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# MOLECULAR BASIS OF CELIAC DISEASE

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■ **Abstract** Celiac disease (CD) is an intestinal disorder with multifactorial etiology. HLA and non-HLA genes together with gluten and possibly additional environmental factors are involved in disease development. Evidence suggests that $CD4^+$ T cells are central in controlling an immune response to gluten that causes the immunopathology, but the actual mechanisms responsible for the tissue damage are as yet only partly characterized. CD provides a good model for HLA-associated diseases, and insight into the mechanism of this disease may well shed light on oral tolerance in humans. The primary HLA association in the majority of CD patients is with DQ2 and in the minority of patients with DQ8. Gluten-reactive T cells can be isolated from small intestinal biopsies of celiac patients but not of non-celiac controls. DQ2 or DQ8, but not other HLA molecules carried by patients, are the predominant restriction elements for these T cells. Lesion-derived T cells predominantly recognize deamidated gluten peptides. A number of distinct T cell epitopes within gluten exist. DQ2 and DQ8 bind the epitopes so that the glutamic acid residues created by deamidation are accommodated in pockets that have a preference for negatively charged side chains. Evidence indicates that deamidation in vivo is mediated by the enzyme tissue transglutaminase (tTG). Notably, tTG can also cross-link glutamine residues of peptides to lysine residues in other proteins including tTG itself. This may result in the formation of complexes of gluten-tTG. These complexes may permit gluten-reactive T cells to provide help to tTG-specific B cells by a mechanism of intramolecular help, thereby explaining the occurrence of gluten-dependent tTG autoantibodies that is a characteristic feature of active CD.

## INTRODUCTION

Celiac disease (CD), or gluten sensitive enteropathy, is a condition in which ingested wheat gluten or related proteins from rye and barley are not tolerated (1). CD, like type 1 diabetes, rheumatoid arthritis, and multiple sclerosis, has a chronic nature where particular HLA alleles are overrepresented among the patients (2). Commonly these disorders are multifactorial; HLA genes and other genes together with environmental factors are involved in disease development. The expression of CD is strictly dependent on dietary exposure to gluten and

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similar cereal proteins (1). Patients go into complete remission when they are put on a gluten-free diet, and they relapse when gluten is reintroduced into the diet. CD is in this respect unique among the chronic inflammatory HLA- associated diseases in that a critical environmental factor has been identified.

CD is primarily a disease of caucasians (1). It is most frequently recognized among Europeans, although there is an increasing awareness of this disorder in the United States. CD commonly presents in early childhood with classic symptoms including chronic diarrhea, abdominal distension, and failure to thrive (3). The general condition of these children is severely impaired. The disease may also present later in life with symptoms that tend to be more vague and include anemia, fatigue, weight loss, diarrhea, constipation, and neurological symptoms (4).

CD patients on a gluten-containing diet have increased levels of serum antibodies to a variety of antigens, including gluten and the autoantigen tissue transglutaminase (tTG) (5, 6). The presence of antibodies to gluten and tTG is strictly dependent on dietary exposure to gluten. Testing of serum antibodies to gluten and tissue tTG is utilized to predict CD, and this provides a great aid in clinical practice (5, 7, 8). The final diagnosis of CD, nevertheless, rests on the demonstration of typical mucosal pathology by histological examination of small intestinal biopsies. The reported prevalence of disease with overt symptoms varies enormously in the populations of Europe and North America. Assessment of the prevalence by biopsy examination of individuals identified by antibody screening has however demonstrated surprisingly similar prevalence rates of about 1:200 to 1:400 throughout Europe and North America (9). Many of the patients identified in these studies have no symptoms or only mild symptoms that are often associated with decreased psychophysical well-being and anemia (4, 9).

The clinical expression of CD is probably influenced by environmental factors. In Sweden an ''epidemic'' of CD in children under the age of two years produced a dramatic fourfold increase in incidence rates in the period 1985–1987 and a similar rapid decline in the incidence rates from 1995–1997 (10). These changes in incidence concur with changes in infant feeding practices and suggest that the amount and timing of the gluten introduction (perhaps in conjuction with the breast feeding duration) is important for precipitation of the disease in children (10). Whether the pattern of gluten feeding in infants affects only the age of onset of the disease or whether it ultimately changes the overall population prevalence is still an open question.

Current treatment of CD is a lifelong exclusion of gluten from the diet. Poor diet compliance by patients and undiscovered disease are associated with complications including increased risk of anemia, infertility, osteoporosis, and intestinal lymphoma (4). Notably, untreated CD is associated with increased mortality. Research into the molecular basis of the disease has already lead to improved diagnosis, and it is hoped this research will lead to better treatment in the future.

## THE CELIAC LESION

The lesion in CD is localized in the proximal part of the small intestine. Villous atrophy, crypt cell hyperplasia, lymphocytic infiltration of the epithelium, and increased density of various leukocytes in the lamina propria characterize the classic textbook type of lesion (1). These alterations represent one end of a spectrum of mucosal pathology that Marsh (11) has classified into three stages: the infiltrative, the hyperplastic, and the destructive lesions. The infiltrative lesion is characterized by infiltration of small nonmitotic lymphocytes in the villous epithelium without any other sign of mucosal pathology. The hyperplastic lesion is similar to the infiltrate lesion but in addition has hypertrophic crypts whose epithelium may be infiltrated by lymphocytes. The destructive lesion is synonymous to the classic lesion described in textbooks. Oral challenge experiments with gluten have demonstrated that these stages are dynamically related (12). The existence of a spectrum of pathological stages in CD is interesting when considering the polygenic nature of CD. In the NOD mouse model of autoimmune diabetes, where at least 14 different loci are involved in the control of the disease, nearly all NOD mice develop insulitis, but many animals do not go on to develop diabetes (13). Notably fewer susceptibility genes are required to produce insulitis than diabetes (13). It is conceivable that in CD different susceptibility genes contribute at different stages to the development of the end-stage disease.

The pathological alterations and the type of cellular infiltrates found in the classical, flat-destructive lesion are well characterized, and the major features are summarized in the following.

### Enterocytes

In CD there is an increased loss of epithelial cells and increased proliferation of epithelial cells in the crypts. Both these factors have been used to explain the villus atrophy found in CD (14, 15). It is not clear whether the two phenomena are causally linked, and if so, which of them is primary or secondary. The increased epithelial cell loss probably reflects increased apoptosis of enterocytes (16), whereas the increased enterocyte proliferation appears to be due to an increased production of keratinocyte growth factor (KGF) by stromal cells (17).

Several molecules with immune function are known to have an altered expression in CD. There is an increased epithelial expression of HLA class II molecules with strong expression of DR and DP molecules, but with little or no expression of DQ molecules (18, 19). The expression of the polymeric Ig receptor is also upregulated (20). Notably, this enhanced expression of the polymeric Ig receptor is accompanied by increased transport of IgA and IgM into the gut lumen (21).

### Intraepithelial Lymphocytes

Three major lineages of intraepithelial lymphocytes (IELs) occur in the normal human small intestine; the most prominent is the TCR$\alpha\beta+$ $CD8^{+}CD4^{-}$ popu-

lation, while the TCRαβ+ $CD8^-CD4^+$ population and the TCRγδ+ $CD8^-CD4^-$ population are also present. Both the TCRαβ+ $CD8^+CD4^-$ and the TCRγδ+ $CD8^-CD4^-$ populations are expanded in CD. In contrast to the TCRαβ+ $CD8^+$ IELs that return to normal when gluten is removed from the diet, the TCRγδ+ IELs appear to remain at an elevated level (22). However, IELs of both the TCRαβ+ $CD8^+$ and TCRγδ+ lineages express the Ki67 proliferation marker, suggesting intraepithelial proliferation of both populations in CD (23). Interestingly, the majority of TCRγδ+ IELs express the Vδ1 TCR variable region (24, 25). Spies and co-workers have demonstrated that γδ T cells expressing this variable region recognize MICA and MICB molecules (26)—molecules that are mainly expressed by intestinal epithelial cells (27). Activated human IELs are able to produce a number of cytokines including IFN-γ, IL-2, IL-8 and TNF-α and are known to have a lytic potential (28). Furthermore, in CD, but not in giardiasis, the IELs stain positive for granzyme B and TiA (a marker characteristic for cytotoxic lymphocytes), indicating that some IELs in the celiac lesion may be activated cytotoxic T cells (29).

## Lamina Propria Leukocytes

A marked infiltration of TCRαβ+ T cells appears in the lamina propria in the active lesion. These T cells are mostly $CD4^+$ and carry a memory phenotype (CD45RO+) (30). Notably, an increased percentage of these lamina propria T cells express the CD25 (IL2R α-chain) activation marker but lack the Ki67 marker associated with proliferation (23). Thus, gluten appears to induce a nonproliferative activation of $CD4^+$ lamina propria T cells. This fits well with the results of several studies reporting increased cytokine production by T cells in the lamina propria (31–33). There seems to be a particular increase in cells producing IFN-γ, whereas no increase appears in cells producing IL-4 or IL-10 (33, 34). mRNA for IFN-γ has been found to be increased more than 1000-fold in untreated disease related to a small increase in the message for IL-2, IL-4, IL-6, and TNF-α (33). Furthermore, the IFN-γ mRNA level of biopsies of treated patients has been demonstrated to reach that of untreated patients by in vitro stimulation with gluten (33). Altogether, these results are consistent with the conception that gluten-reactive T cells in the lamina propria have a cytokine profile dominated by production of IFN-γ.

A characteristic of the CD lesion is an accumulation of IgA-, IgM-, and IgG-producing plasma cells (35). The specificities of the antibodies produced by these cells have been only partly characterized; however, in vitro culture of biopsies has demonstrated that antibodies to gliadin (36) and endomysium (i.e. tTG) (37) are produced.

Just beneath the epithelium in the normal mucosa a high number of macrophage/dendritic-like cells stain positive for CD68 (38). It is conceivable that these cells are involved in sampling of luminal antigens. The expression of the HLA

class II, ICAM-1, and CD25 molecules is increased in these macrophage/dendritic-like cells, suggesting that they are activated in the disease state (18, 23, 39).

### The Extracellular Matrix

In the normal small intestine extracellular matrix formation (ECM) by stromal cells balances ECM degradation mediated by matrix metalloproteinases (MMPs). Increased ECM degradation has been suggested to play a role in the villous atrophy of CD. This is supported by the demonstration of a decreased ratio of cells expressing collagen I and tissue inhibitor of metalloproteinases (TIMP)-1 mRNA to those expressing matrix metalloproteinase (MMP)-1 and -3 mRNA in untreated CD (40). Expression of MMP-1 and MMP-3 mRNA is mainly localized to subepithelial fibroblasts and macrophages. It is likely that the increased expression of metalloproteinases is related to activation of mucosal T cells (see later).

## THE GENETICS OF CELIAC DISEASE

A high prevalence rate (10%) among first degree relatives of CD patients indicates a strong genetic influence on susceptibility to develop CD (41). Familial clustering can be expressed as the ratio of the prevalence in relatives of affected individuals over the prevalence within the population as a whole (42). The ratio $\lambda_s$ based on the sibpair risk is the most commonly used. If this ratio is close to 1, then there is no evidence for genetic factors in susceptibility. In contrast, the $\lambda_s$ value for CD is estimated to be 30–60 (42, 43), which is high compared with other multifactorial disorders like rheumatoid arthritis, type 1 diabetes, and multiple sclerosis. The strong genetic influence in CD is further supported by a high concordance rate of 70% in monozygotic twins (44). The sibship aggregation attributable to HLA ($\lambda_{s\ HLA}$) is estimated to be 2.3–5.5 (42, 43). Using these estimates and assuming a multiplicative model of disease predisposing genes, the overall importance of non-HLA genes has been calculated to be greater than that of HLA genes (42, 43). However, attempts to map predisposing genes by linkage analysis have, with the exception of the HLA, failed to reveal unambiguous candidate genes or chromosomal regions (45–48). This suggests that each of the yet-unmapped predisposing CD genes has only a minor genetic influence. Indications for susceptibility regions at 5qter and 11qter are weak (47). As with other polygenic inflammatory diseases, little is known about the non-HLA susceptibility genes. Conceivably, however, the gene products of many of these genes have immune-related functions. In the case of CD, the HLA genes (see later) and the non-HLA genes shape the immune response to gluten so that immunopathology is produced in the small intestine. Relevant to this are the recent reports that the CTLA-4/CD28 gene region contains a CD susceptibility gene (49, 50), although this finding is not consistent in all populations (51).

## HLA GENES IN CELIAC DISEASE

CD was first found to be associated with the HLA class I molecule B8 (52, 53). Later stronger associations were found to the HLA class II molecules DR3 and DQ2 (54–56). The genes encoding DR3 and DQ2 are in strong linkage disequilibrium, and DR3 and DQ2 are both contained within the B8-DR3-DQ2 or the B18-DR3-DQ2 extended haplotypes. The B8-DR3-DQ2 and the B18-DR3-DQ2 haplotypes are both associated with CD (57, 58). This is significant, as these two haplotypes are conspicuously dissimilar in the regions outside the DR-DQ region.

CD is also associated with DR7 (59, 60), but this association is seen almost only when DR7 occurs together with DR3 or DR5 (61, 62). This is unlike the susceptibility associated with DR3, which is seen irrespective of the accompanying DR allele. In studies to date, most CD patients have been shown to carry either the DR3-DQ2 haplotype or are DR5-DQ7/DR7-DQ2 heterozygous. Evidently, CD patients with these DR-DQ combinations share the genetic information conferring CD susceptibility (63) (see Figure 1, bottom part). The DQA1*0501 and DQB1*0201 alleles of the DR3-DQ2 haplotype (64, 65) are also found when

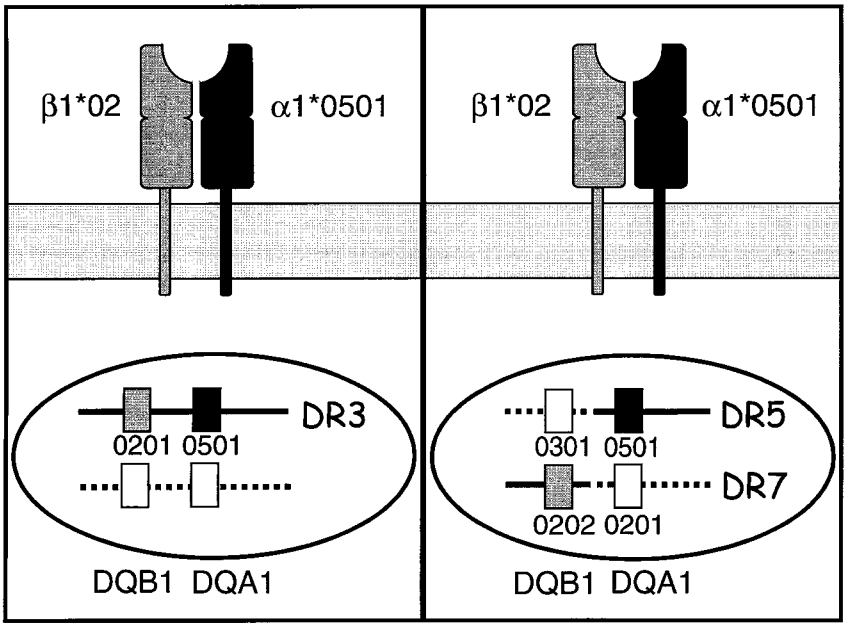


**Figure 1** Patients with CD who are DR3 or DR5/DR7 heterozygous express the same HLA-DQ2 molecule, HLA-DQ(α1*0501, β1*02). The DQA1*0501 and DQB1*02 genes are located in *cis* (on the same chromosome) in DR3 individuals, whereas they are located in *trans* (on opposite chromosomes) in DR5/DR7 heterozygous individuals.

the DR5-DQ7 and DR7-DQ2 haplotypes are combined. The DR5-DQ7 haplotype carries the DQA1*0501 and DQB1*0301 alleles (66), and the DR7-DQ2 haplotype carries the DQA1*0201 and DQB1*0202 alleles (67, 68). Notably, the DQB1*0201 and DQB1*0202 alleles are identical except for the codon of residue 135 located in the membrane proximal domain of the DQβ chain (69). Recombination (crossing over) seems to be an important mechanism for the generation of HLA haplotypes (70). Accumulating evidence suggests that the DR3-DQ2, DR7-DQ2, and the DR5-DQ7 haplotypes have a close evolutionary relationship. Based on microsatellite analysis, fragments of DNA flanking the DQA1 gene of the DR3-DQ2 haplotype have been identified on the DR5-DQ7 haplotype, and fragments of DNA flanking the DQB1 gene of the DR3-DQ2 haplotype have been identified on the DR7-DQ2 haplotype (71, 72). Thus, the genetic information in the DQ subregion of the DR3-DQ2 haplotype is reestablished in DR5-DQ7/DR7-DQ2 heterozygotes, although the sequence information is split between two chromosomes.

It can be argued that susceptibility for CD depends on an interaction between at least two genes on the DR3-DQ2 haplotype that are reunited in DR5-DQ7/DR7-DQ2 heterozygous individuals. Theoretically this gene interaction could involve any HLA-linked genes in the DQ region. However, complete sequencing of an 86-kb genomic fragment spanning the DQ subregion of the DR3-DQ2 haplotype failed to identify genes other than the DQA1 and the DQB1 genes in this region (73). Furthermore, the DQA1 and DQB1 are very good candidates because their products interact by forming a class II heterodimer and because they are situated close to the putative recombination site. This evolutionary consideration together with the fact that most CD patients share a particular pair of DQA1 and DQB1 genes located either in *cis* or in *trans* are strong arguments that the DQA1*0501 and DQB1*0201 alleles jointly confer susceptibility to CD by coding for the DQ(α1*0501, β1*02) heterodimer (Figure 1, top part).

In most populations studied, 90% or more of the CD patients carry the DQ(α1*0501, β1*02) heterodimer, compared to 20%–30% in healthy controls (63). The fraction of patients in different populations that encode this DQ heterodimer by genes in *cis* or in *trans* position depends on the haplotype frequencies of DR3-DQ2, DR5-DQ7, and DR7-DQ2 haplotypes in the given populations (74). In a few patients the DQ(α1*0501, β1*02) heterodimer may be found to be encoded in *cis* position by haplotypes other than DR3-DQ2 or in *trans* position by individuals being heterozygous for combinations other than DR5-DQ7/DR7-DQ2 (63). There is no increase of the DQ(α1*0501, β1*0301) or DQ(α1*0201, β1*02) heterodimers alone in CD demonstrating that susceptibility is dependent on both the DQα and DQβ chains in the DQ(α1*0501, β1*02) heterodimer.

Many studies have reported a particular increased risk for CD among individuals who are DR3-DQ2 homozygous and DR3-DQ2/DR7-DQ2 heterozygous (for references, see 63). This could be explained by a gene dosage effect of the DQB1*02 allele possibly caused by an increased expression of the DQ(α1*0501, β1*0201) heterodimer in such individuals (75). A gene dosage effect of DQB1*02

could also provide an explanation of the high degree of HLA haplotype identity observed among affected siblings (74, 76).

Depending on the populations studied, about 2%–10% of CD patients do not carry the DQ(α1*0501, β1*02) heterodimer. The great majority of these patients carry different subtypes of DR4. The genetic determinant responsible for the HLA association in these individuals is likely to be different from that of the DQ(α1*0501, β1*02)-expressing individuals. To unequivocally identify the responsible molecule encoded by the DR4 haplotype by a genetic approach is, however, difficult. Notably, there is a clear skewing in the representation of the DR4-DQ8 vs the DR4-DQ7 haplotype among these patients (77–79). This implies that DQ8, i.e. DQ(α1*0301, β1*0302), is most probably the molecule responsible for susceptibility. An opposing view is that the susceptibility is mediated by the DR53, i.e. DR(α*, β4*0101), molecule that is carried on most of the DR4, DR7, and DR9 haplotypes (80). The majority of DQ(α1*0501, β1*02)-negative patients would fit into this category. Importantly, however, this model does not account for the observed skewing of the DR4-DQ8 vs. the DR4-DQ7 haplotypes. Moreover, DQ(α1*0501, β1*02)-negative CD patients who carry the DRB1*0701-DQB1*03032 haplotype exist (79), and this haplotype is reported to carry a non-expressed null allele at the DRB4 locus (81). Further studies including typing for the DRB4 null allele are needed to clarify the role of DR53 as a susceptibility molecule in CD.

Genes located in the HLA gene complex other than DQ might also contribute to CD susceptibility. Associations to particular DP alleles have been reported in different populations, but many of these associations can be explained by linkage disequilibrium between the involved DP allele(s) and the DQA1*0501 and DQB1*02 alleles (for further discussion, see 63). Moreover, no independent associations to alleles at the TAP1 and TAP2 loci have been found (82–84). Several studies have consistently indicated that DQA1*0501/DQB1*02-positive individuals carrying the DR5/DR7 genotype have a higher risk to develop disease than do those of the DR3/DRX genotype (X ≠ DR7 and DR3) (84–86). Furthermore, it has been indicated that the risk of the DR3/DR7 genotype is higher than that of the DR3/DR3 genotype (84, 86), although this is not a consistent finding (75, 87). This has led to the suggestion that a gene on the DR7-DQ2 haplotype confers an additive effect to that of the DQA1*0501/DQB1*02 genes (86). To note, a locus with a protective allele of the DR3-DQ2 haplotype would produce the same effect. Studies of Irish CD patients have indicated an additional predisposing role of TNF genes, an association independent of DQ2 that has been demonstrated using a microsatellite polymorphism situated near the TNF genes (88). Moreover, a polymorphism of the TNF-α gene promoter has been demonstrated to be a component of the DR3-DQ2 haplotype (89). A Finnish study failed to reproduce the finding of a DQ2-independent association of the TNF microsattelites (90). These discrepant results may relate to population differences.

Recently, an allele of a locus (D6S2223) that is located 2, 5 Mb telomeric to the HLA-F locus was found by Lie et al (91) to be less frequent among DR3-

DQ2 homozygous CD patients compared to DR3-DQ2 homozygous controls. The same allele of the D6S2223 locus was also found to be underrepresented among DR3-DQ2 homozygous type 1 diabetes patients, and it was transmitted less often than expected from DR3-DQ2 homozygous parents to diabetic siblings (92). These findings suggest that a gene(s) in the vicinity of D6S2223 is involved in the pathogenesis of both CD and type 1 diabetes. In addition, the MIC-A and MIC-B genes are interesting candidate susceptibility genes in CD, as the MIC molecules are ligands for TCR$\gamma\delta$ T cells. The MIC genes are located near the HLA-B locus, and the MIC-A*008 (5.1) allele is in strong positive linkage disequilibrium with HLA-B8 (93, 94). This allele is particularly interesting since it bears a frameshift and a premature stop codon in exon 5 (95) that might affect the expression of the molecule.

Taken together, available data strongly suggest that susceptibility to develop CD is primarily associated to two conventional peptide-presenting DQ molecules: i.e. DQ($\alpha$1*0501, $\beta$1*02) (= DQ2) or to a lesser extent DQ($\alpha$1*03, $\beta$1*0302) (= DQ8). An issue still to be clarified is whether there are additional molecules encoded by unidentified genes in the HLA gene complex that also contribute to the genetic predisposition for CD. However, any effect of these additional genes is likely to be moderate. A key question for the understanding of the molecular basis for CD is therefore to define the functional role of the DQ2 and DQ8 molecules.

## PEPTIDE BINDING MOTIF OF DISEASE-ASSOCIATED DQ MOLECULES

Peptides binding to DQ2 have anchor residues in the relative positions P1, P4, P6, P7, and P9 (96–100). This is the same spacing as previously found for DR molecules, suggesting that DQ2 bound peptides adopt to a conformation similar to that of peptides bound to DR molecules. The peptide-binding motif of DQ2 illustrated in Figure 2 is quite different from other class II–binding motifs that have been identified (101). Notably, the preference for negatively charged residues for the three anchor positions in the middle seems to be unique for DQ2. The binding motif of DQ8 is different from that of DQ2, but DQ8 also displays a preference for binding negatively charged residues at several positions (i.e. P1, P4, and P9) (102, 103). Hence, both the DQ2 and DQ8 molecules share a preference for negatively charged residues at some of their anchor positions.

The peptide-binding motif of DQ2, i.e. DQ($\alpha$1*0501, $\beta$1*02), is different from the motifs of the closely related DQ($\alpha$1*0501, $\beta$1*0301) and DQ($\alpha$1*0201, $\beta$1*02) molecules (96, 99, 104), which do not confer susceptibility to CD (see above). The binding motif of the DQ($\alpha$1*0501, $\beta$1*0301) molecule is clearly different from that of DQ($\alpha$1*0501, $\beta$1*02) with differences at the P4, P7 and P9 pockets (96), whereas the differences between DQ($\alpha$1*0501, $\beta$1*02) and

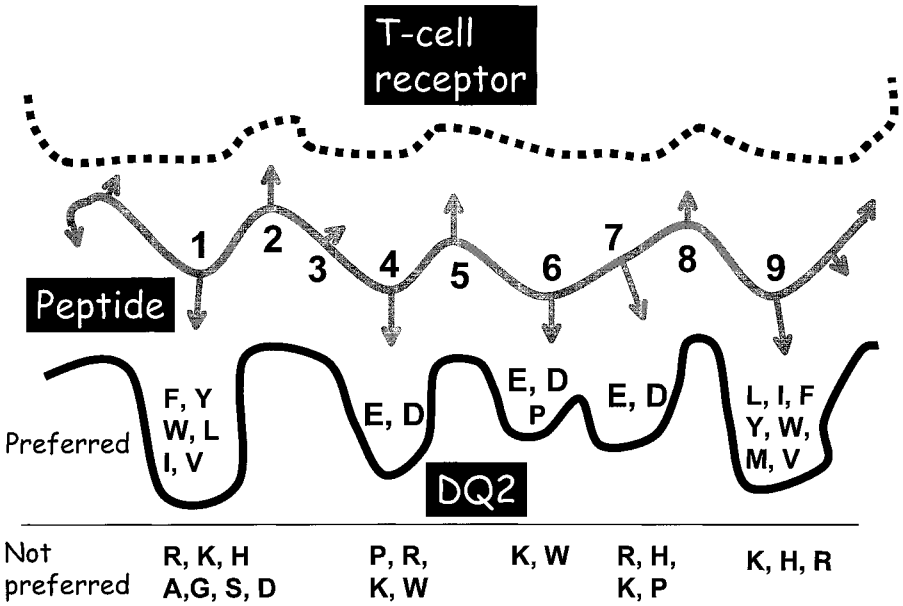


**Figure 2** Schematic depiction of the peptide binding groove of HLA-DQ2 (i.e. HLA-DQ(α1*0501, β1*02) with the peptide-binding motif displayed with the one letter code for the amino acids. A bound peptide and a TCR recognizing the peptide/HLA complex are also indicated. This motif description is based on a compilation of results from references 96–100.

DQ(α1*0201, β1*02) are more subtle (96, 99). The molecules have similar binding motifs with the most apparent difference being an additional anchor residue at P3 for DQ(α1*0201, β1*02) (99, 105).

## PREFERENTIAL PRESENTATION OF GLUTEN-DERIVED PEPTIDES BY DISEASE-ASSOCIATED HLA MOLECULES TO INTESTINAL T CELLS

The DQ2 and DQ8 molecules could confer susceptibility to CD by presenting disease-related peptides in the target organ or alternatively by shaping the T cell repertoire during T cell development in the thymus. This issue has been addressed by studies of T cells derived from the celiac lesion. Stimulation of small intestinal biopsy specimens with a peptic/tryptic digest of gluten induces rapid activation (i.e. expression of CD25, the IL-2 receptor α-chain) of the T cells in the lamina propria of CD patients, but not of non-CD control subjects (106). Gluten-reactive T cells can be isolated and propagated from intestinal biopsies of CD patients but

not from non-CD controls (107–109). These T cells are $CD4^+$ and use the αβ TCR. Importantly, T cells isolated from biopsy specimens of patients carrying the DR3-DQ2 haplotype typically recognize gluten fragments presented by the DQ2 molecule rather than the other HLA molecules carried by the patients (107). Both DR3-DQ2-positive and DR5-DQ7/DR7-DQ2-positive antigen-presenting cells (i.e. carrying the DQA1*0501 and DQB1*02 genes in *cis* or in *trans* position) are able to present the gluten antigen to these T cells (107, 110). Likewise, T cells isolated from small intestinal biopsies of DQ2-negative, DR4-DQ8-positive patients predominantly recognize gluten-derived peptides when presented by the DQ8 molecule (111). It is notable that no DR(α, β1*01)-restricted intestinal T cells specific for gluten have been reported supporting a role of DQ8 rather than the DR(α, β1*01) molecule in conferring susceptibility to CD. Taken together, these results allude to presentation of gluten peptides in the small intestine as the mechanism by which DQ2 and DQ8 confer susceptibility to CD. A thymic effect of the same DQ molecules on the TCR repertoire selection is, however, not excluded by these results.

The DQ2 and DQ8 molecules are not preferential antigen-presenting molecules in the intestinal mucosa irrespective of antigen. T cells specific for astrovirus (a common gastroenteritis virus) are predominantly DR restricted (109), which suggests that the peculiar HLA restriction pattern of the gliadin-specific T cells of the intestine must be related to the antigen. Interestingly, gluten-specific T cells can also be found in the peripheral blood (112). These T cells are restricted either by DR, DP, or DQ molecules, and they do not therefore display the the predominant DQ2 or DQ8 restriction observed for gluten-specific T cells from the intestinal mucosa (112). One explanation for this could be that the majority of gluten-specific T cells of peripheral blood recognize epitopes different from those recognized by T cells of the small intestine.

Studies of lamina propria T cells in situ have, as mentioned above, indicated that gluten reactive T cells have a cytokine profile dominated by IFN-γ. This notion is sustained by the characterization of gut-derived DQ2 and DQ8-restricted gluten-specific T cell clones. These T cells uniformly secrete IFN-γ at high concentrations, and some produce IL-4, IL-5, IL-6, IL-10, TNF-α, or TGF-β in addition (113).

## T CELL RECOGNITION OF DEAMIDATED GLUTEN PEPTIDES

Wheat gluten is a mixture of numerous proteins grouped into the gliadin and glutenin fractions. These proteins serve as a source of nitrogen and carbon for the growing seedling during germination. A vast sequence heterogeneity among gliadin and glutenin proteins probably reflects that these proteins have been subjected to few structural constraints during evolution. Generally, gluten proteins

contain a large percentage of proline and glutamine residues, while many other amino acids, including glutamic and aspartic acid, are unusually scarce. Feeding experiments have demonstrated that the gliadin fraction can precipitate CD (114), whereas the role of glutenins is still inconclusive. Proteins of the gliadin fraction can be subdivided according to their sequence into the α-, γ-, and ω-gliadins (115). A large number of different gliadins exist within each of these gliadin families. Estimates suggest that as many as 50 to 150 different α-gliadin genes may be present in a single wheat cultivar (116). For the work of identifying peptide fragments recognized by T cells, the complexity of this antigen presents a big challenge.

Initially it was difficult to reconcile the DQ2 (and DQ8) binding motifs with presentation of gluten peptides because gluten proteins have an unusual scarcity of negatively charged residues. A clue to help explain this paradox came from the observation that the stimulatory capacity of gliadin preparations for gliadin-specific intestinal T cells was significantly enhanced following treatment at high temperatures and low pH (117). These conditions are known to cause nonspecific deamidation of glutamines to glutamic acid and may thus convert gliadin from a protein with very few peptides with the potential to bind to DQ2/DQ8 into one with many such. An important and general role for deamidation of gluten for T cell recognition was sustained by analysis of the response pattern of a panel of polyclonal, gliadin-specific T cell lines derived from biopsies (118). All the lines responded poorly to a gliadin antigen prepared under conditions of minimal deamidation (chymotrypsin-digestion), compared to the same antigen when further heat-treated in an acidic environment.

The characterization of gluten epitopes recognized by intestinal T cells has extended the knowledge about the importance of deamidation for their T cell recognition. So far five unique epitopes of gluten that are recognized by gut T cells have been identified; three restricted by DQ2 (118, 119) (Table 1 and Figure 3) and two restricted by DQ8 (120, 121) (Table 1). The three DQ2-restricted peptides, one from γ-gliadin and two from α-gliadins (DQ2-γ-gliadin-I, DQ2-α-gliadin-I and DQ2-α-gliadin-II), fail to stimulate T cells in their native form but are potent antigens when a single glutamine residue is exchanged with glutamic

**TABLE 1**

| Epitope | Derived from protein | Presentation element | Recognized by patients | Reference |
|---|---|---|---|---|
| DQ2-γ-I-gliadin | γ-gliadin | DQ2 | Infrequently | (118) |
| DQ2-α-I-gliadin | α-gliadin | DQ2 | Frequently | (119) |
| DQ2-α-II-gliadin | α-gliadin | DQ2 | Frequently | (119) |
| DQ8-α-I-gliadin | α-gliadin | DQ8 | Frequently? | (120) |
| DQ8-I-glutenin | Glutenin | DQ8 | Not known | (121) |

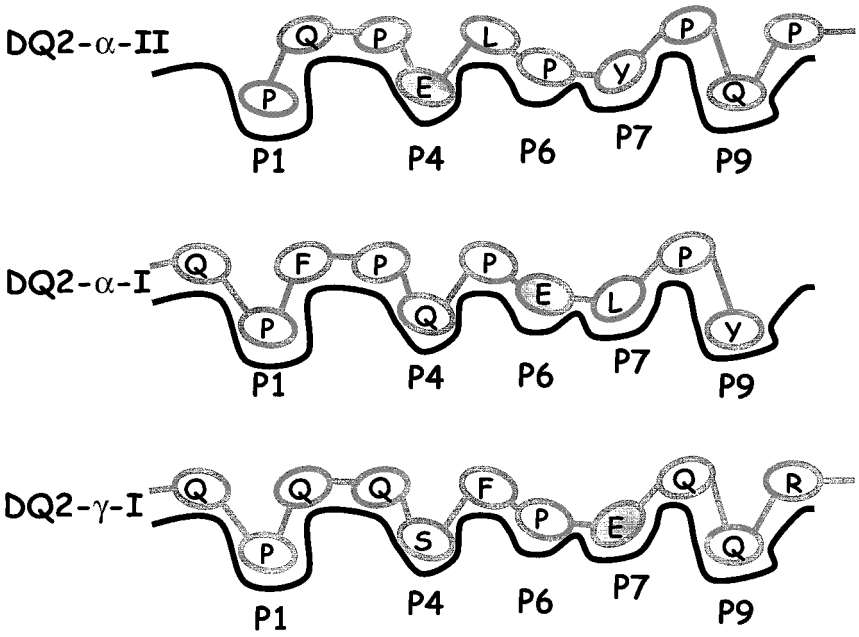


**Figure 3** A cartoon showing the amino acid sequence (one letter code) and binding of the three known gluten epitopes recognized by HLA-DQ2 restricted intestinal T cells of CD patients. All three epitopes contain a glutamic acid (E) residue that has been converted from glutamine (Q) by deamidation. The glutamic acid residues formed by deamidation improve the binding affinity and are critical for T cell recognition in all three epitopes. Notably, glutamic acid residues are accommodated in the P4 pocket for the DQ2-α-II-gliadin epitope (119), in the P6 pocket for the DQ2-α-I-gliadin epitope (119), and in the P7 pocket for the DQ2- γ-I-gliadin epitope (118). In some α-gliadins, the DQ2-α-I-gliadin and DQ2-α-II-gliadin epitopes are part of the same fragment, and it is the very same glutamine that is modified by tTG in both epitopes.

acid in certain positions. The recognition of one of the DQ8-restricted peptides from α-gliadins (DQ8-α-gliadin-I) is augmented by introduction of negatively charged residues (122), whereas this is not seen for another DQ8-restricted peptide of glutenin (DQ8-glutenin-I) (121). These data demonstrate that most, but not all, gluten-specific intestinal T cells from CD patients recognize gluten proteins only after they have undergone deamidation. Moreover, the results with the glutenin epitope demonstrate that intestinal T cells can recognize gluten proteins other than gliadins (121). This raises the question of whether glutenins are also able to precipitate the disease.

## DEAMIDATION IN VIVO IS LIKELY TO BE ENZYMATICALLY MEDIATED BY TISSUE TRANSGLUTAMINASE

The deamidation of gliadin may take place in the acidic environment in the stomach (118). Alternatively, it can be mediated by the enzyme tissue transglutaminase (tTG) as demonstrated by Molberg et al (123) and later also by van de Wal et al (122). tTG is expressed in many different tissues and organs; in the small intestine it is expressed just beneath the epithelium in the gut wall (123). Notably the activity of tTG is elevated in the small intestinal mucosa of CD patients in both the active disease phase and in remission (123a). The enzyme is present both intracellularly and extracellulary, and in the extracellular environment tTG plays a role in extracellular matrix assembly, cell adhesion, and wound healing (124). The calcium-dependent transglutaminase activity of tTG catalyzes selective cross-linking or deamidation of protein-bound glutamine residues (125). Notably, tTG is the same protein that Dieterich et al found to be a major focus of the autoantibody response in CD (6). In contrast to the nonenzymatically mediated deamidation that results in a near random deamidation of the often numerous glutamine residues in gliadin peptides, tTG appears to carry out an ordered deamidation of some few specific glutamines (123). For all the three DQ2-restricted gliadin epitopes recognized by gut T cells and the DQ8-α-gliadin-I epitope, the residues critical for T cell recognition are all specifically targeted by tTG (119, 122, 123). Interestingly, the deamidation of glutamines that are not targeted by tTG (e.g. by acid treatment) can be deleterious for T cell recognition (105, 122). Additional evidence for a role of tTG comes from experiments where T cell lines have been established from biopsies challenged with a minimally deamidated gliadin antigen (chymotrypsin-digested) and then tested for recognition of this antigen or the same antigen treated with tTG (Ø Molberg, S McAdam, KEA Lundin, C Kristiansen, K Kett, EH Arentz-Hansen, LM Sollid, manuscript in preparation). In 14 out of 15 patients, the T cell lines responded better to the antigen that had been subjected to treatment with tTG. Similarly, T cell lines established from two DQ2+ patients by stimulating biopsies with a chymotrypsin-digested recombinant α-gliadin were found to recognize synthetic peptides representing the DQ2-α-gliadin-I and DQ2-α-gliadin-II epitopes, but not the corresponding non-deamidated peptides (Ø Molberg, S McAdam, KEA Lundin, C Kristiansen, EH Arentz-Hansen, K Kett, LM Sollid, manuscript in preparation). Taken together, these results indicate that deamidation in vivo is mediated by tTG.

It is intriguing to hypothesize that tTG plays a central role in the selection of gliadin T cell epitopes. Credence to this idea comes from the observation that the intestinal T cell response to α-gliadin in adults is focused on a single deamidated glutamine (in the related DQ2-α-gliadin-I and DQ2-α-gliadin-II epitopes) that is targeted by tTG (119). Knowledge of the substrate recognition sites of tTG should allow further testing of this hypothesis. Unfortunately, the available information

on sequences targeted by tTG is not presently sufficient to establish the overall substrate specificity of the enzyme.

## HIERARCHIES OF GLUTEN T CELL EPITOPES?

The existence of multiple epitopes in gluten that are recognized by small intestinal T cells of CD patients raises several interesting questions: Are only some of the epitopes pathogenic and thereby relevant to explain the HLA association? Are responses toward some of the epitopes generated during the early phases of disease development, while the responses to others are a result of epitope spreading? Are different epitopes recognized by distinct groups of patients (e.g. children vs. adults)? Are some epitopes more relevant to disease as responses to them are found in the majority of the patients or because there is a higher precursor frequency of T cells in the lesion specific for these epitopes? The answers to most of these questions must await further investigations. At present we know that for the DQ2-α-gliadin-I and DQ2-α-gliadin-II epitopes, intestinal T cell reactivity is found in most if not all adult DQ2+ patients (119), whereas for the DQ2-γ-gliadin-I epitope, intestinal T cell reactivity is found in only a minority of DQ2+ patients (118). Less is known about the DQ8-restricted epitopes because few DQ8-positive patients have been tested so far. However, the DQ8-α-gliadin-I appears to be frequently recognized (120). What causes the variance in responsiveness to the different epitopes and whether this reflects qualitative or quantitative differences between the patients are presently unclear.

Epitope spreading (126) may be a mechanism relevant to CD that could explain the existence of several gluten epitopes. In experimental autoimmune encephalomyelitis where epitope spreading occurs, along with spreading of new antigenic epitopes there is also a ''spreading'' of MHC class II molecules involved in epitope presentation (127, 128). The strict restriction of DQ2 and DQ8 as presentation elements for gluten-reactive T cells of the disease lesion clearly deviates from the picture found in experimental autoimmune encephalomyelitis and may suggest that other mechanisms are operating. Further studies are clearly needed to sort out this question.

The mapping of epitopes of gluten proteins recognized by intestinal T cells is incomplete; the actual number of distinct epitopes is currently a matter of speculation. However, recent results from testing intestinal T cells of Norwegian adults against a panel of recombinant α-gliadins suggest that the number of epitopes might be more limited than initially thought (119). From the sequences represented in a panel of full-length recombinant α-gliadins, there seems to be only a single immunodominant fragment that contains the two related epitopes DQ2-α-gliadin-I and DQ2-α-gliadin-II.

The disease relevance of epitopes defined using peripheral blood T cells must also be questioned because peripheral blood T cell gliadin epitopes do not appear to be limited in their presentation by DQ2 or DQ8 (112) nor to be enhanced by

treatment with tTG (123). Furthermore, a DQ2-restricted epitope of α-gliadin, which was defined by peripheral blood T cells of a CD patient (129) and which induces mucosal changes in peptide feeding experiments (130), fails to be recognized by gluten-reactive polyclonal intestinal T cell lines from six patients even after tTG treatment (119).

## GLUTEN-SPECIFIC T CELLS MAY PROVIDE HELP FOR AUTOANTIBODY PRODUCTION

The IgG and IgA serum antibodies to tTG (also termed anti-endomysial antibodies) are a hallmark of CD, and detection of serum IgA tTG-antibodies is utilized to predict the disease (7, 8). As the B cells producing the tTG antibodies have undergone an isotype switch, it is likely that these are T cell-dependent antibody responses. This poses a problem since the existence of T cells recognizing tTG is doubtful. tTG is expressed ubiquitously in the human body, and staining with sera from untreated CD patients indicates that the antigen is also expressed in fetal thymus (131). Most likely, T cells reactive with tTG are therefore deleted by negative selection in thymus, and if they should exist they would likely have induced serious systemic autoimmunity. Interestingly, gluten seems to drive the antibody production, as the presence of tTG antibodies is strictly dependent on dietary gluten exposure (8). This raises the possibility that gluten-reactive T cells provide help for tTG-specific B cells by a mechanism of intramolecular help (132) analogous to the hapten-carrier system (133). As mentioned earlier, an important physiological role of tTG is the catalysis of isopeptide bond formation between glutamine and lysine residues (125). Indeed, it is the substitution of water rather than lysine in this reaction that results in deamidation. In vitro treatment of gliadin fragments with tTG leads to some gliadin fragments becoming covalently attached to tTG by autocatalysis (6, 123). tTG-specific B cells may selectively bind and internalize gliadin-tTG complexes via specific surface immunoglobulins. The gliadin fragment may finally be processed and presented by DQ2 or DQ8 to the gliadin-specific T cells, thereby providing cognate help for B cell maturation, isotype switching, and antibody secretion. This model can explain why tTG antibody levels in CD are dependent on the presence of gliadin in the diet because its removal will also abolish the T cell help needed for antibody production.

Autocatalysis by tTG should be more likely to occur when the concentration of other amine donors (lysine containing proteins/primary amines) is low. In fact, deamidation is also likely to happen when the amount of primary amines is low or absent (125). Formation of gliadin-tTG complexes and deamidation of gliadin may thus reflect an altered microenvironment in the gut mucosa. The unusual ability of gliadins to act as excellent amine acceptor substrates for tTG may result in a local depletion of lysine/polyamines, and the altered microenvironment may hence be established in situations where increased levels of gluten proteins get access to the subepithelial area.

## PERTURBED ORAL TOLERANCE TO GLUTEN IN CELIAC DISEASE?

Although the concept of oral tolerance is not as firmly established in humans as it is in rodents, it is clearly necessary that mechanisms that allow for tolerance to soluble food antigens exist in humans (134). In keeping with this thinking, oral tolerance to gluten in patients with CD either is not established properly or is broken. A deeper understanding of this issue should shed new light on the mechanism behind oral tolerance in humans. Given the preferential intestinal T cell response to deamidated gluten fragments in CD patients, it is conceivable that deamidation is central to the perturbation of the oral tolerance. Deamidation increases the binding affinity of gliadin peptides for DQ2 from poor but significant binders to epitopes with reasonable, but by no means exceptional, affinity (118, 119). The moderate binding affinity of these epitopes concurs with the finding that they do not carry optimal anchors in all the anchor positions. It is interesting that the modified glutamine residues for the three defined DQ2-restricted gliadin epitopes recognized by intestinal T cells occupy different pockets within DQ2 (Figure 3). This suggests that the altered affinity of the gliadin peptides for DQ2 is a critical factor involved in loss of tolerance rather than recognition of a single ''pathogenic'' motif that binds to DQ2 (119). Concurrent with the increase in affinity for DQ2 caused by deamidation of the gliadin peptide is a change in conformation of the gliadin/DQ2 complex. This is apparent by the failure of the T cells to recognize the unmodified peptides even at higher concentrations that should compensate for their lower affinity for DQ2. However, the simple modification of glutamine residues that act as major T cell receptor contact residues appears not to be sufficient to break tolerance as none of the modified glutamines are found in such positions (105, 119).

Gliadin fragments containing two glutamine residues targeted by tTG may well be deamidated and cross-linked to other proteins that contain lysine. Conditions may exist in the gut, where T cell epitopes are both created and trapped locally by tTG, that prevent the epitopes from being presented by antigen-presenting cells that induce tolerance in the gut. Alternatively, it may prevent these epitopes from spreading systemically, a factor thought to be important in the establishment of oral tolerance (135). In this regard it is interesting that the motif targeted by tTG and shared in the DQ2-α-gliadin-I and DQ2-α-gliadin-II epitopes is repeated within many of the α-gliadins (119).

## MECHANISMS INVOLVED IN FORMATION OF THE CELIAC LESION

The evidence discussed above provides strong evidence that $CD4^+$ TCRαβ+ T cells in the lamina propria are central for controlling the immune response to gluten that produces the immunopathology of CD. The knowledge of the events

downstream of T cell activation is, however, still incomplete. The characterization of mechanisms operating in the model of human fetal gut explant cultures, where activation of T cells induces villous atrophy and hyperplasia of the crypts, has provided interesting clues and indicated some major pathways (136). However, knowing how the immune system usually utilizes a multitude of effector mechanisms for fighting its opponents, it is reasonable to believe that multiple effector mechanisms may well be involved in the creation of the celiac lesion. Adding to the complexity, recent in vitro organ culture studies have indicated that gluten exerts additional immune relevant effects independent of T cell activation (137, 138). Some of these effects have rapid kinetics, and conceivably the direct effects of gluten may facilitate subsequent T cell responses.

Cytokines produced by lamina propria $CD4^+$ T cells may be involved in the increased crypt cell proliferation and the increased loss of epithelial cells. IFN-$\gamma$ induces macrophages to produce TNF-$\alpha$. TNF-$\alpha$ activates stromal cells to produce KGF, and KGF causes epithelial proliferation and crypt cell hyperplasia (17). IFN-$\gamma$ and TNF-$\alpha$ can jointly have a direct cytotoxic effect on intestinal epithelial cells (139). It is also conceivable that IELs and in particular $\gamma\delta$ T cells play a role in the epithelial cell destruction by recognizing MIC molecules induced by stress (26).

Alterations of the extracellular matrix can also distort the epithelial arrangement, as the extracellular matrix provides the scaffold on which the epithelium lies. Enterocytes adhere to basement membrane through extracellular matrix receptors so that modification or loss of the basement membrane can result in enterocyte shedding. Evidence for increased extracellular matrix degeneration in CD exists, and this degeneration may be important for the mucosal transformation found in CD (40). The increased production of metalloproteinases by subepithelial fibroblasts and macrophages is likely to be directly or indirectly induced by cytokines that are released from activated T cells.

Do the autoantibodies play a role in the pathogenesis of CD, or are they just an epiphenomenon? The significant increase in prevalence of CD among IgA-deficient individuals (1) speaks against a role of the antibodies. However, most CD patients also have elevated levels of serum IgG endomysial (i.e. tTG) antibodies (5), and little is known about the antibodies found locally in the mucosa of IgA-deficient CD patients. Interestingly, the endomysial (i.e. tTG) antibodies can, as suggested by Mäki and coworkers (140), be involved in the disease development by blocking interactions between mesenchymal cells and epithelial cells during the migration of epithelial cells and fibroblasts from the crypts to the tips of the villi. tTG is necessary for activation of transforming growth factor-$\beta$ (TGF-$\beta$) (141). Indirect inhibition of TGF-$\beta$ activation by anti-tTG antibodies can be envisaged to have broad effects as TGF-$\beta$ is known to affect the differentiation of the intestinal epithelium (140), to stimulate extracellular matrix formation (142), and to regulate the function of many immune competent cells within the gut microenvironment (143). In addition, tTG has been demonstrated to be involved in attachment of fibroblasts to the extracellular matrix (144), suggesting

that the autoantibodies could also be involved in lesion formation by perturbing important contacts between fibroblasts and extracellular matrix components. The tTG antibodies may in addition modulate the deamidating activity of tTG in either an inhibiting or a promoting fashion (145). Further research is clearly needed to establish whether and how the tTG antibodies play a role in CD pathogenesis.

## HLA ASSOCIATION WITH DISEASE: LESSONS TO BE LEARNED FROM CD

Strong evidence suggests that the primary HLA association in CD is to the classical peptide presenting HLA molecules DQ2 and DQ8. These HLA molecules predispose to disease by presenting gluten peptides to $CD4^+$ T cells in the affected organ, although an effect mediated by shaping of the T cell repertoire in the thymus cannot yet be excluded. This has clear relevance for studies of other HLA-associated diseases where the identity of the HLA molecules involved are less well defined and where the triggering antigens have not been identified.

The DQ2 and DQ8 molecules bind gluten peptides that after specific deamidation become good peptide ligands for DQ2 and DQ8. Exactly why no other class II molecules are able to present gluten peptides in the gut that result in disease is not yet fully understood. Likely related is that peptides that become deamidated in the gut mucosa are particularly effective in inducing a pathologic immune response, and that the DQ2 and DQ8 molecules are especially suited to bind deamidated peptides. The DQ(α1*0501, β1*0301) and DQ(α1*0201, β1*0202) molecules which are related to the predisposing DQ(α1*0501, β1*0201) molecule and which do not predispose to CD have different binding motifs, although the binding motif of DQ(α1*0201, β1*0202) is very similar. Interestingly, DQ(α1*0501, β1*0201) and DQ(α1*0201, β1*0202) expressing B lymphoblastoid cell lines exhibit abilities to present the DQ2-α-gliadin-II epitope that differ according to when the epitope is incorporated into a complex antigen that requires processing as compared with the peptide that is processing independent (105). This might suggest that factors involved in processing and peptide binding act differentially for loading of the gliadin peptides to two DQ molecules and that this is relevant for explaining the HLA association.

Modification of self-proteins analogous to gliadin in CD would create epitopes recognized as nonself. This could be a more general mechanism for breaking of immunological tolerance and precipitation of autoimmune disease. Perhaps as many as 50% to 90% of the proteins in the human body are posttranslationally modified (P Roepstorff, personal communication), and the degree and type of modification are likely to be altered in an inflamed microenvironment. Epitopes harboring a posttranslational modification may go unreported, as the standard use of recombinant proteins and synthetic peptides for the characterization of T cell epitopes means that most in vivo modified epitopes would escape detection. This class of epitopes should not be overlooked, and it will be important to devise

strategies that will identify modified T cell epitopes of potential autoantigens so that their role in autoimmune disease can be clarified.

Another important point illustrated from the studies of CD is how a foreign antigen drives autoantibody production. For most autoimmune diseases, autoantigens have been defined by use of the autoantibodies. It is often inferred that T cells must exist that are reactive with the autoantigen because the antibodies are of the IgG or IgA isotypes whose formation is dependent on T cell help. In some cases this assumption may turn out to be unjustified. It is in my opinion appropriate to intensify the search for unknown foreign agents that might be hosted by the human body (virus, bacteria, etc) and that are capable, after combining with a self-protein, of providing help for autoimmune responses similar to that found in CD.

This review illustrates that the molecular basis of CD is complex. Given the multifactorial etiology of the disease with involvement of several genes and environmental factors, this is not unexpected. Despite the recent advances in understanding of critical steps in disease development, there is much still to be learned about the disease. Several predisposing genes are yet to be identified. Given the difficulty in defining susceptibility genes with modest effects, a combined functional and genetic approch will be required for their identification. The full understanding of multifactorial inflammatory diseases is surely a formidable challenge for scientists. Compared with the other diseases of this nature, however, CD stands out as a disease for which it should be easier to decipher both the actions of the predisposing gene products and how they interact with other gene products and environmental factors. In this situation it is justified to call for intensified research on CD as this can serve as an illuminator for the other multifactorial inflammatory diseases.

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