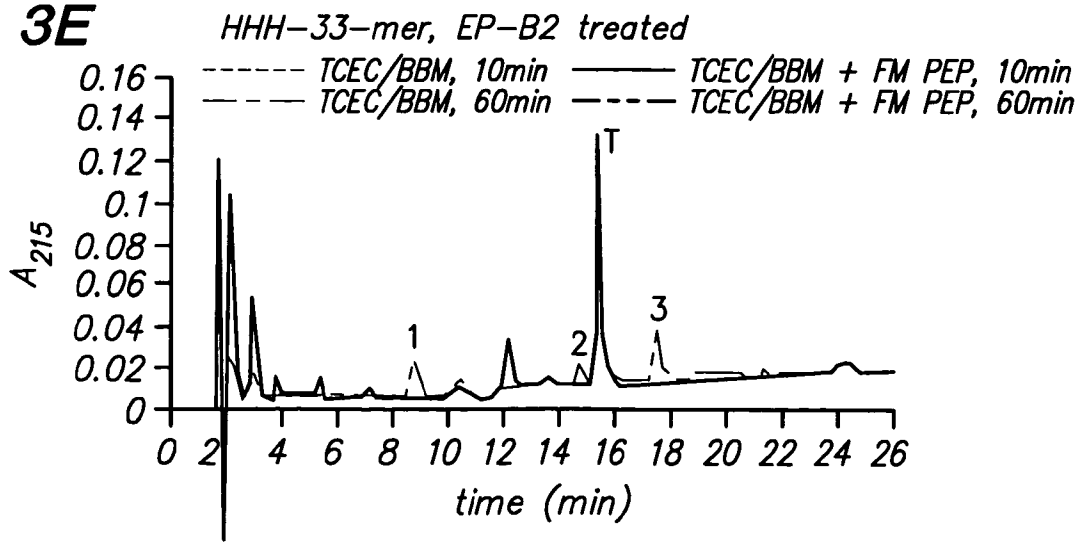


FIG. 3E



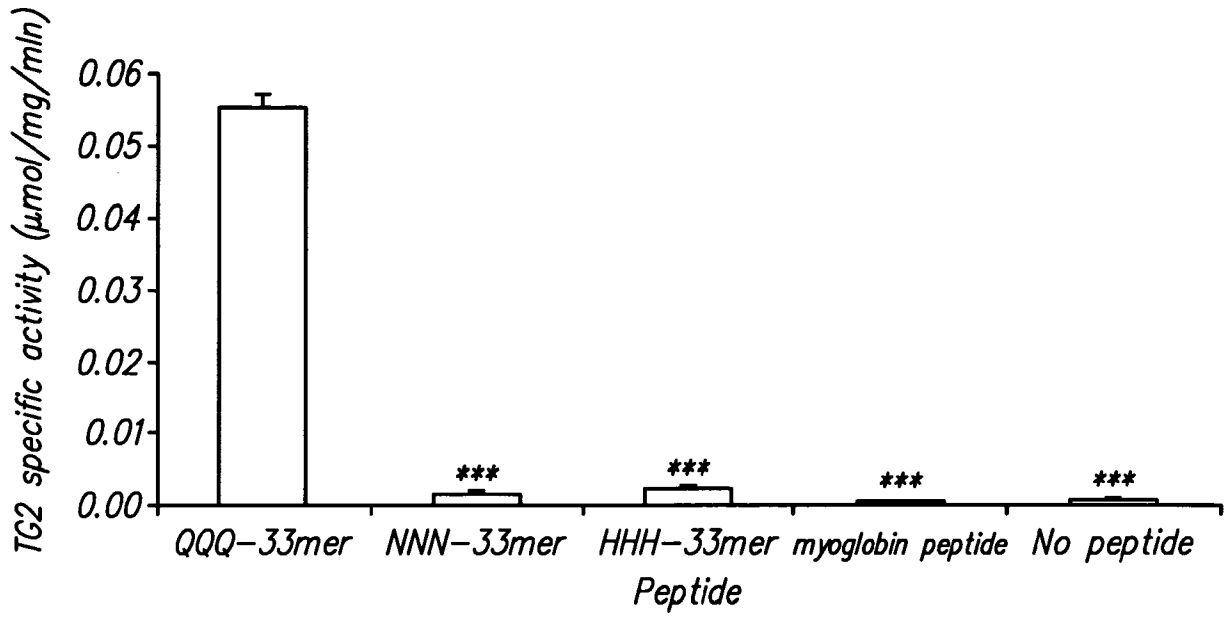

TG2 specific activity (µmol/mg/mln)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
***
***
***
***
QQQ–33mer
NNN–33mer
HHH–33mer
myoglobin peptide
No peptide
Peptide

FIG. 4A

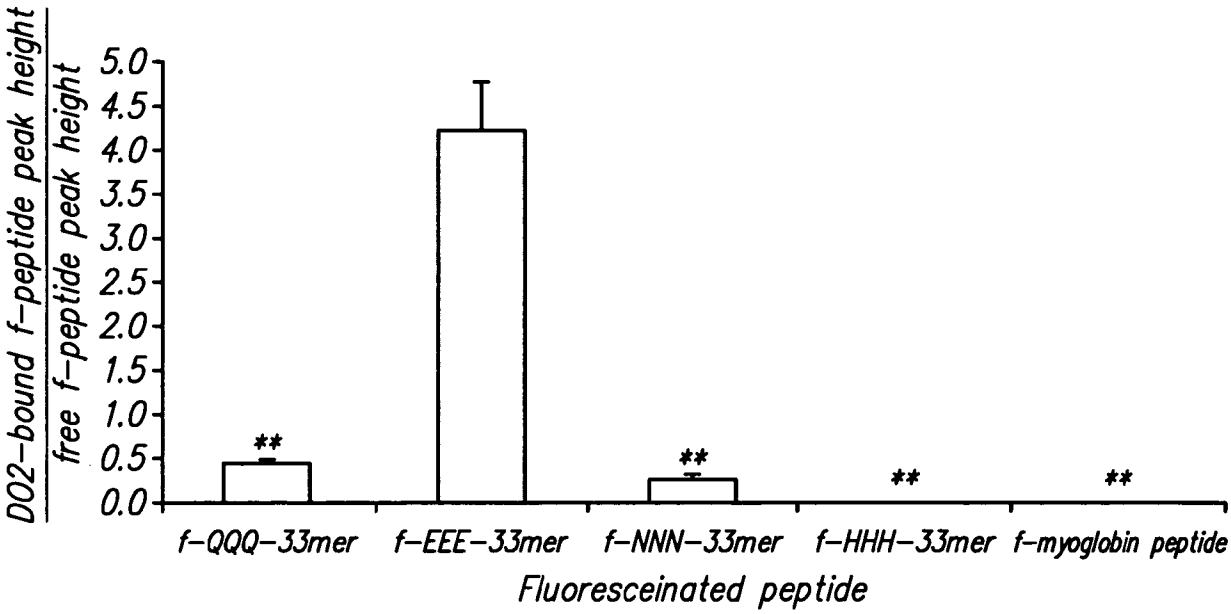

DO2–bound f–peptide peak height / free f–peptide peak height
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
f–QQQ–33mer
f–EEE–33mer
f–NNN–33mer
f–HHH–33mer
f–myoglobin peptide
Fluoresceinated peptide

FIG. 4B

FIG. 5A

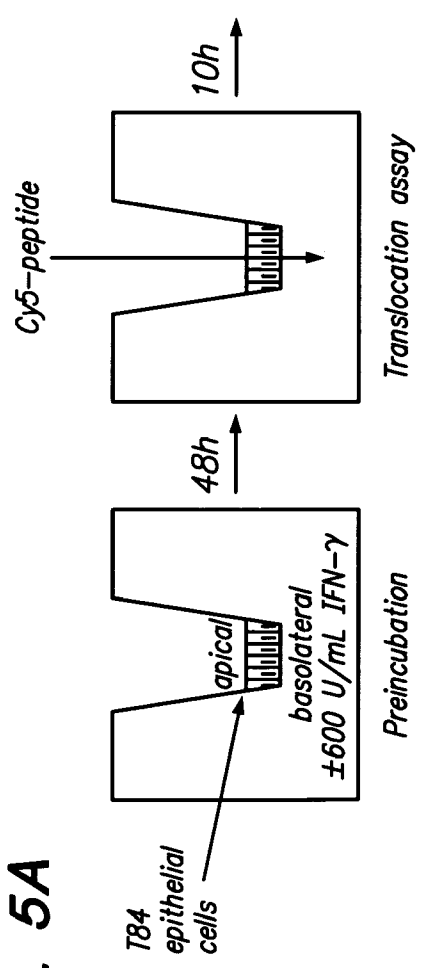
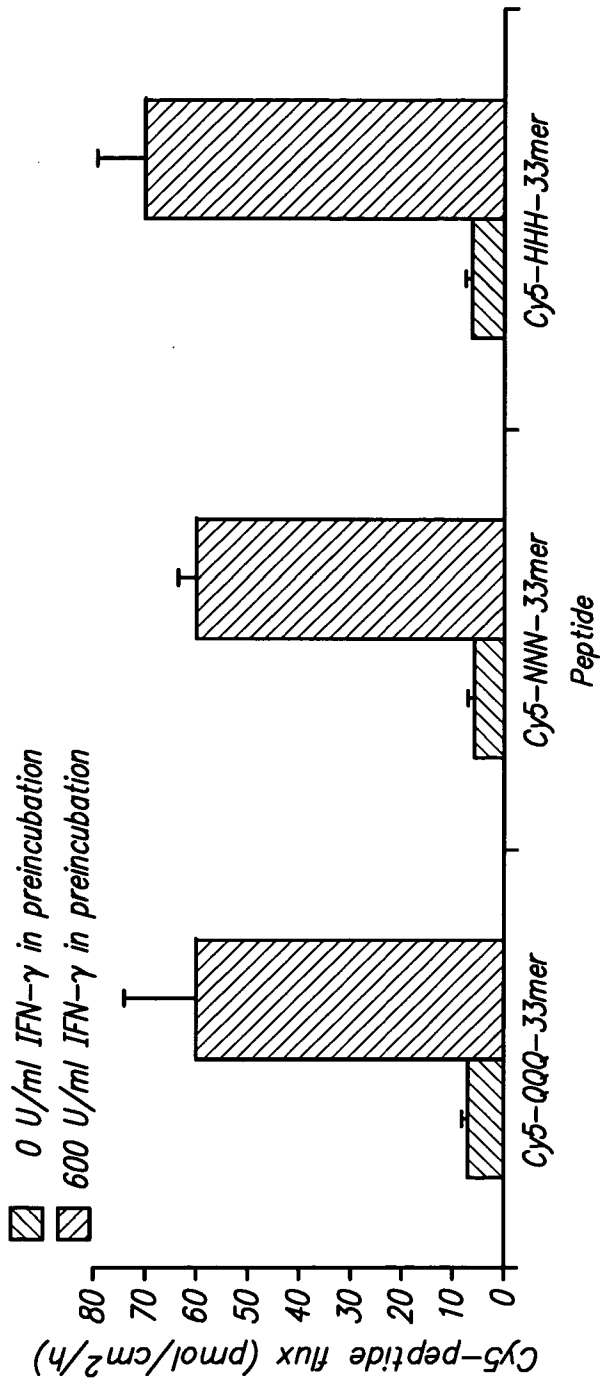

T84 epithelial cells
apical
basolateral
±600 U/mL IFN-γ
Preincubation
48h
Cy5-peptide
10h
Translocation assay
Sample basolateral chamber over time and measure fluorescence in 95-well format to determine flux.
Analyze 0 and 10 h timepoints by LC-MS

FIG. 5B


0 U/ml IFN-γ in preincubation
600 U/ml IFN-γ in preincubation
Cy5-peptide flux (pmol/cm2/h)
0
10
20
30
40
50
60
70
80
Cy5-QQQ-33mer
Cy5-NNN-33mer
Cy5-HHH-33mer
Peptide

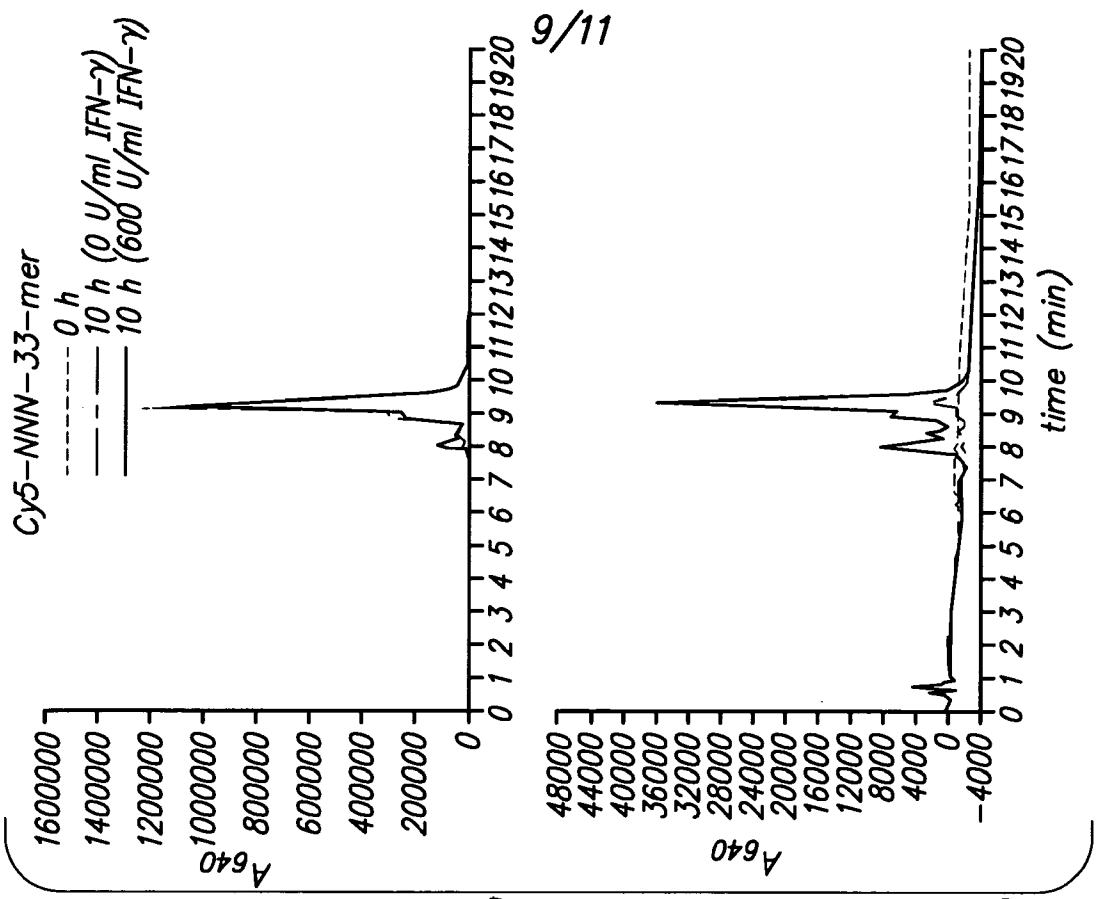

Cy5-NNN-33-mer
0 h
10 h (0 U/ml IFN-γ)
10 h (600 U/ml IFN-γ)
A640
time (min)

FIG. 5D

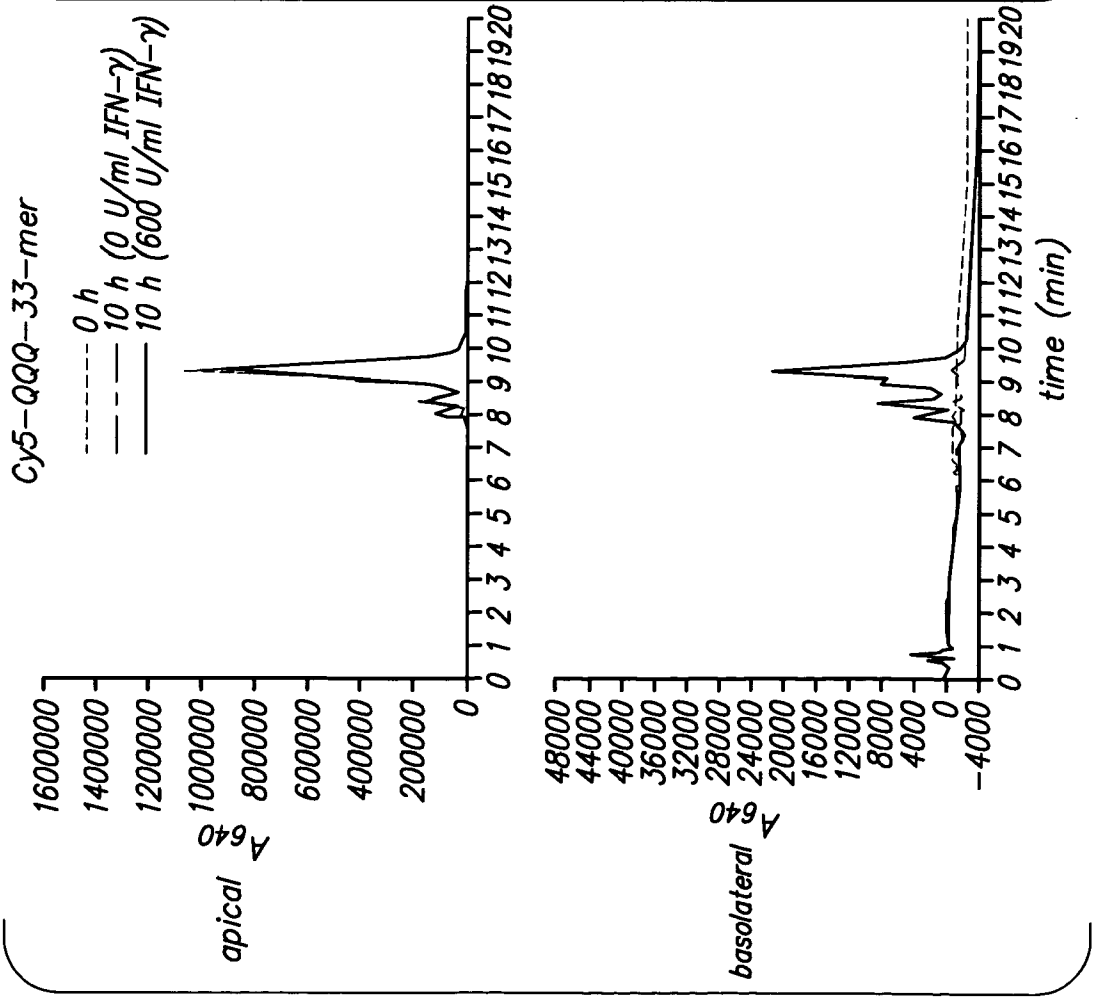

Cy5-QQQ-33-mer
0 h
10 h (0 U/ml IFN-γ)
10 h (600 U/ml IFN-γ)
apical
basolateral
A640
time (min)

FIG. 5C

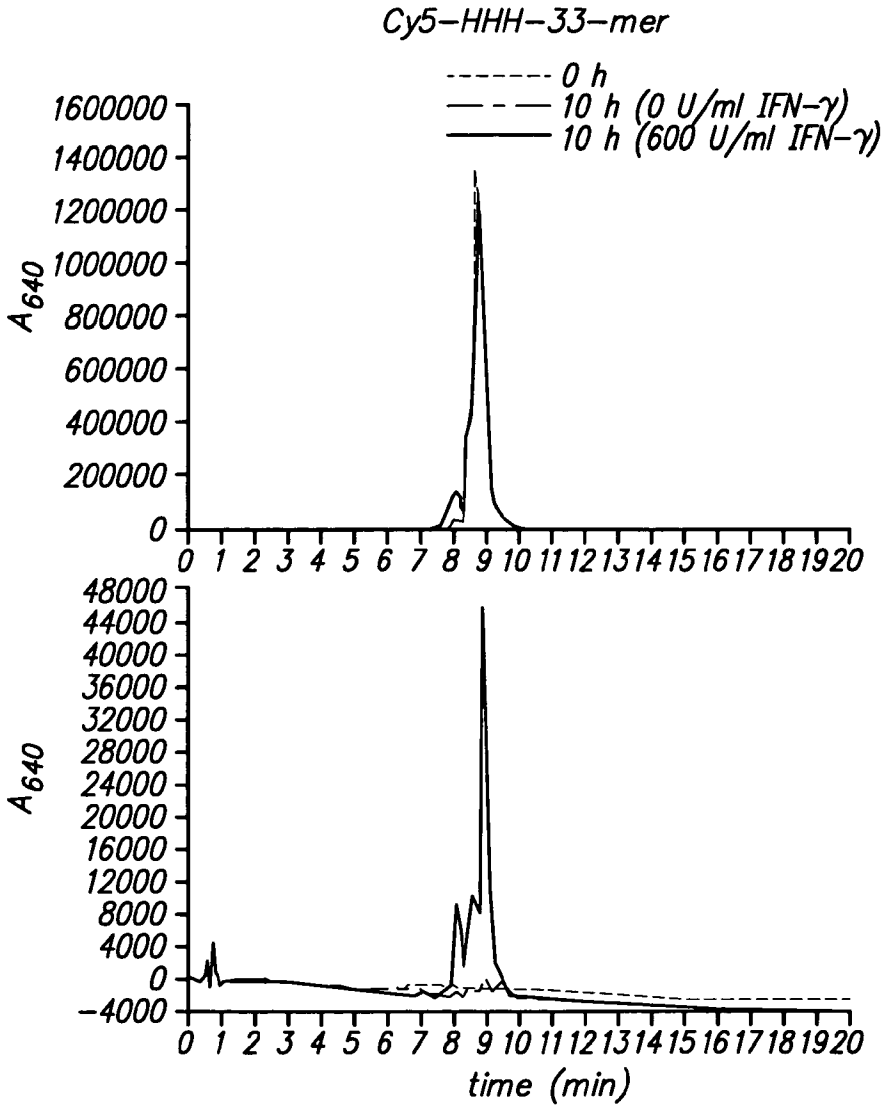

Cy5-HHH-33-mer
0 h
10 h (0 U/ml IFN-γ)
10 h (600 U/ml IFN-γ)
A640
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A640
48000
44000
40000
36000
32000
28000
24000
20000
16000
12000
8000
4000
0
-4000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
time (min)

FIG. 5E

Peptide	Apical concentration 0 h	0 U/ml IFN-γ preincubation		600 U/ml IFN-γ preincubation	
		Apical concentration 10 h	Basolateral concentration 10 h	Apical concentration 10 h	Basolateral concentration 10 h
Cy5-QQQ-33-mer	20 μM (100%)	19.1 μM (95.3%)	0.034 μM (0.2%)	17.0 μM (84.9%)	0.452 μM (2.3%)
Cy5-NNN-33-mer	20 μM (100%)	19.1 μM (95.6%)	0.062 μM (0.3%)	16.2 μM (81.1%)	0.572 μM (2.9%)
Cy5-HHH-33-mer	20 μM (100%)	19.0 μM (95.2%)	0.060 μM (0.3%)	17.2 μM (86.0%)	0.559 μM (2.8%)

% remaining relative to initial apical concentration

FIG. 5F

专利名称(译)	非炎性谷蛋白肽类似物作为腹腔口炎的生物标志物		
公开(公告)号	EP2277046A2	公开(公告)日	2011-01-26
申请号	EP2009746974	申请日	2009-05-13
[标]申请(专利权)人(译)	斯坦福大学		
申请(专利权)人(译)	THE利兰·斯坦福，齐齐哈尔大学董事会		
当前申请(专利权)人(译)	THE利兰·斯坦福，齐齐哈尔大学董事会		
[标]发明人	BETHUNE MICHAEL THOMAS KHOSLA CHAITAN		
发明人	BETHUNE, MICHAEL, THOMAS KHOSLA, CHAITAN		
IPC分类号	G01N33/53		
CPC分类号	A61K38/011 G01N33/6893 G01N2800/065 G01N2800/52		
优先权	61/053949 2008-05-16 US		
外部链接	Espacenet		

摘要(译)

通过检测患者代谢口服施用的谷蛋白肽的能力来评估肠病性疾病的诊断或患有肠病的患者对治疗的响应，在临床试验环境中或在长期疾病管理设置中。模拟。可以以多种方式监测肽代谢。方便地，在口服给药后的一段时间内，在患者样品中检测肽的代谢物的外观，例如，在尿液，血浆，呼吸，唾液等中，谷蛋白肽类似物任选被标记，例如，具有同位素，荧光等标签。