CHAPTER 2

Presence of gluten proteins in breast milk: implications for the development of celiac disease

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ABSTRACT

Aims: Celiac disease (CD) is a multifactorial disease with a strong genetic association and is caused by a T cell mediated immune response to gluten. Although much is known about the molecular mechanism, the trigger for the onset of CD is still unclear. A correlation between a reduced risk of developing CD and breastfeeding has been shown. The preventive mechanism however, remains unclear. In this study we test the hypothesis that T cell stimulatory epitopes originating from dietary gluten appear in the human breast milk.

Method: Breast milk of 23 mothers on a normal diet (mean gluten intake: 17 g/day) and 13 mothers on a strict gluten-free diet was collected in the period from one week until eight months after delivery. The presence of gluten proteins was studied using monoclonal antibody based competition assays specific for the T cell stimulatory epitopes of gliadin (Glia-α9, Glia-α20, Glia-γ1) and glutenin (Glt-156 and High Molecular Weight (HMW)).

Results: T cell stimulatory epitopes of both gliadin and glutenin (Glia-α9 and HMW glutenin) were detected in the breast milk of mothers on a normal diet. Correlation studies revealed that the gluten intake was not correlated with the level of the Glia-α9 T cell stimulatory epitope detected.

Conclusion: Infants are exposed to small levels of gluten via breast milk. It is tempting to assume that these low levels of gluten are responsible for the induction of oral tolerance to gluten.
INTRODUCTION

Celiac disease (CD) is an inflammatory intestinal disorder caused by immune responses induced by dietary gluten proteins (1-3). It is a multi factorial disease with a strong HLA association. Approximately 95% of celiac disease patients are HLA-DQ2 (α1*0501, β1*0202), whereas the remainder is usually HLA-DQ8 (α1*0301, β1*0302). With a prevalence of approximately 1 in 100-200, CD is the most common food induced enteropathy in the western world. A wide range of variable symptoms are associated with CD, including abdominal pain, diarrhea, constipation, vomiting, osteoporosis, growth retardation, and migraine. Many patients, however, have only very mild or no apparent clinical symptoms and are never diagnosed properly. At present, the only possible treatment for CD patients is a strict life-long gluten-free diet (GFD).

Although feasible, a GFD is complicated by the widespread use of gluten in the food industry and as an additive to many products that are not normally associated with gluten or wheat, like medication. Moreover, a strict GFD causes a severe restriction in the patients’ social life (4).

Gluten molecules are the storage proteins of wheat and can be subdivided in the gliadin and glutenin protein families, both involved in CD. The proteins have a high proline content (5) and as a result, gluten proteins are poorly degraded by enzymes of the gastrointestinal tract (6). In the small intestine of CD patients, the partially degraded gluten proteins are modified by the activity of the enzyme tissue transglutaminase (tTG) (7-9). This so called deamidation introduces a negative charge in gluten peptides which facilitates their binding to the disease predisposing HLA-DQ2/8 molecules and facilitates efficient presentation of gluten peptides to CD4+ T cells of the immune system (7-14). The T cell response against gluten is specific for CD patients since no evidence of T cell mediated reactivity against dietary gluten has been reported in normal, non-celiac, mucosa. The gluten reactive T cells have a Th0/Th1 phenotype and usually release the proinflammatory cytokine IFN-γ (15). Although at least 50 T cell stimulatory epitopes in gluten proteins have been identified, a unique 33-mer peptide of α-gliadin seems to be the most immunogenic (6,16). This 33-mer harbours six in part overlapping epitopes and it is resistant to the enzymatic degradation by gastric, pancreatic and brush border enzymes.

Although much is known about the molecular mechanism underlying CD, little is known about the onset and possibilities to prevent the disease. Since only a minority of the genetically predisposed individuals actually develop CD, a threshold of tolerance was suggested (17). This threshold is influenced by both gene dose (18) and gluten exposure (19). A large repertoire of abundant immunogenic gluten peptides in the diet, together with a high copy number of HLA-DQ2, thus may favor the breaking of oral tolerance. In present day practice, gluten is introduced into the diet of infants at the age of 6-7 months.
(20). As there is no restriction in the amount of gluten given, gluten intake at the age of 12 months is between 6 and 9 grams daily (21), while gluten-specific T cells of CD patients are known to respond to microgram amounts. The sudden introduction of grams of gluten may thus play an important role in the breaking of oral tolerance. Another factor influencing the threshold or breaking of tolerance is breastfeeding. Recent studies have shown that breastfeeding offers protection against the development of CD (19,22). Breastfeeding at the time of gluten introduction and ongoing breastfeeding while gluten is already being consumed were associated with a reduced risk of development of CD. The exact preventive mechanism of breastfeeding on the development of CD, however, remains unclear. A tentative explanation might be that small amounts of gluten in breast milk promote the induction of low dose oral tolerance against gluten.

In this study, the presence of gluten in breast milk of mothers on a normal gluten-containing diet (ND), was investigated and compared to a control group of mothers on a GFD.

MATERIALS AND METHODS

Subjects
In 2005 lactating women on a ND were contacted at random at one Child Health Care Center (Nieuw Vennep, the Netherlands). In the year 2004 year, the Child Health Care Centers were attended by 91% of the families with infants in the Netherlands (23). Lactating mothers on a GFD were contacted through a call in ‘Glutenvrij’ a periodical distributed among members of the Dutch Celiac Disease Society (NCV), and a call on the website of the NCV.

Samples
Breast milk of women on a ND and of women on a GFD was collected longitudinally. Both groups of participants were asked to collect three samples of breast milk (morning, afternoon and evening) once a month. The samples were stored at home in labeled tubes in the freezer. After collection, the samples were transported to the laboratory, thawed, subdivided into small portions and stored at -70°C. Before analysis, one portion of each sample was thawed and the whey fraction was obtained by centrifugation at 14,000 rpm for 30 minutes at room temperature, after which the fat was removed.

Competition assays for the quantitative detection of T cell stimulatory epitopes of gluten proteins
Competition assays were performed as described earlier (24-26). For quantification of the gliadin assays, a standard curve was made by the Prolamine Working Group gliadin standard (27) in a concentration range of 10 μg/ml-10 ng/ml. The assays specific for the
detection of T cell stimulatory epitopes of LMW glutenin were calibrated using a 25-mer synthetic peptide as a standard that contains the Glt-156 epitope (14). The HMW-glutenin specific assay was calibrated using a chymotrypsin digest of purified HMW-glutenin proteins (kindly provided by P. Shewry, Rothamsted Research, Hampenden, United Kingdom). Both glutenin standards were used in a concentration range from 1 μg/ml-2 ng/ml.

Food record and gluten calculation
The mothers on a ND recorded a food record on three consecutive days preceding the day of breast milk collection. The last day of recording coincided with the day of breast milk collection. The amounts of food consumed were recorded in household measures and the name of the manufacturer of the products used was precisely written down. To determine the gluten intake, the vegetable protein content of the gluten-containing products was calculated according to the Dutch Food Composition Table (28). Since there are no analyses on gluten content of products, this was calculated by multiplying the grams of vegetable protein of the gluten-containing food by 0.8 as described by Overbeek et al., (29) and by linking a food composition table spreadsheet to food consumption data using MS Access 2000. The products that may contain gluten, but with missing brand information or with a rounded number of zero grams protein in the food composition table, were defined by us as 'risk products'. As an assumption for the gluten content in those risk products an amount of 20 mg gluten per 100 g food product was used, which is the maximum of the Codex Alimentarius norm (30) for gluten-free products.

Data analysis
The data obtained by the competition assays and the commercial gliadin ELISA were imported in the scientific graphing and statistics program Graph Pad Prism version 4.02 (GraphPad Software, Inc. San Diego CA, USA). The significance of the differences detected between the level of gluten epitopes in the breast milk of mothers on a ND vs those on a GFD were assessed with a 2-sided unpaired t-test. P<0.05 was considered to indicate a significant difference. The Glia-α9 epitope is part of the degradation resistant 33-mer of alpha gliadin (16), and therefore the most likely to be detected in the breast milk as compared to Glia-α20, Glia-γ1 and HMW glt. Correlations between grams of gluten intake of the mother and Glia-α9 concentrations in breast milk were carried out using SPSS version 14.0 for Windows and checked by the Pearson correlation test. P<0.05 was considered to indicate a significant correlation.
RESULTS

Subjects
Twenty-three mothers on a ND (mean age 33±5 y) and 13 mothers on a GFD (mean age 34±4 y) responded and joined the study. Twelve of the 13 mothers on a GFD were CD patients diagnosed by small bowel biopsy. One mother showed clinical symptoms of CD and went on a GFD without being diagnosed.

Figure 1: Distribution of breast milk samples collected at different months of lactation.
Breast milk samples were collected longitudinally at different stages of lactation of mothers on a normal diet (ND, n=131) and mothers on a gluten-free diet (GFD, n=116). In both groups, most samples were obtained from month 2 and 3 of the lactation period.

Breast milk samples
Breast milk samples were collected from the first week after delivery up to eight months of lactation. A total of 131 samples of mothers on a ND and 116 samples of mothers on a GFD were obtained. The sample distribution over the various months of lactation was comparable for both groups with the highest number of samples obtained in months 2 and 3 of lactation (Figure 1).

The presence of gliadin- and glutenin-derived T cell stimulatory epitopes in breast milk
The presence of T cell stimulatory epitopes of gluten proteins known to be involved in CD (both from gliadin and glutenin) was determined in the whey fractions of the samples using mAb-based competition assays. In both groups of samples, ND vs GFD, T cell
stimulatory epitopes were detected in the assays specific for the Glia-α9, Glia-α20, Glia-γ1 and HMW-glutenin T cell stimulatory epitopes (Figure 2).

Figure 2: Level of T cell stimulatory epitopes of gliadin and glutenin in breast milk.
Whey fraction of breast milk of mothers on a normal diet (ND) and mothers on a gluten-free diet (GFD) were measured in the mAb-based competition assays specific for T cell stimulatory epitopes of gliadin and glutenin. Mean level per mother of (A) the Glia-α9, (B) the Glia-α20, (C) the Glia-γ1 and (D) the HMW glutenin-derived T cell stimulatory epitope.

For the LMW glutenin-derived T cell stimulatory epitopes no results were obtained since the LMW specific competition assay was not suited to measure the presence of the T cell stimulatory epitopes in breast milk. T cell stimulatory epitopes Glia-α9 and HMW glutenin present in the samples from mothers on a ND were significantly increased (P<0.05) compared to the levels in the samples of mothers on a GFD. The differences between the levels of the T cell stimulatory Glia-α20 and Glia-γ1 epitopes were higher in women on a ND, but did not reach significance (Table 1).
Table 1: Levels of T cell stimulatory epitopes of gliadin and glutenin in breast milk of mothers on 
a normal diet versus mothers on a gluten-free diet.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Normal diet (n=131)</th>
<th>Gluten-free diet (n=116)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean SEM</td>
<td>mean SEM</td>
<td></td>
</tr>
<tr>
<td>Gli-a9 (μg/ml)</td>
<td>0.1324 0.0187</td>
<td>0.0615 0.0088</td>
<td>0.0135</td>
</tr>
<tr>
<td>Gli-a20 (μg/ml)</td>
<td>0.1285 0.0425</td>
<td>0.0549 0.0181</td>
<td>0.5410</td>
</tr>
<tr>
<td>Gli-γ1 (μg/ml)</td>
<td>0.4038 0.0471</td>
<td>0.3669 0.0394</td>
<td>0.7922</td>
</tr>
<tr>
<td>HMW glt (ng/ml)</td>
<td>44.92 6.388</td>
<td>22.92 6.162</td>
<td>0.0351</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean; p indicates a two sided p value for the difference between 
gliadin and glutenin levels detected in breast milk of mothers on a normal diet compared to 
mothers on a gluten-free diet (unpaired t test); P<0.05 indicate a significant difference between 
the compared mean values.

Gluten intake of mothers on a normal, gluten-containing diet
Of the 23 mothers on a ND 22 kept a 3-day food record preceding the day of breast milk 
collection. In total, 48 food records were collected and the mean gluten intake was 
17±3.8 g/day. The median consumption of gluten from risk products was 1.5 mg/day 
which is less than 0.01% of the total gluten intake. Because of this small percentage, the 
glutten intake from risk products was not taken into account in further analysis.

No correlation between gluten intake and level of Gli-a9 detected in breast milk of mothers on a 
normal diet
The correlation between gluten intake and the level of gluten epitopes detected in the 
breast milk of mothers on a ND was studied using the mean levels detected for the 
degradation resistant Gli-a9 epitope. This epitope is part of the degradation resistant 33-
mer of alpha gliadin (16), and therefore the most likely to be detected in the breast milk. 
We checked the correlation between the gluten intake of the mothers on a ND and the 
level of Gli-a9. No correlations were found between the mean level of Gli-a9 epitope 
detected and the amount of gluten consumed in the three or two days preceding the day 
of breast milk collection (r= -0.142, P=0.37; r= -0.131, P=0.42, respectively). In addition, 
no correlation was detected between the mean level of the Gli-a9 epitope and the gluten 
intake during day 2 or day 3 (r= -0.104, P=0.52; r= -0.109, P=0.50, respectively).

DISCUSSION
In this study, breast milk samples were analyzed by antibody based assays specific for the 
detection of T cell stimulatory gluten peptides, for the presence of dietary gluten-derived 
peptides involved in the development of CD. Increased levels of peptides of both gliadin 
and glutenin could be shown in breast milk of mothers on a ND compared to those in
the breast milk of mothers on a GFD. The detected level of Glia-α9 however, did not correlate with the gluten intake of the mothers on a ND. Our result indicates that breast-fed children are exposed to low amounts of gluten-derived T cell stimulatory peptides involved in CD, even before gluten is introduced into the infants’ diet.

The presence of gluten proteins in breast milk has been reported previously (31,32). The mean level of gliadin in breast milk of 178 ng/ml (range: 5-2000 ng/ml) reported in previous studies (31,32) correlates well with the mean levels of T cell stimulatory epitopes of gliadin detected in our study (Glia-α9 mean level 132 ng/ml, range 20.2-392 ng/ml; Glia-α20 mean level 129 ng/ml, range 0-737 ng/ml and Glia-γ1 mean level 404 ng/ml, range 143.7-1059 ng/ml).

Moreover, with the recently developed competition assay specific for the T cell stimulatory epitope of HMW glutenin (25), we could show that next to gliadin HMW glutenin also appears in breast milk (HMW mean level 44.92 ng/ml, range 2.23-104.8 ng/ml). Regarding the presence of LMW glutenin-derived T cell stimulatory epitopes in breast milk, nothing is known. The assay specific for detection of the LMW-derived T cell stimulatory epitope of LMW glutenin (25) was not suitable for detection of this epitope in breast milk (this study).

Gliadin given to mothers on a gluten restricted diet, appeared in the breast milk between 2-4 hours after gliadin intake (31). Similar to our study, basal levels of gliadin were detectable even before intake of gliadin (31). From that study it is not clear however, whether the level of gliadin detected in the breast milk correlated with the gluten intake of the mothers. The basal level detected both in our study and in the study reported previously (31), might be explained by cross reactivity of the gluten specific antibodies used for detection and human proteins that have some similarity with gluten sequences. For example, recently, cross reactivity between human anti-gliadin antibodies and a proline glutamin rich neuronal protein, Synapsin I, has been described (33). Analyses of the human database for the minimal epitopes detected by our antibodies used for the competition experiments, did not reveal any human proteins that might be recognized by our antibodies (result not shown). On the other hand, we did not have detailed information on the strictness of the diet of mothers on a GFD and it is possible that the GFD diet was not kept strictly, either intended or unintended. Future experiments should be aimed at what proteins are detected by gluten specific assays in the breast milk of mothers on a strict GFD.

Upon ingestion, gluten proteins are digested by enzymes of the gastrointestinal tract including pepsin, trypsin, chymotrypsin and brush border enzymes. However, because of the high proline content of the gluten proteins, digestion is not complete and both of α-gliadin (33-mer containing 6 overlapping T cell stimulatory epitopes including the Glia-α9 epitope detected in this study) and of γ-gliadin, degradation resistant peptides have been
described (6,16). Those peptides contain the T cell stimulatory epitopes known to be involved in CD.

Moreover, in a recent study in which gluten degradation in the gastrointestinal system was mimicked in a gastrointestinal model, it was shown that also the HMW glutenin proteins are relatively resistant to degradation, probably even more resistant than gliadin proteins (26).

It has been suggested that the incomplete degradation of gluten in combination with the binding properties of the gluten peptides to HLA-DQ2 and HLA-DQ8 molecules, especially after deamidation by Tg, are the main cause of CD. Until now however, it is not known how those degradation resistant peptides cross the epithelial barrier and reach the lamina propria where, after endocytosis by dendritic cells, they are presented to the immune system. Analysis of the peptides detected in the breast milk in the near future might reveal in which form gluten crosses the epithelial barrier and enters the human body.

The presence of gliadin- and glutenin-derived peptides in breast milk of mothers indicates that infants are exposed to small amounts of gluten through the breast milk before the introduction of gluten into the infants’ diet, which normally is advised from the age of 6 months. It is tempting to assume that those low amounts of gluten peptides induce oral tolerance to gluten as is described for other antigens (34-36).

Lactating mammary glands are part of the integrated mucosal immune system and milk antibodies reflect antigenic stimulation of the mucosal immune system in the gut and in the airways. Secretory lgA from breast milk exhibits antibody specificities for an array of both intestinal and respiratory common pathogens (37) and dietary proteins like cows milk proteins and gluten (38,39). Until the infant develops its own secretory IgA and IgM producing B cell blasts and plasma cells, it is dependent on the maternal secretory antibodies present in breast milk.

Next to antibodies, other various dietary antigens are present in breast milk; however, dietary restriction during pregnancy and breastfeeding has shown no conclusive effect on the development of atopic diseases in the child (40,41). These antigens stimulate the maturation of the infants’ mucosal immune system (41) and under hyporeactive or immunosuppressive conditions, such as low antigen dose and/or presence of down regulatory cytokines as IL-10 and TGF-β (42), activation of the immune system might be skewed towards a Th2 or tolerogenic phenotype.

CD is a disease with a strong genetic association. HLA-DR3, DQ2 positive individuals have a five times higher risk of developing CD than HLA-DR3, DQ2 heterozygous individuals and a more than 11 times higher risk than people with other HLA haplotypes (18). However, since most HLA-DR3, DQ2 positive individuals do not develop CD, it is generally assumed that gluten induces oral tolerance. Recently, dietary gluten specific, CD4+, IL-10 and TGF-β producing regulatory T cells have been described. The cells are
present in the mucosa and inhibit pathogenic gluten reactive T cells (43). It would be very interesting to know to which peptides those regulatory T cells respond and whether those peptides are present in breast milk and involved in the induction of oral tolerance against CD.

In conclusion, in the present study we show that low amounts of T cell stimulatory epitopes of gluten, both from gliadin and glutenin, are present in breast milk of mothers on a ND. Since oral tolerance to food antigens is induced early in childhood, in the period infants are breast-fed, these peptides might be involved in the induction of gluten tolerance. Future experiments should be aimed at the characterization and identification of the gluten peptides present in breast milk.

This knowledge will give some new insights in the way gluten tolerance is induced and will help us to generate novel strategies aimed at the prevention of the onset of CD.

REFERENCES


