Despite sequence homologies to gluten, salivary proline-rich proteins do not elicit immune responses central to the pathogenesis of celiac disease

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Celiac disease (CD) is a T cell-mediated inflammatory enteropathy that manifests itself in genetically predisposed individuals. In its etiology, both genetic and environmental factors are implicated. The major genetic risk factors are carriage of HLA-DQ2 heterodimers, expressed in 90–95% of CD patients, or HLA-DQ8 heterodimers, expressed in the remainder of CD patients. The primary environmental cause of CD is ingested gluten, a heterogeneous mixture of glutamine- and proline-rich storage proteins present in wheat, rye, and barley (10, 12, 22, 46).

According to solubility in alcoholic solutions, gluten is divided into soluble gliadins and insoluble glutenins. Gluten proteins are unusual because they have a high content of proline (P) and glutamine (Q). In the most abundant gliadins, Q comprises 35–38% of the total amino acids, while P ranges from 13–17% (49, 62, 63). The high P content makes dietary gluten partially resistant to mammalian digestive enzymes, resulting in relatively large peptides that reach the small intestine, some of which contain immunogenic domains (47, 48). Without exception, the immunogenic domains contain glutamic acid (E) residues that derive from Q deamidation by the ubiquitous enzyme tissue transglutaminase (TG2) in the lamina propria (39, 58). The introduction of E residues confers negative charges to gluten, and select deamidated domains bind strongly to HLA-DQ2 or HLA-DQ8 and cause T cell stimulation and intestinal inflammation (26, 42, 56).

Interestingly, the otherwise rare feature of a high P and Q content is not only observed in gluten proteins, but also in a group of proteins present in high concentration in human saliva: the proline-rich proteins (PRPs). PRPs are divided into acidic and basic PRPs. As in gliadins, both P and Q constitute ~50% of the total amino acids of PRPs (18). Acidic PRPs are encoded by five different alleles at the PRH1 and PRH2 loci. The majority of PRPs are encoded by four genes: PRB1, PRB2, PRB3, PRB4, some of which show allelic variations resulting in variable length isoforms (27, 32). PRPs are post-translationally modified within the gland and furthermore undergo excessive degradation after secretion in the oral cavity by microbial proteases (16, 37). As a result, human saliva contains a diverse mixture of acidic and basic PRP-derived proteins and peptides (17, 20, 21).

It has been hypothesized that structural mimicry between gluten and other proteins may be a factor in the etiology of CD. Kangoff and coworkers (23) noted earlier structural similarities between a 12-amino acid residue peptide from E1b protein from adenovirus and α-gliadin. On the basis of this observation it was postulated that infection with the virus could possibly predispose individuals to the development of CD through antigenic cross-reactivity (24). In analogy, we postulated that the structural similarities between dietary gluten and salivary PRPs could play a role in CD pathogenesis. In both gluten and PRP protein families, the P and Q residues frequently appear consecutively in the sequence and the XPQ peptide domain is highly prevalent in gliadins as well as in basic PRPs. The XPQ domains are of particular interest since they are prevalent in...
immunogenic portions of the gliadin proteins (51). On the basis of the structural similarities between gliadins and salivary PRPs, their shared destination in the gastrointestinal canal, and the fact that dietary gluten exclusion is not always an effective means to resolve inflammation in CD (29, 45, 61), we hypothesized that salivary PRPs and gliadins may share functional characteristics that are relevant in CD. The present study investigates the functional resemblances between gliadins and PRPs in terms of their ability to elicit inflammatory immune responses in vitro and in vivo.

MATERIALS AND METHODS

Subjects and inclusion/exclusion criteria. The study was approved by the Committee for Clinical Investigations at the Beth Israel Deaconess Medical Center and the Institutional Review Board at Boston University. Parotid secretion (PS) was collected from four groups of subjects: 1) healthy subjects (HC) having no signs (genetic, serological, or histological) of symptoms of CD or gluten sensitivity and presenting in overall good health (n = 19); 2) non-celiac patients (GI) suffering from non-immune-mediated gastrointestinal symptoms and in whom CD was excluded by serological and histological testing (n = 11); 3) celiac disease patients (CD) with positive anti-DGA (deamidated gliadin antibodies) and/or anti-TG2 antibodies (30) and duodenal villous atrophy at diagnosis and who were clinically responsive to a gluten-free diet (GFD) (n = 20); and 4) refractory CD patients (RCD) who were previously diagnosed as CD and met criteria for RCD after a minimum of 6 mo on the GFD (1) (n = 8). Exclusion criteria were illicit drug or excessive alcohol use; unstable or uncontrolled heart, kidney, or liver disease; a clinically defined mental illness; sicca syndrome; or overt signs of severe dental or periodontal health issues. All subjects enrolled were at least 18 years old and consented prior to participation in the study. The demographics of the enrolled patients are shown in Table 1. More detailed clinical characteristics of these patients have been reported earlier (53). The majority of the donors were women, and all patients were Caucasian. The HC group was matched in age, gender, and race to the CD group.

Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Source</th>
<th>HC (n = 19)</th>
<th>GI (n = 11)</th>
<th>CD (n = 20)</th>
<th>RCD (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD</td>
<td>33.6 ± 13.9</td>
<td>41.0 ± 14.8</td>
<td>35.1 ± 16.6</td>
<td>54.1 ± 13.5</td>
</tr>
<tr>
<td>Gender, % M</td>
<td>M 26.3%</td>
<td>M 27.3%</td>
<td>M 15%</td>
<td>M 37.5%</td>
</tr>
<tr>
<td>F</td>
<td>F 73.7%</td>
<td>F 72.7%</td>
<td>F 85%</td>
<td>F 62.5%</td>
</tr>
</tbody>
</table>

HC, healthy controls; GI, (unrelated) gastrointestinal disorders; CD, celiac disease; RCD, refractory CD; M, male; F, female.

Table 2. Structural comparison between E1b (from adenovirus), PRP2 (from human saliva), and gliadin/glutenin immunogenic domains

<table>
<thead>
<tr>
<th>Source Proteins</th>
<th>Peptide</th>
<th>T Cell Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1b (adenovirus)</td>
<td>L R R G M F R P S Q C N</td>
<td>?</td>
</tr>
<tr>
<td>Glia-a-peptide</td>
<td>L G Q G S F R P S Q Q N</td>
<td>Yes</td>
</tr>
<tr>
<td>Glia-a-9 (α-1)</td>
<td>Q P Q P A P A G Q P Q G P P R P</td>
<td>?</td>
</tr>
<tr>
<td>Glia-g-1</td>
<td>Q P Q Q P Q Q S F F Q Q R P F</td>
<td>Yes</td>
</tr>
<tr>
<td>Glt-17</td>
<td>Q Q Q F S Q Q Q Q Q P L P Q</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Boldface sequences: viral (E1b) and salivary (PRB) peptide sequences. *Amino acids in known immunogenic gliadin domains showing homology to E1B or PRB peptide sequences.
tion by using Histopaque (Sigma), essentially as described (8). The were separated from other blood cells by density gradient centrifuga-

medium (2

Grand Island, NY), 100 U/ml penicillin and 100

g/ml streptomycin

were centrifuged and the secreted TNF-

α protein was confirmed. PS from all subjects showed similar

nonspecific cross-reactivity of the gliadin antibody to this

58-kDa region. This band matched salivary amylase, and the

very weak recognition by the anti-gliadin antibody, in the

other hand, the relatively high amount of PS protein showed a

noticeable in the Coomassie-stained gel but showed a strong

(AJP-Gastrointest Liver Physiol

Proliferation evaluation was based on incorporation of [1H]thymidine

(7) wherein each agent was evaluated in triplicate wells. The stimula-
tion index was calculated by dividing the average counts per minute
{(cpm) of the agent (triplicate wells) by the average cpm of media
alone (triplicate wells)).

Statistical analysis. Differences in the stimulation index of DQ2/
DQ8 mice studies and ELISA results were analyzed with the Mann-
Whitney U-test. Significance was set at P < 0.05.

RESULTS

Salivary PRPs show sequence similarity to gliadin immuno-
genic domains. On the basis of the observed structural similarity
between gliadin with PRPs, sequences of 10 well-established epitopes in gluten and gliadins relevant in CD were aligned with the sequences of basic and acidic PRPs. The epitopes showing the highest level of overlap are shown in Table 2. It is immediately evident that the spacing in glutamines and prolines in the PRPs and gliadin peptides are similar and that levels of overlap up to 50% can be observed. The overlap between E1b protein from adenovirus and gliadins occurs in a region located outside the major immunogenic gliadin epitope domains. Salivary PRPs, however, show similarities with sequences within the immunogenic gliadin domains. Therefore, their potential immune stimulatory properties were further explored.

PS protein recognition by anti-gliadin antibody and R5 antibody. Structural similarities between PRPs in PS and gliadins were investigated by immunoblotting with an anti-gliadin antibody. Figure 1A shows the gliadin (0.1 μg), salivary amylase (15 μg), and PS (48 μg) protein patterns in a Coomassie-stained SDS gel. The same samples and amounts were blotted and incubated with a polyclonal anti-gliadin antibody (Fig. 1B). The low loaded amount of gliadin was barely noticeable in the Coomassie-stained gel but showed a strong reaction with the anti-gliadin antibody, as expected. On the other hand, the relatively high amount of PS protein showed a very weak recognition by the anti-gliadin antibody, in the ~58-kDa region. This band matched salivary amylase, and the nonspecific cross-reactivity of the gliadin antibody to this protein was confirmed. PS from all subjects showed similar patterns of weak reactivity in this region, and no group-specific
Recognition of salivary proteins was also investigated with a second antibody, R5, directed at the pentapeptide QQPFP and related sequences comprised in immunogenic gluten epitopes (25, 36, 40, 57).

The Codex Alimentarius and the Food and Drug Administration have recommended the R5 ELISA assay for the detection of gluten contaminations in gluten-free foods, where a value of <20 ppm is regarded as a gluten free. The results show that in

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**Fig. 2.** Schematic presentation of the proposed influence of salivary proline-rich proteins (PRPs) on immune responses in celiac disease (CD). A: in the pathogenesis of CD, dietary gliadins are hydrolyzed to smaller fragments by human digestive enzymes (e.g., pepsin, trypsin), and then deamidated by transglutaminase 2. Deamidated gliadin fragments have higher binding affinity to HLA-DQ2/8 on antigen presenting cells (APCs) and could activate T cell responses. B: on the basis of the structural similarity and shared destination of salivary PRPs and gliadins, we hypothesized that salivary PRPs would also be hydrolyzed by human digestive enzymes, be deamidated, and bind to HLA-DQ2/8 followed by activating T cells (hypothesis 1). In addition, or alternatively, salivary PRPs may compete with gliadins for HLA-DQ2/8 binding and thus indirectly interfere with the immune response triggered by gliadins (hypothesis 2).

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**Fig. 3.** Effect of PS proteins from various sources on TNF-α and IL-10 production by peripheral blood mononuclear cells (PBMCs) from CD patients. PBMCs were isolated from fresh blood of 2 CD patients (CD17, CD20) on a gluten-free diet. Gliadins or PS from CD, refractory CD type 1 patients (RCD), and healthy control (HC) subjects were digested with pepsin and trypsin (PT) and deamidated with tissue transglutaminase (TG2). The level of TNF-α (A and B) and IL-10 (C and D) produced by PBMCs were analyzed after 24-h incubation to the respective samples. PT-TG2-treated H2O alone served as the negative control. Glia: 100 μg/ml; PS: 100 μg/ml; PBMCs: 1 x 10⁶ cells/ml.
all four patient groups, the “gluten-like” protein levels in parotid saliva averaged at <1 ppm (Fig. 1C). The combined results indicated that PS proteins do not contain structural elements that are sufficiently similar to gliadins to be recognized by the polyclonal general anti-gliadin antibody or the monoclonal R5 antibody.

Cytokine secretion by PBMCs under stimulation of PS. We next investigated whether PRPs and gliadins shared immunogenic properties relevant in CD. A two-pronged approach was followed. In the first, it was tested whether salivary PRPs, after PT-TG2 treatment, might exert direct immune stimulatory effects on PBMCs similar to equally treated gliadins (Fig. 2, hypothesis 1). In the second, it was investigated whether PRPs could compete with, or ameliorate, the inflammatory effects of gliadins (Fig. 2, hypothesis 2).

PBMCs from two CD patients (CD17 and CD20) were incubated with gliadins or PS samples from three or four HC, CD, or RCD patients, each at 100 μg/ml. In both patients’ PBMCs, gliadins induced secretion of TNF-α and IL-10 (Fig. 2). Very low induction of IL-21 and IFN-γ was observed and these cytokines were not further considered (data not shown). TNF-α and IL-10 responses to PS proteins, regardless of the donor group, were similar to those of the negative control. These results indicated that PS proteins do not exhibit direct immune-stimulatory effects on PBMCs analogous to gliadins.

Competition of salivary PRPs with gliadins. Having demonstrated that PRPs do not trigger immunity in PBMCs of patients and controls directly, we assessed the ability of salivary PRPs to interfere with the gliadin-induced TNF-α and IL-10 induction through competition, thus antagonizing gluten-triggered inflammation. To test this effect, 25, 50, or 100 μg/ml of PS proteins were added together with 100 μg/ml gliadins (all PT-TG2 treated) to the PBMC culture. Three experiments were carried out, with PBMCs from three CD patients. Gliadins alone exerted the anticipated stimulatory effect, and PS proteins alone had no effect, as in the previous experiment (Fig. 3). Corrected for background effects, we observed no competition between gliadins and PS protein on the gliadin-induced cytokine stimulation (Fig. 4). Noticeably, PT-TG2 alone (without PS or gliadin protein present) induced significant cytokine production in PBMCs, likely due to innate immune activation by the enzymes and/or TG2. In line with this, PT-TG2 effects on human intestinal epithelial cell gene transcription have also been reported (41), emphasizing the importance of proper controls in cell-based experiments with PT-TG2-treated proteins.

Responses in a DQ2/DQ8 transgenic mouse model for celiac disease. Given that only a small fraction of the T cells in PBMC are derived from the small intestine, we investigated the specific effects of PRPs on gliadin-sensitized T cells in a mouse model for CD. AEo DQ2/DQ8 transgenic mice were immunized with total PS proteins, or with fractions of PS proteins that were enriched in acidic or basic PRPs, or with chemically synthesized peptides that were designed based on the basic PRP domain APPAGQPQGPPRPPQ (Table 1). The substitutions made were P11→Y11, theoretically favoring deamidation by TG2 (56), and Q8→E8, mimicking deamidation by TG2 (39, 58). In this experimental approach the mixed genetic background was not expected to affect the results to show a present or absent T cell stimulatory response. Neither

![Image](http://ajpgi.physiology.org/)

Fig. 4. Effect of PS proteins on gliadin-triggered TNF-α and IL-10 production by PBMCs from CD patients. PBMCs from 3 CD patients (CD8, CD9, CD13) were analyzed separately. Gliadins, with or without increasing amount of PS from each patient, were incubated with PBMCs. The levels of TNF-α (A–C) and IL-10 (D–F) produced by PBMCs were analyzed after 24 h incubation. All gliadins and PS were digested with PT and deamidated with TG2. PT-TG2 alone served as the negative control. Glia: 100 μg/ml; PS: 25, 50 or 100 μg/ml; PT-TG2 alone contained 3 levels of enzymes used to treat the PS samples (referred to as 25, 50, 100). PBMCs: 1 × 10⁶ cells/ml. Values represent means ± SD of 3 independent samples.
the salivary protein fractions nor the peptides displayed immunogenic properties in the mouse model (Fig. 5) in contrast to PT-gliadin injection (positive control).

**DISCUSSION**

The results of this investigation demonstrate that gliadins and PRPs, despite structural similarities at the epitope level, differ markedly with respect to their ability to stimulate the immune system. It was recognized at the initiation of this study that salivary proteins would not be likely to trigger the inflammatory responses in CD, given that gluten exclusion from the diet in most cases prevents and resolves intestinal inflammation. However, in RCD patients, inflammation persists, despite strict adherence to a GFD, and to date the inflammatory trigger in RCD is unknown. Given that the gluten-like salivary PRPs are swallowed continuously, it was postulated that RCD patients’ saliva might differ from HC, GI, or CD saliva that could explain such effects on the immune system. We have reported that, structurally, salivary PRP concentrations and isoforms were very similar in HC, GI, CD, and RCD (53). Here we demonstrate that also at the functional level PRPs from RCD patients do not differ from PRPs in HC, GI, or CD patients. This seems to exclude the possibility that point mutations in RCD patients would have rendered their PRPs more gluten-like.

Closer inspection of the structural constraints of gliadin epitopes and their interaction with HLA-DQ2 or HLA-DQ8 on antigen-presenting cells provides possible explanations for the nonstimulatory effects of PRPs. Deamidation of select Q residues by the enzyme TG2 is a critical step in CD pathogenesis, and the spacing between Q and P residues in gliadins is an important determinant dictating TG2 recognition. In the gliadin sequences QP, QG, QXXP, and QXXG (X being any amino acid), the Q is not a target for TG2, whereas in the sequences QXP, QXXF, and QXPF, the Q is frequently deamidated (56). Gliadins and PRPs share foremost the high prevalence of P and Q residues in the XPQ context, and in both acidic and basic PRPs a large number of Q residues appear in the QGPP context. Projecting the known TG2-preferred gliadin sequences onto the PRPs, Q in QGPP may or may not be deamidated, since QXP is preferred by TG2, but QG and QXQP are not. The remainder of the Q residues in PRPs almost all fall into the nonpreferred substrates for TG2 (QXXP). Noticeably, the NH2-termini of basic as well as acidic PRPs contain natural glutamic acid residues as well as aspartic acid residues, and thus negative charges in PRPs are naturally present. Despite this, PRPs did not participate in T cell activation in vitro or in vivo, likely because the structures do not fit well into the HLA-DQ2 and HLA-DQ8 binding pockets.

Thus a key question became whether the TG2-deamidated/naturally deamidated PRP peptides could theoretically bind to HLA-DQ2 or HLA-DQ8. On the basis of the amino acid sequences of immunogenic gluten epitopes and the structural resolution of some gluten epitopes in association with HLA-DQ2 by X-ray crystallography (26), it is known that negatively charged residues (e.g., glutamic acid) are preferred at positions 4, 6, and 7 and large hydrophobic amino acids at positions 1 and 9 (59, 60). HLA-DQ8 has a preference for negatively charged residues at anchor positions 1 and 9 (19, 54). Although some PRPs sequences in the negatively charged domains satisfy theoretic DQ2 binding affinity, they may not satisfy all the requirements for subsequent T cell stimulation. This appears to be supported by the present results.

To detect the antigenic properties of gliadin peptides, in vitro challenge of CD patients’ PBMCs (55), small intestinal biopsies (31), and gluten-specific T cell lines generated from biopsies (35, 38) have been employed. In our study we employed PBMCs. It is recognized that PBMCs may contain a relatively limited number of CD4+ T cells that are derived from the small intestine and that are gliadin reactive. However, PBMCs have been used successfully in other studies to detect gliadin immunogenicity (5, 11, 13, 43) and to study CD patients’ immune responses to a gluten challenge (2–4, 14, 15, 28, 50). Nonetheless, additional investigations e.g., with patient-derived T cell lines, or biopsies would be valuable to...
confirm the results obtained with the PBMCs. Furthermore, it will be of interest to continue investigations with larger cohorts of RCD patients. In our study the number of RCD patients was limited, and an unequivocal conclusion as to whether this patient group may have become sensitized to their own salivary PRPs is not yet justified.

It is of interest to note that the gut-associated lymphoid tissues are exposed to saliva and the proteins it contains, before exposure of the GI system to gluten. It is during this early time frame that T cells are primed to tolerate the proline- and glutamine-rich salivary PRP proteins. It can be hypothesized that oral tolerance to gluten-like salivary PRPs may affect tolerance to dietary gluten proteins introduced later in life (at around 6–12 mo after birth). The ability of the body to discriminate between salivary PRPs and gluten remains intriguing and may offer novel insights into the key structural elements of gliadins precipitating in CD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES
