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Noninflammatory Gluten Peptide Analogs as Biomarkers for Celiac Sprue

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SUMMARY

New tools are needed for managing celiac sprue, a lifelong immune disease of the small intestine. Ongoing drug trials are also prompting a search for noninvasive biomarkers of gluten-induced intestinal change. We have synthesized and characterized noninflammatory gluten peptide analogs in which key Gln residues are replaced by Asn or His. Like their proinflammatory counterparts, these biomarkers are resistant to gastrointestinal proteases, susceptible to glutenases, and permeable across enterocyte barriers. Unlike gluten peptides, however, they are not appreciably recognized by transglutaminase, HLA-DQ2, or disease-specific T cells. In vitro and animal studies show that the biomarkers can detect intestinal permeability changes as well as glutenase-catalyzed gastric detoxification of gluten. Accordingly, controlled clinical studies are warranted to evaluate the use of these peptides as probes for abnormal intestinal permeability in celiac patients and for glutenase efficacy in clinical trials and practice.

INTRODUCTION

Celiac sprue is a common disease of the small intestine, the primary environmental and genetic causes of which are well understood. The environmental cause of this immune disease is dietary gluten from wheat and similar proteins from rye and barley (Dicke et al., 1953). Gastrointestinal digestion of gluten releases proteolytically resistant, immunotoxic peptide fragments, such as the 33-mer from α-gliadin (Shan et al., 2002). These peptides traverse the mucosal epithelium by unknown mechanisms and are deamidated at specific glutamine residues by an endogenous enzyme, transglutaminase 2 (TG2) (Arentz-Hansen et al., 2000; Molberg et al., 1998). Deamidated peptides bind with high affinity to the primary genetic determinant of celiac sprue, human leukocyte antigen (HLA)-DQ2 (Kim et al., 2004; Quarsten et al., 1999), a major histocompatibility complex class II molecule possessed by >90% of patients (Sollid et al., 1989). Upon encountering DQ2-gluten complexes on the surface of antigen-presenting cells, gluten-specific, DQ2-restricted $CD4^+$ T cells mediate a Th1 response comprising the secretion of proinflammatory cytokines such as interferon-γ (IFN-γ) (Nilsen et al., 1995, 1998; Troncone et al., 1998) and the recruitment of $CD8^+$ intraepithelial lymphocytes, ultimately causing villous atrophy and crypt hyperplasia (Jabri et al., 2005). Additionally, $CD4^+$ T cells amplify a humoral immune response to dietary gluten comprising production of both gluten-specific antibodies and TG2-specific autoantibodies (Sollid, 2002). In many affected individuals, this molecular pathogenesis is manifested clinically as nutrient malabsorption, wasting, and/or chronic diarrhea (Alaedini and Green, 2005; Green and Jabri, 2006). Chronic inflammation caused by recurrent exposure to gluten is associated with the increased incidence of bone disorders, neurological complications, and cancer (Catassi et al., 2002; Corrao et al., 2001). 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 induces relapse (See and Murray, 2006).

Whereas researchers are translating many of the above fundamental insights into clinical practices that facilitate initial diagnosis (Anderson et al., 2005; Dieterich et al., 1997; Koskinen et al., 2008; Raki et al., 2006, 2007), there are serious deficiencies in the available spectrum of tools for the long term care of celiac patients (Silvester and Rashid, 2007). The cornerstone of disease management is exclusion of gluten from the diet. However, a gluten-free diet is extremely difficult to maintain due to the ubiquity of gluten in human foods and a majority of patients exhibit incomplete recovery on a gluten-free diet (Ciacci et al., 2002; Cornell et al., 2005; Pietzak, 2005). As a result, there is considerable interest in the development of non-dietary therapies that improve the health and quality of life of celiac sprue patients. The current study was motivated by the need for accurate, noninvasive tools for managing patients who practice dietary gluten control and for assessing the efficacy of new drugs in such patients on an ongoing basis.

Several recent studies have highlighted the potential of orally administered gluten-specific proteases (i.e., glutenases) for treating celiac sprue. Since the proteolytic resistance of gluten peptides stems from their high proline (~15%) and glutamine (~35%) content, efforts are primarily focused on enzymes

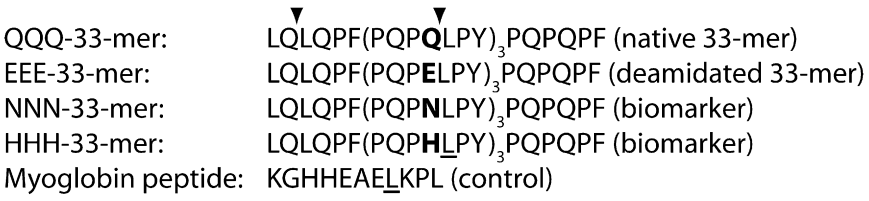


Figure 1. Synthetic Gluten and Biomarker Peptide Sequences

Sequences are shown for the native 33-mer (designated QQQ-33-mer) derived from α2-gliadin, the synthetically deamidated 33-mer (EEE-33-mer), the biomarkers NNN-33-mer and HHH-33-mer, and a noninflammatory 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.

capable of cleaving proximal to these residues. Microbial prolyl endopeptidases (PEP) detoxify immunodominant gluten in vitro (Shan et al., 2002; Stepniak et al., 2006) and are especially effective when complemented with a barley endoprotease, EP-B2, that preferentially cleaves gluten C-terminal to glutamine residues (Gass et al., 2007; Siegel et al., 2006). The therapeutic promise of these oral proteases is further underscored by their ability to digest gluten in the rat stomach (Gass et al., 2006, 2007) and by the ability of EP-B2 to protect a gluten-sensitive rhesus macaque against gluten-induced clinical relapse (Bethune et al., 2008b). To bring therapeutic glutenases to bear on the human condition of celiac sprue, however, safe and effective tools for assessing enzyme efficacy in human celiac patients are needed. A similar need exists for therapies that have alternative modes of action such as larazotide acetate, which is anticipated to reverse tight junction dysfunction in celiac patients (Paterson et al., 2007), or CCX282, an antagonist of the CCR9 chemokine receptor (Peyrin-Biroulet et al., 2008).

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 analog 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 not be recognized by human TG2 or HLA-DQ2. Consequently, it must not stimulate an inflammatory response from disease-specific T cells. We present two examples of biomarkers that meet these criteria and demonstrate their utility in animal models.

RESULTS

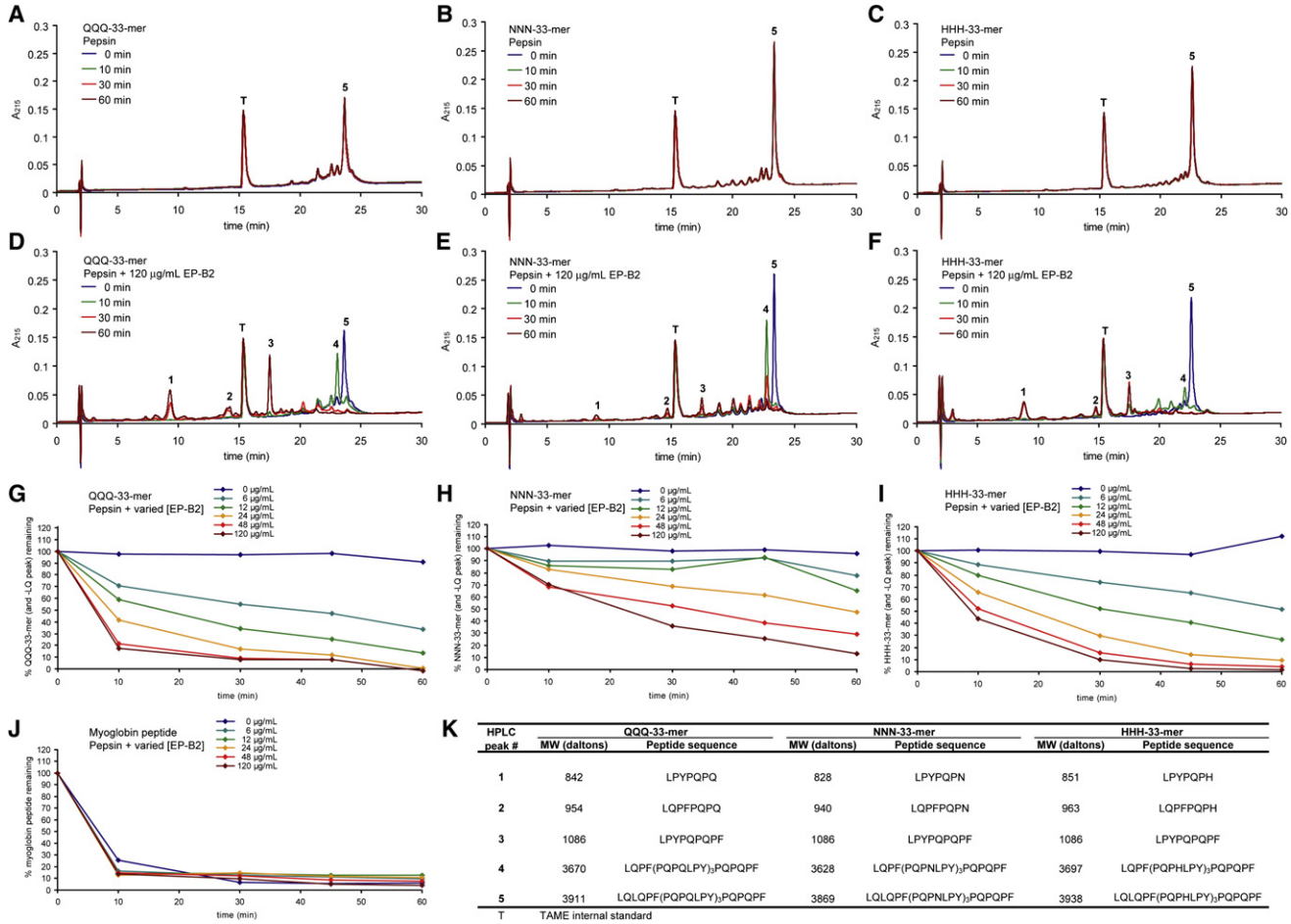
Design of Gluten Peptide-Based Biomarkers

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 analog was that, within the scope of naturally occurring (i.e., dietary) amino acids, this conservative change would minimally perturb recognition by the EP-B2 glutenase (Bethune et al., 2006) (Figure 1, arrowheads). The rationale for the Q→H analog 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-DQ2 (Kim et al., 2004; Quarsten et al., 1999). 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. As controls for our study, we synthesized a peptide derived from myoglobin with moderate proteolytic resistance but unrelated sequence (Piper et al., 2004), as well as EEE-33-mer, the synthetically deamidated analog of the 33-mer (Figure 1). The structures and purity of all peptides were confirmed by liquid chromatography-assisted mass spectrometry (LC-MS) (see Figure S1 available online).

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 (Shan et al., 2002), 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 (Figures 2B and 2C). To test whether the experimental glutenase EP-B2 accelerates 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 (Figures 2E and 2F) in a time- and dose-dependent manner (Figures 2G–2I). In 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 analogs did not substantially alter the sites of susceptibility to EP-B2-mediated digestion.

We next performed simulated duodenal digests to determine whether the biomarkers are resistant to pancreatic proteases



K

HPLC peak #	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	963	LQPFPQPH
3	1086	LPYPQPQPF	1086	LPYPQPQPF	1086	LPYPQPQPF
4	3670	LQPF(PQPQLPY)$_3$PQPQPF	3628	LQPF(PQPNLPY)$_3$PQPQPF	3697	LQPF(PQPHLPY)$_3$PQPQPF
5	3911	LQLQPF(PQPQLPY)$_3$PQPQPF	3869	LQLQPF(PQPNLPY)$_3$PQPQPF	3938	LQLQPF(PQPHLPY)$_3$PQPQPF
T	TAME internal standard					

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 QQQ-33-mer (A and D), NNN-33-mer (B and E), and HHH-33-mer (300 μM) (C and F) after simulated gastric digestion for specified durations with either pepsin alone (A–C) or pepsin and 120 μg/mL EP-B2 (D–F). 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 QQQ-33-mer (G), NNN-33-mer (H), HHH-33-mer (I), and myoglobin peptide (J) 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 percentage 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 (A)–(F). Data shown are representative of three independent experiments.

(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 min 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 min, 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 min, 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 (Shan et al., 2002), 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), suggesting these biomarkers can be used to assess efficacy of combination enzyme therapies (Gass et al., 2007; Siegel et al., 2006). Indeed, addition of FM PEP enabled further digestion of fragments remaining after treatment of the 33-mer and biomarkers with EP-B2 (Figures 3C–3E).

Biomarkers Are Noninflammatory 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,

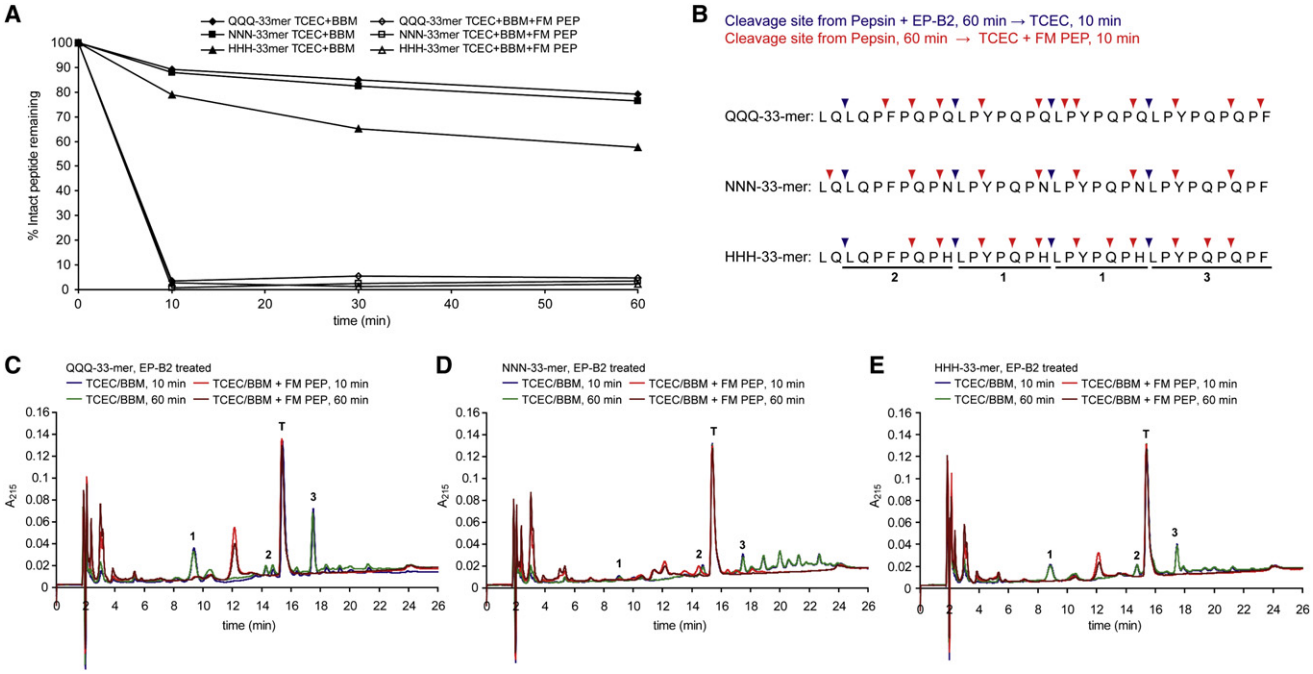


Figure 3. The 33-mer Gluten Peptide and Gluten Peptide-Based Biomarkers Are Similarly Resistant to Cleavage by Pancreatic Enzymes and Susceptible to Cleavage by Prolyl Endopeptidase

(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 PEP from FM PEP. Following treatment with pepsin, each peptide (300 μM) was digested in vitro with pancreatic proteases (TCEC) and rat intestinal 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 percentage 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 QQQ-33-mer (C), NNN-33-mer (D), and HHH-33-mer (E) (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 B. Data shown are representative of two independent experiments.

or stimulate a strong immune response by preexisting 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 NADH (Piper et al., 2002). Consistent with previous results (Shan et al., 2002), the 33-mer was readily deamidated by TG2 (Figure 4A). In 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 and at 37°C to simulate the endocytic environment. After 45 hr, DQ2-bound and free fluorescein-labeled peptides were separated by high-performance size exclusion chromatography (HPSEC) and the ratio of their peak heights was determined by fluorometry. Consistent with previous results (Xia et al., 2006), >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. In all three cell lines and both clones tested, nanomolar concentrations of TG2-treated 33-mer were sufficient to induce proliferation, with EC_{50} values ranging from ~10 to 200 nM (Figure 4C). The low immunostimulatory capacity of the biomarkers toward any of the cell lines and clones precluded response saturation and EC_{50} determination. However, it is apparent that the immunostimulatory capacity of NNN-33-mer was reduced ~1000-fold

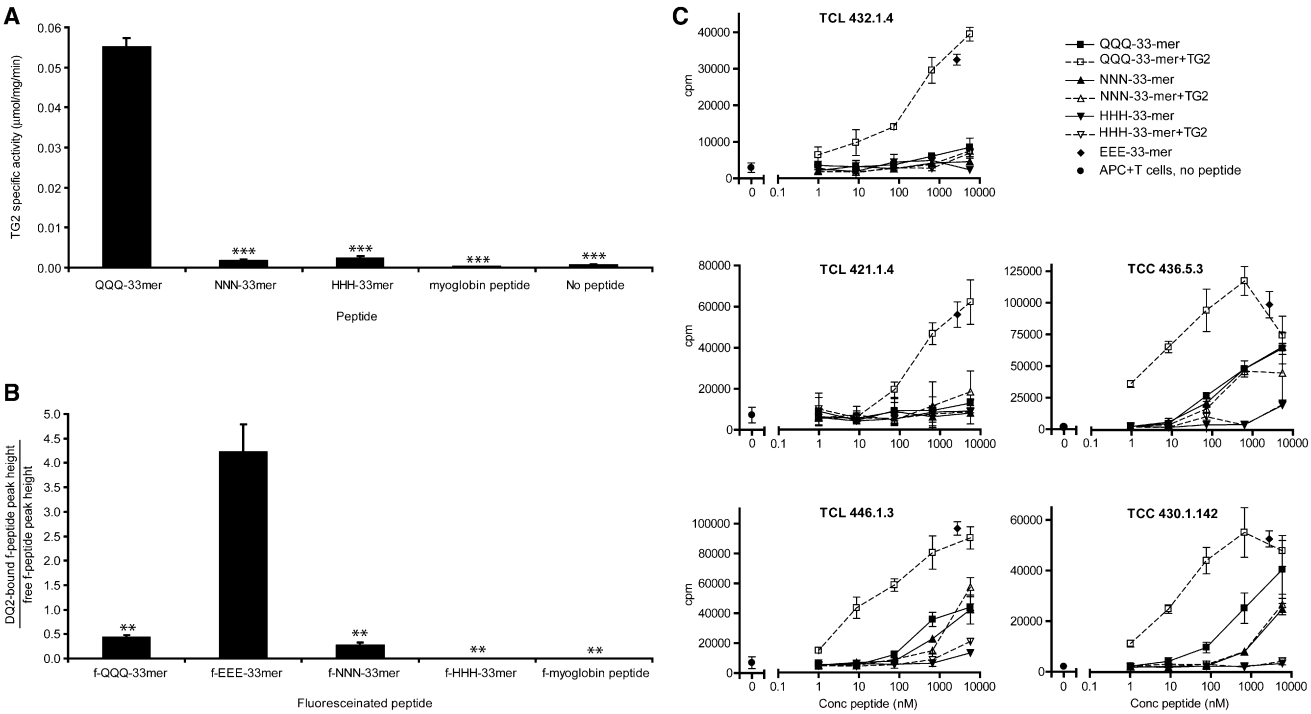


Figure 4. Biomarkers Are Noninflammatory in the Context of Celiac Sprue

(A) Specific activity of 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 ± SD for triplicate assays are shown. Data are representative of three 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-α-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 hr at 37°C. Means ± SD for triplicate assays are shown. Data are representative of three independent experiments. Statistical comparisons were performed with respect to samples containing f-EEE-33-mer. **$p < 0.01$.

(C) Activation of celiac patient-derived intestinal T cell lines (TCL) and T cell clones (TCC) in response to incubation with DQ2-homozygous antigen-presenting cells preloaded with varied concentrations of ±TG2-treated QQQ-33-mer, NNN-33-mer, or HHH-33-mer. Positive (2000 nM synthetically deamidated EEE-33-mer) and negative (no peptide) controls are shown for comparison. Activation is measured in terms of counts per minute (cpm) of [^{3}H]-thymidine incorporated into harvested DNA of proliferating T cells. Means ± SD for triplicate wells are shown.

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.

Biomarker Metabolism Parallels Gluten Digestion In Vivo

The potential for noninflammatory gluten peptide-based biomarkers to report on in vivo gluten detoxification by oral glutenase therapy was evaluated in rats. Fasted rats were administered a meal containing 1 g gluten supplemented with 0, 10, or 40 mg EP-B2 glutenase (for groups 1, 2, or 3, respectively; n = 4 animals/group), as well as [$^{13}C_3$]-HHH-33-mer biomarker, [D_3]-myoglobin peptide control, and vancomycin (a nonabsorbable internal dosing standard). Animals were euthanized 90 min after meal administration and gastrointestinal contents, as well as plasma samples, were collected. Samples were analyzed by HPLC, competitive enzyme-linked immunosorbent assay (ELISA), and/or liquid chromatography-assisted triple quadrupole tandem mass spectrometry (3Q LC-MS/MS).

In all groups, minimal gluten had entered the small intestine by 90 min after meal administration. In contrast, gastric samples from rats receiving no EP-B2 contained abundant gluten (Figure 5A). Virtually all of this material eluted after the HPLC internal standard (>15 min) (Figures 5A and 5B), corresponding to the later retention times reported for larger, immunogenic gluten peptides (Gass et al., 2007). Administration of increasing amounts of EP-B2 caused a progressive shift of the HPLC profile from immunogenic peptides toward smaller, nontoxic metabolites in rats from groups 2 and 3 (Figures 5A and 5B). These results were confirmed by a competitive ELISA using a monoclonal antibody specific for a common gluten sequence (QPQLPY) (Moron et al., 2008). Gastric contents from rats in group 3 (1:25::EP-B2:gluten) had significantly less reactive gluten epitopes compared to control rats from group 1 (Figure 5C). Finally, the amount of intact 33-mer peptide, derived from ingested gluten, was measured in gastric contents by 3Q LC-MS/MS. Because the 33-mer is a product of digestion by pancreatic proteases (Shan et al., 2002), gastric contents were treated ex vivo with trypsin and chymotrypsin prior to mass spectrometric analysis. In agreement with previous results (Gass et al., 2006), the 33-mer was present at ~100 μg/mL in the gastric contents of control rats fed 1 g gluten, but was completely digested in animals receiving EP-B2 with their gluten meal (Figure 5D).

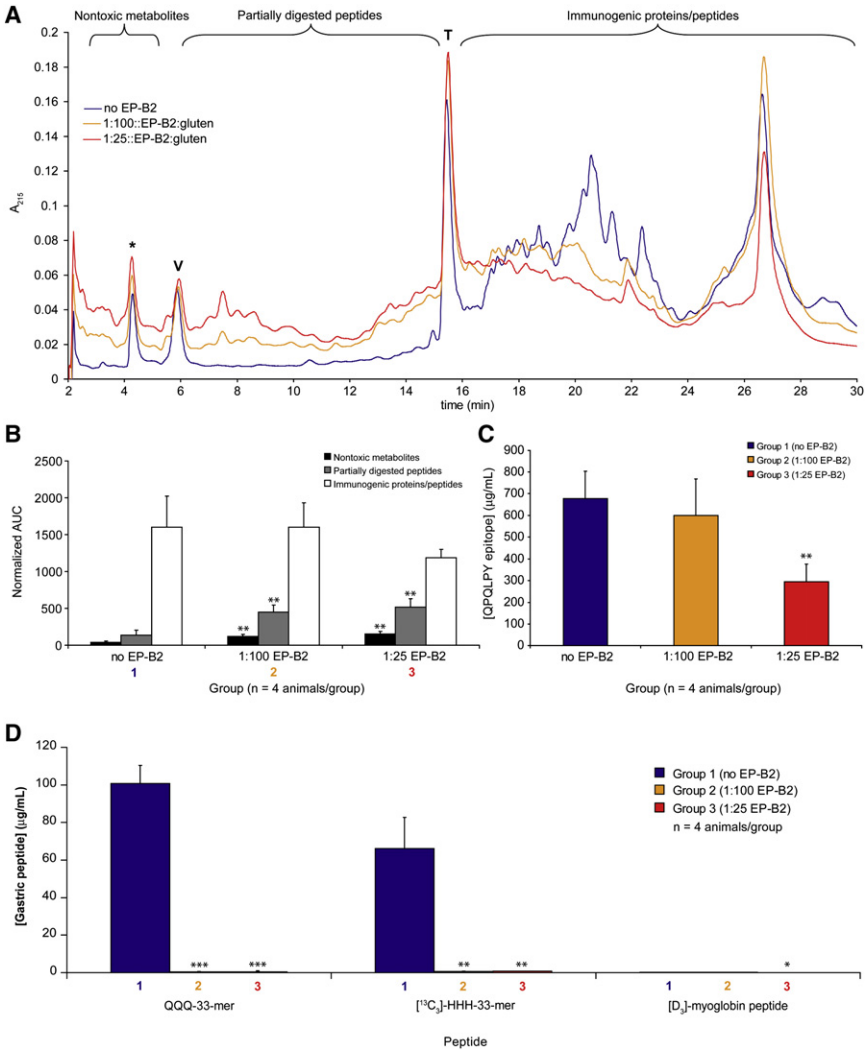


Figure 5. Glutenase-Mediated Biomarker Metabolism Parallels Gluten Digestion In Vivo

Three groups of rats (n = 4 animals/group) were administered a gluten-containing meal supplemented with 0, 10, or 40 mg EP-B2 glutenase (for groups 1, 2, or 3, respectively), 19.7 mg [$^{13}C_3$]-HHH-33-mer biomarker, 3.3 mg [D_3]-myoglobin peptide control, and 10 mg vancomycin dosing standard. Gastric contents were collected after 90 min and analyzed by HPLC, competitive ELISA, and 3Q LC-MS/MS.

(A) Representative HPLC traces of gastric contents from one animal per group. Internal standards for dosing (V) and HPLC analysis (T) correspond roughly to cut-offs between regions of nontoxic metabolites, partially digested peptides, and proteins and peptides retaining immunogenicity (Gass et al., 2007). Asterisk indicates a nongluten peak that was omitted from area-under-the-curve analysis.

(B) Area-under-the-curve analysis quantifying the data from (A). The area under the curve for HPLC trace regions corresponding to nontoxic metabolites (2.7–5.6 min), partially digested peptides (6.3–15.1 min), and immunogenic proteins and peptides (16.0–30.0 min) was normalized to the area under the curve for the vancomycin and TAME internal standards. Means ± SD for each animal group are shown. Data are representative of four similar experiments. Statistical comparisons were performed with respect to group 1 (no glutenase). **$p < 0.01$.

(C) Competitive ELISA analysis quantifying the concentration of peptides containing the gluten peptide sequence QPQLPY in gastric contents. Samples from each animal were tested in triplicate, with a typical error of 5%–10% of the sample mean. Means ± SD for each animal group are shown. Data are representative of four similar experiments. Statistical comparisons were performed with respect to group 1 (no glutenase). **$p < 0.01$.

(D) 3Q LC-MS/MS analysis quantifying the concentration of QQQ-33-mer (derived from ingested gluten), [$^{13}C_3$]-HHH-33-mer biomarker, and [D_3]-myoglobin peptide in gastric contents. Samples from each animal were tested in triplicate, with a typical error of <10% of the sample mean for group 1. Means ± SD for each animal group are shown. Statistical comparisons were performed with respect to group 1 (no glutenase). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

To determine whether biomarker metabolism reflects the reduction in gastric gluten mediated by EP-B2 in vivo, 3Q LC-MS/MS was used to measure gastric levels of [$^{13}C_3$]-HHH-33-mer. The intact biomarker was present at similar levels (~65 µg/mL) as the 33-mer in rats receiving no EP-B2 (Figure 5D). Paralleling the glutenase-mediated reduction observed for bulk gluten and the 33-mer gluten peptide, supplementation of the gluten meal with EP-B2 resulted in complete digestion of the biomarker. In contrast, the control myoglobin peptide was present at substantially lower levels in all animal groups.

In animals that exhibited glutenase-enhanced gastric digestion of the labeled biomarker, the plasma concentration of the labeled metabolite [^{13}C]-leucine was expected to be correspondingly higher. However, 3Q LC-MS/MS detection of [^{13}C]-leucine and [D_3]-leucine in the plasma was hindered by interference from the far more abundant (~250 µM) plasma levels of unlabeled leucine. As an alternative approach, mass spectrometry was used to assay for the presence of intact labeled biomarker peptide in the plasma. The intact biomarker was not detected in any rat plasma sample from this experiment.

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 potentially 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 (Nilsen et al., 1998), has been extensively studied (Bruewer et al., 2005; Madara and Stafford, 1989). 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 (Bethune et al., 2009). To simulate transport under healthy and inflammatory states, media alone or media containing IFN-γ was incubated for 48 hr 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 (Figure 6A). The flux of Cy5-33-mer and both Cy5-labeled biomarker peptides was ~6 pmol/cm^2/hr under basal conditions (Figure 6B). Following exposure of T84 monolayers to IFN-γ, the flux of all three 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 (Matysiak-Budnik et al., 2003). 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 hr 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 and 10 min as a single major peak (Figures 6C–6E). 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 hr, no other breakdown products were identifiable by mass spectrometry, and >95% of each intact peptide remained in the apical chamber of control cells (Figure 6F). Intact Cy5 peptides were present at somewhat lower levels (>80% of initial) after 10 hr 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 (Figures 6C–6E). After 10 hr, 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 (Figure 6F). 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.

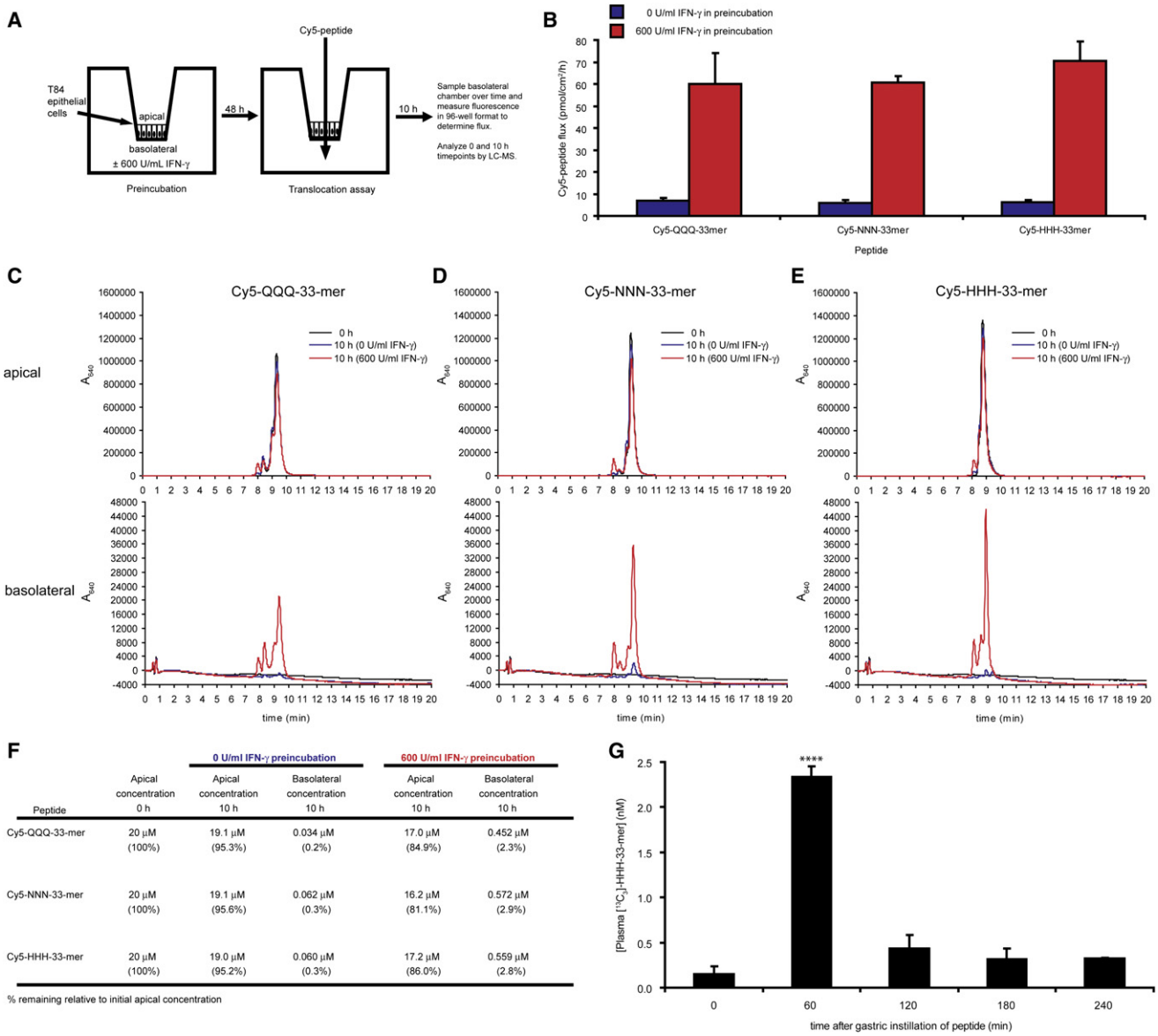
Various rodent models of impaired intestinal barrier function have demonstrated a dependence of inflammation and increased permeability on IFN-γ elevation (Demaude et al., 2006; Luyer et al., 2007; Musch et al., 2002). To examine biomarker transport under basal and inflammatory conditions in vivo, catheterized rats were administered 20 mg [$^{13}C_3$]-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 LC-MS/MS. As predicted by the lack of a serological response to ingested gluten in rodents (March, 2003), biomarker was detected in the plasma of only one of eight control rats administered oral biomarker (four in this study and four in the glutenase study described above). Pretreatment with IFN-γ did not elicit a general increase in biomarker transport in the test animal group relative to controls, as only one of four 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 (Figures 6B and 6F). Additionally, 33-mer dosed together with [$^{13}C_3$]-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 noninflammatory counterpart are transported in parallel in vivo.

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 (Bethune et al., 2008b). To determine if similar absorption of a noninflammatory gluten peptide-based biomarker occurs, a gluten-sensitive macaque with chronic diarrhea and elevated plasma antigliadin antibodies was administered 100 mg [$^{13}C_3$]-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 (Figure 6G), similar to the extent and rate of absorption reported for the 33-mer gluten peptide (Bethune et al., 2008b). Intragastrically administered labeled biomarker was again detected in the peripheral blood of this gluten-sensitive macaque in a repeat experiment (2.2 ± 0.5 nM). In contrast, biomarker transport was not detected in this animal after clinical and serological remission was achieved through treatment with a gluten-free diet, nor was it detected in two identically dosed healthy controls (Figure S2).

DISCUSSION

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 (Catassi et al., 2002; Corrao et al., 2001). 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.

Here we used the disease-causing agent in celiac sprue as a template for designing noninflammatory surrogates as potentially useful 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 (Kim et al., 2004). Additionally, gluten peptides are extraordinarily stable in the relevant physiological compartment, making them ideal scaffolds for drug and biomarker design. Research into the molecular basis for celiac



F

Peptide	Apical concentration 0 h	0 U/ml IFN-γ preincubation: Apical concentration 10 h	0 U/ml IFN-γ preincubation: Basolateral concentration 10 h	600 U/ml IFN-γ preincubation: Apical concentration 10 h	600 U/ml IFN-γ preincubation: 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

Figure 6. 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 hr, 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 ± SD for triplicate assays are shown. Data are representative of two 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 hr. Elution of Cy5-QQQ-33-mer (C), Cy5-NNN-33-mer (D), and Cy5-HHH-33-mer (E) were monitored by their absorbance at 640 nm. Intact Cy5-peptides elute as the major peak between 9 and 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 C–E.

(G) 3Q LC-MS/MS analysis quantifying the concentration of [$^{13}C_3$]-HHH-33-mer in the peripheral plasma of a gluten-sensitive rhesus macaque (FH45) following intragastric biomarker administration. Means ± SD for triplicate assays are shown. Statistical comparisons were performed with respect to the 0 min time point (prior to biomarker administration). ****$p < 0.0001$.

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. In the present study, the 33-mer from α2-gliadin (Shan et al., 2002) was used as a scaffold for biomarker design because its metabolism in the presence and absence of glutenases has been extensively characterized (Bethune et al., 2006; Gass et al., 2006; Shan et al., 2004, 2002) and because its intact transepithelial translocation has been demonstrated (Bethune et al., 2008a; Schumann et al., 2008). Since the 33-mer is among the most stable of immunogenic gluten peptides (Shan et al., 2002), other gluten peptides are likely to be digested more quickly than the biomarkers described. Additionally, the observation that gluten peptide transepithelial flux scales inversely and linearly with size (Bethune et al., 2009) suggests that biomarkers based on smaller gluten peptides may provide a more robust signal in applications measuring peptide transport. Other examples of disease-relevant gluten peptide sequences that are suitable for biomarker design include the 26-mer from γ5-gliadin (Shan et al., 2005) and the p31-49 peptide (Sturgess et al., 1994). Since this latter peptide acts through the innate immune system, biomarkers based on the p31-49 peptide should be modified such that they no longer provoke expression of IL-15 and nonclassical major histocompatibility complex class I molecules MIC and HLA-E from celiac patient intestinal biopsy-derived enterocytes (Hue et al., 2004; Maiuri et al., 2003).

Oral protease therapy is one of the more promising non-dietary treatments being developed for celiac sprue (Bethune and Khosla, 2008), 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 (Cornell et al., 2005). More recently, clinical efficacy of oral EP-B2 was demonstrated in a gluten-sensitive rhesus macaque (Bethune et al., 2008b). 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. In contrast, in the present study, the metabolism of [$^{13}C_3$]-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. Future studies will focus on optimizing noninvasive 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.

In preliminary 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 gluten-sensitive 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 (Bethune et al., 2008b). Biomarker transport was not observed in healthy controls, also consistent with the 33-mer experiment (Bethune et al., 2008b), nor was it observed in the gluten-sensitive animal following treatment with a gluten-free diet. Notwithstanding the clear need for more extensive animal studies and more sensitive detection methods to validate this approach, these data suggest 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 (Paterson et al., 2007; Watts et al., 2005). In analogy to the use of neutralizing antibodies against TNF-α to treat Crohn's disease (Baert et al., 1999), anti-IFN-γ antibodies may represent another candidate for reducing gut permeability and inflammation in celiac sprue. Lastly, strict adherence to a gluten-free diet reduces intestinal permeability in a majority of celiac patients (Duerksen et al., 2005) and this reduction precedes measurable improvements in histology (Cummins et al., 2001). Therefore, biomarkers may be 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 (Friis et al., 1992). This is likely related to the 7.6-fold higher levels of IFN-γ present in treated patients relative to healthy controls (Wapenaar et al., 2004). 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 that 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 (Clayburgh et al., 2004).

Finally, recent studies in cell and tissue culture provide intriguing clues regarding the mechanisms underlying the intact absorption of gluten across the intestinal epithelium (Bethune et al., 2009; Matysiak-Budnik et al., 2008; Schumann et al., 2008). Given the enormous potential for in vivo studies to contribute to our understanding of gluten peptide transport in celiac sprue (Friis et al., 1992), biomarkers are likely to be of considerable benefit to basic research in this area.

SIGNIFICANCE

We used the disease-causing agent in celiac sprue as a template for designing two noninflammatory analogs for use in diagnostic, therapeutic, and basic research applications. By making rational amino acid substitutions in the 33-mer gluten peptide using standard peptide synthesis techniques, we produced biomarkers that retain the resistance to gastrointestinal proteases, susceptibility to therapeutic glutenases, and permeability across enterocyte barriers exhibited by the 33-mer. Unlike this proinflammatory

counterpart, however, they are not appreciably recognized by key players in celiac sprue pathogenesis: transglutaminase, HLA-DQ2, and disease-specific T cells. Animal studies conducted in rats and in gluten-sensitive macaques confirmed that these biomarkers can detect intestinal permeability changes as well as glutenase-catalyzed gastric detoxification of gluten. Accordingly, controlled clinical studies are warranted to evaluate the use of these peptides as probes for abnormal intestinal permeability in celiac patients and for glutenase efficacy in clinical trials and practice. In addition to facilitating diagnosis and management of celiac sprue, these metastable peptides are potentially useful tools for alimentary drug delivery, for basic mechanistic studies of intestinal transport, and for clinical monitoring of intestinal permeability in other diseases of genetic or infectious origin in which the gut barrier is impaired. Finally, the strategy introduced in this study for rational design of peptide-based biomarkers is instructive for the development of additional gluten peptide-based biomarkers for celiac sprue and for other gastrointestinal diseases for which a dietary protein or pathogen trigger is known.

EXPERIMENTAL PROCEDURES

Materials

Peptide synthesis reagents were purchased from Chem-Impex, Peptides International, Anaspec, and Novabiochem. Cy5-NHS ester was purchased from Amersham Biosciences. Isotope-labeled amino acids were purchased from Cambridge Isotope Laboratories. Recombinant IFN-γ was purchased from Peprotech, Inc. Cell culture media, antibiotics, human serum, 5-(and 6-) carboxyfluorescein, and fluorescently labeled dextrans were purchased from Invitrogen. Fetal bovine serum was purchased from Atlanta Biologicals. Gluten flour was purchased from Bob's Red Mill. Thrombin was purchased from Novagen. Protease inhibitor cocktail set 1 was purchased from Calbiochem. Pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, vancomycin, and Nα-p-tosyl-L-arginine methyl ester hydrochloride (TAME) were purchased from Sigma-Aldrich. BBMs were purified from rat intestine as previously described (Kessler et al., 1978) and stored at −80°C until use. The recombinant proenzyme precursor of barley endoprotease EP-B2 (Bethune et al., 2006; Vora et al., 2007), FM PEP (Shan et al., 2004), and human TG2 (Piper et al., 2002) 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 (Xia et al., 2005).

Animals

Twelve 8 week old male Wistar rats weighing between 250 and 300 g (Charles River Laboratories) were singly housed in standard polycarbonate shoebox cages measuring 10.5 × 19 × 8 inches with wire bar lids and microisolator tops (Allentown, Inc.). Eight 8 week old male Wistar rats weighing between 250 and 300 g (Charles River Laboratories) with indwelling jugular vein catheters were housed similarly. Rats were allowed access to rodent chow #5010 (Purina) 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°F 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).

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 (Bethune et al., 2008a). 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. During gluten-free diet treatment, FH45 was administered gluten-free monkey chow #5A7Q (Purina), as described (Bethune et al., 2008a). 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.

Peptide Synthesis, Labeling, and Purification

Peptides were synthesized using Boc/HBTU chemistry on solid phase as previously described (Xia et al., 2005). 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 (LQLQPF(PQPHLPY)$_3$PQPQPF; underlined) and [5,5,5-D$_3$]-leucine was incorporated at position 8 in the myoglobin peptide (KGHHEAELKPL; underlined). For DQ2 binding assays, peptides were labeled at their N terminus on solid phase with carboxyfluorescein, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, and 1-hydroxy-7-azabenzotriazole 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 C_{18} 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 N 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 A_{280} (ε_{280} = 3840 $M^{-1}cm^{-1}$) and A_{652} (ε_{652} = 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 A_{205} (ε_{205} = 27 $(mg/mL)^{-1}cm^{-1}$) as previously described (Scopes, 1974). The concentrations of fluorescein-labeled peptides were determined at pH 9.0 by spectrophotometric measurement of A_{495} (ε_{495} = 80,200 $M^{-1}cm^{-1}$). Working stocks were stored at 4°C and their integrity confirmed periodically by RP-HPLC.

Cell Culture

T84 epithelial cells from the American Type Culture Collection 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 antigen-presenting cell media (RPMI supplemented with antibiotics and 5% [v/v] fetal bovine serum). Every other day, CD114 were split to 0.4 × 10^6 cells/mL. Gluten-specific, DQ2-restricted T cell lines (TCL 432.1.4, TCL 421.1.4, and TCL 446.1.3) and T cell clones (TCC 436.5.3 [DQ2-α-II specific] and TCC 430.1.142 [DQ2-α-I and DQ2-α-III specific]) were isolated from celiac patient intestinal biopsies and expanded as previously described (Molberg et al., 2000). 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.

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 time points. Samples were heat deactivated at 95°C for 5 min, diluted 1:5 in HPLC solvent A (95% H_2O, 5% acetonitrile, and 0.1% trifluoroacetic acid) supplemented with an internal standard (TAME), and analyzed by reverse-phase HPLC. Samples (50 μl) were separated over a C_{18} column (Grace Vydac) 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 (Figures 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 BBM, were added. Recombinant PEP from 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 C_{18} HPLC system (Waters Corporation) coupled to a UV/Vis detector and a ZQ single quadrupole mass spectrometer with an electrospray ionization source. Samples were eluted with a water-acetonitrile 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.

Transglutaminase Deamidation Assay

Coupled spectrophotometric assays for TG2 activity in the presence of each peptide were performed as previously described (Piper et al., 2002). Briefly, each peptide (100 μM) was added to a 200 mM MOPS (pH 7.2) buffer containing 5 mM $CaCl_2$, 1 mM Na_4EDTA, 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 A_{340} was monitored. The specific activity of the enzyme in the presence of each peptide was calculated from the rate of NADH consumption (ε_{340} = 6220 $M^{-1}cm^{-1}$).

HLA-DQ2 Binding Assay

Peptide exchange assays for determining the equilibrium occupancy of each peptide on HLA-DQ2 were modified from previously described methods (Xia et al., 2006). Briefly, recombinant DQ2-αI-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 hr at 4°C, after which protease inhibitor cocktail was added. Thrombin-cleaved DQ2-αI-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, and 0.02% [w/v)] NaN_3) for 45 hr 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). DQ2-bound and unbound fluorescent peptides were separated using a BioSep 3000 size exclusion column (Phenomenex) 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.

T Cell Proliferation Assay

The T cell proliferation assay was modified from previously described methods (Qiao et al., 2005), 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 hr 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 coincubated 48 hr to allow T cells to proliferate in response to DQ2-peptide complex stimulation. After 48 hr, 1 μCi [^{3}H]-thymidine was added to each well and cells were incubated an additional 16 hr. Finally, DNA was collected on a filter mat using a cell harvester and counts per minute resulting from incorporated [^{3}H]-thymidine were measured with a liquid scintillation counter.

Biomarker and Glutenase Dosing to Rats

Procedures for administration of gluten and therapeutics to rats were modified from those previously described (Gass et al., 2006). The study comprised 3 days, including fasting, acclimation to the gluten test meal, and glutenase/biomarker dosing. Adult rats (n = 4 for each of three groups) were fed a commercially available rat chow until day 1 of the study. Rats were then fasted for 12 hr, while drinking water remained freely available throughout the study. On day 2, all three 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, and 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 hr. 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 [$^{13}C_3$]-HHH-33-mer, 3.3 mg [D_3]-myoglobin peptide, and 10 mg vancomycin. Vancomycin was added as a nonabsorbable dosing internal standard, as previously described (Gass et al., 2006). 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 (Gass et al., 2006). Peripheral plasma samples were collected via cardiac puncture at the time of euthanasia and stored at −80°C.

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 × g. Syringe-filtered (0.45 μm) supernatants (100 μl) were diluted 1:5 in 95% H_2O, 5% acetonitrile, and 0.1% trifluoroacetic acid supplemented with an internal standard (TAME) and analyzed by reverse-phase HPLC. Samples (50 μl) were separated over a C_{18} column (Grace Vydac) 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 × g. Supernatants were heat deactivated and processed for HPLC as described for gastric contents.

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 × 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 (Moron et al., 2008). Briefly, equal volumes of coating solution (5 μg/mL gliadin [Sigma-Aldrich] 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 hr 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) and then blocked with blocking buffer (5% [w/v] nonfat milk in PBS [pH 7.2]) for 2 hr 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) diluted 1:10,000 in assay

buffer was added to each standard or sample dilution. Mixtures were incubated with gentle agitation for 2 hr at room temperature, and then added to plate wells in triplicate. After 30 min incubation at room temperature, wells were washed five times with washing buffer and TMB liquid substrate solution (Sigma-Aldrich) 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) was used to fit the 33-mer standard curve to the sigmoidal model:

$$A_{450} = A_{450min} + (A_{450max} - A_{450min}) / [1 + (x/IC_{50})^n],$$

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.

3Q LC-MS/MS Analysis of Intact Peptides and Biomarker Metabolites

The amounts of gluten-derived 33-mer, [$^{13}C_3$]-HHH-33-mer, and [D_3]-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 × 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 hr at 4°C, and centrifuged for 10 min at 4°C, 16,100 × 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 described (Gass et al., 2006) 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 $978.8^{4+} \rightarrow 263.2^{+}$ (30 V cone voltage, 27 eV collision energy) for the quantification assay and m/z $1304.7^{3+} \rightarrow 263.2^{+}$ (40 V cone voltage, 50 eV collision energy) as a confirmatory transition. For [$^{13}C_3$]-HHH-33-mer detection, the transition monitored was m/z $986.75^{4+} \rightarrow 263.2^{+}$ (45 V cone voltage, 32 eV collision energy) for the quantification assay. For [D_3]-myoglobin peptide detection, the transitions monitored were m/z $632.7^{2+} \rightarrow 229.2^{+}$ (35 V cone voltage, 25 eV collision energy) for the quantification assay and m/z $632.7^{2+} \rightarrow 129.2^{+}$ (35 V cone voltage, 33 eV collision energy) as a confirmatory transition. For NNN-33-mer internal standard, the transitions monitored were m/z $968.6^{4+} \rightarrow 263.4^{+}$ (32 V cone voltage, 32 eV collision energy) for the quantification assay and m/z $968.6^{4+} \rightarrow 226.0^{+}$ (40 V cone voltage, 50 eV 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 [D_{10}]-leucine as an internal standard. Samples were injected in triplicate (20 μl each) and eluted from an Atlantis T3 column (3 μm, 2.1 × 100 mm; Waters) with a 20 mM ammonium acetate (pH 5.0)-acetonitrile gradient. For leucine quantification, the transitions monitored were m/z $132.2^{+} \rightarrow 86.2^{+}$ (15 V cone voltage, 10 eV collision energy). For [^{13}C]-leucine quantification, the transitions monitored were m/z $133.2^{+} \rightarrow 86.2^{+}$ (15 V cone voltage, 10 eV collision energy). For [D_3]-leucine quantification, the transitions monitored were m/z $135.2^{+} \rightarrow 89.2^{+}$ (15 V cone voltage, 10 eV collision energy.). For [D_{10}]-leucine internal standard quantification, the transitions monitored were m/z $142.2^{+} \rightarrow 96.2^{+}$ (15 V cone voltage, 10 eV collision energy.)

Peptide Translocation Assays

Peptide translocation assays were performed as previously described (Bethune et al., 2009). 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) at 5×10^4 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 hr 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 molecular weight)-Alexa Fluor-488, 2 μM dextran (70,000 molecular weight)-Texas red, and 20 μM Cy5-labeled peptide to the apical chamber. Labeled dextrans were added to confirm monolayer integrity and size-selective transport (Bethune et al., 2009). Samples of the apical and basolateral media were taken at the 0 hr time point, and basolateral samples were taken every hour over a 4 hr experiment. Fluorescence in collected samples was measured in 96-well format on a Flexstation II 384 (Molecular Devices), monitoring three channels (excitation 490 nm and emission 525 nm for Alexa Fluor-488; excitation 585 nm and emission 620 nm for Texas red; excitation 640 nm and emission 675 nm for Cy5). The slope of basolateral fluorescence units versus time (from 1 to 4 hr) 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/hr).

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 hr 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.

Biomarker Dosing in Rats following IFN-γ Treatment

Adult catheterized rats (n = 4 for each of two groups) were administered a daily dose of vehicle (PBS) or 10^8 U/m^2 IFN-γ intravenously via catheter for 2 days. On day 3, 48 hr 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 [$^{13}C_3$]-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.

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 antigliadin antibodies, intestinal villous blunting, and clinical symptoms indicative of gluten sensitivity (Bethune et al., 2008a). On the day of the study, a dose of 100 mg of isotopically labeled biomarker ([$^{13}C_3$]-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 4 months later with FH45 and two healthy control macaques, HI48 and HK31, on a gluten-containing diet and again with FH45 in clinical and serological remission after 2 months of treatment with a gluten-free diet. In these later experiments, 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.

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.

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00240-3.

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REFERENCES

Alaedini, A., and Green, P.H. (2005). Narrative review: celiac disease: understanding a complex autoimmune disorder. Ann. Intern. Med. *142*, 289–298.

Anderson, R.P., van Heel, D.A., Tye-Din, J.A., Barnardo, M., Salio, M., Jewell, D.P., and Hill, A.V. (2005). T cells in peripheral blood after gluten challenge in coeliac disease. Gut *54*, 1217–1223.

Arentz-Hansen, H., Korner, R., Molberg, O., Quarsten, H., Vader, W., Kooy, Y.M., Lundin, K.E., Koning, F., Roepstorff, P., Sollid, L.M., and McAdam, S.N. (2000). The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J. Exp. Med. *191*, 603–612.

Baert, F.J., D'Haens, G.R., Peeters, M., Hiele, M.I., Schaible, T.F., Shealy, D., Geboes, K., and Rutgeerts, P.J. (1999). Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. Gastroenterology *116*, 22–28.

Bethune, M.T., and Khosla, C. (2008). Parallels between pathogens and gluten peptides in celiac sprue. PLoS Pathog. *4*, e34.

Bethune, M.T., Strop, P., Tang, Y., Sollid, L.M., and Khosla, C. (2006). Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endoprotease. Chem. Biol. *13*, 637–647.

Bethune, M.T., Borda, J.T., Ribka, E., Liu, M.X., Phillippi-Falkenstein, K., Jandacek, R.J., Doxiadis, G.G., Gray, G.M., Khosla, C., and Sestak, K. (2008a). A non-human primate model for gluten sensitivity. PLoS ONE *3*, e1614.

Bethune, M.T., Ribka, E., Khosla, C., and Sestak, K. (2008b). Transepithelial transport and enzymatic detoxification of gluten in gluten-sensitive rhesus macaques. PLoS ONE *26*, e1857.

Bethune, M.T., Siegel, M., Howles-Banerji, S., and Khosla, C. (2009). Interferon-gamma released by gluten-stimulated celiac disease-specific intestinal T cells enhances the transepithelial flux of gluten peptides. J. Pharmacol. Exp. Ther. *329*, 657–668.

Bruewer, M., Utech, M., Ivanov, A.I., Hopkins, A.M., Parkos, C.A., and Nusrat, A. (2005). Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. FASEB J. *19*, 923–933.

Catassi, C., Fabiani, E., Corrao, G., Barbato, M., De Renzo, A., Carella, A.M., Gabrielli, A., Leoni, P., Carroccio, A., Baldassarre, M., et al. (2002). Risk of non-Hodgkin lymphoma in celiac disease. JAMA *287*, 1413–1419.

Ciacci, C., Cirillo, M., Cavallaro, R., and Mazzacca, G. (2002). Long-term follow-up of celiac adults on gluten-free diet: prevalence and correlates of intestinal damage. Digestion *66*, 178–185.

Clayburgh, D.R., Shen, L., and Turner, J.R. (2004). A porous defense: the leaky epithelial barrier in intestinal disease. Lab. Invest. *84*, 282–291.

Cornell, H.J., Macrae, F.A., Melny, J., Pizzey, C.J., Cook, F., Mason, S., Bhathal, P.S., and Stelmasiak, T. (2005). Enzyme therapy for management of coeliac disease. Scand. J. Gastroenterol. *40*, 1304–1312.

Corrao, G., Corazza, G.R., Bagnardi, V., Brusco, G., Ciacci, C., Cottone, M., Sategna Guidetti, C., Usai, P., Cesari, P., Pelli, M.A., et al. (2001). Mortality in patients with coeliac disease and their relatives: a cohort study. Lancet *358*, 356–361.

Cummins, A.G., Thompson, F.M., Butler, R.N., Cassidy, J.C., Gillis, D., Lorenzetti, M., Southcott, E.K., and Wilson, P.C. (2001). Improvement in intestinal permeability precedes morphometric recovery of the small intestine in coeliac disease. Clin. Sci. (Lond.) *100*, 379–386.

Demaude, J., Salvador-Cartier, C., Fioramonti, J., Ferrier, L., and Bueno, L. (2006). Phenotypic changes in colonocytes following acute stress or activation of mast cells in mice: implications for delayed epithelial barrier dysfunction. Gut *55*, 655–661.

Dicke, W.K., Weijers, H.A., and Van De Kamer, J.H. (1953). Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. Acta Paediatr. *42*, 34–42.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E.O., and Schuppan, D. (1997). Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat. Med. *3*, 797–801.

Duerksen, D.R., Wilhelm-Boyles, C., and Parry, D.M. (2005). Intestinal permeability in long-term follow-up of patients with celiac disease on a gluten-free diet. Dig. Dis. Sci. *50*, 785–790.

Friis, S., Dabelsteen, E., Sjostrom, H., Noren, O., and Jarnum, S. (1992). Gliadin uptake in human enterocytes. Differences between coeliac patients in remission and control individuals. Gut *33*, 1487–1492.

Gass, J., Vora, H., Bethune, M.T., Gray, G.M., and Khosla, C. (2006). Effect of barley endoprotease EP-B2 on gluten digestion in the intact rat. J. Pharmacol. Exp. Ther. *318*, 1178–1186.

Gass, J., Bethune, M.T., Siegel, M., Spencer, A., and Khosla, C. (2007). Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. Gastroenterology *133*, 472–480.

Green, P.H., and Jabri, B. (2006). Celiac disease. Annu. Rev. Med. *57*, 207–221.

Hue, S., Mention, J.J., Monteiro, R.C., Zhang, S., Cellier, C., Schmitz, J., Verkarre, V., Fodil, N., Bahram, S., Cerf-Bensussan, N., and Caillat-Zucman, S. (2004). A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity *21*, 367–377.

Jabri, B., Kasarda, D.D., and Green, P.H. (2005). Innate and adaptive immunity: the yin and yang of celiac disease. Immunol. Rev. *206*, 219–231.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., and Semenza, G. (1978). A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. Biochim. Biophys. Acta *506*, 136–154.

Kim, C.Y., Quarsten, H., Bergseng, E., Khosla, C., and Sollid, L.M. (2004). Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. Proc. Natl. Acad. Sci. USA *101*, 4175–4179.

Koskinen, O., Collin, P., Korponay-Szabo, I., Salmi, T., Iltanen, S., Haimila, K., Partanen, J., Maki, M., and Kaukinen, K. (2008). Gluten-dependent small bowel mucosal transglutaminase 2-specific IgA deposits in overt and mild enteropathy coeliac disease. J. Pediatr. Gastroenterol. Nutr. *47*, 436–442.

Luyer, M.D., Buurman, W.A., Hadfoune, M., Wolfs, T., van't Veer, C., Jacobs, J.A., Dejong, C.H., and Greve, J.W. (2007). Exposure to bacterial DNA before hemorrhagic shock strongly aggravates systemic inflammation and gut barrier loss via an IFN-gamma-dependent route. Ann. Surg. *245*, 795–802.

Madara, J.L., and Stafford, J. (1989). Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. J. Clin. Invest. *83*, 724–727.

Maiuri, L., Ciacci, C., Ricciardelli, I., Vacca, L., Raia, V., Auricchio, S., Picard, J., Osman, M., Quaratino, S., and Londei, M. (2003). Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet *362*, 30–37.

March, J.B. (2003). High antigliadin IgG titers in laboratory rabbits fed a wheat-containing diet: a model for celiac disease? Dig. Dis. Sci. *48*, 608–610.

Matysiak-Budnik, T., Candalh, C., Dugave, C., Namane, A., Cellier, C., Cerf-Bensussan, N., and Heyman, M. (2003). Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. Gastroenterology *125*, 696–707.

Matysiak-Budnik, T., Moura, I.C., Arcos-Fajardo, M., Lebreton, C., Menard, S., Candalh, C., Ben-Khalifa, K., Dugave, C., Tamouza, H., van Niel, G., et al. (2008). Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. J. Exp. Med. *205*, 143–154.

Molberg, O., McAdam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., et al. (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat. Med. *4*, 713–717.

Molberg, Ø., McAdam, S., Lundin, K., and Sollid, L. (2000). Studies of gliadin-specific T cells in celiac disease. In Celiac disease: Methods and protocols, M.N. Marsh, ed. (Totowa, NJ: Humana), pp. 105–124.

Moron, B., Cebolla, A., Manyani, H., Alvarez-Maqueda, M., Megias, M., Thomas Mdel, C., Lopez, M.C., and Sousa, C. (2008). Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. Am. J. Clin. Nutr. *87*, 405–414.

Musch, M.W., Clarke, L.L., Mamah, D., Gawenis, L.R., Zhang, Z., Ellsworth, W., Shalowitz, D., Mittal, N., Efthimiou, P., Alnadjim, Z., et al. (2002). T cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na+/K+-ATPase. J. Clin. Invest. *110*, 1739–1747.

Nilsen, E.M., Lundin, K.E., Krajci, P., Scott, H., Sollid, L.M., and Brandtzaeg, P. (1995). Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon gamma. Gut *37*, 766–776.

Nilsen, E.M., Jahnsen, F.L., Lundin, K.E., Johansen, F.E., Fausa, O., Sollid, L.M., Jahnsen, J., Scott, H., and Brandtzaeg, P. (1998). Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. Gastroenterology *115*, 551–563.

Paterson, B.M., Lammers, K.M., Arrieta, M.C., Fasano, A., and Meddings, J.B. (2007). The safety, tolerance, pharmacokinetic and pharmacodynamic effects of single doses of AT-1001 in coeliac disease subjects: a proof of concept study. Aliment. Pharmacol. Ther. *26*, 757–766.

Peyrin-Biroulet, L., Desreumaux, P., Sandborn, W.J., and Colombel, J.F. (2008). Crohn's disease: beyond antagonists of tumour necrosis factor. Lancet *372*, 67–81.

Pietzak, M.M. (2005). Follow-up of patients with celiac disease: achieving compliance with treatment. Gastroenterology *128*, S135–S141.

Piper, J.L., Gray, G.M., and Khosla, C. (2002). High selectivity of human tissue transglutaminase for immunoactive gliadin peptides: implications for celiac sprue. Biochemistry *41*, 386–393.

Piper, J.L., Gray, G.M., and Khosla, C. (2004). Effect of prolyl endopeptidase on digestive-resistant gliadin peptides in vivo. J. Pharmacol. Exp. Ther. *311*, 213–219.

Qiao, S.W., Bergseng, E., Molberg, O., Jung, G., Fleckenstein, B., and Sollid, L.M. (2005). Refining the rules of gliadin T cell epitope binding to the disease-associated DQ2 molecule in celiac disease: importance of proline spacing and glutamine deamidation. J. Immunol. *175*, 254–261.

Quarsten, H., Molberg, O., Fugger, L., McAdam, S.N., and Sollid, L.M. (1999). HLA binding and T cell recognition of a tissue transglutaminase-modified gliadin epitope. Eur. J. Immunol. *29*, 2506–2514.

Raki, M., Tollefsen, S., Molberg, O., Lundin, K.E., Sollid, L.M., and Jahnsen, F.L. (2006). A unique dendritic cell subset accumulates in the celiac lesion and efficiently activates gluten-reactive T cells. Gastroenterology *131*, 428–438.

Raki, M., Fallang, L.E., Brottveit, M., Bergseng, E., Quarsten, H., Lundin, K.E., and Sollid, L.M. (2007). Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. Proc. Natl. Acad. Sci. USA *104*, 2831–2836.

Schumann, M., Richter, J.F., Wedell, I., Moos, V., Zimmermann-Kordmann, M., Schneider, T., Daum, S., Zeitz, M., Fromm, M., and Schulzke, J.D. (2008). Mechanisms of epithelial translocation of the alpha(2)-gliadin-33mer in coeliac sprue. Gut *57*, 747–754.

Scopes, R.K. (1974). Measurement of protein by spectrophotometry at 205 nm. Anal. Biochem. *59*, 277–282.

See, J., and Murray, J.A. (2006). Gluten-free diet: the medical and nutrition management of celiac disease. Nutr. Clin. Pract. *21*, 1–15.

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., and Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. Science *297*, 2275–2279.

Shan, L., Marti, T., Sollid, L.M., Gray, G.M., and Khosla, C. (2004). Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue. Biochem. J. *383*, 311–318.

Shan, L., Qiao, S.W., Arentz-Hansen, H., Molberg, O., Gray, G.M., Sollid, L.M., and Khosla, C. (2005). Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. J. Proteome Res. *4*, 1732–1741.

Siegel, M., Bethune, M.T., Gass, J., Ehren, J., Xia, J., Johannsen, A., Stuge, T.B., Gray, G.M., Lee, P.P., and Khosla, C. (2006). Rational design of combination enzyme therapy for celiac sprue. Chem. Biol. *13*, 649–658.

Silvester, J.A., and Rashid, M. (2007). Long-term follow-up of individuals with celiac disease: an evaluation of current practice guidelines. Can. J. Gastroenterol. *21*, 557–564.

Sollid, L.M. (2002). Coeliac disease: dissecting a complex inflammatory disorder. Nat. Rev. Immunol. *2*, 647–655.

Sollid, L.M., Markussen, G., Ek, J., Gjerde, H., Vartdal, F., and Thorsby, E. (1989). Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. J. Exp. Med. *169*, 345–350.

Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L., and Koning, F. (2006). Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. Am. J. Physiol. Gastrointest. Liver Physiol. *291*, G621–G629.

Sturgess, R., Day, P., Ellis, H.J., Lundin, K.E., Gjertsen, H.A., Kontakou, M., and Ciclitira, P.J. (1994). Wheat peptide challenge in coeliac disease. Lancet *343*, 758–761.

Troncone, R., Gianfrani, C., Mazzarella, G., Greco, L., Guardiola, J., Auricchio, S., and De Berardinis, P. (1998). Majority of gliadin-specific T-cell clones from celiac small intestinal mucosa produce interferon-gamma and interleukin-4. Dig. Dis. Sci. *43*, 156–161.

Vora, H., McIntire, J., Kumar, P., Deshpande, M., and Khosla, C. (2007). A scaleable manufacturing process for pro-EP-B2, a cysteine protease from barley indicated for celiac sprue. Biotechnol. Bioeng. *98*, 177–185.

Wapenaar, M.C., van Belzen, M.J., Fransen, J.H., Sarasqueta, A.F., Houwen, R.H., Meijer, J.W., Mulder, C.J., and Wijmenga, C. (2004). The interferon gamma gene in celiac disease: augmented expression correlates with tissue damage but no evidence for genetic susceptibility. J. Autoimmun. *23*, 183–190.

Watts, T., Berti, I., Sapone, A., Gerarduzzi, T., Not, T., Zielke, R., and Fasano, A. (2005). Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats. Proc. Natl. Acad. Sci. USA *102*, 2916–2921.

Xia, J., Sollid, L.M., and Khosla, C. (2005). Equilibrium and kinetic analysis of the unusual binding behavior of a highly immunogenic gluten peptide to HLA-DQ2. Biochemistry *44*, 4442–4449.

Xia, J., Siegel, M., Bergseng, E., Sollid, L.M., and Khosla, C. (2006). Inhibition of HLA-DQ2-mediated antigen presentation by analogues of a high affinity 33-residue peptide from alpha2-gliadin. J. Am. Chem. Soc. *128*, 1859–1867.