Celiac disease : from basic insight to therapy development

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General discussion

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Gluten as an etiologic factor causing celiac disease has first been described by the Dutch pediatrician Willem Karel Dicke in his PhD thesis defended in Utrecht on 30 May 1950. Much has been learned about the celiac disorder ever since. We know that high proline content renders certain gluten sequences resistant to gastrointestinal proteolysis. In addition, the multiple glutamine residues present in gluten make it an excellent substrate for tissue transglutaminase, an enzyme capable of deamidating glutamine into glutamic acid and thereby introducing a negative charge in gluten peptides. Such modified gluten peptides can bind HLA-DQ2 or HLA-DQ8 molecules expressed by intestinal antigen presenting cells and are displayed to gluten-specific T cells, which result in inflammation. This provides a satisfactory explanation for the observed strong association between HLA-DQ2/8 and the development of celiac disease. Moreover, there is now also evidence that gluten can trigger innate immune responses that contribute to the destruction of the healthy gut architecture. Notwithstanding these unquestionable advances, a number of fundamental questions remain unanswered.

T cell stimulatory gluten epitopes

In the early nineteen-nineties gluten-specific T cells were isolated from biopsies of celiac patients, a very significant breakthrough in celiac disease research. Such T cells allowed a specific search for the gluten-derived peptides that stimulated these T cells. It became apparent that the T cells invariably responded to gluten peptides in the context of either HLA-DQ2 or HLA-DQ8. Moreover, it was found that a large number of gluten peptides existed that had T cell stimulatory properties and that most of those peptides required modification by tissue transglutaminase in order to be able to bind to HLA-DQ2/8 and trigger T cell responses [1,2]. Although many T cell stimulatory gluten peptides have been already characterized it is estimated that gluten and similar proteins from other cereals might contain over a hundred toxic sequences [3]. Ultimately, the identification of all these peptides may be required to allow the development of novel intervention protocols and the generation of cereals that are harmless for patients. It has been shown that computer database searches with dedicated algorithms can be a very efficient method of spotting the potential T cell epitopes [3] and (Chapter 4). Such algorithms have been based on: (i) HLA binding motif (Chapters 3, 4), (ii) the substrate specificity of tissue transglutaminase (tTG) (Chapter 4) and (iii) homology to the known epitopes (Chapter 4). While the specificity of tTG has been well established [3,4] we explored the possibility that further insight into the binding properties of HLA-DQ2 molecule might deepen our understanding of the unique capability of HLA-DQ2 to bind a large repertoire of T cell stimulatory gluten peptides. For this purpose we isolated and characterized an unprecedented number of natural peptides present in HLA-DQ2 (Chapter 4). We found that HLA-DQ2 prefers acidic amino acids not only at p4, p6 and p7, as shown previously [5,6], but virtually at all anchor and non-anchor positions (Chapter 2). Similarly, we observed selection against positively charged amino acids at the p3 non-anchor position and a preference for a proline at the p8 position. As the large majority of T cell stimulatory gluten peptides carry a proline at the p8 position, this unique preference of HLA-DQ2 is highly significant. In ad-dition, we observed that proline was either preferred or tolerated at the p1, p3 and p6, i.e. at all the positions where a proline is frequently found in T cell stimulatory gluten peptides. Finally, we observed that a proline at the p-1 position could be essential for T cell recognition (Chapter 3). Together, these observations indicate a unique role for proline in gluten peptide binding to HLA-DQ2.

Although wheat gluten has been identified as the main symptom-inducing factor in celiac disease it has been observed that consumption of barley and rye by celiac patients also results in appearance of clinical manifestations. Yet, until recently no T cell stimulatory epitopes from cereals other than wheat had been known. In order to identify potential T cell stimulatory peptides from these other cereals we searched

for gluten-homologue sequences in the gluten-like proteins: hordeins from barley, the secalins from rye and the avenins from oats (Chapter 4). Eleven homologous sequences were identified, of which seven were recognized by gluten-specific T cell lines and clones derived from celiac patients. This demonstrated that the detrimental effects of barley and rye consumption could at least partially be ascribed to T cell cross-reactivity towards homologous peptides derived from these cereals. Hordein- and secalin-derived peptides often elicited equally vigorous or even stronger T cell responses than gluten-derived sequences, which implies that at least some of the T cell specificities may be raised primarily against epitopes from barley and rye.

Innate immunity and celiac disease

The default set-up of the intestinal immunity is the generation of tolerance. High concentrations of anti-inflammatory cytokines such as IL-10 and TGF-β contribute to sustaining a tolerogenic milieu. The antigens present in the gut lumen are constantly sampled by intestinal dendritic cells and presented to the T cells in either Peyer's patches or mesenteric lymph nodes, which results in the generation of regulatory $CD4^+$ T cells [7]. Initiation of protective immune reactions requires specific danger signals [8]. It has been proposed that such a signal could be provided by gluten itself, as it has been demonstrated that gliadin is capable of stimulating cytokine production by human macrophage line THP-1 [9] and inducing the maturation of monocyte-derived dendritic cells in vitro [10]. This, however, seems unlikely since despite most people consuming gluten on a daily basis, no stimulation of dendritic cells in vivo or ex vivo has ever been observed in healthy individuals. Also, it is known that potent stimulators of dendritic cells, such as lipopolysaccharide (LPS) or peptidoglycan, although highly abundant in the lumen of the intestine, do not normally evoke immune responses. Further, it has been shown that gliadin and the gliadin-derived fragment p31-43 can induce IL-15 expression only in celiac patients and proinflammatory status seems to be a prerequisite for the innate recognition of gliadin [11–13]. Together, this indicates that gluten itself is not a factor that can directly trigger the onset of celiac disease by induction of an innate response. Instead, other inflammatory stimuli are required to polarize the normally quiescent and tolerogenic dendritic cells so that they became receptive to gluten and could prime gluten-specific Th1 lymphocytes. Once triggered, these DCs and possibly other APCs produce IL-15, which has been found to reprogram intraepithelial lymphocytes (IELs) into NK-like cells that express NK receptors including an activating receptor NKG2D [11]. At the same time IL-15 induces expression of MICA, a ligand of NKG2D, on epithelial cells [11]. In vitro, this has been shown to result in killing of the epithelial cells and devastation of the intestinal architecture [12,13]. An increased number of IEL in the small-intestinal

biopsies of CD patients is a well-established feature of CD. These findings indicate that such innate T cell response may contribute to the intestinal tissue damage and the uncontrolled IEL expansion that can lead to malignant T cell lymphomas [14]. The molecular mechanism through which gluten stimulates innate responses in celiac patients, however, still needs to be elucidated.

What triggers celiac disease?

HLA-DQ2 (DQA1*0501/DQB1*0201) is the most important genetic factor predisposing to celiac disease as about 95% of celiac patients are HLA-DQ2 positive. It has been described that HLA-DQ2 homozygotic individuals have a significantly increased risk of disease development compared to heterozygotes [15]. Moreover, HLA-DQ2 homozygosity predisposes to complications such as refractory celiac disease with aberrant T cells (RCD II) and enteropathy-associated T-cell lymphoma [14]. We have provided evidence that this gene-dose effect could be explained by the observation that the density of the HLA-DQ2 molecules on the cell surface is determined by the HLA-DQ2 gene dose and influences the strength of the gluten-specific T cell response [16]. Thus, a high level of gluten presentation correlates with a high chance of disease development. Consequently, a reduction of the gluten intake may decrease the risk of disease development. This latter hypothesis is supported by the observation that the introduction of relatively large amounts of gluten to infants' diet in Sweden resulted in a dramatic increase in the incidence of celiac disease in children younger than 2 years. This effect disappeared upon removal of the gluten from the follow-up formula [17].

Another implication of the gene-dose effect is that a temporary upregulation of HLA, for example during the course of an infection, might enhance the presentation of gluten peptides and thus increase the risk of disease development. It is in agreement with the several observations of celiac disease onset in patients treated with interferon alpha (IFN-α) for unrelated disorders [18–20]. Type I interferons, including IFN-α, are a group of cytokines with potent antiviral and immunoregulatory functions. They are secreted in response to infections with viruses and intracellular bacteria [21]. It is known that type I interferons serve as a danger signal reinstructing tolerogenic intestinal APCs to promote the differentiation and maintenance of Th1 cells, which results in the deployment of protective immune responses [22]. This is orchestrated by the secretion of IFN-γ and IL-15, cytokines known to be implicated in celiac disease [23,24]. Finally, IFN-α has also been shown in human ex vivo studies to be essential for the development of villous atrophy [25].

Although no single pathogen has been found to be associated with celiac disease it is known that virtually every child experiences episodes of rotavirus gastroenteritis in the first two years after birth [26,27]. Also other enteroviruses are a frequent cause of diarrhea in children [27] and there is evidence that the gradual intro-

duction of gluten during breastfeeding decreases the relative risk of CD, which may in part result from enhanced protection to pathogenic microorganisms due to maternal IgA antibodies in breast milk. It could be therefore speculated that in a predisposed individual any intestinal infection could trigger the onset of celiac disease provided: (i) it results in type I IFN secretion and elicits strong Th1 protective responses (ii) is severe enough to facilitate the breaking of oral tolerance, and last but not least (iii) a sufficient amount of gluten is available to be presented to T cells in context of HLA-DQ2 or DQ8.

Interestingly, there is a growing body of experimental data that provides support for the hypothesis that various autoimmune diseases result from by-stander T cell activation [28]. Celiac disease shares many similarities with common autoimmune disorders and is often associated with them, it cannot be therefore excluded that comparable mechanisms trigger the onset of these diseases [29].

As mentioned, HLA-DQ2 is the most important genetic factor predisposing to celiac disease. Yet it can explain only about 40-50% of the genetic contribution and it is clear that a number of other genes must be implicated [30,31]. Genome-wide and association studies employing microsattelite markers or single-nucleotide polymorphisms (SNPs) are valuable methods to search for regions containing implicated genes. Usually such an identified chromosomal region spans over a relatively large fragment of the chromosome and contains multiple genes. There are two options to identify the real causative genes in such regions. The first is the candidate gene approach while in the second fine mapping of the entire region is performed to identify the gene in question. In the candidate gene approach genes can be selected on the basis of a variety of criteria, for example microarray data pointing to differential expression of certain genes in material from patients and controls (Chapter 5). Unfortunately, the chance of identifying genes that contribute to the disease using the candidate gene approach is limited as evidenced by our study on the gene coding for prolyl oligopeptidase (*PREP*) (Chapter 5), and various other studies [32,33]. In contrast, the fine mapping of the linkage region on chromosome 19, using tag SNPs from the HapMap project, recently allowed the identification of first non-HLA gene significantly associated with celiac disease [30]. The gene in question, *MYO9B*, encodes an unconventional myosin molecule. The way this molecule could be implicated in the disease development remains enigmatic but it has been hypothesized that myosin IXB variants could account for an affected integrity of the intestinal barrier. Such a "leaky gut" would result in increased penetration of gluten peptides through the intestinal epithelium and thereby contribute to an elevated risk of breaking the oral tolerance to gluten. The fact that the association of the *MYO9B* allele with celiac disease could not be reproduced in an English [34] and Swedish/Norwegian cohorts [35] implies that the genetic component of the predisposition may be different in various populations. This could explain a poor repro-

ducibility of genome-wide association scans performed by various research centers [36]. In any case, *MYO9B*, is most likely only one of many genes that are involved in CD. There is evidence that other genes await to be discovered in chromosomal regions 2q23-32, 5q31-33 and 6p21 [37–39]. And what will be the nature of these other contributing genes? Some of them may actually predispose to autoimmunity in general. This would explain the increased prevalence of several autoimmune diseases in celiac patients. Such a pro-autoimmune phenotype could stem from a defective regulatory T cell compartment, a proinflammatory status, hyperresponsiveness to danger signals or other factors that could have an impact on tolerance induction. Alternatively, a decreased protection against intestinal (especially viral) infections could be responsible for recurrent episodes of gastroenteritis associated with local inflammation and as such predispose to an increased risk of celiac disease development.

Finally, it needs to be emphasized that the identification of celiac disease associated genes will just be a first step towards unraveling the complexity of the pathogenesis and heterogeneity of the clinical picture in the celiac disorder. Understanding the underlying mechanisms might prove challenging.

Prevention, management and therapeutic modalities

Given the central role of IL-15 in the pathomechanism of celiac disease this cytokine has been proposed as an obvious target for a potential intervention. It can, however, be anticipated that IL-15 blocking might severely impair the natural protective functions of the intestinal immune system. The disparity between the potential benefits and risk of serious complications seems to disqualify such an approach. Also, one should take into account that such a treatment would neither eliminate the gluten-specific CD4 responses nor eradicate the predisposing factors. Unfortunately, as long as all the genetic and environmental components facilitating the onset of CD are not known the development of an effective strategy to re-establish tolerance may be very difficult if not impossible. Albeit the rational immunotherapeutic modalities do not seem available in the near future there is much that can be done insofar as prevention is concerned. As shown in Sweden establishing the right guidelines regarding introduction of gluten into the diet of infants can significantly decrease the incidence of CD. There is evidence that the introduction of gluten while breastfeeding reduces the risk of CD. Breastfeeding not only protects the digestive tract of an infant against infections, but also facilitates the proper development of the gastrointestinal immune system and helps establishing the oral tolerance. Also during the episodes of frequent in small children gastroenteritis, feeding gluten should be discouraged, particularly in case of individuals at risk i.e. HLA-DQ2 or -DQ8 positive, and with a history of CD in the family.

Thus far a gluten-free diet is the only available treatment in celiac disease. Gluten and gluten-like proteins are responsible for the exceptional baking properties of wheat flour. The quest for generating cultivars characterized by low toxicity for celiac patients while preserving their baking qualities has been a Holy Grail of research on the celiac disorder for already many years. Several approaches have been taken to achieve this goal. One of them aims at the development of non-immunogenic gluten molecules that could subsequently be introduced into the genome of safe cultivars such as rice or maize. Comparison of the T cell stimulatory versus homologous non-stimulatory sequences indicated amino acid residues crucial for T cell recognition. Single amino acids substitutions in such peptides proved very efficient in limiting the T cell reactivity to gluten in vitro. Moreover, such amino acid changes could be introduced by site-directed single-nucleotide substitutions in gluten genes; ccg => cag, for example would result in the replacement of proline by glutamine. Since proline and glutamine are the most abundant amino acids in gluten such changes should not impair the unique baking properties of gluten. Although theoretically conceivable, this approach faces serious limitations. Database searches performed by Vader et al. [3] demonstrated that the number of potentially toxic sequences in wheat gluten might exceed a hundred. The existence of other varieties further enlarges the epitope repertoire. Furthermore, in the recent studies gluten and the gluten-derived peptide p31-43 have been found to stimulate innate immune responses and thereby induce tissue damage in celiac patients [11–13]. Besides, T cell stimulatory sequences are usually found repetitively in multiple gluten proteins. The elimination of all these epitopes will thus be a major task. Therefore, wheat cultivars that display a natural low toxicity should first be selected as starting material for the above-mentioned approach. Recent studies indeed indicate that considerable heterogeneity exist between the toxicity of wheat varieties, opening up the possibility to exploit this for the development of safer wheat.

An interesting alternative to the generation of non-toxic grains is to supplement human gastrointestinal tract with enzymes that are capable of cutting T cell stimulatory sequences. This idea dates back to nineteen fifties when Krainick and Mohn demonstrated in celiac children a decreased toxicity of gluten that had been in vitro digested with papain [40]. Although the subsequent clinical trials with oral papain supplementation did not provide any clear-cut conclusions [41] the concept did not die and in 2002 Chaitan Khosla with his group proposed the application of prolyl oligopeptidase from *Flavobacterium meningosepticum* to degrade the proline-rich gluten molecules [42]. Later on, other bacterial prolyl oligopeptidases were tested. Meanwhile the group of Teodor Stelmasiak in Australia started clinical trials using enzyme preparations from animal intestines but the results were far from satisfactory [43]. It should be noted that the degradation of the toxic gluten sequences would have to be completed before gluten reaches the duodenum. Both bacterial

and animal prolyl oligopeptidases have a pH optimum of pH 7-8 and therefore cannot act in the acidic environment of the stomach. Furthermore, the enzymes are not stable to pepsin and low pH and will thus become inactivated by gastric juice. Additional limitations of bacterial and animal prolyl oligopeptidases result from their relatively low activity and the preference for small substrate peptides [44]. It is clear that none of the mentioned enzymes could be a serious candidate for an oral supplement in management of celiac disease. Instead, a fungal prolyl endoprotease from *Aspergillus niger* (AN-PEP) appeared an interesting alternative [45,46]. We tested this enzyme in vitro and demonstrated that it could efficiently cleave not only all tested gluten peptides but also entire gluten molecules (Chapter 5). The detoxification of the gluten digests was confirmed by the tests with epitope-specific antibodies and patient-derived gluten-specific T cells. Importantly, the enzyme works optimally at pH 4-5, remains active at pH 2 and is entirely resistant to the digestion with pepsin. Due to the high efficiency of AN-PEP we estimate that 10 mg of AN-PEP should be sufficient to detoxify 1 g of gluten. For comparison, in the clinical investigations carried by the Australian group approximately equal amount of enzyme and gluten was used (900 mg of enzyme extract per 3.5 g biscuit) [43]. Prolyl endoprotease form *A. niger* is thus now by far the most promising candidate to be used as oral supplement for treatment of celiac disease.

Since there is no animal model of celiac disease available a clinical trial will ultimately have to be performed to demonstrate whether oral supplementation with AN-PEP can prevent the appearance of clinical manifestations after gluten consumption by celiac patients. Alternatively, AN-PEP may be combined with a blocker of HLA-DQ2 and/or a blocker of tissue transglutaminase to increase the efficiency of the treatment.

Looking to the future

What advances in the studies on celiac disorder are awaiting us in the near future? Development of efficient preventive measures and novel treatment protocols will require a thorough understanding of the mechanisms underlying the pathogenesis. Why do only a fraction of predisposed individuals develop the disease? What are the environmental factors contributing to disease onset? Answering these questions should be the aim of studies in the coming years. There is no doubt that this would be greatly facilitated if an animal model of celiac disease became available. Another fundamental goal that should be further explored is the development of non-toxic cereals and methods that will allow gluten consumption by patients. Enzymatic degradation of gluten seems at present the most tangible approach. Such developments would have a positive impact on the quality of life of millions of celiac patients worldwide. Ultimately the research could lead to protocols that reintroduce mucosal tolerance to gluten. Such therapies are currently being developed in animal

models for a number of autoimmune diseases and if operational they would revolutionize the treatment of celiac patients.

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