Animal Models to Study Gluten Sensitivity

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Abstract

The initial development and maintenance of tolerance to dietary antigens is a complex process that, when prevented or interrupted, can lead to human disease. Understanding the mechanisms by which tolerance to specific dietary antigens is attained and maintained is crucial to our understanding of the pathogenesis of diseases related to intolerance of specific dietary antigens. Two diseases that are the result of intolerance to a dietary antigen are celiac disease (CD) and dermatitis herpetiformis (DH). Both of these diseases are dependent upon the ingestion of gluten (the protein fraction of wheat, rye, and barley) and manifest in the gastrointestinal tract and skin, respectively. These gluten-sensitive diseases are two examples of how devastating abnormal immune responses to a ubiquitous food can be. The well-recognized risk genotype for both is conferred by either of the HLA class II molecules DQ2 or DQ8. However, only a minority of individuals who carry these molecules will develop either disease. Also of interest is that the age at diagnosis can range from infancy to 70–80 years of age. This would indicate that intolerance to gluten may potentially be the result of two different phenomena. The first would be that, for various reasons, tolerance to gluten never developed in certain individuals, but that for other individuals, prior tolerance to gluten was lost at some point after childhood. Of recent interest is the concept of non-celiac gluten sensitivity, which manifests as chronic digestive or neurologic symptoms due to gluten, but through mechanisms that remain to be elucidated. This review will address how animal models of gluten-sensitive disorders have substantially contributed to a better understanding of how gluten intolerance can arise and cause disease.

Introduction

While humans by and large tolerate a vast array of dietary antigens without negative consequences, intolerances do occur. Celiac disease (CD) and its skin manifestation, dermatitis herpetiformis (DH), are two examples of enteric intolerance toward a dietary antigen. Both diseases are characterized by the development of enteropathy after the ingestion of gluten, which is a group of proteins found in wheat, barley, and rye [1, 2]. The development of this intolerance may result from either a failure in the initial development of tolerance to gluten or the loss of tolerance at some point after tolerance to gluten has been initially established.

To better understand the immunologic pathways and mechanisms that inhibit the generation of tolerance to gluten or the loss of tolerance to gluten in adults, there are many different animal models of gluten sensitivity that can be used (Figure 1). These models utilize three primary species, dogs, monkeys, and mice, although a few studies on gluten sensitivity have been done with other species (eg, rabbits and rats) [3]. The rat model has been a useful...
model for gluten digestion and studying the in vivo effects of gliadin on enterocytes [4, 5]. The dog and nonhuman primate models are both spontaneous models of CD, while mouse models are not spontaneous and need gliadin sensitization, chemical and/or drug treatment, and genetic alterations in order to develop features of CD. However, with mice there is a great advantage over the other models in that transgenes can be introduced in order to evaluate the contribution of specific genes to the development of tolerance to gluten. Although every model has certain elements of CD, not all elements of CD have been incorporated into one model yet. Depicted in Table 1 are the four prominent animal species used for modeling gluten sensitivity and which elements are present in each model. This separation of elements allows us to understand the interplay and effect that each element has on the final manifestation of disease.

Currently there are two well-known animal models that spontaneously generate gluten-dependent diarrhea, the dog and the rhesus macaque (Table 1). A recent publication suggests that gluten dependent colic spontaneously develops in horses, making it a third spontaneous model of gluten sensitivity [6]. Common to all three of these spontaneous models is the lack of an association with MHC II. With the dog model, a rigorous study concluded that there definitely was no association with the MHC II, and so far no published reports have addressed this with either the rhesus macaque model or the horse model. All of the mouse models that incorporate celiac-associated MHC II alleles (DQ2 and DQ8), in contrast, do not spontaneously develop gluten-dependent enteropathy. These results bring up the question of how intolerance to gluten develops. One possibility is that genetically susceptible individuals never develop tolerance to gluten. The second possibility is that genetically susceptible individuals are exposed to an environmental influence that causes a loss of tolerance to gluten. All of the animal models listed in Table 1 provide clues as to whether either possibility may be present in celiac patients.

Only one study has been published on the horse model, and in that study, one horse was identified that had substantially higher levels of IgA against tissue transglutaminase (tTG) and IgA against horse endomysium (EMA) than any of the other horses tested [6]. These levels were significantly decreased after 6 months on a gluten-free diet. Similarly, the shortened villi were found to be markedly increased in length after the 6 month gluten-free diet. With only one horse currently identified as celiac-like, much more work needs to be done to determine what other elements of celiac disease are present in this model.

With the dog model, Irish Setters develop wheat-dependent partial-villous atrophy with intraepithelial lymphocyte infiltration that is genetically transferred but not MHC II dependent, and so this model most resembles celiac infants and potentially what occurs in the initial stages of pathogenesis in celiac infants [7–9]. It is noteworthy that the Irish Setter model did not have increased levels of antigliadin antibodies while on a wheat-containing diet, indicating a purely innate (nonadaptive) response to gliadin (gluten) in these animals [10]. This latter point supports the theory that the CD4+ T-cell response to gluten and gliadin may be triggered by or enabled by a strong aberrant innate response to gluten.

With the nonhuman primate (rhesus macaque) model, a subset of monkeys will develop gluten-dependent enteropathy. This enteropathy consists of partial-villous atrophy, increased intraepithelial lymphocytes (IELs), and antigliadin and anti-tTG antibodies. To identify these individuals, juvenile monkeys (<4 years of age) were first screened for the development of clinical symptoms of noninfectious, chronic diarrhea of idiopathic origin [11, 12]. The diarrheic monkeys were then screened for the development of both antigliadin and anti-tTG antibodies. Those that were positive for both were then evaluated by endoscopy and found to have villous atrophy that reversed with a gluten-free diet. In contrast to the dog model, however, a skin rash similar to DH was observed in the rhesus...
model [13]. This dermatitis resolved with a gluten-free diet and had IgA deposits that were tTG+. Co-localization of epidermal transglutaminase with the IgA deposits was not evaluated. It was also not determined if these animals had villous atrophy or increased numbers of IELs in the intestinal epithelium. However, recent publications have shown that the enteropathy in the diarrheic rhesus macaques has many similarities to the innate immune response to gliadin as part of CD in humans. By using healthy controls as a comparison, it was shown that the 33 amino acid gliadin-derived peptide (33mer) could cross the epithelial barrier only in monkeys that displayed celiac-like enteropathy. This gluten-dependent intestinal permeability was not found to occur in healthy controls, suggesting that gliadin-induced intestinal permeability is an aberrant response to gliadin and may precede the development of the focused CD4+ T-cell response to gliadin. Indeed, it is not entirely clear whether the gluten-sensitive enteropathy in the nonhuman primate model is mediated by CD4+ T cells, and, in this respect, is similar to the dog model. If this is true, this result further supports the theory that there can be situations where a gluten-dependent enteropathy occurs in the absence of a CD4+ T-cell response.

Role of CD4+ T cells

While the large animal models may more closely mimic the innate immune responses to gluten, the central role of CD4+ T cells in causing enteropathy has been studied in an adaption of the T-cell transfer model of colitis [14, 15] (Table 1). In this study, C57BL/6 donor mice were raised on a gluten-free chow (GFC) and injected at the base of the tail with gliadin in complete Freund’s adjuvant (CFA) and CD4+ CD45RBlow CD25− T cells were subsequently isolated from the spleens. These cells were then injected intraperitoneally into Rag1−/− recipient mice. This resulted in crypt hyperplasia and villous atrophy when the recipient mice were orally challenged with gluten. Interestingly, this developed primarily in the duodenum and with increased severity in the proximal jejunum, but rarely in the ileum, similar to human CD [16]. When the recipient mice were placed back onto a GFC, these pathologies disappeared, demonstrating the gluten dependence of the pathology. These results also demonstrated that CD4+ T cells (or at least a subset of them) could mediate gluten-dependent intestinal damage. However, as this mouse model used C57BL/6 mice, the model was dependent upon mouse MHC II and therefore could not address the role of DQ2 and DQ8.

Role of MHC II

To address the role of MHC II (specifically DQ8), Black et al [17] used a transgenic mouse that expressed a genomic fragment that contained the celiac-predisposing gene of DQ8 (Table 1). Without sensitization, they did not develop symptoms; however, once sensitized to gluten by intraperitoneal injection with gluten, they did develop a strong T-cell proliferative response as well as an anti gliadin antibody response [17]. A later study with these mice demonstrated that with an intraperitoneal injection with gliadin and an additional 3 weeks of gavage with gliadin, an increase in the number of CD3+ IELs developed in the absence of villous atrophy [18] (Table 1). There was also a gliadin-dependent neuromuscular dysfunction that consisted of altered muscle contraction and ion transport. Studies with transgenic mice that expressed DQ2 with DR3 had similar results, with strong T-cell proliferation to gliadin after sensitization to gliadin, but no development of enteropathy [19]. Thus, DQ2 and DQ8 are necessary but insufficient alone for the development of clinical disease.

Despite the lack of development of substantial villous atrophy, the DQ8 transgenic mouse has provided us with great insight as to why a majority of celiac patients have T-cell responses against deamidated epitopes of gliadin. In the one study by Hovhannisyan et al
[20], it was shown that the B57 polymorphism of DQ8, which lacks an Aspartic acid at the B57 position of DQ8, allowed for the native form of the immunogenic gliadin epitope to bind to DQ8. It was also demonstrated that this very same polymorphism that allowed for binding to the native form of the gliadin epitope also allowed for the binding of deamidated gliadin epitopes and actually resulted in a heteroclitic (stronger) response to the deamidated form of the gliadin epitope. This latter observation may explain why pediatric celiac cases have T-cell responses against a number of gluten-derived epitopes, but a substantial number of adult cases have T-cell responses predominantly against deamidated gliadin epitopes [21].

Role of Tissue Transglutaminase (tTG)

Another arm of the immune system that is associated with the development of CD is the B cell, characterized in humans by the presence of antibodies directed against self and antibodies directed against gluten, both of which are widely used for detection. Many studies have demonstrated that anti-tTG antibodies (predominantly IgA, but also IgG) are closely associated with the development of gluten-sensitive enteropathy; however, it is not clear why these antibodies are produced or even if they have a pathogenic role in the development of CD. One study found that these antibodies are deposited in the small intestine of untreated celiac patients [22] and another study observed that some celiac patients have anti-tTG antibodies that may disrupt or block the enzymatic function of tTG [23]. It is the production of these antibodies that suggests that CD may be an autoimmune disease and demonstrates that not only tolerance against a dietary protein, but also tolerance against a self-antigen, is disrupted in celiac patients. However, the dependence of these antibodies and the disease itself on the continued exposure to an exogenous antigen places CD in a unique position in that it is the only autoimmune disease for which we have detected the exogenous trigger. Animal models have provided information into the pathogenic role of these antibodies in both CD and DH.

A direct analysis of the pathogenic properties of antibodies in DH was addressed using a passive transfer mouse model [24]. This was based on a passive transfer model that the authors had generated for linear IgA bullous disease (LABD). In a previous publication, the authors had determined that in a common subtype of LABD, patients produced IgA that bound to a 97-kDa protein (LABD-97) found in epidermal extracts [25]. Using athymic mice and SCID mice grafted with human skin, the authors demonstrated that purified IgA or IgG antihuman LABD97 injected into these mice resulted in deposits at the basement membrane zone of the human skin grafts, and thereby mimicked the LABD pathology. By contrast, sera from DH patients did not provide granular IgA deposits at the basement membrane zone that are typically found in DH patients [24]. Undaunted, Dr Zone’s group [26] improved this model by later transferring purified antibodies against epidermal transglutaminase into these mice. They found that granular IgA deposits did form at the basement membrane zone with this antibody, supporting the theory that had been earlier proposed by Sardy et al [27], that anti-TGe antibodies comprise the IgA deposits in DH. However, no separation of the dermal/epidermal junction developed as had with the linear IgA model. With the passive transfer model designed by Dr Zone, we see that DH sera, which contains both anti-epidermal transglutaminase and antitissue transglutaminase antibodies, did not result in the production of gluten-dependent blistering or enteropathy; albeit, the transfer of purified anti-TGe did result in antibody deposition in the epidermal/dermal junction.

To address the possibility of anti-tTG antibodies mediating damage in the intestine, a number of studies used different approaches to overexpress anti-tTG antibodies in vivo. One group induced the expression of anti-tTG by using recombinant adeno-associated virus (rAAV) and a sequence for antihuman tTG derived from a phage display library of
antibodies from intestinal lymphocytes of a celiac patient [28] (Table 1). The Fc domains of mouse IgG1 were used to make the final mini-antibody (MB) suitable for in vivo studies in mice, and $10^{10}$ anti-tTG MB rAAV virions were injected into C57BL6 mice [29]. Four weeks after injection, a substantial amount of anti-tTG MB was detected in the sera, and deposits were found in the muscle tissue that was injected, but not in the uninjected contralateral muscle tissue. No deposits were detected in the intestine and no increase in intestinal permeability or clinical symptoms associated with CD were observed to develop in these mice, despite the presence of deposits in the lung and corresponding granulomatous structures. These results would indicate that anti-tTG antibodies by themselves do not trigger the development of enteropathy and, more interestingly, do not bind to small intestinal tissue, suggesting that other phenomena are required for the anti-tTG antibodies to bind to the small intestinal tissue of the mouse.

In a human study, blocking the activity of tTG with antibodies against tTG in vitro decreased gliadin-specific activation of T-cell clones derived from the small intestines of celiac patients [30], suggesting that tTG was necessary for the development of gluten-dependent enteropathy. Mice that overexpress IL15 with the enterocyte-specific T3b promoter and develop enteropathy in a gluten-independent fashion do have a considerable amount of anti-tTG IgA produced [31]. All of these results would suggest, then, that anti-tTG antibodies are produced after (or simultaneously with) the development of small intestinal inflammation, and can develop in the absence of a gluten-driven CD4+ T-cell response.

Of interest was one study that isolated antibodies that bound to transglutaminase 2 (TG2/tTG) [32]. The authors found that there were two classes; class I only bound to tTG, whereas class II bound to tTG, transglutaminase 3 (TG3), and transglutaminase 6 (TG6). TG3 is an epidermal transglutaminase, which is expressed in the skin, and TG6 is a transglutaminase that is expressed in the brain. Adult C57Bl/6 mice were injected intraventricularly with purified single-chain variable fragments (scFvs) that were derived from the blood of celiac patients and fell into the two classes of transglutaminase binding [32]. Both classes caused dramatic motor impairment in the mice and were found to localize to the brains of the mice, specifically localizing to the lateral ventricles the collicoli in the cerebellum, and the brain parenchyma. However, it was not reported whether any of the injected scFvs localized to the small intestinal tissue, or if gluten affected the development of disease.

**Genetic Predisposition Toward Autoimmunity**

These results, with mouse models of CD, would suggest then that a strong CD4+ T-cell response against gliadin and a strong B-cell response to tTG are not sufficient by themselves to cause gluten-dependent enteropathy. Because CD is associated with other autoimmune diseases [33], it is likely that genes that contribute toward the development of autoimmunity are required for the development of the gluten-sensitive diseases; this theory is supported by the finding that DQ2 and DQ8 only provide 30% to 35% of the familial risk for developing CD [34]. Many of the non-HLA candidate genes that have been identified in a Genomic Wide Association Study (GWAS) are involved in immune function, with some specifically associated with T-cell development of the thymus and other autoimmune diseases, such as type 1 diabetes (T1D) [35]. Other studies have shown that CD is tightly associated with T1D, such that celiac patients have over a two-fold greater risk of developing T1D (hazard ratio) [36]. Of interest is that T1D patients have a 20-fold increased risk for developing CD [37, 38]. Also, the mouse model ofT1D, the non-obese diabetic (NOD) mouse, has reduced villous height and an increase in IELs while on a gluten-containing diet, further supporting the theory that many of the mechanistic pathways that contribute to T1D also contributes to the development of CD [39].

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Certainly MHC II is one of these mechanistic pathways that contribute to both diseases, since DQ2-DR3 and DQ8-DR4 haplotypes are associated with both diseases, although T1D is more associated with DQ8 and CD/DH are more associated with DQ2 [40, 41]. However, both diseases also share non-HLA genes [35, 42]. Similar to CD, MHC II alone (found in IDDM1 in humans) is not sufficient for the development of T1D [43]. This has been demonstrated in the NOD mouse model in that the MHC II allele that predisposes toward the development of T1D, H-2\textsuperscript{g7}, while required for disease development, cannot alone generate the development of T1D [44, 45]. Other genes are required to be present, and these have been designated Insulin-Dependent Diabetes (IDD) loci. Although most IDD loci only incrementally contribute to the development of diabetes, two IDD loci (5 and 13) were found to be shared between NOD mice and humans and contribute to the development of invasive insulitis [43]. An alternative interpretation is that the IDD loci contribute to a predisposition toward autoimmunity through a decreased ability to maintain tolerance to self, and thereby contribute to the development of other autoimmune diseases, such as CD.

To test the hypothesis that autoimmune predisposing genes (genetic background) contribute to the development of gluten sensitivity, NOD mice were crossed with DQ8 transgenic mice. The resulting NOD Abo DQ8 mice are capable of developing tolerance to dietary gliadin and do not spontaneously develop the clinical symptoms of gluten-sensitive diseases [46] (Table 1). Similarly, the expression of the DQ2DR3 haplotype on the NOD background did not result in the spontaneous development of either gluten-sensitive disease [19]. These results would suggest then that even on a genetic background that predisposes toward the development of autoimmunity, the expression of DQ2 and DQ8 are still not sufficient to generate clinical symptoms of either gluten-sensitive disease. These results led to a very important conclusion, that something in addition to the combination of a genetic susceptibility to gluten sensitivity and to autoimmunity is required for the development of CD.

**Disruptions to Intestinal Homeostasis**

To address how disruptions to intestinal homeostasis might affect gluten sensitivity in these models, two groups used indomethacin, a COX inhibitor, which was shown to induce intestinal motility in rats [47], and later to transiently increase paracellular intestinal permeability in rats [48]. In the study that used transgenic mice that expressed HLA-DQ8, indomethacin treatment was administered in the drinking water (3 mg/100 mL) for 14 days after oral sensitization to gluten using cholera toxin [49], which consisted of gavaging the mice with gluten and cholera toxin simultaneously once every week for 3 weeks (Table 1). This resulted in decreased villous height without crypt hyperplasia and without an increase in IELs. The triple treatment also resulted in the increased production of IFN\textgamma. In the study with transgenic mice that expressed both DQ8 and human CD4, gluten was injected intraperitoneally with acetic acid into the mice [50]. They were then intragastrically gavaged with 2 mg of gluten 3 times a week for 7 weeks. Twenty-four hours before each gluten gavage, indomethacin was administered by gavage (3.5 mg/kg). It was observed that gliadin plus indomethacin substantially increased the transcellular permeability over indomethacin alone, and that IFN\textgamma production was considerably increased with treatment with both gliadin and indomethacin over either alone. Thus, although the two studies used different lines of transgenic mice and different sensitization protocols, they both came to the same conclusion: that indomethacin does not induce gluten-dependent villous atrophy with an increase in IEL numbers and crypt hyperplasia. Based on the results on gliadin-induced permeability with the rhesus macaque model, it may be that the gliadin-induced permeability that is associated with CD needs to be chronic, as opposed to transiently, induced, or that permeability is a co-phenomenon.
This theory that transient inductions of inflammation are not enough to induce a perpetuating CD4+ T-cell response to gliadin that results in enteropathy was further supported by a study that evaluated the use of polyinosinic/polycytidylic acid (poly [I:C]) with DQ2 transgenic mice. These mice were injected intraperitoneally with poly (I:C) to induce transient small intestinal damage that consisted of villous atrophy for 12 hours [51]. However, no T-cell proliferation that was gliadin specific was detected 72 hours after the poly (I:C) treatment and oral gliadin challenge. Similarly, treatment with methotrexate, which induces severe intestinal inflammation without villous atrophy for 3 to 5 days after treatment [52], also did not result in T-cell proliferation against gliadin, further supporting the findings with indomethacin, that transient disruptions to intestinal homeostasis through chemical means does not result in gluten-dependent enteropathy in genetically predisposed individuals.

Role of IL15

Celiac patients have increased levels of IL15 in the lamina propria [53], and IL15 has also been shown to disrupt the function of regulatory T cells in CD patients [54]. To address the role of IL15 in celiac disease, one study generated a mouse that overexpressed IL15 systemically (using a minimal MHC class I D\(^d\) promoter) and crossed that with mice that expressed DQ8 [53] (Table 1). In this study, the authors found that the feeding of gliadin to mice maintained on a GFC resulted in an increase of CD3+ IELs to 30 per 100 enterocytes, which was 10-fold greater than the temporary disruption to intestinal homeostasis via anti-CD25 in the NOD Abo DQ8 mice [55]. Neither of the parent lines of mice (IL1-5 D\(^d\) alone or DQ8 alone) had increased levels of IELs with gliadin feeding [53, 56]. This result would suggest that an increase in CD3+ IELs that is comparable to that found in celiac patients (>40/100 enterocytes) only occurs when there is an overexpression of IL15 in the lamina propria. However, the DQ8 D\(^d\) IL15 transgenic mice did not develop villous atrophy.

A different study inserted human IL15 behind an enterocyte specific promoter (T3b) (Table 1) [57]. This targeted overexpression of IL15 to the small intestine of mice resulted in inflammation in the small intestine, but not in the large intestine [57], with a villous height-to-crypt ratio of 2.1:1. There was also a marked increase (>10-fold) in CD8\(\alpha\)\(\beta\) + T cells in the small intestinal lamina propria [57]. Of considerable interest was the increased number of plasmablasts with corresponding increases in anti-tTG IgA, despite the lack of a gluten-specific CD4+ T-cell response [58]. Other autoantibodies that were produced were against double-stranded DNA and rheumatoid factor [58]. These results would suggest that the production of anti-tTG IgA and increase in infiltrating CD8+ T cells can all develop in the absence of a gluten-specific T-cell response. Since the promoter constructs for IL15 overexpression are different in the two studies (D\(^d\) vs T3b), it would be of great interest to see if overexpression of IL15 by intestinal epithelial cells, such as the T3b driven expression of IL15, in a DQ8 transgenic (or DQ2/DR3) transgenic mouse model would result in the development of gluten-dependent villous atrophy.

Role of regulatory T cells

One study with NOD Abo DQ8 mice found that a partial depletion of regulatory T cells with injection of anti-CD25 led to an increase in CD3+ IELs and a lower villous-to-crypt ratio [54], both of which were made significant when gliadin sensitization was done after anti-CD25 treatment (\(P<0.01\) and \(P<0.05\), respectively). Much more dramatic was the severity of insulitis that developed with the anti-CD25 and (cholera toxin)/(gliadin sensitization). They did not, however, develop hyperglycemia [54]. These results suggested, then, that with a temporary but severe disruption to the maintenance of regulatory T cells
(CD4+CD25+Foxp3+) in the NOD Abo DQ8 mice via anti-CD25 treatment, some features of gluten-dependent enteropathy developed temporarily [54].

Another study of gliadin-associated regulatory T cells generated a DQ2.5 transgenic mouse deficient in mouse MHC II (MHC II−/−), in which a genomic fragment that contained DQ2.5 was used for microinjection, and crossed that with a transgenic mouse that expressed a gliadin-specific T cell receptor (TCR) [59] (Table 1). They then took CFSE-labeled CD4+ splenic T cells from the HLA-DQ2.gliadin-TCR.MHCII−/− double-transgenic mice and transferred them by intravenous injection into HLA-DQ2 recipient mice. These particular cells expressed both IFNγ and IL-10, but not FoxP3, and localized to the spleen after feeding of deamidated gliadin, as opposed to the mesenteric lymph nodes, as was observed in their ovalbumin/DO11.10 control study. Of great interest was that the splenic CFSE CD4+ T cells in the recipient DQ2 mouse transferred a suppressive effect upon a gliadin-specific DTH response in the recipient HLA-DQ2 mice. This study would suggest that these Tr-1–like splenic cells may be another pathway to suppress inflammatory responses to deamidated gliadin. A different study demonstrated that CD4+CD25+FoxP3+ cells are completely functional in CD when IL15 has been removed through antibody depletion [56]. Thus, it remains to be seen if these splenic Tr-1–like cells are able to suppress inflammatory T-cell responses to deamidated gliadin in humans, especially in celiac patients and their family members.

**Role of Intestinal Microbiota in CD**

A number of papers have demonstrated that there are differences in the microbiota of untreated and treated celiac patients [60–62]. In addition, the treated CD patients also had differences with healthy controls [63]. Most of these differences have been compositional in that one bacterial species is increased in the overall percentage while others decreased. All of these studies showed that the proportion of *Bacteroides* and *Escherichia coli* species were increased in untreated celiac patients and the proportion of *Bifidobacterium* species decreased compared to both treated celiac patients and healthy controls. These changes raised the question of whether the bacteria that expand in untreated celiac patients are causing the disease or whether they are merely opportunistic and take advantage of the change in nutrients due to increased intestinal permeability. One study approached this question by taking germ-free rats raised and maintained on a GFC and administering the various bacterial species of interest [5]. The application of *Bifidobacterium bifidum*, gliadin, and IFNγ together to rat duodenal intestinal loops did not cause a translocation of bacteria. However, the application of *E coli*, gliadin, and IFNγ did result in the translocation of gliadin peptides across the epithelial layer. These results would indicate, then, that gliadin can translocate the epithelial barrier if certain bacterial species are present in greater proportions in the intestinal microbiota. Thus, it is possible that the composition of the intestinal microbiota plays a crucial role in the initial stages of gluten-dependent enteropathy, and may help explain why only a very small percentage of individuals develop gluten-dependent enteropathy.

**Exploring Gluten Dependence in Other Diseases**

As was discussed earlier, when the NOD ABo DQ8 mice are partially depleted of CD4+CD25− Foxp3+ regulatory cells and sensitized to gliadin, the mice develop insulitis [54]. This raises the question of whether gluten tolerance (and intolerance) plays a role in the development of T1D. The gluten connection to the development of T1D comes from the increased risk of developing both diseases. T1D patients have a 20-fold increased risk of developing CD [37, 38], and patients with CD have a 2.4-fold greater chance of developing T1D (hazard ratio) [36]. Although CD and its skin manifestation (DH) are clearly gluten-
sensitive diseases (in that the inclusion of gluten in the diet has relatively immediate consequences), this review would be remiss if it did not cover the work that has been done with the effect of gluten upon the development of T1D in mice and rats. Currently, there are two spontaneous animal models of T1D, the NOD mouse and Bio-Breeding diabetes-prone (BBDP) rats. Of interest is that it has been clearly shown that raising both of these models on a GFC substantially delays the onset, as well reduces the overall incidence, of T1D compared to those raised on a standard gluten-containing chow (GCC) [39, 64–68]. Even the NOD mouse model displays elements of CD with increased numbers of IELs and decreased villous height when the mice are maintained on a GCC[39]. Although there is great controversy as to whether T1D patients are truly affected by dietary gluten, especially if a gluten-free diet can be beneficial for T1D patients, there is irrefutable evidence that dietary gluten does affect the development of diabetes in these two animal models [69, 70]. In both the NOD and BBDP animal models, it has been demonstrated that providing a gluten-containing diet to these animals will substantially increase the incidence of hyperglycemia [66]. However, it is also true that casein and other dietary proteins in the diet will also increase the incidence of diabetes in these animal models [71]. Therefore, if these animals are raised on a hydrolyzed diet, then the development of hyperglycemia is postponed [72, 73]. Clearly, these animal models display the classical symptom of intolerance to gluten in that the development of disease is affected by the consumption of gluten. The most important take-home message from this is that the introduction of gluten-derived protein into the diet triggers an autoimmune response against the islets, especially when regulatory T cells are diminished.

A number of studies have focused on the potential of cross-reactivity, specifically focusing on a wheat-storage globulin protein found in wheat, called Glo-3. This was originally named Glb-1 and was identified using sera from diabetic BBDP rats [74]. It was later identified as Glo-3A [75]. Antibodies against Glb-1 (Glo-3A) were considerably higher in T1D patients than in controls [74]. It was later found that increased sera levels of zonulin, a marker for gut permeability in CD patients, DH patients, and T1D patients [76–78], were associated with increased sera levels of antibodies against Glb1 (Glo-3A) in humans using the Diabetes Autoimmunity Study in the Young (DAISY) [79]. Finally, antibodies against Glo-3A were purified from a patient with both CD and T1D using recombinant Glo-3A and then were used in a cross-reaction study using both rat jejunum and rat pancreas [80]. Interestingly, the antibodies enriched for Glo-3A binding bound to cells in the lamina propria of rat jejunum [80], specifically CD163+ macrophages; but they did not bind to rat pancreas. Thus, the presence of anti-Glo-3A antibodies in T1D patients demonstrates that T1D patients mount an immune response to at least one wheat-derived protein.

What is of great interest is the concept that T1D patients may be intolerant to gluten. This is certainly the case for the T1D patients that are later diagnosed with CD. The intriguing question is whether T1D patients as a whole group are intolerant to gluten or if it is only the one subset of T1D patients that later develops CD that is truly intolerant to gluten. The studies with Glo-3A demonstrate that T1D patients as a group produce an aberrant immune response to at least one wheat-derived protein. Also, the recent study (in 2011) with the partial depletion of CD4+CD25+Foxp3+ cells in gluten-sensitized NOD Abo DQ8 mice would suggest that the development of insulitis and gluten sensitivity are associated with each other [54]. Thus, although studies done on T1D patients have not demonstrated that gluten consumption affects the development of diabetes in humans, the BBDP rat, the NOD mouse, and the NOD Abo DQ8 mouse models all suggest that gluten does play a role in the development of T1D.
Therapies to Establish Tolerance to Gluten

If we proceed with the notion that clinical symptoms of CD can arise as either the inhibition of tolerance to gluten or the disruption/loss of tolerance to gluten, then individualized approaches should be taken when administrating therapies that target the different pathogenic steps (Figure 2). The best example of this would be the administration of therapies designed to re-establish tolerance to gluten-derived proteins (such as gliadin). One group did this by generating a strain of *Lactococcus lactis* to secrete an immunogenic epitope of gliadin [81]. This approach was based on a previous study in which *Lactococcus lactis* bacteria were successfully bioengineered to secrete IL-10 [82]. This study demonstrated that administration of this IL-10 secreting strain successfully ameliorated the development of colitis in IL-10−/− mice and caused a 50% decrease in pathological symptoms in a Dextran Sulphate Sodium (DSS) model of colitis. Advancing this model further, the group bioengineered *Lactis* to secrete ovalbumin [83]. The ability to suppress in an antigen-specific fashion was shown using DO11.10 mice that express TCR specific for ovalbumin. The T-cell response was shown to be decreased via delayed-type hypersensitivity assay (DTH). This reduction was shown to be due to CD4+CD25− regulatory T cells expressing Foxp3 and CTLA4 via cell-transfer assays. Using the same approach, *Lactis* were bioengineered to secrete a deamidated form of a gliadin-derived epitope that was DQ8 restricted and immunogenic for CD [81]. The ability of this strain to induce tolerance to gliadin was evaluated using GFC weaned and maintained NOD Abo DQ8 mice. The gliadin-secreting, *Lactis*–treated, GFC-weaned and maintained NOD Abo DQ8 mice were then parenterally sensitized to gliadin. The administration of gliadin-secreting *Lactis* successfully diminished the T-cell response to gliadin as evaluated by DTH [81]. Analysis of the production of cytokines in the intestine and the phenotype of T cells suggested that the bioengineered *Lactis* strain was inducing CD4+CD25− Foxp3+ T cells that were gliadin specific.

A different study determined the efficacy of different probiotic strains on the development of gluten sensitivity in a DQ8 transgenic mouse model of gluten sensitivity [49]. In this study, the authors found that the administration of *Lactobacillus fermentum*, *Bifidobacterium lactis*, *Lactobacillus plantarum*, and *Lactobacillus paracasei*, along with gliadin and cholera toxin, intragastrically increased the expression of IFNγ and/or TNFα by means of mesenteric lymph nodes or splenic cells of DQ8 transgenic mice sensitized to gliadin. This result supported a previous finding, in which it was determined that the administration of *Lactobacillus casei* alone during gliadin sensitization to DQ8 transgenic mice with gliadin and cholera toxin had an adjuvant effect, resulting in an increased proliferative response to gliadin, with a corresponding increase in IFNγ production [81]. However, when *Lactobacillus casei* was administered to the DQ8 transgenic mice alone, in the absence of gliadin sensitization, IL-10 was increased, while IFNγ was not increased in the spleen and mesenteric lymph nodes [84]. Of interest was that when the group administered *L. casei* in a model of gluten-induced villous damage using gliadin, cholera toxin, and indomethacin, *L. casei* then acted as a probiotic, and reduced villous blunting [85]. This result of reduced villous blunting was similar to that found with the administration of *L. casei* to humans, wherein the small intestine was protected from damage induced by low doses of aspirin [86]. These latter results highlight the fact that chemical perturbations to the intestine will alter how the bacteria in the small intestinal microbiome will interact with the host’s intestinal immune system. It would be reasonable to assume then that probiotic therapy in the absence of severe inflammation-inducing drugs would have a high chance of success in individuals who were diagnosed later in life, due presumably to a loss of tolerance to gliadin. However, the administration of such a therapy might not work in individuals who were diagnosed with CD as very young children, as they presumably never developed tolerance to gliadin.
Another potentially curative therapy is the intradermal injection of immunogenic gluten-derived peptides with the intent of generating tolerance to gluten. This approach was tested with HLA DQ2DR3 transgenic mice and resulted in a suppressed inflammatory CD4+ T-cell response to gliadin [87]. Yet another curative approach would be the administration of immunogenic gluten-derived peptides intranasally, as was successfully done with α-gliadin using DQ8 transgenic mice [88]. As with the bioengineered L. lactis, these approaches probably would have a higher chance of success in individuals who once were tolerant to gluten, but lost it later in life, as opposed to individuals who never developed tolerance to gluten. It is also possible that tolerance to gluten will need to be maintained long term, as sensitivity to gluten may never abate.

Therapies to Block T Cell Activation/Function

The administration of products that only temporarily block celiac disease, such as polymeric blocking molecules, may prove to be effective in both groups of celiac patients. One study done with Abo DQ8 mice demonstrated that the administration of poly(hydroxyethylmethacrylate-co-styrene sulfonate) [P(HEMA-co-SS)], which complexes with gliadin in a relatively specific fashion, resulted in decreased numbers of CD3+ IELs and gliadin-induced barrier dysfunction [89]. As this approach temporarily blocks the activation of gliadin-specific T cells, this therapy would be expected to be effective in both groups of celiac patients (Figure 2).

The use of peptidases that would render gliadin-derived peptides nonimmunogenic is another temporary therapy that may prove to be useful for both groups of celiac patients. One such example is a barley-derived endoprotease called EP-B2. The first animal model study that was done with EP-B2 used a rat model of gluten digestion [4]. In that model, they were able to demonstrate that the administration of EP-B2 resulted in a substantial decrease (~50%) in the release of the immunogenic 33mer in the small intestine. EP-B2 was later provided to gluten-sensitive rhesus macaques and shown to inhibit the development of clinical symptoms when the gluten-sensitive rhesus macaques were challenged with gluten [12]. Of concern, though, was that levels of anti-gliadin IgG and IgA and anti-tTG IgG were not decreased, and in fact were higher than the levels found in gluten-sensitive monkeys maintained on a gluten-containing diet [12]. Thus, although this therapy could theoretically be used for both individuals who never achieved tolerance to gliadin as well as individuals who had lost their tolerance to gluten, the potential for side effects due to ongoing inflammation is great.

Therapies to Block Inflammation Induced by Epithelial Cells

Another approach to temporarily blocking the inflammation would be the administration of antibodies against IL-15, the IL-15 receptor, or downstream (of IL-15) signaling molecules, in order to effectively block the signaling from the IL-15 receptor (Figure 2). Administration of anti-IL15R to the mice that overexpressed IL-15 in the small intestine resulted in the reversal of villous atrophy and inflammation in the intestines of these mice [31]. Another approach was to target IL-15 itself, and this therapy was proven successful with the administration of the anti-human IL-15 antibody to the same line of mice that overexpressed IL-15 driven by the T3b promoter (enterocyte-specific) [90]. Targeting IL-15 or its receptor may prove to be quite useful in those celiac patients that express abnormally high levels of IL-15, as well as refractory celiac patients.

A different approach to blocking the development of inflammatory processes is to block the tight junction disassembly that occurs in celiac patients on a gluten-containing diet [91] (Figure 2). This process has been shown to be due to the gliadin-induced release of zonulin and subsequent rearrangement of zonula occludens-1 (ZO-1) by epithelial cells, resulting in...
disruption of the tight junction assembly [91]. A recent paper has demonstrated that a novel drug called larazotide acetate, also known as AT1001, inhibits the redistribution and rearrangement of ZO-1 and actin, resulting in greater integrity in the tight junctions and less translocation of gliadin peptides [92]. This was determined in vitro using Caco-2 and IEC-6 cells and in vivo treatment of gliadin-sensitized hCD4DQ8 transgenic mice with larazotide acetate and resulted in a decrease in macrophages in the small intestine and an improvement in barrier function parameters, including conductance and transcellular permeability [93]. Administration of this drug would be best if done at (or shortly before) the time of gluten ingestion, as was done with the hCD4DQ8 transgenic mouse study, because the translocation of gliadin peptides occurs within 1 hour after gliadin is administered orally, as demonstrated by the rhesus macaque study [12].

**Therapies Using Non-Immunogenic Wheat Strains**

Of course, there is also the approach of recognizing that a considerable number of celiac patients may be incapable of generating tolerance to gluten, because they never established it when they were infants. For these individuals, the best therapeutic approach may be to completely avoid the immunogenic peptides via the use of ancient strains of wheat. One study used DQ8 transgenic mice to evaluate the toxicity of the gluten-free grains (ie, tef, millet, amaranth, and quinoa) [94]. Mice were sensitized to gliadin by administration of cholera toxin and a peptic trypsic digest of gliadin, spleen, and mesenteric lymph nodes extracted and treated in vitro with a peptic trypsic digest of gliadin, tef, millet, amaranth, or quinoa. Only the peptic trypsic digest of gliadin generated a strong T-cell response, indicating that the four other grains did not have any immunogenic epitopes that cross-reacted with the gliadin derived from *Triticum aestivum* (wheat). Similarly, only gliadin from *T aestivum* induced the production of IFNγ from seven different T-cell lines that were isolated from the small intestines of CD patients. These results would suggest that these four grains are safe for celiac patients to eat.

**Overall Conclusions**

All of these studies with animal models have demonstrated some very important points. The most important point is that intolerance to gluten, as seen in CD and DH, is a conglomeration of both aberrant adaptive and aberrant innate immune responses to gluten. The second point is that the animal models would suggest that intolerance to gluten is initiated early in life, since both of the spontaneous animal models of CD have gluten-dependent villous atrophy, while none of the induced (in adulthood) rodent models do. Indeed, it is only with the introduction of the overexpressing IL-15 transgene into the DQ8 mouse model that the mouse models of gluten sensitivity achieve an element of spontaneous gluten-dependent enteropathy. This then comes to the third point, that the gluten-sensitive diseases are the result of a self-perpetuated intertwining of the aberrant adaptive immune response to gluten with the aberrant innate response to gluten. How and when this intertwining occurs to result in gluten-dependent enteropathy (CD) or gluten-dependent pruritis (DH) will be the crucial questions to address next with the animal models of gluten sensitivity.

**Acknowledgments**

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Abbreviations

CD  celiac disease
DH  dermatitis herpetiformis
T1D  type 1 diabetes
NOD  non-obese diabetic
IDD  insulin dependent diabetes
GFC  gluten-free chow
GCC  gluten-containing chow

References


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Figure 1. Pathogenic Steps of Celiac Disease That Each Animal Model Species Can Address
Depicted in the illustration is each of the seven main pathogenic steps of celiac disease, and their descriptions are listed in the left column of the corresponding table. Listed in the right column are the species of the animal models that mimic the steps.
Figure 2. Specific Animal Models Used to Test Novel Therapies for Celiac Disease
Depicted in the illustration is each of the seven main pathogenic steps of celiac disease. Listed below in the table are the novel therapies generated for targeting specific steps and the species (animal models) that have been used to test the therapies. References are provided in the far right column.
Table 1

Elements of Celiac Disease in the Animal Models. Listed in the left column are the descriptions of each animal model of gluten sensitivity. Listed in the next column to the right is whether gliadin sensitization is necessary to generate the model. Listed in the columns to the right of that are the main elements of celiac disease (MHC II dependency, gluten-dependent enteropathy, antibodies against gliadin and/or tTG, and partial villous atrophy) and whether each of the specific animal models has this element. The references for the original generation or main body of work for each animal model are provided in the far right column.

<table>
<thead>
<tr>
<th>Genetic Background /Transgene</th>
<th>Gliadin Sensitization</th>
<th>MHC II Dependent</th>
<th>Gluten-Dependent Enteropathy</th>
<th>Antibodies To Gliadin and/or tTG</th>
<th>Partial Villous Atrophy</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>Without</td>
<td>Unknown</td>
<td>Yes</td>
<td>Gliadin and tTG</td>
<td>Yes</td>
<td>(6)</td>
</tr>
<tr>
<td>Irish Setter</td>
<td>Without</td>
<td>No</td>
<td>Yes</td>
<td>Gliadin</td>
<td>Yes</td>
<td>(7–10)</td>
</tr>
<tr>
<td>Nonhuman Primate (Monkey)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhesus Macaque</td>
<td>Without</td>
<td>Unknown</td>
<td>Yes</td>
<td>Gliadin and tTG</td>
<td>Yes</td>
<td>(11–13)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar-AVN</td>
<td>Without</td>
<td>No</td>
<td>No</td>
<td>Unknown</td>
<td>No</td>
<td>(4,5)</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57Bl/6 Transfer to Rag1−/−</td>
<td>With</td>
<td>Yes (mouse)</td>
<td>Yes</td>
<td>Gliadin</td>
<td>Yes</td>
<td>(15)</td>
</tr>
<tr>
<td>Abo DQ8</td>
<td>Without</td>
<td>Yes</td>
<td>No</td>
<td>Neither</td>
<td>No</td>
<td>(17,18)</td>
</tr>
<tr>
<td>Abo/DQ8</td>
<td>With</td>
<td>Yes</td>
<td>No</td>
<td>Gliadin</td>
<td>No</td>
<td>(17,18)</td>
</tr>
<tr>
<td>NOD ABo DQ8</td>
<td>Without</td>
<td>Yes</td>
<td>No</td>
<td>Gliadin</td>
<td>No</td>
<td>(46)</td>
</tr>
<tr>
<td>NOD ABo DQ8</td>
<td>With</td>
<td>Yes</td>
<td>Yes</td>
<td>Gliadin and tTG</td>
<td>No</td>
<td>(55)</td>
</tr>
<tr>
<td>DQ2/DR3</td>
<td>Without</td>
<td>Yes</td>
<td>No</td>
<td>Neither</td>
<td>No</td>
<td>(19)</td>
</tr>
<tr>
<td>DQ2/DR3</td>
<td>With</td>
<td>Yes</td>
<td>No</td>
<td>Gliadin and tTG</td>
<td>No</td>
<td>(19)</td>
</tr>
<tr>
<td>DQ2/DR3 + TCR gliadin</td>
<td>Without</td>
<td>Yes</td>
<td>No</td>
<td>Gliadin</td>
<td>No</td>
<td>(19,59)</td>
</tr>
<tr>
<td>DQ2/DR3 + TCR gliadin</td>
<td>With</td>
<td>Yes</td>
<td>No</td>
<td>Gliadin</td>
<td>No</td>
<td>(19,59)</td>
</tr>
<tr>
<td>IL-15/−/− (D3)</td>
<td>Without</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>(54,55)</td>
</tr>
<tr>
<td>IL-15/−/− (T3b) 4</td>
<td>Without</td>
<td>No</td>
<td>No</td>
<td>tTG</td>
<td>Yes</td>
<td>(56,57)</td>
</tr>
<tr>
<td>IL-15/−/− (D3) DQ8</td>
<td>Without</td>
<td>Yes</td>
<td>Yes</td>
<td>Gliadin and tTG</td>
<td>No</td>
<td>(53)</td>
</tr>
<tr>
<td>Anti tTG</td>
<td>Without</td>
<td>No</td>
<td>No</td>
<td>tTG</td>
<td>No</td>
<td>(29,32)</td>
</tr>
</tbody>
</table>

2 Abo-A drastic mutation of the (H-2 Aβ) gene resulting in a lack of expression of the A complex of mouse MHC II (17).

3 IL-15 +/- (D3)-Refers to the IL-15 overexpressing mouse line that has mouse IL-15 behind the minimal MHC I D3 promoter, which results in IL-15 expressed in the lamina propria.

4 IL-15 +/- (T3b)-Refers to another IL-15 overexpressing mouse line that has human IL-15 behind the intestinal epithelial cell specific promoter T3b and results in IL-15 expressed in the intestinal epithelium.