(51) International Patent Classification 6: A61K

(21) International Application Number: PCT/DK99/00255

(22) International Filing Date: 6 May 1999 (06.05.99)

(30) Priority Data:

- 0621/98 DK 6 May 1998 (06.05.98)
- 60/091,545 US 1 July 1998 (01.07.98)

(71) Applicant (for all designated States except US): KÖBENHAVNS UNIVERSITET [DK/DK]; Nørregade 10, P.O. Box 2177, DK–1017 Copenhagen K (DK).

(72) Inventors:


(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS A/S; Sankt Anne Plads 11, P.O. Box 3007, DK–1021 Copenhagen K (DK).

(54) Title: TREATMENT OF CELIAC DISEASE

(57) Abstract

The present invention relates to a method of treating celiac disease comprising interfering with the deamidation of at least one glutamine residue in a gliadin or glutenin molecule. This may be provided by prohibiting or interfering with the deamidation of at least one glutamine residue by derivation of at least one glutamine residue in a gliadin or glutenin molecule in wheat flour by a chemical or enzymatic deamidation of gluten followed by a chemical or enzymatic derivation of the generated carboxyl group(s). In a further aspect, the invention relates to a method of interfering with the deamidation of at least one glutamine residue in a gliadin or glutenin molecule and thereby treating celiac disease, the method comprising administering, to a patient having or suspected of having celiac disease, at least one of the following substances: (a) a substance which is capable of increasing the pH in the gastroduodenal tract of a subject, e.g., an antacidum, an anticholinergic agent, H2–receptor antagonists or a proton pump inhibitor, (b) a substance which is capable of eliminating deamidating bacteria in the gastroduodenal tract of a subject, e.g., an antibiotic or antimicrobial agent, and/or (c) a substance which is capable of interfering with the effect of at least one deamidating enzyme in the gastroduodenal tract of a subject.
FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Albania</td>
<td>ES</td>
<td>Spain</td>
<td>LS</td>
<td>Lesotho</td>
</tr>
<tr>
<td>AM</td>
<td>Armenia</td>
<td>FI</td>
<td>Finland</td>
<td>LT</td>
<td>Lithuania</td>
</tr>
<tr>
<td>AT</td>
<td>Austria</td>
<td>FR</td>
<td>France</td>
<td>LU</td>
<td>Luxembourg</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
<td>GA</td>
<td>Gabon</td>
<td>LV</td>
<td>Latvia</td>
</tr>
<tr>
<td>AZ</td>
<td>Azerbaijan</td>
<td>GB</td>
<td>United Kingdom</td>
<td>MC</td>
<td>Monaco</td>
</tr>
<tr>
<td>BA</td>
<td>Bosnia and Herzegovina</td>
<td>GE</td>
<td>Georgia</td>
<td>MD</td>
<td>Republic of Moldova</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>GH</td>
<td>Ghana</td>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>GN</td>
<td>Guinea</td>
<td>MK</td>
<td>The former Yugoslav</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
<td>GR</td>
<td>Greece</td>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>HU</td>
<td>Hungary</td>
<td>MN</td>
<td>Mongolia</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
<td>IE</td>
<td>Ireland</td>
<td>MR</td>
<td>Mauritania</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>IL</td>
<td>Israel</td>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td>BY</td>
<td>Belarus</td>
<td>IS</td>
<td>Iceland</td>
<td>MX</td>
<td>Mexico</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
<td>IT</td>
<td>Italy</td>
<td>NE</td>
<td>Niger</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>JP</td>
<td>Japan</td>
<td>NL</td>
<td>Netherlands</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>KE</td>
<td>Kenya</td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>KG</td>
<td>Kyrgyzstan</td>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>CI</td>
<td>Côte d'Ivoire</td>
<td>KP</td>
<td>Democratic People's Republic of Korea</td>
<td>PL</td>
<td>Poland</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>KR</td>
<td>Republic of Korea</td>
<td>PT</td>
<td>Portugal</td>
</tr>
<tr>
<td>CN</td>
<td>China</td>
<td>KZ</td>
<td>Kazakhstan</td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>CU</td>
<td>Cuba</td>
<td>LC</td>
<td>Saint Lucia</td>
<td>RU</td>
<td>Russian Federation</td>
</tr>
<tr>
<td>CZ</td>
<td>Czech Republic</td>
<td>LI</td>
<td>Liechtenstein</td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>DE</td>
<td>Germany</td>
<td>LK</td>
<td>Sri Lanka</td>
<td>SE</td>
<td>Sweden</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
<td>LR</td>
<td>Liberia</td>
<td>SG</td>
<td>Singapore</td>
</tr>
<tr>
<td>EE</td>
<td>Estonia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>Slovenia</td>
<td>SK</td>
<td>Slovakia</td>
<td>SN</td>
<td>Senegal</td>
</tr>
<tr>
<td>SZ</td>
<td>Swaziland</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TD</td>
<td>Chad</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>Togo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TJ</td>
<td>Tajikistan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>Turkmenistan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td>Turkey</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>Trinidad and Tobago</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UA</td>
<td>Ukraine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UD</td>
<td>United States of America</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UZ</td>
<td>Uzbekistan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VN</td>
<td>Viet Nam</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YU</td>
<td>Yugoslavia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZW</td>
<td>Zimbabwe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TREATMENT OF CELIAC DISEASE

FIELD OF INVENTION

The present invention is based upon the finding that deamida-
tion of glutamine residues in gliadin is important for immune
activation and presumably tissue destruction in celiac dis-
ease. In its broadest aspect, the present invention thus re-
lates to a method of treating celiac disease comprising in-
terfering with the deamidation of disease eliciting glutamine
residue(s).

GENERAL BACKGROUND

Celiac disease (CD) is a chronic intestinal disease with
chronic diarrhoea, malnutrition and loss of weight as its
most important manifestations. It is elicited by ingestion of
components of wheat, rye, barley and possibly oats flour. The
existing treatment is a life-long exclusion of flour from the
diet, which affects the quality of life of CD individuals.

The incidence of the disease varies but a rate of 1 case of
about every 300 born person in the population has been repor-
ted in several investigations. In some countries, e.g. Den-
mark, the incidence is lower, but with increased investigati-
ons the gap tends to be reduced.

The pathogenesis of the disease has both genetic and environ-
mental components. Monozygotic twins have a concordance rate
of approximately 75%, suggesting an environmental component.
About 10% of first degree relatives are affected. One genetic
component is the HLA association of the disease; more that
90% of the patients have the DQA1*0501 and DQB1*02 alleles.
However, there are more genetic components as the concordance rate between siblings with this particular DQ is only about 30%.

It is known that a group of proteins, the gliadins, in the wheat flour are the pathogenic agents. Gliadins can be classified into several groups (α-type, γ-type and ω-type) and among these at least the α- and the γ-type gliadins are known to cause the disease. Each subcultivar of wheat contains about 30 different α- and γ-gliadins, with some variations in amino acid sequences within each type. Both types are characterised by insolubility in neutral aqueous solutions, a molecular mass around 40 kDa and an N-terminal half built up of repeating sequences being very rich in glutamines and prolines.

The presence of T cells in the small intestinal mucosa that recognize the gliadins is characteristic of individuals with CD. Such T cells can be isolated, propagated in vitro and cloned. It has been demonstrated that presentation of gliadin to these cells is almost exclusively restricted to DQ2. Evidence is accumulating that there is a crucial role for these T cells in the immunopathogenesis of the disease (Scott et al., 1997). In vitro, proliferation of gliadin-specific, HLA-DQ2-restricted T cell clones from the small intestine of celiac disease patients can be initiated by the addition of a water soluble form of gliadin heated at low pH and a HLA-DQ2 carrying antigen presenting cell. The proliferation is registered by the incorporation of radioactive thymidine. The part of the structure of the gliadins (the toxic sequence(s)) recognised by such T cells has, however, been elusive and this has hampered the understanding of the pathogenesis of
the disease and thereby the possibility to develop interfering therapeutics.

SUMMARY OF THE INVENTION

Contrary to previous assumptions that conversion of glutamine to glutamic acid will abolish the harmful effects of gliadin (Van de Kamer, JH and Weijers, HA, 1955), which findings have been generally accepted in the field, it has been found by the present inventors that glutamines in distinct sequences of gliadin have to be deamidated to be toxic. With knowledge about a toxic sequence which has been established by the present inventors and general knowledge about possible mechanisms for deamidation, the demonstrated disease provoking deamidation as well as other disease provoking deamidations can be treated by inhibiting the deamidation of distinct glutamines in gliadin or elsewhere, e.g. in glutenin, another major component of gluten.

In its broadest aspect, the present invention relates to interfering with the deamidation of at least one glutamine residue in a gliadin molecule.

There are several possibilities to interfere with deamidation. One main principle is to modify the gliadins of the food, e.g. the gliadin of the wheat flour making deamidation impossible. This might be achieved by deamidation of sensitive amides in glutamines followed by chemical derivation of the generated carboxyl groups to remove or modify the negative charge either directly in an enzymatic reaction or by deamidation followed by derivation. It might also be possible to generate gliadins which cannot be deamidated by genetic engineering of wheat.
Another main principle is to interfere with the processing of gliadins in the preparative phase of the food or when the gliadins are processed by the CD individual. It will be further analysed whether the fermentation of the dough or the baking process results in deamidation. If a deamidating activity in baker's yeast is found, it might be possible to remove this by genetic manipulation. If deamidation occurs as a result of the baking process per se, it might be more appropriate to derivatise the glutamines of gluten into more heat-stable residues.

Neutralisation of the acidic environment in the stomach by any procedure leading to decreased secretion of hydrochloric acid by the parietal cells, such as by administration of a proton pump inhibitor, a histamine 2 receptor antagonist, an anticholinergic agent, or a passive therapeutic agent like an acid neutralising agent, is likely to prohibit or minimize a deamidation due to the low pH of the stomach. Thus, in one embodiment the substance to be used is capable of increasing the pH in the gastroduodenal tract of a subject. With the available knowledge, pH should be increased above 3.0. Further or alternatively, possible deamidase producing microorganisms may be reduced or eradicated by antibiotic treatment.

Therefore, in a further aspect the invention relates to the use of a substance which is capable of eliminating or decreasing the amount of deamidating bacteria in the gastroduodenal tract of a subject. Finally, in the cases where an enzyme in the digestive secretion(s) or intestinal wall is responsible for the deamidation, substances that interfere with the effects of such an enzyme should be used either alone or as an additional supplement. Thus, the invention also relates to the use of a substance which is capable of inhibiting at
least one deamidating enzyme in the gastroduodenal tract of a subject.

DETAILED DISCLOSURE OF THE INVENTION

5

In the examples it is demonstrated that at least one glutami-

ne in a sequence of gliadin has to be deamidated to be toxic
in celiac disease. In the following the results of the exam-

10

ples will be summarized, and a number of possibilities of in-
terfering with this crucial modification step in order to

provide a new treatment of the disease will be outlined.

Although the mechanism is exemplified with reference to a

15 particular toxic sequence, it is evident that the principle

is not restricted to this particular sequence. Furthermore,

it should be emphasized that apart from treating patients

with manifest CD, the principles described in the present ap-

20 plication may also be used to prevent manifest CD in persons

having latent or silent (not clinical manifest) CD, i.e. in a

person having or suspected of having CD or being in a risk

1.1. Identification of a toxic sequence

A distinct γ-type gliadin (γ-gliadin 36) was purified from

25 gliadin of the strain Kadett. It was digested with chymotryp-
sin followed by heating at low pH. Fragments capable of sti-
mulating a T-cell clone in vitro were purified by chromatoo-

30 graphies on Sephadex G-50, Superdex HR 10/30 and MonoQ. After

partial sequencing and analyses by matrix-assisted laser
desorption/ionization-time-of-flight and electrospray ioniza-
tion trap mass spectrometry, it could be demonstrated

that fragments with varying degrees of deamidated glutamines

in the sequence QQLPQPQPQPSFPQQRPF (SEQ ID NO:1) stimula-
ted proliferation of T cell clones. By using synthetic peptides of varying lengths, it could be demonstrated that the minimal toxic sequence originates from the gliadin sequence QPQQSFPOQQ (SEQ ID NO:17) and that it was stimulatory only after heating at low pH. This finding is in agreement with deamidation of one or more glutamines being crucial for the development of the disease.

1.2. Demonstration of deamidation of a distinct glutamine

Electrospray ionization mass spectrometry of methyl-esterified peptides of different purified fragments demonstrated partial deamidations in many different positions with some positions being more prone to deamidation. Guided by this analysis two synthetic 11-mer peptides [PyQPQQSFPOQQ (Py = pyroglutamic acid) (SEQ ID NO:14) and PyQPQQSFPOEQ (SEQ ID NO:15)] were tested. Both peptides were stimulatory after heating at low pH; however, only the latter was stimulatory prior to this treatment. This result demonstrates that deamidation of one distinct glutamine is critical for recognition by the T cell clone used.

1.3. Evidence that the identified sequence is active in different patients

A further 10 polyclonal gut-derived T cell lines stimulated by gliadin and derived from seven patients were tested for proliferation with SEQ ID NO:1. None of the T cell lines responded positively to the untreated peptide whereas T cells from two patients recognised the peptide treated after heating at low pH. The same T cell lines also responded to SEQ ID NO:15 but not to SEQ ID NO:14 suggesting that the sequence is recognised by T cells from different patients. The results
further demonstrate that also other sequences, capable of stimulating T cells to proliferation, occur.

1.4. Evidence that the requirement of deamidation is general

T cell lines from six patients as well as 25 T cell clones from five patients were tested with chymotrypsin digested gliadin either untreated or treated by heating at low pH. All T cell lines and most clones displayed weak reactivity against the untreated gliadin, while the reactivity was markedly enhanced by heating at low pH. Thus, deamidation appears to be important for recognition by most T cells from the small intestinal mucosa of CD patients.

1.5. Mechanisms of deamidation

The deamidation might either occur as part of the normal processing of baked wheat-containing food before or after it has been ingested or be specific for CD. Deamidation under normal conditions might occur during a. fermentation of the bread, b. baking of the bread, c. exposure to the hydrochloric acid of the stomach, d. exposure to deamidating enzymes during digestion in the gastrointestinal tract, e.g. exposure to deamidating enzymes in the intestinal wall. If the deamidation is specific for CD, a disease-specific deamidation might occur as a result of bacterial infection in the gastrointestinal tract or a change in the effects of a deamidating enzyme in the digestive secretions or intestinal wall.
1.5.1. Physiological deamidation

1.5.1.1. Fermentation of the dough

Gliadin was treated with yeast under conditions corresponding to the normal fermentation of a dough. No significant T-cell proliferation stimulatory activity could be detected.

1.5.1.2. Baking of the bread

A piece of bread was made of flour, water and yeast according to standard procedures. It was homogenised and gliadin prepared according to standard procedures. No significant T-cell proliferation stimulatory activity could be detected.

1.5.1.3. Exposure to the hydrochloric acid of the stomach

Incubation at 37°C, pH 1.8 for 15 minutes of chymotrypsin digested gliadin significantly increased the proliferation stimulatory activity as tested with one T cell line and two T cell clones. These results suggest that sufficient deamidation can occur in the acidic environment of the stomach. Their significance will be further studied in vivo using experimental animals (mice). By infusion of a peptide (GQPQQSFPOQQ, SEQ ID NO:19) in the stomach followed by collection of duodenal juice, the generation of the T-cell stimulatory epitope will be analysed using the T-cell proliferation assay described in Example 2. The obviation of the generation of the epitope will similarly be studied by using mice with low hydrochloric acid secretion (gastrin knock-out mice, mice treated with a substance which inhibits or reduces the secretion of gastric hydrochloric acid).
1.5.1.4. Exposure to deamidating enzymes during digestion in the gastrointestinal tract

No secreted or intestinal microvillus bound enzyme with protein deamidase activity has been described.

1.5.1.5. Exposure to deamidating enzymes in the intestinal wall

It is known from the literature that the enzyme tissue transglutaminase also displays protein deamidase activity. In the intestine this enzyme is localised in subepithelial myofibroblasts and secreted from here to the subepithelial reticular sheet located just beneath the basal lamina of the intestinal epithelium.

Treatment with commercially available purified transglutaminase from guinea-pig liver transformed SEQ ID NO:1 from a non-stimulatory to a potent T cell stimulatory peptide for gut-derived T cell lines from two patients. Separation of the transglutaminase treated peptide by MonoQ-chromatography followed by matrix-assisted laser desorption/ionization-time-of-flight and electrospray ionization ion trap mass spectrometry showed that a fraction containing the peptide QQLPQPEQPQQSPQQPERPP (SEQ ID NO:6) was unstimulatory, whereas a fraction that predominantly contained the peptide QQLPQPEQPQQSPPEQERPP (SEQ ID NO:7) was stimulatory. This result suggests that sufficient deamidation can occur in the intestinal submucosa.

30
1.5.2. Microbiological deamidation

There are several examples that demidating enzymes occur in microorganisms. Transglutaminases have been identified and characterised from *Streptoverticillium* (Kanaji et al., 1993) and from *Bacillus subtilis* (Kobyashi et al., 1998). Furthermore, two protein deamidating enzymes, peptidoglutaminase I and II, have been isolated from *Bacillus circulans*. It might be that enzymes of this type from intestinal bacteria cause the deamidation and that such bacteria constitute a predisposing environmental factor. This possibility is tested by analyses of deamidating activities of intestinal juice under normal and disease conditions. If deamidation activity is found, attempts to isolate and type the responsible microorganism will be undertaken.

**THERAPEUTIC POSSIBILITIES**

Further studies will focus on the following three aspects which all harbour therapeutic possibilities: a. search for deamidase activities b. search for the site(s) of physiological deamidation and c. search for the effect of interfering with deamidation in vitro and in vivo.

2.1. Monitoring of deamidation

For all three aspects it will be important to be able to monitor the degree of deamidation of a natural or synthetic peptide including the toxic sequence of undigested or partially digested gliadin. The following methods can be used:
2.1.1 Ion exchange chromatography

A synthetic peptide without basic or acidic residues (as is the case with the toxic sequence) has a net charge at neutral pH close to zero. This means that it is not retarded on an ion exchanger. Generation of glutamate residues results in one negative charge per deamidated glutamine. A sensitive system using MonoQ-chromatography on the Pharmacia SMART system which allows the demonstration of peptides with one, two or three deamidated glutamines has been developed.

2.1.2 Mass spectrometry

If details on the localisation of glutamine deamidation are required, further analysis using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) or electrospray ionization MS/MS after methyl-esterification to detect mass increments of 15 daltons will be undertaken.

2.1.3 Monoclonal antibodies

The generation of monoclonal antibodies against the peptide PQPQPQSFPQQFRF (SEQ ID NO:9) has been initiated with the aim of obtaining an antibody that is specific for the deamidated peptide. The use of monoclonal antibodies is not sensitive to the occurrence of other substances and might be used for analysis in many different systems, e.g. processing of gliadin or peptide by the stomach or intestinal wall using Western blotting, ELISA or immunohistochemistry. It is expected that such an antibody will detect generation of the toxic sequence also in undigested or partially digested gliadin and thereby be of great importance in defining the site(s) and mechanism of the deamidation.
2.1.4. Proliferation of T cell clones

Deamidation can be demonstrated by the cell proliferative test using T cell clones from the diseased mucosa as originally used for the demonstration of the deamidation. Once biochemical evidence suggests that deamidation takes place, modified peptides can be tested using T cells.

2.1.5. Measurement of liberated ammonia

Deamidation can also be followed by a quantitative analysis of the liberated ammonia. This can be done by a commercially available test from Boehringer Mannheim, Germany (cat.no. 112 732). The test is based on spectrophotometric analysis at 340 nm of the disappearance of added NADH after further addition of 2-oxoglutarate and glutamate dehydrogenase to the sample.

2.2. Mechanism and localisation of deamidation

2.2.1. Demonstration of deamidase activities

Deamidation will be searched for either by looking for deamidase activities using in vitro enzymatic assays with a distinct peptide as substrate in combination with the MonoQ system or using monoclonal antibodies as monitoring system. Deamidase activity will be looked for in pancreatic secretions, intestinal juice and intestinal wall under normal and pathological conditions. The origin and nature of a detected activity will be characterised (normally/pathologically occurring, origin of production (pancreas, intestinal wall, bacteria)).
2.2.2 Localisation of deamidation

The generation of deamidated residues in gliadin will be monitored by monoclonal antibodies specific for the deamidation all through the gliadin processing (fermentation, baking, processing in the stomach, processing in the intestinal lumen and processing in the intestinal wall).

2.3.1. Inhibition of deamidation

As discussed previously, one main principle is to modify the gliadins in the food. One embodiment of the invention is thus a method of treating celiac disease comprising interfering with the deamidation of at least one glutamine residue in a gliadin molecule, comprising prohibiting or minimizing deamidation of at least one, preferably all relevant, glutamine residues by derivation of a gliadin in wheat flour. This may be done by bulk deamidation of wheat flour either by non-enzymatic deamidation using mild alkali or acid treatment, or enzymes (transglutaminase, proteases or peptidoglutaminase) (Hamada JS, 1994). Blocking might be achieved by chemical or enzymatic derivation. The use of transglutaminase may allow the chemical derivation of glutamine amides in a single reaction.

The deamidation of at least one glutamine residue in a gliadin molecule may be interfered with and a patient having or suspected of having celiac disease may be treated by administering at least one of the following substances:

(a) a substance which is capable of increasing the pH in the gastroduodenal tract of a subject,
(b) a substance which is capable of eliminating deamidating bacteria in the gastroduodenal tract of a subject, and/or

(c) a substance which is capable of interfering with the effect of at least one deamidating enzyme in the gastroduodenal tract of a subject.

2.3.1.1. Increasing the pH in the gastroduodenal tract

The pH in the gastroduodenal tract may be increased by administering an antacidum, such as aluminiumoxide hydrate, magnesium carbonate, magnesium hydroxide, magnesium oxide, dihydroaluminium sodium carbonate, magnesium aluminium silicate, aluminium aminoacetate, calcium carbonate or combinations of these substances. In general, these substances or combinations of substances will be administered in combinations and dosages commonly used for anti-ulcer treatment and the effect tested by a clinical trial essentially as outlined below with respect to a proton pump inhibitor.

Alternatively, a substance which is capable of inhibiting or reducing the secretion of gastric acid from parietal cells may be administered in order to increase the pH in the gastroduodenal tract. Examples of substances suitable for this purpose are anticholinergic agents such as butylscopolamine and propantheline, H₂-receptor antagonists such as ranitidine, cimetidine, famotidine and nizatidine, and proton pump inhibitors such as omeprazol, lansoprazol and pantoprazol. In general, these substances will be administered in dosages commonly used for anti-ulcer treatment and the effect tested by a clinical trial essentially as outlined in the following section with respect to a proton pump inhibitor.
The possibility that the acidity of the stomach significantly contributes to demidation in vivo will be tested on patients with CD by the use of a proton pump inhibitor such as omeprazol, lansoprazol or pantoprazol in combination with monitoring of the morphology of the intestine and the clinical symptoms. A prospective, open and uncontrolled pilot experiment consisting of 6 to 12 patients with CD in remission and some patients with so-called refractory CD will be performed.

The patients will be treated with omeprazol, 40 mg x 2 daily, under gluten provocation. Alternatively, lansoprazol in a dosage of 30 mg x 1-2 or pantoprazol in a dosage of 40 mg x 1-2 may be used. The total length of the trial period is planned to be at most 24 weeks. The results will be evaluated as earliest after 6 patients. When the trial has established a positive effect of the treatment, it is planned to establish a prospective, randomised, controlled and blinded trial. The study has been accepted by the Danish Medicines Agency and a response from the local ethical committee is awaited within short. It is described in detail in Example 4.

2.3.1.2. Eliminating deamidating bacteria in the gastroduodenal tract

Enzymes from the intestinal bacteria may cause deamidation. If it is established that this mechanism is of significance, the deamidase(s) from the intestinal microbiological flora should be reduced or eradicated by treatment with an antibiotic or antimicrobial agent, preferably by a non-absorbable and locally acting treatment. The exact choice of antibiotic or antimicrobial agent will depend on the isolation and characterisation of the deamidase producing microorganism(s). It is contemplated that combinations of tetracycline or amoxi-
cillin with metronidazole or clarithromycin for 1-2 weeks will be useful. In general, the antibiotic or antimicrobial agent or combinations of agents will be administered in combinations and dosages commonly used for treating infection by *Helicobacter pylori* and the effect tested by a clinical trial essentially as outlined above with respect to a proton pump inhibitor.

2.3.1.3. Inhibition of at least one deamidating enzyme in the gastroduodenal tract

If the deamidation is caused by an enzyme functioning during the normal processing, it will be possible - dependent on its nature and localisation - either to remove the enzyme or to interfere with its deamidating activity, e.g. by specific enzyme inhibitors. A possible deamidase activity might be removed from baker’s yeast by genetic engineering. The effect of the treatment will be tested by a clinical trial essentially as outlined above with respect to a proton pump inhibitor.

2.3.1.4. Derivation of disease-provoking glutamines

Industrially prepared gluten or a gluten derivative is used as a substrate for the derivation. This derivatised gluten shall subsequently be made suitable for baking by back-addition of wheat starch, or if it is difficult to obtain a wheat starch product completely free from gluten, it is contemplated to use maize, rice or possibly potato starch.
2.3.1.4.1. Deamidation of glutamine followed by derivation of the generated carboxyl groups

The optimal conditions for acid deamidation of gluten are well characterised (Wu et al., 1976). It is also possible to use an enzymatic deamidation with chymotrypsin at pH 10 (Kato et al., 1989). This procedure would be expected to be less random than the acid procedure. It will then be possible to derivatise either by the generation of epsilon-peptides using peptide synthesis methods, e.g. the carbodiimide coupling procedure (Gish, 1970) or to esterify the generated carboxyl groups generating e.g. a methyl ester (Hunt et al., 1986). A chemical derivation has the advantage that it allows the blocking with a substance that does not allow degradation in the intestine (D-lysine, methanol). The reaction may either be performed on solubilised gluten or on a gluten suspension. Solubilisation without deamidation can be achieved by proteolytic treatment, e.g. Pronase E (Babiker et al., 1996). It is thus possible that transamidation with a proper polar substance similarly will solubilise gluten.

2.3.1.4.2 Direct derivation of glutamines

Tissue transglutaminase has earlier been used to introduce lysine (Iwami and Yasumoto, 1986) and lysyl dipeptides (Ikura et al., 1985) into food proteins. This approach has the drawback of high cost of the tissue transglutaminase. A microbial transglutaminase purified from Streptoverticillium (Ando et al., 1989) is used in some other food industrial processing procedures. Transamidation with lysine or a lysine dipeptide will additionally increase the nutritional value of gluten (Friedman and Finot, 1990; Segura et al., 1996).
There are several studies on the metabolism of gamma-glutamyllysine. Several data suggest that gamma-glutamyllysine passes through the intestinal wall unchanged (Raczynski et al., 1975) and is mainly digested in the kidneys (Friedman and Pinot, 1990; Raczynsky et al., 1975). There is a considerably larger amount of the hydrolysing capacity in the liver (9x) and in the kidney (18x) than in the intestine (Raczynsky et al., 1975).

2.3.1.4.3. Further testing of derivatised forms of gluten

The suitability of wheat for baking bread is determined by interaction between glutenins and gliadins of the gluten. Derivatives of gluten can therefore be expected to change these properties. Once derivatised gluten has been generated, it will be tested in standardised baking experiments in collaboration with a flour producer. Having obtained detoxified gluten with preserved baking properties, approval for its use as a food with respect to non-toxicity will be obtained. Finally, the effect of its intake on the disease will be tested by a clinical trial essentially as outlined above with respect to a proton pump inhibitor.

2.3.2. Further considerations regarding inhibition of deamidation

It should be emphasized that for optimal treatment it could be that more than one of the above mechanisms for inhibiting the deamidation of at least one glutamine should be implemented, and consequently more than one of the above described substances should be administered simultaneously or consecutively or more than one of the above described strategies for the inhibition of deamidation of at least one glutamine
should be followed. Each substance or strategy should be tested solely and, if appropriate, in combination, in order to decide whether the substance or strategy is indeed useful. However, once the biological effect of potential value, i.e. the principle of treating celiac disease by interfering with the deamidation of at least one glutamine residue in a gliadin molecule or elsewhere, e.g. in glutenin, has been established, it is within the skill of the person of ordinary skill in the art, by use of methods described in standard textbooks, guidelines and regulations as well as common general knowledge within the field, to select the exact procedure (e.g. dosage) to be implemented for any selected substance or strategy using merely routine experimentation procedures.
### Table 1

List of names of the protein sequences with reference to SEQ ID NOs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>134-153</td>
<td>1</td>
</tr>
<tr>
<td>134-153; Py134</td>
<td>2</td>
</tr>
<tr>
<td>134-153;E140</td>
<td>3</td>
</tr>
<tr>
<td>134-153;E148</td>
<td>4</td>
</tr>
<tr>
<td>134-153;E150</td>
<td>5</td>
</tr>
<tr>
<td>134-153;E140,150</td>
<td>6</td>
</tr>
<tr>
<td>134-153;E140,148,150</td>
<td>7</td>
</tr>
<tr>
<td>135-153</td>
<td>8</td>
</tr>
<tr>
<td>137-154</td>
<td>9</td>
</tr>
<tr>
<td>138-152;Py138</td>
<td>10</td>
</tr>
<tr>
<td>138-152;Py138,E148</td>
<td>11</td>
</tr>
<tr>
<td>138-152;Py138,E140,148,150</td>
<td>12</td>
</tr>
<tr>
<td>140-150</td>
<td>13</td>
</tr>
<tr>
<td>140-150;Py140</td>
<td>14</td>
</tr>
<tr>
<td>140-150;Py140,E148</td>
<td>15</td>
</tr>
<tr>
<td>140-149</td>
<td>16</td>
</tr>
<tr>
<td>141-150</td>
<td>17</td>
</tr>
<tr>
<td>141-150;E148</td>
<td>18</td>
</tr>
<tr>
<td>140-150;G140</td>
<td>19</td>
</tr>
<tr>
<td>142-150</td>
<td>20</td>
</tr>
<tr>
<td>143-150</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>22</td>
</tr>
</tbody>
</table>

### LEGENDS TO FIGURES

Figure 1

A) MonoQ chromatography of a stimulatory fraction with molecular weights between 2-3 kD obtained by successive steps of gel filtration of chymotrypsin digested γ-36 gliadin. Aliquots of fractions were tested for their capacity to activate
the gut-derived DQ2-restricted gliadin-specific T cell clone 4.32 in a proliferative assay by measuring incorporation of $^3$H-thymidine (bars). The last peak, mainly due to a constituent of the buffer, was not tested for recognition by the T cell clone.

B) MonoQ chromatography of acid/heat treated Py135-153 peptide. Aliquots of fractions were tested for recognition by the T cell clone 4.32 (bars).

Figure 2

Proliferation of the gut-derived DQ2-restricted T cell clone 4.32 in response to synthetic peptides of a γ-type gliadin.

The peptides in the upper panel were tested in the untreated form at 10 μM (black bars) or in the acid/heat-treated form at concentrations of 10, 1, 0.1 μM (open bars). The lower panel depicts the responses against the peptides 140-150;Py140 (SEQ ID NO:14) and 140-150;Py140,E148 (SEQ ID NO:15). The concentrations of the peptides were as above, except that the 140-150;Py140,E148 peptide (SEQ ID NO:15) was also tested at 1 and 0.1 μM in the untreated form.

Figure 3

Analysis of the proliferative responses of four gut-derived T cell lines from four different CD patients to untreated and acid-heat treated chymotrypsin digested Kadett gliadin.
Figure 4

Recognition of chymotrypsin-digested gliadin by a gut-derived DQ2-restricted T cell line from an untreated celiac disease patient (CD412). Gliadin in various concentrations was incubated for 2 hours at 37°C with 100 μg/ml guinea pig TGase. The acid-heat treated gliadin was incubated at pH 1.8, 98°C, freeze-dried and resuspended at pH 7.4. The various samples were incubated with APC (allogenic HLA-matched T cell depleted PBMC (irradiated 25 Gy)). T cell proliferation was measured as $^3$H-thymidin incorporation 48-72 hours after antigenic stimulation. Data are from one representative experiment (out of three). Similar data were obtained with gut-derived T cell lines from five other CD patients.

Figure 5

A) Kinetic analysis of TGase mediated deamidation of gliadin peptide 134-153 (SEQ ID NO:1). The peptide was incubated with TGase for various time periods and then analyzed by ion exchange chromatography. Depicted are the chromatographs of the peptide after 0, 30, 60 and 180 min incubation with TGase. The fractions of the 180 min chromatography were divided, and one aliquot was tested for recognition by the DQ2-restricted T cell transfectant 60.6. Fractions 15 and 28 from the other aliquot were methyl-esterified and sequenced by ESI MS/MS to determine the number and positions of deamidated glutamine residues. The peak corresponding to fractions 38-46 in the chromatographs contains mainly impurities in the Tris-HCl buffer. Recognition of each of the fractions by DQ2-restricted T cell transfectant 60.6 was measured by quantification of IL-2 release.
B) The upper part denotes the amino acid sequence of gliadin peptide 134-153 (SEQ ID NO:1) prior to TGase treatment. The lower part denotes the number and positions of deamidated glutamine residues in chromatography fractions 15 and 28 of peptide 134-153 (SEQ ID NO:1) after 180 min TGase treatment. The number and positions of deamidated glutamine residues in each fraction were assessed by ESI MS/MS analysis.

Figure 6

A) T cell recognition and DQ2 binding of the synthetic gliadin-peptide 138-152;Py138 (SEQ ID NO:10) and variants with Gln to Glu amino acid substitutions. Peptide binding to DQ2 was measured in a cell free assay with purified HLA-molecules and binding affinity is given as IC₅₀, i.e. the amount of gliadin peptide necessary for 50% inhibition of binding of a labeled indicator peptide. The synthetic peptides were made with Gln to Glu substitutions in the same positions (140, 148 and 150) which were deamidated upon incubation of gliadin peptide with TGase (Figure 6B). Recognition of peptides by the DQ2-restricted T cell transfectant 60.6 was measured by IL-2 release in response to peptide stimulation.

B) Effect of acid/heat treatment of peptide 138-152;Py138, E140,E148,E150 (SEQ ID NO:12) on T cell recognition and DQ2 binding. Acid/heat treatment of the peptide was done at pH 1.8, 98°C for 60 min.
EXAMPLES

EXAMPLE 1

IDENTIFICATION OF A GLIADIN T CELL EPITOPE IN CELIAC DISEASE

Materials and Methods

Preparation of gliadin and gliadin peptides

A distinct γ-type gliadin (γ-gliadin 36) was purified from gliadin of the wheat strain Kadett as described in Sjöström H et al., 1992. Carboxymidomethylation and chymotrypsin digestion (1:200 w/w, 24 h) was performed as described in Stone, RA et al., 1989 followed by heating (98°C) in an acetic acid-HCl solution, pH 1.8 for 60 min (acid/heat treatment). Fragments capable of stimulating a T-cell clone in vitro were purified by successive chromatographies on Sephadex G-50, Superdex Peptide HR 10/30 and MonoQ equilibrated with 5 mM Tris/HCl buffer, pH 6.5 and developed with a gradient ending at 50 mM NaCl. The two latter were run on a SMART system (Pharmacia, Sweden).

Synthetic peptides

Peptides quality controlled by HPLC (> 80% purity) and MS were purchased from Neosystem (Strasbourg, France) or Research Genetics (Huntsville, AL).

T cells and proliferative assays

The T cell clone 4.32 from CD patient 280 (Lundin KEA et al., 1993) and T cell lines and 25 DQ2-restricted T cell clones
from the CD patients 370, 372, 380, 387 (Molberg et al., 1997), 410, 411, 412 (Molberg et al., 1998A) were derived from small intestinal biopsies challenged in vitro with pepsin and trypsin digested gliadin. Proliferative T cell assays using DR3, DQ2 positive B lymphoblastoid cell lines as antigen presenting cells were performed as described elsewhere (Lundin KEA et al., 1993).

Mass spectrometry (MS) analysis

Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) (Karas M et al., 1988) mass spectra were obtained on a Voyager Elite (PerSeptive Biosystems, Framingham, MA) or on a Bruker Reflex (Bruker-Franzen Analytik GmbH, Bremen, Germany) mass spectrometer. Electrospray ionization (ESI) (Penn JB et al., 1989) MS/MS spectra were recorded on an API III (Perkin Elmer Sciex, Perkin Elmer, Ontario, Canada) triple quadrupole mass spectrometer (Hunt DF et al., 1986) or an ESQUIRE (Bruker-Franzen Analytik GmbH) ion trap mass spectrometer (Cooks GL et al., 1991). The extent of deamidation within peptides was determined by methyl-esterification (Hunt DF et al., 1986) of MonoQ fractions followed by MALDI-TOF mass spectrometry to detect mass increments of 15 daltons, each of which represents one acidic group. The position of Glu residues within MonoQ fractions of the heat/acid treated synthetic peptides was similarly assessed by ESI MS/MS analysis of methyl-esterified fractions. C-terminal sequencing was done by MALDI-TOF analysis of peptides degraded by carboxypeptidase Y (Thiede B et al., 1995).
DQ2 peptide binding assay

A competitive peptide binding assay using affinity purified DQ(a1*0501,a1*02) molecules, a $^{125}$I-labeled indicator peptide KPLLIIAEDVGEY (SEQ ID NO:22) (MB 65kDa 243-255Y) and various unlabeled gliadin peptides was performed as described (Johansen BH et al., 1994).

Results and discussion

Separation on Sephadex G-50 revealed that all fractions with a molecular weight above approximately 2 kD were active. Fractions corresponding to molecular weights between 2-3 kD were pooled, acid/heat treated and further purified. The stimulatory activity of the fractions of the final MonoQ-Sepharose chromatography was centered around three defined positions (Fig. 1a). The dominating peptides in these positions were found by MALDI-TOF MS to have molecular masses of 2418.2 dalton, 2419.2 dalton and 2420.2 dalton. Methyl-esterification indicated that the dominating peptides in these fractions had 2, 3 and 4 carboxyl groups (including the carboxyl group of the C-terminal amino acid). Altogether this suggested that the active fractions contained the same peptide with one, two or three deamidated glutamine residues and that deamidation of at least one glutamine was crucial for acquisition of T cell stimulatory capacity.

Partial sequence of the peptide was obtained by ESI MS/MS (Hunt DF et al., 1986) and by C-terminal sequencing using carboxypeptidase Y followed by MALDI-TOF analysis. The information on peptide mass, fragment masses and partial sequence matched the sequence of a γ-type gliadin (Sugiyama T et al., 1986) in the SWISSPROT database (amino acids 134-153 of
GDB2_WHEAT; accession no. P08453) when accounting for modification of an N-terminal glutamine to a pyroglutamic acid and deamidation of 1, 2 or 3 of the other glutamines. The sequence was confirmed by testing a synthetic peptide QQLPQQQPQQSQFQQRPF (SEQ ID NO:1). This peptide was barely stimulatory for 4.32 in an untreated form, but became stimulatory after acid/heat treatment. Similar treatment of overlapping and truncated peptides allowed determination of the minimal stimulatory epitope (141-150) (SEQ ID NO:17). A longer peptide (140-150) (SEQ ID NO:13) gave slightly better stimulation (Fig. 2, upper panel).

Testing of MonoQ fractions of acid/heat treated 134-153 peptide (SEQ ID NO:1) revealed activity in the same positions as were found for the γ-36 fragments (Fig. 1b). MALDI-TOF analysis of active fractions demonstrated identical masses with those of the purified γ-36 fragments. Methyl-esterification and MS analysis of active fractions corroborated the varying numbers of glutamic acid residues. ESI MS/MS analysis of methyl-esterified peptides of two T cell stimulatory MonoQ fractions (Nos. 4 and 17) demonstrated partial deamidations in many different positions with some positions being more prone to deamidation. Guided by this MS/MS analysis two synthetic 11-mer peptides were tested; one with a glutamic acid position at position 148 and one with a glutamine at this position (140-150;Py140,E148 [SEQ ID NO:15] and 140-150;Py140 [SEQ ID NO:14]). Both peptides were stimulatory following acid/heat treatment; however, only 140-150;Py140,E148 (SEQ ID NO:15) was stimulatory prior to this treatment (Fig. 2, lower panel) thus demonstrating that deamidation of Q148 was critical for recognition by this T cell clone.
The peptides were next tested for binding to DQ2 in a cell free binding assay (Johansen BH et al., 1994). Peptide 138-152;Py138,E148 (SEQ ID NO:11) bound significantly better than the 138-152;Py138 (SEQ ID NO:10) peptide (IC50 values 6.2 μM vs. 61.4 μM) indicating deamidation to be relevant for binding to DQ2. Alignment of the 141-150 sequence with the DQ2 binding motif (Johansen BH et al., 1996; van de Wal Y et al., 1996; Vartdal F et al., 1996) suggests that the Q148 residue is the P7 residue. At this position negatively charged residues are preferred for DQ2 binding (Johansen BH et al., 1996; van de Wal Y et al., 1996; Vartdal F et al., 1996; Koelle DM et al., 1997).

The immuno-relevance of the 134-153 peptide (SEQ ID NO:1) was analyzed by testing a further 10 polyclonal gut-derived T cell lines derived from seven patients. None of the T cell lines responded positively to untreated peptide whereas T cells from two patients recognized the acid/heat treated peptide. The same T cell lines also responded to the 140-150;Py140,E148 peptide (SEQ ID NO:15) but not to the untreated 140-150;Py140 peptide (SEQ ID NO:14) suggesting the 140-150;Py140,E148 epitope (SEQ ID NO:15) to be a common epitope for intestinal T cells of different CD patients (Table 2). Moreover these data corroborate the previous evidence for the existence of several distinct DQ2-restricted gliadin epitopes (Lundin KEA et al., 1997).
Table 2 ³H thymidine incorporation after treatment with peptides from γ-gliadin

<table>
<thead>
<tr>
<th>T cell line</th>
<th>Crude gliadin</th>
<th>γ-gliadin peptide 135-153 (SEQ ID NO:8)</th>
<th>γ-gliadin peptide 141-150 (SEQ ID NO:17)</th>
<th>γ-gliadin peptide 141-150;E148 (SEQ ID NO:18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>T</td>
<td>NT</td>
<td>T</td>
</tr>
<tr>
<td>370-R</td>
<td>25164</td>
<td>&lt;0</td>
<td>18522</td>
<td>&lt;0</td>
</tr>
<tr>
<td>412-R</td>
<td>11436</td>
<td>866</td>
<td>21448</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

Crude gliadin (3 mg/ml) was incubated with DQ2-expressing B-lymphoblastoid cells for 16-20 hours and peptides [135-153 (SEQ ID NO:8)] at 25 μM and [141-150 (SEQ ID NO:17)] and [141-150;E148 (SEQ ID NO:18)] at 10 μM) for 4 hours before addition of the gut-derived T cell lines. ³H thymidine incorporation was measured 48-72 hours after antigen stimulation and values are given as Δcpm: Proliferation of [T cells + antigen presenting cells + antigen] - [T cells + antigen presenting cells without antigen]. Peptides were tested in non-treated (NT) or acid/heat-treated (T) forms.

To further address the general relevance of deamidation for recognition of gliadin by T cells of the celiac lesion, T cell lines from six patients as well as 25 T cell clones from five patients were tested with chymotrypsin digested gliadin either untreated or acid/heat treated. All T cell lines and most clones displayed weak reactivity against the untreated gliadin, while the reactivity was markedly enhanced by acid/heat-treatment (Fig. 3). Thus deamidation appears to be important for recognition of gliadin by most T cells from the small intestinal mucosa of CD patients.

The deamidation of gliadin could theoretically take place ex vivo (i.e. during the fermentation or during the baking process) or in vivo. Experiments failed to support the former
possibility whereas there has been evidence for the latter. Incubation at 37°C, pH 1.8 for 15 minutes of chymotrypsin digested gliadin created the epitope recognized by 4.32. Similar results were obtained with one T cell line (CD380-R) and one clone (CD387.9-E) that recognize other DQ2-restricted gliadin epitopes. This suggests that sufficient deamidation can occur in the acidic environment of the stomach. The deamidation could also be caused by enzymatic processes. Recently evidence has been obtained that tissue transglutaminase can deamidate gliadins and create the epitope described here (Example 2). Alternatively, enzymes of intestinal bacteria may cause the deamidation (Ogawa M et al., 1978) and such bacteria may constitute a predisposing environmental factor in CD.

An interesting hypothesis for development of CD could be that the intestinal immune system becomes tolerant to non-deamidated gliadin peptides and that a process leading to deamidation of gliadin could contribute to the break of tolerance. In this respect it is interesting that the gastric H+ production is half of the adult level at the age of three months and reaches adult levels only at two years of age (Agunod M et al.). Factors that modulate the degree of deamidation of gliadin later in life could likewise be involved in the breaking of a tolerance to gliadin.

Negatively charged residues are preferred as anchors for binding of peptides to DQ2 in the positions P4, P6 and P7 (Johansen et al., 1996; van de Wal et al., 1996; Vartdal F. et al., 1996). No known motifs of other human class II alleles demonstrate such a clear preference for negatively charged residues at P7 as DQ2 (Rammensee HG et al., 1995). Proteins with a high content of negatively charged amino
acids should hence be particularly likely sources of DQ2-restricted epitopes. Native gliadins have a very low content of negatively charged residues (usually < 2% of the residues). However, gliadins have a very high content of glutamine (usually > 35% of the residues). This could explain the drastic effect of deamidation for creation of DQ2-restricted T cell epitopes, and provides an explanation as to why gliadins are involved in DQ2-restricted T cell recognition. The present results furthermore indicate that recognition of deamidated gliadins is central in the pathogenesis of CD. Thus interference with processes leading to deamidation of gliadin is possibly a target for therapy of CD.

The fact that T cells can recognize epitopes only after their modification has implications for immunology in general and T cell mediated diseases in particular. The methods traditionally employed for the characterization of T cell antigens are unlikely to report modified epitopes. The present observations illustrate that taking the possibility of antigen modification into consideration can lead to novel and important findings.

EXAMPLE 2

TISSUE TRANSGLUTAMINASE SELECTIVELY MODIFIES GLIADIN PEPTIDES

Materials and Methods

Immunohistochemistry

Cryostat sections from healthy controls and untreated CD patients were stained using an indirect three colour immuno-fluorescence technique as described in Halstensen TS et al.,
1992. Images were digitally analyzed using a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) and a Hamatsu C5810 imaging system (Hamatsu, Shizouka, Japan). The following mouse mAbs were used: anti-TGase (TG 100, IgG1; Neomarkers, Fremont CA), anti-DQ (SPV-L3, IgG2a; gift of H. Spits, DNAX Research Institute, Palo Alto, CA) and anti-CD3 (RIV9, IgG3; Sanbio, Am Uden, The Netherlands). Secondary goat anti mouse antibodies (Southern Biotechnology, Birmingham, AL) were biotinylated anti-IgG3, FITC-conjugated anti-IgG2a and cy.3-conjugated anti-IgG1. The biotinylated anti-IgG3 was visualized by an AMCA conjugated streptavidin (Vector, Burlingame, CA).

Preparation of gliadin antigens

Digestion of crude gliadin (Sigma St. Louis, MO; G-3375) with pepsin (Sigma; P-7012) and trypsin (Sigma; T-7418) was done as described in Lundin KE et al., 1993. α-chymotrypsin (Sigma; C-3142) digestion of gliadin from the wheat strain Kadett was performed at 200:1 (w/w) in 0.1 M NH₄HCO₃ with 2 M urea at 37°C for 24h and stopped by incubation in boiling water for 5 min. Synthetic gliadin peptides quality controlled by HPLC (> 80% purity) and MS were purchased from Research Genetics (Huntsville, AL, USA).

Generation of antigen specific T cells

PT-gliadin specific, gut-derived-T cell lines were established from small intestinal biopsies essentially as described in Lundin KE et al., 1993, with the exception that isolation of some T cells from the gliadin-challenged biopsy material was done without enrichment for CD25 expressing cells. Gut-derived T cell lines of three DQ2+CD patients (CD370, 380 and 387) and two DQ8+ patients (CD282 and CD360)
on a gluten free diet and three untreated DQ2+ patients (CD410, 411 and 412) were tested. In addition, DQ2-restricted T cell clones (370.E-14, 380.E-3, E-11 and E-27, 387.R-3, R-12 and R-16, 411.R-1, 412.R-2, R-3 and R-5) (Molberg et al., 1997; Molberg et al., 1998B) made from these T cell lines were used. Generation of gut-derived astrovirus-specific T cell lines (Molberg et al., 1998A and Molberg et al., 1998B), gut-derived DQ8 restricted T cell clones (Lundin KE et al., 1994), PPD-reactive T cell clones (RN4.26 and 5.28 from PBMC) (Lundin et al. unpublished) and P/T-gliadin-specific T cell clones from PBMC (Gjertsen HA et al., 1994) have been described elsewhere.

Generation of a gliadin specific DQ2-restricted T cell transfectant

Vα and Vβ gene segments from the gliadin specific gut-derived T cell clone 4.32 (Lundin et al., 1993) were amplified from genomic DNA and cloned into the T cell receptor expression vectors pTαcass and pTβcass, respectively (Kouskoff V et al., 1995). These vectors allow for functional expression of murine or human variable region T cell receptor spliced to murine constant regions (Madsen, L., Svejgaard, A. and Fugger, L. unpublished observations). Transfection of these two plasmids together with CD4:pBss and a Neo selection marker into the T cell receptor deficient mouse T cell hybridoma BW 58α/β- (Letourner F et al., 1989) allowed for the selection and cloning of a stable transfectant (60.6) expressing human CD4 and chimeric T cell receptor.
T cell assays

T cells (5x10^4) were added to 5x10^4 APC (either HLA matched allogenic Epstein Barr virus transformed B-LCL irradiated 80 Gy or HLA matched allogenic PBMC irradiated 25 Gy) which had been incubated for 16-20h with complex antigen or 4h with peptides in a volume of 100 μl of RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 15% pooled human serum. Assays were performed in 96 well U-bottom plates (Nunc, Roskilde, Denmark) and T cell proliferation was measured as ^3H thymidine incorporation 48-72 hours after antigenic stimulation. Activation of the 60.6 T cell transfectant was measured as IL2 release following an 18 hour incubation of 2.5x10^4 transfectant cells with 5x10^4 DQ2+ B-LCL as APC. The concentration of IL2 was quantified by time-resolved fluorometry, using Delfia reagents (Wallac, Finland) and murine anti-IL2 antibodies (JES6-1A12 and JES6-5H4, Pharmingen, CA, USA). Parallel assays using the original T cell clone and the transfectant confirmed that the chimeric T cell receptor expressed by the transfectant maintained its specificity both in terms of MHC restriction and peptide specificity (data not shown).

Tissue transglutaminase assays

PT-gliadin (1-1000 μg/ml), chymotrypsin-digested gliadin (0.1-1000 μg/ml) or gliadin peptides (0.2-100 μM) were incubated with 100 μg/ml guinea pig TGase (Sigma; T-5398) at 37°C for 2h in PBS with 0.8 mM CaCl_2. This concentration of TGase and CaCl_2 was found to give optimal T cell stimulation and it is comparable to what is described in other systems (Aeschliman D et al., 1994; Larre C et al., 1993). After 2 hours in-
cubation, gliadin/TGase mixtures were incubated with APC and tested in T cell assays as above.

DQ2 peptide binding assay

A competitive peptide binding assay using affinity purified HLA-DQ(α1*0501,β1*02) molecules, a ¹²⁵I-labeled indicator peptide KPLLIAEDVEGEY (SEQ ID NO:22) (Mycobacterium bovis 65kDa Heat shock protein 243-255Y) and various unlabeled gliadin peptides, was performed as described in Johansen BH et al., 1996.

SDS-PAGE of gliadin peptides incubated with TGase

Peptides 134-153 (SEQ ID NO:1), 134-153;E148 (SEQ ID NO:4), 134-153;E140,E148,E150 (SEQ ID NO:7) were synthesized with an extra N-terminal Y (i.e. 133-153, Y133 etc.) and radiolabeled with ¹²⁵I using the chloramine T method as described in Johansen B et al., 1996. TGase was ¹²⁵I-labeled as control. Trace amounts of ¹²⁵I-peptides (20000 cpmp) were mixed with their corresponding unlabeled peptides in various concentrations and incubated with 100 μg/ml unlabeled TGase at 37°C for 2h in PBS with 0.8 mM CaCl₂. The samples were boiled in Laemmli buffer with 2-mercaptoethanol for 5 min and separated by SDS-PAGE (15%-gel).

Ion exchange chromatography

Mono-Q chromatography in the SMART system (Pharmacia, Uppsala, Sweden) was run in 5 mM Tris-HCl buffer purified by treatment with DEAE-Sephadex A50 (buffer A, pH 6.5). The column was eluted by a gradient ending at 0.05 M NaCl in buffer A. Synthetic peptide 134-153 (SEQ ID NO:1) (1 mg/ml) was dis-
solved in buffer A supplemented with 0.8 mM CaCl₂ and incubated with 1 mg/ml TGase.

Mass spectrometry

Fractions collected from ion exchange chromatography of TGase treated peptides were subjected to methyl-esterification (Hunt DF et al., 1986) of acid residues (C-terminus and Glu) followed by MALDI-TOF mass spectrometry (Karas M et al., 1988) to determine the number of Glu residues. ESI (Fenn JB et al., 1989) ion trap (Cooks GL et al., 1991) tandem mass spectrometry (Hunt DF et al., 1986) was used to determine the position of Glu residues. Peptide ions were isolated in the ion trap and fragmented by collisions with He gas creating a series of N- and C-terminal fragments with differing peptide chain lengths. By comparison of the fragmentation patterns of the native peptide with the TGase treated fractions, mass increments of 15 Da could be assigned to methyl-esterified Glu residues in these fragment ions. MALDI-TOF mass spectra were obtained on a Bruker Reflex mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany). ESI tandem mass spectra were recorded on an ESQUIRE ion trap mass spectrometer (Bruker-Franzen Analytik).

Results

Expression of TGase in the small intestinal mucosa

Immunofluorescence staining of small intestinal biopsy specimens from control subjects and untreated CD patients demonstrated expression of TGase mainly in the subepithelial region of the mucosa. The CD patients displayed a higher expression level than control subjects and they also had some
TGase expression in the brush border. Notably, in the untreated CD patients the subepithelial region staining positive for TGase was heavily infiltrated with CD3 positive T cells and large cells expressing DQ molecules (data not shown).

Effect of TGase on gut-derived, gliadin-specific T cell reactivity

A panel of T cell lines and clones (from eight different CD patients) isolated from duodenal biopsies challenged ex vivo with pepsin-trypsin digested gliadin (PT-gliadin, an antigen known to provoke disease in vivo (Frazer AC, 1959) were tested. It was found that addition of TGase to proliferative assays enhanced reactivity and sensitivity to PT-gliadin for all these gut-derived T cells (data not shown). None of the gut-derived T cells were reactive to TGase alone. The effect of TGase was not dependent on type of antigen presenting cells (APC) as results with B lymphoblastoid cells lines (B-LCL) and T cell depleted peripheral blood mononuclear cells (PBMC) both demonstrated enhanced responses (10 fold and 30 fold). Further, the effect of TGase on T cell recognition appeared to be specific for gliadin. The reactivity of two astrovirus-specific gut-derived DR2 restricted T cell lines (one from an untreated CD patient), and two DR3-restricted PBMC-derived T cell clones specific for PPD (Purified Protein Derivative) from Mycobacterium tuberculosis were not sensitive to TGase mediated antigen modification (data not shown).
The TGase effect is restricted to gut-derived, gliadin specific T cells

The most established property of TGase is to catalyze protein crosslinking via the formation of isopeptide bonds between Gln and Lys residues (Folk JE et al., 1983; Aeschlimann D et al., 1994). Gliadins are rich in Gln residues, but typically have very few Lys residues (Wieser H, 1995). Nonetheless, gliadin-gliadin complexes are known to form in the presence of TGase (Larre C et al., 1993). Since particulate antigens are more efficiently taken up by APC (Watts C, 1997) TGase could enhance gut-derived T cell recognition of gliadin by catalyzing the formation of gliadin complexes. However, with the exception of one single DQ2-restricted T cell clone, no increase in PT-gliadin specific proliferation of PBMC derived T cells from six CD-patients (Gjertsen HA et al., 1994) was detected upon addition of TGase (data not shown). This argues that increased uptake of gliadin mediated by TGase cannot account for the observed enhanced proliferation of gut-derived T cells. Furthermore, these data also suggest that gliadin-specific T cells resident in peripheral blood and gut mucosa may recognize distinct epitopes and, most importantly, that TGase could be instrumental in the creation of gliadin epitopes recognized by gut-derived T cells.

The effect of TGase on complex gliadin antigens

The PT-gliadin recognized by gut-derived T cells has been subjected to digestion at low pH, a condition known to facilitate non-enzymatic deamidation of Gln residues (Hamada JS, 1994). Interestingly, TGase can catalyze substrates to react with water to be deamidated when amine acceptors are in deficit (Folk JE, 1983). Indeed, such Gln deamidations have been
demonstrated with gliadin as a substrate (Larre C et al., 1993). The possibility that TGase mediated deamidation of gliadin could be important for recognition by gut-derived T cells was tested by analyzing the effect of TGase on gut-derived T cell recognition of a water-soluble chymotrypsin digested gliadin. Digestion with chymotrypsin was performed at neutral pH, thereby avoiding spontaneous non-enzymatic deamidation. Chymotrypsin-digested gliadin only weakly stimulated gut-derived T cells from six CD patients (four DQ2+ and two DQ8+). However, it could be converted to an efficient antigen by incubating it in acid (pH 1.8) for 1 hour at 98°C. Notably, TGase treatment of the chymotrypsin-digested gliadin generated a more potent antigen for all of the gut-derived T cells than the acid/heat treatment (Figure 4 and data not shown).

The effect of TGase on gliadin peptides

A detailed molecular analysis of the actions of TGase on gliadin focused on a recently characterized gliadin epitope recognized by gut-derived DQ2-restricted T cells from three different CD patients (Example 1). This epitope was originally identified within a 20mer fragment isolated from a purified γ-type gliadin. In its unmodified form this fragment was determined to have the sequence QQLPQPQFPQOFPQQRRPF (SEQ ID NO:1), a sequence that matched the residues 134-153 of the SWISSPROT entry GDB2_WHEAT (accession No. P08453). Acid/heat treatment of this fragment resulted in a complex mixture of peptides with varying numbers of deamidated Gln residues. Of these, the Glu at position 148 was essential for recognition by gut-derived T cells, as without this deamidation the 134-153 peptide (SEQ ID NO:1) was barely stimulatory. The minimal T cell epitope was mapped to residues 141-150 (SEQ ID NO:17).
Gut-derived T cell lines from two patients (CD370 and CD412), as well as a T cell transfectant (60.6) expressing a chimeric T cell receptor containing variable regions cloned from the gliadin peptide-specific gut-derived T cell clone 4.32 recognized the acid/heat treated peptide 134-153 (SEQ ID NO:1). These T cells were used to demonstrate that TGase treatment transformed peptide 134-153 (SEQ ID NO:1) from a non-stimulatory to a potent T cell stimulatory peptide.

Ion exchange chromatography of TGase treated peptide 134-153 (SEQ ID NO:1) resulted in two distinct and dominant peaks (Figure 5A). Fractions collected from the chromatography were aliquoted. One aliquot was tested for recognition by the T cell transfectant 60.6 and revealed that the T cell stimulatory capacity of the peptide was retained in the peak corresponding to fraction 28. Fractions 15 and 28, corresponding to the two peaks, were then subjected to methyl-esterification of acid residues (C-terminus and Glu) followed by Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight (MALDI-TOF) and ElectroSpray Ionization (ESI) ion trap mass spectrometry to determine the number and positions of the deamidated glutamine residues. Both fractions contained one dominating peptide, with two and three deamidations, respectively (Figure 5B). In the non-stimulatory fraction 15, Q140 and Q150 were identified as the deamidated glutamine residues. In the T cell stimulatory fraction 28, both these residues were also deamidated, but in addition a third residue was deamidated, predominantly at Q148.

Incubation of synthetic $^{125}$I-labeled peptides encoding T cell epitopes (i.e. peptide 134-153;E140 [SEQ ID NO:3] and 134-153;E140,E148,E150 [SEQ ID NO:7]) with TGase and subsequent analysis of this material by SDS-PAGE indicated that a sig-
significant fraction of each peptide became covalently coupled to the enzyme (data not shown). This demonstrates that TGase and gliadin peptides may form hapten-carrier like complexes that can be involved in production of TGase autoantibodies (Dieterich W et al., 1997)

DQ2 binding affinity of TGase modified gliadin peptides

The proposed peptide-binding motif for DQ2 predicts a preference for negatively charged residues in relative position 7 (Johansen BH et al., 1996; van de Wal Y et al., 1996). Testing of synthetic peptides in a cell free DQ2 binding assay demonstrated a 10-fold increase in binding affinity of peptides with a E148 substitution (Figure 6A and Example 1), indicating that position 148 could be accommodated into DQ2 pocket 7. Although the binding affinity to DQ2 of acid/heat treated peptide 138-152;Py140,E140,E148,E150 (SEQ ID NO:12) was similar to peptide 138-152;Py138,E148 (SEQ ID NO:11) and peptide 138-152;Py138,E140,E148,E150 (SEQ ID NO:12), the latter was a more potent antigen for the T cell transfectant (Figure 6). This implies that while non-enzymatic deamidation of the peptide generates a complex mixture of deamidated variants, of which only a minority are advantageous for T cell recognition, the enzymatic deamidation by TGase preferentially acts on residues favorable for T cell recognition and binding to DQ2.

Discussion

The data suggest that the repertoire of antigen-specific HLA class II restricted CD4+T cells can be shaped by modified antigens, and further, that organ-specific T cells in an inflammatory disease can recognize epitopes generated by a spe-
cific enzyme activated by the inflammation. This type of phenomena may widely go unreported as the standard use of synthetic peptides for the characterization of T cell epitopes means that the majority of in vivo modified epitopes would escape detection. As the present data indicate that the fine specificity of peripheral blood T cells and organ-specific T cells recognizing the same complex antigen are different, this emphasizes the dangers of sampling PBMC when studying the immunology of other organs.

The spatial relationship, revealed by immunohistochemistry, between abundantly expressed extracellular TGase and DQ-expressing cells just underneath the mucosal epithelium of CD patients suggests that TGase might be involved in processing of gliadin prior to binding to DQ2 molecules. There is an infiltrate of T cells in the same region, and ex vivo studies of gliadin challenged biopsies have demonstrated that some of these T cells are gliadin specific (Halstensen TS et al., 1992). Thus, the subepithelial region appears to be a micro-environment where TGase mediated modification, DQ2 binding and T cell recognition of gliadin might occur.

Detailed analysis of one T cell epitope (within residues 134-153 of a γ-gliadin) recognized by DQ2-restricted gut-derived T cells from three CD patients revealed that TGase mediated an ordered and specific deamidation of Gln residues which was important for DQ2 binding and critical for T cell recognition. The data suggest that peptide 134-153 (SEQ ID NO:1) is not the only gliadin peptide which can be transformed into an efficient T cell epitope through TGase modification. All the gut-derived T cell clones displayed enhanced reactivity to TGase modified gliadin, but none of them recognized TGase treated peptide 134-153 (SEQ ID NO:1). Fur-
thermore, analyses with different purified gliadins have demonstrated that these T cell clones are heterogeneous with respect to fine specificity (Molberg, unpublished observations), inferring that TGase mediated deamidation potentiates several different gliadin epitopes.

It is intriguing that the DQ2 binding epitopes recognized in CD are created by an enzyme which is the main focus of the autoantibody response in this disease (Dieterich W et al., 1997). It has previously been proposed that a prerequisite for production of these disease-associated autoantibodies is that hapten-carrier like complexes of gliadin and TGase are formed in vivo. This will provide (gliadin specific) T cell help to TGase specific B cells (Sollid LM et al., 1997). Indeed, the present data indicate that, in addition to generating gliadin fragments recognized by gut-derived T cells, TGase can also catalyze formation of complexes between gliadin T cell epitopes and TGase. Notably, in this model autoantibody production to TGase does not involve break of T cell tolerance to the abundantly expressed TGase protein.

Introduction of negative charges at defined positions in peptides is likely to affect the repertoire of HLA molecules able to bind them. Both DQ2 and DQ8 have been predicted to preferentially bind peptides with negatively charged residues (Johansen BH et al., 1996; van de Wal Y et al., 1996; Kwok WW et al., 1996). The extreme skewing towards DQ2 and DQ8 restriction of gliadin-specific gut-derived T cells may therefore reflect that TGase deamidation favour selective binding of gliadin to these HLA molecules.

While this report describes the enzymatic deamidation of a foreign antigen, it could be envisaged that self-antigens
could also be substrates for similar types of modifications. Mass spectrometric analysis of peptide fragments eluted from HLA molecules has identified post-translationally modified self proteins (Meadows L et al., 1997). Notably, autoreactive human CD8+T cells specifically recognizing modified peptides have been described in Meadows L et al., 1997; Skipper JC et al., 1996. In a mouse arthritis model, it has also been demonstrated that modified self peptides are recognized by the class II restricted autoreactive T cells (Michaelsson E et al., 1994).

Future work should be aimed at elucidating whether enzymatic modification of antigens is a general mechanism for the immune system to define epitopes or if it is a process invariably associated with the kind of pathological immune activation described in this study.

EXAMPLE 3

MODIFICATION OF GLUTAMINES IN WHEAT GLUTEN

Materials and Methods

Chemically deamidated gluten is available as a food additive from several food companies, as acid hydrolysis of gluten is an accepted method to change the properties of gluten. The Amylum Group (Belgium) thus prepares and sells SWP 050, a soluble wheat protein especially developed for its emulsifying properties, and it is recommended for use in meat, sauces and dressings. Another product of The Amylum Group, SWP 100, is completely soluble above pH 5.5 and has a high emulsifying capacity and an excellent emulsion stability. This product is recommended for coffee creamers, soups, sauce, bread and
meat. Corresponding products can also be obtained from other gluten producers, e.g. Midwest grain products, Inc. Atchinson, KS, USA and Kröner Stärke, Germany.

Enzymatically digested gluten for calf feeding is industrially produced and available from The Amylum Group, Belgium (Solpro) and from Hyproca Dairy, The Netherlands (Novolat 80). The Amylum Group also has a corresponding product, SWP 500, for use as a food additive. Microbial transglutaminase is used in the food industry to induce crosslinks between proteins and thereby give a better texture and improve the baking strength. It is produced as a commercial product (Activa™) for food by Ajinomoto, Japan.

The derivation of glutamine amide groups can be monitored by the analysis of liberated ammonia. Ammonia is analysed by the quantification of the disappearance of NADH after the addition of 2-oxo-glutarate, NADH and glutamate dehydrogenase to the sample. The test is commercially available from Boehringer Mannheim, Germany.

Results and discussion

Treatment of the soluble constituents of Solpro (3.25 mg/ml) with Activa™ (0.5%) for 2 hours in the presence of 20 mM lysine at 50°C resulted in the development of ammonia. Assuming a glutamine content of 30% on a mass basis, it could be calculated that 32% of the glutamines were changed, presumably into the corresponding epsilon lysine peptide. A longer incubation did not increase the substitution grade.

Treatment of suspended ordinary gluten (2.5 mg/ml) with Activa™ (0.5%) for two hours at 50°C in the presence of 20 mM
lysine resulted in the development of ammonia. Assuming a glutamine content of 30% on a mass basis, it could be calculated that 14% of the glutamines were changed, presumably into the corresponding epsilon lysine peptide. A longer incubation did not increase the substitution grade.

It can thus be concluded from these experiments that it is possible to derive a substantial part of the glutamines in gluten. It might be that this substitution grade is sufficient for the preparation of a gluten that is non-toxic to coeliac patients. A further optimisation of this process may allow the preparation of a gluten that is non-toxic to coeliac patients. It should be stressed that it is probably not necessary to change all glutamines to interfere with the pathological immunological reaction.

EXAMPLE 4

CLINICAL STUDY OF THE EFFECT OF OMEPRAZOL

Materials and Methods

The studies comprise a prospective, open, and uncontrolled pilot series of 6-12 patients with celiac disease in remission as well as individual patients with so-called refractory celiac disease. The patients are treated with omeprazol (40 mg x 2 daily) with gluten provocation, and the total study period for each patient is at the most 16 weeks. The results are evaluated at the earliest when 6 patients have entered and at the latest when 12 patients have entered. If the studies indicate a positive effect of omeprazol, subsequent prospective, randomized, controlled and blinded studies are planned (specific protocol). The results are collected from
two clinical, gastroenterologic centres (the University Hospitals in Copenhagen Rigshospitalet and Hvidovre Hospital), 3-6 patients from either centre. The study is expected to begin on 1 May 1999 and will last 18 months.

Criteria for inclusion

6-12 patients having an earlier diagnosed celiac disease (cryptohypertrrophic villus atrophy with clinical remission/normal villus architecture after gluten-free diet). Some patients having refractory celiac disease (villus atrophy without any other known cause despite treatment with an adequate gluten-free diet) may also be included.

Criteria for exclusion

Age < 18 years, newly diagnosed celiac disease, other chronic gastro-intestinal or liver diseases, diabetes mellitus, epilepsy, current prednisolone treatment, immunosuppressive/ cytostatic treatment, pregnant (fertile women must use safe contraceptive agents (P pills, IUD), and lactating, lack of cooperability, need for assistance from an interpreter, ongoing treatment with proton pump inhibitors or H2-blockers, known hypersensitivity or intolerance to omeprazol, anticoagulant treatment with vitamin K antagonist or a heparin composition.

Monitoring of the patients as well as examinations and time schedules will be apparent from the table below.

T = 0: Patients in remission. Start with one tablet of omeprazol 40 mg x 2 (Losec®, Astra) for 3 days. On the 4th day, increasing gluten exposition is started, beginning with 1/2
slice of bread a day and doubling the amount of bread every 3rd day up to a total of 4 slices of white bread (Trier, 1998).

5 Refractory celiac disease:
The patients are started on a Losec® treatment (no provocation).

At normal biopsy after 8 weeks:
10 Losec® is discontinued, and the protocol is followed unchanged for another 8 weeks.

Exit from the study:

15 1. Clinical recurrence: Increasing symptoms: Moderate (bothering) to severe (preventing daily tasks) discomfort (stomach pain, diarrhoea, discomforting gases, other gastro-intestinal problems, unspecific complaints) ascribable to the gluten provocation.

20 2. Weight loss of more than 5% of the initial value.

3. The hemoglobin concentration below 6.0 mmol/litre for women (ref.: 7.1-9.9) and below 7.0 mmol/litre for men (ref.: 8.1-10.9).

4. The serum albumin concentration below 500 mg/litre (ref.: 540-800).

30 5. Significant villus atrophy at 8 weeks.

The project medicine (Losec® tablets, Astra A/S) is labelled with the administration procedure, name, address and tele-
phone number of the main investigator and Astra A/S. Counting of the remainder of the medicine is carried out at each medical control.

5 Events and side effects as well as serious events and serious side effects are reported in accordance with the guidelines of the Danish Medicines Agency.

Suspected side effects of the drug are recorded, and moderate to severe, optionally serious symptoms with a possible or probable causal connection with the project medicine will result in exit from the study.

In all cases, a renewed biopsy of the small intestine is aimed at when ending the study prior to 8 weeks.

In patients with refractory celiac disease, the omeprazol treatment is continued if there is a beginning histological remission after 8 weeks, and a renewed biopsy is taken after 26 weeks.
<table>
<thead>
<tr>
<th>Time (weeks) Exam.</th>
<th>0</th>
<th>2wk</th>
<th>3wk</th>
<th>4wk</th>
<th>5wk</th>
<th>6wk</th>
<th>8wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical control</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Height/weight</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>HLA type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Biopsy (#)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Blood screen (*)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Serology (**)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Symptom monit. (***)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

#: 4-6 routine biopsies with a forceps from the 2nd or 3rd piece of duodenum in connection with gastroscopy.

*: CRP, Leuc.+diff., Hgb, MCV,
Fe**, Ca**, Mg**, Zn**, b-folate, se-B12,
K+, Na+, creatinin, carbamide,
BS, TSH (only 0), PTH (0, 4wk, 12wk).
10 alat, bas.phosph.,bilirubin, PP, albumin,

**: Antibodies to endomysium, gliadin antibodies (Statens Seruminsttitut) as well as IgA (only T=0).
***: "Diary" 2 days prior to control (defaecation: frequency and consistency, discomforting gases, stomach pain, general symptoms (protocol)).

5 Analysis of data:

Primary endpoint is the histology of the small intestine. All biopsies are graded histologically as follows (Smecuol et al., 1997):

10 Type I: Normal mucosa.

Type II: Shortened and broadened villi with a decreased crypt/villus ratio compared to type I, and increased cellularity of lamina propria.

Type III: Very short, almost eradicated villi with an inversely crypt/villus ration, cubic or pseudostratified epithelium, long twisted crypts, as well as tight mononuclear infiltration of lamina propria.

Type IV: Total villus atrophy.

All biopsies are treated routinely and are examined and evaluated blinded by a trained pathologist (EH).

Secondary endpoints are:

Blood test values: Hgb concentration, Se-iron, Se-albumin

30 Serological results: Increase in gliadin and antibody titer against endomysium

Symptom development: Change in the total score.
Symptoms are assessed at a total score two days before beginning the study, and prior to each medical control (protocol). Statistic evaluations are made non-parametrically.

Ethical considerations:

Patients are included after informed written and oral information and consent.

When there is no effect of the treatment with the proton pump inhibitor, a state of malabsorption lasting a few weeks is caused to the patient; this may give temporary discomfort which will disappear when the gluten-free diet therapy is started. The malabsorption may cause a modest, temporary state of deficiency with respect to certain nutrients. With the selected time schedule and the selected exit criteria, such a temporary state of deficiency will not result in lasting discomfort or other late consequences. The study makes it possible to investigate a novel therapeutic concept which, if it works, will improve the daily life of all patients with celiac disease.

The planned extra biopsies of the small intestine are deemed to be harmless since complications (haemorrhage, perforation) are extremely rare in connection with this (routine) procedure.

Thus, the project is carried out in accordance with the Helsinki Declaration II.
REFERENCES


Frazer, AC. Gluten-induced enteropathy, the effect of partially digested gluten. Lancet 1959:252-255.


immobilized chymotrypsin at alkaline pH. J. Food Science 5:1345-1347.


Molberg, Ø et al. CD4+ T cells with specific reactivity against astrovirus isolated from normal human small intestine. Gastroenterology. 1998A; 114:115-122.


CLAIMS

1. A method of treating celiac disease comprising interfering with the deamination of at least one glutamine residue in a gliadin or glutenin molecule.

2. A method according to claim 1 comprising prohibiting deamination of at least one glutamine residue by derivation of at least one glutamine residue in a gliadin or glutenin molecule in wheat flour.

3. A method according to claim 1 or 2 comprising prohibiting or interfering with the deamination by at least one of the following methods:
   (a) chemical deamination of gluten followed by chemical derivation of the generated carboxyl group(s);
   (b) chemical deamination of gluten followed by enzymatic derivation of the generated carboxyl group(s);
   (c) enzymatic deamination of gluten followed by chemical derivation of the generated carboxyl group(s);
   (d) enzymatic deamination of gluten followed by enzymatic derivation of the generated carboxyl group(s).

4. A method according to claim 3 wherein gluten is deamidated by acid treatment and the generated carboxyl group(s) chemically coupled, e.g. with lysine, a lysine peptide or an amine selected from the group consisting of methylamine, ethylamine, putrescine, spermine and spermidine.
5. A method according to claim 3 wherein gluten is deamidated by acid treatment and the generated carboxyl group(s) enzymatically modified e.g. with a γ-glutamylmethylamidase synthetase (EC 6.3.4.12)

6. A method according to claim 3 wherein gluten is deamidated by enzymatic treatment with e.g. transglutaminase or chymotrypsin and the generated carboxyl group(s) chemically coupled e.g. with lysine, a lysine peptide or an amine selected from the group consisting of methylamine, ethylamine, putrescine, spermine and spermidine.

7. A method according to claim 3 wherein gluten is deamidated by enzymatic treatment with e.g. chymotrypsin and the generated carboxyl group(s) enzymatically modified e.g. with a γ-glutamylmethylamidase synthetase (EC 6.3.4.12)

8. A method according to claim 2 wherein at least one glutamine residue of gluten is directly modified using transglutaminase e.g. in the presence of lysine, a lysine peptide or an amine selected from the group consisting of methylamine, ethylamine, putrescine, spermine and spermidine.

9. A method of interfering with the deamidation of at least one glutamine residue in a gliadin or glutenin molecule and thereby treating celiac disease, the method comprising administering, to a patient having or suspected of having celiac disease, at least one of the following substances:

(a) a substance which is capable of increasing the pH in the gastroduodenal tract of a subject,
(b) a substance which is capable of eliminating deamidating bacteria in the gastroduodenal tract of a subject, and/or

(c) a substance which is capable of interfering with the effect of at least one deamidating enzyme in the gastroduodenal tract of a subject.

10. A method according to claim 9 wherein the substance in (a) is selected from the group consisting of antacida, such as aluminium oxide hydrate, magnesium carbonate, magnesium hydroxide, magnesium oxide, dihydroaluminium sodium carbonate, magnesium aluminium silicate, aluminium aminoacetate, calcium carbonate and combinations thereof.

11. A method according to claim 9 wherein the substance in (a) is capable of inhibiting or reducing the secretion of gastric acid from parietal cells.

12. A method according to claim 11 wherein the substance is selected from the group consisting of anticholinergic agents such as butylscopolamine and propantheline, H₂-receptor antagonists such as ranitidine, cimetidine, famotidine and nizatidine, and proton pump inhibitors such as omeprazol, lansoprazol and pantoprazol.

13. A method according to claim 9 wherein the substance in (b) is an antibiotic or antimicrobial agent.

14. A method of modifying at least one glutamine residue in a gliadin or glutenin molecule and thereby prohibiting the exposure of the deamidated gliadin or glutenin to the immune system of a patient having or suspected of having celiac disease.
15. Use of a substance for the preparation of a composition for interfering with the deamidation of at least one glutamine residue in a gliadin or glutenin molecule.

16. Use according to claim 15 wherein the substance is capable of increasing the pH in the gastroduodenal tract of a subject.

17. Use according to claim 16 wherein the substance is selected from the group consisting of antacida, such as aluminiumoxide hydrate, magnesium carbonate, magnesium hydroxide, magnesium oxide, dihydroaluminium sodium carbonate, magnesium aluminium silicate, aluminium aminoacetate, calcium carbonate and combinations thereof.

18. Use according to claim 16 wherein the substance is capable of inhibiting or reducing the secretion of gastric acid from parietal cells.

19. Use according to claim 18 wherein the substance is selected from the group consisting of anticholinergic agent such as butylscopolamine and propantheline, H₂-receptor antagonists such as ranitidine, cimetidine, famotidine and nizatidine, and proton pump inhibitors such as omeprazol, lansoprazol and pantoprazol.

20. Use according to claim 15 wherein the substance is capable of eliminating deamidating bacteria in the gastroduodenal tract of a subject.

21. Use according to claim 20 wherein the substance is an antibiotic or antimicrobial agent.
22. Use according to claim 15 wherein the substance is capa-
ble of interfering with the effect of at least one deamidat-
ing enzyme in the gastroduodenal tract of a subject.
Fig. 2
<table>
<thead>
<tr>
<th>T</th>
<th>APC</th>
<th>Gliadin</th>
<th>Acid/Heat</th>
<th>TCL 380-R</th>
<th>TCL 410-R</th>
<th>TCL 411-R</th>
<th>TCL 412-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Graph showing results](chart)

**Fig. 3**
Fig. 4
Fig. 5A

Fig. 5B
Fig. 6A

- P_Y-PQQPQQSFQSPAQPQPQRP  (SEQ ID NO:10)
- P_Y-PQQPQQSFQSPAQPQPQRP  (SEQ ID NO:11)
- P_Y-PQQPQQSFQSPAQPQPQRP  (SEQ ID NO:12)

Fig. 6B

- Unmodified  P_Y-PQQPQQSFQSPAQPQPQRP  (SEQ ID NO:12)
- Acid/heat-treated P_Y-PQQPQQSFQSPAQPQPQRP  (SEQ ID NO:12)
SEQUENCE LISTING

Københavns Universitet
Treatment of celiac disease

20718PC1
22
FastSEQ for Windows Version 3.0
1
20
PRT
Artificial Sequence

<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln</td>
<td>Gln</td>
<td>Leu</td>
<td>Pro</td>
</tr>
<tr>
<td>Gln</td>
<td>Arg</td>
<td>Pro</td>
<td>Phe</td>
</tr>
</tbody>
</table>

VARIANT
(1)...(1)
pyroglutamic acid

<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln</td>
<td>Gln</td>
<td>Leu</td>
<td>Pro</td>
</tr>
<tr>
<td>Gln</td>
<td>Arg</td>
<td>Pro</td>
<td>Phe</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln</td>
<td>Gln</td>
<td>Leu</td>
<td>Pro</td>
</tr>
<tr>
<td>Gln</td>
<td>Arg</td>
<td>Pro</td>
<td>Phe</td>
</tr>
</tbody>
</table>

| 4 | 20 | PRT |
|    |    |     |

| 2 | 20 | PRT |
|    |    |     |

| 3 | 20 | PRT |
|    |    |     |
| <400> | 4 |
|-------|
| Gln | Gln | Leu | Pro | Gln | Pro | Gln | Gln | Ser | Phe | Pro | Glu | Gln |
| 1    | 5   | 10  | 15  |
| Gln  | Arg | Pro | Phe |
| 20   |
| <210> | 5 |
| <211> 20 |
| <212> PRT |
| <213> Artificial Sequence |

| <400> | 5 |
|-------|
| Gln | Gln | Leu | Pro | Gln | Pro | Gln | Gln | Ser | Phe | Pro | Glu | Gln |
| 1    | 5   | 10  | 15  |
| Glu  | Arg | Pro | Phe |
| 20   |
| <210> | 6 |
| <211> 20 |
| <212> PRT |
| <213> Artificial Sequence |

| <400> | 6 |
|-------|
| Gln | Gln | Leu | Pro | Gln | Pro | Gln | Gln | Ser | Phe | Pro | Glu | Gln |
| 1    | 5   | 10  | 15  |
| Glu  | Arg | Pro | Phe |
| 20   |
| <210> | 7 |
| <211> 20 |
| <212> PRT |
| <213> Artificial Sequence |

| <400> | 7 |
|-------|
| Gln | Gln | Leu | Pro | Gln | Pro | Gln | Gln | Ser | Phe | Pro | Glu | Gln |
| 1    | 5   | 10  | 15  |
| Glu  | Arg | Pro | Phe |
| 20   |
| <210> | 8 |
| <211> 19 |
| <212> PRT |
| <213> Artificial Sequence |

| <400> | 8 |
|-------|
| Gln | Leu | Pro | Gln | Pro | Gln | Gln | Pro | Gln | Gln | Ser | Phe | Pro | Glu | Gln |
| 1    | 5   | 10  | 15  |
| Arg  | Pro | Phe |
|      |
| <210> | 9 |
| <211> 18 |
| <212> PRT |
| <213> Artificial Sequence |

| <400> | 9 |
|-------|
| Pro | Gln | Pro | Gln | Gln | Pro | Gln | Gln | Ser | Phe | Pro | Gln | Gln | Gln | Arg | Pro |
| 1    | 5   | 10  | 15  |
| Phe  | Ile |
<210> 10
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(1)
<223> pyroglutamic acid

400> 10
Gln Pro Gln Gln Gln Ser Phe Pro Gln Gln Gln Arg Pro
1  5  10  15

<210> 11
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(1)
<223> pyroglutamic acid

400> 11
Gln Pro Gln Gln Gln Gln Ser Phe Pro Glu Gln Gln Arg Pro
1  5  10  15

<210> 12
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(1)
<223> pyroglutamic acid

400> 12
Gln Pro Glu Gln Pro Gln Gln Ser Phe Pro Glu Gln Gln Glu Arg Pro
1  5  10  15

<210> 13
<211> 11
<212> PRT
<213> Artificial Sequence

<400> 13
Gln Gln Pro Gln Gln Ser Phe Pro Gln Gln Gln Gln
1  5  10

<210> 14
<211> 11
<212> PRT
<213> Artificial Sequence
<220>
<221> VARIANT
<222> (1)...(1)
<223> pyroglutamic acid

<400> 14
Gln Gln Pro Gln Gln Ser Phe Pro Gln Gln Gln Gln
 1 5 10

<210> 15
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<221> VARIANT
<222> (1)...(1)
<223> pyroglutamic acid

<400> 15
Gln Gln Pro Gln Gln Ser Phe Pro Glu Gln Gln Gln
 1 5 10

<210> 16
<211> 10
<212> PRT
<213> Artificial Sequence

<400> 16
Gln Gln Pro Gln Gln Ser Phe Pro Gln Gln Gln
 1 5 10

<210> 17
<211> 10
<212> PRT
<213> Artificial Sequence

<400> 17
Gln Pro Gln Gln Ser Phe Pro Gln Gln Gln Gln
 1 5 10

<210> 18
<211> 10
<212> PRT
<213> Artificial Sequence

<400> 18
Gln Pro Gln Gln Ser Phe Pro Glu Gln Gln Gln
 1 5 10

<210> 19
<211> 11
<212> PRT
<213> Artificial Sequence

<400> 19
Gly Gln Pro Gln Gln Ser Phe Pro Gln Gln Gln Gln
 1 5 10
<210> 20
<211> 9
<212> PRT
<213> Artificial Sequence

<400> 20
Pro Gln Gln Ser Phe Pro Gln Gln Gln
1  5

<210> 21
<211> 8
<212> PRT
<213> Artificial Sequence

<400> 21
Gln Gln Ser Phe Pro Gln Gln Gln
1  5

<210> 22
<211> 14
<212> PRT
<213> Artificial Sequence

<400> 22
Lys Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu Tyr
1  5  10