UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

NIKA JAPELJ

VPLIV AMINOKISLINSKIH SUBSTITUCIJ V ALFA-GLIADINSKIH PEPTIDIH NA NJIHOVO IMUNOGENOST PRI CELIAKIJI

EFFECT OF AMINO ACID SUBSTITUTIONS IN ALPHA-GLIADIN PEPTIDES ON THEIR IMMUNOGENICITY IN COELIAC DISEASE

Ljubljana, 2014

Diplomsko nalogo sem opravljala na Rayne Institut-u, Department of Gastroenterology (King's College London) pod mentorstvom prof. dr. Boruta Božiča, mag. farm., spec. med. biokem. in somentorstvom prof. dr. Paul-a J Ciclitira-e, dr. med.

Zahvala

Iskreno se zahvaljujem mentorju, prof. dr. Borutu Božiču, da je usmeril mojo pot v London in mi omogočil začetke raziskovalnega dela tam, pa tudi za vso strokovno pomoč in namige pri pisanju diplomske naloge.

Posebej hvala Tanji za potrpežljivo uvajanje v laboratorijsko delo, ves trud pri popravljanju diplomske naloge, znanje, ki ga je z veseljem delila z mano, se posebej pa za prijateljski odnos in olajšanje mojih začetkov ob prihodu.

Zahvaljujem se sošolcem za medsebojno bodrenje med učenjem in slabih rezultatih, dobro voljo na predavanjih in popestritev mojih študentskih let.

Hvala londonskim prijateljem, ki so mi v zadnjem letu lepšali dneve.

Zahvala tudi Janu za sodbudo in prave odločitve v preteklih letih.

Nenazadnje hvala Karin za pozitivno energijo in čustveno podporo v dobrih in slabih trenutkih ter staršem, ki so mi s finančno in moralno podporo omogočili študij.

Izjavljam, da sem diplomsko nalogo samostojno izdelala pod mentorstvom prof. dr. Boruta Božiča, mag. farm., spec. med. biokem.

Nika Japelj

Content

List of Figures

Abstract

Coeliac disease (CD) is a chronic inflammatory disease that affects small intestine which occurs among genetically predisposed individuals when exposed to gluten proteins in wheat or homologous proteins from barley and rye. It is mediated by gluten specific T-cells which recognize gluten proteins present on the HLA-DQ2 or HLA-DQ8 molecules. Both gluten fractions, gliadins and glutenins, have been shown to be CD toxic. Most immunogenic peptides have been found in α-gliadins. Immunodominant peptides based on the amino acid sequence 57-72 of α-gliadin and their counterparts with amino acid substitutions were assessed for their CD immunogenicity. Small intestinal biopsies from CD patients were obtained and cultured over night involving the organ culture with medium containing peptic-tryptic digested gluten. T-cells were subsequently isolated from the duodenal biopsies, restimulated and tested with selected peptides in T-cell proliferation assays. It was demonstrated that deamidation of immunodominant peptides at position 65 of α-gliadin resulted in increased T-cell stimulation. We identified modified peptide with single point amino acid substitution in the core epitope that resulted in lower immunogenicity of the peptide. Further was assessed that peptide with two amino acid substitutions at the core epitope completely abrogated the T-cell response to the immunodominant peptide. Our results suggest that careful selection of amino acid substitutions affects the peptide binding to either HLA-DQ2 molecules or T-cell receptor (TCR) of gluten-sensitive T-cells. Our results imply that modification of immunodominant epitope within α-gliadin might generate CD non-toxic peptides that could potentially be used in immunotherapy in CD.

Key words: coeliac disease, α-gliadin, T-cell response, immunogenicity of gluten peptides

Povzetek

Celiakija je kronična vnetna bolezen tankega črevesja, ki se pojavi pri genetsko predispoziranih posameznikih, ko so izpostavljeni proteinom glutena iz pšenice ali homolognim proteinom iz ječmena ali rža. Posredujejo jo gluten-specifične celice T, ki prepoznajo proteine glutena predstavljene na HLA-DQ2 ali HLA-DQ8 molekulah. Obe frakciji glutena, gliadini in glutenini, sta dokazano toksični za bolnike s celiakijo. Največ imunogenih peptidov se nahaja v α-gliadinih. Testirali smo imunodominantne peptide z aminokislinsko sekvenco 57-72 znotraj α-gliadina in njihove substituente z aminokislinkimi zamenjavami, z namenom oceniti njihovo imunogenost. Bolnikom celiakije smo odvzeli biopte tankega črevesa in jih kultivirali čez noč v prisotnosti glutena razgrajenega z pepsinom in tripsinom. Pozneje smo izolirali celice T, jih restimulirali in jih testirali z izbranimi peptidi v testih proliferacije celic T. Ugotovili smo, da se z deamidacijo na mestu 65 α-gliadina T celični odziv poveča. Peptid z aminokislinsko substitucijo v epitopu na ustreznem mestu, je povzročil zmanjšanje odziva celic T. Z dvema aminokislinskima substitucijama v epitopu peptida smo imunski odziv celic T popolnoma izničili. Predvidevamo, da smo z aminokislinskimi substitucijami na pravih mestih vplivali na vezavo peptida na HLA-DQ2 molekulo oziroma T-celični receptor. Naši rezultati nakazujejo, da lahko z aminokislinskimi substitucijami znotraj epitopov α-gliadinov ustvarimo peptide, ki niso toksični za paciente s celiakijo in jih morebiti lahko uporabimo v imunoterapiji celiakije.

Ključne besede: celiakija, α-gliadin, T celični odziv, imunogenost peptidov glutena

List of Abbreviations

AGA	Anti-gliadin antibodies
APC	Antigen-presenting cells
APL	Altered peptide ligand
ASM	Autologous serum medium
CD	Coeliac disease
CD4	Cluster of differentiation 4
cpm	Counts per minute
CV	Coefficient of variation
DGP	Anti-deamidated gliadin peptide
HLA	Human leukocyte antigen
IEL	Intraepithelial lymphocyte
EATL	Enteropathy associated T-cell lymphoma
ELISA	Enzyme-linked immunosorbent spot
EMA	Antiendomysial IgA antibodies
DMSO	Dimethyl sulphoxide
GFD	Gluten free diet
^{3}H	Tritiated thymidine
HLA	Human Leukocyte Antigen
HMWG	High molecular weight glutenin
IEL	Intra-epithelial lymphocytes
INF γ	Interferon Gamma
IL	Interleukin
LMWG	Low molecular weight glutenin
MHC	Major histocompatibility complex
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
psi	Pound-force per square inch
PT gluten	Peptic-tryptic digest of industrial gluten
RPM	Revolutions per minute

RPMI	Rosewell Park Memorial Institute
SD	Standard deviation
SI	Stimulation Index
SIL	Small intestinal lymphocytes
TCR	T-cell Receptor
tTg	Tissue transglutaminase
WGO	World Gastroenterology Organization

1 INTRODUCTION

1.1. COELIAC DISEASE IN GENERAL

Coeliac disease (CD) is a chronic inflammatory disease that affects the small intestine which is caused by gluten-sensitive intestinal T-cell, when exposed to gluten proteins in wheat or homologous proteins from barley and rye. The condition occurs in genetically predisposed individuals, carrying the genes encoding HLA-DQ2 or HLA-DQ8 molecules. Other names for the condition inclute: sprue, coeliac sprue, gluten-sensitive enteropathy and gluten intolerance. The prevalence in Europe is between 0.5 and 2% of the population; it is one of the most common inflammatory disorders of small intestine. There is currently no cure for CD. The only available treatment is a strict gluten-free diet which affects patients' quality of life (1).

CD patients suffer malnutrition and malabsorption. Symptoms differ between children and adults. In children it manifests as diarrhoea, abdominal distension, muscle wasting and failure to thrive, whereas in adults besides diarrhoea, it presents also with anemia, osteoporosis, neurological or psychological symptoms. Long-term untreated disease increases the risk of malignancy and mortality. In a few cases, even after more than 12 months of a strict gluten-free diet, these individuals continue to have an abnormal small intestinal mucosa with all the symptoms typical for CD. This is called refractory CD. In type I, intraepithelial lymphocytes (IEL) with normal phenotype are found, whereas in type II, IEL with monoclonal phenotype have been identified. These later group of patients have a high risk of developing enteropathy associated T-cell lymphoma (EATL) with a 50% year mortality (1).

1.2. PATHOGENESIS OF COELIAC DISEASE

1.2.1. Adaptive immune system

APC molecules do not normally bind native gluten peptides on their surface; deamidation is needed. The enzyme which catalyzes deamidation is tissue transglutaminase (tTg), also called transglutaminase 2. Normally it is present within of cells in an enzymatically inactive form. If tolerance to native peptides is lost or there is cause tissue damage, tTg is released. When the pH is low (pH<7, that may occur with inflammation) or no primary lysine residues are available, the enzyme catalyzes deamidation of proteins with glutamine residues to become negatively charged glutamic acid residues (Figure 1). High calcium concentrations are necessary for enzyme activity. Deamidated peptides are then presented by HLA-DQ2 or HLA-DQ8 to T cells in the intestine. Activation of T cells leads to IFN-γ production and consequently higher HLA-DQ2/8 expression and this results in higher presentation of gluten peptides to gluten-sensitive T-cells. If gluten is further presented in the gastrointestinal tract, this becomes a self-amplifying loop that causes further tissue damage. This is a start for second self-amplifying loop: higher release of tTg and therefore increased peptides presentation to the T-cells. The final result is a strongly enhanced T cells response, with more IFN-γ induced tissue damage and increased release of tTg. The immune response is thus highly activated and indeed amplified (2)(3).

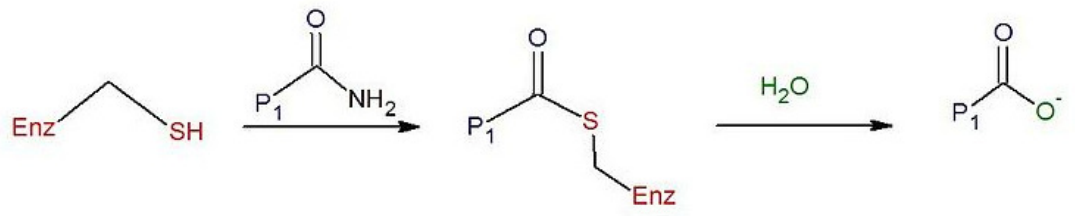

Figure 1: *Reaction of deamidation catalyzed by TG-2 when pH is low*

1.2.2. Innate immune system

Peptide 31-49 from α-gliadins is thought to activate the innate immune system with a mechanism that remains not completely understood. Production of IL-15 by epithelial cells and macrophages is upregulated. IL-15 induces expression of NKG2D, activating innate immune receptor on IELs, and also increased expression of MICA, the epithelial ligand of NKG2D. Binding MICA to NKG2D receptor boosts the cytotoxicity of IEL that in turn induce epithelial damage. This again leads to tTg release and deamidation of gluten peptides and at the end increased CD4+ T-cell activity (2)(3).

1.3. GENETICS OF COELIAC DISEASE

1.3.1. HLA genes

CD is a high heritable disease. The risk of developing the condition in first-degree relatives is up to 20% (4).DQ2 or DQ8 haplotypes of human leukocyte antigen (HLA) are needed to develop CD. They are also found in 25%-40% of non-coeliac European population, it is thus not sufficient condition for diagnosis (5).
HLA molecules are glycoproteins expressed on cell membranes, which have an important role in CD by presentation deamidated gluten peptides to helper T-cells. HLA-DQ2 heterodimer is coded with the alleles DQA1*05, DQB1*02 in cis or trans conformation. Individuals who are homozygous for DQ2.5 develop CD 4-6x more often than those who are DQ2.5 heterozygous. Individuals homozygous for DQ2.5 are also associated with an increased risk of develop RCD II and EATL; any association for HLA-DQ2.5 heterozygousy is less clear. More rarely CD patients possess the HLA-DQ8 molecule (5-12% coeliac patients). HLA-DQ8 is coded with DRB1*04, DQA1*0301, DQB1*0302 alleles. Also for HLA-DQ8 individuals, association with development of RCDII and EATL is unclear (6).

1.3.2. Non HLA genes

The fact that individuals without CD may carry either DQ2 or DQ8 haplotypes indicates that other genes outside of MHC region are also important in disease development. For example genes encoding components for T-cell receptor and molecules involved in T and B-cell activation, have been analysed. The 4q27 region of chromosome was found to be the most significant CD associated non HLA-DQ2/8 region. This area contains IL-2 and IL-21 genes which encode cytokines important for T-cell activation (6)(7). The strong genetic influence in CD is further supported by studies on twins. One study, based on Italian Twin Registries, reported around 70% concordance between monozygotic twins, whereas in dizygotic concordance is just 11%, the same as the risk first-degree relatives have (3).

1.4. DIAGNOSIS OF COELIAC DISEASE

The diagnosis of the condition is based on abnormalities seen on small intestinal biopsies and determining antibodies in patients serum (Figure 2).

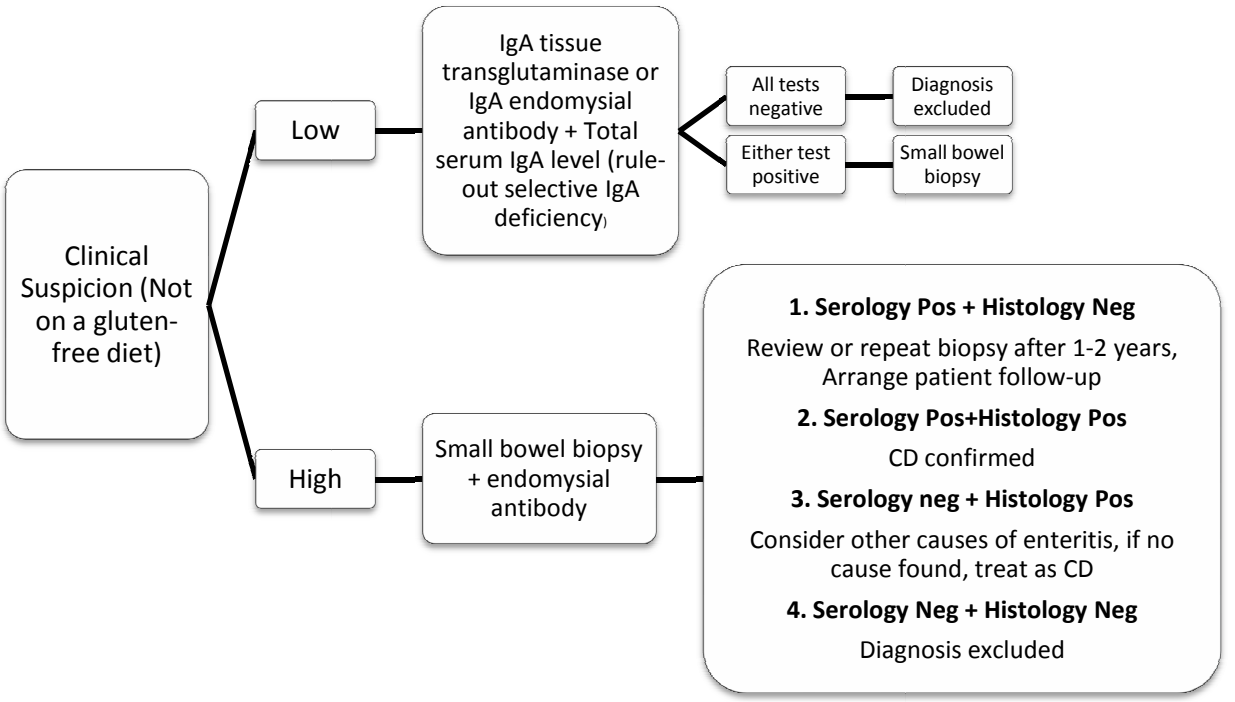

Figure 2: *World Gastroenterology Organization (WGO) Global Guideline for diagnosis of CD*

1.4.1. Serology

We can confirm the diagnosis with serum antibodies in patients where small intestinal biopsies reveal an enteropathy. We can divide serological tests for coeliac disease in two groups:

1.) Autoantibodies:

- **Antiendomysial IgA antibodies (EMA) test**- Antibodies are directed against non-collagenous extracellular matrix proteins made by fibroblasts. These antigens are generated by tissue transglutaminase, which change the certain amin-acid residue by transforming glutamine to glutamic acid. To detect antibodies, an indirect immunofluorescence test is used. The test is

sensitive and the most specific to screen for CD (both 98%). In case there is lack of overall IgA, we have to detect AGA IgG

- **Anti tissuetransglutaminase (tTg) IgA antibody test:** Antibodies are detected by ELISA where human recombinant tTg serves as antigen. It is cheaper and simpler in comparison EMA testing. Test has 97% specificity and 94% sensitivity.

2.) Antibodies targeting the offending agent:

- **Anti-deamidated gliadin peptide (DGP) antibody test:** Antibodies are isotype IgG or IgA. They are detected with sandwich ELISA. Synthetic deamidated gliadin peptides are used as antigen. The sensitivity of test is 89% and specificity 66%, which makes them less clinically relevant (2) (5).

To confirm CD in patients with discovered enteropathy the anti-tTg or EMA test is used. Detecting IgG is helpful when patients have IgA deficiency or for some EMA-negative and tTg-negative patients. Patient has to be on normal diet before test.
To select patients for duodenal biopsy single assays detecting IgA and IgG subtypes of tTg and DGP is suggested. If there is any suspicion of cases, a combination of tests should be use.
For high-risk population, any of these three tests can be used.
DGPs are useful as a diagnostic aid in children under age of 3 years (lower performance of anti-tTg) and in IgA-deficient patient (5).

1.4.2. Intestinal Biopsy

Several biopsies should be taken from second part of duodenum in case of patchy mucosal changes. The typical histological changes comprise:

- Blunted or atrophic villi
- Crypt hyperplasia
- Intraepithelial lymphocyte infiltration (>25/100 cells)
- Epithelial changes
- Mononuclear cell infiltration of the lamina propria

1992 March classified histological mucosal damage in 5 stages: pre-infiltrative, infiltrative, hyperplastic, destructive or hypoplastic lesion. The classification was upgraded by Oberhuber. He divided stage 3 to tree subgroups depending on villous atrophy (1).

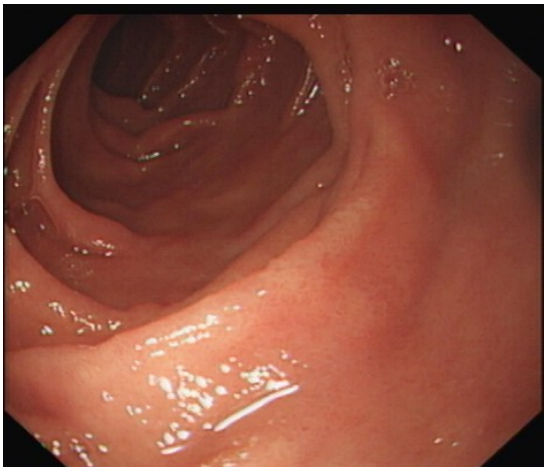

Figure 3: *Picture of small intestine surface took during endoscopy*

1.4.3. Genetic Testing

Because of high- negative predictive value (HLA-DQ2 and HLA-DQ8 are also present in 30%-40% of non-coeliac population in Europe), HLA typing can be used to help to exclude possible patients in equivocal cases. It is also helpful to choose which family member should be screened with serological testing or for the patients which are already on GFD (5).

1.5. CEREAL TOXICITY AND TOXIC GLUTEN PROTEINS

Cereals are part of true grasses family (Gramineae). This family includes twelve subfamilies. Important for CD is subfamily Pooideae. All CD toxic grains fall within Triticeae group within Pooideae subfamily: wheat (Triticum), rye (Secale) and barley (Hordeum). Oats (Avena) belongs to the separate tribe, the Avenae group. The nontoxic cereals (millet, corn, sorghum and rice) evolved separately within the grass family (Triticae) (6) (5).

CD is triggered by gluten in wheat, barley and rye. Gluten is a heterogeneous mixture of proline- and glutamine- rich storage proteins of cereals such as wheat, barley, rye and oats, connected with non-covalent bonds. They are responsible for baking characteristics of flour; when the flour is mixed with water, they form a visco-elastic gluten network. Gluten proteins can be further divided into two subcomponents: prolamins and glutenins. Prolamins have a high content of proline and glutamine; they are soluble in ethanol. They present a significant source of nitrogen, sulphur and carbon for developing plant endosperm. Prolamins are mainly monomeric proteins and contain only intramolecular disulphide bonds. Dependant on the cereals they are termed gliadins from wheat, hordeins from barley, secalins from rye and avenins from oats. We can further divide gliadins according to their decreasing electrophoretic mobility into α, β, γ and ω subfractions or to α, β and ω subfraction according to their N- terminal amino acid sequences. The second group is termed glutenins. They comprise subunits, which are connected with intermolecular disulphide bonds, to form polymers. Glutenins are insoluble in neutral aqueous solution, saline or ethanol. They are further categorised into high molecular weight (HMW) and low molecular weight (LMW) group, the classification of which depends on their relative electrophoretic mobility. The HMW group contains proteins that are important for the baking quality of the flour (elasticity and strength of the dough) (5).

The gluten content of food for individuals with CD is regulated by Codex Alimentarius (CODEX STAN 118-1979). It was revised in 2008 and ratified in January 2009.Codex sets the regulatory standards for the maximum permitted amounts of gluten in food in the European Union*:*

- GLUTEN FREE FOOD: Food or ingredients that are naturally free of gluten, which contain 20 mg/kg of gluten or less. These foods are specially produced, prepared or processed for people on a gluten-free diet or for normal consumption (1).

-VERY LOW GLUTEN: Food which contains from 21 to 100 mg/kg of gluten. This include foods containing ingredients made from wheat, barley, rye or oats that have been specially processed to remove gluten (8).

Assessing protein toxicity is mainly directed against wheat gluten, while barley and rye because of their similarities with gluten proteins have not been involved in many toxicity

testing. Recent studies confirm the possible toxicity of oats in the certain CD suffers (around 5%). Two avenin peptides were described as T-cells epitopes in few patients. Immunotoxicity was still showed lower than in wheat, barley and rye what is probably due to lower content of gluten in oats and lower proline content of avenins (9).

Both groups of storage proteins (gliadins and glutenins) are CD toxic. Toxicity of glutenin fraction was in previous studies difficult to evaluate since they are difficult to extract and purify. It has been demonstrated that *in vitro* HMW-GS trigger T-cells response in a low percentage of patients while *in vivo* it is highly disease activating. It suggests that HMW-GS might be involved in an innate immune mechanism at the early stage of the disease (10).

In gliadins toxic peptides were found in the α, γ, ω subfractions. The most CD toxic are thought to be α-gliadins. They harbour peptides which can stimulate adaptive and innate immune response. It is thought that the only innate immune system stimulating peptide is α-gliadin peptide corresponding to the sequence p31-49. A peptide which is often tested in toxicity studies is 33mer peptide (amino acid sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF). It was identified as a primary activator of T-cells. It is highly resistant to degradation in GIT and encodes six T cell epitopes which partially overlap one another (11).

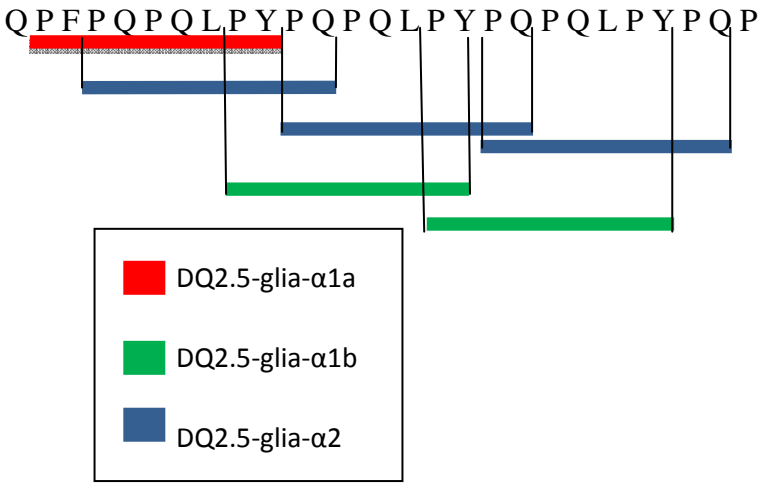

Figure 4: *Illustration of CD toxic 33mer peptide and T-cell epitopes it harbour (length of the peptides is not equivalent to the real one)*

A harmonized nomenclature of epitopes has been suggested (12). Further on, these epitope names are used. There were 31 epitopes discovered to be CD toxic. They are found in more than 50 different glutenins and gliadins peptides, encoded by different allels. The recently described Sollid nomenclature involves epitopes being divided into DQ2.5, DQ2.2, DQ8 and DQ8.5 restricted epitopes.

Table I: *The list of DQ2.5 restricted gliadin epitopes recognized by T-cells of CD patients*

Epitope	***Previous names (P1-P9)***	***Peptide-binding register***
DQ2.5-glia-α1a	DQ2-α-I, α9	P F P Q P **E** L P Y
DQ2.5-glia-α1b	DQ2-α-III	P Y P Q P **E** L P Y
DQ2.5-glia-α2	DQ2-α-II, α2	P Q P **E** L P Y P Q
DQ2.5-glia-α3	glia-α20	F R P **E** Q P Y P Q
DQ2.5-glia-γ1	DQ2-γ-I	P Q Q S F P **E** Q Q
DQ2.5-glia-γ2	DQ2-γ-II, g30	I Q P **E** Q P A Q L
DQ2.5-glia-γ3	DQ2-γ-III	Q Q P **E** Q P Y P Q
DQ2.5-glia-γ4a	DQ2-γ-IV	S Q P **E** Q **E** F P Q
DQ2.5-glia-γ4b	DQ2-γ-VIIc	P Q P **E** Q **E** F P Q
DQ2.5-glia-γ4c	DQ2-γ-VIIa	Q Q P **E** Q P F P Q
DQ2.5-glia-γ4d	DQ2-γ-VIIb	P Q P **E** Q P F C Q
DQ2.5-glia-γ5	DQ2-γ-VI	Q Q P F P **E** Q P Q
DQ2.5-glia-ω1	DQ2-ω-I	P F P Q P **E** Q P F
DQ2.5-glia-ω2	DQ2-ω-II	P Q P **E** Q P F P W

1.6. PEPTIDE BINDING

1.6.1. HLA-DQ2 and peptide binding

MHC class II molecules are glycoproteins located on the surface of cell membranes of an antigen presenting cells; they present protein fragments to CD4+ T-cells. They comprise an α and β chain, composed of extracellular (α_1, α_2, β_1, β_2), transmembrane and cytoplasmic domains. Alpha1 and β_1 form the groove for binding peptide (the cleft between two antiparallel α-helices). The peptide binding side is open at the end, it thus allows peptides

different length to bind to the groove. Two different mechanisms are important for binding peptides with MHC class II:

A. Hydrogen bonds between residues of MHC and carbonyl oxygen and amide nitrogen of the peptide residues
B. Pockets are formed by extensive polymorphic residues of the MHC. They can catch amino acid side chains of peptides at the P1, P4, P6, P7 and P9 positions and are responsible for more specific peptide binding for each molecule.

The majority of CD patients have genes for HLA-DQ2 (human specific MHC). X-ray crystal structure of HLA-DQ2 complex with gluten peptide permits characterisation of the interactions between these molecules and gluten peptides that were recently identified. There are seems to be hydrogen bond at positions 2, 4, 6 and 9. The most important are the two at position 2 and 4. In contrast with other MHC class II molecules, at HLA-DQ2 has no hydrogen bond at position 1. A proline residue at this position is not able to form hydrogen bonds.

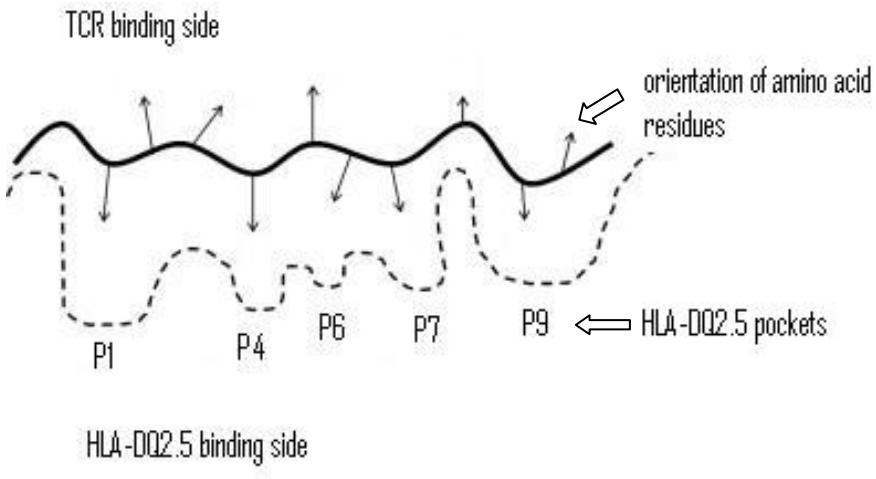

Figure 5: *Schematic presentation of peptide binding to the HLA-DQ2.5 groove. Five positions interact with that groove, others bind to TCR on T-cells.*

1.6.2. Peptide and TCR binding

CD4+ T-cells express a TCR receptor which binds to the MHC class II-peptide complex. It can consist of an α- and β- chain or an γ- and δ- chain. The function of the latter is not fully understood. All the chains have three domains: one extracellular Ig-like, another a

transmembrane and the third a cytoplasmic tail. Peptides form polyproline type II helical conformation while they are bound with the variable Ig domain of TCR. With only few peptide side chains they are bound to TCR whereas other chains bind to MHC class II molecule. Two signals are needed for full T cell activation. The first is an TCR-peptide-MHC interaction. Interaction between co-stimulatory molecules expressed on the surface of T-cells and their counterparts on APC present the second signal. The second signal can enhance or modulate the first signal. It is especially important at the initial stage of the immune reaction when T-cells are not fully activated. The peptides can cause full T-cell stimulation, blocking of the stimulation or induction of limited T-cells responses (13).

1.7. METHODES FOR ASSESSING THE TOXICITY OF GLUTEN FRACTIONS AND PEPTIDES

There are a variety of different *in vitro* methods to investigate the pathogenesis, including small-intestinal mucosal biopsy organ culture and culture of T cell lines. But still no animal model exist which would be appreciated specially for initial testing of new possible candidate drug for treatment of the condition (14).

1.7.1.In Vitro Methods

The most reliable in vitro model is the organ culture of small intestinal tissue obtained from CD patients. The first method for culturing human mucosal biopsies using *in vitro* organ culture system was described in 1969. Tissue samples are taken from the small intestine and incubated with culture medium under special conditions (95% O_2-5% CO_2) at 37°C. Biopsies can be kept viable *in vitro* for up to 24 hours with this method (14).
The organ culture method was subsequently used evaluating peptide toxicity in CD. Biopsies are incubated with gluten, gluten fractions and peptides. Histological changes of small intestine after incubation are evaluated: there is a decrease of surface cell height of villous enterocyte, decreases in the villus height: crypt depth ratio, increases in the intraepithelial lymphocyte count...In comparison with T cell studies, duodenal biopsies contain many different cell types, that provides numerous options for study, e.g.

contribution of different cytokines. IL-15 was discovered to play an important role in disease mechanism. With this method immunological effects of gluten challenge have been defined: T-cells in lamina propria of treated coeliac patients do not express CD25. When they are exposed to gluten challenge, this expression is induced. This was the important observation for isolation of gluten specific T-cells from small intestinal biopsies (15)(16).

CD4+ T-cells play the most important role in adaptive immune response in the disease pathology. The first isolation of gluten-specific T-cells from small intestinal biopsy specimens was reported in 1993 for measure immunogenic effects of proteins and peptides in CD. To isolate T-cells, small intestinal biopsies are first incubated in the presence of CD toxic antigen and then disrupted to release T-cells. To cultivate T-cells *in vitro*, repeateded antigen restimulation is required. IL-2 receptor CD25 increases in number after activation with antigen. If later IL-2 is added as stimulant, only growth of antigen-specific lines is increased (T-cells with CD25). Irradiated peripheral blood mononuclear cells (PBMCs) are used as "feeder" cells for T-cell support and as antigen presenting cells (APC). Proliferation of T-cells in culture when stimulated with antigen can be determined by measuring interferon (INF-γ) or interleukin (IL-4) production or with a T-cell proliferation assay. In the latter test T-cells are incubated with antigen, APCs and tritiated thymidine. The thymidine is incorporated into the nuclei of dividing cells and measured by scintillation counter (16) (17).

1.7.2.In Vivo methods

Small intestinal *in vivo* gluten challenge was previously the only method for disease diagnosis. Nowadays it is used for purposes of research (short-term challenge) or long-term if there is a doubt about diagnosis or for determines suitability of individual food for CD patients. In long-term challenge patients are eating gluten containing food for two to six weeks follows by taking small-intestinal and at the end biopsies are taken to assess histological changes. In short-term challenge gluten fraction or peptides are applied directly to the small bowel by infusion cannula attached to a Quinton multiple biopsy capsule. Everything is done under X-ray control. Later biopsies from duodenum are taken in time intervals to check time- and dose- dependent histological changes: villus height to

crypt depth, enterocyte cell height and intra-epithelial cell counts. Method enables to use relatively small amounts of gluten equivalent (15). In the previous study it was shown that *in vivo* challenge with HMW-GSD gives different results than T-cell study with the same peptides (10). Method is invasive and time consuming but still remains as a golden standard for assessing CD toxicity (10).

Thus *in vivo* challenge of the small intestine is invasive and time consuming, other methods have been utilised. In the method where gluten peptides were directly infused to the rectum, it was shown that the number of intra-epithelial lymphocytes increases. Later oral gluten challenge has been used as a potential method. Peptides were injected into the buccal mucosa from treated coeliac patients. The increase in CD4+ T-cells in response to peptides was seen. This method is less invasive and could be more acceptable technique for *in vivo* studies.

2. PURPOSE OF THESIS

Alpha-gliadins have been reported to be the most immunogenic peptides within wheat gluten. They harbour several major CD triggering epitopes. The elimination of these epitopes from α-gliadins would be an important step for CD toxicity elimination.
The purpose of our study is to test gliadin peptides that potentially lack coeliac toxicity. Candidate α2-gliadin peptides (corresponding to residues 57-72), which contained one or more amino acid substitutions within the T-cell epitopes, were tested in T-cell assays to evaluate their toxicity for patients with CD.

The study intends to test the hypotheses:

I. Deamidation of the amino acid residue at position 65 of α-gliadin peptide increase toxicity for individuals with CD

II. Any amino acid substitution within the core epitope of α-gliadin eliminate CD toxicity

III. Studies involving overlapping T-cell epitopes result in the increased immunogenicity of α-gliadin peptides

3. PATIENTS, MATERIALS AND METHODS

3.1. REAGENTS, CHEMICAL SOLVENTS

- Industrial gluten (Vital wheat gluten, NW676, Roquette UK Ltd, Sallow Road, Weldon Industrial Estate, Corby Northants NN17 5JX, UK)
- Hydrocloric acid (around 36%, BDH Chemicals, UK)
- Double Processed Tissue Culture Water (Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Pepsin Agarose from porcine gastric mucosa (P0609-10KU, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Trypsin from Bovine Pancreas Agarose (T1763-50UN, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Sodium hydroxide 1.0 N (Sigma, UK)
- Transglutaminase from guinea pig liver (T5398-10UN, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- $CaCl_2$ (Sigma, UK)
- Phosphate buffered saline tablets (P4417, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Heat inactivated Fetal Bovine Serum 500ml (70259OF, Gibco, Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK)
- RPMI 1640 with L glutamine (E15-840, PAA, PAA Laboratories Ltd, Termare Close, Houndstone Business Park, Yeovil, Somerset BA22 8YG UK)
- Hepes buffer 1M (HO887, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Plasmocin 50mg (ant-mpt, InvivoGen, 5 rue Jean Rodier F-31400 Toulouse, France)
- AmphotericineB 100ml (P11-001, PPA, UK)
- Water Double Processed tissue culture water (Sigma, UK)

- Heparin (Mucous) Injection B.P. 1000 Units/ml, heparin sodium (PL0043/0041R, Leo Laboratories Ltd, Longwick Road, Princes Risborough, Buckinghamshire, HP27 9RR, UK)
- Lymphocyte Separation Medium Light Sensitive (LSM 1077, PAA, UK)
- Dimethyl SulphoxideHybri-Max (D2650, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Albumin from human serum (A4327, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- IL-2, Human, Recombinant (17908-10KU, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Albumin, chicken egg- ovalbumin (Sigma, UK)
- Lectin from Phaseolus vulgaris (red kidney bean) (L2769-2MG, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Thymidine, [6-^{3}H], 5mCi (NET355, Perkin Elmer, PerkinElmer Life & Analytical Sciences, Chalfont Road, Seer Green, Bucks, HP9 2FX, UK)

3.2. SOLUTIONS

tTg mix solution

135 µL	$CaCl_2$ (2mM in DW)
10 µL	Digested gluten (10g/L in DW)
125 µL	Transglutaminase from guinea pig liver (200 µg/ml in phosphate buffered saline (PBS))

Organ culture medium (10ml)

1,5ml	Heat inactivated Fetal Bovine Serum
8ml	RPMI 1640 with L glutamine
100µL	Hepes buffer 1M
10µL	Plasmocin 25g/L
100µL	Amphotericin 0,25 g/L

Autologous serum medium (ASM) for 50ml

500µL	Hepes buffer 1M
44ml	RPMI 1640 with L glutamine
50µL	Plasmocin 25g/L
500µL	Amphotericin 0,25g/L

3.3. EQUIPMENT AND ACCESORIES

Following equipments were used:

- Electronic pipette (4-000-205-E, Drummond Scientific Company, Broomall, PA, USA)
- Centrifuge (Sorvall RT7, Thermo Fisher Scientific)
- Magnetic stirrer hotplate (SM3, Stuart Scientific, UK)
- Gammacell 1000 Elite (GC-1000, Nordion International Inc, Kanata, Ontario, Canada)
- Harvester 96 (Mach, Tomtec, 706 Harborview, Orange, CT 06477)
- Liquid scintillation counter (Wallac 1450 Microbeta plus, Perkin Elmer)
- Heat sealer (Wallac 1295-012,Perkin Elmer)
- Neubauer counting chamber (1110000, LO-Laboroptik GmbH)
- Microliter glass syringe (Hamilton Syringe 500ul 750LT, ESSLAB LTD)
- Modular incubator chamber (3886047, Billups-Rothenberg Inc, P.O. BOX 977, Del Mar California 92014 (CA)
- Water bath (JB1, Grant Instruments Ltd, Barrington, Cambridge, CB2 5QZ, England)
- Cryo 1C Freezing Container (5100-0001, Thermo Scientific Nalgene, USA)
- Scales (GR-200, A& D Instruments LtD, Japan)
- Microscope (CK, Olympus, Japan)
- Vortex (K-550-GE, Scientific Industries Inc, Bohemia, NY, USA)
- Water-Jacketed CO2 Incubator (51201069, Napco 6000, US)
- Gas Jacketed Incubator (BB 6220, Heraeus, Switzerland)

- Fridge
- Freezer (-20°C and -56°C)
- Laminar flow hood

Following accessories were used:

- Micropipettes (0,5-10µL, 20-200µL, 100-1000µL)
- Macropipettes (1ml, 10ml)
- Plastic Pasteur pipettes
- Polypropylene conical tubes (15ml, 50ml)
- Nunc 1.8 ml Cryotube (368632, Nunc, A/S, Kamstrupvej 90, DK-4000, Roskilde, Denmark)
- Bijou tube 7 ml (129A, Sterilin Ltd)
- Universal 30 ml bottle (128B, Sterilin Ltd)
- 24 Well flat bottomed plate (353047, BD Falcon)
- 96 Well U bottomed plate (353077, BD Falcon)
- Organ Culture Dish (353037, BD Falcon)
- NALGENE Cryo 1°C Freezing container
- Forceps
- Disposable scalpels
- 5 ml syringe
- 70 µm Cell strainer (352350, BD Falcon)
- Sample bag, for microbeta (1450-432, PerkinElmer, UK)
- Filtermat A, for microbeta(1450-421, Perkin Elmer, UK)
- Blowtorch

3.4. PATIENTS

Small intestinal biopsies were taken during the diagnostic procedures or follow-up testing in subject with suspected or known CD. All six volunteers read and signed Informed Consent Form. The study was approved by the St Thomas' Hospital Research Ethics Committee (reference numbers 05/Q0207/167).Volunteers were diagnosed according to British Society of Gastroenterology guidelines. Six volunteers all typified as DQ2 positive, included four females and two males (Table III). Median age was 40.5 years, range 32-59. If it was possible, we choose CD patients which are not on the gluten free diet and are more suitable for our research. Biopsies were taken from the second part of the duodenum, collected in tube with organ culture. From all patients also around 60ml of blood was taken and saved in tubes with heparin, to prevent blood coagulation. As soon as possible biopsies samples and blood were transport to the lab. T-cells were from one to four weeks in culture prior to testing in T-cell proliferation assay, depends of the T-cell growth.

Table II: *Details of CD patients included in study: sex, age, time on a gluten-free diet, DQ status and weeks of T-cell line in culture prior to testing in T-cell proliferation assay*

Subject	Sex	Age at time of biopsy	GFD	DQ status	weeks of T-cell line in culture
Patient A	female	40	4 months	DQ2	4 weeks
Patient B	female	41	0	DQ2	1 week
Patient C	female	32	11 years	DQ2	1 week
Patient D	female	59	0	DQ2	1 week
Patient E	male	34	0	DQ2	3 weeks
Patient F	male	55	0	DQ2	2 weeks

3.5. EXPERIMENTAL WORK

3.5.1. Antigen preparation

Wheat gluten was initially digested with pepsin and trypsin. 1g of industrial gluten was dissolved in 50ml 0.1M HCl and pH adjusted to 2 if necessary. The mixture was then kept in the incubator until temperature reached 37°C and then 4000U of pepsin added. For 2 hours mix was stirred in the incubator at 37°C. Following 20 minutes of centrifugation at 3000rpm (1200 x g), supernatant's pH was adjusted to 7.8 with 1M NaOH. 25U of trypsin was added and again stirred for 2h in the incubator (at 37°C). With centrifugation for 20 minute at 2000rpm (500 x g), enzyme was removed. Supernatant's pH was adjusted to 7.0 with 1M HCl and air dried in the glass petri dish at 37°C. Later, gluten fractions were incubated with tTg prior to incubation with APC.

tTg mix solution was prepared as follows:

-digested gluten was dissolved in distilled water to make 10g/L and irradiated for 45 minutes (gluten solution)

- $CaCl_2$ was mixed with distilled water to make 2mM solution ($CaCl_2$ solution)

-5 PBS tablets were dissolved in 1L distilled water and irradiated. To that mixture, tTg was added to a final concentration 0,5U/ml. Smaller aliquots were made and frozen to -56°C (tTg solution)

All three solutions were mixed in ratio, incubate for 4h at 37°C and irradiated for 30 minutes:

125µl tTg solution (200 µg/ml)
135µL $CaCl_2$ solution (2mM)
10µL gluten solution (10mg/ml)

Ovalbumin (OVA) was digested and pre-treated with tTg at the same way as gluten.

3.5.2. Isolating peripheral blood mononuclear cells (PBMCs) from blood

Samples were centrifuged at 2000rpm (500 x g) for 15 minutes, to obtain plasma and cell layers. After removing supernatant (plasma), cell layer was mixed and divided in 10ml aliquots in 50ml centrifuge tubes. In each aliquot 28ml RPMI was added and mixed thoroughly. To separate white cells from others, we pipetted 13 ml Lymphoprep, placed pipette tip at the bottom of tube and slowly released 10ml Lymphoprep while slowly lifting the pipette. Mix was centrifuged at 3000rpm (1200 x g) for 20 minutes. The white cells which were lying on the RPMI layer were collected and centrifuged again at 2000rpm (500 x g) for 15minutes. The supernatant was removed and the cell pellet resuspended in 10ml ASM. To count the PBMCs 20µL of mix was taken and stained with the same volume of tryptan blue.

***Equation 1**: Calculation of numbers of cells from standard haemocytometer chamber*

Number of cells = 2(1:1 dilution of tryptan blue) **x 10^4** (count 10 squares) **x the original volume of liquid from which sample was removed (ml) x number of cells per square**

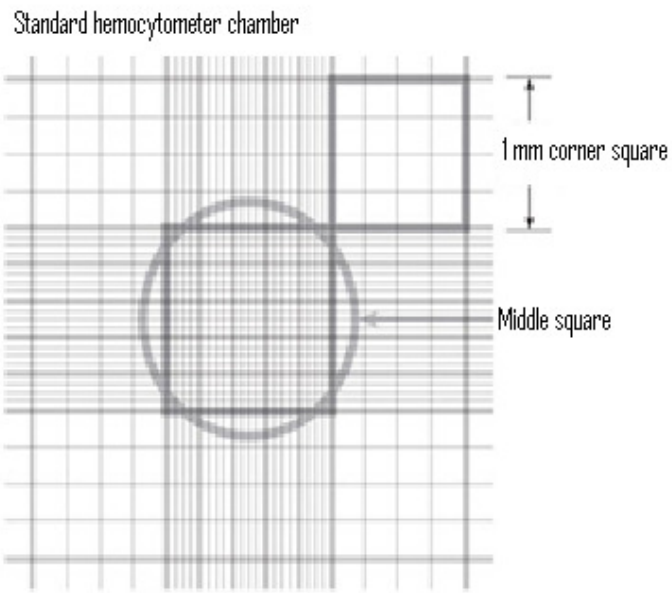

***Figure 6:** Standard haemocytometer chamber for counting PBMCs and T-cells*

4x 10^6 cells were irradiated (22 Gy) and placed into a wells of a 24 well plate to combine with the SIL fraction the following day as a support for the T-cells. The remainded of cells were frozen down (See Freezing of cells for liquid nitrogen storage) and later used as APC for the T-cells in the restimulation and proliferation assays.

3.5.3. Obtaining autologous plasma for culture medium

Following separation of the cells and plasma, plasma was placed in the water bath at 56˚C for 35minutes to inactivate complement. Heated inactivated plasma was centrifuged at 2000rpm (500 x g) for 15 minutes. The supernatant was collected and used to serve as autologous plasma medium.

3.5.4. Freezing PBMCs for liquid nitrogen storage

Suitable amount of empty cryotubes and cells in the centrifuge tube were placed on ice for 20 minutes. Twenty percentage dimethyl sulphoxide (DMSO) in autologus plasma medium was added then to cells drop-wise, gently mixing after each drop. The volume of 20% DMSO depends on the volume of ASM in which PBMCs are resuspended (V_{DMSO}: V_{ASM}= 1:1). The cryotubes contained around 5x10^6 cells. The tubes were placed in iso-propanolol and frozen to -50˚C overnight. The following day cryo tubes were transferred to liquid nitrogen to store.

3.5.5. Small intestinal organ culture

Approx 1,5ml organ culture medium was placed in bijou tube to sterilized antigen (gluten) to reach final concentration 5mg/ml and placed in incubator. To prepare organ culture dish in advance we placed sterile grid over centre well, added 1,5ml sterile water to outer well and kept dish in incubator at 37˚C. During the diagnostic procedures biopsies were taken. Following biopsies for routine histology we collected biopsies for research in bijou tube with 100µl organ culture medium. In laboratory biopsies were placed on the grid (7 biopsies per grid). The orientation of biopsies has to be with the serosal side down (redder side with red dot) and mucosal up. Biopsies should be touching (cell contact) to obtain an

improved yield of cells. Organ culture medium with the digested gluten was injected then into the central well to get capillary effect that covers the biopsies. It was important that the tissue was not completely submerged in organ culture medium. Dishes were placed into the organ culture chamber. In this chamber gas mixture of 95% O2/ 5% CO2 was flowed through for 1 minute. Then we sealed the chamber and waited until the pressure 2 psi was reached. Chamber was kept in incubator (37°C) overnight.

3.5.6. Isolating T-cells from *in vitro* gluten-challenged duodenal biopsies

To isolate T-cells, biopsies were chopped into small pieces with scalpel blades. The whole time biopsies need to be in ASM. Mixture was passed through a 70µm cell strainer and washed with ASM. We collected pieces on the strainer (debris fraction) separate to the mixture which passed trough the strainer (SIL fraction). Irradiated PBMCs from the day before were added to SIL fraction, to provide more cell-to-cell contact. Cells were washed twice in 10ml ASM warmed to 37°C and centrifuging at 1500rpm (300 x g) for 7 minutes. Cell pellets were resuspended in 4ml ASM and divided into two wells in 24-well plate.

3.5.7. Thawing PBMC cells

RPMI squeezing pipettes and 15ml centrifuge tubes need to be cooled down on ice first. We took vials with PBMC from liquid nitrogen storage and placed them under tepid water until the suspension melts. RPMI was then added drop-wise up to volume of 10ml whilst constant hand-shaking. Thawed T-cells were immediately centrifuged for 7 minutes at 1500rpm (300 x g), resuspended in another 10 ml ice-cold medium and once again spin down. The pellet was then resuspended in ASM and cells counted. PBMCs were irradiated (22 Gy) to prevent their proliferation.

3.5.8. Maintenance of T-cell lines in culture

Three days after, T-cells had been isolated and then every 2-3 days depending on colour change and cell growth rate, medium was refreshed by removing half the volume culture supernatant and replacing it with fresh culture medium. Concurrently to every ml ASM 100µl IL-2 was added to serve as a T-cell growth factor. If cells in well were overcrowded, we split cell suspension into two wells, in each well half volume. On day 7 cultures were

restimulated. First, PBMCs were thawed, irradiated and incubated for 18 hours with deamidated peptic-tryptic digested gluten. The number of PBMCs we kept, was depending on the number of T-cells in the culture (number of PBMC: number of T-cells= 1:1)

Ratio has to be as follows:

2x10^6 PBMC in 1ml ASM + 1ml tTg mix

The following day PBMC cells with antigen presented on their surface were combined with T-cells and kept in fresh culture medium. Restimulation cycle was repeated once a week.

3.5.9. T-cell transformation assay

We used autologous PBMC as antigen presenting cells. First we thawed them, counted with haemocytometer and irradiated (22 Gy). Then PBMC were suspended in ASM to reach the right concentration ($5x10^4$ cells in 50µl ASM).In 96 well U-bottom plate 50µl per well of these suspension was given. Depending on the size of peptide, incubation time at 37˚C varied. Whole proteins needed 18h of incubation, meanwhile for small peptides 4h was sufficient for PBMCs to present antigen on their surface. The final concentration of complex antigen had to be 100µg/ml and 10µg/ml for small peptide. We used digested gluten as a positive control, OVA as negative control and ASM without any peptides as background. Small tested peptides were obtained synthetically and had 95% purity. In the Table III, amino acid sequences for tested peptides are shown. They were marked as peptide 1, 2, 3, etc for the simplicity of peptide labelling in text and tables. Tested peptides harbour T-cell epitopes or their counterparts with amino acid substitutions (Table IV, Table V)

Table III: Marking peptide amino acid sequences with numbers (1-8)

Mark of peptide	Peptide description
1	Q L Q P F P Q P Q L P Y P Q P Q
2	Q L Q P YP Q P Q L P Y P Q P Q
3	Q L Q P FP Q P E L P Y P Q P E
4	Q L Q P Y P Q P E L P Y P Q P E
5	Q L Q P F P Q P EL S Y P Q P E
6	Q L Q P F P Q P KL S Y P Q P E
7	Q P EL P Y P Q P E L P Y P Q
8	Q L Q P F P K P K L P Y P K P Q

Table IV: Amino acid sequence comparison between peptide 1 and peptide 2 and epitopes they harbour

Peptide 1	Q	L	Q	P	**F**	P	Q	P	Q	L	P	Y	P	Q	P	Q
Peptide 2	Q	L	Q	P	**Y**	P	Q	P	Q	L	P	Y	P	Q	P	Q
α2-gliadin	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**

Epitope DQ2.5-glia-α1a

Epitope2.5-glia-α2

Table V: Amino acid sequence comparison between peptide 3 and peptide 7 and epitope they harbour

peptide 3	Q	L	Q	P	F	P	Q	P	E	L	P	Y	P	Q	P	E					
peptide 7							Q	P	E	L	P	Y	P	Q	P	E	L	P	Y	P	Q
α2-gliadin	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**

Epitope DQ2.5-glia-α1a

Epitope DQ2.5-glia-α2

Epitope DQ2.5-glia-α1b

The T-cells to be tested for antigen reactivity were counted using haemocytometer. Cells were subsequently centrifuged at 1500rpm (300 x g) for 7 minutes, resuspended in ASM to reach concentration $5x10^4$ cells/ 100µl/ well and added to the pre-pulsed APC. After 48h of incubation, tritiated thymidine was added (1µCi/well in 20µl ASM) and plate incubated for further 18hour. Plates were harvested with a Tomtec Cell Harvester. The level of thymidine incorporation was measured using a Wallac 1450 Microbeta Plus liquid scintillation counter. We calculated the stimulation indices (S.I.) for each antigen by dividing the mean counts per minute (cpm) for the T-cells plus APC plus test antigen by the cpm for T-cells plus APC alone.

***Equation 2**: Calculation of SI*

$$S.I. = \frac{\text{Mean cpm for T cells plus APC plus antigen}}{\text{Mean cpm for T cells plus APC}}$$

4 RESULTS

Biopsies from six different coeliac disease patients were taken and T-cells raised to test different gluten peptides and their variants for coeliac toxicity. The tested antigens comprised: digested and tTg treated gluten (GLU), ovalbumin and eight different 16mer α-gliadin peptides.

The stimulation indices (SI) were calculated by counting of thymidine incorporation into the gluten specific T-cells. T cell lines with SI 2 or more were considered positive (CD toxic). All the T-cells proliferation assays were undertaken in triplicates. Where coefficient of variation (CV) was 0.2 or more, the value which deviate the most was excluded and SI calculated again.

Digested gluten served as a positive control. If the SI for a cell line was positive (SI≥2), it was accepted as gluten specific. From the tested lines, 6 of them were gluten specific (results presented in the table VII). OVA tested negative in all the proliferation assays. Between one to eleven α-gliadin peptides were tested for each line, depending on the number of T cells available. We calculated for all the peptides fractions how many times each peptide was tested and in how many cases it was positive. We also recorded percentage of positive results for each peptide.

Table VI: *Results of 6 proliferation assays: six gluten sensitive T cell lines were tested with gluten (GLU) and peptides 1-8, depending on cell numbers available. Grey background indicates positive T-cell response (stimulation index ≥2)*

	GLU	1	2	3	4	5	6	7	8
patient A	5.61	2.81	2.00	14.82	13.61	2.57	0.83	2.36	1.04
patient B	8.88	2.12	1.90	1.90	3.02	1.85	1.44	1.74	1.67
patient C	4.36	3.49	1.82	6.66	16.71	8.89	1.17		
patient D	2.42	1.98		1.73	2.48	1.38			
patient E	3.6			1.60				2.93	1.07
patient F	7.41					1.63			
% of positive responses		3/4 75%	1/3 33.00%	2/5 40%	4/4 100%	2/4 50%	0 0%	2/3 66.00%	0 0%

4.1. DEAMIDATION OF DOMINANT GLUTEN EPITOPES

Peptide 1 (QLQPFPQPQLPYPQPQ) and 2 (QLQPYPQPQLPYPQPQ) are immunodominant peptides. In T-cell proliferation assays they tested positive or border line negative (all SI around 2). Peptide 1 which harbours overlapping DQ2-glia-α1a and DQ2-glia-α2 epitopes tested positive in 3 out of 4 (75%) and peptide 2 (DQ2-glia-α1b and DQ2-glia-α2 epitopes) in 1 out of 3 (33%) gluten sensitive cell lines. Deamidated peptides 1 and 2 were tested to demonstrate the importance of deamidation (glutamine to glutamic acid) at position 65 of α2-gliadine. Peptide 3 (QLQPFPQPELPYPQPE) was positive in 2 out of 5 (40%) assays, peptide 4 (QLQPYPQPELPYPQPE) was in all samples tested positive (100%). For two T-cell lines (patient A and patient C) stimulation indices for peptide 4 were high above the cut-off for positivity (SI=2).

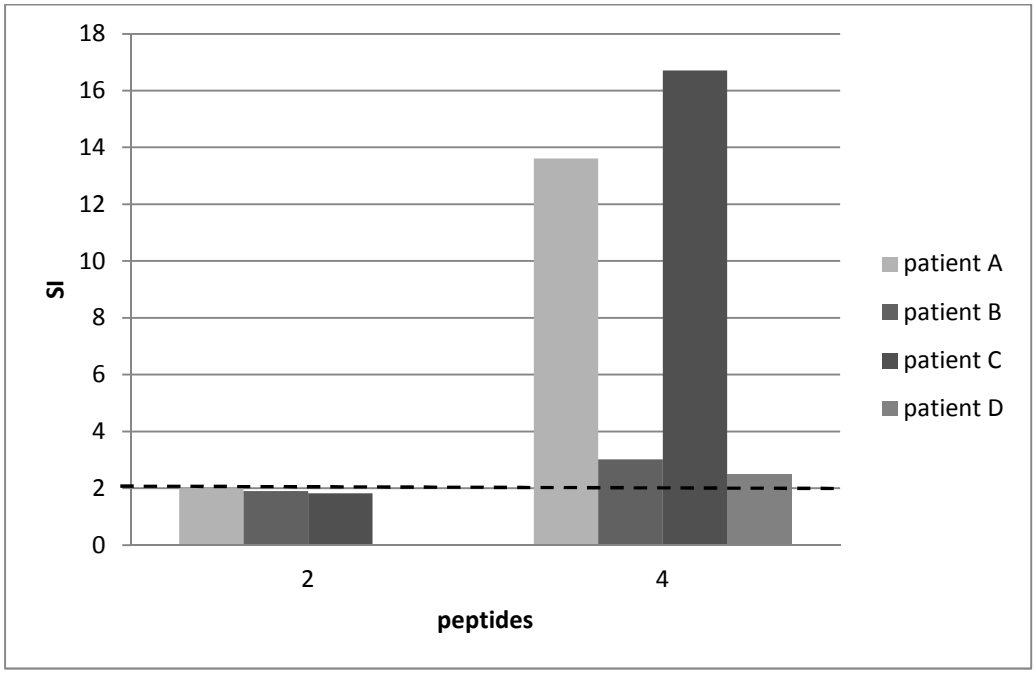

Figure 7: *Results of proliferation assays for peptides 2 and 4 demonstrating fall of SIs when peptides are deamidated*

4.2. AMINO ACID SUBSTITUTIONS WITHIN T-CELL EPITOPE

In peptide 5, 6 and 8 amino acid substitutions in the T-cell epitope were introduced. If we compare the SI for peptide 3 (QLQPFPQPELPYPQPE) and 5 (QLQPFPQPELSYPQPE), which differ just in one amino acid residue, in all samples we can see a reduction of T cell response in peptide 5 (indices fall) (Figure 8). Peptide 6 (QLQPFPQPKLSYPQPE) has two amino acids substitutions introduced in comparison with peptide 3 (QLQPFPQPELPYPQPE). First proline to serine substitution at position 67 and another glutamine to lysine at position 65. In comparison with peptide 5 (one amino acid substitution), in peptide 6 (two amino acid substitutions) all indices decreased. Not only that, these substitutions resulted in complete elimination of T cell response; The SI were negative in all tested T-cell lines (100%).Peptide 8 (QLQPFPKPKLPYPKPE), which serve as a negative control, has three amino acid substitutions. Three inner glutamines are substitute with lysine. It was negative in three out of three samples (100%).

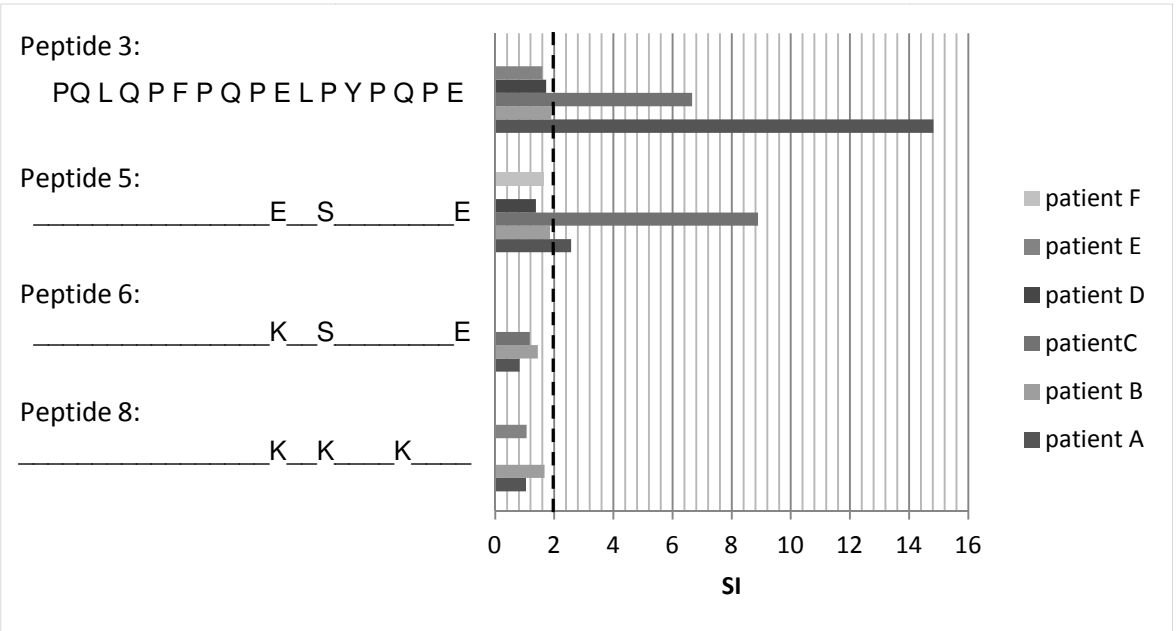

Figure 8: *Results of proliferation assays for deamidated immunodominant peptide (peptide 3) and its counterparts with amino acid substitutions*

4.3. OVERLAPPING T-CELL EPITOPES

Peptide 7 (QPELPYPQPELPYPQ) harbours three α-gliadin epitopes which overlap DQ2.5-glia-α1a, whole DQ2.5-glia-α1b and DQ2.5-glia-α2 epitope. The N-terminal amino acid region is the same as in deamidated immunodominant peptide (peptide 3), but the sequence is rapted six amino acids residues into the C-terminal end. This peptide tested positive in 2 out of 3 (66%) proliferation assays (Figure 10).

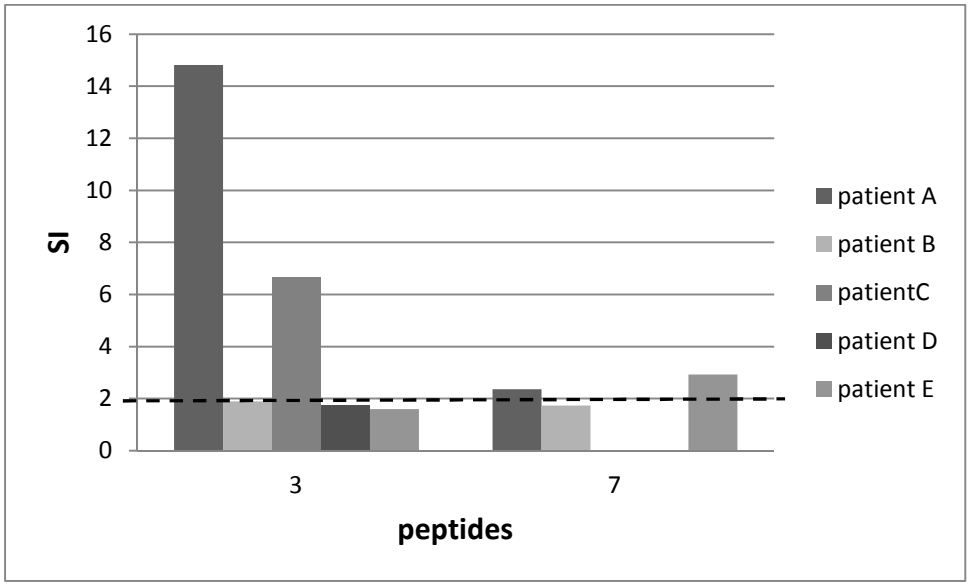

Figure 9: *Results of proliferation assay for peptide 3 and peptide 7*

4.4. PROOF OF CONCEPT ON PATIENT A

The results of a gluten sensitive T cell line from patient A are particularly interesting. For both immunodominant peptides, peptide 1 and peptide 2, positive response of T cells was shown. The SI for peptide 1 was 2.81 and for peptide 2 was 2.00. With deamidation at position 65, index becomes five times higher (from 2.81 to 14.82) for peptide 3 and increases almost seven times (from 2.00 to 13.61) for peptide 4. Proline to serine substitution at peptide 5 causes a drastic fall of SI (to 2.57) compared with peptide 3. Two amino acid substitutions (peptide 6) resulted in abrogation of coeliac disease toxicity; SI was negative (SI=0.83) (Figure 11).

SI for peptide 7 positive (SI=2.36). Peptide 8 was confirmed to act as a negative control also with an SI of patient A for that peptide (SI=1.04).

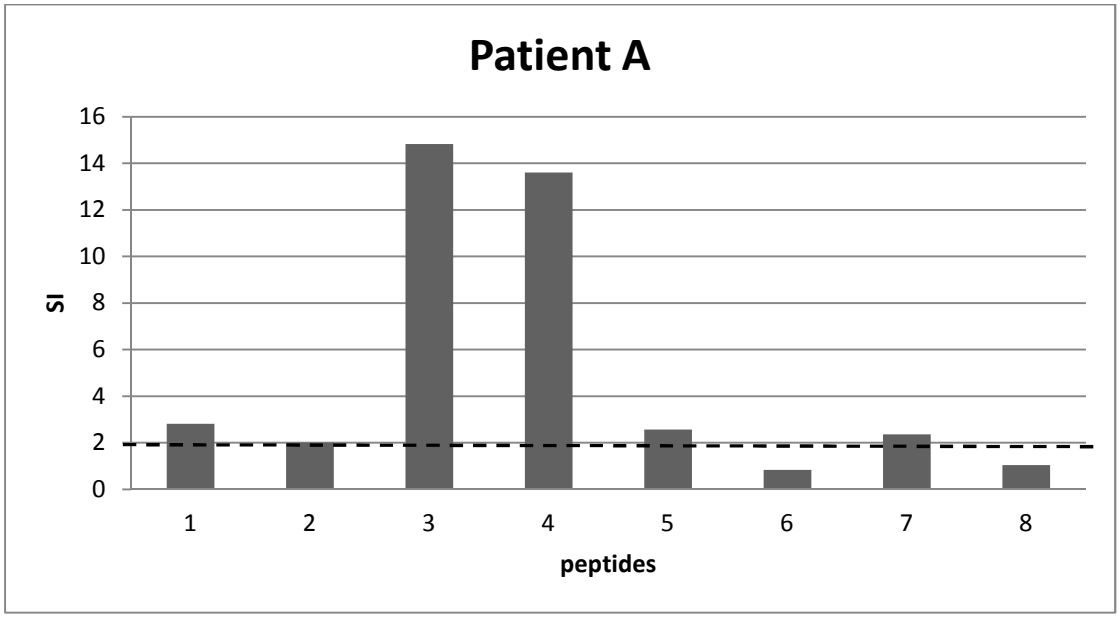

Figure 10: *Results of proliferation assays for T-cell line of patient A*

5 GENERAL DISCUSSION

5.1. GLUTEN SPECIFICITY OF T-CELLS

Gluten is as a protein that is digested in the stomach with pepsin to peptides and in the duodenum further to smaller peptides by trypsin. To imitate digestive system, we exposed industrial gluten to both enzymes. First pH, which is necessary for enzyme activation, was regulated. Digested gluten was used as a prestimulating antigen of biopsies and after exposure to tTg, for T-cell restimulations. In some other studies they used gliadin fraction only to expand T-cell line (18). The reason why we stimulated biopsies and T-cells with a whole gluten fraction is assumption that there are immunogenic determinants presented also in high and low molecular glutenins. Use of gliadin fraction only, a fraction of glutenin specific cells would not be stimulated. With every restimulation, more T-cells with a response directed toward gluten are isolated. To check gluten specificity, every experiment was also undertaken with medium only as a base line and with digested gluten as a positive control. In few cell lines, a sufficient number of cells was obtained within the first week after isolation. Proliferation assays with these cells were made therefore without restimulation. All these T-cell lines were found to be non-gluten-specific. There are several reasons for the presence of non-gluten-specific T-cells in small intestine e.g. small intestinal bacterial overgrowth (SIBO) or Helicobacter pylori infection. At the beginning of our study, the same problem with gluten specificity occurred even after more additional restimulations. There can be more reasons. Firstly, the industrial gluten we used was out to date and therefore possibly unable to stimulate gluten sensitive T-cells to proliferate. Secondly, IL-2 is used as a culture supplement as it is growth factor for T-cells. It can therefore happen, that IL-2 trigger T-cell stimulation which includes also cells which are not gluten-specific. Thirdly, optimised irradiation of PBMC is crucial for the APC to exert their antigen-presentation functions as opposed to proliferation. To reach this, a dose of 22 Gy should be used. Every year, irradiation time has to be prolonged due to our cesium source losing some of activity. If the length of radiation is incorrect, PBMC can proliferate or be unable to do their antigen-presentation. However, six cell lines were established as gluten specific and were tested for selected peptides.

5.2. IMMUNODOMINANT T-CELL EPITOPES

The subjects of our research were α-gliadins. Eleven peptides corresponding to α-gliadin residues 63-77 (peptide 8) or 57-72 (all other peptides) were synthesised and tested in T-cell proliferation assays. It has been suggested that there are four DQ2.5 restricted α-gliadin epitopes (12). Our peptides harbour three of these epitopes and their variants: DQ2.5-glia-α1a, DQ2.5-glia-α1b and DQ2.5-glia-α2. The epitopes in our candidate peptides overlap. The selection of peptides was based on these variants of immunodominant epitopes, where one or more nucleotide substitutions in genes encoding a codon led to a different amino acid in the expressed peptide. Therefore smaller genetic mutation is needed and by that bigger chance that this mutation naturally occurs. Main amino acids substitutions that occurred in our peptides are proline to serine and glutamic acid to lysine.
In comparison with a previous study, the same peptide sequences were studied but single point amino acid substitution with alanine was introduced in different positions of immunodominant epitope (19).

Peptide 1 and peptide 2 differ in one epitope. While peptide 1 harbours DQ2.5-glia-α1a and DQ2.5-glai-α2 epitope, peptide 2 harbours DQ2.5-glia-α1b and DQ2.5-glai-α2 epitope. These two epitopes (DQ2.5-glia-α1a and DQ2.5-glia-α1b) differ by one amino acid- phenylalanine is substituted with tyrosine. In our study, peptide 1 showed higher SI then peptide 2 in all tested samples. This was not the case when the peptides were deamidated. While deamidated peptide 2 (peptide 4) was toxic in all the tested cell lines, was deamidated peptide 1 (peptide 3) in 60% negative. It implies that each cell line responds different. A T-cell line from one patient can be sensitive to certain immunodominant epitope but fail to respond to others and the opposite. Our results confirm the observations of previous study (20). They tested T-cell lines with α-2 gliadin, DQ2.5-glia-α1a epitope and DQ2.5-glia-α2 epitope. In two cell lines out of three there was response to α-2 gliadin, while response was not elicited by these two epitopes. That indicates that response was triggered by DQ2.5-glia-α1b epitope.

Tested immunodominant peptides (peptide 1 and peptide 2) are part of the α-gliadin derived 33mer peptide, which was identified as highly immunogenic for majority of CD individuals. How is it possible that that long peptide survive gastrointestinal digestion? If we look at our peptides, which are the product of gluten digestion, we notice that they harbour significant number of proline residues. These residues are the most resistant to proteolysis by gastric, pancreatic and intestinal enzymes. Gliadin epitopes are usually located in regions rich in proline residues. This is probably the reason why relatively big gluten peptides come to small intestine and remain there for sufficiently period whilst exposed to tTg. The latter then deamidates them that in turn makes them even more immunogenic.

5.3. EFFECT OF DEAMIDATION OF DOMINANT GLUTEN EPITOPES ON T-CELL STIMULATION

Our results for peptides 2 and 4 show, that with deamidation of α-gliadin peptide at position 65, SI of stimulation increases drastically. Although comparison between SI for peptides 1 and 3 is not so clear, indexes are lower than their deamidated counterparts in all studied cell lines. Particularly striking are indices from patient A. Glutamic acid at position 65 of DQ2.5-glia-α1a epitope (peptide 3) results in more than five times higher SI than glutamine at the same position (peptide 1). Similarly, for DQ2.5-glia-α1b epitope deamidation of the peptide leads to more than six time higher SI (peptide 4: peptide 2). That demonstrates that deamidation at position 65 is preferential either for HLA-DQ2 or TCR binding. With T-cell proliferation assay, we cannot say exactly which of these two binding was effected. In previous studies the structure of DQ2 complex with DQ2.5-glia-α1a gluten epitope was determined (21). They evaluated the increase of immunogenicity as a result of negative charged residues at the position 65. X-ray crystallography was used. It was discovered that negative charged residue is involved in hydrogen-bonding network with HLA-DQ2.5 cleft at position6 (P6). The amide group of glutamine cannot form as strong hydrogen bonds as carboxyl group of glutamic acid. First, amide nitrogen is not good hydrogen-bond acceptor. Additionally, also carboxyl oxygen of glutamine is not as good acceptor as negatively charged carboxylate oxygen. Consequently, the bond between

negative charged epitope and DQ2.5 cleft is stronger. As our results demonstrate, more stable gliadin-DQ2 complex results in higher T-cell stimulation.(21)

Enzyme which catalyzes that deamidation in human intestine of CD patients is tTg. The question is, how tTg chooses its targets and why it deamidates specially gluten peptides and especially at certain positions. At previous study, they tried to discover preferred substrates of tTg by using 5-biotinamido-pentylamine as a substrate for transamidation with tTg (22). Peptides which were targeted by tTg, were crosslinked with biotin compound, then purified and sequenced. It was shown that amino acid proline determines specificity of the enzyme. Even though proline in gluten peptide sequences is not involved in bonding HLA-DQ molecule or TCR on T-cells, it plays important role in disease pathogenesis. tTg targets typically the Q in QXP motif and not QXXP and QP motif. T-cell epitopes are rich in these sequences and present a perfect target for enzyme (22). Immunodominant epitope we have studied (DQ2.5-glia-α1a) has QLP sequence on the right position (65, 66, 67) that when it is deamidated, it becomes a good DQ2 and TCR binder. Glutamine to glutamic acid substitutions on other positions (59 and 63) of this epitope have also been studied (Arentz-Hansen et al. 2000). Interestingly, it was shown that deamidation at other positions leads to increase in affinity of the peptide for DQ2, but does not trigger T-cell response. The same test was made with peptide harbouring DQ2.5-glia-α2 epitope. After tTG exposure, two new peaks were created and recognized bytandem mass spectrometry. Deamidation was identified at positions 65 and 72. Another glutamine at position 70 was synthetically deamidated. In contrast with deamidations at positions 65 and 72, in this case T-cell response did not increase. (18)

We observed that also some native (non-deamidated) α-gliadin peptides triggered positive T-cell responses. SI for peptide 1 in T-cell lines from patient A, B, C were more than 2 and also SI for peptide 2 in T-cell line from patient A. It demonstrates that also not deamidated α-gliadin peptides can trigger T-cell stimulation. Our results confirm the observations that deamidation of glutamine with tTgat position 65 and 72, transform peptides that they bind DQ2 molecules more likely, but it is not necessity for their binding (19). Negative charged residues (glutamic acid instead of glutamine) are better for binding DQ2 cleft, but it may not be essential for T-cell stimulation.

The notion that tTg is very important factor in disease development, might prompt one to consider tTg inhibition as therapeutic strategy. As our results demonstrate, also non-deamidated α-gliadin peptide can trigger T-cell stimulation. We can therefore conclude that potential enzyme inhibitor may not abrogate T-cell response completely. Additionally, tTg is also involved in major biological pathways (apoptosis, cell adhesion, signal transduction, collagen assembly and wound repair mechanism). Its inhibition may cause harmful systemic effects. Therefore inhibiting of tTg may not be a good approach for coeliac treatment.

5.4. EFFECT OF AA SUBSTITUTIONS AT IMMUNODOMINANT GLUTEN EPITOPE ON T-CELL

5.4.1. Proline to serine substitution at position 67 of α2-gliadin (peptide 5)

Substitution of proline to serine at position 67 of α2-gliadin peptide (amino acids 57-72) caused downregulation of T cell stimulation in our study. In the T-cell line from patient A this substitution caused more than five times lower SI in comparison with peptide 3 which has, except for this substitution, the same amino acid sequence.

Our results demonstrate that with substitution at position 67, TCR or HLA-DQ2 binding was affected. If we consider that DQ2.5-glia-α1a epitope of the peptide 3 is positioned within the DQ2 cleft, position 67 where substitution was made in peptide 5 points toward TCR. It has been demonstrated that there are numerous different ligands, which are able to bind to TCR. But the question is, which peptide is an “optimal ligand” and has the biggest affinity for the receptor. Two factors are important to form strength interactions between peptide-HLA complexes with the TCR: physical proximity and chemical (charge) complementarities. Proline at position 67 seems to be the perfect ligand for the TCR. On the other hand, serine is charged the same as proline, but has smaller side chain. This single amino acid substitution leads to the modification in TCR contact and weaker signal. Peptide, in which single or multiple amino acid is substituted and TCR contact manipulated, is called altered peptide ligand (APL) (23). Different functions of T-cells can

be affected: proliferation, production of cytokines or cytotoxicity. Each function is activated separately, depends on the strength of TCR stimulation. To reach the maximum level of activation, it has to bind perfectly to TCR, ligand has to be present in the appropriate concentration and costimulatory signal has to be available. In our case, proliferation was affected. This is one of the most complex functions and requires full T cell activation which was decreased with amino acid substitution. (11)

In a previous study they demonstrated that DQ2.5-glia-α1a epitope and DQ2.5-glia-α2 epitopes bind to a different frame to HLA-DQ2 (18) . Peptide 5 harbours both epitopes with one amino acid substitution. If we consider that DQ2.5-glia-α2 epitope binds to HLA-DQ2, position 67 binds to P6 pocket of HLA-DQ2. In the immunodominant α-gliadin peptide (peptide 1) position 67 is proline. Proline is the only natural amino acid whose amide hydrogen is lost because of participating of the secondary amide in a polypeptide backbone. Consequently, it cannot form hydrogen bonds. Even though, proline has important role in binding to DQ2. Proline rich peptides naturally occur in a left-handed polyproline II helical conformation, due to proline structural rigidity. This conformation is preferred for HLA-DQ2 ligands. In our peptide (peptide 6) proline is substituted with serine. It causes decrease, but not abrogation of T-cell response in all cases. If proline at this position is substituted with lysine it resulted in complete elimination of T-cell response (18). Lysine is a positive charged amino acid. It was demonstrated in a previous study that positive charged residues inhibit binding to P6 (24). The same happened if proline is substituted with alanine (19). It indicates that even though serine does not form perfect conformation for HLA-DQ2 binding, it is still a better ligand in comparison with alanine. The reason might be in serine properties. Its hydroxyl group can participate in hydrogen bonds as donor or acceptors and can form these bonds with the HLA-DQ2 molecule, while alanine cannot.

5.4.2. Proline to serine substitution at position 67 and glutamic acid to lysine substitution at position 65 of α2-gliadin (peptide 6)

With introduction of two substitutions in peptide 3, the T cell response was completely abrogated in all studied T-cell lines. These two substitutions led to 17-fold decrease of SI when comparing peptide 3 and 6 in T-cell line from patient A. In comparison with peptide

5, one additional amino acid substitution in peptide decreases SI at this cell line for factor three.

In peptide 6, glutamic acid at position 65 of α-gliadin is changed to lysine. As it was previously described, glutamic acid at position 65 participates in a strong hydrogen bond. Negative charged amino acid residues are therefore preferential (21). Lysine is a positive charged amino acid and its atoms can serve just as hydrogen donors and not as hydrogen acceptors as glutamic acid. It cannot form any hydrogen interactions with HLA-DQ2 that result in less stable complex. Our results confirm previous studies (18) (19) (21). In the study of Arentz-Hansen and colleagues, substitution with the same amino acid was made. In an HLA-DQ2 peptide binding assay DQ2.5-glia-α1a epitope was checked with lysine substitutions at positions 63, 64 or 65. Substitution at position 65 resulted in drastically decrease of binding. It is surmised that substitution at this position with any non-negative charged residue results in decrease of peptide toxicity (18).

We showed that with two substitutions at the right positions of the immunodominant peptide, T-cell response can be eliminated. In our study the toxic effect of peptides was evaluated just with T-cell proliferation (SI). Further investigations are required to confirm if HLA-DQ or TCR binding is affected. Once this is shown, new approaches for CD treatment can be investigated. One approach could be the peptide, which can bind to TCR but contact is modified i.e. TCR binding antagonist. Antagonist does not induce any of T-cell function (proliferation, cytolysis, cytokine release) and at the same time prevents normal antigen from binding. The elimination of T-cell response with peptide 6 indicates that this peptide could be a good TCR antagonist. It is known that T-cells, in contrast with HLA-DQ molecules, can recognize many different gluten peptides. The recognition also differs from patient to patient. This is the reason why forming an HLA-DQ blocking peptide could be more appropriate approach.

HLA-DQ- peptide binding is one of the crucial roles in coeliac disease pathogenesis. X-ray crystal structures of complexes comprising of HLA-DQ2 with certain gliadin epitopes have previously been described. This makes it possible to develop an optimal HLA-DQ2 blocking peptide. By blocking the presentation of CD-toxic peptides, disease expression could be controlled. It would be essential to form the peptide which has a strong affinity for the HLA-DQ2 molecule but hinders contact with TCR. It can be achieved by making a smaller peptide on TCR side, allowing the receptor to dock on to peptide-MHC complex,

but not allow recognition. Another possibility might be big and bulky peptide, which prevents docking any TCR to the peptide-MHC complex. The option could be dimeric peptide or peptide with cyclic structure on TCR side, which sterically hinder TCR binding. The same principle was previously tested in other autoimmune diseases including type 1 diabetes and rheumatoid arthritis. As distinct, delivery of the drug to the target place in coeliac disease would be easier. At the same time as gluten product are consumed, drug could be taken and compete for HLA-DQ binding with gluten peptides. The major problem for peptides as drugs is their *in vivo* stability and their rapid inactivation by gastrointestinal enzymes. The second consideration is in case using peptides harbouring T-cell epitopes not problematic. They are originally more stable from proteolysis (25).

In our study, peptides were tested in T-cell proliferation assays. T-cell study is the first step for testing CD peptide toxicity. Before using peptides in any of these approaches, further tests are needed to demonstrate safety of peptides for CD patients. First is testing peptides in organ culture where innate and adaptive immune systems are involved. If safety of peptides is confirmed, they will need to be tested by in vivo feeding study. This would be required to prove that peptide is safe for consumption for coeliac patients.

5.4.3. Three inner glutamines substituted with lysine (peptide 8)

In peptide 8, indices were negative in all tested lines. This peptide serves as a negative control for comparison with the SI of other peptides. Three substitutions are introduced to the immunodominant peptide (peptide 1), that is at the positions 63, 65 and 70 of α-gliadin peptide. As the model of Vardal, Arentz-Hansen and Bergseng suggests these are one of the crucial positions for DQ2 binding (24) (18) (13). First two bind to the P4 and P6 pockets. We supposed that with these substitutions immunodominant epitope is transformed to the extent where is not able to bind to APC.

5.5. EFFECT OF OVERLAPPING CD TOXIC MOTIFS (peptide7)

Peptide 7 also contains amino acid sequence found in 33mer peptide but the N-terminal end starts at another position (p63). In contrast, all other tested peptides start with p57. Peptide 7 harbours sequences which are commonly recognized gliadin epitopes: part of the

DQ2.5-glia-α1a, whole DQ2.5-glia-α1b and DQ2.5-glia-α2 epitope which are overlapping. 9mer motif with DQ2.5-glia-α2 epitope repeats twice. Peptide 7 is deamidated at positions 65 and 72, what is important for its binding. Presumably, peptide is binding to the pocket where DQ2.5-glia-α1b epitope or DQ2.5-glia-α2 epitope bind. A T-cell line from patient A tested positive with peptide 7. However, the SI was lower in comparison to SI with peptide 3 (deamidated immunodominant peptide which harbours whole epitope DQ2.5-glia-α1a). Surprisingly, in one out of three T-cell lines the SI resulted negative. This was unexpected because DQ2.5-glia-α2 epitope was tested as highly toxic in previous studies (18) (19). This indicates how T-cell response to the same peptide can differentiate between cell lines from different CD patients. However, peptide was tested positive in two T-cell lines and is accepted as toxic for coeliac patients.

6 CONCLUSION AND FUTURE PERSPECTIVES

The present study using modified immunogenic peptides, demonstrate that with substitutions in the correct positions of peptide, T-cell response can be blocked. From our results we can conclude:

I. With single point amino acid substitution in the core epitope of α-gliadin, immunogenicity of a peptide can be decreased.

II. With carefully selected two amino acid substitutions in the core epitope of α-gliadin, peptides become non-immunogenic.

III. Modification of immunodominant epitope within α-gliadin might generate CD non-toxic peptides that could potentially be used in immunotherapy in CD.

IV. Gluten-specific T-cells from different patients can recognise diverse gluten epitopes and not all epitopes are found toxic for every gluten-specific T-cell line.

V. With deamidation gluten peptides become more immunogenic.

In our study, just α- gliadins have been tested. Because of the similar properties and mechanism of binding, similar approach could be made also for other gliadins, to make more peptides for immunomodulation. Since our peptides were tested just on the DQ2+ CD patients, studies on DQ8+ patients remain to be established.

7 REFERENCES

1. Ludvigsson JF, Leffler D a., Bai JC, Biagi F, Fasano a., Green PHR, et al.: The Oslo definitions for coeliac disease and related terms. Gut 2012 Jan [cited 2014 Jan 28];62(1):43–52.

2. Koning F, Schuppan D, Cerf-Bensussan N, Sollid LM: Pathomechanisms in celiac disease. Best Pract Res Clin Gastroenterol 2005 Jun [cited 2014 Jan 28];19(3):373–87.

3. Tjon JM-L, van Bergen J, Koning F: Celiac disease: how complicated can it get? Immunogenetics 2010 Oct [cited 2014 Jan 28];62(10):641–51.

4. Dubé C, Rostom A, Sy R, Cranney A, Saloojee N, Garritty C, et al.: The prevalence of celiac disease in average-risk and at-risk Western European populations: A systematic review. Gastroenterology 2005 Apr [cited 2014 Jan 26];128(4):S57–S67.

5. Bai JC, Fried M, Corazza GR, Schuppan D, Farthing M, Catassi C, et al.: World Gastroenterology Organisation global guidelines on celiac disease. J Clin Gastroenterol 2013 Feb;47(2):121–6.

6. Van Heel D a, Hunt K, Greco L, Wijmenga C: Genetics in coeliac disease. Best Pract Res Clin Gastroenterol 2005 Jun [cited 2014 Jan 28];19(3):323–39.

7. Hunt K a, Zhernakova A, Turner G, Heap G a R, Franke L, Bruinenberg M, et al.: Newly identified genetic risk variants for celiac disease related to the immune response. Nat Genet 2008 Apr [cited 2014 Jan 24];40(4):395–402.

8. Celiac Support Organisation: Commission regulation (EC) No 41/2009 concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten http://www.csaceliacs.info/celiac_disease_defined_european_union_commission.jsp

   Date of access: 15.1.2014

9. Real A, Comino I, de Lorenzo L, Merchán F, Gil-Humanes J, Giménez MJ, et al.: Molecular and immunological characterization of gluten proteins isolated from oat cultivars that differ in toxicity for celiac disease. PLoS One 2012 Jan [cited 2014 Jan 28];7(12):e48365.

10. Dewar DH, Amato M, Ellis HJ, Pollock EL, Gonzalez-Cinca N, Wieser H, et al.: The toxicity of high molecular weight glutenin subunits of wheat to patients with coeliac disease. Eur J Gastroenterol Hepatol 2006 May;18(5):483–91.

11. Mitea C, Salentijn EMJ, van Veelen P, Goryunova S V, van der Meer IM, van den Broeck HC, et al.: A universal approach to eliminate antigenic properties of alpha-gliadin peptides in celiac disease. PLoS One 2010 Jan [cited 2014 Jan 24];5(12):e15637.

12. Sollid LM, Qiao S-W, Anderson RP, Gianfrani C, Koning F: Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. Immunogenetics 2012 Jun [cited 2014 Jan 27];64(6):455–60.

13. Bergseng E: Peptide binding to HLA-DQ2 and developmend of blocking agents for the treatment of celiac disease, Doctoral thesis. University of Oslo, 2007.

14. Browning TH, Trier JS: Organ culture of mucosal biopsies of human small intestine. J Clin Invest 1969 Aug;48(8):1423–32.

15. Ciclitira PJ, Ellis HJ, Lundin KE: Gluten-free diet--what is toxic?. Best Pract Res Clin Gastroenterol 2005 Jun [cited 2014 Jan 28];19(3):359–71.

16. Lindfors K, Rauhavirta T, Stenman S, Mäki M, Kaukinen K: In vitro models for gluten toxicity: relevance for celiac disease pathogenesis and development of novel treatment options. Exp Biol Med (Maywood) 2012 Feb [cited 2014 Jan 27];237(2):119–25.

17. Wieser H, Koehler P: The Biochemical Basis of Celiac Disease. Cereal Chem 2008 Jan; 85:1–13.

18. Arentz-Hansen H, Körner R, Molberg O, Quarsten H, Vader W, Kooy YM, et al.: The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J Exp Med 2000 Feb 21;191(4):603–12.

19. Ellis HJ, Pollock EL, Engel W, Fraser JS, Rosen-Bronson S, Wieser H, et al.: Investigation of the putative immunodominant T cell epitopes in coeliac disease. Gut 2003 Feb;52(2):212–7.

20. Arentz–Hansen H, Mcadam SN, Molberg Ø, Fleckenstein B, Lundin KE a., Jørgensen TJD, et al.: Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. Gastroenterology 2002 Sep [cited 2014 Jan 24];123(3):803–9.

21. Kim C-Y, Quarsten H, Bergseng E, Khosla C, Sollid LM: Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. Proc Natl Acad Sci 2004 Mar 12;101(12):4175–9.

22. Dørum S, Arntzen MØ, Qiao S-W, Holm A, Koehler CJ, Thiede B, et al.: The preferred substrates for transglutaminase 2 in a complex wheat gluten digest are Peptide fragments harboring celiac disease T-cell epitopes. PLoS One 2010 Jan [cited 2014 Mar 7];5(11):e14056.

23. Bielekova B, Martin R: Antigen-specific immunomodulation via altered peptide ligands. J Mol Med (Berl) 2001 Oct [cited 2014 Feb 13];79(10):552–65.

24. Vartdal F, Johansen B, Thorpej CJ, Stevanovic S, Eriksen JE, Thorsby E. The peptide binding motif of the disease associated HLA-DQ (αl* 0501, βl* 0201) molecule. 1996;2764–72.

25. Sollid LM, Khosla C: Future therapeutic options for celiac disease. Nat Clin Pract Gastroenterol Hepatol 2005 Mar [cited 2014 Jan 24];2(3):140–7.

8. APPENDIX

8.1. Example of data analysis

patient D	1	2	3	SD	MEAN	CV	SI
blank	221	262	221	23.67136	234.6667	0.100872	
gluten	524	498	676	96.14572	566	0.169869	2.411931
ovalbumin	152	326	280	90.16282	252.6667	0.356845*	1.076704
peptide 1	440	396	312	65.03332	382.6667	0.169948	1.630682
peptide 3	329	399	245	77.10599	324.3333	0.237737*	1.382102
peptide 4	481	565	291	140.3757	445.6667	0.314979*	1.899147
peptide 5	271	312	178	68.661	253.6667	0.270674*	1.080966

*Where CV was 0.2 or more, the value which deviate the most was excluded and SD, MEAN, CV and SI calculated again

patient D	1	2	3	SD	MEAN	CV	SI
blank	221	262	221	23.67136	234.6667	0.100872	
gluten	524	498	676	96.14572	566	0.169869	2.411931
ovalbumin	326	280		32.52691	303	0.10735	1.291193
peptide 1	440	396	312	65.03332	382.6667	0.169948	1.630682
peptide 3	329	399		49.49747	364	0.135982	1.551136
peptide 4	481	565		59.39697	523	0.11357	2.228693
peptide 5	271	312		28.99138	291.5	0.099456	1.242187