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Immune Responses to Cereal Prolamin Proteins in Coeliac Disease

A thesis submitted for the degree of Doctor of Philosophy

by

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University of Dublin

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TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION

CHAPTER 4. COELIAC T CELL LINE RESPONSES TO THE PROLAMINS; GLIADIN, SECALIN, HORDEIN AND AVENININ

DECLARATION

I declare that this thesis is my own work and has not been submitted previously for any degree at this or any other university and except where otherwise stated, it is entirely my own work.

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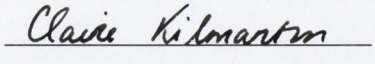

Claire Kilmartin

This thesis is dedicated to Mam and Dad

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- Feighery L, Lynch S, **Kilmartin C**, Abuzakouk M, Weir D, Jackson J, Feighery C. Flow-cytometric detection of lactase expression in normal and coeliac intestinal epithelium. *Eur J Gastroenterol Hepatol* 2001;13:897-902

Poster presentations

- **Kilmartin C**, Lynch S, Feighery L, Abuzakouk M, Feighery C. Effects of gliadin and other stimuli on enterocyte lactase and HLA-DR expression in duodenal biopsy culture.
  - Irish Society of Immunology, Dublin, Ireland, September 1999.

- **Kilmartin C**, Lynch S, Abuzakouk M, Feighery C. Immune responses to gliadin but not to avenin in organ culture studies of coeliac biopsies.
  - Irish Society of Gastroenterology, Dublin, Ireland, Summer 2000.
  - Digestive Disease Week, Atlanta, Georgia, May 2001.
  - 11th International Congress of Immunology, Stockholm, Sweden, July 2001.

Oral Presentations

- **Kilmartin C**, Lynch S, Abuzakouk M, Feighery C. Immune responses to gliadin but not to avenin in organ culture studies of coeliac biopsies.
  - British Society of Immunology, Harrogate, Yorkshire, England. Winter, 2000.

- **Kilmartin C**, Lynch S, Abuzakouk M, Weiser H, Feighery C. Avenin fails to induce a Th1 response in coeliac tissue following *in-vitro* culture.
  - 10th International Symposium on Coeliac Disease 2002, Paris, France, June 2002.

ABBREVIATIONS

Abbreviation	Meaning
aa	Amino acid
AGA	Anti-gliadin antibody
Ap-1	Activation protein-1
APC	Antigen presenting cell
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
Con A	Conconavalin A
CPM	Counts per minute
Ct	Threshold cycle
DAG	Diacylglycerol
DH	Dermatitis herpetiformis
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EATL	Enteropathy associated T cell lymphoma
EB/AO	Ethidium Bromide/Acridine orange
EBV	Epstein-Barr virus
ECP	Eosinophil cationic protein
EDTA	Ethylene diaminetetra-acetic acid
eIF2	Eukaryotic initiation factor 2
ELISA	Enzyme linked immunosorbent assay
EMA	Endomysial antibody
ER	Endoplasmic reticulum
Erk	Extracellular receptor-activated kinase
ESPGAN	European society of pediatric gastroenterology and nutrition
FADD	FAS-associated death domain
FAM	6-carboxylfluorescein
FCS	Foetal calf serum
FF	Frazer's Fraction
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gram

GALT	Gut associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFD	Gluten free diet
GVHD	Graft-versus-host-disease
h	Hour
HEX	Hexachloro-6-carboxyfluorescein
HLA	Human leukocyte antigen
HMW	High molecular weight
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
ICE	Interleukin-1β-converting enzyme
ICOS	Inducible co-stimulator
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
JOE	2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein
KGF	Keratinocyte growth factor
L	Ligand
l	Litre
LAT	Linker of activation in T cells
LFA	Leukocyte function associated antigen
LMW	Low molecular weight
LP	Lamina propria
LPH	Lactase-phlorizinhydrolase
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharide
M	Molar
MAdCAM-1	Mucosal addressin cell adhesion molecule 1

MAP	Mitogen activated protein
MBP	Major basic protein
ME	2-Mercaptoethanol
MFI	Median Florescence Intensity
MHC	Major histocompatibility complex
MIF	Migration inhibition factor
min	Minute
ml	Mililitre
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
M_r	Molecular weight
mRNA	Messenger RNA
NAC	Non-amplification control
NK	Natural killer cell
NTC	No template control
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDARs	Pre-developed assay reagents
PE	Phycoerythrin
PEP	Prolyl endopeptidase
PHA	Phytohaemagglutenin
pI	Isoelectric point
PIP_2	Phosphatidylinositol 4, 5 biphosphate
PKC	Phosphokinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PT	Peptic tryptic
PWM	Pokeweed mitogen
R	Receptor
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Room temperature

RT-PCR	Reverse transcription PCR
SI	Stimulation index
SLP	Second linker protein in T cells
S-poor	Sulphur poor
S-rich	Sulphur rich
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TAMRA	6-carboxytetramethylrhodamine
TAP	Transporter associated protein
TCC	T cell clone
TCL	T cell line
TCR	T cell receptor
TET	Tetrachloro-6-carboxylfluorescein
TGF	Transforming growth factor
Th	T helper cell
Tm	Melting temperature
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain
TRAF 2	TNF receptor-associated factor-2
Treg	T regulatory cell
tTG	Tissue transglutaminase
TUNNEL	Terminal deoxynucleotidyl nick-end labelling
UTR	Untranslated region
WGA	Wheat germ agglutinin
ZAP-70	Zeta associated protein 70

SUMMARY

Coeliac disease is an inflammatory disease of the small intestine, precipitated in susceptible individuals by gliadin, the alcohol soluble (prolamin) fraction of wheat gluten. There is a strong genetic influence on susceptibility to develop coeliac disease. Ninety to ninety-five percent of patients are HLA-DQ2 positive which is coded by DQA1*0501 and DQB1*0201 genes either in *cis* or in *trans*. The disease is characterised by crypt hyperplasia, villous atrophy and increased inflammatory cells in the epithelium and lamina propria. The pathogenesis is currently explained by the immunological hypothesis, which proposes that coeliac disease is being driven by an abnormal T cell response to gliadin. The enzyme tissue transglutaminase enhances T cell recognition of gliadin. In addition to gliadin, similar prolamin fractions of barley and rye (hordein and secalin respectively) are thought to activate the disease process. The safety of oats and its prolamin avenin is more controversial.

The aim of this thesis was to develop *in vitro* assays to determine the 'toxicity' of the prolamin avenin. Additional studies were also carried out with hordein and secalin. The following were investigated as indicators of their toxicity and / or immogenicity: (1) brush border lactase expression, (2) mucosal endomysial antibody (EMA) production, (3) pro-inflammatory cytokine production and (4) T cell proliferation.

In the initial experiments, intestinal biopsies from coeliac patients were cultured in the presence of gliadin or gluten. Enterocytes, isolated from the tissue, were then assessed flow cytometrically for lactase and HLA-DR expression. We found that culture with the wheat proteins for periods up to 48 hours had no effect on these markers. We then stimulated biopsies with the T cell mitogens, OKT3 and PMA. T cell activation and cytokine production have been associated with mucosal damage in coeliac disease. While, prolonged culture with the mitogens increased enterocyte HLA-DR expression, lactase levels remained unchanged. We found that TNF-α reduced lactase expression on enterocytes following 48 hours culture. We proposed that this was due to the pro-apoptotic nature of this cytokine. The effect could be blocked in the majority of cases with an antibody to TNF receptor 1. The results indicate that enterocyte lactase reduction is a slow process *in vitro* and is therefore unsuitable as a marker of prolamin 'toxicity'.

In our second set of investigations we assessed mucosal EMA production following biopsy culture with gliadin. We found that EMA was produced at the mucosal site and was specific for coeliac patients. However, antibody production was not induced by gliadin. Our results also indicated that mucosal EMA production was not a function of the length of the gluten free diet nor was it dependant on the inflammatory infiltrate in the mucosa. We concluded that mucosal EMA production was not a good marker of prolamin immunogenicity *in vitro*.

In a subsequent study we used the cytokines IFN-γ and IL-2 as evidence of immune activation *in vitro*. Duodenal biopsies from coeliac and control patients were cultured with peptic tryptic (PT) gliadin or PT avenin for 4 hours. Cytokine mRNA was quantified by TaqMan PCR and secreted cytokine protein was measured in the culture supernatant by enzyme-linked immunosorbent assay. After culture with PT gliadin, an increase in IFN-γ mRNA was observed in all nine patients with coeliac disease. Increased IFN-γ protein was also found in four of these patients. Smaller increases in IL-2 mRNA were detected in six subjects with increased IL-2 protein found in two patients. In contrast to PT gliadin, there was no significant IFN-γ or IL-2 response when coeliac biopsies were cultured with PT avenin. Similarly, biopsies from normal controls did not respond to PT gliadin or PT avenin stimulation. The findings of this study are in keeping with *in vivo* studies, which report that oats are safe for consumption by coeliac patients.

A disadvantage of the organ culture approach described above is the restriction on the number of experiments, which can be performed. It was impossible to compare the response to two different cereal fractions in the same individual. For this reason we established intestinal T cell lines from coeliac patients for our next study. We found that the T cells proliferated and produced IL-2 and IFN-γ in response to gliadin, secalin, hordein and avenin. We discovered that tissue transglutaminase enhanced T cell recognition of gliadin, secalin and hordein but did not affect avenin to the same extent.

Until now our knowledge of immune responses to prolamin proteins has been limited to gliadin. The findings of this thesis have helped define the immunogenicity of secalin, hordein and avenin in coeliac disease.

CHAPTER 1

General Introduction

1.1 GUT ASSOCIATED LYMPHOID TISSUE

The intestinal epithelium is continuously protected from luminal antigens by the gut associated lymphoid tissue (GALT). The GALT encompasses the Peyer's patches, mesenteric lymph nodes (MLNs) and the lymphocytes of the lamina propria and epithelium, which are dispersed throughout the intestine (Köhne *et al.* 1996).

Antigens can gain access to the GALT via two routes; through M cells, microfolded epithelial cells overlying the Peyer's patches and through villus epithelial cells (Köhne *et al.* 1996) (Figure 1.1). Peyer's patches are structurally similar to other secondary lymph nodes i.e. they consist of a varying number of follicles with a central dome of B cells surrounded by T cells and macrophages (Janeway and Travers 1997b). Naïve T and B cells encounter antigen in the Peyer's patch and become activated. They then leave via MLNs, lymphatics and the thoracic duct and re-circulate back to mucosal surfaces where they act as effector cells throughout the length of the intestine (Köhne *et al.* 1996; Mowat and Viney 1997).

Lymphocyte homing to the mucosa is achieved by the upregulation of adhesion molecules belonging to the integrin family, namely $\alpha4\beta7$ and $\alpha E\beta7$ (Shaw and Brenner 1995). $\alpha4\beta7$ is expressed on most lamina propria lymphocytes (LPLs). This integrin binds to its endothelial ligand, mucosal addressin cell adhesion molecule 1 (MAdCAM-1), which is selectively expressed by venules in mucosal tissues (Shaw and Brenner 1995). $\alpha4\beta7$ is important for the movement of blasts from the Peyer's patches into the intestinal mucosa (Hamann *et al.* 1994). $\alpha E\beta7$ is expressed on 40% of LPLs and nearly all intraepithelial cells (IELs). This integrin ligates to E cadherin, which is expressed on enterocytes (Kilshaw 1999), and is thought to be involved in maintaining IELs within the epithelial layer (Cepek *et al.* 1994).

1.2 ORAL TOLERANCE

Under normal circumstances, cells of the GALT do not mount an active immune response against dietary antigens and normal gut flora. This form of peripheral tolerance is known as oral tolerance and can be defined as "the specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral route" (Faria and Weiner 1999). Oral tolerance is a T cell dependant

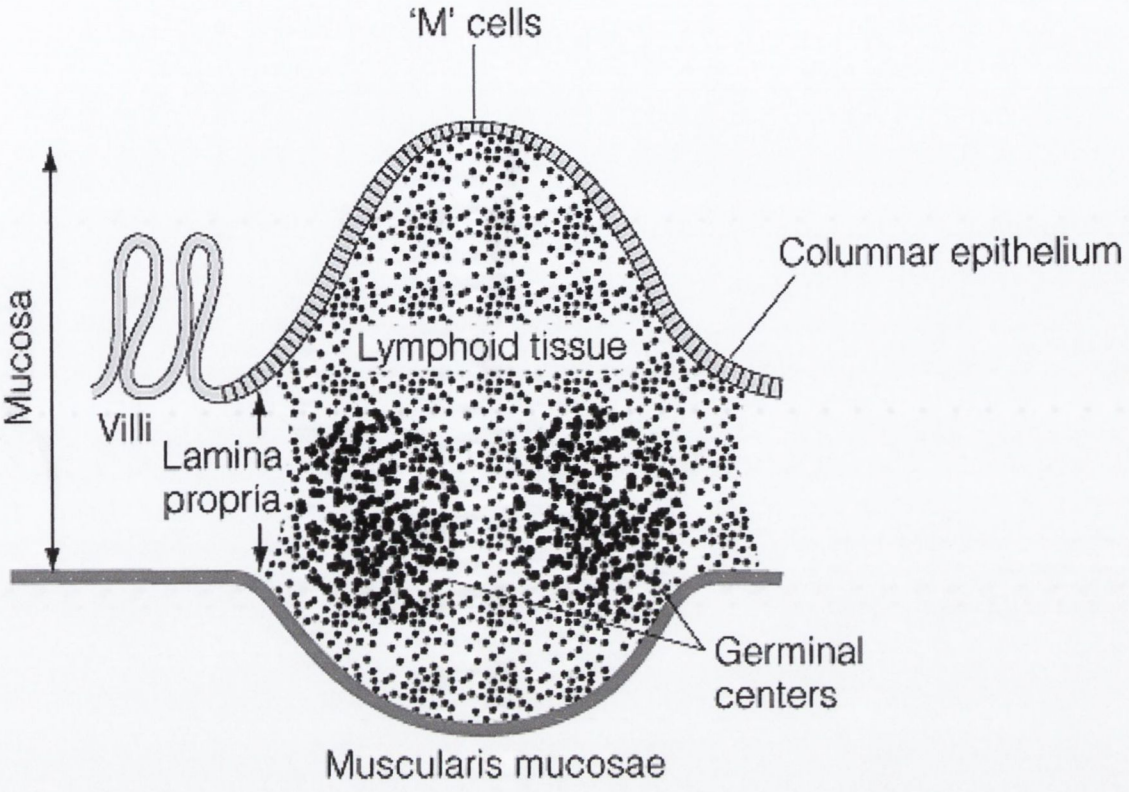


Figure 1.1 M cells and an underlying Peyer's patch. Antigens gain access to the GALT via M cells. Naïve T and B cells encounter antigen in the Peyer's patch and become activated. B cells are localised in the germinal centres and are surrounded by T cells and macrophages.

phenomenon and is mediated by more than one mechanism. The following are some of the main processes thought to be involved; clonal deletion, anergy, regulatory T cells and cytokines.

1.2.1 Mechanisms of oral tolerance

Clonal deletion

Clonal deletion of $CD4^+$ T cells via apoptosis has been demonstrated following oral administration of ovalbumin (OVA) to T cell receptor (TCR) transgenic mice (Chen *et al.* 1995). A 10-20% reduction in $CD4^+$ T cells was noted in the spleen, thymus and all other lymphoid organs when mice were fed high doses of OVA (Chen *et al.* 1995). Recent studies have indicated a possible role for FAS mediated apoptosis in oral tolerance induction (Marth *et al.* 1998).

Anergy

Anergic T cells are induced when antigen is presented in the absence of co-stimulatory molecules (Mueller *et al.* 1989). These non-proliferating cells do not secrete interleukin-2 (IL-2) nor express the IL-2 receptor (IL-2R) (Melamed and Friedman 1993). This anergic state, however, can be reversed *in vitro* by incubation with IL-2 (Melamed and Friedman 1993).

Regulatory T cells

Initially a population of suppressor $CD8^+$ T cells were thought to be involved in tolerance induction. It was discovered that $CD8^+$ splenocytes from a tolerised mouse could transfer tolerance to a naïve animal (Mowat 1985). However, in mice that were injected with anti-CD8 antibodies and in β_2 microglobulin deficient mice oral tolerance was also induced (Vistica *et al.* 1996). Similarly in mice depleted of $CD8^+$ cells tolerance to OVA was normal (Barone *et al.* 1995).

Oral tolerance, however, cannot be achieved in CD4 depleted mice (Barone *et al.* 1995; Garside *et al.* 1995) and most workers now agree that $CD4^+$ T cells are essential for oral tolerance. It was thought that T helper (Th) 2 cells were mediating tolerance by downregulating Th1 responses (see section 3.1.2 for a description of Th1 and Th2 cells). More recently populations of Th cells that secrete suppressive cytokines have been discovered; Th3 cells (which produce significant amounts of transforming growth factor

beta (TGF-β) and varying amounts of IL-4 and IL-10) and T regulatory (Treg) 1 cells (which produce high levels of IL-10, significant amounts of TGF-β and little or no IL-4) (Battaglia *et al.* 2002).

Regulatory cytokines

The suppressor cytokines, which have received most interest, are IL-4, IL-10 and TGF-β. Rizzo *et al.* (1999) carried out experiments with IL-4 knock-out mice and concluded that the cytokine was essential for oral tolerance that is induced by regulatory cells. It has been proposed that IL-4 is a differentiation factor for TGF-β secreting Th3 cells (Inobe *et al.* 1998). Others have argued that IL-4 does not play a role in tolerance (Powrie *et al.* 1996). IL-10 was also reported to be involved in tolerance induction by regulatory cells and to protect against autoimmunity (Rizzo *et al.* 1999). Others have reported that oral tolerance remains intact in IL-10 deficient mice (Fowler and Powrie 2002).

TGF-β is involved in tolerance induction in mice and is regulated by IL-12. Anti-IL-12 administration enhances TGF-β production and increases suppression of proliferatative responses following antigen challenge (Marth *et al.* 1996). Mice that are tolerant to haptenized colonic proteins have increased levels of TGF-β and decreased levels IL-12 in their colons. Tolerance is abrogated by the administaion of anti-TGF-β or IL-12 (Neurath *et al.* 1996). TGF-β can also induce bystander suppressive effects, which are defined as the inhibition of a T cell response to an antigen co-administered with the tolerogen (Powrie *et al.* 1996).

1.2.2 Factors affecting oral tolerance

Intestinal absorption and antigen presentation

M cells are adapted for antigen uptake (Köhne *et al.* 1996). They preferentially transcytose insoluble, particulate antigen across the cell (Mayer 2000). While major histocompatibility complex (MHC) class II containing vesicles have been found in these cells (Allan *et al.* 1993), it is more likely that antigen is taken up and processed by professional antigen presenting cells (APCs) once it has passed through the M cell (Faria and Weiner 1999).

Intestinal epithelial cells (IECs) constitutively express both classical and non-classical MHC molecules and can act as APCs (Kaiserlian *et al.* 1989; Mayer and Schlien 1987). IECs have been shown to preferentially activate $CD8^+$ suppressor cells. It is thought that the molecule CD1d is involved in $CD8^+$ T cell activation rather than MHC class I or II molecules (Panja *et al.* 1993). Epithelial cells stimulate $CD4^+$ T cells via MHC class II-mediated pathways. IECs lack co-stimulatory molecules, which would promote anergy in these $CD4^+$ T cells (Sanderson *et al.* 1993).

Dose of antigen

The tolerance mechanism induced is often determined by the dose of antigen administered. High doses of antigen promote clonal deletion or anergy of reactive T cells (Chen *et al.* 1995; Friedman and Weiner 1994). In addition several dosing regimens appear to favour anergic responses (Friedman 1996). Lower doses of antigen activate Tregs that secrete suppressive cytokines (Friedman and Weiner 1994).

Genetics

It has been reported that tolerance to OVA in mice depends on genetic make-up (Lamont *et al.* 1988). The genes involved have not been fully investigated. MHC genes seem to play a role, although they are not the only genes involved (Mayer 2000).

Age

The age at which animals first encounter antigen is important in determining whether tolerance occurs after feeding. Animals exposed to antigen during the very early stages of life do not develop tolerance (Hanson 1981). Intestinal permeability is greater in younger animals and increased exposure to luminal antigens may account for lack of tolerance (Mayer 2000). In addition, the immune system in these animals has not yet fully matured (Faria and Weiner 1999).

A breakdown in oral tolerance may arise due to abnormalities in some of the above. Inflammatory bowel disease (IBD) reflects a breakdown in tolerance to normal gut bacterial flora and may be the result of an immunoregulatory defect (Groux and Powrie 1999). Another example where tolerance is abrogated is coeliac disease. Coeliac disease is an inflammatory disease of the proximal small intestine that results from an intolerance to certain dietary cereals.

1.3 COELIAC DISEASE

1.3.1 History of coeliac disease

Gee was the first to recognise that diet was responsible for the chronic indigestion and steatorrhea seen in patients with the "Coeliac Affection" (Gee 1888). Consequently, many diets such as the banana diet were recommended to alleviate patients' symptoms. While the diets may have been effective in this regard, they failed to identify the cause of the condition. In his MD thesis for the university of Utrecht, Dicke demonstrated that the anorexia, steatorrhoea and increased amount of faeces seen in coeliac patients was in fact caused by wheat and that removal of the cereal from the diet caused the signs and symptoms to disappear (Dicke 1950). This finding was later supported by Rubin *et al.* (1962), who introduced wheat into the ileum of coeliac patients and noted that it caused distension, diarrhoea, steatorrhoea and abdominal discomfort. At the area of wheat injection and distally throughout the length of the ileum there was loss of villi, increased lymphocytes in the lamina propria, and increased mitosis in the crypts. There was no evidence of histological damage in control patients.

Dicke together with van de Kamer and Weijers demonstrated that wheat flour and not starch was responsible for the increased faecal fat in patients (Dicke *et al.* 1953). Other starch containing foods such as cornflour, maize starch, rice flour and potatoes were also harmless. Rye flour and oats were also reported to have an unfavourable effect on patients (Dicke *et al.* 1953). Results were confirmed by Anderson *et al.* (1952) who reintroduced wheat flour, starch or gluten into the coeliac diet after a wheat-free period and noted that wheat flour and wheat gluten caused a loss of appetite, diarrhoea, loss in weight and a change in temperament while wheat starch did not.

1.3.2 Clinical symptoms

In childhood typical symptoms include chronic diarrhoea, failure to thrive, abdominal distention, poor appetite and irritability (Kelly *et al.* 1990). In adults the classic signs of steatorrhoea, weight loss and fatigue are now rarely encountered. There has been an increase in patients presenting with milder or atypical symptoms (Maki and Collin 1997).

Mucosal damage in the coeliac small intestine results in the malabsorption of nutrients. Coeliac patients are at a high risk of developing osteopenia or osteoporosis (Mora *et al.* 1999; Valdimarsson *et al.* 1996) as a result of impaired calcium and vitamin D uptake. Iron deficiency anemia is quite common among patients, which may result in fatigue (Carroccio *et al.* 1998). Women with untreated coeliac disease may present with infertility, spontaneous and recurrent abortion and low birth weight. Infertility among men is also common (Sher *et al.* 1994). Some patients with coeliac disease also present with neurological manifestations. Gluten ataxia is the most common. Patients may also present with epilepsy, posterior cerebral calcifications, intellectual deterioration and brain atrophy (Collin *et al.* 1991; Gobbi *et al.* 1992). Gluten sensitivity may manifest as an IgA mediated skin disease called dermatitis herpetiformis (DH). DH patients have coeliac disease and their skin rash is gluten dependent (Fry 2002).

1.3.3 Diagnosis

Biopsy

In 1970 the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) laid down criteria for the diagnosis of coeliac disease (Meeuwisse 1970). Three biopsies were necessary for diagnosis: an initial biopsy showing a flat mucosa, a second demonstrating an improvement in architecture while taking a gluten free diet (GFD) and a final biopsy showing a deterioration of the mucosa with gluten challenge. In 1990 the criteria were revised due to the increased use of sensitive and specific serologic tests (Working group report 1990). Now if typical or atypical symptoms are present and serology is positive it is sufficient to take a single biopsy to confirm diagnosis. A clinical and serological improvement on a GFD is adequate to monitor recovery. A demonstration of mucosal improvement on a GFD may be required in the case of asymptomatic patients (Farrell and Kelly 2001).

Serology

Anti-gliadin antibodies (AGAs)

Coeliac patients produce antibodies that are directed towards gliadin (Maki 1995). Enzyme linked immunosorbent assays (ELISAs) detecting serum IgA and IgG antibodies were first used in the early 1980s to diagnose coeliac disease. However, they had only a moderate sensitivity (IgA; 75-90%, IgG; 69-85%) and specificity (IgA; 82-95%, IgG; 73-90%) (Farrell and Kelly 2001). Gliadin antibodies are found in other gastrointestinal

diseases such as IBD, cows milk protein intolerance and also in healthy individuals (Uibo *et al.* 1993). Positivity for gliadin antibodies seems to increase with age in the normal population (Uibo *et al.* 1993). There is a better correlation between gliadin antibodies and villous atrophy in children than in adults (Maki 1995).

Anti-reticulin antibodies

Coeliac patients were found to have serum antibodies that bound to antigens in rat kidney, liver and stomach (Seah *et al.* 1971). Immunofluorescence staining produced a distinctive staining pattern. IgA class reticulin antibodies gave a sensitivity of 97% and a specificity of 98% (Maki 1995).

Endomysial antibodies

Antibodies in coeliac patients' serum were found to react not only with rodent tissues but also primate and human tissues. These became known as endomysial antibodies (EMAs) because they bind to the endomysium of monkey oesophagus; the connective tissue, which lines the smooth muscle bundles (Chorzelski *et al.* 1983). In the laboratory the EMAs, which are fluorescently labeled, bind to the endomysium producing a distinctive honeycomb pattern. Human umbilical cord has also proved to be an effective substrate and thus the use of material from endangered species is avoided (Corroccio *et al.* 1996). The test has a sensitivity of 94% and a specificity of 99% (James and Scott 2000). EMAs are a useful predictor of coeliac disease. Many latent coeliacs who are EMA positive with a normal mucosa later develop a flat mucosa (James and Scott 2000). However, the technique of indirect immunofluorescence for IgA EMA is both subjective and labour intensive.

Anti-tissue transglutaminase antibodies

The target antigen of the EMA was recently identified as tissue transglutaminase (tTG) (Dieterich *et al.* 1997). Since then an IgA ELISA using tTG as a substrate has been developed to screen for coeliac disease (Schuppan and Hahn 2001). The ELISA method is less subjective and laborious than the EMA immunoflorescence technique. Initial tTG ELISAs were based on guinea pig tTG and were reported to have a sensitivity of 90% and a specificity of 96% (Schuppan and Hahn 2001). Human recombinant tTG has now replaced guinea pig and has improved the sensitivity (96%) and specificity (99%) of the original ELISA test (Burgin-Wolff *et al.* 2002; Carroccio *et al.* 2002b; Sblattero *et al.* 2000). ELISAs with human tTG have been reported to be comparable (Burgin-Wolff *et*

al. 2002) and sometimes even more sensitive (Kumar *et al.* 2001; Sblattero *et al.* 2000) than the EMA immunofluorescence test.

1.3.4 Epidemiology

In the 1950s the incidence of coeliac disease was reported to be as low as 1/8000 in England and Wales and 1/4000 in Scotland (Davidson and Fountain 1950). Patients were diagnosed based on classic symptoms such as steatorrhoea, weight loss and failure to thrive. Later, diagnosis improved with the use of malabsorption tests and peroral biopsy techniques. Consequently, the number of reported cases grew to 1/450-500 in Ireland (Mylotte *et al.* 1973) and Scotland (Logan *et al.* 1986) in the 1970's.

At present there is an increased awareness of atypical forms of coeliac disease. The iceberg analogy is often used to describe the full spectrum of the disease (Figure 1.2). Above the waterline are typical overt patients who have been diagnosed by biopsy demonstrating a flat mucosa. Under the surface are patients with 'atypical' or 'silent' coeliac disease. Atypical patients have a diagnostic biopsy and positive serology but present with atypical symptoms such as anemia, infertility and osteoporosis (Murray 1999). Silent coeliacs also have a classic mucosal lesion and are EMA positive but are asymptomatic (Farrell and Kelly 2001). These patients are usually identified after serological screening of high-risk populations. Latent coeliac disease refers to patients presenting with a normal biopsy while on a normal diet who either before or since have had a flat biopsy that recovers with a GFD. These patients may have atypical symptoms such as anemia, delayed puberty, infertility and oral ulcers. Biopsies do not show signs of early enteropathy, however serum is EMA positive (Holmes 2001; Troncone *et al.* 1996). Potential coeliacs are patients who do not have a biopsy consistent with overt coeliac disease yet have immunological abnormalities similar to those found in coeliac patients. Markers include the presence of EMA, high numbers of IELs in particular those expressing the $\gamma\delta$ TCR and increased expression of CD25 (α chain of the IL-2R) and co-stimulatory molecule, B7, in the lamina propria (Holmes 2001; Troncone *et al.* 1996).

Now coeliac disease is reported to be one of the most frequent genetically based diseases occurring in 1 of 130-300 in the European population (Catassi *et al.* 1996; Kolho *et al.* 1998). Early screening in the US reported that the incidence of coeliac disease was

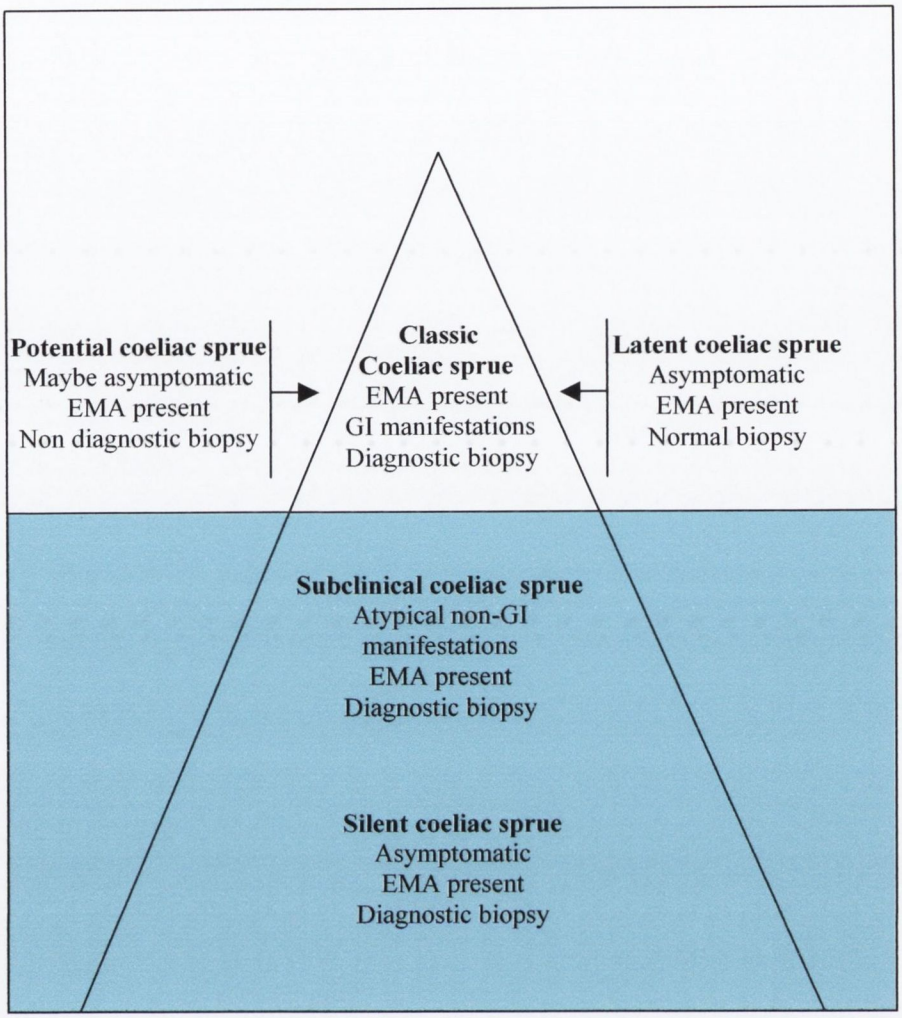


Figure 1.2 The coeliac iceberg model (taken from Farrell and Kelly 2001).

much lower than that of Europe (Rossi *et al.* 1993; Talley *et al.* 1994). However, better awareness in recent epidemiological studies has led to the conclusion that coeliac disease is as frequent in the US as in Europe (Berti *et al.* 2000; Not *et al.* 1998). Coeliac disease affects many ethnic groups. Cases have been reported in South America (Gandolfi *et al.* 2000), North Africa (Catassi *et al.* 1999), and Asia (Shahbazkhani *et al.* 2000; Yachha *et al.* 2000), although there is a greater prevalence in Caucasians.

In adults, peak incidence is in the fifth decade (Feighery 1999). A gradual rise in the age at diagnosis is apparent. This may be due to delayed introduction of gluten into the infant diet and increased diagnosis of older children and adults with atypical coeliac disease (Kennedy and Feighery 2000; Murray 1999). It is thought that the timing of gluten introduction and the quantity is important in precipitating the disease in children (Taminiau 1996). Females are more commonly affected than males. A female to male ratio of 3:1 has been observed (Feighery 1999).

1.3.5 Genetics

The MHC is a group of genes responsible for tissue compatibility. In humans this gene region is called the human leukocyte antigen (HLA) complex (Elgert 1996b). T cells recognise antigen bound to either HLA class I or class II molecules. The class I molecules are present in most nucleated cells. They bind endogenous peptides synthesised in the cytosol and present them to $CD8^+$ T cells. The HLA class II molecules are normally found on APCs (monocytes, macrophages, dendritic cells and B cells). These molecules bind exogenous proteins taken up by the cell by endocytosis. Peptides bound by HLA class II molecules are recognised by the TCR of $CD4^+$ T cells (Thorsby 1999). The HLA molecules are heterodimers of an α and β chain and are encoded by genes on the short arm of chromosome 6. An exception is the β chain of the class I molecules (β_2 microglobulin) which is encoded by a gene on chromosome 15 (Thorsby 1999). The polymorphic genes in the class I region encode the HLA-A, HLA-B and HLA–C molecules. The class II region includes genes encoding the monomorphic α chain and the polymorphic β chains of the DR molecules, as well as the polymorphic α and β chains of the DQ and DP molecules (Figure 1.3) (Janeway and Travers 1997a). Because of the polypmorphic nature of the genes many different variants of HLA molecules exist (Janeway and Travers 1997a).

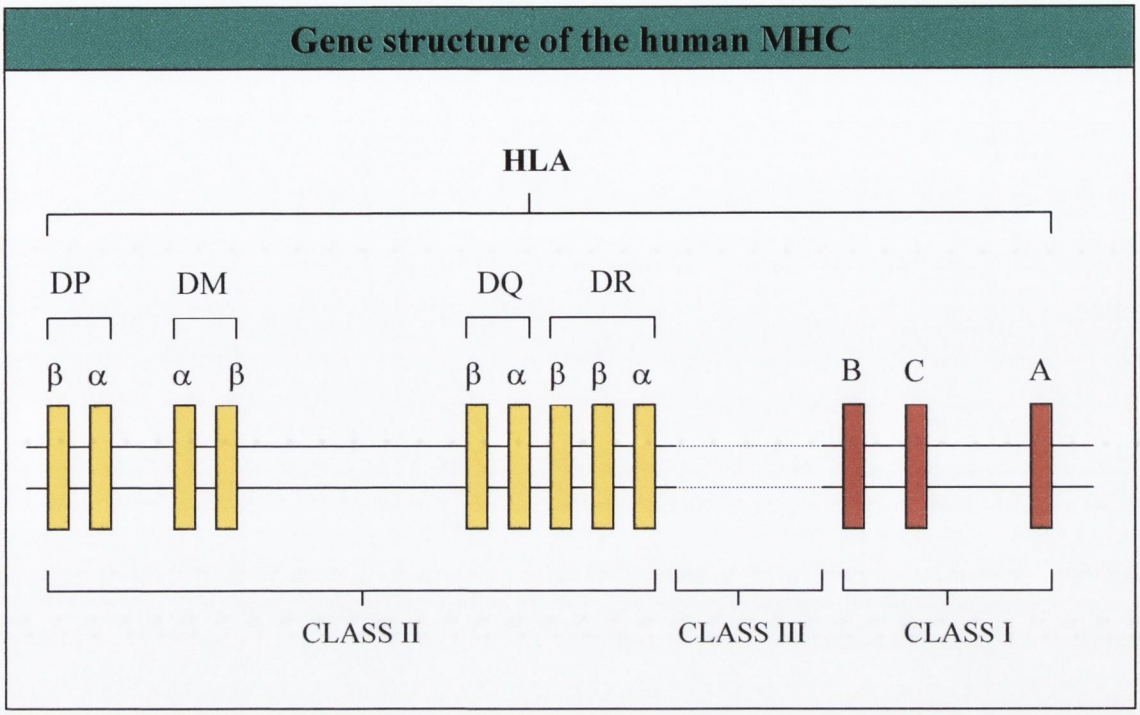


Figure 1.3 The genetic organisation of the major histocompatibility complex in humans. Depicted are the class I genes (HLA-A, -B and –C) and class II genes (HLA-DP, -DM, -DQ and –DR). The class III region encodes important molecules involved in immunity such as TNF and complement factors (adapted from Janeway and Travers 1997a).

There is a strong genetic influence on susceptibility to develop coeliac disease. A 70% concordance rate in monozygotic twins and a prevalence rate of 10% among first degree relatives implies a large degree of heritability (Ellis 1981; Polanco *et al.* 1981). The association between coeliac disease and the HLA antigen was established with Falchuck's observation of an increased frequency of HLA-B8 in coeliac patients (Falchuk *et al.* 1980). Later associations with DR3 (Keuning *et al.* 1976) and DR7 (Betuel *et al.* 1980) were reported. DR7 was frequently found in association with DR3 or DR5 (Mearin *et al.* 1983). More recently it was shown that the strongest HLA association was with DQ2 (Corazza *et al.* 1985; Tosi *et al.* 1983). Most coeliac patients carry the DR3-DQ2 haplotype or alternatively are heterozygous DR5-DQ7 / DR7-DQ2 (Sollid and Thorsby 1993). When sequenced it was discovered that these patients encode the same DQα and DQβ chains. The DQA1*0501 and DQB1*0201 can be encoded in cis by haplotype DR3-DQ2 or in trans by DR5-DQ7 / DR7-DQ2 (Figure 1.4). An increased risk for coeliac disease among individuals who are DR3-DQ2 homozygous and DR3-DQ2 / DR7-DQ2 heterozygous has been reported. It was proposed that the presence of a second copy of the DQB1*0201 allele resulted in the increased expression of the DQ(α1*0501 β1*0201) heterodimer (Ploski *et al.* 1993). HLA-DQ2 is present in 90-95% of patients (Peña *et al.* 1998). A small number of coeliac patients do not carry DQ2 but possess the DQ8 heterodimer DQ(α1*0301 β1*0302) together with DR4 (Spurkland *et al.* 1992).

The prevalence of HLA DQ2 is high in the normal population (20-30%) (Peña *et al.* 1998). This would suggest the involvement of non-HLA linked genes in conferring susceptibility to coeliac disease. A study carried out in western Ireland showed evidence for linkage of at least one non-HLA locus to coeliac disease (Zhong Fei *et al.* 1996). The polymorphic transporter associated protein (TAP) genes located in the MHC region have been analysed. The genes encode a protein involved in the loading of HLA class I molecules with antigen. Studies did not find definitive evidence for an association between TAP genes and coeliac disease (Djilali-Saiah *et al.* 1994; Meddeb-Garnaoui *et al.* 1995; Tighe *et al.* 1994). Associations with the cytokine gene tumor necrosis factor (TNF), also located in the MHC region, have been detected. The TNF haplotype TNF E (Peña *et al.* 1998) and the microsatellite alleles TNFb3 and TNFa2 (McManus *et al.* 1996) were found to be increased in coeliac patients. It was hypothesised that both could play a role in TNF secretion.

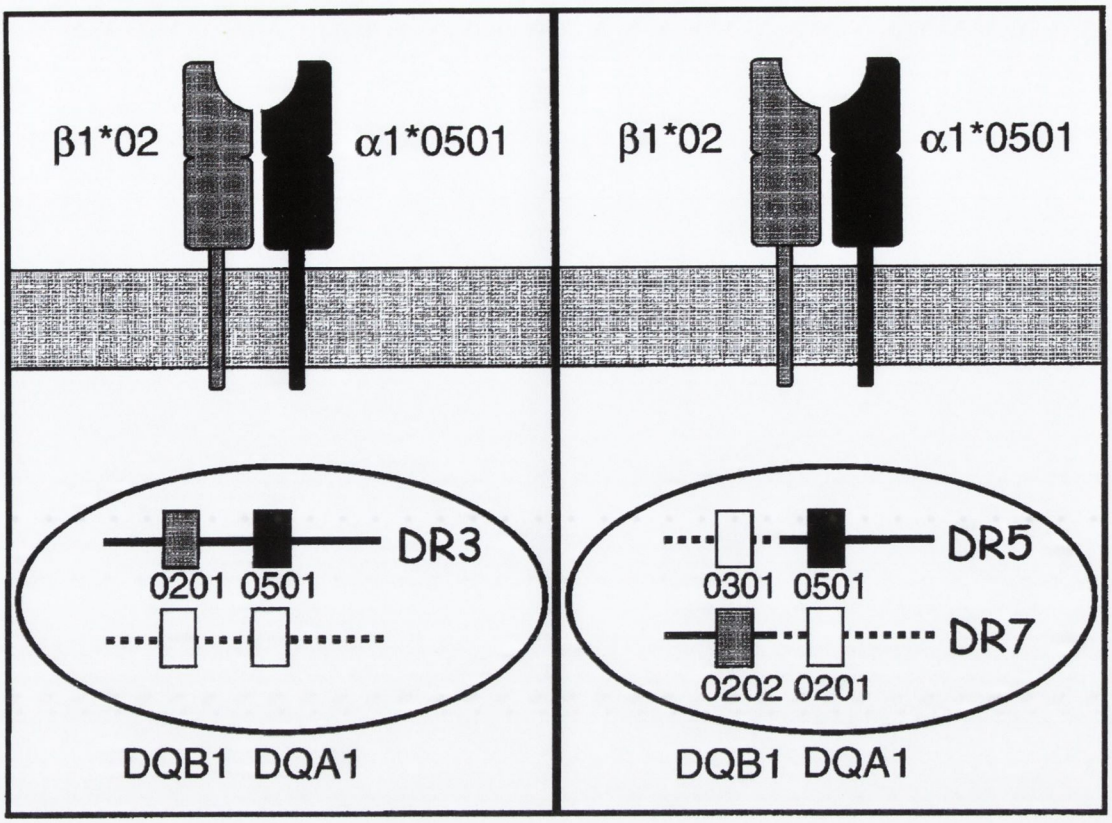


Figure 1.4 Cis and trans expression of HLA-DQ(α1*0501, β1*02) in coeliac patients. Those who are DR3 or DR5/DR7 heterozygous express the same HLA-DQ2 molecule, HLA-DQ(α1*0501, β1*02). The DQA1* 0501 and DQB1*02 genes are located in cis in DR3 individuals, whereas they are located in trans in DR5/DR7 heterozygous individuals (taken from Sollid 2000).

CTLA-4 plays a significant role in oral tolerance (Fowler and Powrie 2002) and the + 49 (A/G) single nucleotide polymorphism in exon 1 has been associated with autoimmunity (Nithiyananthan *et al.* 2002). A Finnish (Holopainen *et al.* 1999) and an Italian / Tunisian (Clot *et al.* 1999) study failed to find an association between CTLA-4 and coeliac disease. However, results from two Scandinavian (Popat *et al.* 2002; Torinsson Naluai *et al.* 2000) and one French group (Djilali-Saiah *et al.* 1998) indicated that there was an association between the A allele of the single nucleotide polymorphism in exon 1 and risk of coeliac disease.

The TGM2 gene encoding tTG was also investigated to see if a particular polymorphism was associated with coeliac disease. A certain isoform could result in increased or altered enzymatic activity in coeliac patients. No differences were observed in the coding sequence (Aldersley *et al.* 2000) or the exonic sequence including splice sites (Popat *et al.* 2001). The dinucleotide repeat polymorphism in the reporter region was also analysed. This site was examined as a marker of linkage and association and it was concluded that polymorphisms in the tTG gene are not linked to coeliac disease. It was hypothesised that polymorphisms in genes coding for proteins that regulate transcription could be involved in the increased tTG activity observed (van Belzen *et al.* 2001).

1.4 THE COELIAC MUCOSAL LESION

1.4.1 Morphology of the lesion

The coeliac lesion is localized in the proximal part of the small intestine and is characterized by villous atrophy, crypt hyperplasia and increased lymphocyte density in the epithelium and lamina propria. Marsh carried out a time course study on treated patients challenged with a peptic tryptic (PT) digest of gliadin and described the progression of the lesion (Marsh 1992; Marsh and Crowe 1995). The first stage is an infiltration of $CD8^+$ cells into the surface and crypt epithelium (type I) (Figure 1.5). While the villous architecture and crypt height remain unaltered, IELs increase to numbers of more than 40 IELs/100 epithelial cells. Crypt hypertrophy follows lymphocyte infiltration (type II). At this stage there is no obvious change in villous architecture. This lesion may be seen in treated patients with incomplete mucosal recovery or in patients that are progressing towards an advanced lesion (Oberhuber 2000). In the next stage there is a heightened loss of enterocytes from the surface to

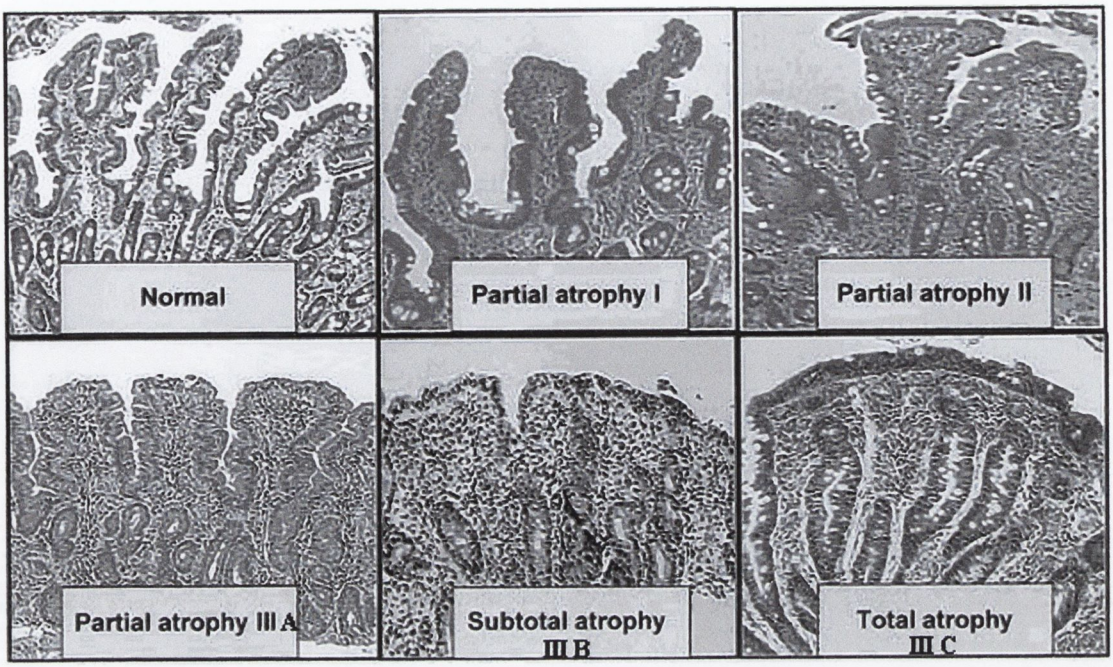


Figure 1.5 Progression of the coeliac mucosal lesion (taken from Fasano and Catassi 2001).

produce the classic flat lesion (type III). The villous atrophy is sometimes classified as partial (III A), sub-total (III B) or total (III C) (Working group report 2001). It is thought that the flat lesion is due to a loss of mature epithelial cells from the surface due to damage and a compensatory increase in enterocyte proliferation in the crypts. The enterocytes that lie on the surface are immature epithelial cells. They are small and cuboidal, have irregular nuclei and poorly developed microvilli (Strober 1976). This type of lesion is not specific for gluten sensitivity. Patients presenting with tropical sprue, autoimmune enteropathy, hypogammaglobulinaemia and food protein hypersensitivity have a similar histology (Oberhuber 2000).

1.4.2 Intraepithelial lymphocytes

IELs are one of the largest populations of lymphocytes in the body and are found between IECs close to the lumen (Cerf-Bensussan *et al.* 1993). IELs have a limited TCR repertoire compared to peripheral blood (Van Kerckhove *et al.* 1992). Eighty to 90% utilize the TCRαβ. A small, yet significant percentage (5%) express TCRγδ. Proportions of γδ cells in the gut have been reported to be higher than those found in peripheral blood (Brandtzaeg *et al.* 1989). Seventy to 90% of IELs are $CD8^+$. The CD8 molecule may be expressed on the surface of T cells as a homodimer or a heterodimer. The heterodimeric αβ form of the CD8 receptor is mainly expressed with only 5% expressing the homodimeric CD8αα form (Trejdosiewicz and Howdle 1995). All CD8αβ IELs express the TCRαβ, but the CD8αα population consists of both TCRαβs and γδs with all TCRγδ IELs being CD8αα (Guy-Grand and Vassalli 1993). The proportion of $CD4^+$ cells is low; a population of $CD4^+CD8\alpha\alpha^+$ T cells exist all bearing the TCR αβ (Lefrancois 1991). There is also a less than 5% minority of $CD4^-CD8^-$ cells (Trejdosiewicz and Howdle 1995).

All subsets of IELs express the adhesion molecule αEβ7 (Cerf-Bensussan *et al.* 1992). They also express α4β7. IELs have an activated phenotype expressing HLA-DR (Abuzakouk *et al.* 1996) and the memory T cell marker, CD45RO (Cerf-Bensussan *et al.* 1993).

Enumeration and phenotype of IELs in coeliac disease

The density of IELs is significantly raised in active coeliac disease. Ferguson and Murray (1971) quantified the number of IELs per 100 villous epithelial cells.

Lymphocyte counts in active coeliac patients were above the normal range of 40 IELs per 100 villous epithelial cells. IEL counts in patients on a GFD were lower than the untreated patients but higher than in controls. A recent report stated that the normal limit used in this study was too high and a limit of 25 IELs per 100 epithelial cells was more accurate (Hayat *et al.* 2002).

Marsh (1980) did not agree with a method of relating lymphocyte densities to epithelial cells because the epithelium in coeliac disease is affected by increased proliferation and reductions in surface area. He used an ocular graticule to obtain the total number of IELs overlying $125\mu m^2$ of muscularis mucosae. He found that areal densities of IELs in coeliac specimens pre-GFD were significantly reduced when compared to controls. Arial densities after GFD did not differ from controls. However, most researchers still use IELs per 100 enterocytes as a measure of IEL density.

Increased IEL densities have been reported in pediatric coeliac patients (Mavromichalis *et al.* 1976), DH patients (Fry *et al.* 1972) and latent coeliacs (Marsh *et al.* 1990). IEL counts in these patients are also dependant on gluten intake. Increased IEL counts are not specific for coeliac disease and have been reported in other enteropathies and autoimmune diseases (Ferguson and Murray 1971; Mavromichalis *et al.* 1976). Therefore, a raised IEL count with normal villous architecture does not always denote latent coeliac disease (Johnston *et al.* 1999).

IELs are larger during the active phase of coeliac disease and decrease in size when gluten is withdrawn from the diet (Marsh 1980). The $CD8^+$ IELs have increased expression of CD45RO, from 24% in controls to 55% in untreated patients (Halstensen *et al.* 1990). IELs have a higher rate of mitosis (Marsh 1980) and increased expression of the proliferation marker Ki67 (Halstensen and Brandtzaeg 1993). CD5 expression, which is upregulated during T lymphocyte activation is increased on $CD8^+$ IELs (Malizia *et al.* 1985; Selby *et al.* 1981). CD7 expression is also upregulated on $CD4^+$ IELs (Malizia *et al.* 1985).

The activated phenotype and the reduction in IEL numbers following gluten withdrawal would suggest that these cells are activated by gliadin. However it is difficult to persuade most IELs to undergo antigen expansion (Ebert 1989). It has been suggested that activation of IELs is only secondary to cytokine release from lamina propria T cells

(Halstensen and Brandtzaeg 1993). The fact that gluten specific $CD8^+$ clones have not been isolated from coeliac patients supports this theory.

In coeliac patients the percentage of double negative ($CD4^-CD8^-$) IELs is increased (Verkasalo *et al.* 1990) and persists during gluten elimination. It has also been observed that the number of cells expressing TCRγδ are also elevated in the epithelium of coeliac patients (Spencer *et al.* 1991). $TCR\gamma\delta^+$ numbers remain constant during gluten elimination while $TCR\alpha\beta^+$ cells return to normal (Spencer *et al.* 1991). Increased γδ levels have also been found in the jejunum of DH patients (Savilahti *et al.* 1992) and patients with latent coeliac disease (Maki *et al.* 1991). As with increased IEL density, increases in TCR $\gamma\delta^+$ IELs are not specific for coeliac disease (Spencer *et al.* 1991). The persistence of γδ T cells in coeliac tissue after the mucosa has recovered suggests that they are not involved in epithelial damage. In fact it has been suggested that γδ T cells can secrete keratinocyte growth factor (KGF) and therefore promote epithelial cell growth (Boismenu and Havran 1994). Antigen specific γδ TCR cells appear to be anergic and may be involved in oral tolerance (Barrett *et al.* 1993). In humans the natural killer cell marker (NK) markers CD56 and CD94 are expressed on $CD3^+$ and $CD3^-$ IELs. During the active phase of coeliac disease the frequency of CD94 is increased over controls (Jabri *et al.* 2000). Expression of other NK markers are not modified. CD94 could be induced *in vitro* by TCR activation or by IL-15.

1.4.3 Lamina propria cells

An increased number of inflammatory cells infiltrate the lamina propria of coeliac and DH patients. The lamina propria volume of untreated coeliac mucosa is 2.3 times above that of control mucosae (Dhesi *et al.* 1984) and accommodates large numbers of T cells, plasma cells, eosinophils, mast cells, macrophages and neutrophils.

T cells

As in peripheral blood, $CD4^+$ cells dominate the lamina propria T cell population. The ratio of CD4:CD8 is similar in both populations (Mowat and Viney 1997). Lamina propria T cells are mainly $TCR\alpha\beta^+$ (Trejdosiewicz and Howdle 1995) and have the phenotype of memory cells; CD45 RO^+ (Smart *et al.* 1988), $CD44^+$ (Mowat and Viney

1997) and L-selectin $^{+}$ (Kanof *et al.* 1988). Up to 50% express αEβ7 (Cerf-Bensussan *et al.* 1993).

Lamina propria $CD4^{+}$ cells display an activated phenotype in coeliac tissue. Expression of CD25 on $CD4^{+}$ cells is increased from 2% in controls to 15% in untreated patients (Halstensen and Brandtzaeg 1993). CD25 is not upregulated on CD8 positive cells, however these cells show enhanced expression of the proliferation marker Ki67 whereas $CD4^{+}$ cells do not. $CD25^{+}$ T cells were isolated from gluten challenged coeliac biopsies and cell lines and clones established (Lundin *et al.* 1993). These T cells proliferated in response to gluten (Lundin *et al.* 1993) secreting large amounts of interferon gamma (IFN-γ) on activation (Nilsen *et al.* 1995). Interestingly the T cells only proliferated when gliadin was presented by DQ2 or DQ8 class II molecules (Lundin *et al.* 1993). Gliadin specific lines have not been isolated from normal controls (Molberg *et al.* 1997).

Pro-inflammatory cytokines, produced by gliadin-specific lamina propria T cells, most likely result in mucosal damage (see section 1.5.4). They may also play a role in recruiting other inflammatory cells that contribute to intestinal damage.

Plasma cells

There is a twofold increase in the total number of lamina propria plasma cells in untreated coeliac patients as revealed by immunoperoxidase staining (Scott *et al.* 1984). Individual populations of A, M and E isotypes are particularly increased over that of the control jejunum (Scott *et al.* 1984). Perfusion of the small intestine found a two-fold increase in the secretion rate of IgA and a fivefold increase in IgM (Colombel *et al.* 1990). This study failed to detect an increase in the IgG plasma cell or IgG secretion rate (Colombel *et al.* 1990). These *in vivo* results correlate with results from organ culture studies with coeliac biopsies. Jejunal biopsies from untreated coeliacs secreted more total IgM and IgA but not IgG during culture than biopsies from control subjects (Wood *et al.* 1986).

Plasma cell counts fall with the introduction of a GFD (Scott *et al.* 1984) and conversely gluten challenge increases plasma cell numbers in adult coeliacs and DH patients (Lancaster-Smith *et al.* 1975). Jejunal antibodies to gliadin are mainly IgA and IgM. This is in contrast to serum where IgA and IgG predominate (O'Mahony *et al.* 1991). Anti-gliadin IgA in the jejunal fluid is almost entirely polymeric and linked to the

secretory component as opposed to serum where the IgA is mainly dimeric and not linked to the secretory component (Colombel *et al.* 1990).

There is an increased incidence of IgA associated with complement in the basement membrane zone and lamina propria of active coeliac patients. This was put forward as a possible cause for mucosal damage in untreated patients (Scott *et al.* 1977).

Eosinophils

Numerous eosinophils congregate in the upper part of the lamina propria of active coeliac patients (Colombel *et al.* 1992). Deposition of granule components by activated eosinophils may contribute to mucosal damage (Colombel *et al.* 1992). Extracellular major basic protein (MBP) deposition is significantly greater in patients with coeliac disease than normal controls (Colombel *et al.* 1992; Tally *et al.* 1992). Immunohistochemical studies of jejunal biopsy specimens demonstrated an augmented extracellular deposit of eosinophil cationic protein (ECP) in the lamina propria of active coeliac patients (Hallgren *et al.* 1989). Jejunal fluid concentrations (Hallgren *et al.* 1989) and basal secretion rates (Lavo *et al.* 1989) of ECP are also increased in active coeliacs.

Mast cells

Studies with mast cells in coeliac disease have given conflicting results in the past. A decrease in the number of lamina propria mast cells has been reported in patients with coeliac disease. Mast cell numbers were said to increase after gluten withdrawal (Kosnai *et al.* 1984; Suranyi *et al.* 1986). The majority of reports however indicate an increase in mast cell numbers in the lamina propria during the active phase of coeliac disease which returns to the normal range in treated patients (Strobel *et al.* 1983; Wingren *et al.* 1986). Gluten induces the degranulation of mast cells (Dollberg *et al.* 1980). Histamine secretion in patients with coeliac disease increases forty minutes after gliadin perfusion to the jejunum (Lavo *et al.* 1989).

Neutrophils

Twenty-fold increases of neutrophils have been reported in the lamina propria of untreated coeliac mucosae compared to controls (Dhesi *et al.* 1984). Perfusion fluid concentrations of myeloperoxidase (MPO), a neutrophil granule component, is 3.5 times

higher in active coeliacs. However, extracellular deposition of MPO was barely detectable by immunohistochemical staining in these patients (Hallgren *et al.* 1989).

Macrophages

Following *in vitro* gluten challenge of treated coeliac biopsies a population of $CD4^+$ $CD45RO^+$ macrophages that express CD25 and high levels of HLA class II molecules can be seen beneath the surface epithelium (Halstensen and Brandtzaeg 1993). Maiuri *et al.* (1996a) noted that just two hours after *in vitro* gliadin challenge, macrophages in the lamina propria overexpress HLA-DR and intercellular adhesion molecule 1 (ICAM-1). Twelve to twenty four hours following challenge there is a dramatic migration of macrophages to the subepithelial compartment of the lamina propria leaving the deep layer of the lamina propria deficient in $CD68^+$ cells.

1.4.4 Enterocytes

Enterocytes begin life in the epithelial crypts and migrate along the crypt and villous walls maturing in the process. They increase in height and in brush border enzyme activity. After about five days the enterocyte reaches the villous tip and is extruded into the gut lumen (Strober 1976). In coeliac disease the proliferative capacity of the epithelial cells is increased as evidenced by increased uptake of bromidine deoxyuridine by enterocytes in coeliac biopsies (Kelleher *et al.* 1994). There is also an increased loss of epithelial cells into the gut lumen (Strober 1976). Expression of HLA-DR is increased on the surface (Marley *et al.* 1987) and is most likely caused by IFN-γ and TNF-α secretion (Sturgess *et al.* 1992). The majority of cells in the active lesion are immature with reduced height, irregular nuclei and poorly developed microvilli. It is hypothesized that the flat coeliac lesion is most likely due to an increased rate of epithelial cell death coupled with a compensatory increase in epithelial cell proliferation in the crypts (Strober 1976).

IECs play an important role in coeliac disease in transporting gliadin to the underlying lamina propria. There are two possible mechanisms by which gliadin reaches lamina propria T cells; between the epithelium (paracellularly) or through the epithelium (transcellularly).

Paracellular transport

It is well established that there is increased permeability in the coeliac intestine (see permeability hypothesis section 1.5.2). Recently it was discovered that gliadin can increase permeability in the intestine by controlling the protein zonulin, which is a regulator of tight junctions in the small intestine (Fasano *et al.* 2000). It was shown that gliadin induces zonulin dependent actin polymerisation which results in tight junction disassembly in coeliac and control intestinal tissues (Fasano *et al.* 2000). Immunofluorescence staining and immunoblotting showed enhanced zonulin expression in the coeliac submucosa compared to controls. This leads to increased intestinal permeability and the potential for increased movement of gliadin into the submucosa (Fasano *et al.* 2000).

Transcellular transport

Gliadin uptake by enterocytes after instillation has been demonstrated, although the mechanism by which this occurs is not yet known. Enterocytes express HLA class II molecules, with HLA-DR expression being greater than DP and DP greater than DQ (Marley *et al.* 1987). Expression is greatest in villous enterocytes and declines towards the crypt cells. In active coeliacs HLA class II expression is increased, with both surface and crypt enterocytes staining positive for all class II molecules (Marley *et al.* 1987). Gliadin can upregulate HLA-DR expression on enterocytes *in vitro* within 2 hours (Maiuri *et al.* 1996a). Theories that enterocytes might present gliadin via MHC class II molecules were confirmed when gliadin was found co-localised with HLA-DR in endocytic vesicles in coeliac patients (Zimmer *et al.* 1995).

1.5 THE AETIOLOGY OF COELIAC DISEASE

1.5.1 Missing enzyme hypothesis

The missing enzyme hypothesis postulates that coeliac patients are deficient in an enzyme involved in gliadin digestion. Peptides are hydrolysed, partially in the gastrointestinal lumen and chiefly in the brush border and cytosol of intestinal enterocytes (Kim *et al.* 1974). When gluten was treated with gastric enzymes, pepsin and trypsin, the fraction was still deleterious to coeliac patients. Yet gliadin treatment with an extract of pig's intestinal mucous membrane rendered the fraction nontoxic

(Frazer 1956). It was concluded therefore, that coeliac patients were deficient in an enzyme found in the intestinal wall rather than the stomach (Frazer 1956).

Coeliac patients were screened for a brush border enzyme deficiency. Whole biopsy homogenates were used in preliminary experiments, which made it impossible to determine what cell types were showing enzymatic activity. Results from these experiments were inconsistent, with some workers demonstrating impaired mucosal digestion of gliadin peptides (Cornell and Townley 1973; Pittman and Pollitt 1966) and others showing no such impairment (Douglas and Booth 1970).

Analysis of individual enzymes found that there were depressed levels of peptidases in treated coeliac patients (Andersen *et al.* 1983a; Andria *et al.* 1980; Sjöström *et al.* 1981). Mucosal damage in active coeliac patients results in many brush border enzyme abnormalities, which normalise on a GFD. However, enzyme levels may not recover at the same rate. It is possible that these results were due to incomplete mucosal recovery rather than a primary enzyme deficiency (Davidson and Bridges 1987). Bruce *et al.* (1984) determined enzymatic activity by measuring the release of glutamine and glutamic acid from gliadin digested by brush border fractions. They found no difference between levels that were released from treated coeliacs verses controls.

It is possible that the brush border region may not be involved at all. It has been proposed that peptide hydrolysis may be defective at the lysosomal, cytosolic or basolateral regions in coeliac patients (Peters and Bjaarnason 1984). Alternatively, the missing enzyme may not be the much sought after peptidase but rather a carbohydrase (Davidson and Bridges 1987).

1.5.2 Intestinal permeability hypothesis

The permeability hypothesis suggests that coeliac patients have a primary defect in mucosal permeability, allowing enhanced gliadin entry into enterocytes, initiating organelle damage, and/or enhanced entry into the submucosal spaces, triggering immunological events in the lamina propria (Bjarnason and Peters 1984).

Sugar absorption was a method commonly employed to measure intestinal permeability. Two saccharides, one large (e.g. cellobiose) and one small (e.g. mannitol) were

administered simultaneously to patients. The urine excretion ratio was then measured (Cobden *et al.* 1978; Cooper and Ukabam 1982; Hamilton *et al.* 1982). Studies with active coeliac patients saw increased permeability to the large molecule and decreased permeability to the small molecule (Cobden *et al.* 1978; Cooper and Ukabam 1982; Hamilton *et al.* 1982). The difference in size meant that these two molecules were probably being absorbed by different routes. The larger possibly by intercellular tight junctions and the smaller by transcellular pores (Cobden *et al.* 1978). It is possible that epithelial damage allowed for better absorption of the larger molecule while reduced surface area, and therefore a reduced number of aqueous pores, hindered absorption of the smaller sugar (Cobden *et al.* 1978).

The question that needed to be answered was whether the increased permeability was a primary defect or just a result of mucosal damage. Cooper and Ukabam (1982) found that the excretion ratio of patients on a GFD correlated with the histology grade of the jejunum, indicating that permeability was a factor of damage. Cobden *et al.* (1978) agreed, reporting that treated patients excreted levels that were intermediate of active patients and controls. Hamilton *et al.* (1982) found that the cellobiose:manitol ratio returned to normal within 5 months of starting a GFD. Importantly, permeability increased in treated coeliacs challenged with gluten (Hamilton *et al.* 1982).

Bjarnason *et al.* (1985) felt that sugar absorption was an inaccurate measurement of permeability (e.g. increased transit times in the intestine and partial hydrolysis by the brush border also affected sugar absorption). They measured ^{51}Cr-ethylene diaminetetra-acetic acid (EDTA) excretion and found permeability was markedly increased in untreated coeliacs and DH patients compared to controls. However, unlike the aforementioned experiments increased permeability was shown to persist in patients on a GFD, including those with a normal mucosa (Bjarnason *et al.* 1985). These results closely resembled those of *in vitro* experiments, which measured ^{51}Cr-EDTA and ^{57}Co-cyanocobalamin uptake by coeliac and control intestinal biopsies (Bjarnason and Peters 1984). Scanning electron microscopy studies (Stenling *et al.* 1984) also found that treated coeliac patients with a normal mucosa had persisting enterocyte lesions, supporting the idea that coeliac patients have a defect in mucosal permeability.

1.5.3 The lectin hypothesis

The lectin hypothesis was coined based on the observation that immature intestinal cells have incomplete glycoproteins on their cell surface, which bind plant lectins with great affinity (Burger 1973). Weiser and Douglas (1976) proposed that coeliac patients have incomplete glycoproteins on the surface of their enterocytes, possibly due to a hereditary biochemical defect or due to infection or surgery. Gluten binds to the surface of the enterocyte and acts as a toxic lectin. Cell death increases as does cell turnover resulting in a flat mucosal lesion.

A carbohydrate-containing component of wheat and a PT digest of gliadin were reported to bind with great affinity to a fraction of coeliac intestinal cells (Douglas 1976) and to undifferentiated K562(S) cells (Auricchio *et al.* 1984a) respectively. K652 (S) cells are a subline, from a patient with chronic myelogenous leukaemia, that differentiate in the presence of butyric acid. Binding in both cases was inhibited by free carbohydrate suggesting that gliadin was binding in a lectin-like fashion.

Stern *et al.* (1988) measured the binding of gliadin fractions to immature rat small intestinal microvillous membranes. Binding of gliadin was found to be weak and non-specific. The saccharide mannan had no effect on gliadin binding nor did enzyme pre-treatment of the cells. In a study using affinity dotting and blotting techniques, binding affinity of wheat proteins to various glycoproteins was analysed (Rühlmann *et al.* 1993). While certain glycoproteins bound the wheat proteins, several displayed no binding reaction at all. Of those wheat proteins that bound, binding was not inhibited by saccharides or enzymatic N-deglycosylation of the ligand glycoproteins. Colyer *et al.* (1987) examined the binding of ^{125}I-Frazer's Fraction (FF)-III to rat and human enterocytes. In experiments carried out with rat lymphocytes binding was not inhibited by a range of saccharides, while binding of the lectin Conconavalin A (Con A) was inhibited. These experiments argue against the theory that the interaction of gliadin with the enterocyte surface membrane is lectin mediated.

Contamination of gluten preparations by the lectin, wheat germ agglutinin (WGA) is thought to be responsible for the positive results. The possibility also exists that the gliadin receptor is not a carbohydrate receptor or that it is a different glycoprotein to the ones used in the above studies.

1.5.4 Immunological hypothesis

The hypothesis receiving most attention at the moment is the immunological hypothesis, which proposes that coeliac disease is being driven by an abnormal T cell response to gluten. The first evidence for cell mediated immunity to gliadin came from Ferguson *et al.* (1975) who cultured jejunal biopsy specimens in the presence of the protein and assayed for migration inhibition factor (MIF) production (i.e. a "factor", produced by the biopsy which could inhibit migration of normal human peripheral blood leucocytes). The addition of α gliadin to the culture medium triggered secretion of MIF. This was not observed in non-coeliac or treated coeliac tissue.

Confirmation came years later with the report that gluten could induce CD25 expression on lamina propria T cells in jejunal coeliac biopsies (Halstensen *et al.* 1993). These $CD25^+$ cells were $CD4^+$ $CD8^-$, $CD45RO^+$ and often $\alpha E\beta7^+$ (Halstensen *et al.* 1993). Maiuri *et al.* (1996a) carried out time-course experiments on coeliac biopsies, challenged with gliadin *in vitro*. Immune activation was noted as early as 1 hour after challenge with enhanced expression of HLA-DR on villous enterocytes. After 2 hours ICAM-1 was upregulated on the lamina propria T cells and macrophages. As time progressed there was increased migration of lamina propria mononuclear cells, mostly $CD4^+$, to the subepithelial compartment. The number of cells in the intraepithelial compartment also increased. These cells were positive for CD8 and αEβ7. As previously mentioned clones were established from the $CD25^+$ T cells in the coeliac lesion (Lundin *et al.* 1993). These cells proliferated to gluten (Lundin *et al.* 1993) and secreted Th1 or Th0 cytokines on activation (Nilsen *et al.* 1995).

It is likely that gliadin induced T cell activation is responsible for mucosal damage coeliac disease. Explants of human foetal small intestine in which T cell activation was induced led to villous atrophy and crypt hyperplasia (da Cunha Ferreira *et al.* 1990). Cytokines, in particular IFN-γ, are probably key players in initiating damage. Supernatants from gliadin reactive T cell clones produce a significant reduction in enterocyte height (Przemioslo *et al.* 1995), which can be blocked by preincubation with anti-IFN-γ (Przemioslo *et al.* 1995). IFN-γ can also trigger macrophage production of TNF-α. Both cytokines, together, are toxic to enterocytes. TNF-α also induces production of KGF, which causes epithelial proliferation and crypt hyperplasia (Bajaj-Elliott *et al.* 1998). Cytokines from activated T cells also induce matix

metalloproteinase (MMP) production and increased levels have been detected in coeliac tissue (Daum *et al.* 1999). MMPs can degrade the extracellular matrix, leading to loss of villous enterocytes .

It is not certain whether antibodies play a role in coeliac pathogenesis or not. Halstensen *et al.* (1992) demonstrated that gluten induces IgG-mediated subepithelial complement activation, which might damage the epithelium. Another hypothesis was that coeliac disease associated tissue antibodies mediated damage by binding to their antigens in the subepithelial lamina propria thereby inhibiting the physiological functions of fibroblasts in the process (Maki 1995). We will discuss later how IgA antibodies, specific for tTG, inhibit TGF-β induced intestinal crypt epithelial cell differentiation (Halttunen and Mäki 1999). The prevalence of coeliac disease in IgA deficient patients (Collin *et al.* 1992) argues against the role of these antibodies in pathogenesis. Sollid *et al.* (1997) have proposed that antibody production in coeliac disease is an epiphenomenon of T cell activation.

1.6 TISSUE TRANSGLUTAMINASE, THE AUTOANTIGEN

1.6.1 Tissue transglutaminase

tTG is one of six enzymes, which constitutes the transglutaminase family. Other members include, three intracellular isoenzymes of epidermal transglutaminase and two extracellular transglutaminases; factor XIII and prostate transglutaminase (Piacentini and Colizzi 1999). These Ca^{2+} dependent enzymes crosslink proteins by catalysing the formation of ε-(γ-glutamyl)-lysine or ε-(γ-glutamyl) polyamine bonds (Folk and Finlayson 1977). Their active site contains a cystine residue that reacts with the γ-carboxyamide of a glutamine residue in a protein, forming a γ-glutamyl thioester and releasing ammonia. The transient acyl-enzyme intermediate then reacts with any nucleophilic primary amine. When an amine is not available, the acyl-enzyme intermediate reacts with water to yield a glutamic acid residue (Figure 1.6) (Greenberg *et al.* 1991). Transglutaminases have, however, a broad specificity for primary amine acceptors, whereas specific donor substrates are limited (Folk 1980).

tTG is an intracellular 85kDa protein. It has 687 amino acids which form four domains; an N-terminal β sandwich domain (amino acid (aa) 1-139), a central catalytic core region

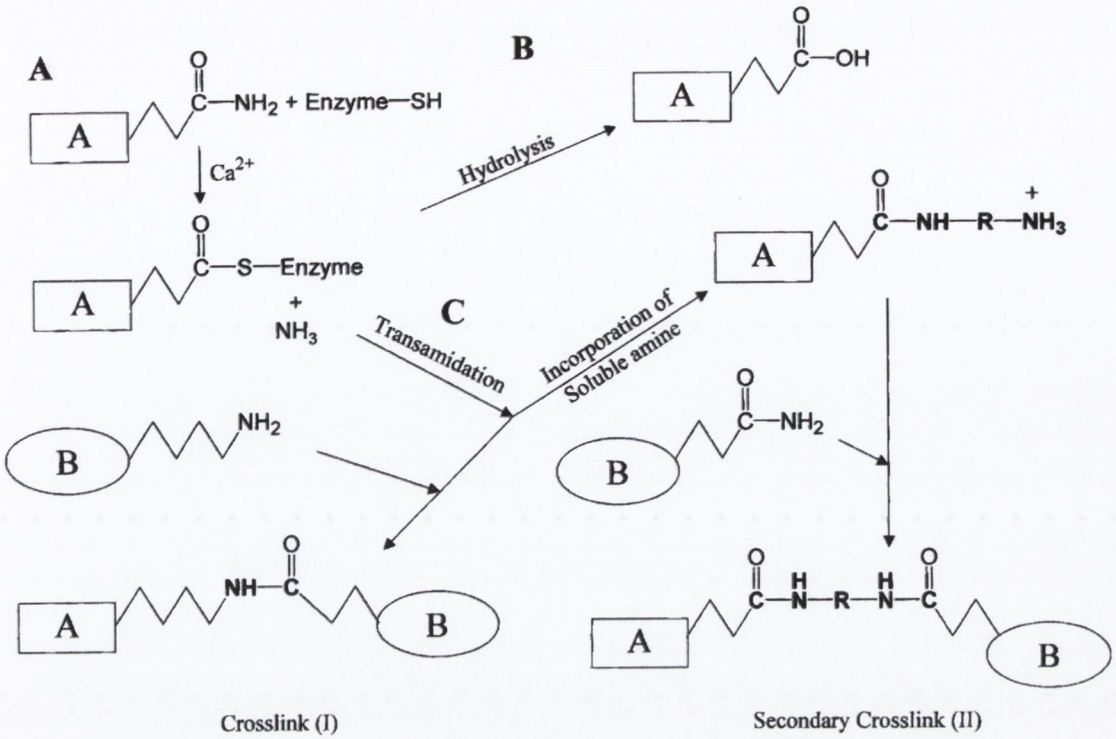


Figure 1.6 Reactions catalysed by tTG. **(A)** The enzyme reacts with a glutamine residue in a protein to form a glutamyl thioester and ammonia. This active thioester reacts with a primary amine **(C)** to form a glutamyl-lysine protein (crosslink (I)). If the primary amine is a polyamine it can be further polymerised by transglutaminase to form crosslink II. **(B)** When amine is not available the acyl-enzyme intermediate reacts with water releasing glutamic acid in the substrate protein (taken from Griffin *et al.* 2002).

(aa 140-454) and two β-barrel regions (aa 479-585 and aa 586-687) (Casadio *et al.* 1999) (Figure 1.7). The amino acid sequences are highly conserved among the species. Enzymatic activity requires Ca^{2+} binding to the region aa 446-453 and a Cys active site located at aa 227 (Gentile *et al.* 1991). The protein also binds GTP at its N terminal region (Lai *et al.* 1996) resulting in a conformational change in the enzyme, which inhibits enzymatic activity (Achyuthan and Greenberg 1987). It is possible that varying concentrations of Ca^{2+} and GTP may regulate tTG activity (Achyuthan and Greenberg 1987).

The ε-(γ-glutamyl)-lysine bonds formed by tTG are stable and resistant to proteolysis (Greenberg *et al.* 1991). The enzyme can therefore stabilize apoptotic bodies by catalysing intracellular protein crosslinking and thus preventing leakage of the cellular contents (Piacentini and Colizzi 1999). tTG may be released during cellular wounding and may stabilise the extracellular matix by crosslinking matrix proteins (Upchurch *et al.* 1991).

1.6.2 Tissue transglutaminase in coeliac disease

In 1985 Bruce *et al.* demonstrated increased tTG activity in jejunal biopsies from treated and untreated coeliac patients compared to normal controls and patients with IBD. Patients in remission showed lower activities than patients in relapse, though these results were not statistically significant (Bruce *et al.* 1985). Immunofluorescence staining later showed tTG expression was mainly located in the subepithelial region of the mucosa. Coeliac expression of the enzyme was higher than that found in normal controls and some tTG was also detected on the brush border of coeliac patients (Molberg *et al.* 1998).

A breakthrough in coeliac disease research came with the identification of tTG as the antigen recognised by EMAs (Dieterich *et al.* 1997). IgA antibodies from coeliac patients recognised an 85kDa protein in human fibrosarcoma cells which when sequenced corresponded to tTG. Pretreating coeliac serum with tTG almost abolished EMA binding to monkey oesophagus (Dieterich *et al.* 1997). Subsequent studies speculated that tTG was not the sole antigen of EMAs (Brusco *et al.* 1999; Lock *et al.* 1999). A 55 kDa nuclear antigen expressed in intestinal cells, endothelial cells and fibroblasts (Natter *et al.* 2001) and the cytoskeletal protein, desmin, (Teesalu *et al.* 2001)

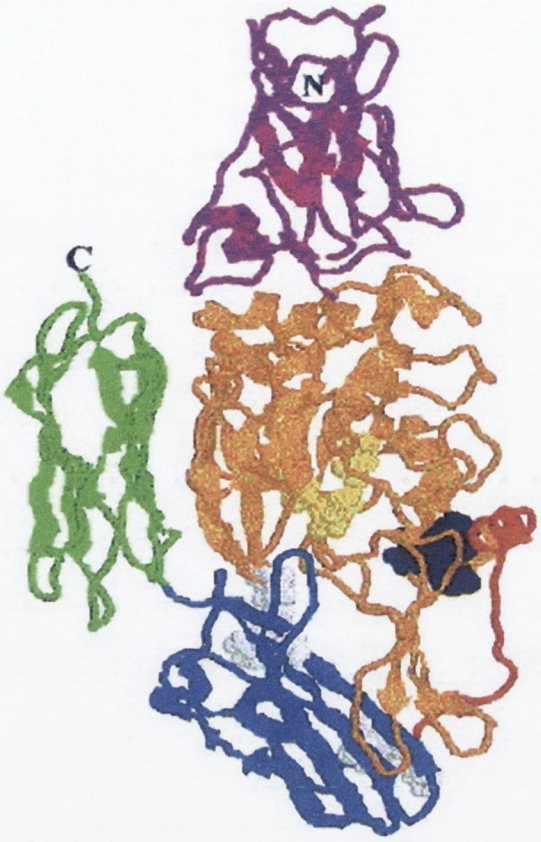


Figure 1.7 The three dimensional structure of tTG. Domains I-IV are coloured respectively in magenta, orange, blue and green. Amino acids involved in the active site, the Ca^{2+} binding site and in the interaction with GTP are coloured yellow, black and light grey respectively (taken from Griffin *et al.* 2002).

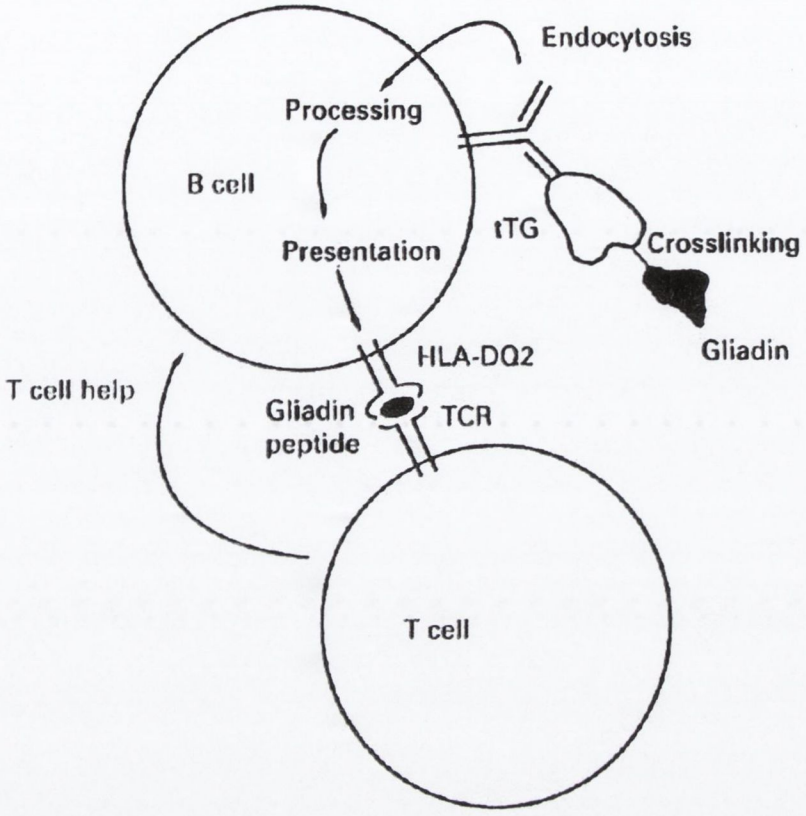


Figure 1.8 Model proposed for the production of anti-tTG antibodies following gliadin intake. tTG binds gliadin and the complex is taken up by tTG specific B cells. Gliadin peptides are presented to gliadin reactive T cells, which in turn aid anti-tTG antibody production by B cells (taken from Sollid *et al.* 1997).

have been suggested as additional autoantigens. However tTG is still recognised as the predominant if not the sole autoantigen in coeliac disease.

The dominant epitopes of tTG autoantibodies occupy the N and C terminus of the molecules. (Seissler *et al.* 2001) (Figure 1.7). It is possible that binding of antibodies affects the enzymatic activity of tTG. Coeliac disease associated IgA antibodies, specific for tTG, inhibit TGF-β induced intestinal crypt epithelial cell differentiation (Halttunen and Mäki 1999). tTG activates latent TGF-β by crosslinking it via the TGF-β-binding protein 1 to the extracellular matrix (Nunes *et al.* 1997). Blocking tTG activity may interfere with the role of TGF-β in epithelial cell differentiation and regulation of MMP activity (Halttunen and Mäki 1999).

Gliadin peptides can become covalently bound to tTG and may perhaps form hapten-carrier complexes (Molberg *et al.* 1998). It has been hypothesised that tTG specific B cells take up the tTG-gliadin complex and present a gliadin peptide to gliadin reactive T cells. The T cells in turn aid antibody production by tTG specific B cells. Therefore when gliadin is removed from the diet T cells can no longer provide help for tTG specific B cells and antibody production declines (Sollid *et al.* 1997) (Figure 1.8). tTG greatly enhances PT gliadin induced proliferation of DQ2 (Molberg *et al.* 1998) and DQ8 (Molberg *et al.* 1998; van de Wal *et al.* 1998a) restricted T cell clones from coeliac patients. This will be discussed in more detail in chapter 4.

1.7 CEREALS

Cereals are members of the monocotyledonous grass family, Gramineae, which comprises four subfamilies (Figure 1.9). The subfamily Pooideae includes the Aveneae tribe or oats and the Triticeae tribe to which wheat, barley and rye belong. Panicoideae contains the tropical cereals maize, sorghum and the millets. Rice is usually classified in the subfamily Bambusoideae and ragi and tef in the subfamily Chloridoideae (Shewry *et al.* 1992b).

1.7.1 Cereal prolamin proteins

Cereal grains are composed of an outer husk or bran, a germ and an endosperm or flour (Bell *et al.* 1981) (Figure 1.10). In 1950 Dicke discovered that wheat and rye flour, but

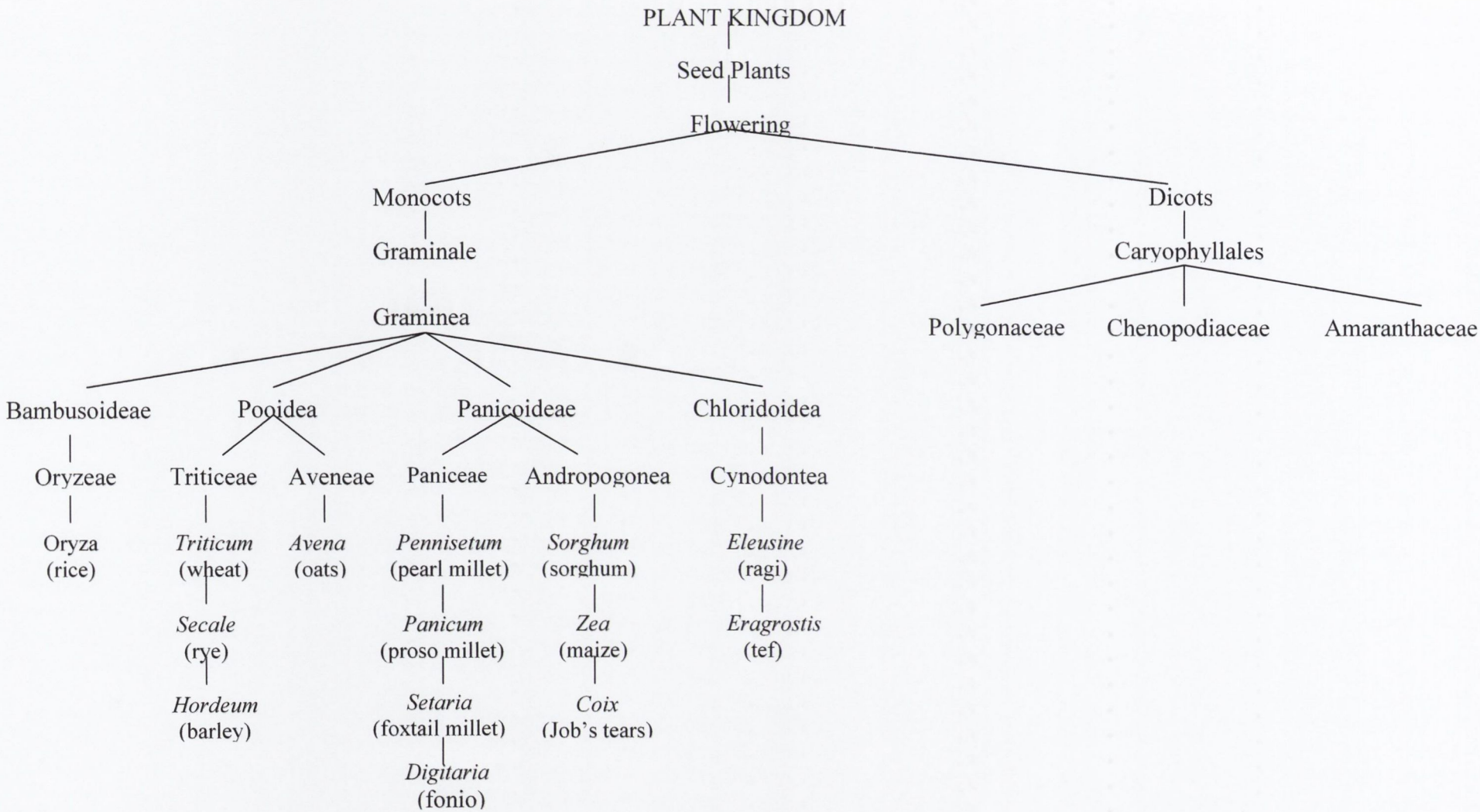


Figure 1.9 The taxonomic relationships of cereals (taken from Shewry *et al.* 1992b).

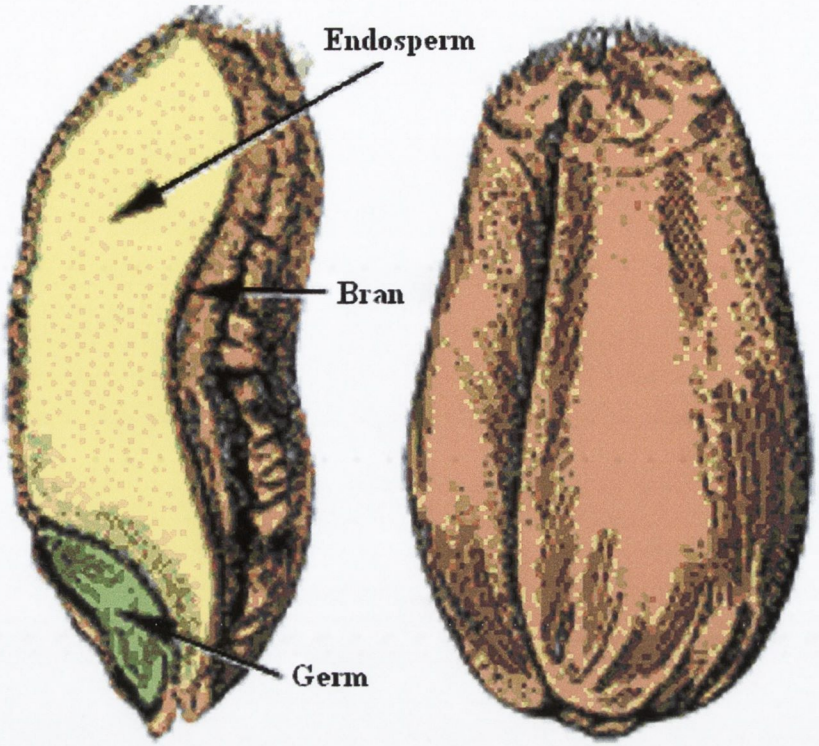


Figure 1.10 A longitudinal section of a cereal grain showing the bran, germ and endosperm. The albumin, globulin, prolamin and glutelin proteins are found in the endosperm.

not starch, was responsible for the streatorrhoea seen in coeliac patients (Dicke 1950). There are four main endosperm proteins, which have traditionally been classified according to their solubility. The albumins which are soluble in water, globulins, which are insoluble in water but soluble in salt solutions, prolamins, which are soluble in ethanol, and glutelins which are insoluble in aqueous or saline solutions and ethanol (Osborne 1924). Together with van de Kamer and Weijers, Dicke found that the alcohol soluble or prolamin fraction of wheat flour triggered symptoms in patients (Dicke *et al.* 1953). The prolamins occur mainly as monomers while the glutelins form aggregates due to the formation of intermolecular disulphide bonds. The glutelins are closely related to the alcohol soluble prolamins and it is now usual to classify them as such (Shewry and Tatham 1990).

Synthesis and function of prolamins

The prolamins are storage proteins, unique for cereals. They are stored in membrane bound vesicles called protein bodies, distributed throughout the endosperm. Following synthesis by endoplasmic reticulum (ER) bound polysomes, a signal peptide directs them to the lumen of the ER, where they are surrounded by a membrane of ER origin. In younger endosperms they are transported via the Golgi apparatus into protein bodies of vacuolar origin (Kreis and Shewry 1989). Disulphide bond formation may take place in the ER as the enzyme protein, disulphide isomerase, which catalyses disulphide bond formation, has been found associated with the ER (Roden *et al.* 1982). They do not undergo post-translational modification except cleavage of a signal peptide. There is no evidence of glycosylation. The protein bodies are then deposited in the endosperm where they remain until germination. The prolamins have an unusually high content of amide nitrogen due to the large percentage of glutamine present (Osborne 1924). During germination the prolamins are degraded to provide a source of nitrogen, sulphur and carbon for the developing seedling (Kreis and Shewry 1989).

Properties of prolamins

Prolamins are encoded by highly polymorphic genes and as a result proteins differ in their molecular weight (M_r), isoelectric point (pI) and amino acid composition. This difference occurs both within individual plants and between genotypes of the same species (Shewry *et al.* 1992b). The sequences of all prolamins, however, can be divided into domains, which differ in amino acid composition and conformation. At least one of these domains contains repeat motifs rich in glutamine and proline residues (Shewry *et*

al. 1994). Structure analysis shows that these repetitive domains form β reverse turns. It is thought that this secondary structure may be important for the packaging of proteins into proteins bodies (Shewry *et al.* 1994). The proportion of proline and glutamine in prolamin proteins is very high. Glutmine content in wheat, barley and rye prolamins ranges from 35 to 37% and proline ranges from 17 to 23%. Oat prolamins are also rich in glutamine but have a lower content of proline. The prolamin proteins of rice, maize and millet do not contain as much glutamine and proline as those in the Pooideae tribe but are richer in amino acids alanine and leucine (Wieser 1994).

The prolamins of the different cereals have been allocated the following names; gliadin (wheat), secalin (rye), hordein (barley), avenin (oats), zein (maize), kafirin (millet) and oryzin (rice).

1.7.2 Nomenclature of wheat, barley and rye prolamins

Three groups of prolamins are recognised based on their amino acid composition; the sulphur rich (S-rich), sulphur poor (S-poor) and high molecular weight (HMW) prolamins. Table 1.1 shows the families of prolamins in wheat, rye and barley that belong to each group.

S-rich prolamins

S-rich prolamins account for 80% of the total prolamin fraction. Their M_r is generally 36-44,000 with the exception of the 75,000 γ-secalins. All of the S-rich prolamins have an N-terminal domain consisting of proline and glutamine rich repeat motifs. In some cases this domain is preceded by a short N terminal sequence (Shewry *et al.* 1994). The repetitive sequences are based on a Pro-Gln-Gln-Xaa tetrapeptide, which forms β reverse turns (see Appendix I for amino acid abbreviations). The C terminus contains most or all of the cysteine residues and is predominantly α-helical (Shewry *et al.* 1994). The S-rich prolamins are further divided into three groups based on sequence comparisons; the γ-type, the α-type, the aggregated or low molecular weight (LMW) type.

γ-type S-rich prolamins

The γ-type S-rich prolamins are considered the ancestral type. They are monomeric proteins present in wheat (γ-gliadins), barley (γ-hordeins) and rye (γ-secalins) but are

Table 1.1 Groups of S-rich, S-poor and HMW prolamins present in wheat, rye and barley (taken from Shewry *et al.* 1992b).

	Wheat	**Rye**	**Barley**
S-rich prolamins			
γ-type	γ-gliadins	Mr 40 000 Mr 75 000 γ-secalins	γ-hordein
α-type	α- and β- gliadins	-	-
Aggregated type	LMW glutenin	-	B hordein
S-poor prolamins	ω-gliadins	ω-secalins	C hordein
HMW prolamins	HMW glutenins	HMW secalin	D hordein

also present in the more distantly related oats and meadow grasses (Shewry *et al.* 1994). An N terminal sequence of 12 residues precedes a repetitive region based on the consensus motif Pro-Gln-Gln-Pro-Phe-Pro-Gln (Figure 1.11). The C terminal region contains eight cysteine residues involved in disulphide bond formation (Shewry and Tatham 1990).

α type S-rich prolamins

α-type S-rich prolamins i.e. α and β gliadins are only present in wheat. Their repetitive domain is less conserved than the γ-gliadins. It consists of interspersed repeats of the motifs Pro-Gln-Pro-Gln-Pro-Phe-Pro and Pro-Gln-Gln-Pro-Tyr (Figure 1.11). The C terminus contains two poly-Gln tracts (Shewry and Tatham 1990). α-gliadins have six cysteine residues which form three inter-chain disulphide bonds which are also conserved in γ-gliadin (Shewry *et al.* 1994). One subgroup of α-gliadins, called A gliadin has the unusual property of aggregating under certain conditions of pH and ionic strength to form fibrils (Shewry and Tatham 1990). An unpaired cysteine, which might have been inserted or deleted, may form inter-molecular disulphide bonds resulting in a polymeric protein (Shewry *et al.* 1994).

The aggregated LMW S-rich prolamins

The aggregated S-rich prolamins are present as polymers stabilised by interchain disulphide bonds. They have M_rs of 65,000 to 90,000 and occur in barley (B hordeins) and wheat (LMW subunits of glutenin) but not in rye. The LMW glutenins have a short non-repetitive N-terminal sequence, which may contain a single cysteine residue followed by a proline rich repetitive domain of interspersed repeats based on two consensus motifs; Pro-Gln-Gln-Pro-Pro-Pro-Phe-Ser and Gln-Gln-Gln-Gln-Pro-Val-Leu (Shewry and Tatham 1990).

The S-poor prolamins

The ω-gliadins of wheat, the ω-secalins of rye and C hordeins of barley form the S-poor prolamin group and range in M_r from 40,000 to 70,000 (Shewry *et al.* 1992b). In each case a large repetitive region is flanked by a short N terminus and C terminus. Cysteines are absent and glutamine, proline and phenylalanine account for about 80% of the total residues (Shewry *et al.* 1994; Shewry and Tatham 1990). Secondary structure indicates β-turns to be present in the central repetitive region. They lack α helical or β sheet structure (Tatham *et al.* 1985). Studies have focussed on C hordein. It has a unique N-

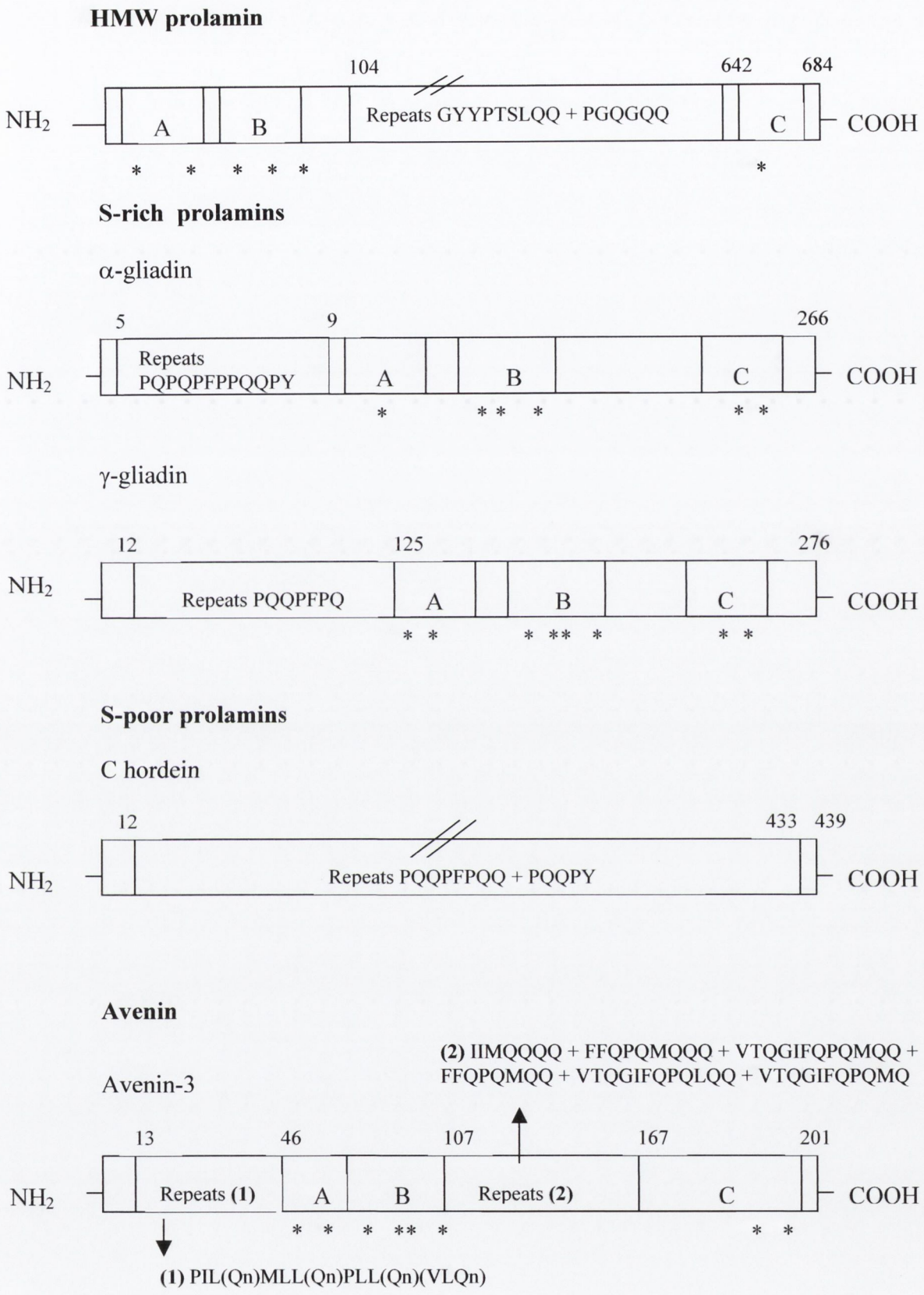


Figure 1.11 Schematic comparison of the structures of prolamins. * indicates the position of cysteine residues, n indicates any number (adapted from Shewry *et al.* 1992b ; Shewry *et al.* 1994 ; Egorov *et al.* 1994b). See appendix 1 for amino acid abbreviations.

terminal domain of 12 residues and a C-terminal domain or 6 residues (Figure 1.11). These flank a repetitive domain of over 400 residues. The repetitive sequences consist mainly of octapeptides (Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln) with at least three pentapeptides (Pro-Gln-Gln-Pro-Tyr) close to the N-terminus (Shewry and Tatham 1990). Limited sequence information is available for ω gliadins and ω secalins: however comparisons suggest that their amino acid sequences are similar to C hordein (Shewry *et al.* 1992b).

The HMW prolamins

The HMW prolamins of wheat are characterised into two types based on their M_r values and sequences; x-types (M_r = 83-88,000) and y-types (M_r = 67-74,000) (Shewry and Tatham 1990). They contain high quantities of glycine, glutamine and proline residues. The HMW glutenins have a non-repetitive N-terminus (81-104 residues) and C-terminus (42 residues) (Figure 1.11). The central region consists of repetitive domains based on three different consensus motifs. Hexapeptides (Pro-Gly-Gln-Gly-Gln-Gln) and nonapeptides (Gly-Tyr-Tyr-Pro-Thr-Ser-Pro/Leu-Gln-Gln) are present on both x- and y-type subunits and tripeptides (Gly-Gln-Gln) in x-type only (Shewry and Tatham 1990). The non-repetive domains contain most or all of the cysteine residues. There are three (x-type) or five (y-type) in the N terminal domain and one in the C-terminal domain (Shewry *et al.* 1992a). The N- and C- terminal domains are predominantly α-helical in structure, while the central repetitive region forms β-turns (Shewry *et al.* 1994). The HMW subunits are stabilised by interchain disulphide bonds. The presence of cysteine residues in the N- and C- terminal domains allows for the formation of head to tail polymers (Shewry and Tatham 1990).

Although the HMW prolamins of barley (D hordein) and rye (HMW secalin) have not been characterised in detail, evidence suggests that they are closely related to the HMW subunits of wheat (Shewry and Tatham 1990).

1.7.3 Evolutionary relationships

Sequence similarity between the different prolamins indicates that they evolved from a common ancestral protein 30aa in length. It has been postulated that this gene triplicated to give regions A, B and C (Kreis *et al.* 1985) (Figure 1.11). Divergence of the prolamins probably arose through the insertion of sequences between A, B and C and in

the case of the S-poor prolamins, loss of all of region A and B and most of region C. It is possible that recombination between introns could have altered the domains. Point mutations at splice boundries and variable splicing could also have given rise to new proteins (Kreis and Shewry 1989).

Regions related to A, B and C are present in three other types of seed proteins e.g. inhibitors of proteases and α-amylases (from cereals including barley, rye, wheat, maize and finger millet), storage globulins (from lupins, castor bean, oilseed rape and brazil nut) and 2S albumin (from sunflower).

1.7.4 The oat prolamin

Avenins are encoded by tightly clustered multigenes. Like other prolamins they are stored in protein bodies in the endosperm (Pernollet *et al.* 1982) and become hydrolysed during germination (Kim and Mossé 1979). Kim *et al.* (1978) found that they could be extracted from oats in 45% ethanol and could be classified into α, β and γ proteins based on electrophoretic mobility.

To date eleven avenins have been isolated (Egorov *et al.* 1994b). Their amino acid length varies from 162 to 203 residues. Protein sequences are very similar, differing only by point mutations, deletions and insertions. M_rs measured by SDS-PAGE range from 21,400 to 30,000. Avenins are rich in glutamine / glutamic acid (33-38%), hydrophobic amino acids (about 50%) and proline (about 10% - although lower than that of gliadins, secalins and hordeins). They are poor in basic amino acids (about 4%), glycine and hydroxyamino acids. Eight cysteine residues were found per avenin protein. Nearly half of the proteins were N terminally blocked (Egorov *et al.* 1994b). Avenin-9 and -3 have been characterised in more detail.

The amino acid sequence of avenin-9 was found to be 182 aa long, had an N-terminal aa sequence (residues 1-10) and contained the conserved regions A (residues 43-67), B (residues 86-121) and C (residues 150-174) found in other prolamin proteins. Its repeat region consisted of repeats of Pro-Phe-Val-(Gln)n where n = 3, 4, or 5 (Egorov 1988). Identification of disulphide bonds was unsuccessful (Egorov *et al.* 1994a). However, eight cysteine residues were present and no free SH groups identified (Egorov *et al.* 1994b). The sequence of avenin-3 was found to be 201 amino acid residues in length

(Figure 1.11). Similar to the S-rich and HMW prolamins, the sequence could be divided into five regions; an N terminal region (residues 1-13) two tandem repeat regions (residues 10-46 and 107-167), a conserved region and a C terminal region containing 8 cysteine residues involved in disulphide bond formation. All 8 cysteine residues are conserved in γ-gliadin (Figure 1.11). Conserved regions A, B and C are also present in avenin-3 (Egorov *et al.* 1994a).

Our knowledge of prolamin sequences advanced with introduction of molecular cloning (Sturgess *et al.* 1991). Studies based on 3 avenin cDNA clones found that the amino acid sequences were very similar (Chesnut *et al.* 1989). The proteins were 203, 201 and 162 amino acids in length and could be divided into domains similar to previously isolated avenin proteins: an N terminal region of 9 or 13 amino acids, 2 regions of imperfect glutamine repeats separated by an amino acid conserved region and a C terminal region. Eight Cys residues involved in disulphide bond were found in the conserved (6 cys residues) and the C terminal region (2 cys residues) (Chesnut *et al.* 1989). Homology to α/β gliadin, γ-gliadin, B-hordein and γ-secalin was found 50 base pairs (bp) upstream of the initiating codon and also in the repetitive regions. The conserved region showed homology to the non-repetitive regions of these prolamins and homology was also seen in the 3' coding region and the 3' untranslated region (Chesnut *et al.* 1989). The N terminal ends of the avenins are rich in β turns due to the presence of repetitive repeats (Pernollet *et al.* 1987).

Oats and rice differ from other cereals in that their major storage proteins are not prolamins but rather globulins. Approximately 10% of the oat seed storage protein is prolamin (Peterson and Smith 1976). The high globulin, low prolamin composition results in oats having a higher nutritional value than most cereals. Cereals with high prolamin content are deficient in amino acid lysine and tryptophan (Peterson and Smith 1976). Despite the predominance of globulin proteins in the mature seed, the concentration of avenin mRNAs is approximately equal to that of globulin mRNAs during development (Chesnut *et al.* 1989). It is unlikely that protein turnover is responsible as it would be inefficient to degrade protein before germination. Reduced rates of translation due to an inefficient translation initiation site and the presence of hairpin loops have been suggested as reasons for reduced prolamin protein production (Shotwell *et al.* 1990). Subsequently it was reported that avenin genes terminate translation with a non favoured UAA(G) sequence whereas globulin genes terminate

with a favoured stop codon UGA(A). It is possible that this may cause the ribosomes to stall at UAA(G) resulting in queuing of ribosomes and slowing of the overall rate of synthesis (Boyer *et al.* 1992).

1.8 TESTING CEREAL TOXICITY AND IMMUNOGENICITY

1.8.1 *In vivo* testing of wheat proteins

When dough is washed to remove starch granules, the cohesive mass that remains is called gluten. Gluten can be divided into two fractions, one soluble (gliadin) and the other insoluble (glutenin) in aqueous alcohol. In an effort to identify the harmful component of wheat flour for coeliac patients, van de Kamer, Weijers and Dicke prepared the following fractions from wheat; gluten, gluten washwater, gliadin, glutenin, ash, crude fibre and fat (Van de Kamer *et al.* 1953). Ash, crude fibre and fat did not produce any reaction in a patient that was very sensitive to wheat. However, gluten and gliadin caused a severe reaction while the reaction to glutenin was not as dramatic. Gluten washwater also gave a slight reaction. Further investigation of gliadin and gluten in seven coeliac patients showed that these fractions caused increased faecal fat excretion.

Sckenk and Samloff (1968) challenged patients simply by reintroducing gluten-containing foods back into the diet of patients that had been on a GFD. In 3 of 4 patients a gluten containing diet induced villous atrophy, the accumulation of inflammatory cells in the lamina propria, lipid droplets in the surface epithelium and steatorrhoea. In one patient lipid accumulation in the enterocytes did not occur but was noted after supplementation of the diet with gluten. The gluten containing diet alone caused villous blunting and an increase in inflammatory cells in this patient. Ross *et al.* (1955) found that administration of 10g of gluten per day to 12 coeliac patients in recovery phase, induced steatorrhoea and other signs of deterioration. In another trial gliadin was shown to be harmful by lowering fat absorption in 4 children while glutenin exerted a similar effect in an additional 3 patients (Sheldon 1955).

Separation of gliadin into subfractions by ion exchange chromatography produced 3 eluates that corresponded to α gliadin (Kendall *et al.* 1972). The other eluates were combined and called pre- and post-α gliadin. Reduced xylose excretion was used to

demonstrate toxicity. Pre-and post-α gliadin induced minor changes while α gliadin noticeably reduced xylose excretion.

In *in vivo* studies, digestion with the enzymes pepsin and trypsin did not alter the harmfulness of gluten (Frazer *et al.* 1959). This excluded the possibility that incomplete protein breakdown in the stomach was responsible for the toxicity. Toxicity was also retained after autoclaving this soluble fraction. The conclusion was that the harmful effect of gluten was due to the presence of a peptide that could not be handled as effectively by patients with coeliac disease as by normal controls. Amino acid content and toxicity was investigated following the sequential digestion of gliadin with pepsin, trypsin, cotazym and intestinal peptidase (Bronstein *et al.* 1966). After digestion with pepsin and trypsin there were a few amino acids but the majority of the digest was peptides. Following peptic-tryptic-cotazym (PTC) and peptic-tryptic-cotazym-intestinal peptidase digestion the preparation had undergone virtually complete digestion to amino acids. Steatorrhoea was produced with the ultrafiltrate of the PT digest, the PTC digest and also acidic peptides isolated from the PT digests.

Many *in vitro* studies described later focussed on identifying toxic peptides in A-gliadin. Peptides found to be toxic *in vitro* contained the sequences Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro. To determine the *in vivo* toxicity of these motifs, patients were challenged with gliadin and then peptides aa 3-21, 31-45 and 202-220 which contained β-reverse turns and the putatively toxic tetrapeptide motifs Pro-Ser-Gln-Gln and / or Gln-Gln-Gln-Pro (Sturgess *et al.* 1994). The peptides were administered in random order with a period of recovery in between. Gliadin and peptide 31-45 induced a significant change in the villous height and crypt depth, a reduction in enterocyte height and an increase in the number of $CD3^+$ IELs while the other peptides did not. This study while implicating peptide 31-45, also proved that β-turn conformations and the previously mentioned tetrapeptides were not sufficient for coeliac toxicity.

1.8.2 *In vivo* testing of other cereals

There have been very few investigations into the toxicity of barley and rye. The early Dutch studies found that rye, similar to wheat, caused increased fecal fat excretion in patients (Dicke *et al.* 1953). Later, changes in the coeliac intestinal mucosa after rye and barley consumption were investigated (Anand *et al.* 1978). Five patients receiving

60g/day barley remained symptom-free but showed signs of morphological damage and reduced enterocyte lactase, sucrase and maltase activity. Of the two patients receiving the same quantity of rye, one developed diarrhoea and abdominal discomfort while the other remained symptom-free. Both showed signs of mucosal damage and reduced levels of disaccharidase activity. Baker and Read (1976) investigated the effects of barley on ten patients. Six of the 10 patients challenged with 120g/day developed gastrointestinal symptoms and an impairment of xylose excretion. One patient could not tolerate beer. In all six patients symptoms returned to normal 72 hours after the study. Four patients remained asymptomatic and showed no reduction in xylose excretion throughout the trial. It is therefore generally accepted that species falling within the genus *Triticum* are almost certainly toxic. Spelt wheats, durum wheats, kawmut and einkorn are also included in this (Kasarda 1994). Triticale, a hybrid of wheat and rye, developed to achieve a high protein, high yield cereal, is not recommended for coeliac use (Bell *et al.* 1981). With the exception of wheat starch all products of wheat including bran and germ, which may be contaminated with traces of endosperm, are not recommended for coeliac consumption (Bell *et al.* 1981).

Rice and maize, found to have no effect on fecal fat excretion in early trials, are considered safe for coeliac consumption (Dicke *et al.* 1953; Van de Kamer *et al.* 1953). Within the grass family sorghum, the millets, Job's tears, teff ragi and sugar cane are more closely related to maize and rice than wheat and are therefore unlikely to be toxic (Kasarda 1994). While wheat, barley and rye are monocots, buckwheat, amaranth and quinoa are dicots. Their very distant relationship would suggest that the latter cereals do not contain the harmful amino acid sequences found in wheat (Thompson 2001). No consensus has been reached on the toxicity of cereals outside the genus *Triticum* and opinions vary as to whether certain cereals should be included in the GFD or not (Thompson 2000). Clinical trials carried out with oats will be discussed in chapter three.

1.8.3 *In vitro* models of coeliac disease

Organ culture of coeliac small intestinal mucosa

Browning and Trier (1969) were the first to describe a method for maintaining small intestinal mucosal biopsies in culture. The method involved orientating biopsies villi-up on a stainless steal mesh and placing them in the central well of a plastic culture dish. Culture medium, supplemented with foetal calf serum (FCS) and antibiotics, was added

to the well until a thin layer was drawn over the villous surface. The dish was kept at 37°C in 95% O_2/5% CO_2 for the culture period (Browning and Trier 1969). ^3H thymidine incorporation confirmed cells in the crypts were proliferating and migrating up the villous (Browning and Trier 1969).

The preservation of coeliac biopsies *in vitro* enabled researchers to assess the effects of gliadin or gluten on the coeliac mucosa, without causing distress to the patient. These gliadin-induced mucosal changes could be classified as biochemical, morphometric and immunological. These changes were used as parameters to determine the *in vitro* toxicity or immunogenicity of gliadin fractions and peptides.

Biochemical changes

Enzyme analysis revealed reduced levels of alkaline phosphatase in untreated coeliac mucosa compared to normal control mucosa (Falchuk *et al.* 1974). The enzyme levels in the untreated tissue increased when biopsies were cultured in gliadin-free medium. So too did the regularity of the epithelial cells. However, addition of gliadin to the culture medium prevented the enzymatic improvement. Gliadin did not affect normal controls or coeliac patients in remission.

Morphometric changes

Howdle and colleagues disagreed with the sensitivity of the biochemical assay, finding no significant difference in alkaline phosphatase or α glucosidase activity in biopsies cultured with gluten. This was true of normal controls, treated and untreated coeliac patients (Howdle *et al.* 1981b). A more sensitive method for measuring histologic change was proposed. This involved measuring enterocyte height (Howdle *et al.* 1981a). It was discovered that enterocyte height in biopsies from untreated coeliac patients increased following culture in gluten free medium and decreased in the presence of gluten.

Culture with α gliadin or FF-III also induced marked signs of enterocyte deterioration in untreated coeliac biopsies (Fluge and Asknes 1981). Reduced enterocyte height, disorganisation of crypt architecture and deterioration of surface epithelial cells were noted. In this report changes were only observed in untreated patients. Patients in remission or normal controls showed no signs of enterocyte deterioration following challenge.

β, γ and ω fractions of gliadin reduced the enterocyte height and increased the thickness of the layer of necrotic epithelial cells in active coeliac biopsies. α gliadin was toxic to a lesser extent. This result may have been due to protein contamination as Howdle and colleagues (1984) later found that all gliadin fractions (α, β, γ and ω) reduced enterocyte height in biopsies from untreated and treated coeliac patients but not controls.

Weiser *et al.* (1983) took *in vitro* gliadin analysis to the peptide level. A peptide, corresponding to residues 3-55 of α type gliadin, was purified from a PT gliadin digest. Chymotrypsin digestion of this peptide produced peptides 3-24 and 25-55. Both were active by organ culture testing. Interestingly, both contained the Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro motifs. De Ritis *et al.* (1988) cleaved A-gliadin with cyanogen bromide to obtain 3 peptides, which spanned gliadin (1-127, 128-246, 247-266). Further digestion with chymotrypsin yielded smaller peptides; 1-30, 31-55, 1-55 and 56-68. Peptides 247-266 and 56-68 did not inhibit epithelial cell recovery whereas culture with peptides 1-127, 128-246, 1-55, 1-30 and 31-55 prevented the recovery of active coeliac biopsy specimens. These peptides had no effect on biopsies from patients with inactive disease. The highest level of sequence homology between the toxic peptides was Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro.

The measurement of enterocyte height following organ culture was also used to test the toxicity of synthetic gliadin peptides. Three peptides corresponding to residues 31-49, 202-220, and 3-21 of A-gliadin were synthesised (Shidrawi *et al.* 1995). While culture with medium alone improved the enterocyte height in treated and untreated patients, this was not observed when peptide 31-49 was added to the medium. No significant enterocyte changes were observed with the other two peptides or with OVA. The findings do not support the role for the Pro-Ser-Gln-Gln or Gln-Gln-Gln-Pro motifs. Pro-Ser-Gln-Gln was not present in toxic peptide 31-49 and was present in the other two non-toxic peptides. Non-toxic peptide 3-21 also contained the motif Gln-Gln-Gln-Pro.

Mauri *et al.* (1996b) also investigated the *in vitro* activities of peptides containing Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro. Peptides corresponding to residues 31-55, 31-43 and 44-55 of A-gliadin were synthesised and tested on treated and untreated coeliac biopsies. Peptide 31-43 contained the motif Gln-Gln-Gln-Pro and peptide 44-55 the motif Pro-Ser-Gln-Gln. Peptide 31-55 contained both. Peptides 31-43 and 31-55 prevented the increase in enterocyte height seen in coeliac biopsies cultured in gluten free medium. This result was observed in both treated and untreated biopsies. However, a higher

concentration was needed for activity in the case of the treated patients. Peptide 44-55 also induced toxic and immunological changes in treated and untreated coeliac biopsies at a higher concentration than the other two.

Immunological changes

Gliadin induces immunological changes in coeliac tissue during *in vitro* organ culture. Immune activation in biopsies may be used as a readout system to determine the safety of certain cereal proteins for coeliac patients. The following are some examples of immunological changes induced by gliadin in coeliac biopsies maintained in culture.

Gliadin stimulation of coeliac intestinal tissue results in the up-regulation of HLA-DR on enterocytes after just 2 hours (Maiuri *et al.* 1996a). ICAM-1 expression can be seen on cells in the sub epithelial compartment. Gliadin induces T cell and macrophage migration to the lamina propria sub epithelial compartment and stimulates infiltration of the surface epithelium (Maiuri *et al.* 1996a).

Culture with PT gliadin also results in an increase in the number of $CD25^+$ lamina propria T cells (Halstensen *et al.* 1993). This T cell activation is supported by the fact that *in vitro* gliadin stimulation also results in increased cytokine production. Nilsen *et al.* (1998) found that gliadin stimulation of treated coeliac biopsies results in an increase in IFN-γ mRNA. IL-2, IL-4, IL-6 and TNFα mRNA also increase after stimulation (Nilsen *et al.* 1998).

In vitro production of EMA following gliadin challenge will be discussed in chapter 2.

Developing Intestine from Rat Foetus

The intestines of seventeen-day-old rat foetuses were first proposed as a good *in vitro* test system in 1979 (De Ritis *et al.*). At this stage of development there are no villi present in the intestine and undifferentiated epithelial cells line the lumen. Villi form after 48 hours *in vitro* culture in medium alone. A PTC digest of gliadin and one of its subfractions (fraction 9) was shown to inhibit the development of the foetal rat intestine and cause degenerative changes (De Ritis *et al.* 1979). There was good correlation with human intestinal mucosa from patients with active coeliac disease. Gliadin did not have any effect on 21-day-old foetuses suggesting that gliadin was having a direct effect on immature enterocytes (De Ritis *et al.* 1979). Prolamins from rye, barley, oats and

sorghum also slowed down the *in vitro* development of the foetal rat intestine (Auricchio *et al.* 1984b). *Durum* wheat gliadins (Auricchio *et al.* 1982) as well as the prolamins of maize and rice (Auricchio *et al.* 1984b) did not prevent development and morphogenesis of the 17-day-old intestine.

K652 (S) cells

In the presence of PT gliadin K652(S) cells were shown to agglutinate to form a continuous layer with high resistance to shearing forces (Auricchio *et al.* 1984a). Once the cells differentiated in the presence of butyrate, they showed a marked decrease in agglutinating activity with PT gliadin. Agglutination by gliadin was inhibited by the saccharides mannan, D-mannose and N-acetyl-D-glucosamine. Mannan was also able to dissociate cells after agglutination (Auricchio *et al.* 1984a). *Durum* wheat was unable to induce agglutination at high concentrations (Auricchio *et al.* 1984a). K652(S) cells were used to further investigate the apparent "safety" of *durum* wheat (De Vincenzi *et al.* 1995). When PT digests of bread and *durum* wheat gliadin were fractionated into fractions, A, B and C, only fraction C was active in causing agglutination of K652(S) cells in both cases. However, fraction B from the *durum* wheat was able to inhibit the agglutinations caused by fraction C whereas fraction B from bread wheat was not (De Vincenzi *et al.* 1995). The inhibition was due to a 1157.5 Da peptide of 10 amino acids. It most likely competed with toxic peptides for binding to cells but was insufficient to trigger events that led to agglutination or toxicity (De Vincenzi *et al.* 1997).

Peptides aa 31-43 and aa 44-55 of A-gliadin were also tested for their ability to agglutinate K652(S) cells. The peptides contain the putative "toxic" amino acid sequences Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln respectively. Peptide aa 31-55 which contains both sequences was also tested. Both peptides 31-43 and 44-55 were active in agglutinating cells. However 44-55 agglutinated cells at a concentration 2 times greater than the peptide 31-43. Peptide 31-55 was the most active in agglutinating cells (De Vincenzi *et al.* 1994). The 1157.5 Da peptide isolated from *durum* wheat prevented agglutination of cells by the peptides 31-43 and 44-55 and also by PT digests of prolamins from wheat, rye, barley and oats (De Vincenzi *et al.* 1998).

CaCo-2 cells

Caco-2 cells are derived from a human adenocarcinoma, which is capable of expressing some differentiated characteristics of small intestinal enterocytes such as tight junctions,

microvilli, brush border enzymes and transport across the surface membrane (Rousset 1986). A growth inhibition of 50% was observed in Caco-2 cells when they were incubated with a PT digest of bread wheat gliadin. The colony forming ability of differentiated cells was also inhibited. Prolamin proteins from rye, oats and barley had a similar effect whereas those from *durum* wheat had none. The alkaline phosphatase activity of cells was also reduced by bread wheat gliadin but not by *durum* wheat (Giovannini *et al.* 1995). PT digest from wheat, barley, rye and oat prolamins were later found to decrease the incorporation of ^{14}C-thymidine and ^{14}C-uridine into cells compared to BSA and *durum* wheat. There was also a reduction, though less marked, in ^{35}S-methionine, ^{3}H-Glucosamine and ^{3}H-N-acetylmannosamine indicating that DNA and RNA synthesis and (glyco)protein synthesis was inhibited by these toxic cereal proteins (Giovannini *et al.* 1996). Cell viability tests showed no differences between treated and untreated cells. This suggests that while the toxic cereal proteins do not cause an immediate cytotoxic effect they do impair cell metabolism, which causes cell damage over an extended period (Giovannini *et al.* 1996). Peptides 31-43, 44-55 which contain the sequences Gln-Gln-Gln-Pro and Pro-Ser-Gln-Gln respectively and peptide 56-68 which lacks both amino acid sequences were tested with CaCo-2 cells. Peptide 31-43 was more effective at inhibiting cell growth than 44-55. Peptide 56-68 had no effect on cell proliferation. 31-43 was also more effective than 44-55 at inhibiting colony formation and reducing alkaline phosphatase activity (Giovannini *et al.* 1997).

Foetal Chick Intestine

Mothes and colleagues proposed the foetal chick intestinal assay as a model to study the effects of gliadin in coeliac disease (Mothes *et al.* 1985). Culture with gliadin inhibited an increase in disaccharidase activity in the developing intestine (Mothes *et al.* 1985). Fraction 9 and a dodecapeptide corresponding to residues 75-86 of A-gliadin significantly inhibited an increase in sucrase activity in explants and in the culture medium (Cornell and Mothes 1993). Others used this model to demonstrate the toxicity of gliadin peptides containing the sequences Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro (Kocna *et al.* 1991).

CHAPTER 2

Lactase and EMAs as *in Vitro* Markers of Prolamin Toxicity or Immunogenicity

2.1 INTRODUCTION

2.1.1 Brush border disaccharidases

Disaccharides and oligosaccharides are hydrolysed by enzymes located in the brush border membrane of the small intestine. Four enzymes carry out this function; sucrase-isomaltase, lactase phlorizinhydrolase, maltase-glucoamylase and trehalase (Gudmand-Høyer and Skovbjerg 1996). The enzymatic activity of each enzyme varies along the length of the small intestine. The highest activity of sucrase-isomaltase occurs in the middle of the intestine with less activity at the ligament of Treitz and in the distal ileum. Lactase shows maximum activity in the proximal intestine, which declines distally. In contrast, the activity of maltase increases along the small intestine and has its highest activity in the distal ileum. Trehalase, like lactase, has its highest activity in the proximal part of the jejunum (Asp *et al.* 1985; Skovbjerg 1981).

2.1.2 Lactase-phlorizinhydrolase

The lactase-phlorizin hydrolase (LPH) enzyme complex has 2 enzymatic active sites: one for lactase hydrolysis and the other for phlorizin hydrolysis. LPH occurs in the membrane as a dimer with two polypeptide chains of identical molecular weight. Each subunit carries both lactase and phlorizin hydrolase active sites (Mantei *et al.* 1988; Skovbjerg *et al.* 1981). The protein consists of five domains; a cleaved signal sequence, a 'pro' portion, the mature LPH containing the enzyme active sites, a membrane spanning hydrophobic segment near the C terminus and a short hydrophilic cytosolic segment at the C terminus. The C (in) – N (out) orientation of LPH is unusual to other brush border hydrolases (Mantei *et al.* 1988).

2.1.3 Lactase deficiency

Primary lactase deficiency

Primary lactase deficiency occurs as a rare congenital type and as the more common adult type (Villako and Maaroos 1994). Congenital lactase deficiency is due to homozygosity for an autosomal recessive gene. This form of lactase deficiency is evident in the new born after he/she is fed milk. In adult type hypolactasia patients are not completely deficient in lactase activity. Enzymatic activity is about 10 times lower

than that of the normal population. Studies of first-degree relatives of patients with primary hypolactasia show that there is a strong genetic association with the condition (Gudmand-Høyer and Skovbjerg 1996; Villako and Maaroos 1994).

The frequency with which lactose malabsorption occurs varies between different populations and races. The lowest frequency occurs in North West Europe, while almost 100% of the population in South-East Asia are lactase deficient (Gudmand-Høyer and Skovbjerg 1996). Lactase deficiency tends to correlate with traditional non-milk drinking areas (Flatz 1987).

Secondary lactase deficiency

In patients with secondary hypolactasia, lactase deficiency is the result of damage to enterocytes of the small intestine. Diseases of the intestinal mucosa such as giardiasis and tropical sprue and diet related factors e.g. protein deficiency and food sensitive enteropathies may cause lactase loss from the brush border (Villako and Maaroos 1994).

Symptoms of lactase deficiency

The symptoms of lactase maldigestion are abdominal pain, bloating, flatulence and diarrhoea (Dunphy *et al.* 1965). Some patients may present with steatorrhea. Diarrhoea is caused by unhydrolysed lactose, which draws water into the intestine by osmosis (Gudmand-Høyer and Skovbjerg 1996). Colonic bacteria hydrolyse lactose to produce fatty acids, carbon dioxide, hydrogen and methane, contributing to abdominal complaints (Arola and Tamm 1994).

Measuring lactase deficiency

Lactase deficiency may be detected non-invasively by the oral tolerance test. A dose of lactose is ingested and the development of typical symptoms e.g. watery stools is recorded (Desai *et al.* 1967). This test is regarded as unreliable as these symptoms are not specific for lactase deficiency. The number of false positives may be reduced by direct instillation of lactose into the duodenum (Arola 1994). A number of more reliable tests for lactase deficiency are carried out in the laboratory. The enzymatic activity of lactase is detected by homogenising intestinal biopsies and measuring the production of glucose from lactose (Andersen *et al.* 1983a). This method does not give an overall picture of lactase activity in the entire intestine (Bjarnason *et al.* 1996). A stool pH of less than 6 is also a positive indicator of hypolactasia. In addition stools may be

analysed for the presence of lactose using chromatography (Soeparto *et al.* 1972). Breath tests detect increased levels of hydrogen and methane in exhaled air in hypolactasia (Bjørneklett and Jenssen 1982; Levitt and Donaldson 1970). Variations on the breath test include the measurement of $^{14}CO_2$ and $^{13}CO_2$ after ingestion of lactose labelled with ^{14}C or ^{13}C (Arola 1994). Lactose intake is normally accompanied by an increase in blood glucose levels. An increase in blood glucose of less than 1.1 mmol after a lactose load is indicative of hypolactasia. This test has a high rate of false positives (Bjarnason *et al.* 1996). Hypolactasia may be diagnosed by urinary tests measuring the renal excretion of galactose following ingestion of lactose. Low levels of galactose are indicative of hypolactasia (Arola *et al.* 1982). Intact lactose in urine is inversely related to the rate of intestinal hydrolysis. Lactose is sometimes measured with reference to lactulose (Bjarnason *et al.* 1996). Though not used routinely, histology is a useful method of measuring lactase expression. In lactase persistent individuals lactase is expressed uniformly on enterocytes. In hypolatasia there is patchy distribution of the enzyme (Srinivasan *et al.* 1999). This method is advantageous in that one can also observe mucosal damge, which may be responsible for reduced lactase expression. However, this method is comparable to measuring enzymatic activity in that the results are not representative of the entire intestine.

2.1.4 Secondary hypolactasia in coeliac disease

Reduced expression of disaccharidases and other brush border enzymes have been observed in coeliac patients with varying degrees of mucosal damage (Andersen *et al.* 1983b; O'Grady *et al.* 1984; Peters *et al.* 1978). Biochemical (Andersen *et al.* 1983a; O'Grady *et al.* 1984), histochemical (Maiuri *et al.* 1991; Mercer *et al.* 1990; Srinivasan *et al.* 1999), urinary (Weser and Sleisenger 1965) and flow cytometric (Feighery *et al.* 2001) methods have shown hypolactasia in untreated coeliac patients. Lactase appears to be the disaccharidase most sensitive to mucosal damage (O'Grady *et al.* 1984). Treatment with a GFD results in recovery of the enzyme. There is an inverse correlation between histology grade and lactase expression or activity, supporting the theory that enzyme loss is secondary to mucosal damage (Feighery *et al.* 2001; Mercer *et al.* 1990; Nieminen *et al.* 2001). Recovery of lactase is slower than other disaccharidases (O'Grady *et al.* 1984) and may remain depressed for years particularly in older patients (Peña *et al.* 1972). Once morphology and lactase levels have returned to normal,

reintroduction of gluten into the diet depresses enzyme expression (Peña *et al.* 1972; Srinivasan *et al.* 1999)

2.1.5 T cell mitogens

OKT3

It has been known for some time that polyspecific antiserum raised against human T cells has mitogenic properties (Brochier *et al.* 1976). The constituents of the antiserum were elucidated by generating monoclonal antibodies against determinants on the surface of the T cell. This was achieved using the hybridoma technique, which generated a panel of monoclonal antibodies recognising several cell membrane components (Kung *et al.* 1979). The antibodies were designated OKT and of these, OKT3, an IgG2 immunoglobulin was the most potent T cell mitogen (Kung *et al.* 1979). It is effective at 10^{-12}M strengths and active over large concentration ranges in contrast to the mitogens, phytohaemagglutenin (PHA) and ConA (Chang *et al.* 1981; Van Wauwe *et al.* 1980).

OKT3 induces T cell activation by binding to CD3 on the surface of T cells (Borst *et al.* 1983). CD3 is associated with the TCR and is known to play a critical role in T cell activation (see section 3.1.1). OKT3 induces T cell proliferation, IL-2 secretion and upregulation of the IL-2R (Schwab *et al.* 1985). A down-modulation and internalisation of CD3 and the TCR follows binding of OKT3 (Gebel *et al.* 1989; Telerman *et al.* 1987).

Mitogenic responses to OKT3 are accessory cell dependent. Results of initial experiments proposed that the function of accessory cells was to provide Fc receptors for the antibody to bind (Landegren *et al.* 1984). HLA class II molecules were also deemed important when it was observed that anti-MHC class II antibodies inhibited monocyte dependent OKT3 induced T cell activation (Racioppi *et al.* 1990). Binding of OKT3 to CD3 with accessory Fc receptors causes the loss of CD3 from the surface and induction of IL-2R. However, another signal is required to induce production of IL-2. This is probably provided by HLA-class II antigens (Schwab *et al.* 1985). MHC engagement controls a signal responsible for production of IL-1β and IL-6 in monocytes (Racioppi *et al.* 1990). IL-1 may upregulate IL-2 production in T cells following OKT3 stimulation (Racioppi *et al.* 1990; Schwab *et al.* 1985).

Phorbol esters

Croton oil has the ability to induce epidermal tumors in mice. Esterified derivatives of the tetracyclic diterpene phorbol are responsibe for its mitogenic properties. Phorbol 12-myristate 13-acetate (PMA) is the most potent mitogen of the eleven active diesters found in the oil (Blumberg 1980). Phorbol esters act on many different cell types. However, particular emphasis has been placed on mitogenesis in T lymphocytes (Blumberg 1980).

Phorbol esters consist of a lipophilic acyl chain and a hyrophilic phorbol nucleus. The acyl chain is responsible for the insertion and possibly the orientation of the molecule in the phospholipid bilayer of the cell membrane. This allows the phorbol nucleus to reach its cellular target, phosphokinase C (PKC). There is a direct relationship between the length and lipophilic property of the acyl side chain and the biological activity of the phorbol ester (Dobbs and Katz 1988; Favero *et al.* 1990).

Endogenous, diacylglycerol (DAG) binds to PKC at the cell membrane and synergises with inositol triphosphate (IP_3) to activate the enzyme (see section 3.1.1) (Kishimoto *et al.* 1980). PMA has a similar structure to DAG and binds to the same binding site on PKC (Burns and Bell 1991; Cabot 1984). Derivatives of the core region with the same acyl chain differ in their ability to induce lymphocyte mitogenesis, indicating that the hydrophilic nucleus, not the acyl chain binds to PKC (Favero *et al.* 1990). The hydroxyl group in the C4 position is important for the induction of mitogenesis (Favero *et al.* 1990). PKC in resting T cells is normally found in the cytosol however after phorbol ester stimulation the enzyme is translocated to the membrane (Isakov and Altman 1987).

Phorbol ester binding to PKC initiates biochemical reactions, which result in cell activation and proliferation (Touraine *et al.* 1977), production of IL-2 (Hirano *et al.* 1984), upregulation of the IL-2R (Isakov and Altman 1987) and down modulation of CD4 (Favero *et al.* 1990).

Phorbol estsers have an ability to synergistically enhance the response of cells to Ca^{2+} ionophores and anti-CD3 antibodies (Berry *et al.* 1989; Dobbs and Katz 1988; Isakov and Altman 1987). It is thought that treatment of T cells with anti-CD3 antibodies causes transient activation of PKC and that an additional stimulus from e.g. PMA is required for prolonged PKC activation (Berry *et al.* 1989).

Lipopolysaccharide (LPS)

LPS is a component of the outer membrane of gram-negative bacteria. It is composed of a saccharide component and a lipid component called lipid A. LPS activates monocytes and macrophages to produce, TNF-α, IL-1 and IL-6 (Dobrovolskaia and Vogel 2002). T cells, in a mononuclear cell population cultured with LPS, express the activation marker CD25 and produce IL-2 and IFN-γ mRNA (Ulmer *et al.* 2000). However this T cell activation is monocyte dependent. LPS cannot stimulate purified T lymphocytes. Cell-to-cell contact is necessary for T cell stimulation. The interaction is not MHC dependent; co-stimulation signals through CD28 and/or CTLA-4 are required. IL-12 is also important for optimal T cell activation by LPS (Ulmer *et al.* 2000).

2.1.6 Cytokine mediated enterocyte damage

The coeliac mucosa is characterised by villous atrophy, crypt hyperplasia and by increased numbers of inflammatory cells in the intra-epithelial spaces and lamina propria region. Evidence suggests that this inflammatory cell infiltrate is primarily responsible for the observed musocal damage. T cell stimulation with pokeweed mitogen (PWM) or anti-CD3 antibody can induce crypt cell proliferation and rapid loss of villus epithelial cells in human foetal small intestine maintained in culture (da Cunha Ferreira *et al.* 1990). Similarly mice loose the columnar morphology of their enterocytes and develop numerous apoptotic bodies in their crypt region when injected intraperitoneally with anti-CD3 antibody (Merger *et al.* 2002).

T cells mediate enterocyte damage through cytokines. Deem *et al.* (1991) showed that stimulated LPLs produced soluble factors that were cytotoxic to the colonic epithelial cell line HT-29. Also supernatants from activated coeliac T cell clones reduced enterocyte height in intestinal biopsies (Przemioslo *et al.* 1995). Attention has focussed on the cytotoxic properties of TNF-α and IFN-γ. Addition of either anti-TNF-α or anti-IFN-γ resulted in nearly complete inhibition of HT-29 killing by T cell supernatants (Deem *et al.* 1991). Similarly, mucosal alterations induced by T cell clone supernatants could be blocked by pre-incubating biopsy tissue with anti-IFN-γ (Przemioslo *et al.* 1995).

In vivo, normal mice repeatedly injected with IFN-γ showed villous epithelial cell damage and acceleration of epithelial renewal (Guy-Grand *et al.* 1998). Also anti-IFN-γ

treatments abrogate enteropathy in the T cell mediated graft-versus-host-disease (GVHD) in mice (Mowat 1989).

How IFN-γ mediates changes in cell morphology is unclear. IFN-γ can cause rearrangements of filamentous actin in epithelial cells. This may lead to increases in paracellular permeability seen in T84 monolayers treated with the cytokine (McKay and Baird 1999). IFN-γ also upregulates cell surface markers on enterocytes. Workers showed that addition of IFN-γ or TNF-α to small intestinal biopsy cultures increased HLA-class II expression by enterocytes, leading to uniform staining throughout the length of the villus (Sturgess *et al.* 1992). IFN-γ sensitises intestinal epithelial cells to apoptosis via FAS/FAS ligand (L) by upregulating FAS expression on these cells (Martin and Panja 2002; Ruemmele *et al.* 1999b). IFN-γ may also induce apoptosis by upregulating TNFR-1 expression (Guy-Grand *et al.* 1998). The observed synergy between IFN-γ and TNF-α may be due to the upregulation of TNF receptors by IFN-γ. IFN-γ may also mediate enterocyte apoptosis by upregulating Bak expression, a member of the Bcl-2 family of proteins that acts as a promoter of apoptosis (Chernavsky *et al.* 2002).

Mice injected with TNF-α develop intestinal lesions within minutes of administration (Guy-Grand *et al.* 1998). The cytokine induces enterocyte apoptosis, which starts at the tip of the villous and progresses towards the crypt (Guy-Grand *et al.* 1998; Piguet *et al.* 1999). The apoptotic enterocytes become detached and there is shrinkage of the villi. Blocking apoptosis also inhibits detachment. Therefore apoptosis induces detachment and not the reverse (Piguet *et al.* 1999). Garside *et al.* (1993) also detected apoptosis of crypt cells, both *in vivo* and *in vitro*, following TNF-α treatment. It was subsequently reported that TNF increases expression of the cell death mediator, p53, in crypt enterocytes. The authors hypothesised that TNF-α may induce a p53 dependent apoptosis in crypt cells and a p53 independent apoptosis in differentiated cells from the villi (Piguet *et al.* 1998). TNF-α cytotoxicity has also been demonstrated *in vitro*; in the rat intestinal epithelial cell line IEC-18 (Chang and Tepperman 2001), in the transformed rat jejunal intestinal crypt cell line IEC-6 (Ruemmele *et al.* 1999a) and in the colonic cell line HT-29 (Gitter *et al.* 2000).

TNF binds to its receptors TNFR-1 and -2 and induces caspase activity in target cells. One study reported that capase-3 was activated in cells in response to TNF-α stimulation and that inhibition of this enzyme reduced apoptosis (Chang and Tepperman 2001). In another study, inhibition of caspase-3 failed to block TNF-α induced apoptosis in IEC-6 cells but treatment with the interleukin-1β-converting enzyme (ICE)-caspase inhibitor completely abolished the apoptotic response to TNF-α (Ruemmele *et al.* 1999a). TNF-α also sensitises enterocytes to FAS induced apoptosis by upregulating FAS expression on the intestinal epithelial cells (Ruemmele *et al.* 1999b).

In addition to mediating apoptosis, TNF-α may disrupt the epithelial cell barrier by altering the epithelial tight junctions (Rodriguez *et al.* 1995). Degradation of tight junctions and apoptosis contribute to the increased paracellular leakage across the epithelial layer observed following TNF-α treatment (Gitter *et al.* 2000).

IL-15 has recently emerged as a possible mediator of enterocyte damage in coeliac disease. IL-15 positive cells are increased in untreated coeliac tissue and antibodies to this cytokine can block gliadin induced enterocyte apoptosis in these biopsies (Maiuri *et al.* 2000). The same study also demonstrated that IL-15 can induce enterocyte expression of Ki67, transferrin receptor and FAS in treated coeliac biopsies. Guy-Grand *et al.* (1998) also showed that mice that were injected with IL-12 demonstrated increased enterocyte cell death and shrinkage of villi. This IL-12 mediated epithelial cell damage required the presence of IELs and was dependent on IFN-γ secretion to carry out its cytotoxic effects.

2.1.7 *In vitro* production of endomysial antibodies

Cultured jejunal biopsies from untreated coeliac patients produce more IgG and IgM AGAs than control biopsies. Coeliac tissue also secretes more total IgM and IgA than controls (Ciclitira *et al.* 1986). Recently an investigation into EMA production at mucosal sites was conducted. EMAs were detected in the culture medium of untreated coeliac biopsies irrespective of gliadin challenge while cultures from non-coeliac patients were negative. In treated coeliacs EMAs were not detected in the cultures challenged with medium alone. Importantly, gliadin induced EMA production in 17/23 of these biopsies after 24 hours culture (Picarelli *et al.* 1996). A subsequent investigation showed that gliadin induced EMA production in treated coeliac biopsies is time dependent (Picarelli *et al.* 1999). EMAs are absent at 6 hours post gliadin challenge, at 24 hours 64% of the supernatants are positive and 100% are positive for EMA at 72 hours. This indicates new EMA synthesis in response to gliadin challenge.

This *in vitro* evidence of mucosal EMA production was soon supported by *in vivo* studies (Picarelli *et al.* 2002). All fecal supernatanats from patients with untreated coeliac disease were found to be positive for EMA. EMAs were not detected in supernatants from coeliacs on a GFD or from control subjects (Picarelli *et al.* 2002).

In vitro EMA production was used as a readout system to determine the immunogenicity of the gliadin peptide aa31-43. The peptide was capable of inducing EMA production in all eleven treated coeliac biopsies tested. It proved to be a more potent stimulator than a PT digest of gliadin, which induced EMA production in 10/11 biopsies (Picarelli *et al.* 1999). Biopsy culture with the 31-43 peptide was later investigated as a diagnostic tool for coeliac disease (Carroccio *et al.* 2002a). 82/91 newly diagnosed coeliacs secreted EMA into the supernatant after culture with medium alone. After 48 hours culture with the peptide this number was increased to 87. Because of the already high sensitivity and specificity of serum EMAs, their detection in cultured intestinal mucosa with the addition of peptide did not significantly improve diagnosis in patients with coeliac disease. However, the EMA assay after biopsy culture identified 7 patients with grade 1 lesions who were negative for serum EMAs. Therefore mucosal EMA was reported to improve sensitivity in patients with mild mucosal lesions (Carroccio *et al.* 2002a).

2.1.8 Aim of this chapter

The aim of this study was to develop an *in vitro* system that would identify cereal prolamins, which are toxic or immunogenic in coeliac disease. It has been proposed that gliadin causes enterocyte damage in coeliac patients either by direct toxicity or by activating T cells, which in turn secrete cytotoxic cytokines. One of the consequences of enterocyte damage is a reduction in brush border lactase expression. In view of this, lactase was investigated as an indicator of enterocyte damage and therefore of cereal toxicity or immunogenicity. Biopsies from coeliac patients were cultured in the presence of gliadin or gluten and lactase expression measured by flow cytometry. As a positive control lactase was also measured following biopsy challenge with various mitogens and cytotoxic cytokines, which are potent mediators of enterocyte damage *in vitro*.

In vitro EMA production has recently been reported to be a sensitive marker of gliadin immunogenicity. EMA secretion was therefore also assessed as a possible indicator of cereal immunogenicity in this study.

2.2 MATERIALS AND METHODS

2.2.1 Patients involved in the lactase study

Eight duodenal biopsies were taken from each of 14 coeliac patients and 23 controls by endoscopy. The mean age of the control group was 43 years (range: 18 to 75 years) and the coeliac group 44 years (range: 28 to 65 years). The male:female ratio was 1:2.7 and 1:1.8 for the control and coeliac group respectively. Ethical approval for the study was granted by the Ethics Committee Board of St. James's Hospital, Dublin, Ireland. Serological and histological details of the coeliac patients are given in Table 2.1. Histology was normal in the disease control patients.

Table 2.1 Coeliac patient details

Patient	Gender	Serum EMA	Histology grade
1	Male	-	0
2	Male	-	0
3	Male	-	1
4	Female	-	1
5	Female	-	1
6	Female	-	0
7	Male	-	2
8	Female	-	1
9	Female	-	1
10	Female	-	0
11	Female	+	3
12	Male	-	1
13	Female	(+)	0
14	Female	-	1

Histology was graded as follows: 0 = normal biopsy; 1= raised intraepithelial lymphocyte (IEL) count; 2 = partial villous atrophy with raised IELs; 3 = subtotal villous atrophy with raised IELs. (+), weak positive.

2.2.2 Preparation of gliadin and the PT digest of gluten

Gliadin (Sigma, USA) was dissolved in 0.1 M acetic acid and prepared as a stock solution of 10mg/ml and stored at -20°C. The stock gliadin preparation was diluted in RPMI-1640 (Gibco BRL, Scotland) to give a final concentration of 1mg/ml.

100g of gluten (Sigma, USA) was digested in 1l of 0.2 M HCl with 2g pepsin (Sigma, USA) for 2h at 37°C. The solution was adjusted to pH 8 with 2 M NaOH. Gluten was then digested with 2g of trypsin (Sigma, USA) for 4h at 37°C with continuous stirring. The proteases were inactivated by heating at 100°C for 30min. The sample was freeze-dried and stored at -70°C. PT gluten was diluted in RPMI-1640 to give a final concentration of 1mg/ml.

2.2.3 Organ culture

Duodenal biopsies were cultured within 15min of endoscopy. The biopsies were orientated villous side up on a nylon gauze and placed in the central well of a 2 ml organ culture dish (Falcon). The well was filled with a volume of RPMI-1640, supplemented with 15% heat inactivated foetal FCS (Sigma, USA) and 1% antibiotic/antimycotic solution (Gibco BRL, UK), sufficient to reach the cut surface of the biopsy (Figure 2.1(A)). In the majority of cases, four biopsies were cultured with RPMI-1640 and four were stimulated with one of the stimulants listed in Table 2.2. In eleven normal controls and 6 coeliac patients, biopsies were divided to facilitate culture with two or more stimuli. The culture dishes were then placed in an organ culture chamber, gassed with a mixture of 95% O_2 / 5% CO_2 and incubated at 37°C for 24h (Figure 2.1(B)). Gas was renewed at 24h if the culture period was extended to 48h.

2.2.4 Isolation of epithelial cells, intraepithelial lymphocytes and lamina propria lymphocytes

Biopsies were placed in calcium and magnesium free HBSS (Gibco BRL, Scotland) containing 1mM dithiothreitol (DTT) (Sigma, USA) and 1mM EDTA (Sigma, USA) and continuously agitated for 1h at 37°C. The supernatant was then removed, cells were washed twice and resuspended in RPMI-1640.

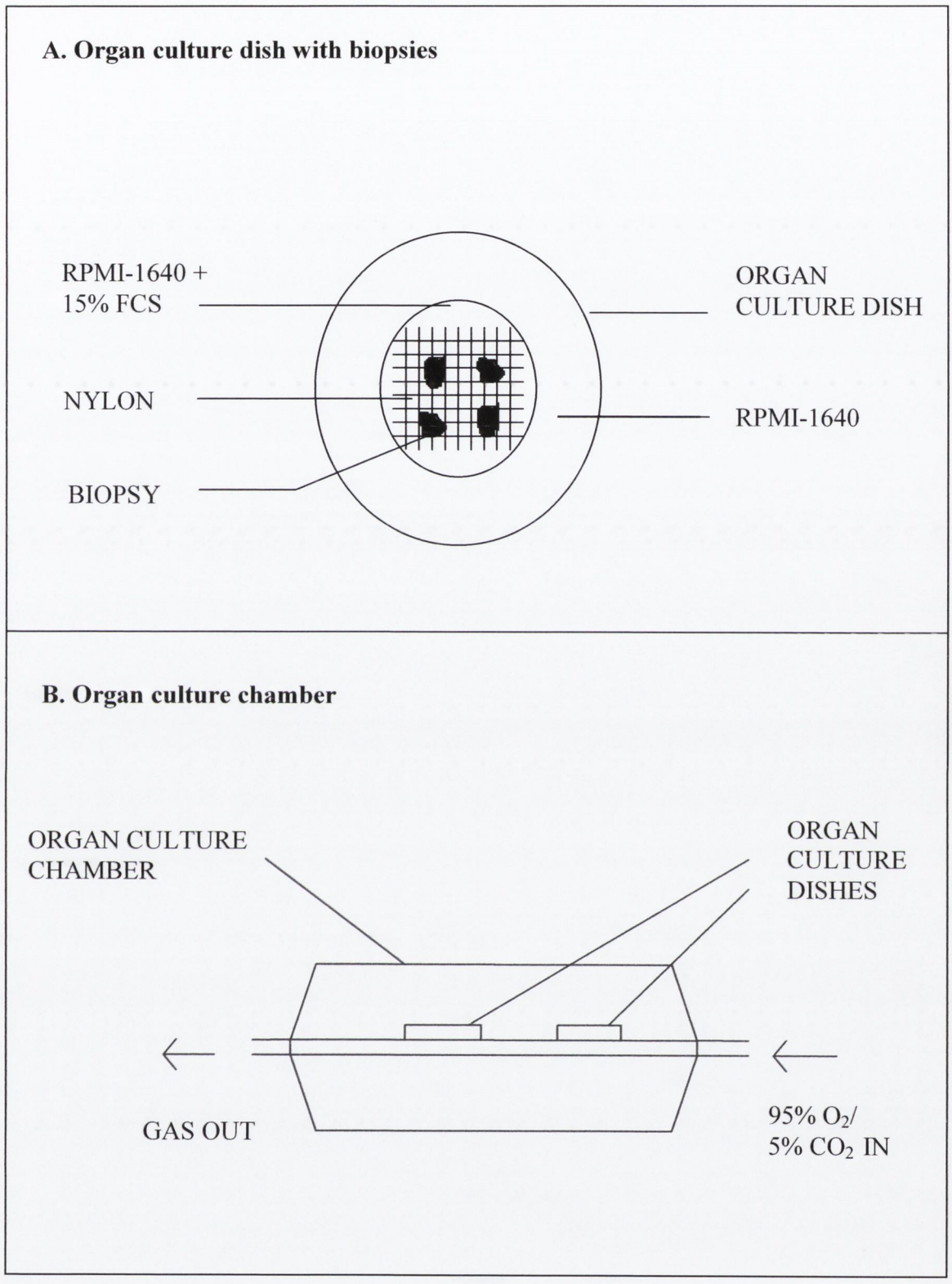


Figure 2.1 The Organ culture system

The remaining lamina propria was washed twice in RPMI-1640 by shaking. The medium was removed and the tissue teased out with a sterile scalpel. The tissue was collagenase (128 U/ml) treated for 2.5h at 37°C with continuous agitation. The medium was removed and cells washed twice and resuspended in RPMI-1640.

Table 2.2 Stimulants and patients used in the organ culture experiments

Stimulant	Concentration	Supplier	Coeliacs	Controls
RPMI-1640		Gibco, Scotland	2	5
Gliadin	1mg/ml	Sigma, USA	3	1
Gluten	1mg/ml	Sigma, USA	4	1
OKT3	30μg/ml, 60μg/ml	ATCC, USA	2	8
PMA	25,75 ng/ml, 1μg/ml	Sigma, USA	2	8
LPS	10,20,50μg/ml	Sigma, USA	1	3
TNF-α	20ng/ml	R&D Systems, UK	3	5
a-TNFR1+TNF-α	15μg/ml+20ng/ml	R&D Systems, UK	4	4
IFN-γ	1μg/ml	R&D Systems, UK	2	1
MIF	12ng/ml	R&D Systems, UK	2	0
PWM	7μg/ml	Sigma, USA	0	1

PMA, Phorbol myristate acetate; TNF-α, tumour necrosis factor alpha, a-TNFR1, anti-tumour necrosis factor receptor 1; LPS, lipolysaccharide, IFN-γ, interferon gamma; MIF, migration inhibition factor; PWM, pokeweed mitogen; ATCC, American Tissue Culture Collection; R & D, Research and Development.

2.2.5 Cell number and viability

Cells were diluted 1:20 in 0.1% ethidium bromide and acridine orange (EB/AO). The cells were counted in a haemocytometer under ultra violet light. Viable cells stained green while non-viable cells appeared orange. The cell suspension was adjusted to a concentration of 1 x 10^6cells/ml.

2.2.6 Cell staining for flow cytometry

Cells were dual stained for cell surface markers using the mouse anti-human monoclonal antibodies listed in Table 2.3. 5 µl of the primary antibodies were incubated with 1 x 10^5 cells (100µl aliquots) for 30min at 4°C. Cells were washed twice with PBS (1% BSA). Directly labelled cells were fixed in 0.5 ml of 0.5% paraformaldehyde (BDH, UK). In the case of anti-lactase, anti-HLA-DR, anti-TNFR-1 and OKT3, where the antibody was unlabelled, cells were incubated with 100µl of a 1:50 dilution of phycoerythrin (PE) conjugated rabbit anti-mouse antibody for 30 min at 4°C. Cells were washed again with PBS (1% BSA) and blocked with 100µl normal mouse serum at a 1:100 dilution for 30 min at 4 °C. Following another wash, 5µl of Ber-EP4 labelled with fluorescein isothiocyanate (FITC) was added and allowed to incubate for 30min. Cells were washed again and resuspended in 0.5% paraformaldehyde.

Table 2.3 Antibodies used in the flow cytometric experiments

Antibody	Florescent label	Supplier
Control IgG1 / IgG2a	FITC/PE	BD, Belgium
Control IgG1	Pure	BD, Belgium
CD3	PE	BD, Belgium
CD25	FITC	BD, Belgium
Ber-EP4	FITC	Dako, Denmark
Lactase M-LAC 4	Pure	Swallow, London (gift)
HLA-DR	Pure	BD, Belgium
TNFR1	Pure	R&D Systems, UK
OKT3	Pure	ATCC, USA
Rabbit anti-mouse	PE	Dako, Denmark
Rabbit anti-mouse	FITC	Dako, Denmark

BD, Becton Dickinson; PE, phycoerythrin; FITC, fluorescein isothiocyanate; ATCC, American Tissue Culture Collection

2.2.7 Flow cytometric analysis

Flow cytometric analysis was performed using a FACScan analyser (Becton Dickinson, Belgium) using the settings listed in Appendix III. 10,000 cells were acquired for each sample. Cells were viewed on the basis of size (forward scatter; FSC) and granularity (side scatter, SSC) and a gate drawn around the lymphocyte population (Figure 2.2 (A)). $CD3^+$ and $CD25^+CD3^+$ cells were viewed on an FL1/FL2 dot plot. Quadrants were set using the isotype matched control antibodies IgG1/IgG2a (Figure 2.2 (B-D)). The number of $CD25^+$ T cells were calculated as a percentage of the total $CD3^+$ population. The extent of T cell activation in stimulated biopsies was determined by comparing the percentage of $CD25^+$ cells from stimulated biopsies with those cultured in RPMI-1640 alone.

Epithelial cells were visualised on the basis of size (FSC) and staining with Ber-EP4 (FL1) (Figure 2.3 (A)). A gate was drawn around the epithelial cell population. Quadrants were set using the appropriate control antibody. The number of lactase positive enterocytes were viewed on a FL-1/FL-2 dot plot. Lactase positive enterocytes were expressed as a percentage of the total enterocyte population (Figure 2.3 (D)). Due to the high constitutive expression of HLA-DR (Figure 2.3 (C, F, I)) levels were measured in median fluorescence intensity values (MFI) rather than percentage positive cells. Changes in lactase or HLA-DR were determined by comparing expression from stimulated biopsies with expression from biopsies from the same individual cultured in RPMI alone.

2.2.8 TNF-α blocking studies

The effect of TNF-α on lactase expression was blocked using an antibody to TNF R-1. Biopsies were cultured with anti-TNF R-1 for 6h. The medium was removed and biopsies were cultured with 20ng/ml TNF-α for 42h. These biopsies were compared to biopsies cultured with TNF-α and biopsies cultured with medium for the same period of time.

2.2.9 Subjects involved in the endomysial antibody study

EMA secretion was investigated in biopsy supernatants from 15 coeliacs and 5 controls. The coeliac male: female ratio was 1:1.5 and the mean age was 49.5 years (range 32-65).

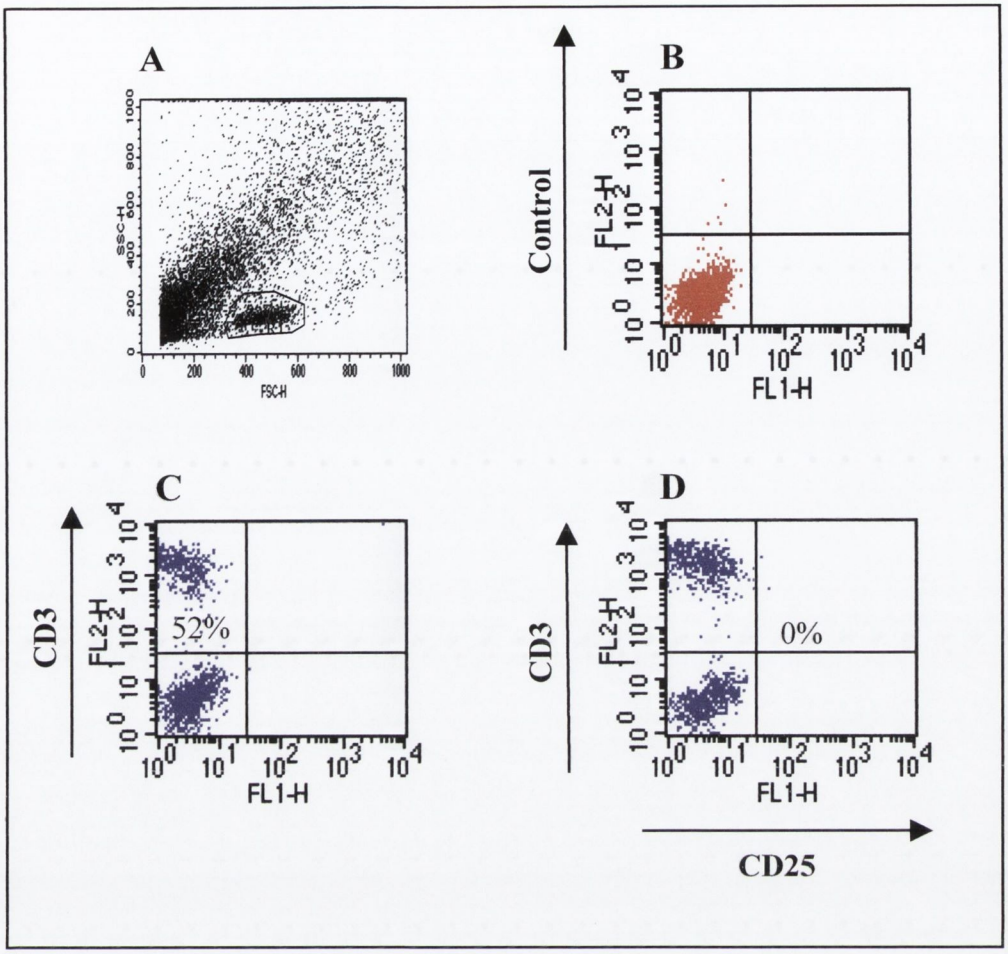


Figure 2.2 Flow cytometric analysis of IELs and LPLs from a coeliac patient. (A) Size vs granularity plot. A gate was drawn around lymphocytes in the FSC region 400-600. FL1/FL2 plots of (B) control antibody (C) $CD3^+$ and (D) $CD25^+ CD3^+$ cells.

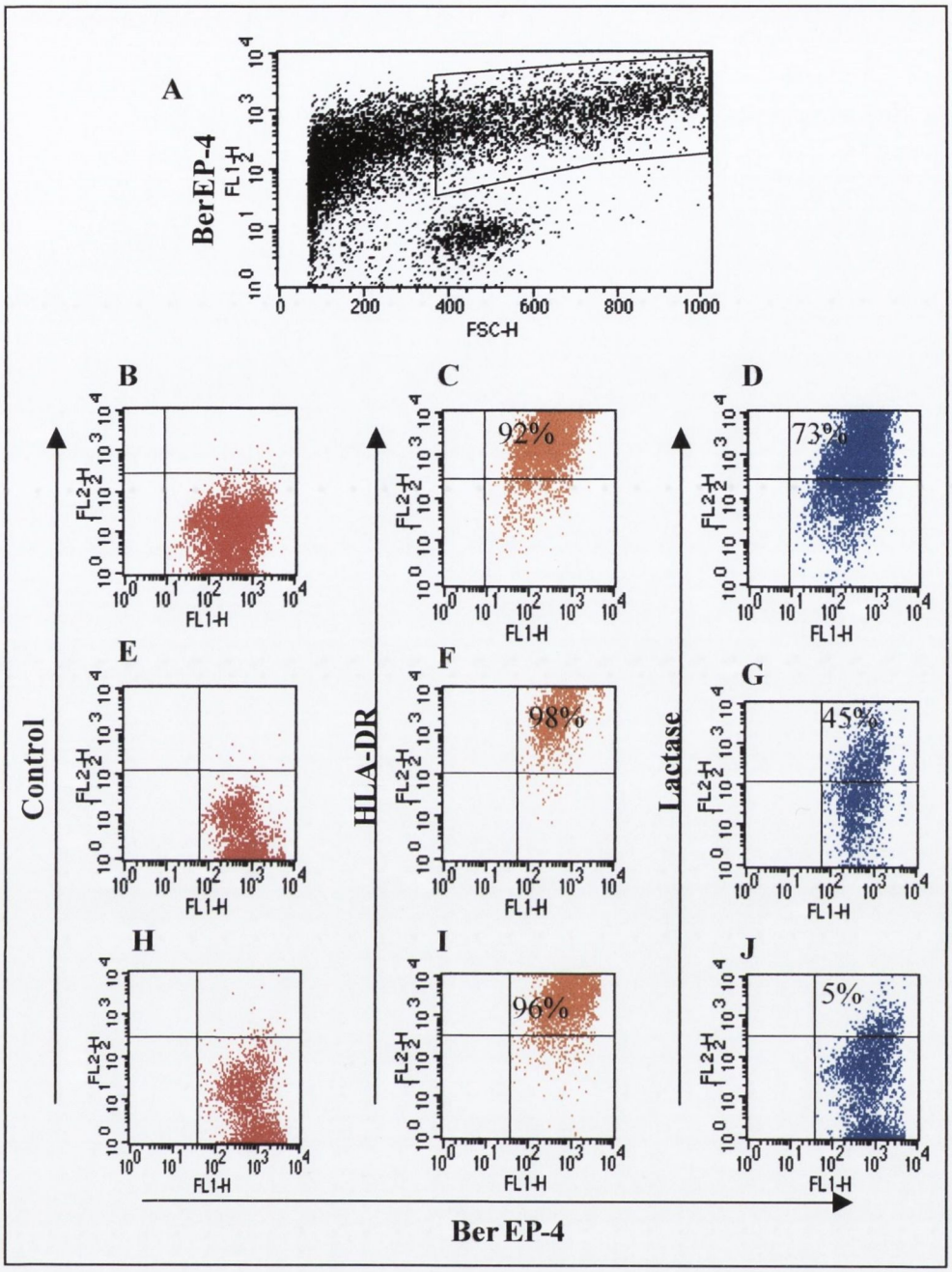


Figure 2.3 Flow cytometric analysis of enterocytes. (A) An FSC vs FL-1 plot with a gate drawn around the enterocyte population. The FL-1 / FL-2 plots represent control, HLA-DR and lactase expression on enterocytes from a normal control (B, C and D), a treated coeliac patient (E, F and G) and an untreated coeliac patient (H, I and J) following 24h culture with RPMI-1640.

The male:female ratio in the normal control group was 1:4 with a mean age of 55.8 years (range 24-67). Biopsies were cultured using the organ culture system described previously. Four biopsies from each patient were cultured with RPMI-1640 and four with a PT gliadin digest (see sections 3.2.1 and 3.2.2 for preparation). The biopsies were cultured for the following durations; 4h (9 coeliacs and 2 controls), 24h (2 coeliacs and 2 controls) and 48h (4 coeliacs and 1 control). After each time point the supernatants were removed and stored at -20°C.

2.2.10 EMA detection in organ culture supernatants

Monkey oesophagus slides (The Binding Site, UK) were air-dried for 20 min. A positive control serum sample and test serum samples were diluted in 1:5 in PBS(0.02% BSA). Culture supernatants were tested undiluted. 70µl of each sample was added to a section on the slide. The slides were incubated for 20min and washed twice in PBS with gentle agitation. Excess liquid was blotted off and FITC labelled anti-human-IgA (diluted 1:50 in PBS) added to the sections. The slides were incubated for a further 20min and washed as before. The slides were then mounted in glycerol mountant and read using fluorescence microscopy. Slides were graded as positive or negative depending on the staining pattern on the monkey oesophagus tissue.

2.3 RESULTS

2.3.1 Cell viability

Over 90% of cells were viable after culture periods of up to 48h. This was the case for biopsies cultured with RPMI-1640 and for those cultured with a stimulus.

2.3.2 Effect of organ culture on lactase, HLA-DR and CD25 expression

Firstly, we examined whether culturing the biopsies in medium alone would have any effect on enterocyte lactase or HLA-DR expression or on the number of $CD3^+CD25^+$ IELs. Enterocytes were analysed by flow cytometry for lactase expression before and after 24h culture with RPMI-1640 (15% FCS). We found that organ culture had little effect on lactase levels (Figure 2.4). A mean 3.5% (median 2%) reduction in lactase positive enterocytes was seen in 2 coeliacs and 5 control patients (range: 12% decrease to 5% increase).

Culture with RPMI-1640 did not cause significant alterations in enterocyte HLA-DR or CD25 expression on $CD3^+$ IELs. HLA-DR MFI was reduced by an average of 434 (range: 1217 decrease to 675 increase). $CD25^+CD3^+$ cells were increased by a mean 0.3 % following culture (range: 3% decrease to 5.8% increase).

2.3.3 Effect of gliadin and gluten on lactase, HLA-DR and CD25 expression

Biopsies from 3 coeliacs and 1 control were cultured with 1mg/ml gliadin for 24h and biopsies from 4 coeliacs and 1 control cultured with the same quantity of PT gluten for 48h. The wheat proteins caused little change in lactase expression in these 7 coeliacs and 2 controls (Figure 2.5). Taken together gliadin and gluten caused a mean 5.7% (median 5%) reduction in lactase expressing enterocytes (range: 11% decrease to 2% increase). Gliadin or gluten did not induce HLA-DR expression or activation of $CD3^+$ IELs in this subject group. There was a mean reduction in HLA-DR MFI of 70 (range: 1002 decrease to 631 increase) and a mean 0.4% reduction in $CD25^+CD3^+$ IELs (range: 1.08% decrease to 1.37% increase).

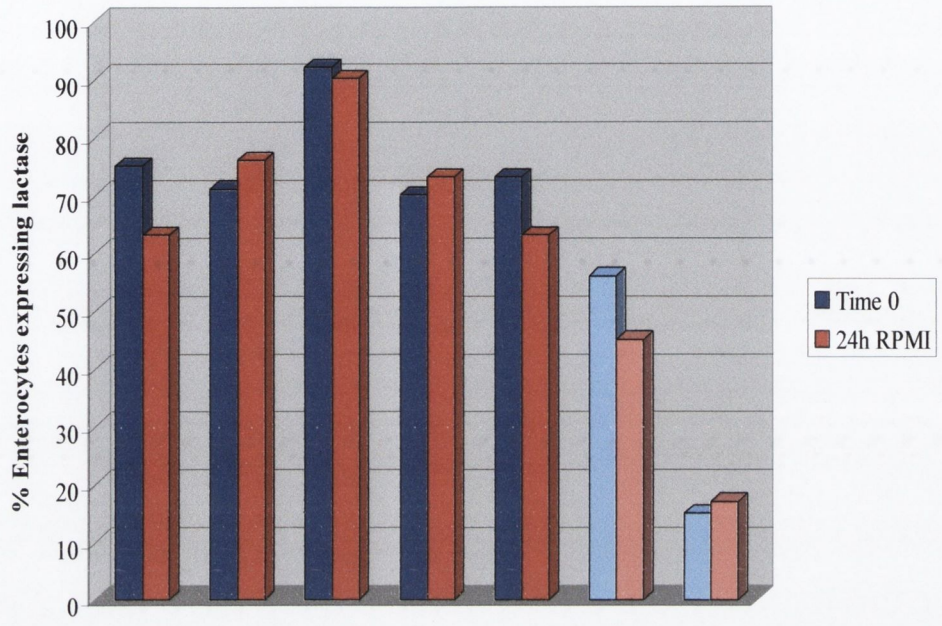
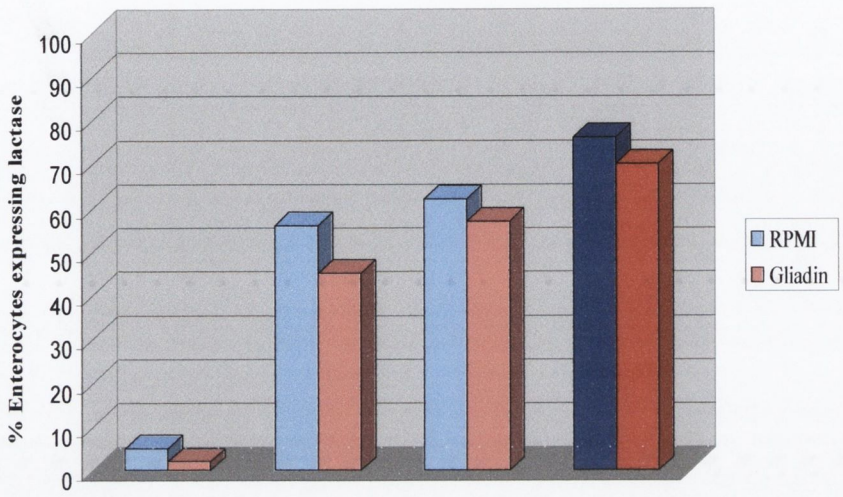


Figure 2.4 Lactase expression in 5 controls and 2 coeliac patients before and after 24h culture with RPMI. Dark coloured bars represent controls, light coloured bars coeliac patients. The graph shows that culture with RPMI had little effect on lactase expression in either group.

A



B

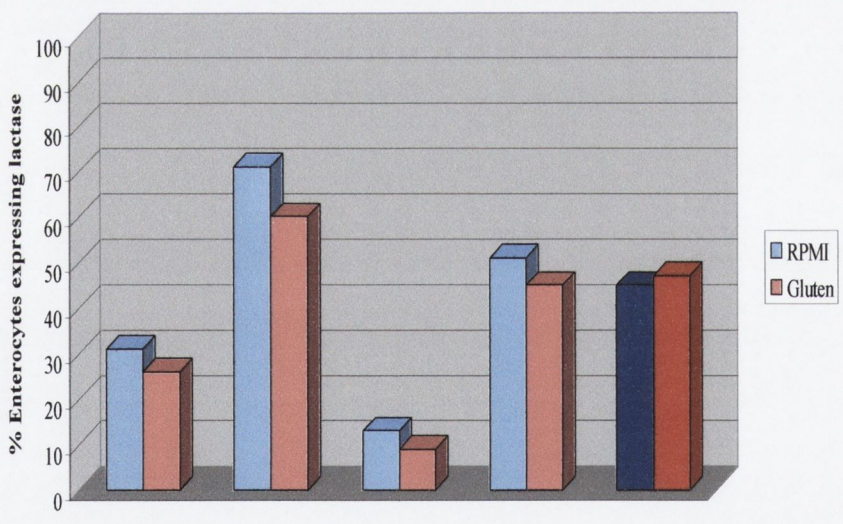


Figure 2.5 Lactase expression following 24h culture with gliadin (A) and 48h culture with gluten (B). Coeliacs are represented by light coloured bars and controls by dark coloured bars.

2.3.4 Effect of T cell activation on lactase, HLA-DR and CD25 expression

In order to assess the effect of T cell activation on lactase expression, biopsies were cultured for different periods of time with varying concentrations of PMA and OKT3. Biopsies from a control and a coeliac patient were cultured with 30μg/ml OKT3 and 25ng/ml PMA for 24h. The number of $CD25^+CD3^+$ IELs increased by 1.44% in the coeliac patient and by 7.64% in the control. Lactase expression was not greatly affected by this T cell activation. A 12% decrease was noted in the coeliac patient and a 9% decrease in the control patient.

We then increased the T cell stimulus; biopsies from 3 normal controls were cultured with 60μg/ml OKT3 and 75ng/ml PMA for 24h. Results are shown in Figure 2.6. There was a mean 15% increase in $CD3^+CD25^+$ IELs (range: increase of 6% to 29%) and an 11% increase in $CD3^+CD25^+$ LPLs (range: 5% increase to 19% increase). This T cell activation corresponded to a mean 7% reduction in lactase positive enterocytes (range: 3% increase to 13% decrease).

Biopsies from 4 controls and 1 coeliac patient were subjected to extreme T cell activation by culturing them with 60μg/ml OKT3 and 1μg/ml PMA for 48h. We were unable to isolate lymphocytes from the lamina propria of stimulated biopsies following culture. In the IEL population, the fluorescence intensity of CD3 was reduced in biopsies cultured OKT3/PMA (Figure 2.7). $CD25^+CD3^+$ IELs increased by a mean 47% (Figure 2.8 (A)) and the average enterocyte HLA-DR MFI increased by 2077 (Figure 2.8 (B)). Despite this pronounced activation there was little alteration in lactase levels: mean 8% (median 5%) reduction (range: 17% decrease to 1% decrease) (Figure 2.8 (A)).

OKT3 and the detection antibody PE-anti-CD3 are both directed against CD3. To investigate whether OKT3 interfered with the binding of the PE labelled anti-CD3 antibody, biopsies from a normal control were cultured with OKT3 for 48h. Bound OKT3 was labelled on IELs with an FITC rabbit anti-mouse antibody. IELs were then stained with the PE labelled anti-CD3 antibody. 58% of the T cell population bound both OKT3 and the PE-labelled anti-CD3 antibody. The remainder of the cells bound the PE-labelled detection antibody alone.

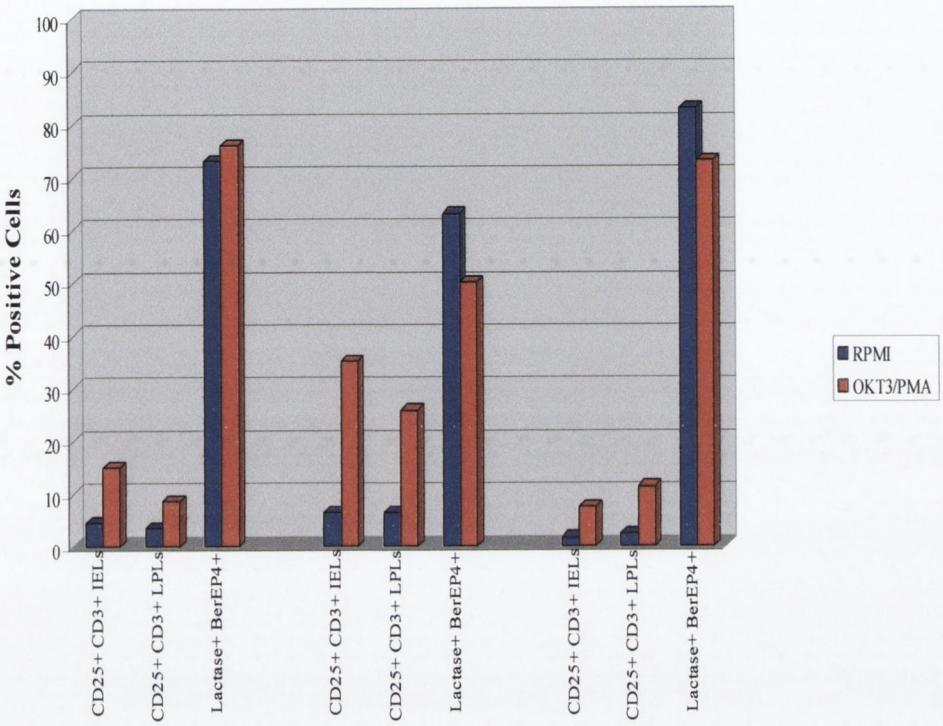


Figure 2.6 The effect of T cell activation on lactase expression in 3 control subjects. The graph shows the increase in $CD3^{+}CD25^{+}$ IELs and LPLs following stimulation with OKT3/PMA for 24h and the corresponding change in enterocyte lactase expression.

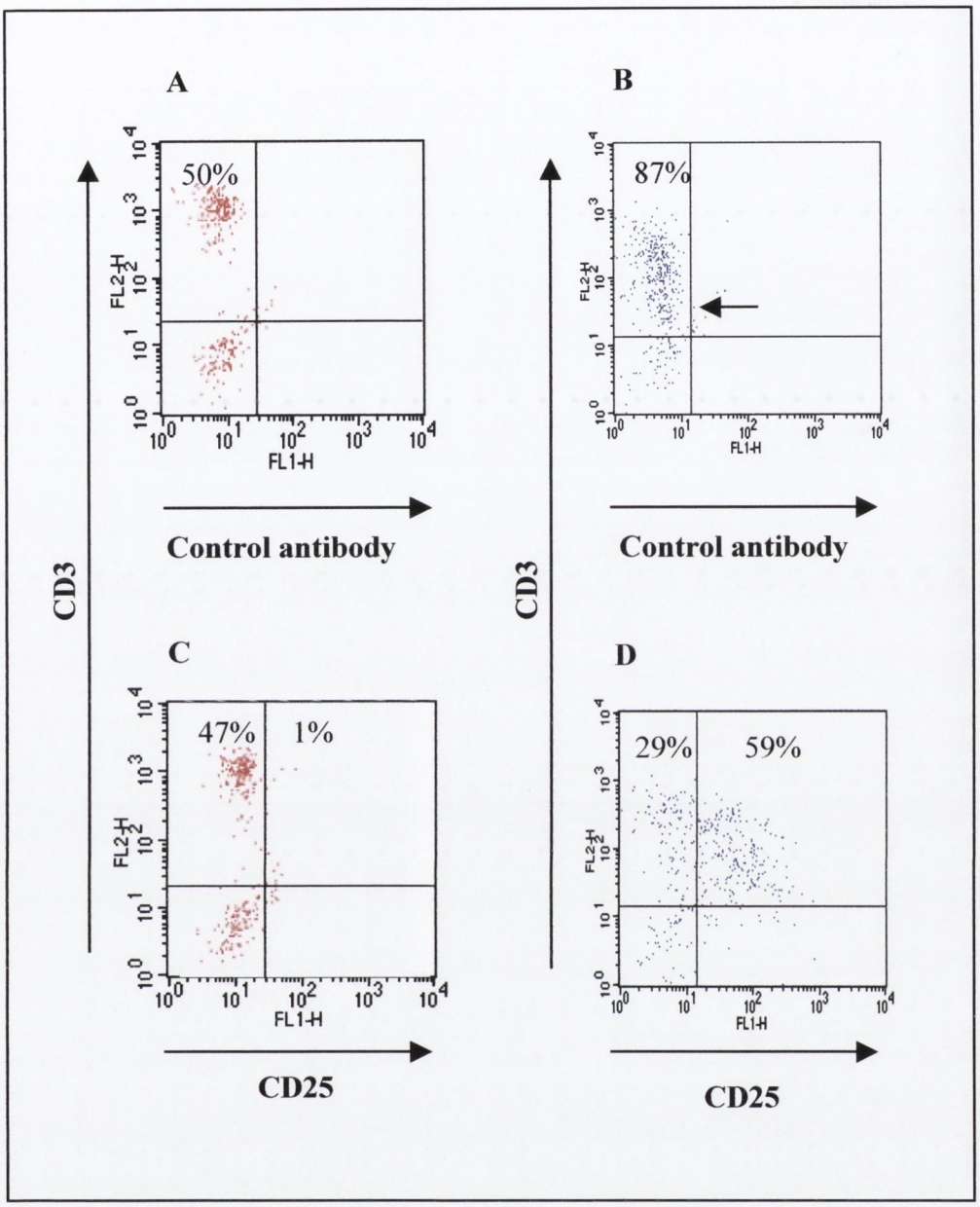


Figure 2.7 CD3 and CD25 expression on IELs from a control patient following culture with RPMI (A and C) and OKT3 / PMA (B and D). The fluorescence intensity of CD3 was reduced in biopsies cultured with OKT3/PMA as indicated by the arrow (B). A dramatic increase in $CD25^{+}CD3^{+}$ cells was observed after 48h culture with OKT3/PMA (D).

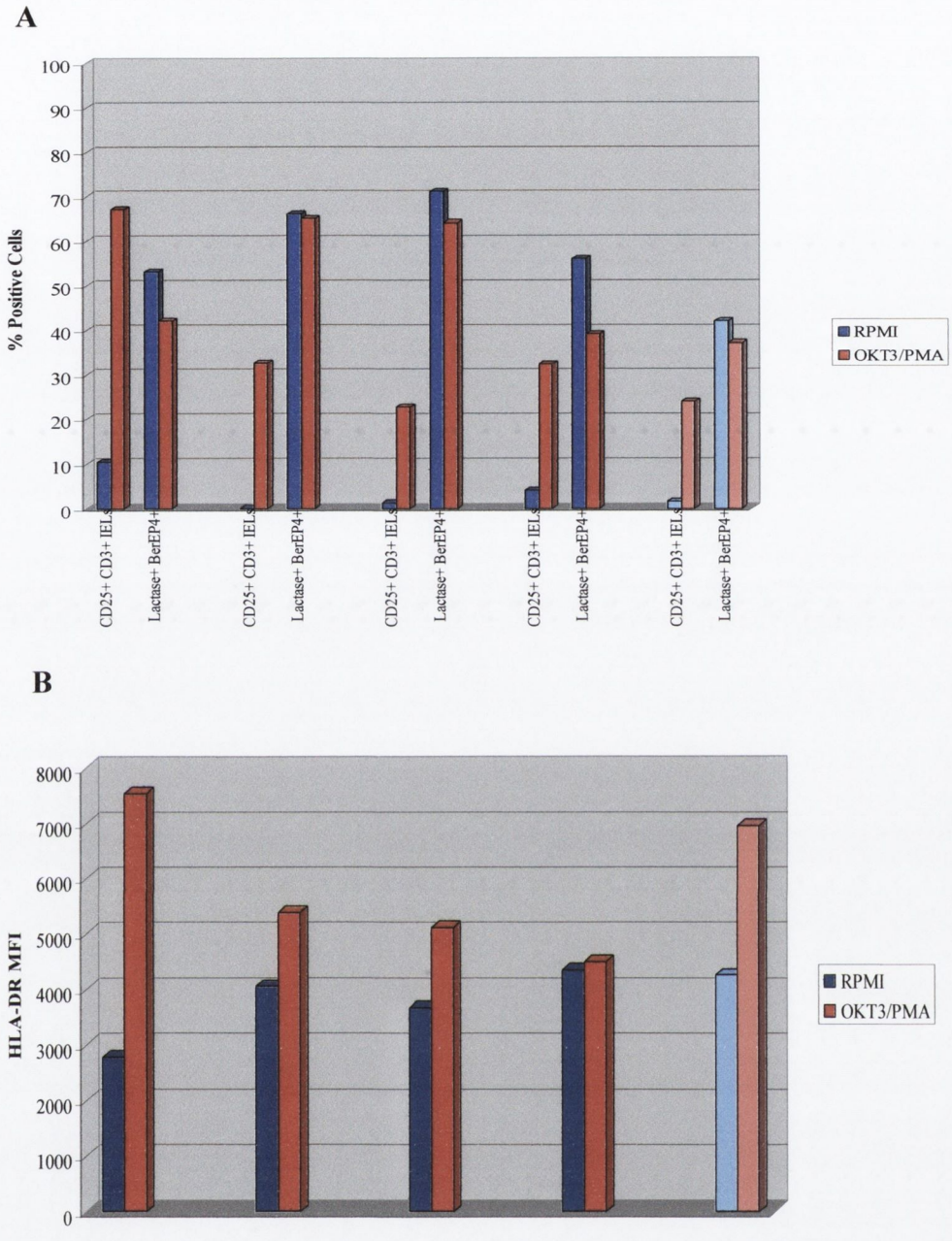


Figure 2.8 (A) Increase in $CD25^{+}CD3^{+}$ IELs and the concomitant changes in lactase expression following 48h culture with OKT3/PMA. (B) The associated increase in enterocyte HLA-DR following 48h T cell activation with OKT3/PMA. Light coloured bars indicate coeliac patients, dark coloured bars, normal controls.

2.3.5 Effect of TNF-α on enterocyte lactase and HLA-DR expression

Culture with 20ng/ml TNF-α for 24h had little effect on the lactase levels of 1 coeliac patient and 1 control. However, when the culture period was extended to 48h a notable reduction in lactase expression was observed in these two subjects, an additional coeliac patient and 3 controls (Figures 2.9 and 2.10). TNF-α reduced the number of lactase positive enterocytes in these patients by a mean 20% (median 21%) (range; 28% reduction to 9% reduction). TNF-α had no effect on lactase levels in 1 coeliac and 1 normal control (Figure 2.10 (A)). Taking all eight experiments together, lactase positive enterocytes were reduced by a mean 15% (median 19%) (range; 0% to 28% decrease). TNF-α increased the expression of HLA-DR in just two of these eight subjects (Figure 2.10 (B)).

2.3.6 Blocking the effect of TNF-α using anti-TNF R-1 antibody

In 3 coeliacs and 2 controls prior incubation with antibody to TNF R-1 blocked the ability of TNF-α to reduce lactase-expressing enterocytes (Figure 2.11), whereas in two subjects this antibody failed to inhibit the effect of TNF-α. In another control TNF-α did not reduce lactase expression (Figure. 2.12).

2.3.7 Staining enterocytes for the presence of TNF R-1

Enterocytes from biopsies cultured in RPMI-1640 were stained for the presence of TNF R-1. The enterocytes showed very poor staining for the receptor. In two coeliac patients 6% and 7% of enterocytes were labelled with the TNF R-1 antibody (Figure 2.13). One control had 13% TNF R-1 positive enterocytes. In two additional controls, 2% of enterocytes were positive for TNF R-1.

2.3.8 Additional experiments to investigate the effect of LPS, PWM, IFN-γ and MIF on enterocyte lactase expression

Biopsies from two control patients were cultured with 10μg/ml LPS, another with 20μg/ml and an additional one with 50μg/ml for 48h. The highest increase in $CD25^{+}CD3^{+}$ IELs after culture with LPS was 11%. Lactase expression was not significantly altered with a 0% and 7% reduction seen in patients cultured with 10μg/ml LPS, a 1% increase seen with the patient cultured with 20μg/ml and a 2% decrease in the patient cultured with 50μg/ml.

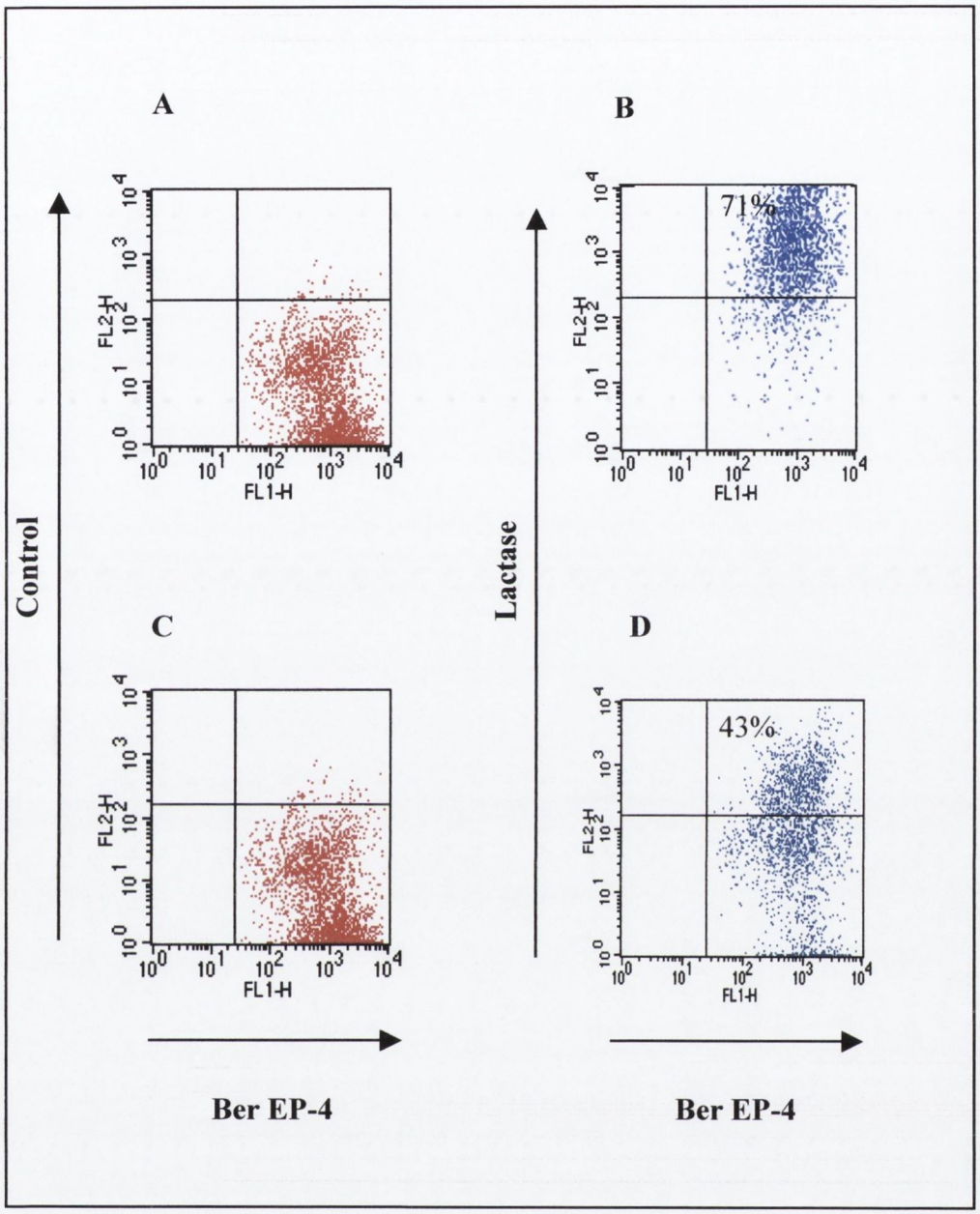


Figure 2.9 Enterocyte lactase expression in a control following biopsy culture with RPMI and TNF-α for 48h. Control (A) and lactase (B) antibody staining on enterocytes from biopsies cultured with RPMI. Control (C) and lactase (D) antibody staining on enterocytes from biopsies cultured with TNF-α.

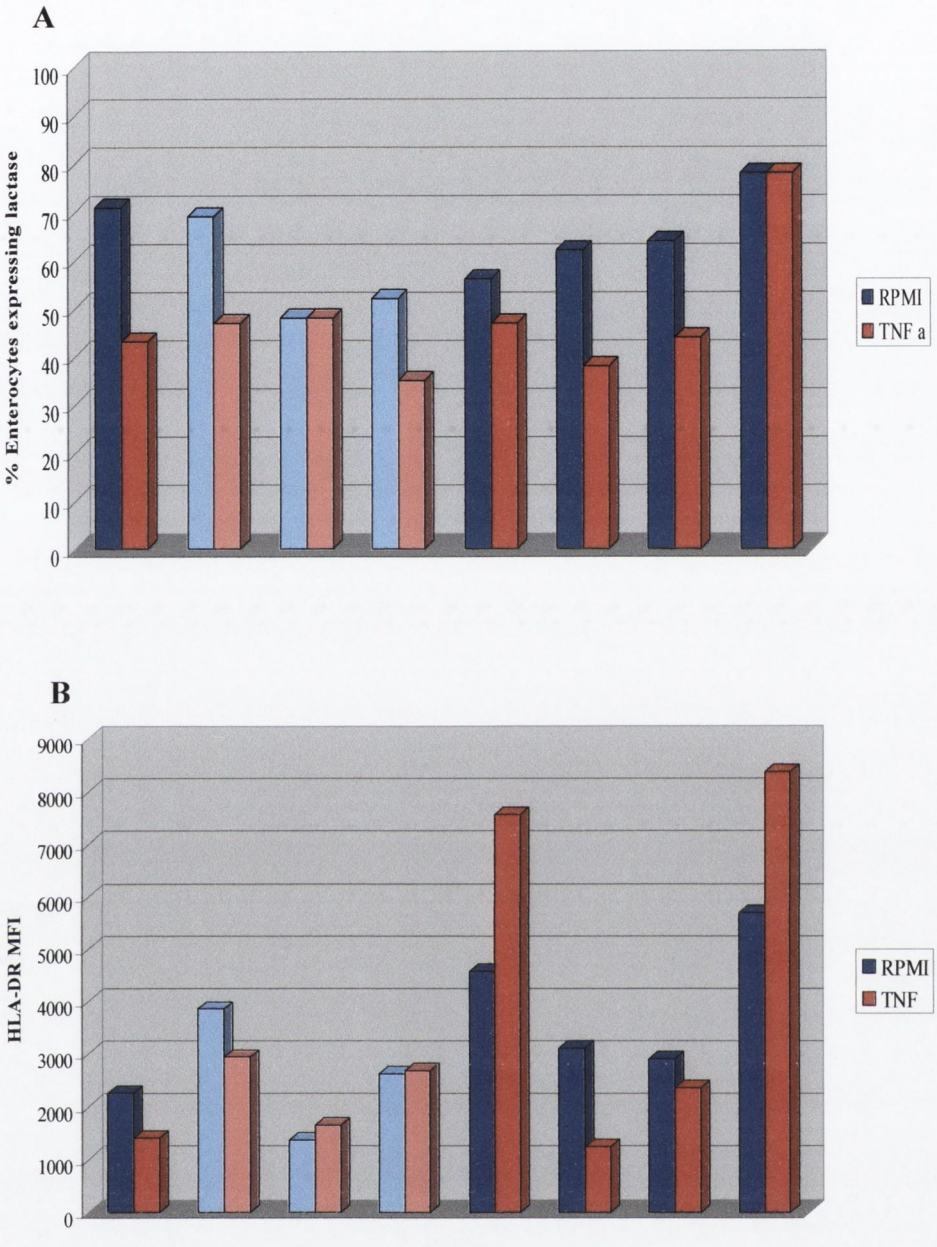


Figure 2.10 (A) Reduction of lactase expressing enterocytes in coeliac and control biopsies following 48h culture with TNF-α. TNF-α reduced lactase expression in 6/8 subjects. **(B) Concomitant changes in enterocyte HLA-DR following stimulation with TNF-α.** Light coloured bars indicate coeliac patients, dark coloured bars normal controls.

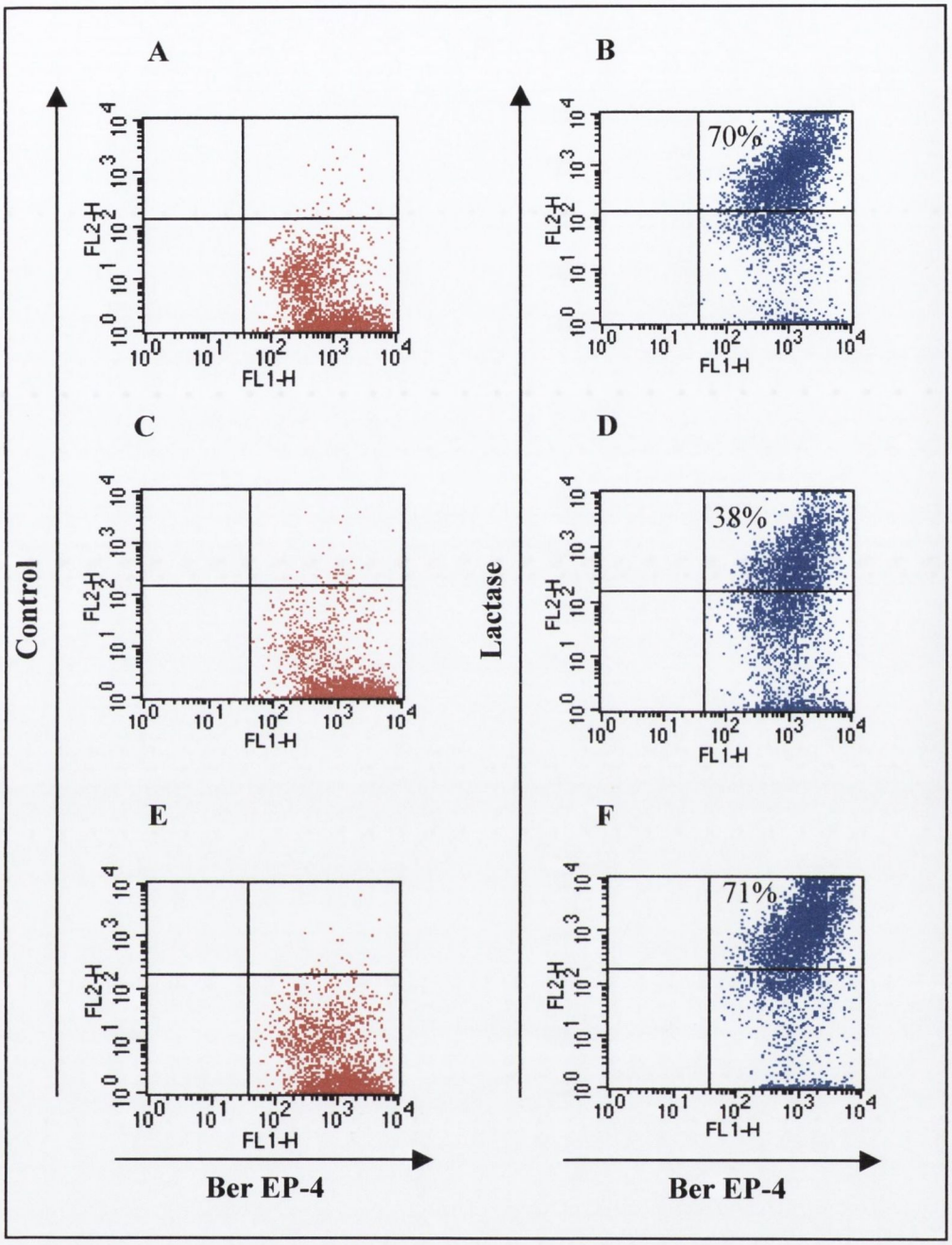


Figure 2.11 FACScan plots showing the inhibitory effect of anti-TNF R-1 on TNF-α's ability to reduce lactase expressing enterocytes in a coeliac patient. Control and lactase expression following biopsy culture with RPMI (A and B), TNF-α (C and D) and both anti-TNF R1 and TNF-α (E and F).

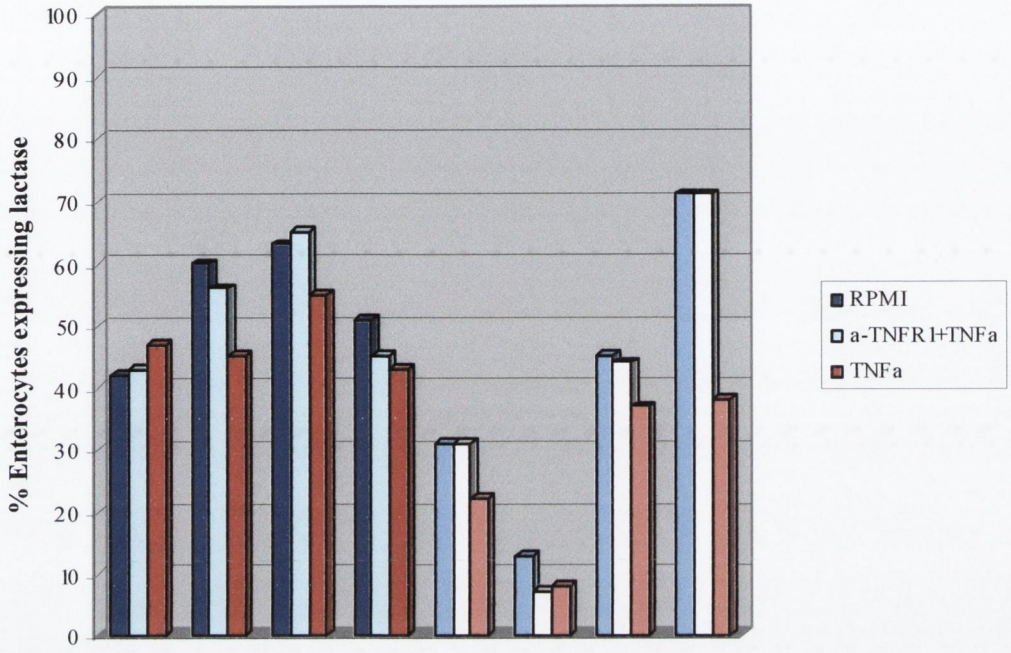


Figure 2.12 The inhibitory effect of anti-TNFR-1 on TNF-α's ability to reduce lactase expression on enterocytes. Anti-TNFR-1 blocked the action of TNF-α in 5 subjects. Activity was not blocked in 2 cases. TNF-α did not reduce lactase expression in one normal control. Light coloured bars indicate coeliac patients, dark coloured bars normal controls.

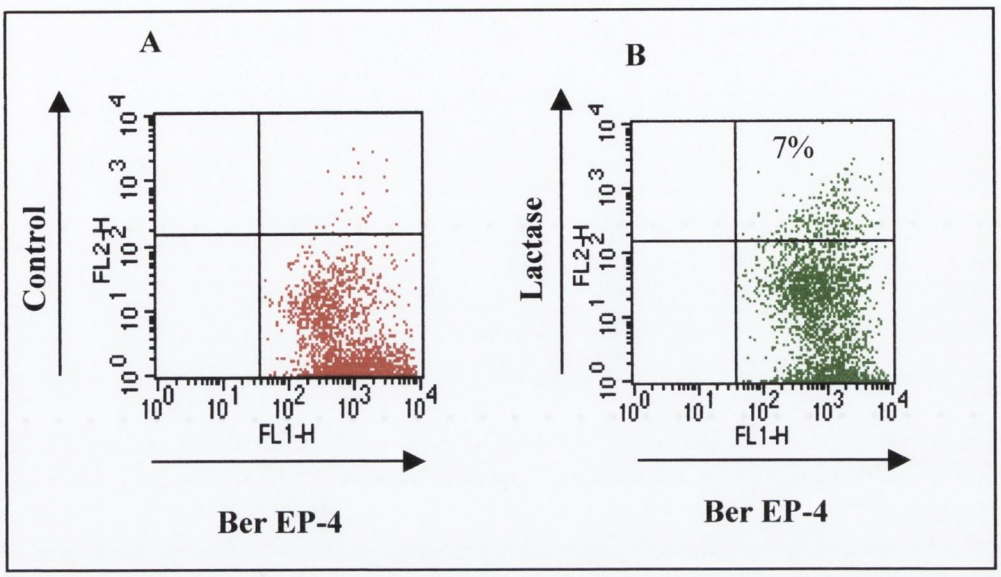


Figure 2.13 FACScan plots showing control staining (A) and TNF R-1 staining (B) on enterocytes from a coeliac patient.

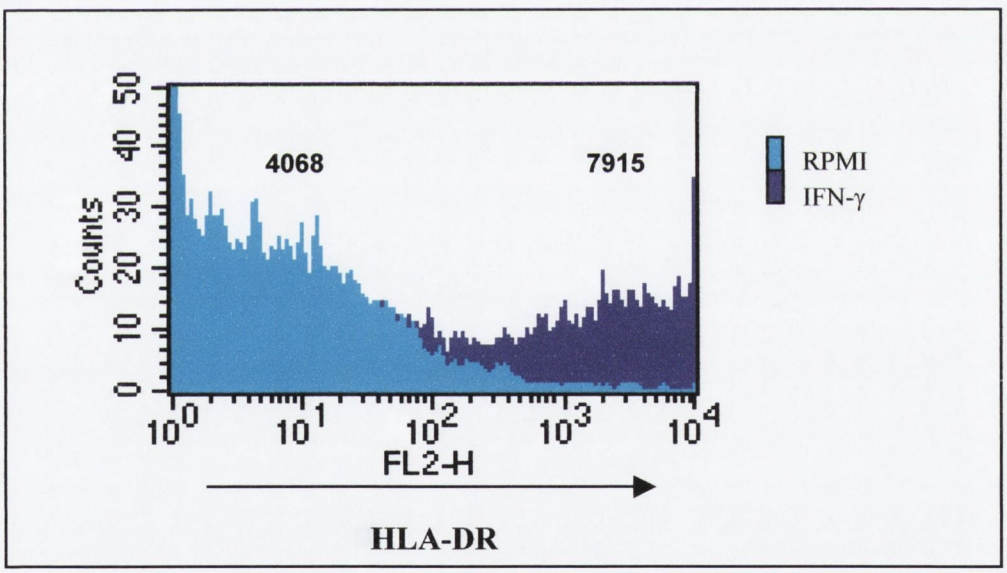


Figure 2.14 Histogram plot showing the increase in enterocyte HLA-DR MFI following coeliac biopsy culture with IFN-γ. Results are shown as MFI values.

A 12% increase in $CD25^{+}CD3^{+}$ IELs was observed in a control patient following 48h culture with PWM. A reduction in lactase expression was not observed.

Biopsies from 2 coeliac patients were cultured with MIF for 48h. A small reduction of 4% and 11% lactase positive enterocytes was noted in these patients' biopsies.

Lactase was reduced by 2% in a control and by 10% in a coeliac patient cultured with 10μg/ml IFN-γ for 48h. The HLA-DR was dramatically increased in these subjects. An MFI increase of 2515 was seen in the control and an increase of 3847 in the coeliac patient (Figure 2.14) after culture with IFN-γ. In a time course experiment with biopsies from a coeliac patient no increase in HLA-DR was noted 2h after culture with IFN-γ. At 6h an MFI increase of 1418 was observed.

2.3.9 Endomysial antibody detection in organ culture supernatants

All 5 controls had a normal histology and their biopsy supernatnants were EMA negative following culture with RPMI alone or with gliadin. The histology grade and EMA results of the coeliac patients are shown in Table 2.4. Gliadin could not stimulate EMA production in coeliac biopsies that were initially EMA-negative. The patients that produced mucosal EMA after 4h were serum EMA positive and had a severe grade 3 lesion (patients 1 and 2). Following 24h and 48h culture, those with milder lesions produced EMA (patients 10,11,12 and 13). Mucosal EMA production at these time points was independent of serum EMA.

Table 2.4 Coeliac patient EMA results in organ culture supernatants and serum

Patient No.	Culture time (h)	Histology grade	EMA following culture with RPMI	EMA following culture with gliadin	Serum EMA
1	4	3	+	+	+
2	4	3	+	+	+
3	4	1	-	-	-
4	4	0	-	-	-
5	4	2	-	-	-
6	4	2	-	-	-
7	4	1	-	-	-
8	4	1	-	-	-
9	4	2	-	-	-
10	24	1	+	+	-
11	24	2	+	+	+
12	48	0	+	+	-
13	48	1	+	+	-
14	48	2	-	-	nd
15	48	1	-	-	-

Histology was graded as follows: 0 = normal biopsy; 1= raised intraepithelial lymphocyte (IEL) count; 2 = partial villous atrophy with raised IELs; 3 = subtotal villous atrophy with raised IELs; nd, not determined.

2.4 DISCUSSION

2.4.1 Lactase as an *in vitro* marker of gliadin toxicity and/or immunogenicity

In this study we investigated lactase expression as an *in vitro* indicator of gliadin toxicity or immunogenicity. We demonstrated that enterocyte lactase could be assessed objectively and semi-quantitatively by flow cytometry. We also showed that lactase was not a sensitive marker of enterocyte damage and therefore unsuitable as an indicator of prolamin-induced toxicity.

Duodenal biopsies were maintained *in vitro* using the organ culture technique, which has proved invaluable in the field of coeliac disease research. In this study we found that biopsies cultured with RPMI-1640 alone retained their cell viability, tissue morphology and normal cellular functions for periods up to 48 hours. Brush border lactase was also preserved and levels after 24 hours culture were comparable to those before culture (Figure 2.4).

Enterocytes originate in the intestinal crypts and migrate upwards proliferating several times before they reach the villi as differentiated epithelial cells. Cell death is necessary to maintain a normal balance in the mucosa and it is thought that differentiated villus epithelial cells die by apoptosis and are extruded into the lumen (Ramachandran *et al.* 2000). Brush border enzymes are synthesised continuously as the enterocytes migrate up the villi. In undifferentiated crypt cells the brush border enzymatic activity is low, increasing to maximal levels in the mature epithelial cell (Nieminen *et al.* 2001). In active coeliac disease there is increased apoptosis and cell loss from the villus. Crypt cell proliferation increases to compensate for the heightened cell loss. Due to this high turnover most of the epithelial cells in the active coeliac mucosa are immature and crypt like, expressing little or no brush border enzymes (Moss *et al.* 1996).

In coeliac disease, increased cell turnover and reduced lactase expression is ultimately gliadin induced. Enzyme recovery occurs after gluten withdrawal from the diet and reintroduction depresses enzyme expression (Peña *et al.* 1972; Srinivasan *et al.* 1999). It was suggested that lactase loss is a sensitive indicator of persisting ingestion of gluten. Bramble *et al.* (1985) carried out a time course study and noted that *in vivo* gluten challenge significantly reduced lactase activity in treated patients in just 4 hours.

Reductions were more pronounced in those patients who had normal or near normal mucosal morphology. However, in our organ culture experiments, gliadin and PT gluten had little effect on lactase expression even after 48 hours (Figure 2.5). Culture caused a mean 5.7% reduction in lactase expressing enteroyctes. Organ culture studies show that gliadin induces enterocyte apoptosis in untreated coeliac biopsies (Maiuri *et al.* 2000). However, in treated coeliac biopsies while gliadin causes upregulation of transferrin receptor and FAS on enterocytes (Maiuri *et al.* 2000) it does not induce enterocyte apoptosis (Maiuri *et al.* 2003). Since reduced lactase expression is a function of enterocyte apoptosis (Moss *et al.* 1996) it is not surprising that we did not detect enzyme changes in our biopsies, the majority of which were from treated patients.

Biopsy culture with gliadin or PT gluten did not increase HLA-DR expression on enterocytes. This was unexpected as HLA class II molecules are increased in the active coeliac mucosa on both crypt and mature epithelial cells (Marley *et al.* 1987). Also Maiuri *et al.* (1996a) demonstrated gliadin-induced upregulation of HLA-DR on enterocytes *in vitro*. In addition, the IELs isolated following gliadin/PT gluten activation did not express CD25. This concurs with earlier reports that IELs in the active coeliac mucosa do not express this activation marker (Abuzakouk *et al.* 1996; Halstensen *et al.* 1993). It could be argued that the gliadin or PT gluten preparations did not reduce lactase expression because they were not immunogenic or toxic. However, when we induced marked T cell activation, which is associated with enterocyte damage, there was still no change in lactase expression.

2.4.2 Effect of T cell activation on enterocyte lactase and HLA-DR expression

McDonald and Spencer showed that by activating T cells in the human intestinal lamina propria *in vitro*, they produced a lesion with coeliac disease like characteristics, including increased cell proliferation, villous atrophy and increased HLA-DR expression on enterocytes (MacDonald and Spencer 1990). We activated IELs and LPLs with a combination of OKT3 and PMA. Sarnacki *et al.* (1992) found that OKT3 induces proliferation and expression of CD25 on IELs, which contradicts previous reports suggesting that activation through the CD3-TCR pathway is difficult to elicit in IELs. When we cultured biopsies with concentrations of OKT3 and PMA that are stimulatory for peripheral blood mononuclear cells (PBMCs) (30μg/ml OKT3 and 25ng/ml PMA), the number of $CD25^{+}CD3^{+}$ IELs increased by just 1.44%. This is in agreement with a

study, which found that IELs require significantly larger amounts of OKT3 to induce proliferation than enriched $CD8^+$ PBMCs (Sarnacki *et al.* 1992). When biopsies were stimulated with higher concentrations of the mitogens (60μg/ml OKT3 and 75ng/ml) the number of $CD3^+$ IELs expressing CD25 increased by a mean 15%. $CD3^+CD25^+$ cells in the lamina propria increased by 11%. This level of T cell activation did not significantly alter lactase expression. A mean 7% reduction in lactase positive enterocytes was observed (Figure 2.6).

Prolonging the culture period to 48 hours and increasing the PMA concentration to 1μg/ml resulted in a mean 47% increase in $CD3^+CD25^+$ IELs (Figure 2.8). Despite this pronounced T cell activation, lactase positive enterocytes were reduced by just 8% (Figure 2.8). Lymphocytes could not be isolated from the lamina propria following 48 hours culture with OKT3/PMA. Mauiri *et al.* (1996a) showed that when coeliac tissue is cultured with PT gliadin there is a dramatic migration of T cells and macrophages in the lamina propria to the subepithelial compartment and infilatration of T cells into the surface epithelium. It is possible that, in our experiments, activated mononuclear cells migrated to the subepithelial compartment and were isolated as IELs when the epithelial layer was stripped from the biopsy. Tissue damage caused by T cell activation may have also contributed to mononuclear cell loss from the lamina propria.

T cells activated with OKT3/PMA had reduced levels of CD3 on their surface. It is known that OKT3 reduces CD3 and TCR expression on the surface of T cells (Gebel *et al.* 1989). It could be argued that the reduced CD3 fluorescence was due to OKT3 blocking the binding of the PE labelled anti-CD3 detection antibody. This was not the case. We found that all IELs that bound the OKT3 antibody also bound the PE-anti-CD3 detection antibody.

Increased HLA-DR was also observed following 48 hours culture with OKT3/PMA. This increase was most likely due to IFN-γ secretion from activated T cells. IFN-γ increases enterocyte HLA-DR in biopsies cultured *in vitro* (Sturgess *et al.* 1992). Before culture, expression of class II molecules is predominantly on the enterocytes at the villus tips. Following culture with IFN-γ there is uniform expression with increased staining on crypt cells (Sturgess *et al.* 1992). We observed increased expression of HLA-DR on enterocytes after just 6 hours culture with IFN-γ. Increased levels of the cytokine have

been detected in both IELs and LPLs in coeliac tissue (Kontakou *et al.* 1994; Olaussen *et al.* 2002).

Increased HLA-DR expression by IFN-γ in coeliac disease may enhance transcytosis and delivery of antigenic material to the lamina propria (McKay and Baird 1999). Bendix *et al.* (1997) found that after 5 days culture in the presence of gliadin there was significant binding of gliadin to HT-29 cells, which was enhanced considerably in the presence of IFN-γ. Inappropriate entry of gliadin into the mucosal compartment could trigger or enhance the inflammatory response in coeliac disease.

2.4.3 Effect of TNF-α on enterocyte lactase expression

IFN-γ is a potent activator of macrophages, which are important sources of TNF-α. The cytotoxicity of TNF-α is well documented. The cytokine exists both as a secreted 17kDa form and as a 26kDa cell associated form. The native structure of TNF-α is a trimer with a total molecular mass of 52 kDa (Fiers 1991). Although cells of the monocyte/macrophage lineage are considered to be the major sources, TNF-α is also synthesised by a variety of other cell types including lymphocytes, NK cells, mast cells and endothelial cells (Tracey *et al.* 1989). TNF-α has pleiotrophic effects on a large number of different cell types (Fiers 1991).

Immunohistochemical studies found increased TNF-α in the mucosa of untreated coeliacs compared to treated coeliacs or controls (Przemioslo *et al.* 1994). Gluten stimulation of treated coeliac biopsies increases mRNA levels of the cytokine (Nilsen *et al.* 1998). In addition, the high secretor phenotype of TNF-α, TNF-E, is significantly associated with coeliac disease (Peña *et al.* 1998).

TNF-α is a member of a growing family comprising at least 10 cytokines, which are primarily involved in the regulation of cell proliferation and apoptosis. Pender *et al.* (1998) found that blocking TNF-α following lamina propria T cell activation with PWM prevented severe injury in explant cultures of human fetal small intestine. In our study T cell activation, which most likely resulted in TNF-α secretion, did not injure the epithelium to the extent of causing lactase reduction. Culture with TNF-α for 24 hours had no affect on lactase expression either. Only after prolonged culture with the

cytokine for 48 hours did we observe a mean 20% reduction in lactase positive enterocytes in 6 of 8 subjects (Figure 2.10).

TNF-α did not cause lactase reduction in 2 cases. The reason for this may be due to the fact that TNF-α can induce a variety of other pro-inflammatory cytokines known to antagonize apoptosis, inducing IL-1, IL-6 and granulocyte macrophage colony stimulating factor (Ksontini *et al.* 1998). TNF-α also induces activation of NF-κB, which is responsible for the inhibition of apoptosis in a variety of cell types, including B lymphocytes, mouse fibroblasts and human malignant epithelial cell lines (Van Antwerp *et al.* 1996).

2.4.4 TNF receptor mediated apoptosis

TNF-α mediates its activity through its receptors. There are two types of TNF receptor, which are differentiated by their size and by monocolonal antibody recognition; TNF R-1 (p55) and TNF R-2 (p75). Both receptors bind TNF-α as well as TNF-β although the latter with lower avidity (Fiers 1991). TNF R-1 and TNF R-2 have been detected on intestinal epithelial cells (Kaiser and Polk 1997). We found poor expression of TNF R-1 on enterocytes isolated from duodenal biopsies. Flow cytometry may not be sensitive enough to detect low levels of receptor expression. However, it has been shown that there is no correlation between the number of TNF receptors and the magnitude or direction of the response (Fiers 1991).

Studies have shown that TNF R-1 is primarily responsible for TNF-induced apoptosis of target cells (Tartaglia *et al.* 1993a; Tartaglia *et al.* 1993b). The intracellular portion of TNF R-1 contains a "death domain" of about 70 amino acids that is required for the signalling of apoptosis and NF-κB activation. Death receptors belong to the TNF-receptor gene superfamily. The best characterised death receptors are FAS and TNF R-1. Others include CAR-1, DR3, DR4 and DR5 (Ashkenazi and Dixit 1998). In the initial step TNF binds to the extracellular domain of TNF R-1 and induces receptor receptor trimerisation. A TNF molecule has three potential interaction sites with its receptor allowing for receptor crosslinkage (Fiers 1991). The death domain of the TNF R-1 binds an adapter protein TNFR-associated death domain (TRADD). TRADD functions as platform recruiting FAS-associated death domain (FADD), TNFR-associated factor-2 (TRAF 2) and receptor-interacting protein (RIP), to form the TNF R-

1 signalling complex which activates signalling cascades leading to apoptosis (Ashkenazi and Dixit 1998, Baker and Reddy 1998).

In this study we pre-incubated biopsies with a mouse monoclonal antibody to human soluble TNF R-1. This antibody can also block activities mediated by the cell surface TNF R-1. It has no cross reactivity with TNF R-2. Pre-incubation with TNF R-1 blocked TNF-α mediated lactase reduction in 5 of 7 cases (Figure 2.12). This would indicate that TNF-α was acting directly on the enterocytes and primarily through TNF R-1. In two cases anti-TNF R-1 did not block TNF-α induced lactase reduction. It is unlikely that the antibody itself contributed to apoptosis as the level of lactase reduction with a combination of anti-TNF-R1 and TNF-α was comparable to the reduction observed with TNF-α alone.

In the two cases where lactase reduction was not blocked by anti-TNF R-1, TNF-α may have mediated enterocyte damage through another pathway. TNF-α induces MMP production. As previously mentioned, blocking TNF-α production following T cell activation prevents mucosal injury *in vitro* but also reduces the level of MMPs in the supernatant, especially stromelysin-1. Authors postulated that TNF-α induced stromelysin-1 is a potent mediator of enteropathy in stimulated explants (Pender *et al.* 1998). A further possibility is that apoptosis was occurring through TNF R-2. Ruemmele *et al.* (1999a) found that costimulation of both TNF-Rs is significantly more potent in inducing IEC-6 cell apoptosis compared to TNF-R1 alone. However cross-talk between the two receptors has been proposed rather than independent signalling via TNF R-2 (Ruemmele *et al.* 1999a). TNF-α induced lactase reduction may also have been mediated through FAS. TNF-α upregulates FAS expression on enterocytes (Ruemmele *et al.* 1999b). There is evidence for FAS mediated enterocyte apoptosis in coeliac disease (Ciccocioppo *et al.* 2001; Maiuri *et al.* 2001).

2.4.5 Effect of IFN-γ, MIF, LPS and PWM on lactase expression

Unlike TNF-α, IFN-γ did not reduce lactase expression, despite its reported cytotoxic properties. Mowat and Garside (1994) found that mice injected intraperitoneally with TNF-α rapidly develop crypt hypertrophy and marked villous atrophy. In contrast, IFN-γ only induces crypt hyperplasia. Villous atrophy does not occur, even after prolonged

administration. It is not until a small amount of TNF-α is administered with IFN-γ that villous atrophy is provoked (Mowat and Garside 1994). We did not test the synergistic effect of these cytokines in our study.

MIF, a cytokine that has been shown to promote the production of pro-inflammatory cytokines such as TNF-α and IL-8 by macrophages (Cunha *et al.* 1993) may act in synergy with TNF-α to augment proinflammatory responses in disease (Donelly *et al.* 1997; Mikulowska *et al.* 1997). In coeliac disease intracellular levels of MIF are considerably higher in intestinal epithelial cells compared to control subjects (O'Keeffe *et al.* 2001). Biopsy culture with MIF caused slight reductions in lactase expression. LPS, another potent inducer of TNF-α secretion by macrophages did not reduce lactase expression either. It would appear that large amounts and prolonged exposure to TNF-α is necessary for reduced lactase expression *in vitro*.

It has been mentioned previously that the lectin PWM can induce mucosal damage in fetal gut explants. Cyclosporin inhibits the ability of PWM to mediate mucosal damage indicating that T cell activation and not direct action of PWM is responsible for enterocyte damage (Lionetti *et al.* 1993). Though T cell activation increased by 12% following culture with PWM there was no reduction in lactase expression.

2.4.6 Conclusion of lactase study

From our results it would appear that lactase loss is a slow process *in vitro*. This is in contrast to the *in vivo* situation where gliadin can induce lactase reduction in 4 hours (Bramble *et al.* 1985). It is possible that the process of enterocyte renewal is slower *in vitro* than *in vivo*. However, rapid crypt cell proliferation in the coeliac mucosa has been demonstrated *in vitro* by ^{3}H uptake (Trier and Browning 1970). The epithelial cell population in the biopsies of patients with severe coeliac lesions is renewed every 24 hours rather than every 4-6 days as in a normal person. Alternatively, apoptotic enterocytes may not be removed effectively *in vitro*, which may account for the slow loss of lactase positive enterocytes from the mucosa. *In vivo* apoptotic epithelial cells are most likely removed by peristalsis. It is unlikely that apoptotic enterocytes were included in our assessment of lactase expressing enterocytes. In all cases cell viability was over 90%: therefore apoptotic cells were probably extruded into the biopsy culture

supernatant. Also during FACS analysis cellular debris was excluded during the gating procedure.

To summarise, lactase reduction is difficult to induce in the *in vitro* situation. Potent stimulation of duodenal biopsies with mitogens and cytotoxic cytokines revealed that it is a slow process. We therefore conclude that enterocyte lactase expression is not suitable as a marker of gliadin or prolamin induced disease activity *in vitro*.

2.4.7 *In vitro* endomysial antibody production as a marker of gliadin immunogenicity

It is apparent from our results that EMA production occurs at mucosal sites and is coeliac disease specific (Table 2.4). The EMAs do not originate from the serum as biopsies from patients who were serum EMA negative secreted the antibody into the culture supernatants (patients 10, 12 and 13). Similarly, Vogelsang *et al.* (1999) reported that EMA found in duodenal mucosa of coeliac patients could not be detected in the gastric mucosa of the same patient.

In the original report by Picarelli *et al.* (1996), EMA was detected in the culture medium of untreated coeliac biopsies irrespective of gliadin challenge. In treated coeliacs EMA was not detected in the cultures challenged with medium alone. Vogelsang's group also found that following culture with medium alone, the only biopsies that secreted EMA where those of untreated coeliacs and treated coeliacs with positive serum EMA (Vogelsang *et al.* 1999). This group suggested that memory B cells were secreting the antibody and that EMA production was a function of the length of the GFD. This was not the case in our study. Biopsies from treated coeliacs, with a grade 0 or 1 histology who were serum EMA negative secreted the antibody when cultured with medium alone. Also some biopsies with a grade 2 histology did not produce EMA. Biagi *et al.* (2000) also found that spontaneous EMA production was not dependent on histology. They detected EMA production in 4/7 coeliac biopsies following culture in medium. In two patients EMAs were probably detectable due to the short duration of their GFD. In another two patients EMA was still detectable though they had followed a diet for 14 and 28 months, which improved their histology and cleared EMA from their serum.

When biopsies were cultured in the presence of PT gliadin we found, in contrast to Picarelli *et al.* (1996), that gliadin could not induce EMA production in biopsies that were initially EMA negative. Biopsies that secreted EMA following culture with gliadin also secreted the antibody when cultured with medium alone. The gliadin preparation used in this study was immunogenic as it induced IFN-γ production in coeliac biopsies (see chapter 3). The results published by Biagi *et al.* (2000) and Vogelsang *et al.* (1999) were similar to ours. Both found that gliadin could not induce mucosal EMA production *in vitro*.

It could be argued that 4 hours culture with gliadin was insufficient time to induce EMA production. Picarelli's group could not detect EMA in biopsy culture supernatants following 6 hours culture with gliadin (Picarelli *et al.* 1999). However, prolonging the culture period to 48 hours had no effect on EMA production (patients 14 and 15). Carroccio and colleagues (2002a) found that gliadin only induced *in vitro* EMA production in patients who were on a GFD for a short period of time and had a high inflammatory infiltrate in the mucosa. The authors proposed that a threshold number of memory cells are necessary for gliadin induced EMA production (Carroccio *et al.* 2002a). This does not explain the lack of EMA production in our specimens. Patient 15 had a high IEL count and patient 14 a high cellular infiltrate with partial villous atrophy.

We conclude that while EMA production occurs at coeliac mucosal sites, it was not possible to induce its production by *in vitro* challenge of treated coeliac biopsies with gliadin. It is clear that EMA detection in organ culture supernatants cannot be considered a marker of gliadin immunogenicity.

CHAPTER 3

Avenin Fails to Induce a Th1 Response in Coeliac Tissue following *in Vitro* culture

3.1 INTRODUCTION

3.1.1 T cell activation

The TCR/CD3 complex

The recognition of MHC-peptide complexes by the TCR is central to T cell activation. The TCR consists of a heterodimer designated TCRαβ. On a minority of cells the receptor is composed of two different chains termed TCRγδ. Each chain is composed of a constant and variable region similar to an immunoglobulin molecule (Clevers *et al.* 1988). A hydrophobic region spans the membrane, which contains positively charged amino acid residues. The chains have a short cytoplamic tail of 5-12aa, which is too small to transduce signals (Abbas *et al.* 2000b). The TCR associates with CD3 forming the TCR-CD3 complex (Figure 3.1). CD3 is composed of 5 polypeptide chains, which associate to form 3 dimers; γε, δε and ξξ. In some cases the latter is found as a heterodimer, ξη (Goldsby *et al.* 2000a). The CD3 chains, γ, δ and ε are structurally similar. They have an extracellular region, which contains a single immunoglobulin-like domain. The transmembrane region contains negatively charged residues, which enable the chains to associate with the TCR. Unlike the TCR chains, these CD3 proteins have long cytoplasmic chains, which contain one copy of a conserved sequence motif. This site is called an immunoreceptor tyrosine based activation motif (ITAM) and it interacts with tyrosine kinases involved in signalling. The ξ and η chains have a different structure to the aforementioned chains. Both have a very short external domain, a transmembrane region and large cytoplasmic domain. The transmembrane region also contains negatively charged amino acids. Unlike the other CD3 chains, the ξ and η chains contain three copies of ITAMs in their cytoplasmic tails (Goldsby *et al.* 2000a; Qian and Weiss 1997)

Adhesion and costimulatory molecules

CD4 and CD8 are important accessory molecules in T cell activation. They stabilise the interaction between T cell and APC by binding to MHC molecules. They also provide accessory activation signals. CD4 is expressed as a monomer and binds to MHC class II molecules. CD8 may be expressed as an αα homodimer or an αβ heterodimer as discussed in section 1.4.2. It stabilises T cell-APC interactions by binding to MHC class I molecules (Goldsby *et al.* 2000a). In addition to CD4 and CD8 several adhesion molecules stabilise cell-to-cell contact. These molecules bind to specific ligands on the

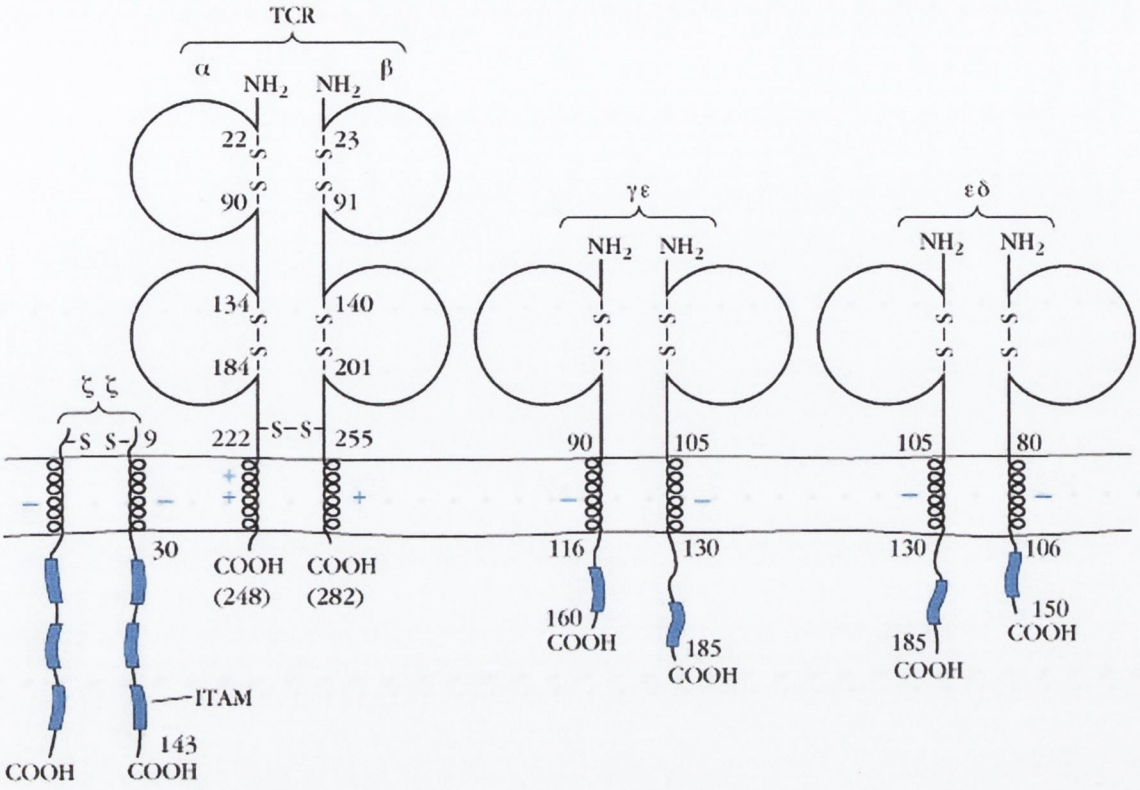


Figure 3.1 Schematic diagram of the TCR-CD3 complex. The complex consists of the T cell antigen binding receptor and the CD3 chains; γε, δε and ξξ (alternatively ξη). The external regions of the TCR and the γ, δ and ε chains of CD3 are similar to the immunoglobulin fold. Ionic interactions enable the CD3 and TCR chains to associate with each other. Each CD3 chain has one ITAM (blue) except ξ, which has three (taken from Goldsby *et al.* 2000a).

APC. Primary adhesion molecules include CD2, which binds to leukocyte function associated antigen (LFA) -3, LFA-1 which complexes ICAM-1 and ICAM-2, and ICAM-3, which interacts with DC-SIGN (this is unique to T cell-dendritic cell interactions) (Janeway *et al.* 2001b).

Signals in addition to those generated by the TCR-CD3 complex are required for full activation of the T cell. These are referred to as co-stimulatory signals. The best-characterised co-stimulatory molecule is CD28, which binds to B7.1 and B7.2 on APCs. CD28, expressed on naïve and resting T cells, delivers a positive activation signal (Frauwirth and Thompson 2002). It also enhances the expression of CD40L on T cells. CD40L interacts with CD40 on APCs, which in turn upregulates the expression of B7 molecules on APCs (Tseng and Dustin 2002). Another co-stimulatory molecule expressed on activated T cells is inducible co-stimulator (ICOS), which binds to ICOSL on APCs (Frauwirth and Thompson 2002). The T cell response is regulated by a CD28 related protein called CTLA-4. CTLA-4 binds to the B7 molecules with significantly greater avidity than CD28. In contrast to CD28, CTLA-4 delivers an inhibitory signal, which limits the magnitude and duration of T cell activation (Tseng and Dustin 2002).

Signal transduction

When the TCR engages with MHC-peptide complexes an immunological synapse is formed. Integrin and non-integrin adhesion molecules mediate the initial adhesion. In the second step, engaged TCR complexes become enriched in a central cluster, surrounded by a ring of integrins (Germain 2001). This clustering brings the protein tyrosine kinase Fyn into contact with the cytoplasmic tails of the CD3 chains (Figure 3.2). Clustering also brings the CD4 and CD8 molecules into close proximity to the TCR. Another tyrosine kinase called Lck is associated with these co-receptors (Janeway *et al.* 2001a). The membrane associated phosphatase, CD45, activates Fyn and Lck by removing an inhibitory phosphate (Germain 2001). Activated Fyn and Lyk then proceed to phosphorylate tyrosine residues in the ITAMs of the TCR-CD3 complex. Phosphorylation of the ξ chain creates docking sites for the tyrosine kinase, zeta associated protein ZAP-70. Once bound, ZAP-70 is phosphorylated by Lyk or Fyn and acquires its tyrosine kinase activity (Qian and Weiss 1997). Activated ZAP-70 phosphorylates "adapter" proteins, which serve as docking sites for other signalling molecules. Two important adapter proteins involved in T cell activation are LAT (linker of activation in T cells) and SLP-6 (second linker or adapter protein in T cells) (Kane *et*

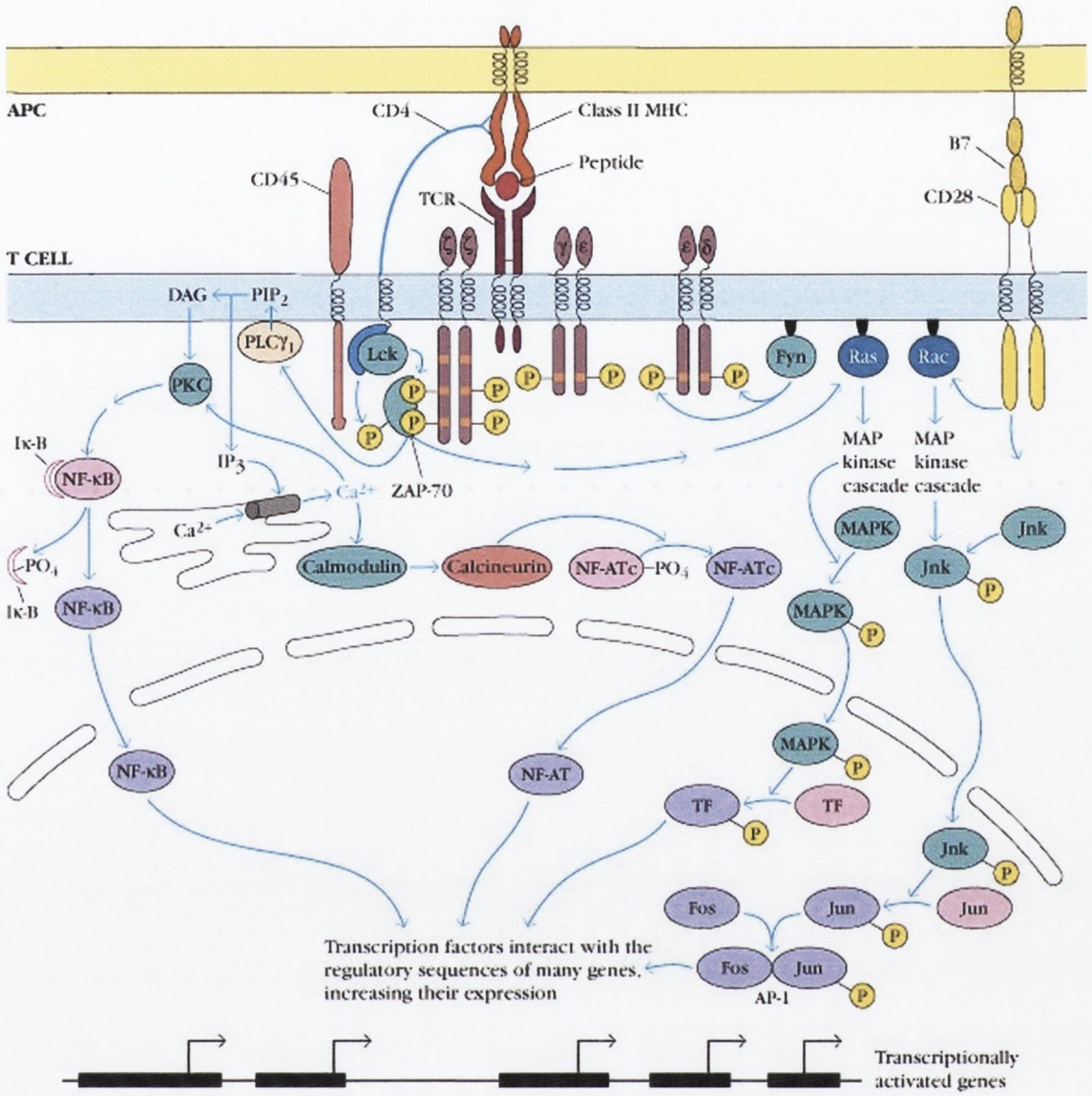


Figure 3.2 Overview of signal transduction during T cell activation (taken from Goldsby *et al.* 2000b).

al. 2000). Four different signal transduction pathways are initiated from this point; a Ras pathway, a Rac pathway, a phospholipase C (PLC)-γ-1 Ca^{2+} dependant pathway and a PLC-γ-1 DAG pathway (Abbas *et al.* 2000a).

Once adapter protein LAT is phosphorylated it recruits to the membrane another adapter protein such as Grb-2. Grb-2 is phosphorylated and recruits Sos, which exchanges GDP for GTP in the guanine nucleotide binding protein, Ras. This form of the Ras protein (Ras.GTP) activates the mitogen-activated protein (MAP) kinase cascade. The MAP kinase pathway results in the activation of extracellular receptor-activated kinase (Erk) and transcription of Fos, which is a component of the activation protein (AP-1) transcription factor (Abbas *et al.* 2000a). The adapter proteins also recruit and activate another GTP exchange protein called Vav. When Vav is activated it acts on another guanine binding protein called Rac. Rac.GTP triggers the MAP kinase cascade resulting in the activation of the c-Jun N-terminal kinase (JNK). Activated JNK phosphorylates c-Jun, which is the second component of the AP-1 transcription factor. Vav and Rac may also be involved in changes in the actin cytoskeleton (Abbas *et al.* 2000a; Janeway *et al.* 2001a).

Tyrosine phosphorylated adapter proteins such as LAT also recruit the enzyme PLC-γ-1 to the plasma membrane. Here the enzyme is phosphorylated by ZAP-70. Active PLC-γ-1 catalyses the hydrolysis of a membrane phospholipid called phosphatidylinositol 4, 5 biphosphate (PIP_2) generating IP_3 and DAG (Acuto and Cantrell 2000) (Abbas *et al.* 2000a). IP_3 diffuses to the ER, where it stimulates the release of Ca^{2+} by binding to IP_3-gated Ca^{2+} release channels in the ER membrane. An overall increase in cytosolic free Ca^{2+} results. Free Ca^{2+} binds to a regulatory protein called calmodulin. This binding alters the conformation of calmodulin allowing it to bind to various target proteins. One of these targets is the enzyme calcineurin (Alberts *et al.* 1994). Calcineurin dephosphorylates the transcription factor NFAT allowing it to translocate to the nucleus and bind to target sequences (Janeway *et al.* 2001a). DAG, the second breakdown product of PIP_2, activates the enzyme PKC. The rise in Ca^{2+} induced by IP_3 causes PKC to translocate from the cytosol to the plasma membrane. DAG is hydrophobic and remains in the membrane where it is formed. DAG activates PKC by inducing a conformational change that makes the catalytic site of the kinase accessible to substrate. Activation of the transcription factor NFκB is dependent on PKC (Abbas *et al.* 2000a).

3.1.2 Cytokines

Cytokines are a diverse group of signalling molecules that act as modulators of inflammation, immunity and haematopoiesis. Many cells are capable of synthesising and responding to these proteins. Cytokines are not usually stored in the cell and are newly synthesised following cell activation. Production is short-lived due to the transient nature of cytokine mRNA. In addition, the synthesis of certain cytokines may be controlled by RNA processing and by post-transcriptional mechanisms (Abbas *et al.* 2000c). Once synthesised, most cytokines are rapidly secreted, while some remain anchored in the plasma membrane. Cytokines act at picomolar and nanomolar concentrations on cytokine receptors expressed by target cells. They function in an autocrine (same cell), paracrine (nearby cell) or endocrine (cell distant to site of production) manner (Goldsby *et al.* 2000b). Their actions are often pleiotropic (have multiple, overlapping biological activities) and redundant (have the same functional effects) (Elgert 1996a).

Function

Cytokines are involved in many aspects of innate immunity. Some cytokines are secreted, primarily by mononuclear phagocytes, in response to infectious agents. These cytokines promote the recruitment of phagocytic cells to the site of infection. Cytokines also function in adaptive immunity. Cytokines secreted by T lymphocytes are involved in the growth and differentiation of lymphocytes or regulate effector cells such as B cells, macrophages, eosinophils and endothelial cells. Cytokines also participate in hematopoiesis (Abbas *et al.* 2000c; Goldsby *et al.* 2000b). The functions of some cytokines are summarised in Table 3.1.

Classifications

There is no amino acid sequence motif or three-dimensional structure to allow us to group cytokines into different classes. Therefore they are often grouped according to their biological activities. Some cytokines e.g. IL-1 and TNF promote inflammation and are known as pro-inflammatory cytokines. They stimulate the production of proteins, which promote an inflammatory response such as cyclooxygenase-2 and inducible NO synthase (Dinarello 2000). Other cytokines suppress the activity of pro-inflammatory cytokines and are classified as anti-inflammatory cytokines e.g. IL-4, IL-10 and IL-13 (Dinarello 2000).

Table 3.1 Selected functions of some cytokines (adapted from (Goldsby *et al.* 2000b).

Cytokine	Source	Target	Function
IL-1	Monocytes, B cells, macrophages, dendritic cells	Th cells B cells NK cells	Co-stimulates activation Promotes maturation and expansion Enhances activity
IL-2	Th1 cells	Th and Tc cells T cell clones NK cells	Induces proliferation Supports long-term growth Enhances activity
IL-3	Th cells, NK cells, mast cells	Hematopoietic cells Mast cells	Supports growth and differentiation Growth and histamine secretion
IL-4	Th2 cells, mast cells, NK cells	Antigen primed B cells Activated B cells T cells	Co-stimulates activation Proliferation and differentiation, Class switch to IgG1 and IgE Th2 differentiation, proliferation
IL-5	Th2 cells, Mast cells	Activated B cells Eosinophils	Proliferation and differentiation Class switch to IgA Growth and differentiation
IL-6	Monocytes, macrophages, Th2 cells,	B cells Plasma cells Hepatocytes	Differentiation into plasma cells Stimulates antibody secretion Synthesis of acute phase proteins
IL-7	Bone marrow, thymic stromal cells	Lymphoid stem cells, Resting T cells	Differentiation into progenitor T and B cells Increases IL-2 and IL-2 receptor
IL-8	Macrophages, Endothelial cells	Neutrophils	Chemokine; chemotactically attracts, induces adherence to vascular endothelium
IL-9	Th cells	Some Th cells	Acts as mitogen, supports proliferation in absence of antigen
IL-10	Th2 cells	Macrophages Antigen-presenting cells	Suppresses cytokine production and thus indirectly reduces cytokine production by Th1 cells Down-regulates class II MHC expression
IL-11	Bone marrow stromal cells	Plasmacytomas Progenitor B cells Megakaryocytes Hepatocytes	Supports growth Promotes differentiation Promotes differentiation Synthesis of acute-phase proteins

IL-12	Macrophages, B cells	Activated Tc cells	Acts synergistically with IL-2 to induce differentiation into CTLs
		NK and LAK cells and activated Th1 cells	Stimulates proliferation
IL-13	Th cells	Macrophages	Inhibits activation and release of inflammatory cytokines; important regulator of inflammatory response
IL-15	T cells	Macrophages	Initiates and maintains inflammation
IL-16	T cells and eosinophils	CD4+ T cells	Chemotaxis; induces expression of class II MHC; induces synthesis of cytokines; suppresses antigen-induces proliferation
IL-17	T cells	Macrophages	Initiates and maintains inflammation
IL-18	Activated macrophages	T cells NK cells	Induces IFN-γ production Enhances NK cell cytotoxicity
IFN-α	Leukocytes	Uninfected cells	Inhibits viral replication
IFN-β	Fibroblasts	Uninfected cells	Inhibits viral replication
IFN-γ	Th1, Tc, NK cells	Uninfected cells Macrophages Many cell types Proliferating B cells Th2 cells Inflammatory cells	Inhibits viral replication Enhances activity Increases expression of class I and class II MHC molecules Induces class switch to IgG2a; blocks IL-4 induced switch to IgE and IgG1 Inhibits proliferation Mediates effects important in delayed-type hypersensitivity
TGF-β	Platelets, macrophages, lymphocytes, mast cells	Monocytes and macrophges Activated macrophages Epithelial, endothelial, lymphoid cells	Chemotactically attracts Induces increased IL-1 production Inhibits proliferation, thus limiting the inflammatory response and promoting wound healing
TNF-α	Macrophages	Tumor cells Inflammatory cells	Cytototoxic effect Induces cytokine secretion
TNF-β	Th1 and Tc cells	Tumor cells Macrophages and neutrophils	Cytotoxic and other effects similar to TNF-α Enhances phagocytic activity

Disease may occur when there is an imbalance between the effects of pro-inflammatory and anti-inflammatory cytokines (D'Elios and Del Prete 1998). Cytokines may also be grouped as (i) interleukins; an antiquated term to describe cytokines made by leukocytes, that act mainly on leukocytes, (ii) interferons; cytokines which respond to viral infections (iii) tumor necrosis factors; cytotoxic and anti-tumor cytokines, (iv) colony stimulating factors; growth promoting cytokines and (iv) chemokines; cytokines involved in chemotaxis and inflammation (Elgert 1996a).

Cytokines produced by helper T cells are often classified as Th1 or Th2. Th1 cytokines are involved in cell-mediated immune responses and delayed type hypersensitivity reactions e.g. IL-2, IFN-γ and TNF. Th1 cytokines are important in phagocytic defence and promote macrophage activation (Romagnani 1996). Th2 cytokines provide help for humoral immune responses e.g. IL-4, IL-5, IL-6, IL-10 and IL-13. These cytokines act as growth and differentiation factors for B cells as well as eosinophils and mast cells (Romagnani 1996). Each subset amplifies itself and cross-regulates the other. IFN-γ inhibits proliferation of Th2 cells. Th2 cytokines, notably IL-4, IL-13 and IL-10, antagonise the actions of IFN-γ, inhibiting macrophage functions (Abbas *et al.* 2000c). It is now clear that there are many subpopulations of T cells, which have a heterogeneous pattern of cytokine production. IL-3 and GM-CSF, for example, are released by both Th1 and Th2 subsets. T cells producing cytokines from both subsets i.e. IL-2, IL-3, IL-4, IFN-γ and TNF-α have been designated Th0 cells (Goldsby *et al.* 2000b). Cytotoxic T cells may also be differentiated based on their cytokine profile. $CD8^+$ Tc1 and Tc2 cells have been defined (D'Elios and Del Prete 1998).

Th1 or Th2 cells develop from the same Th precursor cell. The cytokine profile that a Th cell develops, depends on a number of factors encountered at this naïve precursor stage. Cytokines themselves are important differentiating factors. IL-4 is a major inducer of Th2 cells, whereas IL-12 favours Th1 development. IFNs also promote Th1 development by enhancing IL-12 secretion by macrophages and by enhancing the expression of IL-12 receptors (Romagnani 1996). Binding of cytokines to their receptors results in the triggering of intracellular signalling proteins, such as the Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (Janeway *et al.* 2001a). IL-12 selectively activates STAT 4, which promotes the differentiation of T cells into Th1 cells. IL-4 activates STAT 6, which stimulates transcription of Th2 cytokines and is important in the development of Th2 cells (Abbas *et al.* 2000c).

Microbes can also promote Th1 development by inducing IL-12 production in macrophages. Other factors, which play a role in Th cell differentiation are the concentration of antigen, the site of antigen presentation, the physical form of the immunogen and the co-stimulators expressed on APCs (Abbas *et al.* 2000c).

Cytokine receptors

Cytokines initiate their actions by binding to high-affinity receptors on the surface of target cells. The expression of cytokine receptors is regulated by external signals. Cytokines themselves can enhance or downregulate the expression of their receptors. The cytokine receptors frequently have two or even three polypeptide chains. Their structure consists of an extracellular domain which is responsible for cytokine binding and a cytoplasmic domain, which is responsible for initiating intracellular signalling (Abbas *et al.* 2000c). Some receptor chains are shared by different cytokines. The receptors can be classified into five families based on conserved amino acid sequence motifs found in the extracellular domains. They are as follows; (i) type I cytokine receptors, (ii) type II receptors, (iii) TNF receptors, (iv) immunoglobulin superfamily receptors and (v) chemokine receptors (Goldsby *et al.* 2000b).

Cytokine binding induces receptor aggregation, which brings together the cytoplasmic portions of the receptor molecules (Figure 3.3). The JAK kinases, which are associated with the cytoplasmic domains of the receptor, phosphorylate each other and become active. They in turn phosphorylate tyrosine residues in the cytoplasmic portions of the receptors. The Src homology-2 (SH_2) domains of STAT proteins recognise the phosphorylated residues and become attached to the receptors. The STAT proteins are then phosphorylated by the receptor-associated JAK kinases. The phosphorylated STAT proteins then dimerise and dissociate from the receptor. The STAT dimers form a complex with other cytoplasmic proteins and migrate to the nucleus. Here they bind to specific sequences in the promoter regions of cytokine-responsive genes and activate transcription. The genes activated depend on the STAT protein involved (Ihle 1995; Ihle and Kerr 1995; Janeway *et al.* 2001a).

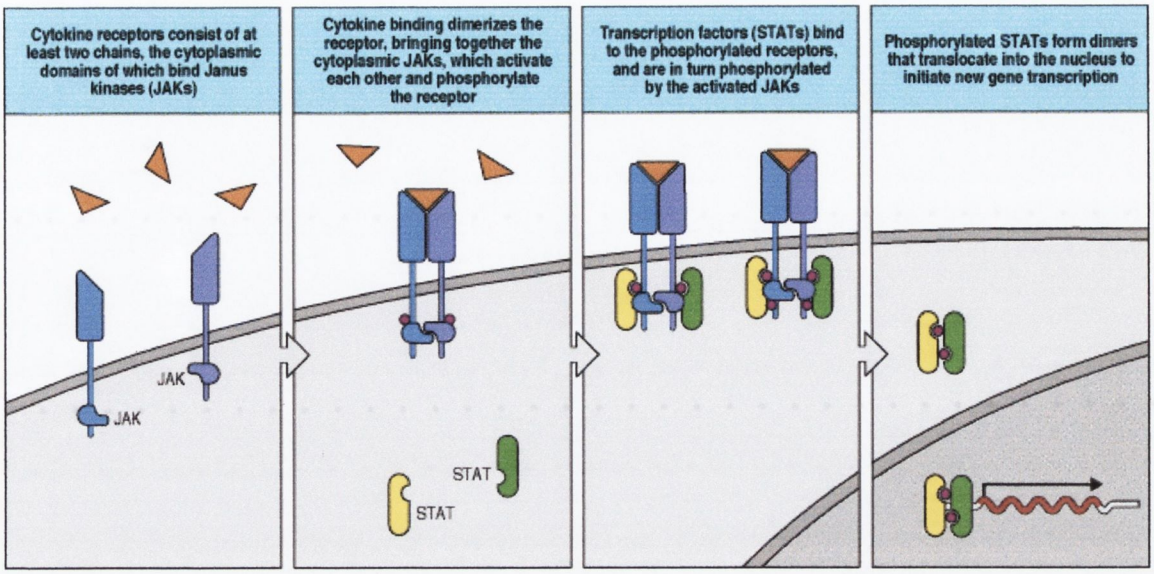


Figure 3.3 Cytokine signalling (taken from Janeway *et al.* 2001a).

3.1.3 TaqMan PCR

Principle of Real-Time polymerase chain reaction (PCR)

Real-time TaqMan PCR is based on detection of a fluorescent signal produced during the amplification of a PCR product. Three oligonucleotides are used in the PCR reaction: a forward primer, a reverse primer, and a probe (Figure 3.4). The probe is an oligonucleotide, labelled with a reporter dye attached at the 5' end and a quencher dye attached at the 3' end. The fluorescent reporter dye can be FAM (6-carboxylfluorescein), TET (tetrachloro-6-carboxylfluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein), HEX (hexachloro-6-carboxyfluorescein), or VIC®. The reporter dye is quenched by TAMRA (6-carboxytetramethylrhodamine). When the probe is intact, the quencher dye absorbs the emission of the reporter dye. During PCR amplification, if the target of interest is present, the probe specifically anneals to the target. During the extension phase of the PCR reaction, the hybridised probe is hydrolysed by the 5' nuclease activity of the Taq polymerase, separating the quencher from the reporter. This results in an increase in fluorescence emission of the reporter dye. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

Normalisation

It is important to correct for inefficiencies in complementary DNA (cDNA) synthesis and RNA input. One method of correcting variations is to normalise to a housekeeping gene. Housekeeping genes β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rRNA (ribosomal RNA) and hypoxanthine-guanine phosphoribosyltransferase are commonly used (Giulietti *et al.* 2001).

Quantification

The cycle number at which the reporter dye emission rises above background levels is called the threshold cycle (C_t). The C_t is inversely proportional to the copy number of the target template, the higher the template concentration, the lower the threshold cycle measured. Two different methods are commonly used to quantify results obtained by TaqMan PCR: the standard curve method and the comparative threshold method (Giulietti *et al.* 2001). In the standard curve method a sample of known concentration is used to construct a standard curve. This is used to quantify an unknown sample. In the comparative Ct method, formulas are used to calculate expression levels relative to a

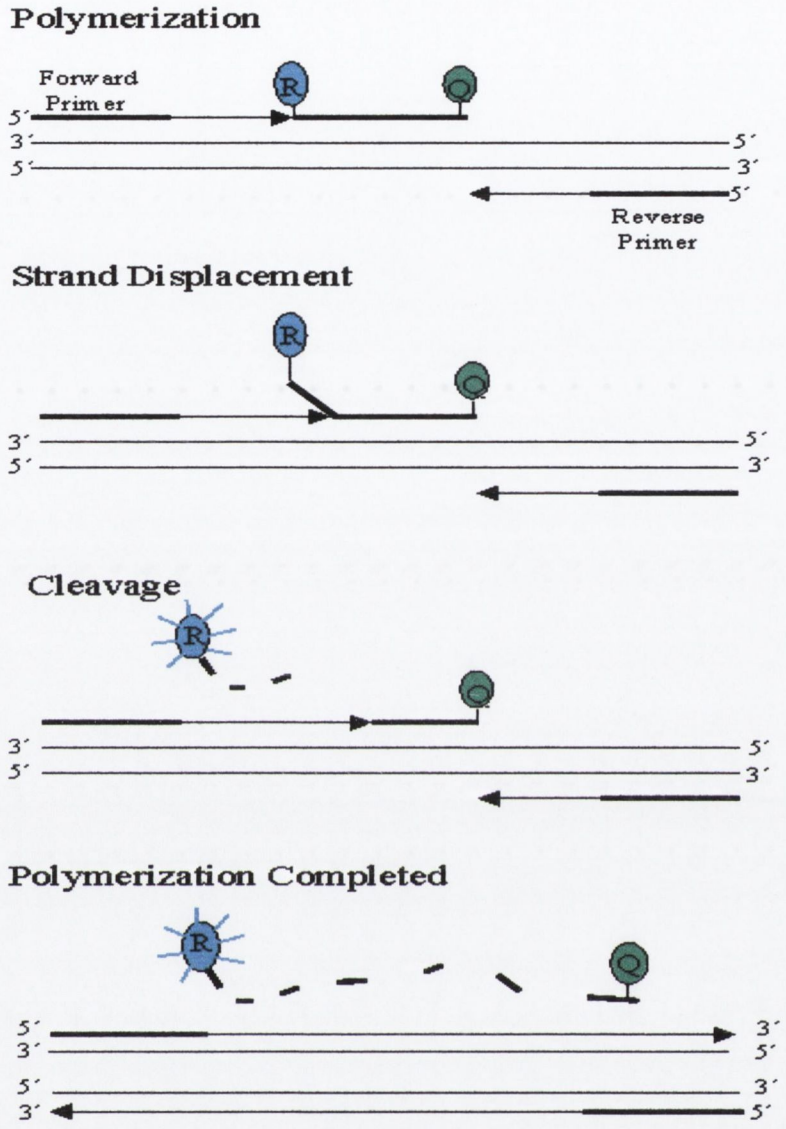


Figure 3.4 TaqMan system. Primers and probe anneal to the target. The 5' to 3' nuclease activity of the Taq polymerase cleaves the probe, separating the reporter and the quencher. The reporter dye fluoresces and the signal measured. Once the probe has been displaced polymerisation continues along the strand.

calibrator. The untreated control may be used as a calibrator. Alternatively, the same mRNA in another organ may be used as a calibrator. The amount of target, normalised to a housekeeping gene and relative to the calibrator is then given by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta C_t$ (sample) - ΔC_t (calibrator), and ΔCt is the C_t of the target gene subtracted from the C_t of the housekeeping gene (Livak and Schmittgen 2001).

Advantages of TaqMan PCR

One of the many benefits of TaqMan real-time PCR is its precision. PCR products are determined during the exponential phase of the reaction rather than the end-point. Also the requirement of three oligonucleotides to anneal to the DNA makes detection extremely specific. Quantitative real-time reverse transcription PCR (RT-PCR) is the method of choice used to quantify the mRNA expression of cytokines, which are often expressed at very low levels. Another advantage of this system is that a large number of samples can be processed in a short period of time (2-3 hours). Classical techniques used to detect PCR products are laborious and quite often hazardous. TaqMan PCR products are quantified in a closed tube, which minimises sample contamination (Giulietti *et al.* 2001).

3.1.4 Oats in the coeliac diet

In addition to the gliadin fraction of wheat, the prolamin fractions of barley and rye, hordein and secalin respectively, are thought to activate coeliac disease. Consequently, coeliac patients are advised to avoid these cereals in their diet. The need to avoid oats in the GFD is more controversial. Dicke (1953) found that a large quantity of oatmeal (140g/day) increased faecal fat excretion in a paediatric patient. He therefore concluded that oats should be avoided by coeliac patients. Baker and Reid (1976) later challenged patients in remission with smaller amounts of oats (60g/day). During a study period of 28-100 days patients had reduced D-xylose excretion and gastrointestinal symptoms. Moulton (1959) investigated whether the quantity of oats was important in causing the adverse effects seen in coeliac patients. He challenged four coeliac children with 46, 70, 163 and 169g of oatflakes for 45, 96, 22 and 23 days respectively. He found that quantities of up to 70g of oatflakes could be consumed without ill effect. Larger amounts resulted in increased fat excretion. However, these levels were still within normal limits. The first study advocating the safety of oats assessed its effects on jejunal morphology (Dissanayake *et al.* 1974). 40-60g/day had no effect on the surface:volume ratio or disaccharidase expression in 4 coeliac children. All of these studies were based on small numbers of patients, they lacked a control group and the duration of oats challenge was brief. Also the tests used to determine oat toxicity i.e. increased fecal fat excretion and reduced D-xylose excretion were unreliable. Importantly, oats were not tested for contamination with wheat.

Trials in the past eight years have been more comprehensive and the issue of oats toxicity has been re-evaluated. A Finnish study monitored 52 treated and 40 newly diagnosed coeliac patients for 6 and 12 months respectively (Janatuinen *et al.* 1995). Patients in both groups were randomly assigned to a GFD or a GFD plus oats. Following an average consumption of 49.9±14.7g/day by treated patients and 46.6±14.7g/day by newly diagnosed patients there was no worsening of villous architecture in either of the diet groups. Of significance, was the fact that oats did not effect symptomatic or mucosal healing in the newly diagnosed patient group. A subsequent publication, by the same authors, reported that oats did not trigger anti-gliadn or anti-reticulin antibody production or affect their rate of disappearance in newly diagnosed patients (Janatuinen *et al.* 2000). Coeliac patients who remained in the study for five years had improved villous architecture and no significant changes in serum

antibody levels (Janatuinen *et al.* 2002). An oats trial was also conducted in Ireland, where patients consumed 50g oats/day for 12 weeks (Srinivasan *et al.* 1996). All patients remained asymptomatic with normal morphology, EMA and AGA titres. Two of these patients were sensitive to a microchallenge of gluten. The Irish and Finnish trials challenged adult patients with oats known to be free from wheat contamination. Hoffenberg *et al.* (2000) assessed the effects of a standard, commercially available oat product on 10 newly diagnosed coeliac children. Again a decrease in symptoms, biopsy score, IEL count and anti-tTG levels were noted, while patients consumed oats.

Similar findings were made in patients with DH (Hardman *et al.* 1997; Reunala *et al.* 1998). These two studies were conducted for 3 and 6 months and the amount of oats consumed was similar to previous trials with coeliac patients. Again, oats did not alter mucosal morphology. Skin rash flare-ups were seen in both studies. This was attributed to dietary lapses as rashes were also noted in the control population (Reunala *et al.* 1998).

Despite good clinical evidence that oats is well tolerated by coeliac and DH patients, the inclusion of oats in the GFD is still debated. A recent *in vitro* study found that culture with avenin, the prolamin faction of oats, did not induce EMA production in coeliac biopsies (Picarelli *et al.* 2001). However an earlier study reported that avenin activated a T cell immune response in cultured jejunal biopsies (Leone *et al.* 1996). It has been suggested that the apparent safety of oats is explained by the low content of avenin in oats.

3.1.5 Aim of this chapter

The purpose of the present study was to investigate the immunogenicity of oat avenin in comparison with wheat gliadin when added to duodenal biopsies in 4h organ culture experiments. IFN-γ and IL-2 cytokine events were measured as objective evidence of T cell activation (Nilsen *et al.* 1998). Cytokine mRNA was quantified by TaqMan PCR, an accurate method of detecting mRNA even when expressed at low levels. Cytokine protein was measured by ELISA.

3.2 MATERIALS AND METHODS

3.2.1 Production of gliadin and avenin

The avenin and gliadin preparations were a gift from Herbert Weiser and were prepared as follows. Kernels of the German wheat cultivar Rektor were milled to flour with an ash content of 0.55 % using a laboratory mill (Brabender Quadrumat Junior). Oats flour was provided by Kölln Flockenwerke (Elmshorn, Germany); PCR analysis (Allmann *et al.* 1993) revealed that the flour was free from wheat contaminations. Both flours were defatted with light petroleum (boiling range 40 – 60°C) and air-dried. 50g of the defatted flours were extracted stepwise three-times with 200ml of a salt solution (0.4 mol/l NaCl + 0.067 mol/l NaK phosphate, pH 7.6) and three-times with 200 ml 60 % (v/v) aqueous ethanol using an Ultra Turrax homogeniser for 5min at room temperature (RT). The suspensions were centrifuged for 15min at RT and 40,000xg. The supernatants of the three ethanol extracts were combined, concentrated to about 200ml using a vacuum evaporator at 40°C, dialysed against 0.01 mol/l acetic acid until free from chloride and freeze-dried.

3.2.2 Peptic tryptic digestion

The stepwise enzymatic hydrolysis of gliadin and avenin was performed with pepsin (Sigma P3286) and trypsin (Sigma T1763) both attached to agarose according to Bolte *et al.* (1996). In preliminary experiments different ratios of proteins (gliadin, avenin) to enzymes (pepsin, trypsin) were tested and controlled by RP-HPLC on C18 silica gel (Müller and Wieser 1995). On a preparative scale 1g of gliadin and avenin was dissolved in 50 ml of diluted HCl (pH 2.0) and 100 mg (4000 U) of pepsin were added. The mixtures were magnetically stirred at 37°C and after 2h centrifuged for 20min at RT and 6,000xg. The pH of the supernatants was adjusted to 7.8 with 0.1 mol/l NaOH and 1ml of the trypsin suspension (25 U) was added. After 2h incubation at 37°C and under magnetical stirring the pH was adjusted to 7.0 with 0.1 mol/l HCl, and the digests were centrifuged for 20min at RT and 6,000xg. The supernatants were then freeze-dried. Control RP-HPLC on C18 silica gel indicated complex peptide patterns for both gliadin and avenin digests.

3.2.3 Patients

Eight duodenal biopsies were taken from seventeen coeliac patients and sixteen disease control patients by gastrointestinal endoscopy. The diagnosis of coeliac disease was based on a typical histological lesion and positive serology (IgA EMAs and AGAs) and positive histological and serological response to a GFD. Ethical approval was granted by the Ethics Committee Board of St. James's Hospital, Dublin, Ireland. Biopsies from both groups of patients were randomly assigned to culture with gliadin or avenin. The coeliac patients are listed according to the prolamin with which their biopsies were cultured in Table 3.2; biopsies from nine coeliac patients (patients 1 to 9) were cultured with PT gliadin; biopsies from eight coeliacs (patients 10-17) were cultured with PT avenin. Duration of GFD, demographic, serological and histological details are also given in Table 3.2.

Biopsies from eight normal controls were also cultured with PT gliadin (patients 18-25) and biopsies from a further eight normal controls were cultured with PT avenin (patients 26-33). The controls included 9 males, 7 females, with a mean age of 43 years (range 23 to 74). The histology of the duodenal mucosa was normal in twelve of these sixteen subjects but was not available for the remaining four patients.

3.2.4 Organ culture

Organ culture was carried out as described in section 2.2.3. Four biopsies were cultured in the presence of 5mg/ml PT gliadin or 5mg/ml PT avenin for 4h. The prolamin-stimulated biopsies were compared to biopsies cultured in medium alone.

Table 3.2 Details of coeliac patients used in the study

Patient No	Stimulant	Gender	Age	Gluten Free Diet (years)	Serum EMA	Histology grade
1	Gliadin	Male	54	> 1.5	-	1
2	Gliadin	Female	21	2	-	1
3	Gliadin	Female	64	1.5	-	2
4	Gliadin	Female	30	0	nd	3
5	Gliadin	Female	52	1	-	2
6	Gliadin	Female	46	1	+	3
7	Gliadin	Female	41	> 1	-	1
8	Gliadin	Male	39	> 13	-	2
9	Gliadin	Male	55	1	-	1
10	Avenin	Female	31	1	-	2
11	Avenin	Female	43	> 15	-	0
12	Avenin	Female	25	4.5	-	1
13	Avenin	Female	35	> 2	-	1
14	Avenin	Female	45	1	-	0
15	Avenin	Female	50	6	-	2
16	Avenin	Male	41	0	+	2
17	Avenin	Female	28	> 5	-	0

EMA, endomysial antibody; nd, not determined; histology grade: 0=normal, 1= raised IELs, 2=partial villous atrophy with raised IELs, 3=subtotal villous atrophy with raised IELs.

3.2.5 RNA extraction

Total RNA was extracted from the biopsies using the guanidium thiocyanate method. All steps were carried out on ice. Whole biopsies were denatured in 500µl of denaturing solution (Appendix II) using a tube pestle. 50µl of 2M sodium acetate pH4 was added and the solution gently mixed. 500µl of saturated phenol was added to the eppendorf tube followed by 200µl of chloroform/isoamyl alcohol (Appendix II). The solution was mixed and incubated on ice for 15min with intermittent mixing. Following incubation the tube was centrifuged at 10,000xg at 4°C for 12min. The aqueous phase was transferred to a fresh tube, 500µl of isopropanol was added and the tube incubated overnight at -70°C. The mixture was centrifuged at 10,000xg for 10min. The supernatant was removed and the RNA pellet was dissolved in 200µl of denaturing solution. 200µl of isopropanol was added and the mix incubated at -70°C for 30min. Following centrifugation at 10,000xg for 10min, the supernatant was removed and 400µl of 75% ethanol added. The tube was incubated on ice for 15min, then centrifuged at 10,000xg for 5min. The supernatant was removed and the pellet allowed to air dry. The RNA pellet was then resuspended in RNase free water and stored at -70°C.

3.2.6 Reverse transcription

RNA samples were heated at 85°C for 2min and placed on ice. 7.5µl total RNA was then reverse transcribed into cDNA in a 30µl reaction mixture containing 5mM $MgCl_2$, avian myeloblastosis virus reaction buffer (50mM Tris-HCl pH 8.3, 50mM KCl, 10mM $MgCl_2$, 10mM DTT, 0.5mM spermidine), 1U/µl RNasin® ribonuclease inhibitor, 1U/µl avian myeloblastosis virus reverse transcriptase, 5ng/µl random primers, 1mM deoxynucleotide triphosphates (Promega, USA). The reaction was performed at 42°C for 1h. cDNA samples were stored at -70°C.

3.2.7 TaqMan primer and probe design

The TaqMan primers and probe for IFN-γ and IL-2 were designed using the software package Primer Express. The DNA sequences of IFN-γ Genbank Accession J00219 and IL-2 Genback Accession K02056 were imported into Primer Express. The introns were deleted and the intron/exon boundaries labelled. The primer and probe sets were chosen

so that the primers or probe traversed an intronic juction. This was to avoid amplification of contaminating genomic DNA. The guidelines adhered to when designing the primers and probes are listed in Appendix IV.

The primers and probes for IL-2 and IFN-γ were synthesised by Perkin Elmer (Table 3.3). The probes were labelled at the 5′ end with the fluorogenic reporter dye 6-FAM and labelled at the 3′ end with the quencher dye 6-TAMRA. GAPDH primers and probe were commercially available from Perkin Elmer as pre-developed assay reagents (PDARs). The GAPDH probe was labelled at the 5′end with VIC® and at the 3' end with 6-TAMRA .

Table 3.3 Cytokine primer and probe sequences

Primers and Probes		Sequence	Amplicon
IFN-γ	Probe	5′ TGTCACTCTCCTCTTTCCAATTCTTCAAAATGC 3′	96 bp
	Forward Primer	5′ TTCAGATGTAGCGGATAATGGAAC 3′	
	Reverse Primer	5′ GAGACAATTTGGCTCTGCATTATTT 3′	
Il-2	Probe	5′ATGCTCACATTTAAGTTTTACATGCCCAA 3′	88 bp
	Forward Primer	5′ GAATTAATAATTACAAGAATCCCAAACT 3′	
	Reverse Primer	5′ AGATGTTTCAGTTCTGTGGCC 3′	

3.2.8 Optimising the primer concentration

The primer concentrations were optimised for IL-2 and IFN-γ primer pairs. Reactions were carried out in duplicate in 25µl of master mix with concentrations of forward and reverse primers shown in Table 3.4. For both IFN-γ and IL-2 the lowest primer concentrations which gave the lowest Ct value were 300nM forward with 300nM reverse.

Table 3.4 The concentration of primers used in the optimisation experiments.

50nM F : 50nM R	300nM F : 50 nM R	900nM F : 50nM R
50nM F : 300nM R	300nM F : 300 nM R	900nM F : 300nM R
50nM F : 900nM R	300nM F : 900nM R	900nM F : 900nM R

F represents the Forward primer and R the Reverse primer

3.2.9 TaqMan PCR reaction

Reactions were carried out in a MicroAmp Optical 96-well reaction plate (Perkin Elmer, USA). The 25µl reaction mixture for IFN-γ and IL-2 consisted of the components listed in Table 3.5. GAPDH PCR reactions were also carried out in 25µl with the components listed in Table 3.6. The Universal PCR Master Mix (Perkin Elmer,USA) contained PCR buffer, $MgCl_2$, glycerol, deoxynucleotide triphosphates, AmpliTaq Gold® and uracil-N-glycosylase. A number of controls were included on each plate, a no template control (NTC); where cDNA was omitted from the reaction mix, a non-amplification control (NAC); where SDS was added to prevent DNA amplification, and a positive control: cDNA from PBMCs which had been stimulated with PMA/OKT3 for 4h. The reaction was carried out using the following cycling parameters: 2min at 50°C, 10min at 95°C, 40 cycles of 15s at 95°C and 60s at 60°C.

Table 3.5 Reaction components for IFN-γ and IL-2 TaqMan PCR

	Volume	Final concentration
Forward primer	x* µl	300nM
Reverse primer	y* µl	300nM
TaqMan probe	0.5 µl	100nM
cDNA	2 µl	≈20ng
TaqMan Universal PCR Master Mix	12.5 µl	1X
RNase free water	10-(x+y)	

* volume varies depending on the stock concentration

Table 3.6 Reaction components for GAPDH TaqMan PCR

	Volume	Final concentration
TaqMan Universal PCR Master Mix	12.5 µl	1X
RNase free water	9.25 µl	
20X GAPDH primers and probe	1.25 µl	1X
cDNA	2 µl	≈20ng

3.2.10 Calculating results

Accumulation of PCR product was detected by monitoring the increase in fluorescence of reporter dye using the ABI Prism 7700 Sequence Detection System®. The software calculates a ΔRn value as follows: $\Delta Rn = (Rn^{+}) - (Rn^{-})$, where Rn^{+} is the emission intensity of the reporter and Rn^{-} is the fluorescence emission of the baseline. The computer generates an amplification plot where ΔRn values are plotted against the cycle number (Figure 3.5). During the initial stages of the PCR, ΔRn values do not exceed baseline. The cycle number at which ΔRn number passes a fixed threshold is called the threshold cycle (Ct). The threshold is set in the exponential region of the amplification plot. There is an inverse relationship between the amount of template present and the Ct value.

The results were calculated by the comparative Ct method, which uses the following formula:

$$\Delta\Delta Ct = \Delta Ct\,(\text{sample}) - \Delta Ct\,(\text{calibrator})$$

where ΔCt = Ct of the target (IFN-γ or IL-2) - Ct of the internal control (GAPDH)

In these experiments the calibrator was resting PBMCs (mean of 3 samples). Relative quantitation values for IFN-γ and IL-2 mRNA were then calculated using the formula:

$$2^{-\Delta\Delta Ct}$$

3.2.11 ELISA

The organ culture supernatants were analysed for IFN-γ and IL-2 by ELISA development kits from Mabtech, Sweden and DuoSet, R&D Systems, Europe respectively.

IFN-γ

A 96 well ELISA plate was coated overnight at 4°C with 2μg/ml capture antibody in PBS. The wells were washed three times with PBS/0.05% Tween and blocked with the same solution for 1h at RT. The wash was repeated and the IFN-γ standards and neat test samples added in triplicate to the plate. Following 2h incubation the unreacted

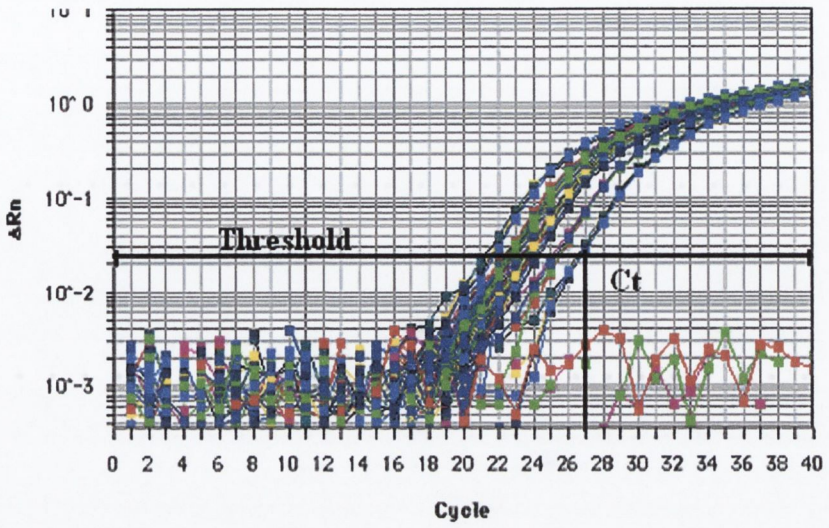


Figure 3.5 A Real Time amplification plot. ΔRn is plotted against cycle number. The graph shows the threshold level and the Ct (threshold cycle), which is the cycle number at which ΔRn number passes the threshold.

cytokine was removed by washing. 1μg/ml of a biotinylated anti-IFN-γ antibody in PBS/Tween was added and incubated for 1h at RT. Unbound antibody was removed by washing. Streptavidin-alkaline phosphatase was added to each well and allowed to react for 1h. Finally the substrate solution p-nitro-phenylphosphate (Appendix II) was introduced into the wells and incubated for 30min. The spectrophotometrical absorbance was measured at 405nm.

IL-2

An ELISA plate was coated in 4μg/ml capture antibody, diluted in PBS and allowed to incubate overnight at RT. The wells were washed three times with PBS/0.05%Tween and blocked with block buffer (Appendix II) for 1h at RT. The washing step was repeated and the IL-2 standards and neat test samples added. Following 2h incubation, the wells were washed and 50ng/ml of biotinylated anti-IL-2 antibody diluted in reagent diluent (Appendix II) was added and allowed to incubate for a further 2h. Excess antibody was removed by washing and streptavidin-horseradish peroxidase diluted in reagent diluent was added to the wells and allowed to react for 20min. The washing step was repeated and tetramethylbenzidine added for a further 20min. The reaction was stopped with 1M H_2SO_4 and the spectrophotometrical absorbance measured at 450nm.

Standard curves

A standard curve was established for each cytokine assay using recombinant standards to quantify the levels of cytokine in the culture supernatants. The absorbance value obtained for each standard was plotted against cytokine concentration using the computer program MultiCalc. The concentration of cytokine in the culture supernatant was determined from the standard curve. An example of a standard curve for IL-2 is shown in Figure 3.6.

3.2.12 Statistical analysis

Statistical differences were evaluated using the Wilcoxon signed-rank test. A p value of less than 0.05 was regarded as statistically significant.

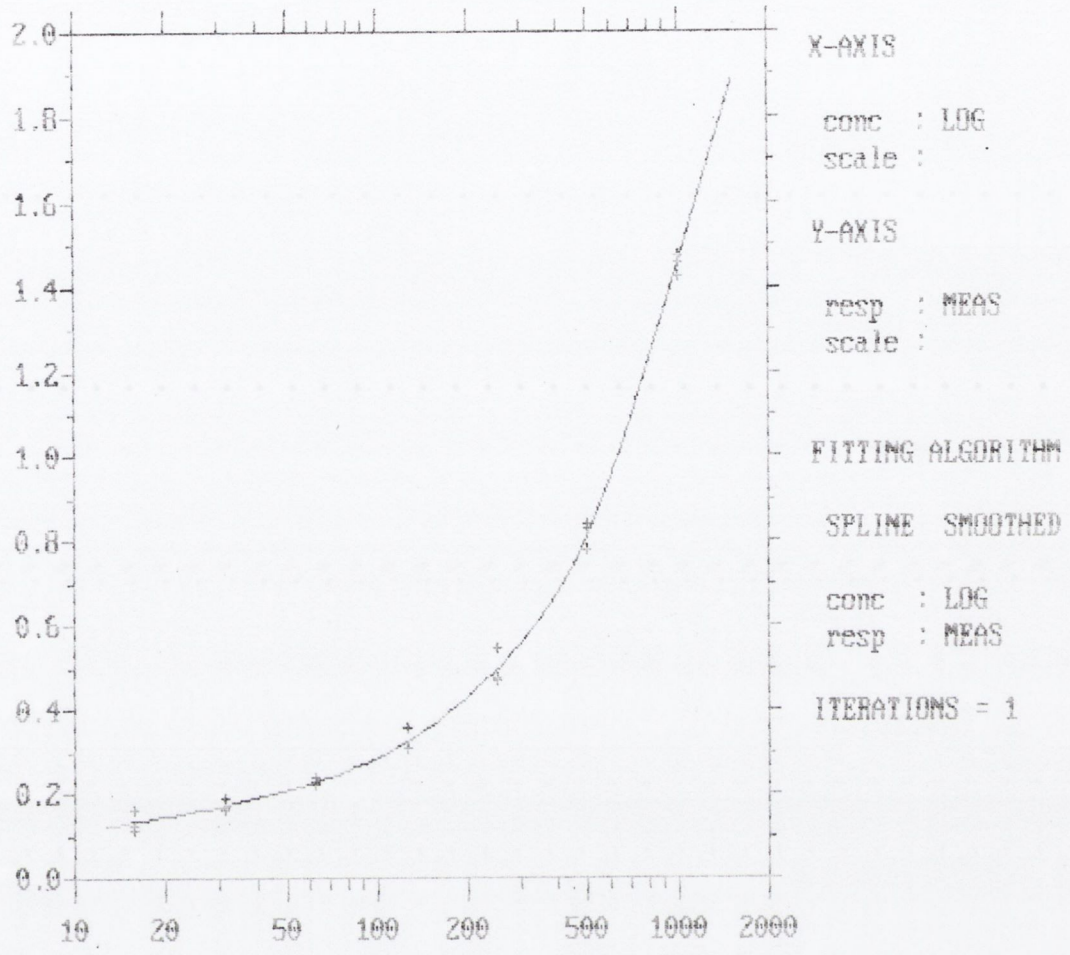


Figure 3.6 An ELISA standard curve for measuring IL-2 protein concentrations. The curve was established using dilutions of recombinant IL-2. IL-2 levels are represented on the x-axis and absorbance values on the y-axis. The absorbance values of unknown samples were read and the concentration of IL-2 in these samples determined from the standard curve.

3.3 RESULTS

3.3.1 Gliadin induced cytokine production in coeliac tissue

A significant increase in IFN-γ mRNA production ($p=0.0077$) was observed in coeliac tissue following 4h culture with PT gliadin (Figure 3.7(A)). Marked increases in the cytokine were observed in four of the nine patients (patients 1, 2, 7 and 8) and a less marked response was observed in the remaining five patients. Although a 4h culture is sub-optimal for cytokine protein production, increased levels of IFN-γ protein were detected in the culture supernatant in four of the patients (Figure 3.7(B)). There was good agreement between protein and mRNA levels, with the four highest producers of IFNγ mRNA also secreting protein into the culture medium.

A significant increase in IL-2 mRNA ($p=0.0357$) was also observed in coeliac specimens after gliadin stimulation (Figure 3.7(C)). IL-2 mRNA levels post stimulation were on average 14 times lower than those observed for IFN-γ mRNA. The highest producers of IL-2 mRNA (patients 1, 2 and 7) also produced high levels of IFN-γ. IL-2 was found in the supernatant of only two patients (patients 1 and 7) after gliadin stimulation (Figure 3.7(D)). Moreover, it can be seen from Figure 3.7(D) that patient 1 spontaneously secreted high levels of IL-2 into the supernatant.

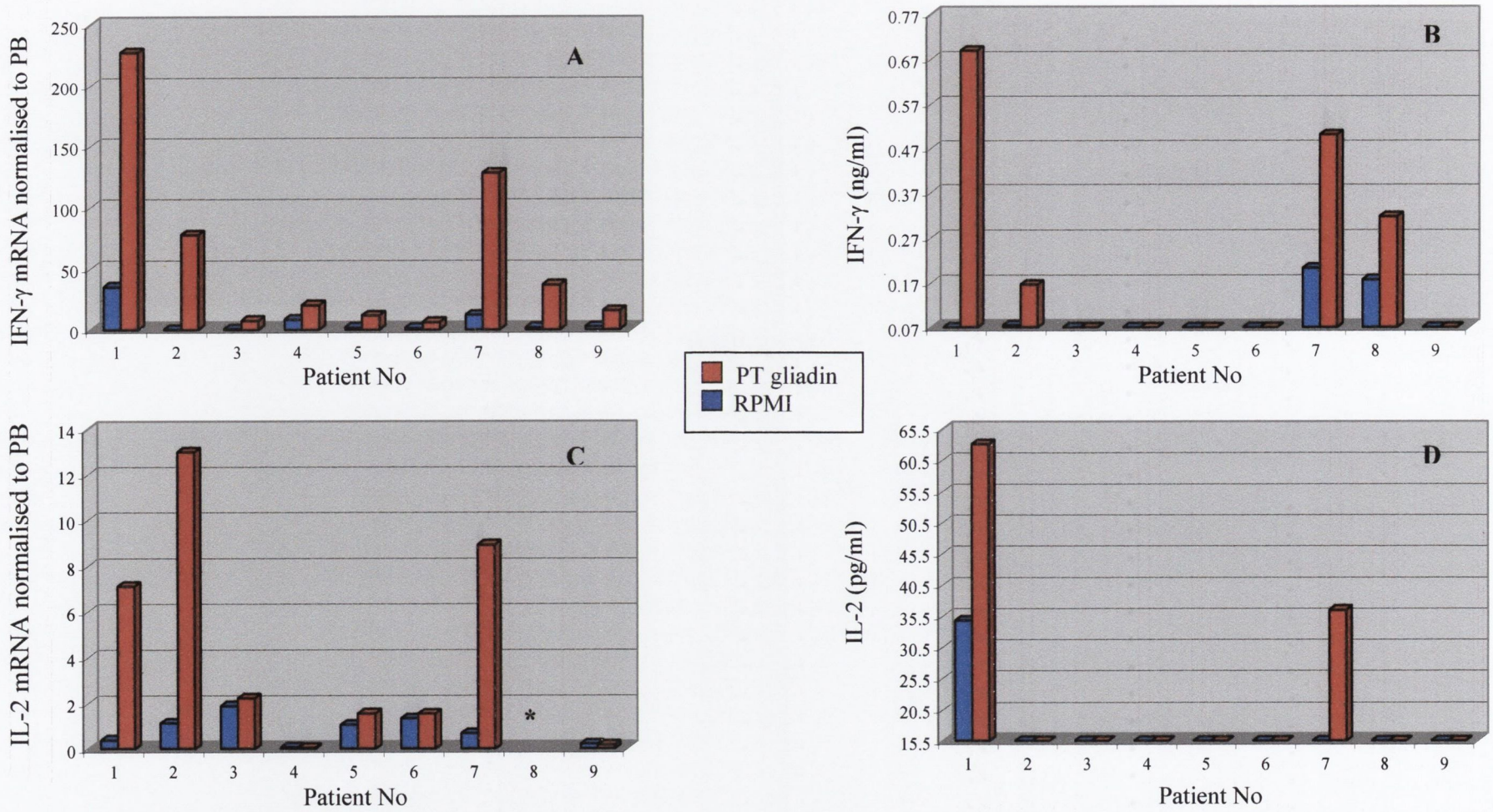


Figure 3.7 Cytokine production by duodenal biopsies from coeliac patients 1-9 cultured with PT gliadin or with RPMI alone. (A) IFN-γ mRNA normalised to peripheral blood (PB); (B) IFN-γ protein; (C) IL-2 mRNA normalised to PB and (D) IL-2 protein. * IL-2 mRNA was not determined in this patient.

3.3.2 Absence of cytokine response to avenin in coeliac tissue

Unlike PT gliadin, PT avenin did not trigger an IFN-γ mRNA ($p=0.7353$) response in coeliac tissue (Figure 3.8(A)). Non-responders to avenin included a patient with marked mucosal inflammation who was EMA positive (patient 16). IFN-γ protein was undetectable after biopsy culture with avenin (Figure 3.8(B)). Furthermore, avenin did not induce significant IL-2 mRNA production ($p=0.2076$, Figure 3.8(C)). IL-2 mRNA levels were slightly raised in three of the eight patients, but a similar response was also seen in the normal control group (Figure 3.10(C)). No IL-2 protein was detected in the medium of avenin stimulated cultures (Figure 3.8(D)).

3.3.3 Absence of cytokine response to gliadin and avenin in normal control tissue

In contrast to coeliac patients, the IFN-γ mRNA response to gliadin in normal controls was not statistically significant ($p=0.8658$). There was a slight increase in just two of eight patients following stimulation (Figure 3.9(A)). IFN-γ protein was not detected in the supernatants (Figure 3.9(B)). Similarly there was no significant IL-2 mRNA produced ($p=0.2367$) in response to PT gliadin (Figure 3.9(C)). IL-2 protein levels in the supernatants were all below quantification levels (Figure 3.9(D)). In a similar manner, avenin failed to induce a significant IFN-γ or IL-2 mRNA or protein response when cultured with normal tissue (Figure 3.10).

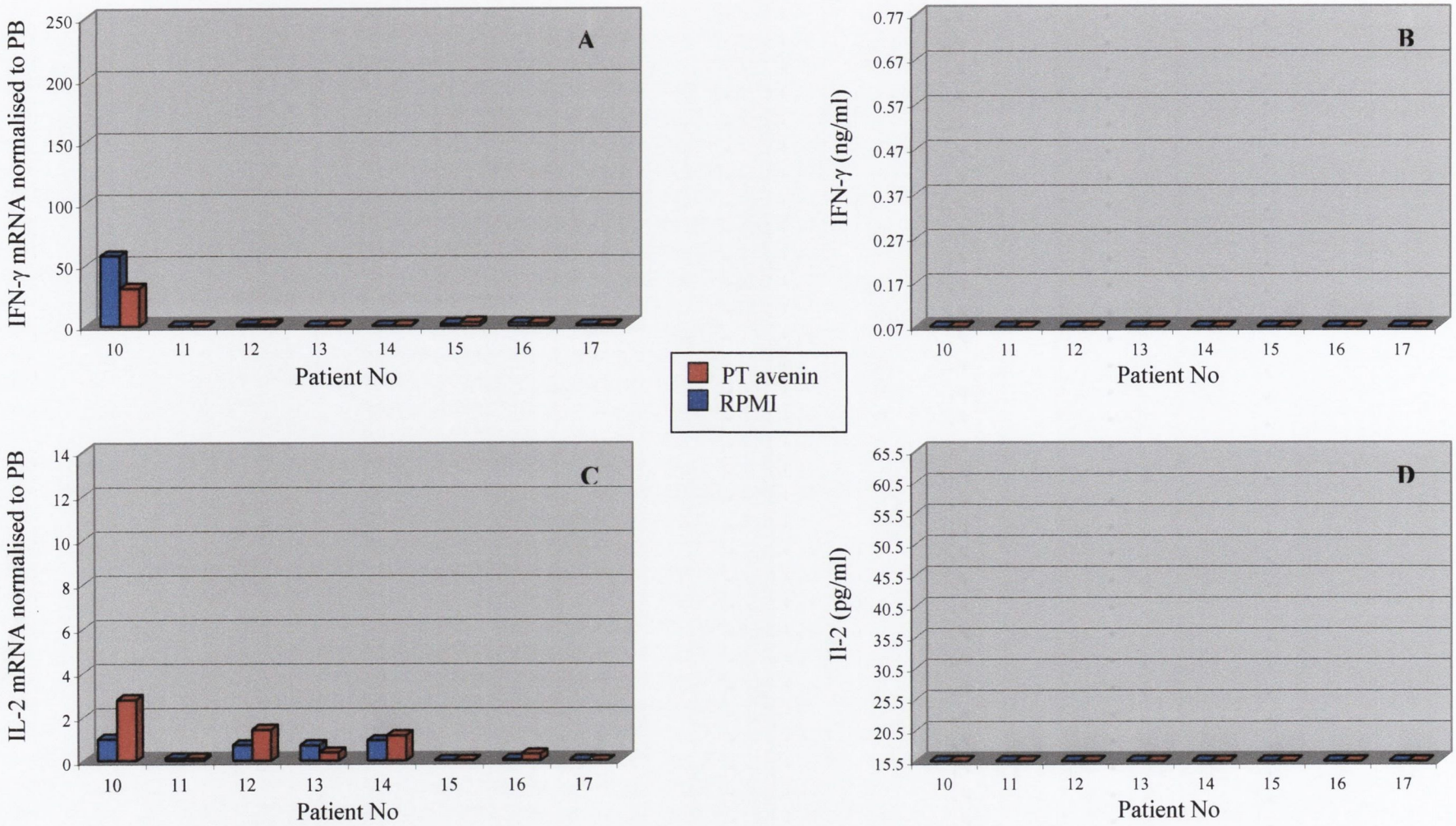


Figure 3.8 Cytokine production by duodenal biopsies from coeliac patients 10-17 cultured with PT avenin or with RPMI alone. (A) IFN-γ mRNA normalised to peripheral blood (PB); (B) IFN-γ protein; (C) IL-2 mRNA normalised to PB and (D) IL-2 protein.

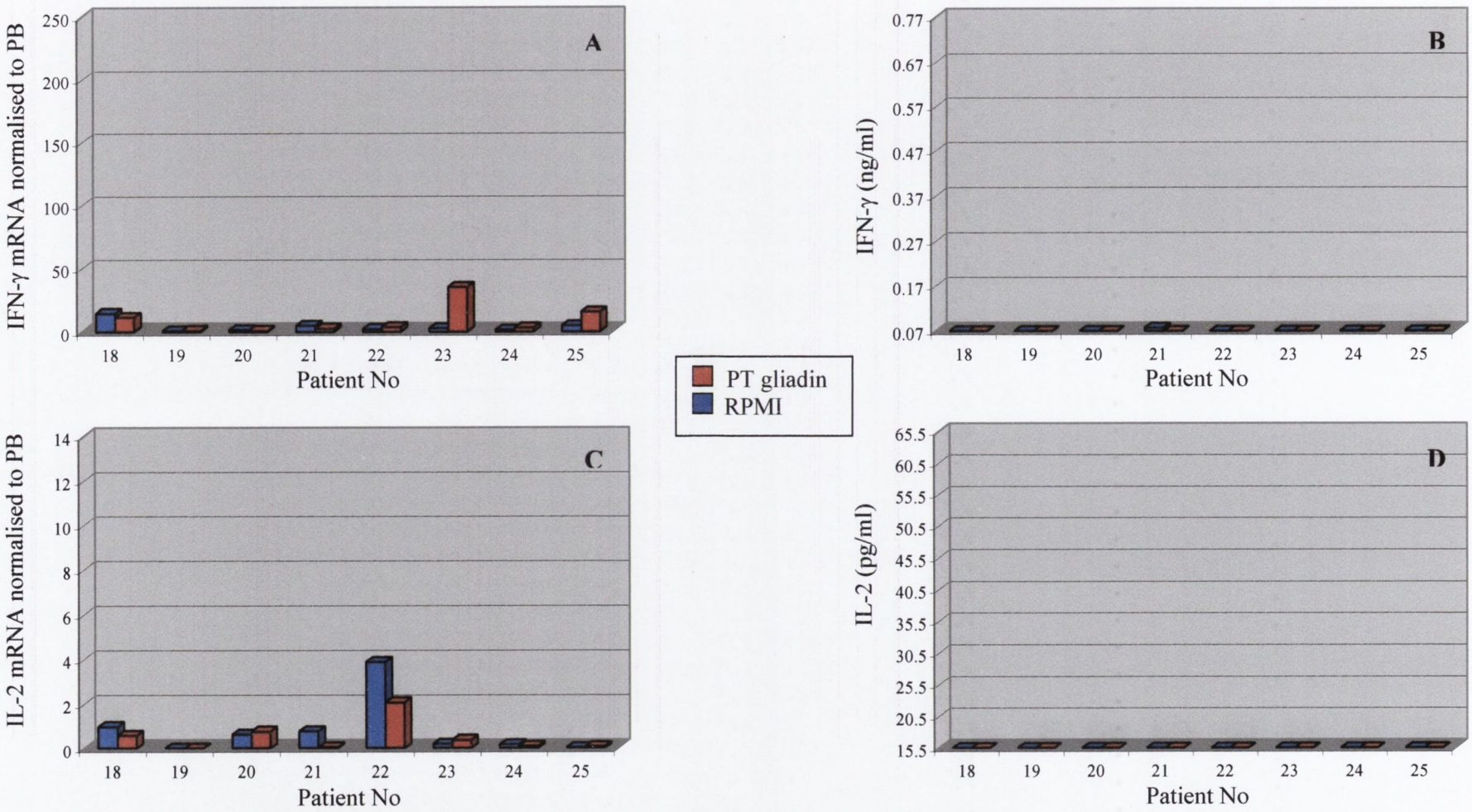


Figure 3.9 Cytokine production by duodenal biopsies from normal control patients 18-25 cultured with PT gliadin or with RPMI alone. (A) IFN-γ mRNA normalised to peripheral blood (PB); (B) IFN-γ protein; (C) IL-2 mRNA normalised to PB and (D) IL-2 protein.

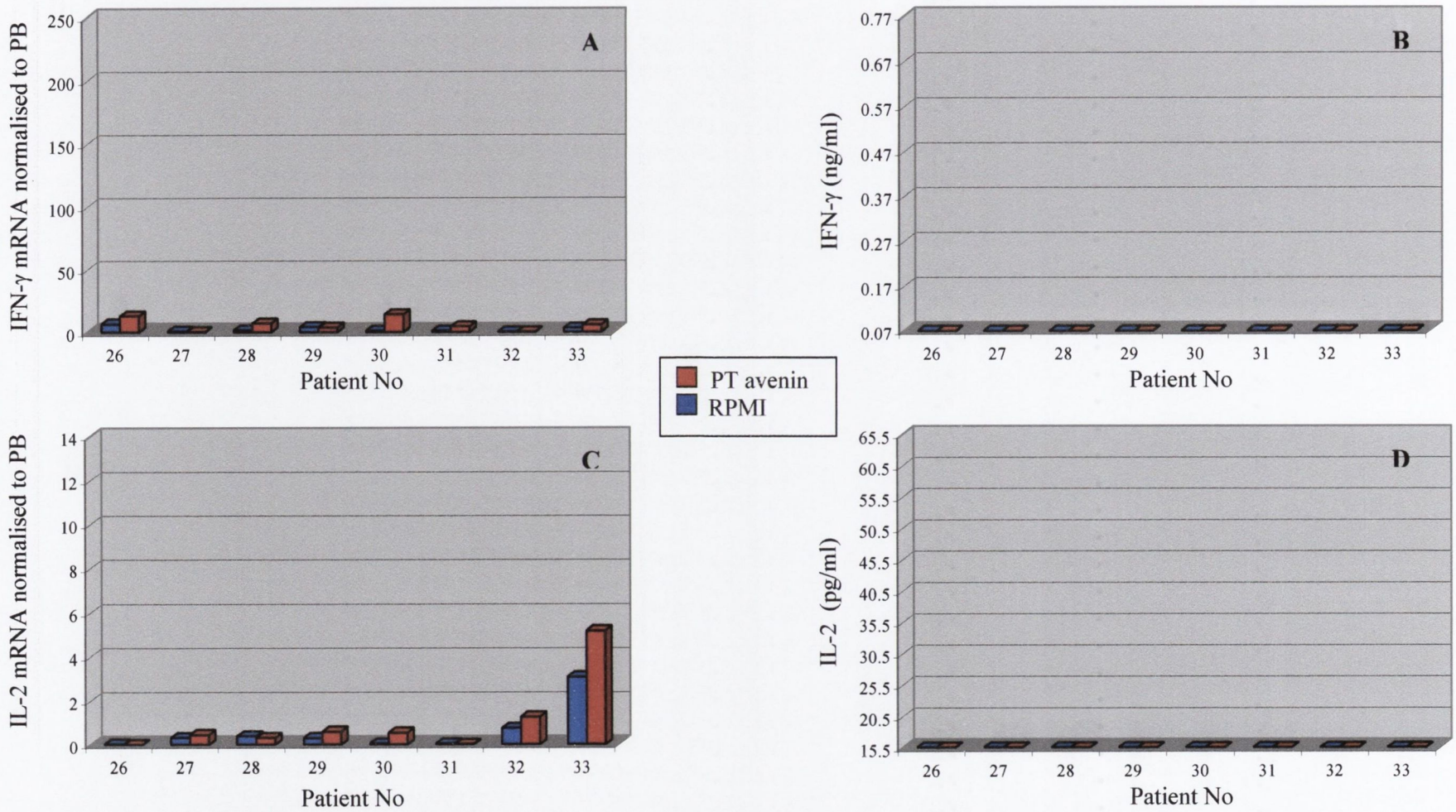


Figure 3.10 Cytokine production by duodenal biopsies from normal control patients 26-33 cultured with PT avenin or with RPMI alone.

(A) IFN-γ mRNA normalised to peripheral blood (PB); (B) IFN-γ protein; (C) IL-2 mRNA normalised to PB and (D) IL-2 protein.

3.4 DISCUSSION

3.4.1 Cytokine responses to gliadin and avenin

The purpose of this study was to determine whether avenin could cause immune activation of cultured duodenal biopsies taken from patients with coeliac disease. The parameters investigated included quantification of IFN-γ and IL-2 mRNA and also measurement of secreted cytokines in culture supernatants. Avenin failed to induce a statistically significant cytokine response in the 8 coeliac patients studied: no significant increase in the level of mRNA or protein for IFN-γ or IL-2 was observed. In contrast, gliadin addition to duodenal biopsy cultures from a further 9 treated coeliac patients caused immune activation in these subjects.

In the gliadin stimulated coeliac biopsy cultures, the production of IFN-γ was more prominent than that of IL-2. An increase in IFN-γ mRNA was observed in all nine patients with very high levels present in four subjects. Moreover, an increase in IFN-γ protein was found in these four patients. In contrast, although IL-2 mRNA was increased in six subjects after gliadin stimulation, the mean IL-2 message level was 14-fold lower than quantities of mRNA for IFN-γ. Furthermore, only low levels of IL-2 protein were present and an increase in this cytokine was found in two subjects following gliadin activation. These findings were specific for biopsies from coeliac subjects and no cytokine products were observed in the gliadin stimulated biopsy cultures from control subjects.

3.4.2 Regulation of cytokine expression

The four-hour culture period was chosen based on published cytokine mRNA kinetic experiments (Nilsen *et al.* 1998). Nilsen *et al.* found optimal mRNA production for IFN-γ and other cytokines (including IL-2, IL-4, IL-6, TNF-α) following gluten stimulation of duodenal organ cultures was between two and six hours. However, such short-term cultures are not optimal for cytokine protein production and in their study Nilsen *et al.* (1998) were unable to detect cytokines in the culture supernatant after two or six hours of gluten stimulation. The failure to detect soluble cytokines might be explained by delayed synthesis of these proteins or because secreted cytokine bound rapidly to

specific receptors. Theoretically, translational control of cytokine mRNA could also account for the lack of protein detected.

Cytokine expression may be regulated at a number of stages in its synthesis; at transcription, splicing, message turnover, translation, protein processing and protein degradation (Sullivan *et al.* 2000). In the case of IFN-γ there is evidence for transcriptional and translational control. The IFN-γ promoter region contains a 'proximal element' of 25bp, which contains putative binding sites for the transcription factors ATF-2, jun, CREB, ATF-1 and Oct-1 (Alune *et al.* 1997). An NF-AT site in the IFN-γ promoter, which is not included in the 'proximal element', has also been described (Campbell *et al.* 1996). IFN-γ transcription is negatively regulated by methylation. A target for DNA methylation was found in the proximal promoter element which, when methylated, inhibits the binding of transcription factors to this region (Agarwal and Rao 1998; Ye and Young 1997).

Recently it was reported that translation of IFN-γ mRNA is regulated by a pseudoknot in the 5' untranslated region (UTR) (Ben-Asouli *et al.* 2002). This pseudoknot activates the kinase, PKR, which in turn phosphorylates the α chain of eukaryotic initiation factor 2 (eIF2). Phosphorylation of eIF2 has a negative effect on IFN-γ protein synthesis (Ben-Asouli *et al.* 2002). In the current study, there was good agreement between IFN-γ mRNA and protein production following gliadin stimulation. The four highest producers of IFN-γ mRNA also secreted the protein. Therefore, in our system, translational regulation of IFN-γ mRNA was not apparent.

IL-2 production is regulated both transcriptionally and post-transcriptionally. The IL-2 promoter / enhancer region consists of approximately 300bp. A number of transcription factor binding sites have been identified in this region including NF-κB, Oct, NF-AT and AP-1 sites. A CD28 response element has also been identified which binds transcription factors activated during co-stimulation (Agarwal and Rao 1998; Jain *et al.* 1995). There is complex interplay between the transcription factors and occupancy of each binding site is required for optimal activation of the IL-2 promoter. The region between –600 and –300bp of the transcriptional start site may also play an important role in IL-2 gene regulation (Agarwal and Rao 1998).

T cell activation results in increased levels of IL-2 mRNA. This induction is followed by decline in IL-2 mRNA formation while transcription continues to occur (Shaw *et al.* 1988). Two methods of post-transcriptional regulation have been proposed; (1) mRNA degradation and (2) splicing of IL-2 precursor transcripts.

The decline in IL-2 synthesis after induction was initially thought to be due to an enhanced rate of IL-2 mRNA degradation (Shaw *et al.* 1988). Cycloheximide, an inhibitor of translation, causes superinduction of IL-2 mRNA. It was thought that the mechanism responsible for degrading IL-2 mRNA was sensitive to cycloheximide and in its presence IL-2 mRNA stabilised (Shaw *et al.* 1988). AUUUA motifs in the 3' UTR of a number of cytokine mRNA species contribute to their instability (Akashi *et al.* 1994). Such sequences are also found in IL-2 mRNA. However, Gerez *et al.* (1995) found that in the presence of cycloheximide little if any stabilisation of IL-2 mRNA occurs. It was also shown that that sequences other than the AUUUA motif are involved in regulating mRNA stability (Sachs 1993).

Subsequent investigations found that expression of IL-2 is regulated at the level of mRNA splicing (Gerez *et al.* 1995). During induction, the flow of precursor transcripts into mature mRNA becomes blocked and unspliced precursor transcripts accumulate. It was proposed that the shutoff was caused by a repressor protein blocking the formation of active mRNA. In the presence of cycloheximide, processing of IL-2 precursor transcripts is greatly facilitated resulting in superinduction of mRNA (Gerez *et al.* 1995; Kaempfer 1998).

In our study, IL-2 protein was not detectable in all cases where IL-2 mRNA was found. While IL-2 is regulated transcriptionally and post-transcriptionally, there is no evidence for translational control. Efrat *et al.* (1984) showed that IL-2 protein could be accounted for entirely by the amount of active IL-2 mRNA present. Therefore, the most likely explanation for an absence of IL-2 protein in our study is delayed synthesis or binding to receptors as previously mentioned.

The finding of secreted cytokines is in keeping with reports of increased tissue expression of several key immune response molecules (HLA-DR and ICAM-1) within two hours of gluten stimulated biopsy culture (Maiuri *et al.* 1996a). IFN-γ is known to

cause up-regulation of both molecules on epithelial cells (Ishii *et al.* 1994; Kaiserlian *et al.* 1991; Paolieri *et al.* 1997; Sturgess *et al.* 1992).

3.4.3 Mucosal damage and cytokine production

Some coeliac patients showed evidence of mucosal damage and two patients were EMA positive. However, spontaneous or gliadin induced IFN-γ production did not correlate with the severity of the patients' mucosal lesion or EMA status. For example, patients 1, 2, 7 and 8 produced the highest levels of IFNγ mRNA following gliadin stimulation and yet all had normal or mildly damaged mucosae and were EMA negative. In contrast, patients 4 and 6 whose biopsies showed severe villous blunting, produced little IFN-γ mRNA, even after gliadin stimulation. These results are of interest, since in earlier studies, levels of cytokine mRNA and protein were higher in patients with untreated coeliac disease (Kontakou *et al.* 1995a; Kontakou *et al.* 1994; Lahat *et al.* 1999; Maiuri *et al.* 2000; Nilsen *et al.* 1998; Przemioslo *et al.* 1994). The different findings may relate to the time point chosen for cytokine detection and the relatively small number of study subjects. Although there was a trend for more severe histological damage in biopsies cultured with gliadin, since each prolamin result was compared with spontaneous cytokine synthesis, this did not affect the validity of the results.

3.4.4 The cytokine profile in coeliac intestinal tissue

The finding of a marked IFN-γ mRNA and protein response in coeliac tissue is in keeping with several previous reports. These studies were based on investigation of fresh biopsy tissue (Kontakou *et al.* 1994), intestinal tissue after organ culture (Nilsen *et al.* 1998) and T cells cloned from duodenal biopsies (Nilsen *et al.* 1995; Przemioslo *et al.* 1995; Troncone *et al.* 1998). A study using radioactive *in situ* hybridisation found (Kontakou *et al.* 1994) an increased number of IFN-γ mRNA producing cells in the lamina propria of untreated coeliac biopsies when compared with controls. IFN-γ mRNA was not detected in IELs or enterocytes. Nilsen *et al.* (1998) measured the level of IFN-γ mRNA production by quantitative RT-PCR and reported that production was increased 1000-fold in untreated patients. Using immunohistochemistry the authors observed that the IFN-γ positive cells located to the lamina propria and not the epithelium, confirming the results of Kontakou *et al.*

In vitro gluten challenge of biopsies from treated coeliac patients increased IFN-γ mRNA to the levels of untreated patients (Nilsen *et al.* 1998). *In vivo* challenge with the gliadin peptide aa31-49 also induced IFN-γ mRNA production (Kontakou *et al.* 1995b). In addition, IFN-γ was produced by PBMCs from coeliac subjects in response to unfractionated gliadin (O'Keeffe *et al.* 1999) or gliadin peptide stimulation (Anderson *et al.* 2000).

IL-2 mRNA production in the coeliac mucosa was also determined by Kontakou's group using *in situ* hybridisation (1995a). The median number of IL-2 expressing cells was significantly increased in the lamina propria of untreated coeliac patients compared with treated patients and controls. Cytokine analysis in the epithelial compartment showed no significant differences between treated and untreated coeliac patients or controls. Nilsen *et al.* (1998) found that IL-2 mRNA could not be measured by quantitative RT-PCR in untreated or treated coeliac patients. However, when biopsies from the treated patients were *in vitro* challenged with gluten, the number of IL-2 transcripts increased.

The release of pro-inflammatory cytokines from activated T cells results in the recruitment of inflammatory cells including macrophages. It was shown by *in situ* hybridisation that the expression of TNF-α and IL-6 mRNA in the small intestinal mucosa of patients with untreated coeliac disease is significantly increased and occurs mainly in the lamina propria (Kontakou *et al.* 1995a). Few positive cells were detected in the epithelium. An immunohistochemical study found increased TNF-α and IL-6 in both the lamina propria but also in the epithelium of untreated coeliacs when compared to treated patients and normal controls (Przemioslo *et al.* 1994). Nilsen *et al.* (1998) found that TNF-α and IL-6 mRNAs were undetectable in active and treated coeliac patients. When the latter group were *in vitro* challenged with gluten increases in both cytokines were detected.

Maiuri *et al.* (2000) examined IL-15 expression by immunohistochemistry and semiquantitative PCR. IL-15 mRNA was detected in controls as well as coeliac samples and no difference was found between the two groups. By immunohistochemistry, however, they detected a significantly increased number of lamina propria cells that stained positive for IL-15 in untreated coeliac disease patients, but not in treated patients or controls (Maiuri *et al.* 2000). IL-15 production is regulated at translation, which maybe why the two results did not correlate (Bamford *et al.* 1996). Using

immunohistochemistry Maiuri *et al.* (2000) demonstrated that gliadin induced IL-15 positive cells after 3 hours of *in vitro* challenge of treated celiac disease biopsy specimens.

Thus, like many other disorders such as Crohn's disease, rheumatoid arthritis and insulin dependent diabetes mellitus (D'Elios and Del Prete 1998), the pro-inflammatory cytokine response seems dominant in coeliac disease. This is keeping with the concept that a delayed type hypersensitivity response to gluten is central to the pathogenesis of coeliac disease. The Th1 response does not appear to be driven by IL-12 (Nilsen *et al.* 1998). Increased levels of IL-18 and IFN-α, both of which promote Th1 cell differentiation, have been detected in coeliac tissue (Monteleone *et al.* 2001; Salvati *et al.* 2002). Because of the predominance of pro-inflammatory cytokines, a study was undertaken to investigate whether there was a deficiency in anti-inflammatory cytokines, IL-4 or IL-10 in coeliac patients (Beckett *et al.* 1996). Immunohistochemical staining and *in situ* hybridisation detected IL-4 and IL-10 protein and mRNA in the lamina propria of treated and untreated coeliacs and disease controls, but not in the epithelium. There were no differences in IL-4 or IL-10 between these groups with either method indicating that levels of IL-4 and IL-10 are not increased in coeliac patients to compensate for increased pro-inflammatory cytokine production (Beckett *et al.* 1996). A subsequent immunohistochemistry study found IL-4 staining in the lamina propria and epithelial layer of both coeliac patients and disease control subjects. Again no consistent difference in IL-4 staining was observed between the coeliac and disease control patients (Hansson *et al.* 2002). Nilsen *et al.* (1998) found that IL-4 and IL-10 mRNA was undetectable in untreated and treated coeliac patients. When biopsies from these patients were challenged with gluten the levels of IL-4 increased slightly whereas IL-10 did not.

3.4.5 The methodology

Organ culture of treated coeliac intestinal biopsies, in the presence of gluten or gluten fractions, is a traditional *in vitro* experimental approach used in the study of coeliac disease. Until recently, these studies relied on detailed morphometric evaluation of the tissue, examining features such as reduced enterocyte height (Fluge and Asknes 1981; Howdle *et al.* 1981a) and increased infiltration of the epithelial layer by lymphocytes (Leone *et al.* 1996). However, in these experiments sizable, properly orientated biopsies are crucial to the success of the study. With the increasing use of endoscopic forceps

rather than the Crosby capsule, to obtain biopsies, few studies now rely on morphometry to reflect tissue changes. In more recent studies, immunohistochemical staining of biopsy tissue has been employed and changes in the level of expression of molecules such as HLA-DR, ICAM-I, CD25 (Maiuri *et al.* 1996a) and various cytokines (Maiuri *et al.* 2000; Przemioslo *et al.* 1994) have been investigated. However, a level of subjectivity is inevitable in this experimental procedure. It was for this reason that in this study, objective, quantitative methods were used, employing realtime PCR for determination of mRNA and ELISA for meaurement of secreted cytokines. A disadvantage of this approach is the restriction on the number of experiments which can be performed: a total of four biopsies were required to obtain sufficient mRNA for analysis of one culture condition e.g. studying the effect of avenin. Thus, it was not possible to compare the response to two different cereal fractions in the same individual.

3.4.6 Conclusion

In earlier organ culture studies of prolamin toxicity, a concentration of 1mg/ml of protein was commonly employed and found to be sufficient to induce a mucosal response (Howdle *et al.* 1981a; Maiuri *et al.* 2000; Maiuri *et al.* 1996a; Maiuri *et al.* 1996b; Shidrawi *et al.* 1995). In the present study, even when 5mg/ml of avenin was added to the biopsy cultures, there was no evidence of cytokine production. An equivalent amount of gliadin did activate the coeliac mucosa.

Because avenin accounts for only 5-15% of the total protein in oats (whereas wheat, barley and rye prolamins constitute 40-50%), it has been suggested that larger quantities of oats may still be toxic to coeliac patients. However, the findings of this study argue against this and demonstrate that purified avenin is not immunogenic to the coeliac mucosa. These results concur with several studies, which reported that oats caused no adverse clinical effects and did not result in immune activation (Hoffenberg *et al.* 2000; Janatuinen *et al.* 2000; Srinivasan *et al.* 1996). One recent study showed that coeliac patients who consumed very large quantities of oats i.e. 93g/day for 2 years, which corresponds to 1.3-2.0g avenin/day, did not show any deterioration in small intestinal histology nor demonstrate raised levels of antibodies (Størsrud *et al.* 2003). Workers have previously shown that 0.1-0.5g gliadin/day for 4-6 weeks causes mucosal damage and positive serology in patients (Catassi *et al.* 1993; Srinivasan *et al.* 1996). Moreover,

our results are in agreement with a short clinical challenge study of two patients with DH given 2.5g of purified avenin for 5 days without adverse effect (Hardman *et al.* 1997).

In conclusion, the findings of this study give evidence that the immunogenic sequences in gliadin are not present in avenin and support the belief that oats are safe for consumption by coeliac patients.

CHAPTER 4

Coeliac T cell line Responses to the Prolamins; Gliadin, Secalin, Hordein and Avenin

4.1 INTRODUCTION

4.1.1 The generation of T cell lines

T cell lines (TCLs) may be propagated using a variety of methods. Normal T lymphocytes may be fused with a T cell tumor line to create immortalised cells or hybridomas. Alternatively, TCLs may be established by serial antigen stimulation with a specific antigen. Finally, T cells may be expanded non-specifically with a polyclonal activator (e.g. mitogen) and IL-2. The methodology behind the latter will be discussed in more detail.

Before generating a TCL using a polyclonal activator, T cells are sometimes given a primary challenge with antigen. *In vitro* culture with antigen increases the number of activated antigen specific T cells in the population (Rees 1990). Following challenge, the T cell population is expanded using e.g. PHA. This method has advantages over repeated antigen challenge as it induces rapid expansion of the TCL and also rules out the need for a constant supply of MHC matched APCs. Long-term growth of T cells depends on the constituents of the culture medium. One of the most important is the serum, which contains essential nutrients. Human serum is preferable to FCS as bovine proteins may cause cross priming of cells. IL-2 is also essential for maintaining T cells *in vitro*. T cell growth induced with recombinant IL-2 may be rapid initially but may decline with prolonged use. This can be overcome by the addition of MHC mismatched PBMCs which supply IL-2 and other growth factors (Rees 1990). Other cytokines such as IL-15 may be added to the culture medium. IL-15 has been shown to protect activated $CD4^+$ T cells from apoptosis and maintain them in a quiescent state (Dooms *et al.* 1998). 2-Mercaptoethanol (ME) in the culture medium reduces oxidants released by macrophages in the culture system, which inhibit the growth of T cells (Noelle and Lawrence 1980). Mycoplasma infection alters the growth and the functional capacity of T cells. Addition of a mycoplasma inhibitor to the culture medium protects against such infection.

The T cells are usually restimulated every 7 days with PHA, IL-2 and allogeneic PBMCs. They are diluted with medium containing IL-2 mid-week only, ensuring that cells are in a non-proliferative state at the end of the cycle. Cells must be in a resting

state before freezing, cloning and use in T cell assays. Clones may be generated by limiting dilution (Molberg 1998).

4.1.2 T cell lines and clones from coeliac patients

Methodology

The generation of gliadin specific intestinal TCLs from coeliac patients was first described in detail in 1993 (Lundin *et al.*). The methods used were similar to those described above. Duodenal biopsies were *in vitro* challenged with PT gluten for 18 hours, then digested with collagenase. $CD25^+$ cells isolated from the tissue were expanded with PHA, IL-2 and autologous PBMCs. TCLs could not be established from untreated patients and there was no T cell growth from two treated coeliac biopsies whose histology was completely normal. However TCLs were generated from four of the six patients who were on a GFD. Two of these lines showed strong proliferative responses to gluten. T cell clones (TCCs) were established from the gluten reactive lines by limiting dilution.

The same group later published slight variations to the protocol (Molberg *et al.* 1997). Following isolation, cells were cultured in the presence of autologous PBMCs, PHA and IL-2. Weekly stimulation thereafter was carried out with allogeneic PBMCs, PHA and IL-2. In contrast to the original study, biopsies from untreated coeliac patients were *in vitro* challenged with PT gliadin before cell isolation. Gliadin specific TCLs could be established from untreated patients using this method. Challenge probably increased the number of gliadin reactive T cells in the active coeliac lesions. TCLs could be established from all of the treated coeliac biopsies, which had some degree of villous atrophy or raised IELs. The TCLs from the control individuals were expandable, but none of them were gliadin–specific (Molberg *et al.* 1997).

Subsequent studies showed that isolating $CD25^+$ cells was unnecessary for the successful generation of an intestinal TCL from coeliac patients. TCLs derived from cells selected with anti-CD25 mAbs or those lines derived from non-selected cells showed no difference in reactivity to gliadin (Molberg 1998).

Variations of the original method have been employed by other groups to generate gliadin specific TCLs from coeliac patients. A Dutch group used a combination of

antigen and PHA stimulation to develop a coeliac intestinal TCL (van de Wal *et al.* 1998b). A biopsy from a coeliac patient was digested with EDTA and DTT and the cells added to autologous monocytes, which had been cultured with PT gluten. After 5-10 days IL-2 was added to the culture. Cells were restimulated every 8 days with IL-2, PHA, PT gluten and autologous monocyte enriched, irradiated PBMCs as APCs (van de Wal *et al.* 1998b).

Antigenic restimulation was the sole method used by an Italian group to create TCLs from two coeliac patients (Troncone *et al.* 1998). Biopsies were cultured with PT gliadin and digested with collagenase. The cellular suspension was incubated with autologous PBMCs and IL-2 for 1 week. The TCLs obtained were restimulated very 14 days with PT gliadin, autologous PBMCs and IL-2. TCCs were established by limiting dilution in the presence of autologous PBMCs, PT gliadin and IL-2 (Troncone *et al.* 1998).

Phenotype

The TCLs and TCCs isolated by the Norwegian group were $CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$ and $TCR\alpha\beta^+$ (Lundin *et al.* 1993; Molberg *et al.* 1997). TCLs from two untreated coeliac patients contained a small percentage of $CD8^+$ cells (Molberg *et al.* 1997). TCCs generated by gliadin restimuation were of the same phenotype: $CD3^+$, $CD4^+$, $CD8^-$ and $TCR\alpha\beta^+$ (Troncone *et al.* 1998).

HLA restriction

The gliadin specific TCLs and TCCs generated from coeliac patients were HLA-DQ restricted. Gluten or gliadin reactivity of TCLs was partially or fully blocked by an anti-DQ monoclonal antibody. Some inhibition by anti-HLA-DR was noted in some TCLs. Gliadin specific responses of the TCCs were inhibited by anti-HLA-DQ and not by mAbs against HLA class I, HLA-DR or HLA-DP (Lundin *et al.* 1993; Molberg *et al.* 1997; Troncone *et al.* 1998).

TCCs isolated from HLA-DR3,DQ2 positive patients were tested for gluten or gliadin reactivity using a panel of B-lymphocyte cell lines (B-LCLs) as APCs. When B-LCLs homozygous for the HLA-DR3/DQ2 haplotype (carrying the DQA1*0501 and DQB1*0201 genes in *cis* position) were used as APCs, strong gluten-specific responses were observed. Those APCs which carried either the DQA1*0501 or DQ*0201 alone induced a week gluten response. If the genes were carried in the *trans* position, a gluten

response was also induced (Lundin *et al.* 1993). TCCs generated from a DR4, DQ7/DR4,DQ8 patient were restricted by DQ8 (Lundin *et al.* 1994). Interestingly, TCCs isolated from a HLA-DR3/4, DQ2/8 coeliac patient could be divided into those, which were HLA-DQ2 restricted or those, which were HLA-DQ8 restricted. A gliadin peptide that stimulated proliferation in HLA-DQ8 restricted clones did not do so in HLA-DQ2 restricted clones (van de Wal *et al.* 1998b).

In contrast to intestinal TCCs, clones derived from peripheral blood are not exclusively HLA-DQ restricted; both HLA-DR and HLA-DP restricted TCCs were also found. Authors postulated that the difference in HLA restriction between intestinal and peripheral blood clones might be due to a high mucosal expression of HLA-DQ molecules on intestinal macrophages (Jensen *et al.* 1995).

Gliadin reactivity

The coeliac intestinal TCLs and TCCs recognised PT gluten and PT gliadin from various different wheat varieties (Lundin *et al.* 1993). The cells did not proliferate in response to non-cereal proteins, pepsin or trypsin. TCLs were tested with rye and were found not to proliferate. The TCCs did not recognise proteins from rye, barley and oats. This was also the case for peptides covering the NH_2 58 aa residues of α-gliadin (Lundin *et al.* 1993).

TCCs were shown to have heterogeneous reactivity patterns when tested against mixed and purified pools of gliadins (Lundin *et al.* 1997). Three crude fractions of gliadin were tested. Fraction 0 was unfractionated and consisted of ω, γ and β gliadins. Fraction 1 consisted of mainly β gliadins and fraction 2 mainly α and β gliadins. Most of the T cell clones recognised all of the fractions. Some discriminated between them. The following gliadins were purified from the crude fractions: α-39, γ-36 and γ-47. Different patterns of recognition were observed, some recognising all three gliadins, some two and some individual gliadins alone. Authors postulated that this heterogenous pattern of reactivity may reflect the many α-type, γ-type or ω-type gliadins that have been isolated from a single wheat strain (Lundin *et al.* 1997). While the amino acid sequences of these gliadins are similar they are not identical (Wieser *et al.* 1987).

In an effort to define the gliadin epitope, gliadin peptides from a PT digest of gluten were tested against a panel of DQ8 restricted TCCs. Six of 8 TCCs recognised the

gliadin peptide aa 206-216. Notably DQ2 restricted clones did not respond to this peptide (van de Wal *et al.* 1998b). Another DQ8 restricted clone from the same patient was found to react with a glutenin peptide aa 707-742 (van de Wal *et al.* 1999). The minimal T cell stimulatory core of the peptide was aa 724-734. The T cells reacted to a large number of naturally occurring variants of this peptide (van de Wal *et al.* 1999).

Gliadin is commonly digested with pepsin and trypsin to render it water-soluble. It was noted that the proliferation of TCCs was increased when gliadin was treated with pepsin followed by heating at 98°C. Under these conditions glutamine residues are converted to glutamic acid residues (Lundin *et al.* 1997). Chymotrypsin proteolysis, carried out at a neutral pH also increases water solubility of gliadin with minimum deamidation of glutamine residues. Recombinant gliadins digested with chymotrypsin poorly stimulated TCCs (Arentz-Hansen *et al.* 2000b). Therefore authors postulated that deamidation of glutamine residues is important for T cell recognition of gliadin.

Tissue transglutaminase-gliadin interaction

Tissue transglutaminase greatly enhances PT gliadin induced proliferation of DQ2 (Molberg *et al.* 1998) and DQ8 (Molberg *et al.* 1998; van de Wal *et al.* 1998a) restricted TCCs. The high glutamine content in gliadin makes it an excellent substrate for tTG, compared to proteins such as OVA, elastin and collagen, which show negligible substrate activity (Bruce *et al.* 1985). tTG can crosslink gliadin in the presence of Ca^{2+} to form high molecular weight proteins (Szabolcs *et al.* 1987), which could lead to more efficient uptake by APCs. Increased gliadin uptake was not responsible for the enhanced T cell proliferation, since PBMCs did not react to gliadin after tTG treatment (Molberg *et al.* 1998).

As mentioned previously tTG catalyses the deamidation of substrates in the absence of amine acceptors (section 1.6.1). Mass spectrometry showed that tTG can deamidate gliadin at particular glutamine residues converting them to glutamic acid (Molberg *et al.* 1998; van de Wal *et al.* 1998a). Tissue transglutaminase deamidation of gliadin is very specific, unlike deamidation achieved by acid heat treatment (Sjöström *et al.* 1998). Only glutamine residues that enhance T cell recognition are targeted. In a DQ8 restricted pepsin digested fragment of gliadin (aa 206-217) tTG deamidated glutamine residues 208 and 216. Substitution of glutamic acid for glutamine at these two positions increased T cell activation, whereas substitutions at other glutamine residues abolished

this effect (van de Wal *et al.* 1998a). Two DQ2 restricted, overlapping (yet not cross-reactive) peptides of gliadin α-9 (aa 57-68) and α-2 (aa 62-75) were both deamidated by tTG at glutamine 65. Again substitution of glutamic acid residues at various positions revealed that deamidation of residue 65 was necessary for T cell recognition (Arentz-Hansen *et al.* 2000a).

Another HLA-DQ-2 restricted γ-gliadin peptide (aa 134-153) was deamidated by tTG at glutamines 140 and 150 but predominantly at glutamine 148. The negatively charged glutamic acid at residue 148 enhanced peptide binding to HLA-DQ2 (Molberg *et al.* 1998). HLA-DQ2 and DQ8 have a preference for binding negatively charged peptides. HLA-DQ2 has four major binding sites at positions P1, P4/7, P6 and P9. The P1 and P9 pockets are quite large and accommodate a wide range of side chains. P4 and 7 have a preference for binding acidic residues (Vartdal *et al.* 1996). DQ8 shares some sequence similarity with DQ2, binding negatively charged side chains at P1, P4 and P9 (Godkin *et al.* 1997). Binding studies identified residues S145, P147, E148 and E150 as the primary anchor residues of the γ-gliadin peptide corresponding to positions P4, P6, P7 and P9 (Quarsten *et al.* 1999). The affinity of truncated and/or glutamic acid and lysine substituted peptides for DQ2 was used to characterise the binding of the α-gliadin peptides to DQ2. The deamidated glutamine at residue 65 of the α gliadin peptides (aa 57-68 and aa 62-75) binds to DQ2 in P6 and P4 pockets respectively (Arentz-Hansen *et al.* 2000a).

The studies identifying peptides, which react with TCCs have all been carried out with adult coeliac patients. An investigation was conducted to see if gliadin specific responses were similar in coeliac children. A panel of TCCs from coeliac children was generated and their responses to gliadin characterised (Vader *et al.* 2002b). The T cell response was heterogeneous, directed against a diverse set of gliadin and glutenin peptides. Responses were detected to deamidated and importantly to non-deamidated peptides. The α-2 and α-9 peptides, which were reported to be immunodominant in adult coeliac clones, only induced simulation in some of the young patient clones (Vader *et al.* 2002b).

4.1.3 Aim of this chapter

As discussed, gliadin T cell epitopes have been well defined with the use of TCLs and TCCs. However, not much is known about T cell responses to other prolamins in coeliac disease. In this study we aimed to determine whether secalin, hordein or avenin could induce T cell responses in coeliac patients. TCLs were generated from coeliac intestinal mucosa. Proliferation and cytokine responses were measured following prolamin stimulation. We also investigated whether tTG could enhance T cell recognition of secalin, hordein and avenin.

4.2 MATERIALS AND METHODS

PROTOCOL 1

4.2.1 Patients

Eight duodenal biopsies were taken from 11 coeliac patients by endoscopy and transferred to sterile RPMI-1640 (Gibco, Scotland) without antibiotics or serum. Seven of the coeliac patients were untreated with a grade 2 or 3 histology. The male:female ratio was 1:2.5 and mean age, 43 years. The remaining patients were on a GFD with a grade 0 or 1 mucosal lesion. The male:female ratio was 1:4 and mean age was 51 years.

4.2.2 Preparation of T cell culture medium

RPMI was supplemented with 15% heat inactivated human serum from clotted whole blood (Sigma, USA), 1% antibiotic/antimycotic solution (Gibco, Scotland) and 0.01M 2-ME (Sigma, USA). The solution was filtered through a 0.45μm syringe filter, aliquoted into 50ml aliquots and stored at 4°C.

4.2.3 Preparation of PBMC feeder cells

Blood was collected into 10ml heparinised evacuated tubes. It was diluted 1 in 2 with HBSS (Gibco, Scotland) supplemented with 1% antibiotic/antimycotic solution. The diluted blood was then layered onto Lymphoprep (Nycomed) and centrifuged at 400g for 30min. The mononuclear cells at the interface were removed with a sterile plastic pipette. The cells were washed twice in HBSS, resuspended in culture medium at a concentration of $1x10^6$ cells/ml and stored overnight at 4°C. On the following day PBMCs were incubated with 5μg/ml mitomycin C (Sigma, USA) for 1h at 37°C. Cells were washed 3-6 times with HBSS and resuspended in culture medium at $1x10^6$ cells/ml.

4.2.4 Antigen challenge and isolation of cells from biopsy specimens

Four biopsies were placed into two wells of a 24 well plate, containing 2ml of culture medium and 500μg/ml of PT gliadin (see sections 3.2.1 and 3.2.2 for preparation). Biopsies were incubated for 24h at 37°C in a CO_2 incubator. After antigen challenge the

biopsies were removed and placed in a 50μm medicon (Dako, Denmark) along with the culture medium containing emigrant cells. Biopsies were ground in a Medimachine (Dako, Denmark) for 1min. The cell suspension was removed with a sterile syringe. The medicon was washed twice with fresh culture medium to remove all cells. The cells were spun at 350g for 7min and resuspended in 1ml of culture medium. 50μl of the cells were plated into each well of a 96 well flat-bottomed plate. 50μl of allogeneic, mitomycin C treated PBMCs and 10 IU/ml of IL-2 (R&D, UK) were added to the biopsy cells. The plate was incubated in a CO_2 incubator for 7 days. The cells were then transferred well for well into a 24 well plate containing 1ml of mitomycin C treated autologous PBMCs at $1x10^6$ cells/ml, 10 IU/ml IL-2 and 1μg/ml PHA (Sigma, USA) in culture medium. The cells were incubated at 37°C in a CO_2 incubator for a further 7 days. 10 IU/ml IL-2 was added on days 3, 4 and 5 of the 7 day stimulation cycle.

4.2.5 Proliferation assay to determine the potency of PHA and IL-2

PBMCs were isolated from whole blood as described. $1x10^5$ cells in 100μl of RPMI (supplemented with 15% human serum (Sigma, USA) and 1% antibiotic/antimycotic solution) were added to each well of a 96 well plate. The cells were cultured in the presence of 1μg/ml PHA for 72h. Cells were also cultured for the same period of time with 3μg/ml PHA and IL-2 at 20, 10 and 5 IU/ml. Two different batches of IL-2 were tested (both R&D, UK). All tests were carried out in triplicate. The plates were incubated for 48h in a CO_2 incubator. After such time 10μl of a 30μCi solution of ^3H (Amersham, UK) was added to the wells. Cells were cultured for a further 24h before harvesting onto glass fibre filters using an automated cell harvester. The filters were then dried and placed into scintillation fluid. Thymidine incorporation in each well was measured as counts per minute (CPM) using a liquid scintillation counter.

PROTOCOL 2

4.2.6 Patients

Patient 1 was a 41 year old female coeliac patient with a grade 1 mucosal lesion. Patient 2 was a 73 year old male treated coeliac with a grade 2 histology. Patient 3 was 38 years old, female with a grade 1 mucosal lesion. Patient 4 was a 65 year old male treated

coeliac patient with a grade 2 lesion. Patient 4 was also diagnosed with enteropathy associated T cell lymphoma (EATL).

4.2.7 Preparation of culture medium

Culture medium was prepared as before with the following changes: the concentration of 2-ME was reduced to 100 μM. Also 2.5μg/ml of a mycoplasma inhibitor, Plasmocin (Invivogen, UK), was added to the medium.

4.2.8 Preparation of PBMC feeder cells

PBMCs were isolated from whole blood. Instead of treating with mitomycin C, cells were γ irradiated at 25Gy. The feeder cells were spun at 350g for 7min and resuspended in culture medium at $1x10^6$ cells/ml. 10 IU/ml IL-2 and 1 ng/ml IL-15 was added to the feeder cells before culture.

4.2.9 Antigen challenge and isolation of cells from biopsy specimens

Eight biopsies were obtained as before and transferred into 2 wells of a 6 well dish, containing 2.5ml culture medium and 1mg/ml PT gliadin (see sections 3.2.1 and 3.2.2 for preparation). Biopsies were incubated at 37°C in a CO_2 incubator for 24h. The biopsies were then torn into pieces with two sterile needles. The pieces were added along with the culture medium to a 50μm medicon. The biopsies were ground for 2 min in a Medimachine and the cell suspension removed as before. The cells were centrifuged and resuspended in 2ml of feeder cells containing cytokines. 125μl of the cell suspension was plated into each well of a 96 well round-bottomed plate. The plate was incubated at 37°C in a CO_2 incubator. On day 3 the cells were split 1 in 2 with culture medium, 10 IU/ml IL-2 and 1ng/ml IL-15. On day 7 of the culture, cells were transferred well for well to a 48 well plate containing 0.5ml of irradiated PBMCs, 10 IU/ml IL-2, 1ng/ml IL-15 and 3μg/ml PHA. Cells were split 1:3 with fresh medium containing IL-2 and IL-15 on days 3 and 4. If necessary, cells were split with culture medium alone on day 5 and 6.

4.2.10 Restimulation and expansion of T cell lines

7 days after the first stimulation with PHA, cells were removed and centrifuged at 350g for 7min. They were resuspended in fresh medium. The cells were seeded at $1x10^6$

cells/ml into a 24 well plate containing $1x10^6$ irradiated feeder cells, 10 IU/ml IL-2, 1ng/ml IL-15 and 3μg/ml PHA. Cells were split with fresh culture medium and cytokines on day 3 and 4 of the cycle. Cells were split with culture medium alone on days 5 and 6.

4.2.11 Freezing cells

Cells were removed, placed in a cool tube and centrifuged at 350g for 7 mins at 4°C. The supernatant was removed and the cells placed on ice. A solution of ice-cold RPMI supplemented with 50% FCS (Sigma, USA) was added slowly to the cell pellet. Ice-cold RPMI with 20% FCS and 20% dimethylsulphoxide (DMSO) was added dropwise at a 1:1 ratio to give a final concentration of $3x10^6$ cells/ml. The cells were immediately aliquoted to chilled cryovials and placed in a –70°C freezer for 24h. The cells were then transferred to liquid nitrogen.

4.2.12 Thawing cells

A cryovial was removed from liquid nitrogen and immersed in a 37°C water bath until the suspension fully melted. The cells were removed and transferred to a tube on ice. 8ml of ice-cold medium with 20% FCS was added dropwise and the suspension shaken gently. The cells were centrifuged and resuspended in warm culture medium.

4.2.13 Growing APCs

B cells from an Epstein-Barr virus (EBV) transformed line were used as APCs. The HLA defined VAVY cell line (European Collection of Cell Cultures) is homozygous HLA-DQA1*0501, DQB1*0201. These cells were grown in a vented $75cm^2$ tissue culture flask (Nunc, UK) in RPMI-1640 (2mM glutamine) supplemented with 10% FCS and 1% antibiotic/antimycotic solution and incubated at 37°C in a CO_2 incubator. Cells were diluted every 3-4 days in fresh medium. Before addition to the T cell proliferation assay, the B cells were subjected to 75Gy γ-irradiation, centrifuged and resuspended in T cell culture medium at a concentration of $1x10^6$ cells/ml. PBMCs from a homozygous HLA-DQ2 coeliac patient were also used as APCs in one experiment. These cells were γ-irradiated at 25Gy before addition to the proliferation assay.

4.2.14 T cell proliferation assay

Preparation of antigen

PT gliadin, PT avenin, PT secalin and PT hordein were diluted in 2mM $CaCl_2$ in PBS to concentrations of 2000µg/ml, 400µg/ml and 80µg/ml. Another set of dilutions was prepared in the same way and 150µg/ml guinea pig tTG (Sigma,USA) added to each one. The proteins were incubated at 37°C for 2h.

Incubation of antigen with APCs

75µl of APCs were plated out in a 96 well round bottomed plate ($5x10^4$ cells/well). 25µl of each antigen preparation was added to the APCs to give a final concentration of 500µg/ml, 100µg/ml and 20µg/ml for each protein. Each dilution of antigen was tested in triplicate.

Addition of T cells and measurement of proliferation by ^{3}H-Thymidine incorporation

T cells were tested on day 7 of the stimulation cycle. The T cells were centrifuged and resuspended in culture medium at $1x10^6$ cells/ml. 50µl ($5x10^4$ cells) was added to each well. 10µg/ml PHA acted as a positive control. Wells containing T cells and T cells plus APCs served as negative controls. The plate was incubated for 48h. After such time 20µl of a 50µCi/ml ^{3}H thymidine solution was added to each well. The plate was incubated for a further 24h. Cells were harvested as previously described.

4.2.15 T cell cytokine assay

1ml of T cells ($1x10^6$ cells) and irradiated APCs ($1x10^6$ cells) were cultured with 500µg/ml PT gliadin, PT avenin, PT secalin and PT hordein which had or had not been treated with 150µg/ml tTG. 3µg/ml PHA served as a positive control. T cells cultured with APCs alone or APCs and tTG acted as negative controls. Supernatants were removed after 24h and 72h, centrifuged to remove cells and stored at -20°C. IFN-γ and IL-2 ELISAs were carried out as previously described (section 3.2.10).

4.2.16 Flow cytometric analysis of the T cell lines

T cells were analysed on day 7 of the cycle. The cells were removed from the culture plate, centrifuged and resuspended at $1x10^6$ cells/ml in RPMI. Staining and flow

cytometric analysis was carried out as described in section 2.2.6 and 2.2.7. The antibodies used are listed in Table 4.1 and the flow cytometer settings in Appendix III.

Table 4.1 Antibodies used to characterise T cell lines

Antibody	Fluorescent label	Supplier
Control IgG1 / IgG2a	FITC / PE	BD, Belgium
CD3	PE	BD, Belgium
CD3	FITC	BD, Belgium
TCR-αβ	FITC	BD, Belgium
TCR-γδ	FITC	BD, Belgium
CD4	FITC	BD, Belgium
CD8	FITC	BD, Belgium
CD19	PE	BD, Belgium
CD25	FITC	BD, Belgium
CD56	PE	BD, Belgium

4.2.17 HLA determination

HLA type was determined by the Blood Transfusion Service Board using a DNA-PCR-SSO method.

4.2.18 Statistics

Proliferative responses to antigen were regarded positive if the stimulation index (SI) was above 3. SI was calculated as the mean CPM in the presence of antigen divided by the mean CPM in the absence of antigen.

4.3 RESULTS

4.3.1 T cell growth using protocol 1

We were unsuccessful in generating a TCL from duodenal tissue using protocol 1. Following two weeks culture, cells were low in number and did not appear viable under the light microscope. Staining with EB/AO confirmed that just 1-3 x 10^4 cells were viable at the end of two weeks culture. The cells were also analysed by flow cytometry at this point. The population lay in the FSC region 200-400. Cells appeared to be positive for CD3 but negative for CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$ and CD25.

It was thought that residual mitomycin C was contributing to the poor T cell viability and proliferation. Mitomycin C treated PBMCs were washed six times rather than three before addition to the cultures. However this had no effect of T cell proliferation. In two cases PBMC feeder cells were γ-irradiated rather than treated with mitomycin C. An increase in T cell proliferation was not observed. Various other changes were made to the original method; the concentration of PHA was increased from 1μg/ml to 3μg/ml, IL-2 was not added to the culture medium on days 3, 4 and 5 and cells were filtered through a 50μm filter prior to culture. These changes to the method did not increase T cell proliferation. Proliferation assays were carried out with PBMCs to test the potency of the PHA and IL-2. PBMCs cultured with 1μg/ml PHA alone had a CPM of 3,534 (SI=10). PBMCs also proliferated when cultured with PHA and 2 different batches of IL-2 (Figure 4.1). These experiments also confirmed that the human serum (Sigma, USA) used was able to sustain the growth of cells.

4.3.2 T cell growth using protocol 2

T cell expansion was achieved using protocol 2. Proliferation was not apparent during the first week of culture. One could see a reduction in the number of enterocytes as the first week progressed. In the second week there was rapid T cell proliferation. By the end of week 2, cells were 90% viable with counts of 4-5 x 10^7 cells. Cells were stained for FACS analysis at the end of the stimulation cycle. Figure 4.2 is a representation of the type of cells observed. Two populations of cells were noted: one in the FSC 200-400 region and one in the 600-900 region (Figure 4.2 (A)). Cells in the upper FSC region were $\geq$ 93% positive for CD3 and expressed CD4, CD8, TCR$\alpha\beta$ and CD25. Cells in the

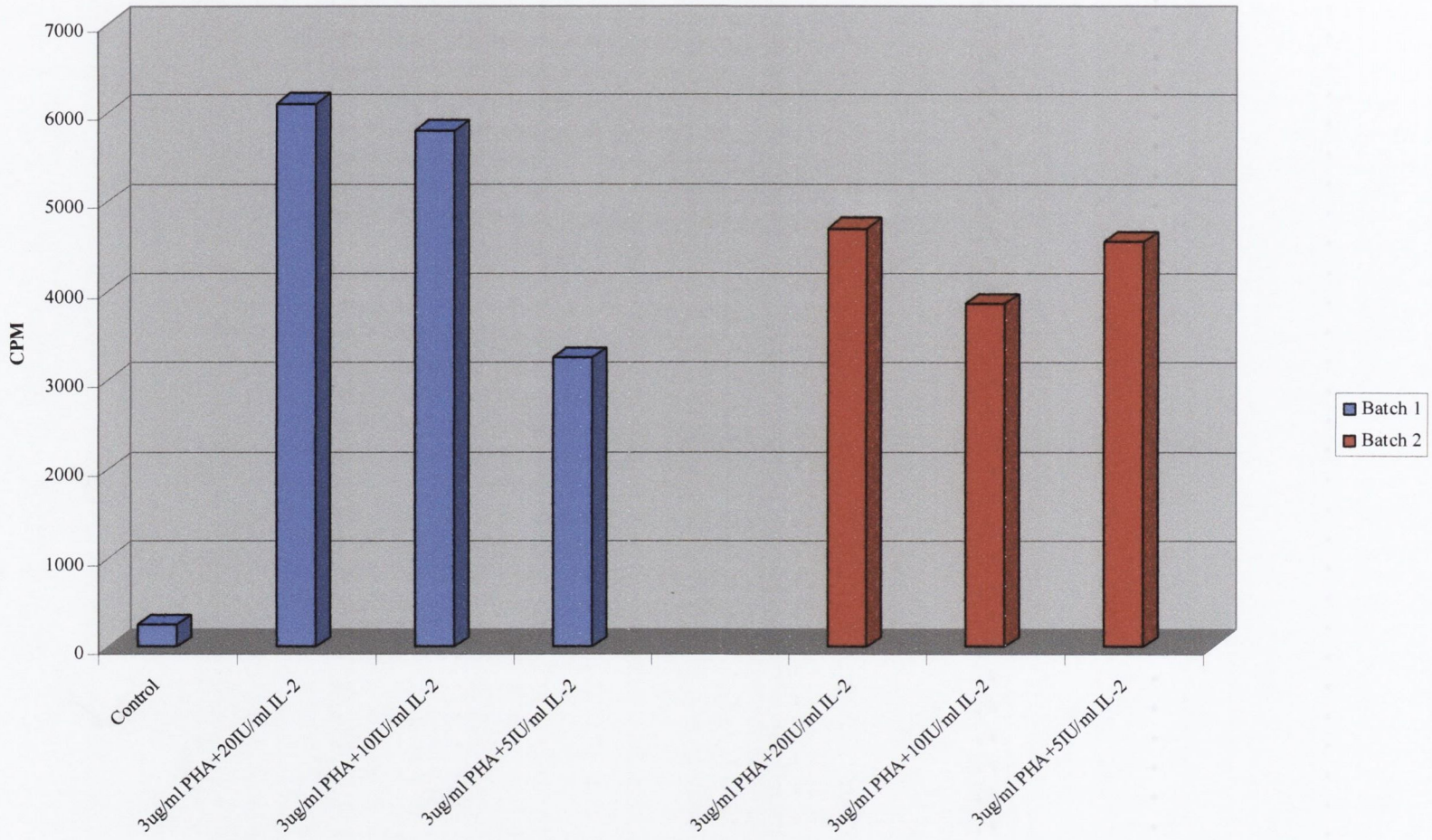


Figure 4.1 Proliferative response of PBMCs to PHA and two different batches of IL-2.

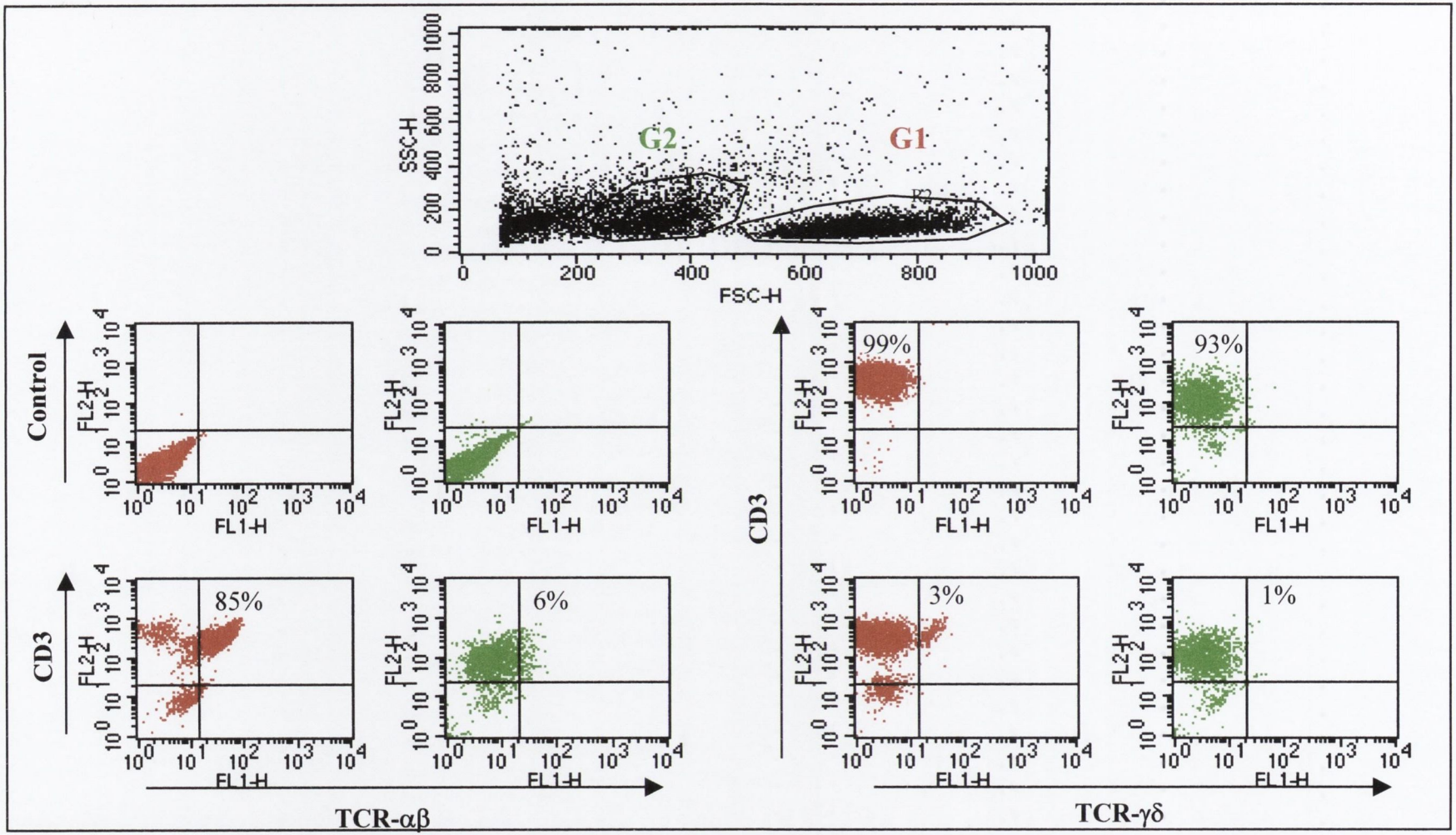


Figure 4.2 (A) FACS plots of a T cell line showing two populations of different size; gate 1 (G1) and gate 2 (G2). The phenotype of the cells in each population is also shown. G1 cells are in red and G2 cells are in green.

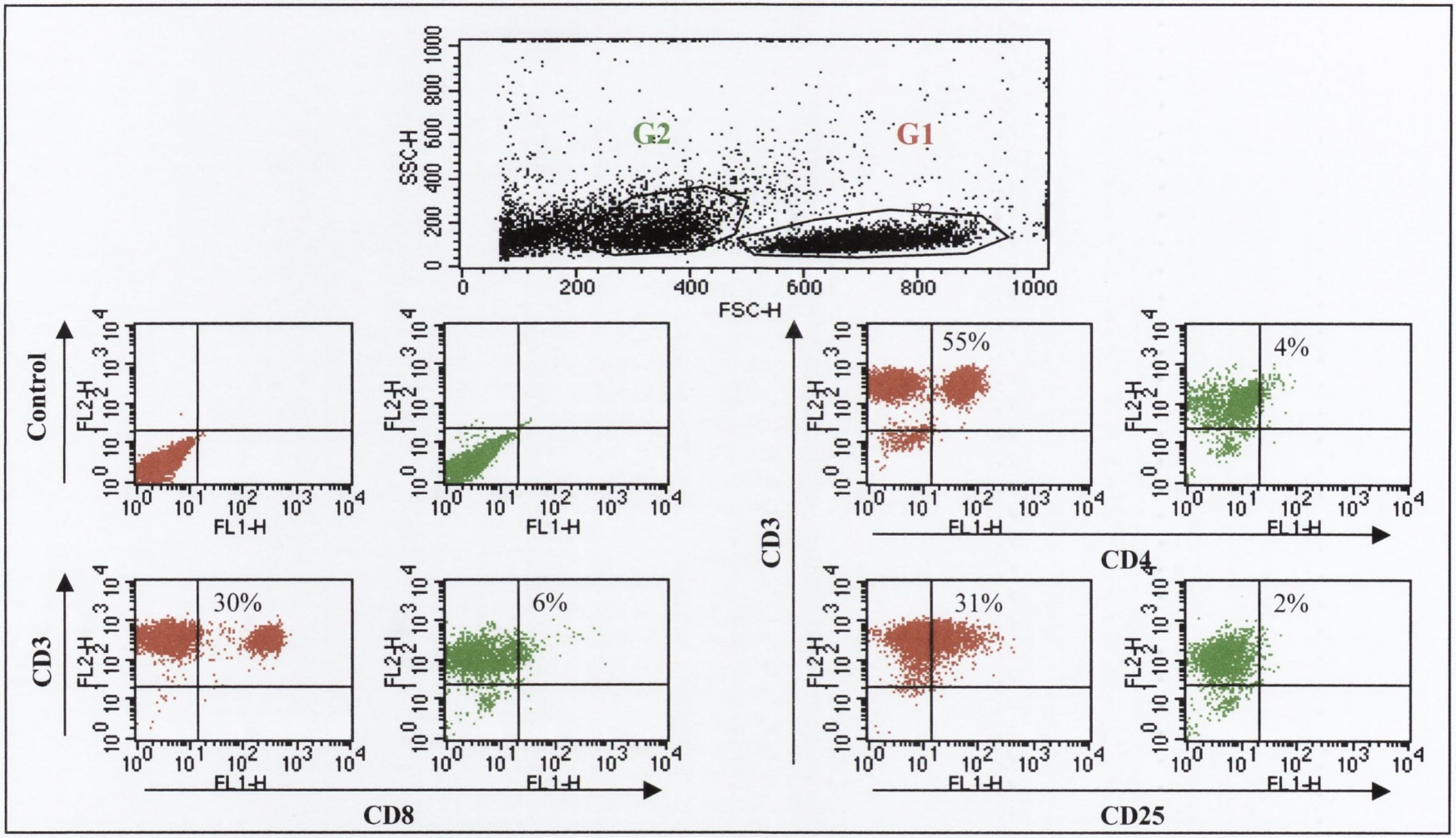


Figure 4.2 (B) FACS plots of a T cell line showing two populations of different size; gate 1 (G1) and gate 2 (G2). The phenotype of the cells in each population is also shown. G1 cells are in red and G2 cells are in green.

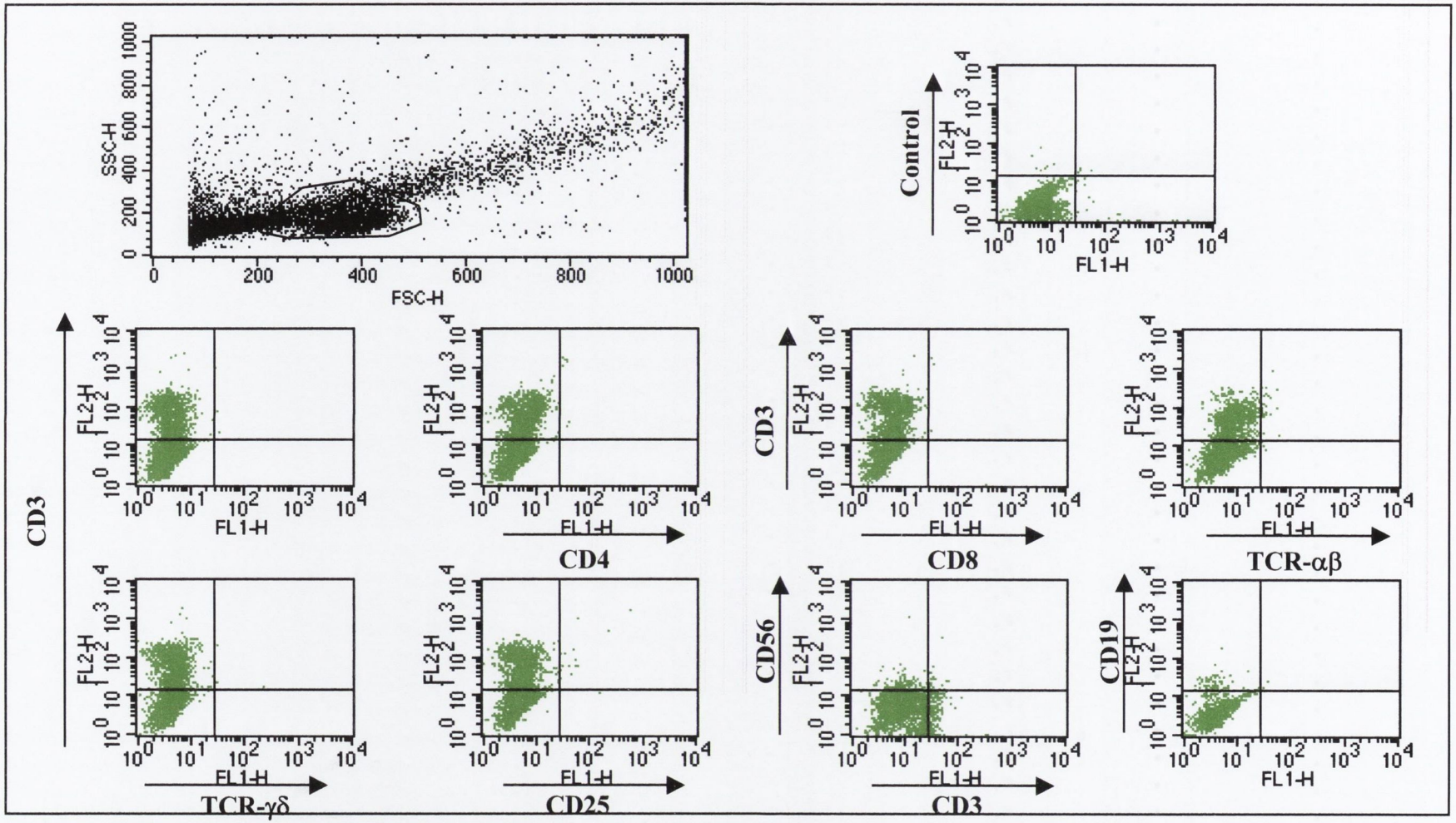


Figure 4.3 FACS plots of γ-irradiated PBMCs which were kept in culture for 7 days. The cells are CD3+ and are negative for all the other cell surface markers shown.

lower FSC region, while being $CD3^+$ were negative for all other T cell markers (Figure 4.2 (A) and (B)). This latter group was most likely apoptotic feeder cells. To confirm this, γ-irradiated PBMC feeder cells were cultured in the presence of PHA, IL-2 and IL-15 for one week. FACS analysis revealed a population of cells in the FSC 200-400 region which were $CD3^+$ but negative for other T cell markers (Figure 4.3).

4.3.3 HLA type

All four patients from whom the cell lines were derived were HLA-DQ 2 positive.

4.3.4 T cell line 1

FACS analysis

TCL 1 was analysed by flow cytometry on day 7 of the stimulation cycle. Cells in the upper FSC region were gated (Figure 4.4). 98% of these cells were $CD3^+$. The majority of this population were $CD4^+$ (74%) with some $CD8^+$ cells (20%). Cells mainly expressed the TCRαβ (78%). 1% stained positive for TCRγδ. 4% expressed both CD56 and CD3 surface markers. There were no B cells in the population. 35% expressed CD25.

Proliferation assay

A proliferation assay with TCL 1 was carried out with gliadin, using PBMCs from a homozygous DQ2 coeliac patient as APCs. In this experiment significant proliferation was induced by the highest concentration (500μg/ml) of gliadin that was both treated (SI=5) and untreated (SI=4) with tTG (Figure 4.5). Lower concentrations of gliadin did not induce significant T cell proliferation. T cells did not proliferate in the presence of tTG alone.

A second proliferation assay was performed with TCL 1 using HLA-DQ2 positive B-LCLs APCs. The proliferative response to gliadin was greater than when PBMCs were used as APCs (Figure 4.6). All four prolamins induced significant T cell proliferation. Treatment with tTG increased the stimulatory capacity of gliadin, secalin and hordein but not avenin. CPMs for the negative controls were low (APCs alone: CPM=191 and APCs and T cells: CPM=1337). The CPM following PHA stimulation was 64,807.

Cytokine assay

ELISA was used to measure IFN-γ and IL-2 secretion following T cell culture with gliadin, avenin, secalin and hordein. The supernatants were initially tested neat. Samples that registered above the standard curve were diluted by 1.5 and re-tested. All four prolamins induced IFN-γ secretion in TCL 1 (Figure 4.7 (A)). At 24h, gliadin induced the highest level of IFN-γ production. Responses to avenin and secalin were slightly lower and comparable to each other. Hordein induced the lowest level of IFN-γ production at this time point. At 72h marked levels of IFN-γ were noted in the supernatants of cells stimulated with secalin and hordein. Considerable IFN-γ was also noted in response to gliadin at this time point. Avenin induced the lowest level of IFN-γ production at 72h. tTG treatment of the prolamins did not result in an obvious increase in IFN-γ secretion. At 24h, slight increases in IFN-γ production were noted when gliadin, secalin and hordein were treated with tTG. No such increase was seen with tTG treatment of avenin. At 72h, tTG treatment increased IFN-γ secretion by hordein only. The level of IFN-γ secreted in response to APCs or tTG alone was too low to be quantified. The IFN-γ detected in response to PHA was 2ng/ml at 24h and 4ng/ml at 72h.

IL-2 protein levels following prolamin stimulation were higher at 24h than at 72h. At the later time point, IL-2 concentrations were close to background levels (Figure 4.7 (B)). At 24h the untreated prolamin, which induced the highest level of IL-2 secretion was secalin followed by gliadin and avenin. Hordein induced the lowest level of IL-2 secretion. At 24h tTG treatment increased the prolamins' capacity to induce IL-2 secretion in all cases except avenin. tTG-gliadin stimulated the highest level of IL-2 secretion followed by tTG-secalin and tTG-hordein. There was a poor IL-2 response to tTG treated avenin. Low background levels of IL-2 were detected in T cell / APC co-cultures. The IL-2 secretion in response to tTG was marginally higher than the secretion induced by APCs and T cells alone. 617pg/ml of IL-2 was secreted in response to PHA at 24h. At 72h IL-2 was undetectable in the supernatants of PHA stimulated cells.

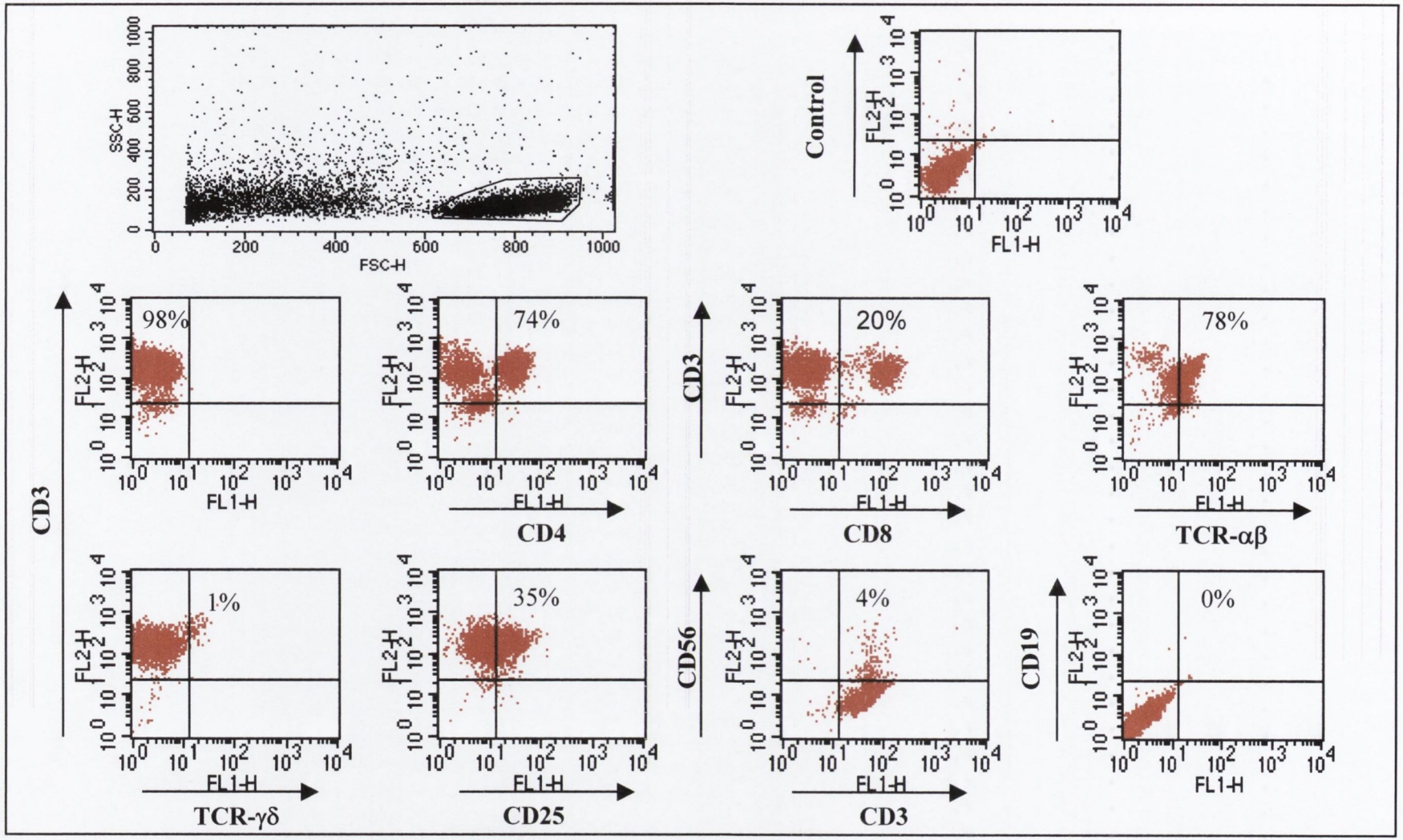


Figure 4.4 FACs plots showing the phenotype of cells in T cell line 1

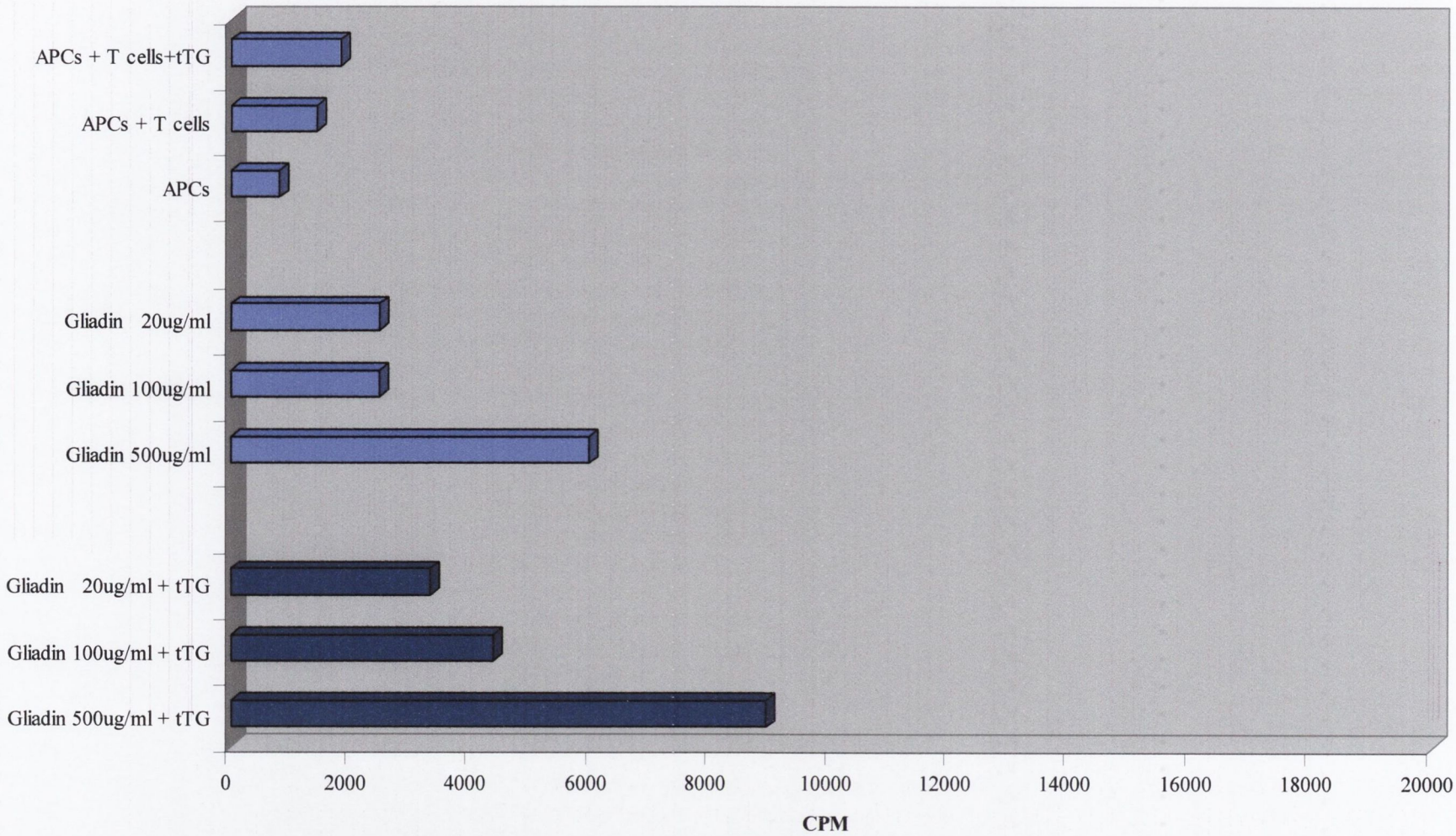


Figure 4.5 Proliferative response of T cell line 1 to gliadin when PBMCs from a DQ2 positive patient were used as APCs.

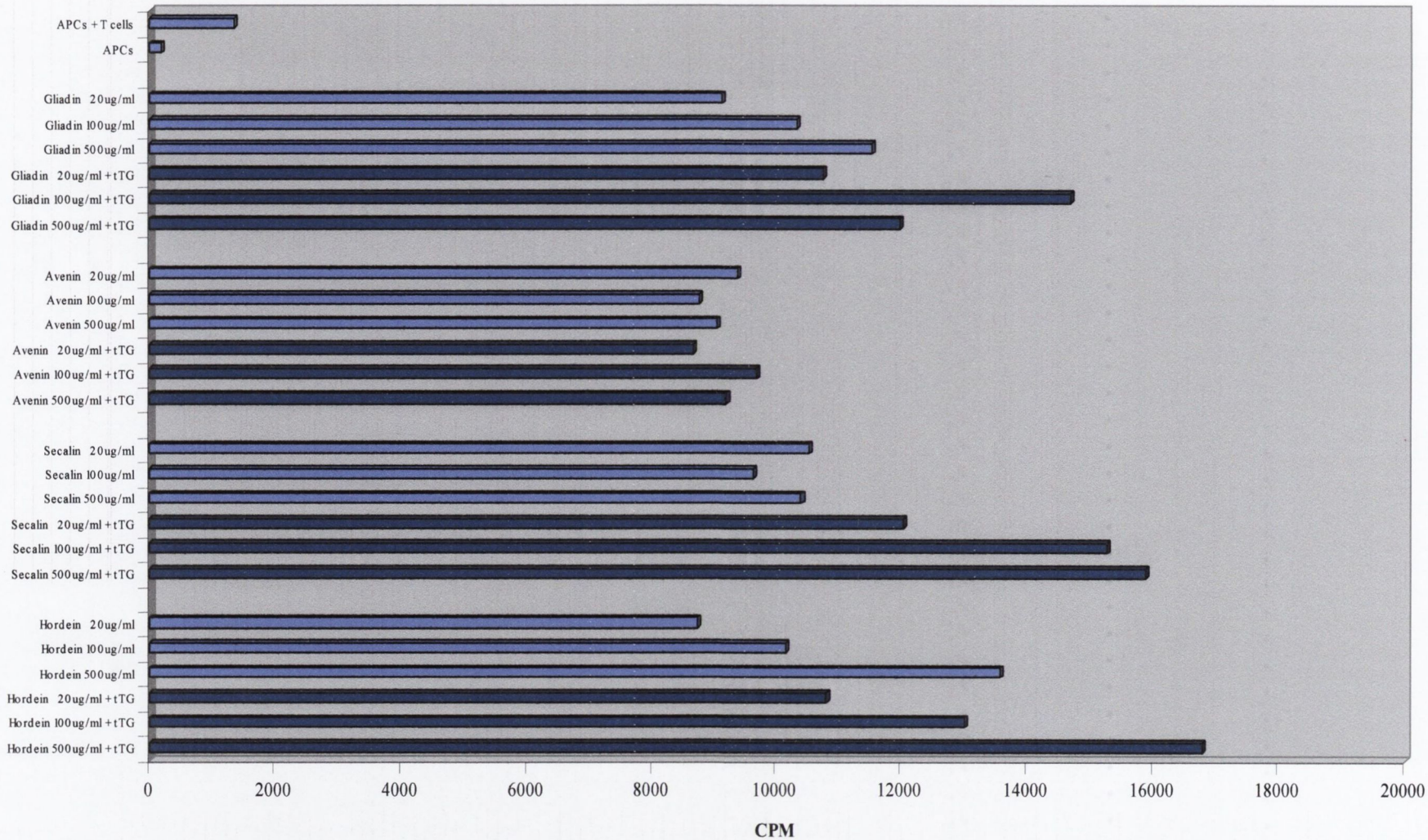


Figure 4.6 Proliferative response of T cell line 1 to prolamin proteins when DQ2 positive B cells were used as APCs.

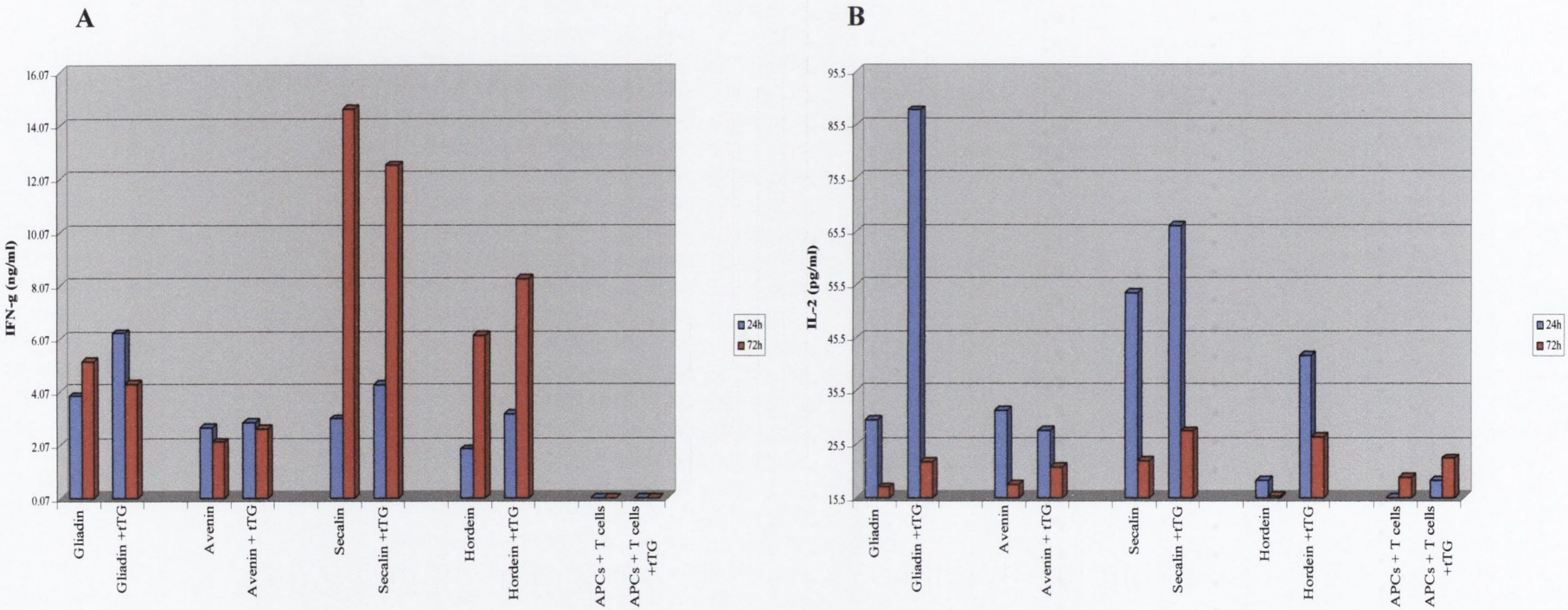


Figure 4.7 T cell line 1: IFN-γ **(A)** and IL-2 **(B)** response to prolamin proteins at 24h and 72h.

4.3.5 T cell line 2

FACS analysis

Cells were analysed by flow cytometry on day 7 of the stimulation cycle. 2 populations were noted in the upper FSC region. The first population in the 700-900 region were 98% $CD3^+$ (Figure 4.8 (A)). They were mainly $CD4^+$ (79%) with a small population of $CD8^+$ cells (15%). 85% expressed the TCRαβ and 62% CD25. 1% of the population expressed TCRγδ, 1% expressed CD56 and 1% both CD56 and CD3. The population of larger sized cells expressed roughly the same percentage of cell surface markers as the previous population (Figure 4.8 (B)). A higher percentage expressed CD25 (85%).

Proliferation assay

The proliferation assay was carried out using HLA-DQ2 positive B-LCLs as APCs. All four prolamins induced considerable proliferation of T cell line 2 (Figure 4.9). tTG clearly enhanced T cell recognition of secalin. Slight increases in proliferation were noted with tTG treatment of gliadin. tTG did not alter the T cell response to hordein or avenin. The CPM values for the negative controls were very low (APCs alone: CPM=487 and APCs and T cells: CPM=1257). The CPM value of the positive PHA control was 29,994.

Cytokine assay

TCL 2 secreted very low levels of IFN-γ in response to the cereal prolamins (Figure 4.10). The highest level of secretion was in response to gliadin and tTG-gliadin at 24h. IFN-γ secretion in response to PHA was 9ng/ml at 24h and 5ng/ml at 72h. IL-2 secretion in response to each prolamin was close to background levels with the exception of tTG-gliadin at 24h. The IL-2 response to tTG secalin at 24h was slightly higher than background levels. PHA induced a positive IL-2 response with 109pg/ml detected at 24h and 23pg/ml at 72h.

4.3.6 T cell line 3 and 4

FACs analysis

TCL 3 had a smaller percentage of $CD4^+$ (52%) cells and a higher percentage of $CD8^+$ (40%) cells compared to TCLs 1 and 2 (Figure 4.11). The cells were mainly $TCR\alpha\beta^+$ (83%) with a small percentage of cells expressing TCRγδ (2%). 20% of the population

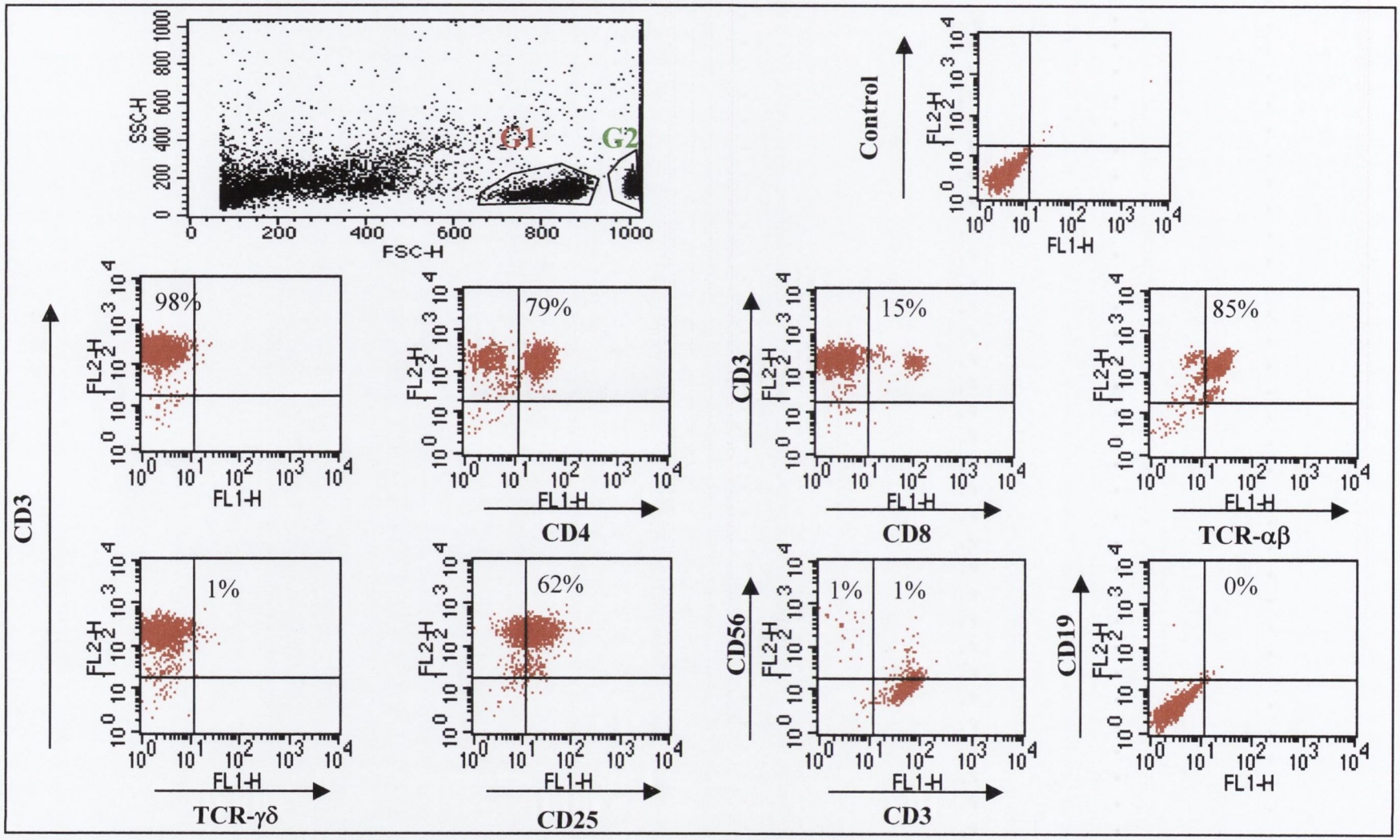


Figure 4.8 (A) FACS plots of T cell line 2. Dots plots show the phenotype of cells in gate 1 (G 1).

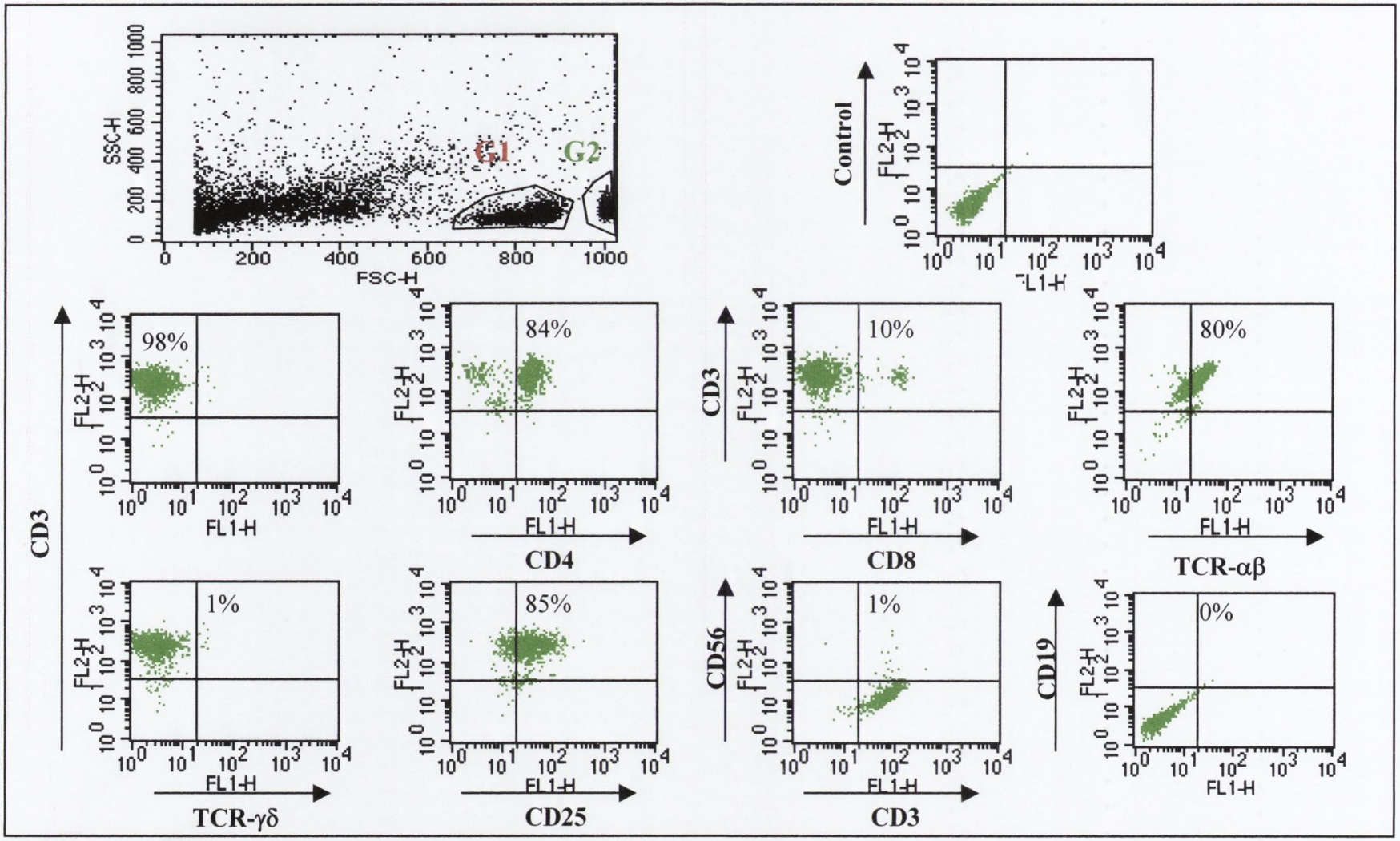


Figure 4.8 (B) FACS plots of T cell line 2. Dots plots show the phenotype of cells in gate 2 (G 2).

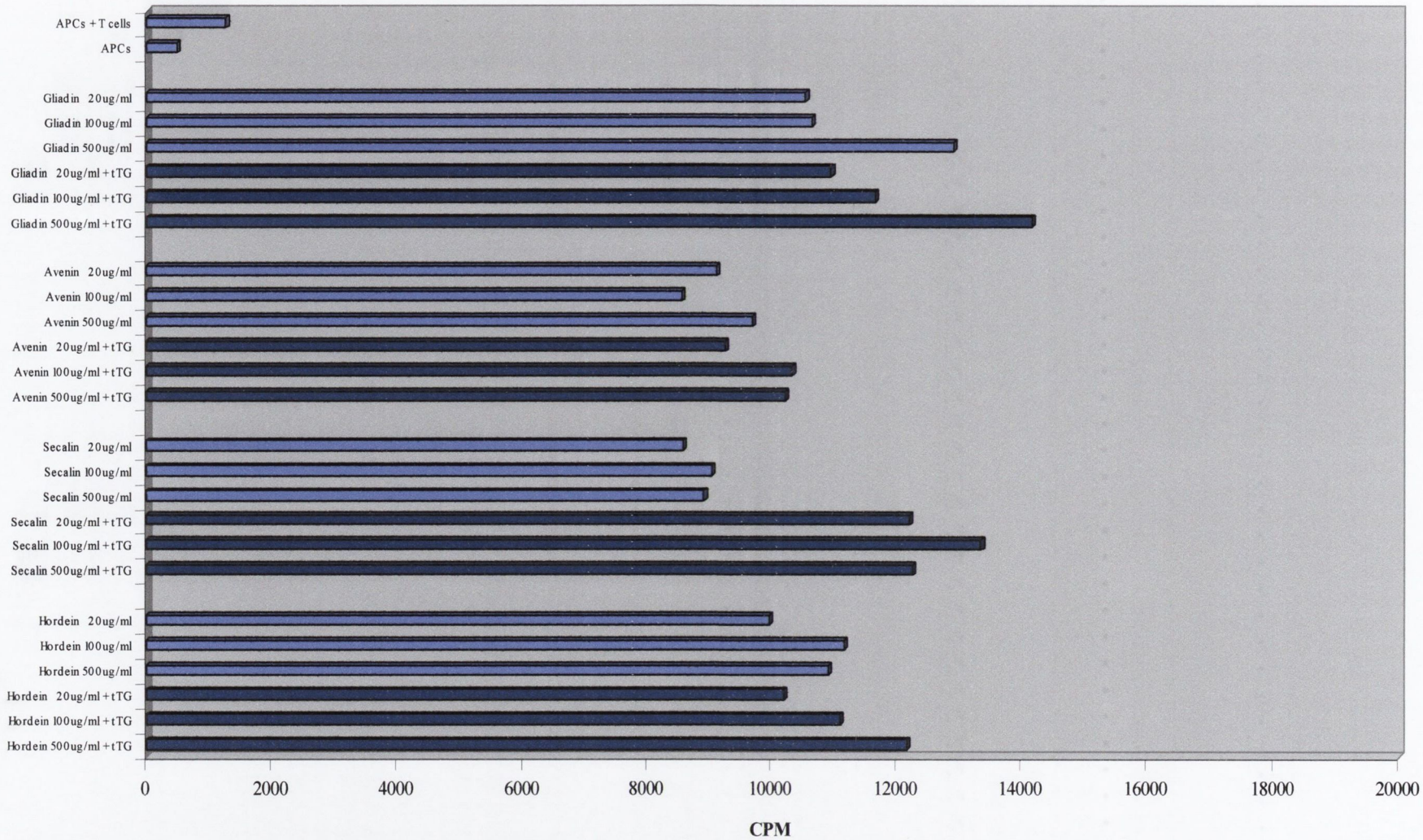


Figure 4.9 Proliferative response of T cell line 2 to prolamin proteins.

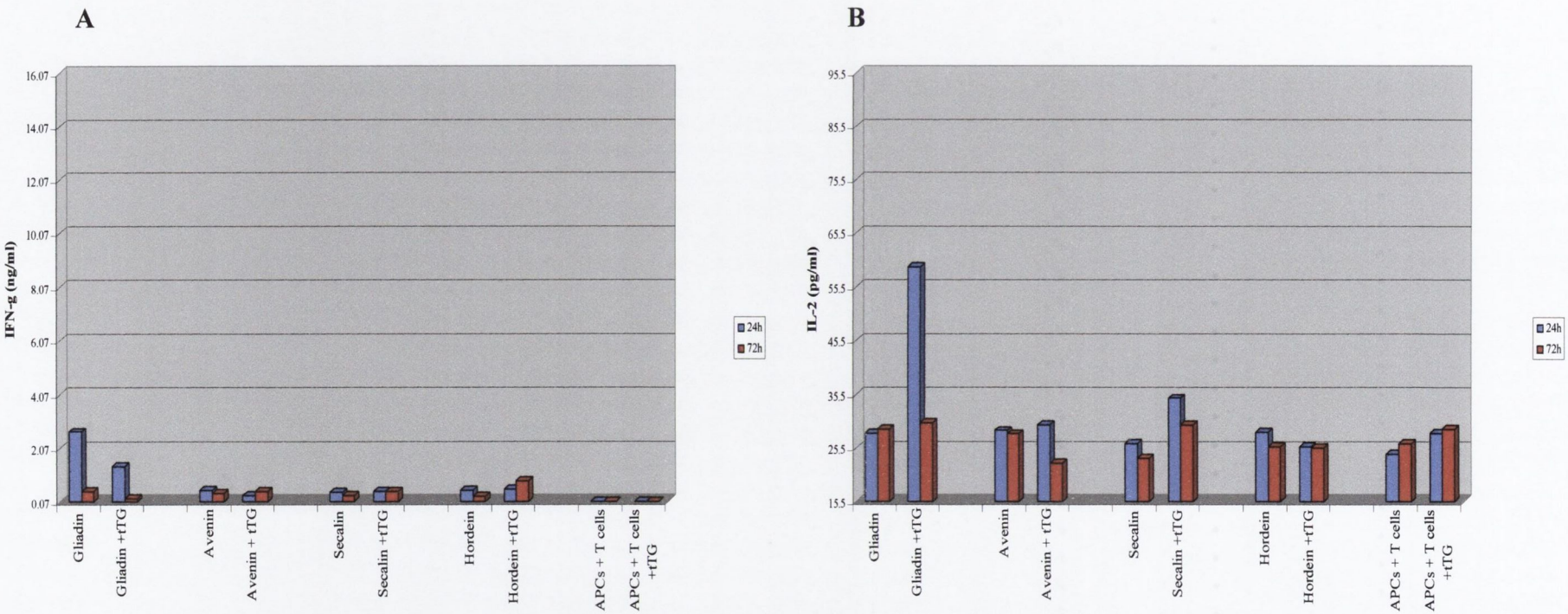


Figure 4.10 T cell line 2: IFN-γ **(A)** and IL-2 **(B)** response to prolamin proteins at 24h and 72h.

expressed CD25. 2% expressed NK T cell markers and 1% NK markers. T cell line 4 was similar to 3 with higher population of $CD8^+$ (53%) and a lower population of $CD4^+$ (54%) cells (Figure 4.14). 68% expressed TCR$\alpha\beta$ and there were no TCR$\gamma\delta^+$ cells. 18% of the T cells were positive for CD25. There were no NK, NK-T cells or B cells present.

Proliferation assay

Proliferation assays with TCL 3 and 4 were carried out using HLA-DQ2 positive B-LCLs as APCs. TCLs 3 and 4 did not proliferate in response to any of the untreated prolamins or to those prolamins treated with tTG (Figure 4.12 and 4.15). The CPM value of cells cultured with PHA was 25,642 for TCL 3 and 21, 013 for TCL 4.

Cytokine assay

TCL 3 did not secrete IFN-γ in response to any of the prolamins (Figure 4.13 (A)). There was a positive IFN-γ response to PHA: 2.1 pg/ml at 24h and 3.88 pg/ml at 72h. Gliadin and tTG treated gliadin triggered IL-2 secretion and was detected at both 24h and 72h (Figure 4.13 (B)). At 72h, IL-2 was detected in the culture medium in response to tTG hordein. The amount of IL-2 detected following cell culture with PHA was 126pg/ml at 24h and 164pg/ml at 72h. TCL 4 secreted a minute amount of IFN-γ in response to tTG-secalin (Figure 4.16 (A)). There was no IFN-γ or IL-2 response to any of the other prolamins (Figure 4.16). IFN-γ was detected in response to PHA at 24h (4.442ng/ml) and at 72h (1.1ng/ml). Similarly IL-2 was detected in response to PHA stimulation at 24h (275pg/ml) and at 72h (184.17pg/ml).

4.3.7 Proliferation assays with T cell lines established from Norwegian coeliac patients

The four prolamins were also tested on three intestinal T cell lines generated from Norwegian coeliac patients in Prof L Sollid's laboratory. Autologous EBV transformed B cells were used as APCs in these assays. Prolamins were treated with human recombinant tTG in this case and a proliferation assay carried out as before. A preparation of tTG treated PT gliadin, and chymotrypsin digested gliadin, both known to induce T cell proliferation, were used as a positive controls. All of the untreated prolamins induced proliferation in the three TCLs (Figures 4.17, 4.18 and 4.19). In lines 437.1.4 and 432.2.1 gliadin produced the highest level of proliferation of the four prolamins. tTG greatly enhanced T cell recognition of gliadin. The T cell proliferation

in response to tTG-gliadin was higher than that observed with our TCLs (note the change in scale). tTG also augmented the proliferative response to hordein in all three cell lines but not to the same extent as gliadin. Significant increases in proliferation were also noted with tTG treatment of secalin in lines 437.1.4 and lines 432.2.1, with less marked increases in line 422.1.3. The effect of tTG on avenin was less obvious. Slight increases in proliferation were noted in lines 422.1.3 and 437.1.4 following tTG treatment of avenin. tTG treatment of avenin did not enhance proliferation of TCL 432.2.1.

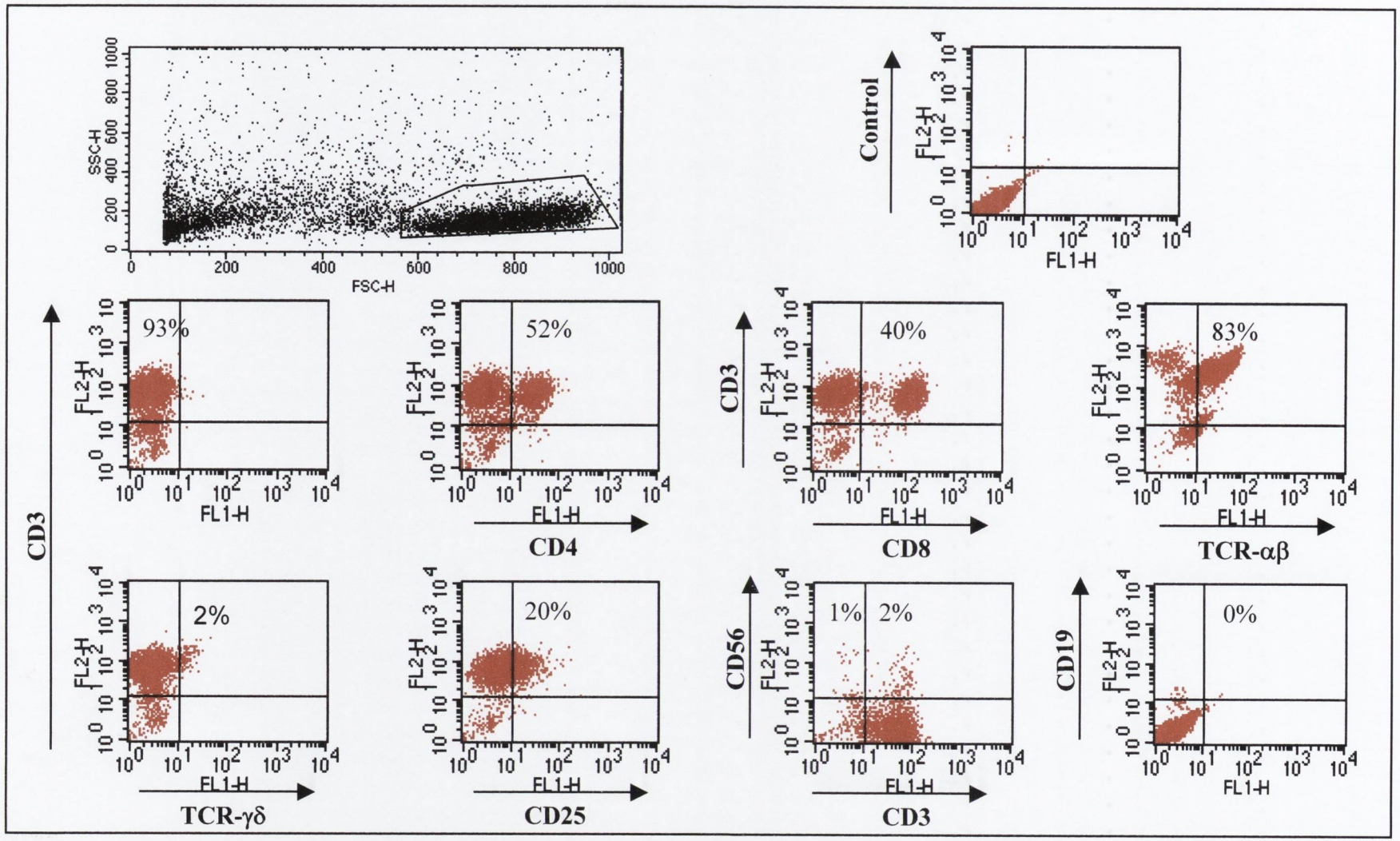


Figure 4.11 FACS plots showing the phenotype of cells in T cell line 3.

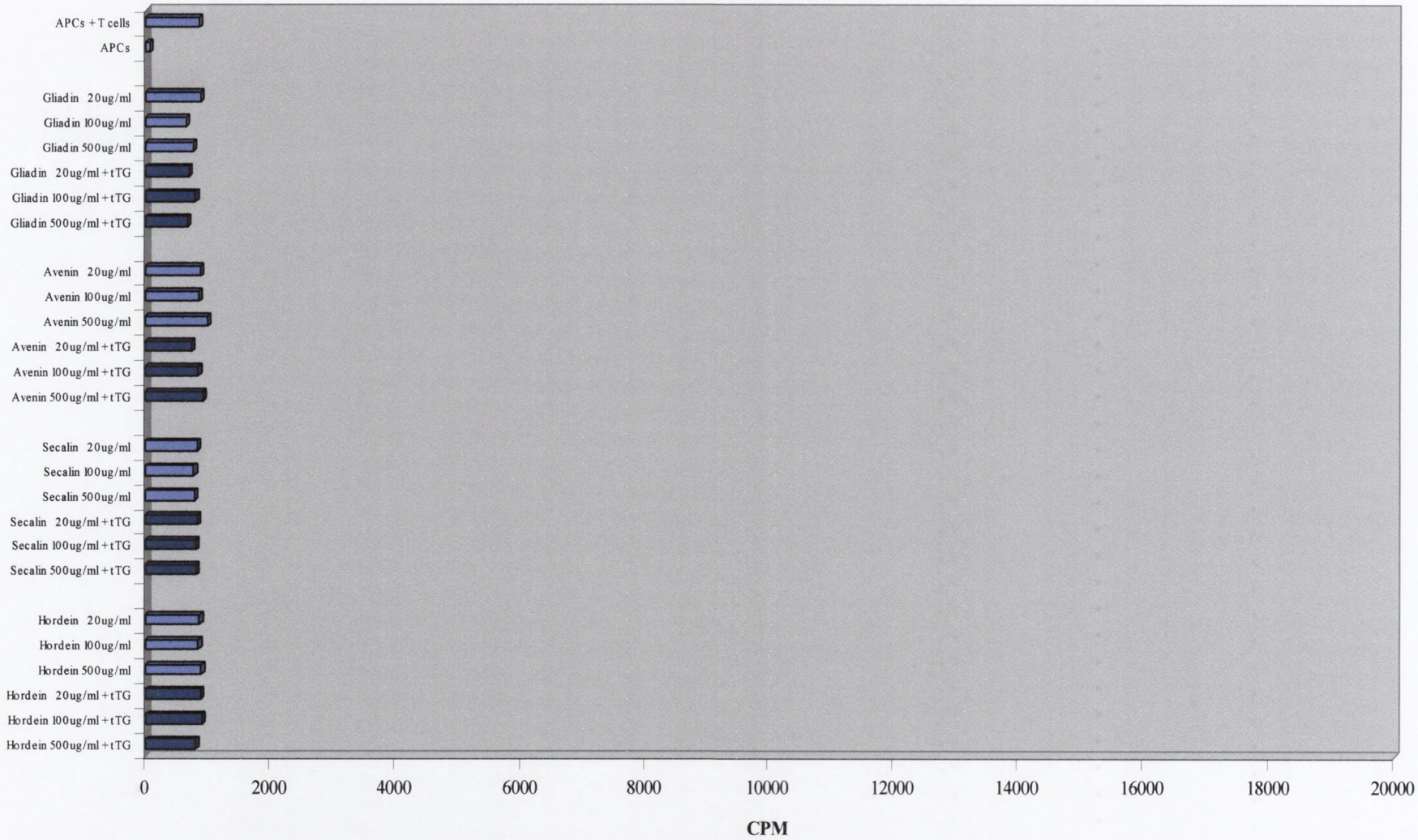


Figure 4.12 Proliferative response of T cell line 3 to prolamin proteins.

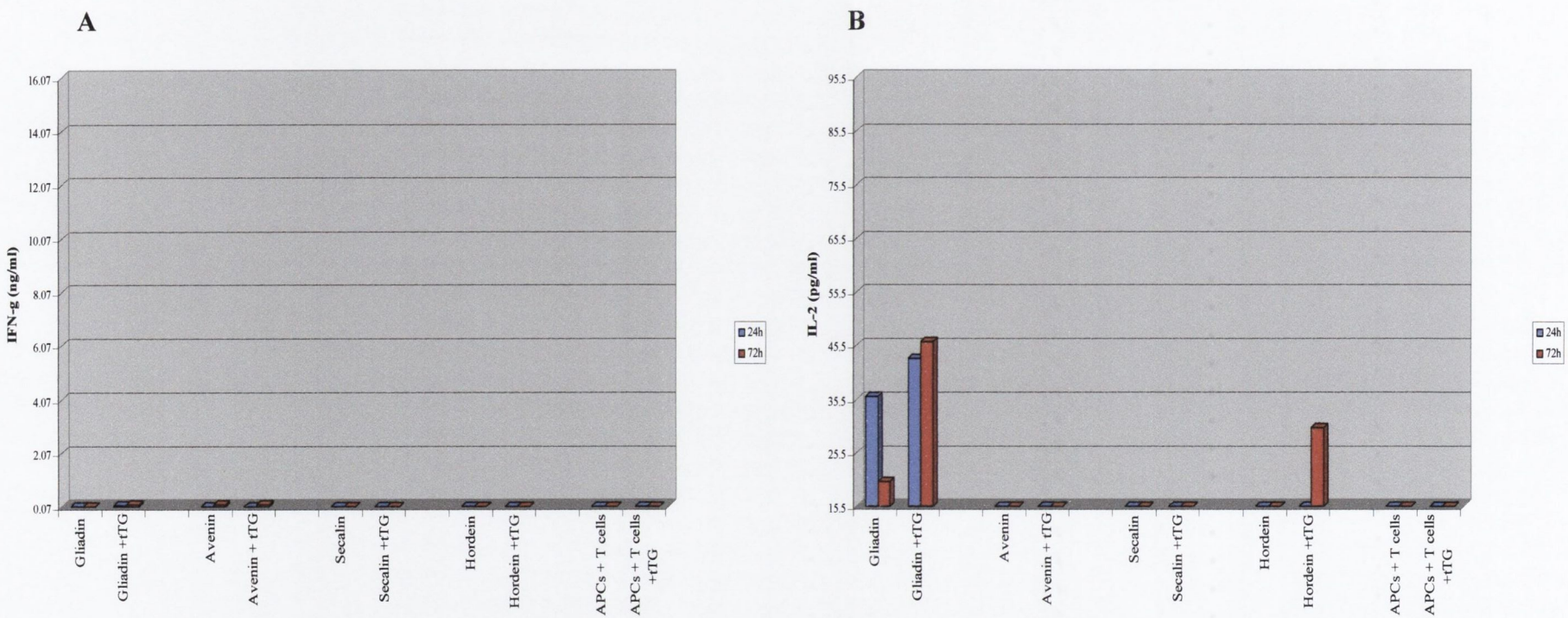


Figure 4.13 T cell line 3: IFN-γ **(A)** and IL-2 **(B)** response to prolamin proteins at 24h and 72h

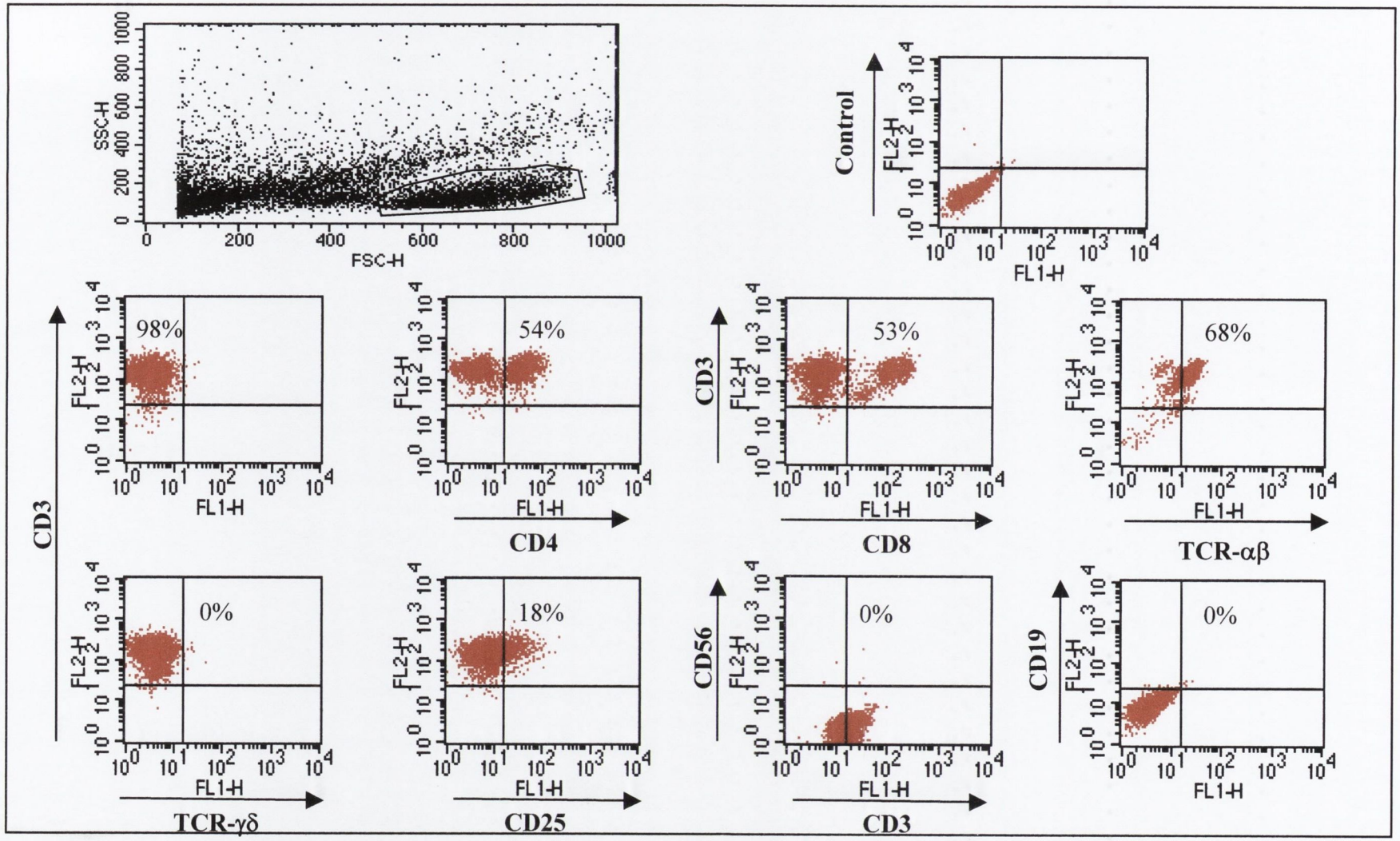


Figure 4.14 FACS plots showing the phenotype of cells in T cell line 4.

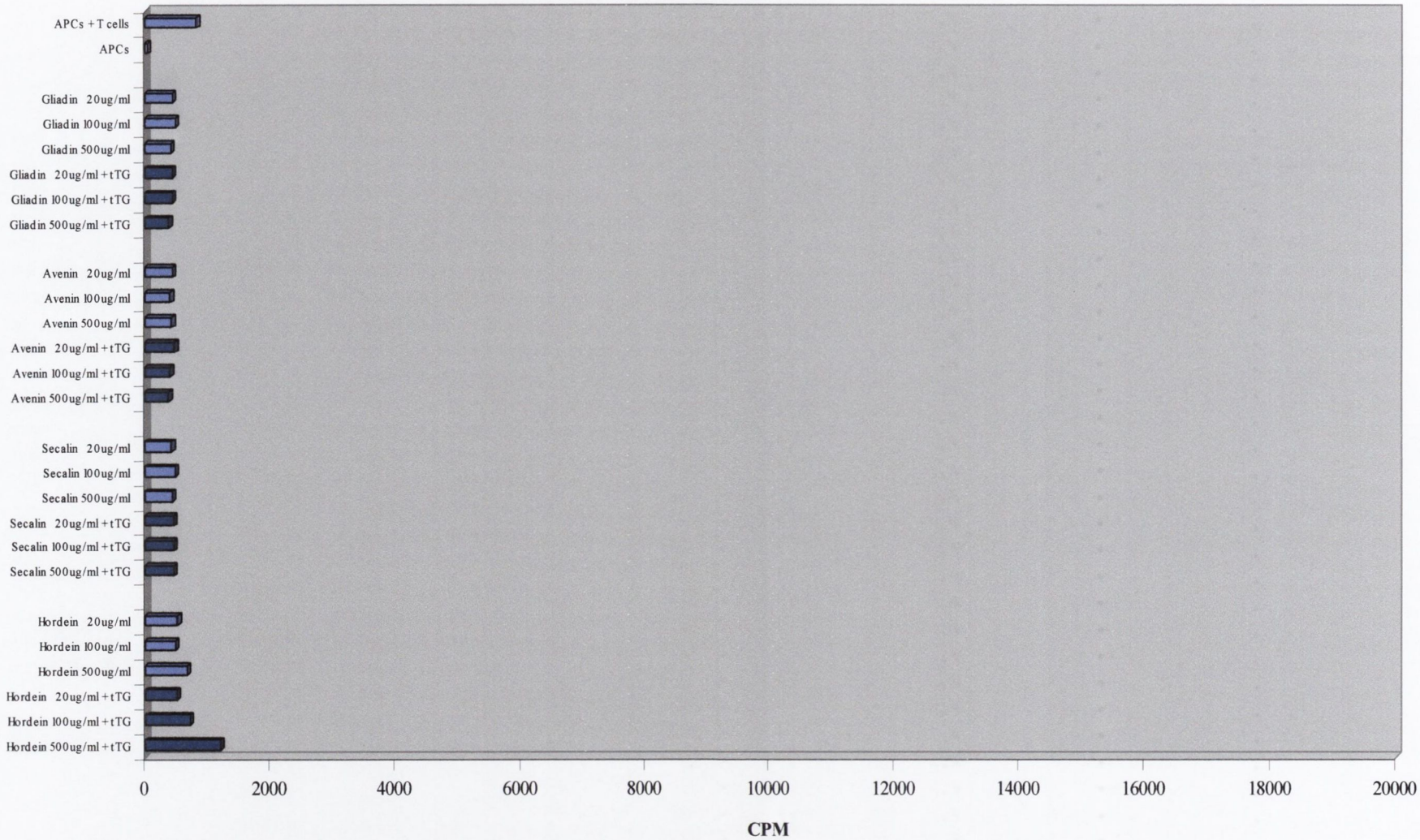


Figure 4.15 Proliferative response of T cell line 4 to prolamin proteins.

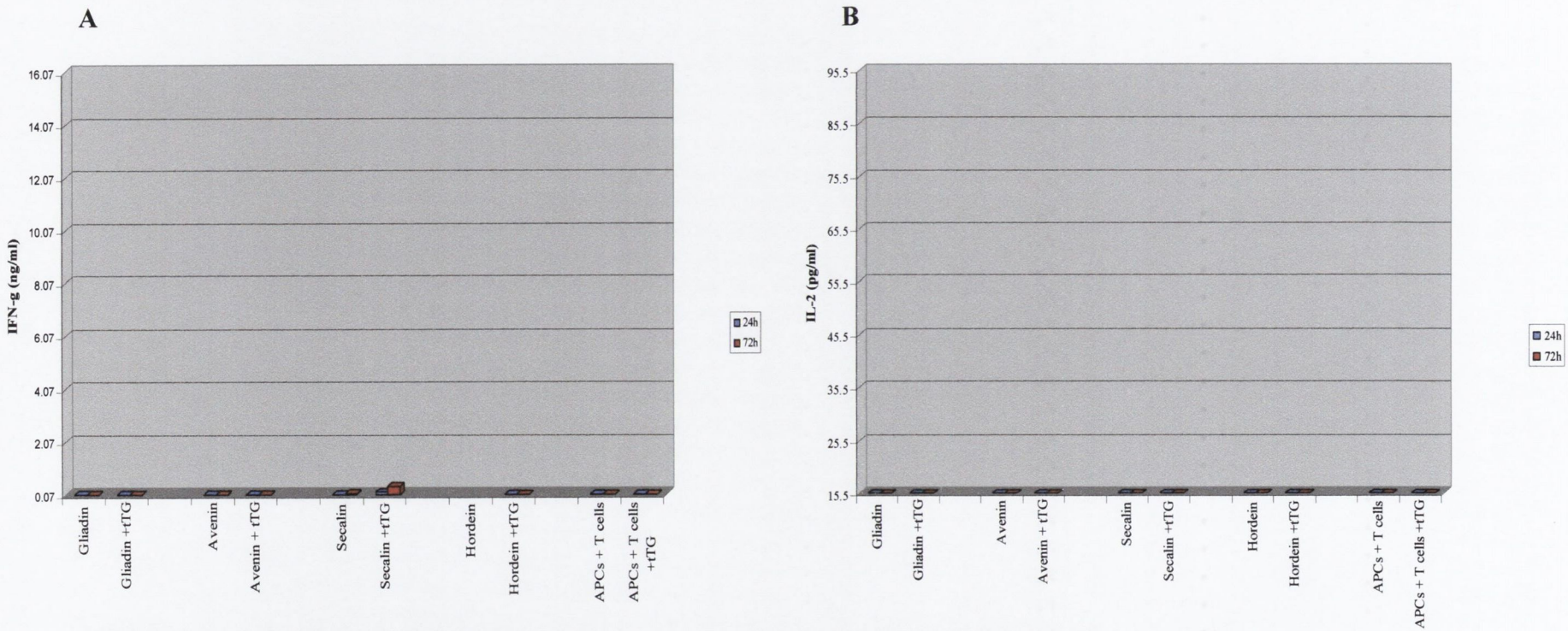


Figure 4.16 T cell line 4: IFN-γ **(A)** and IL-2 **(B)** response to prolamin proteins at 24h and 72h.

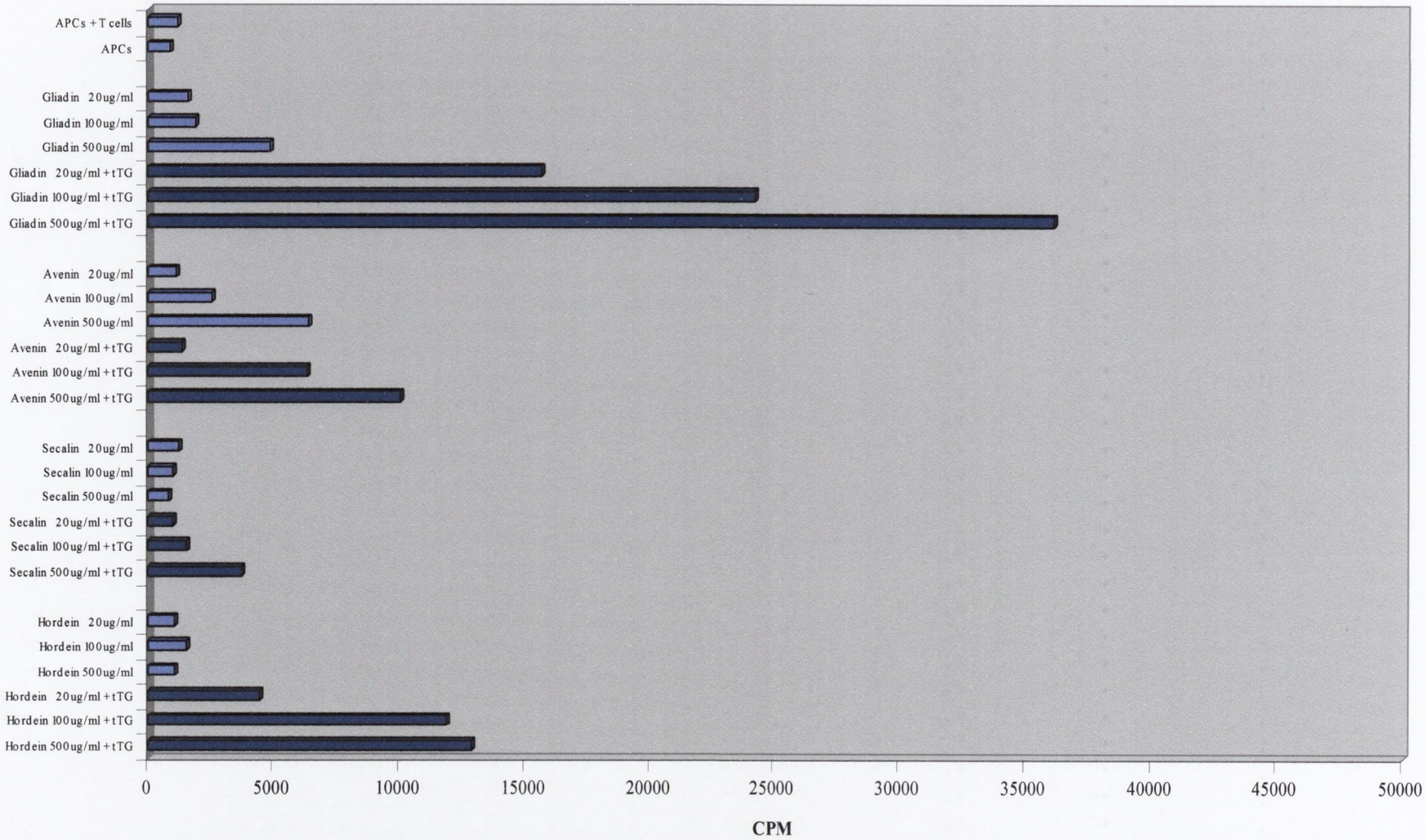


Figure 4.17 Proliferative response of T cell line 422.1.3 to prolamin proteins

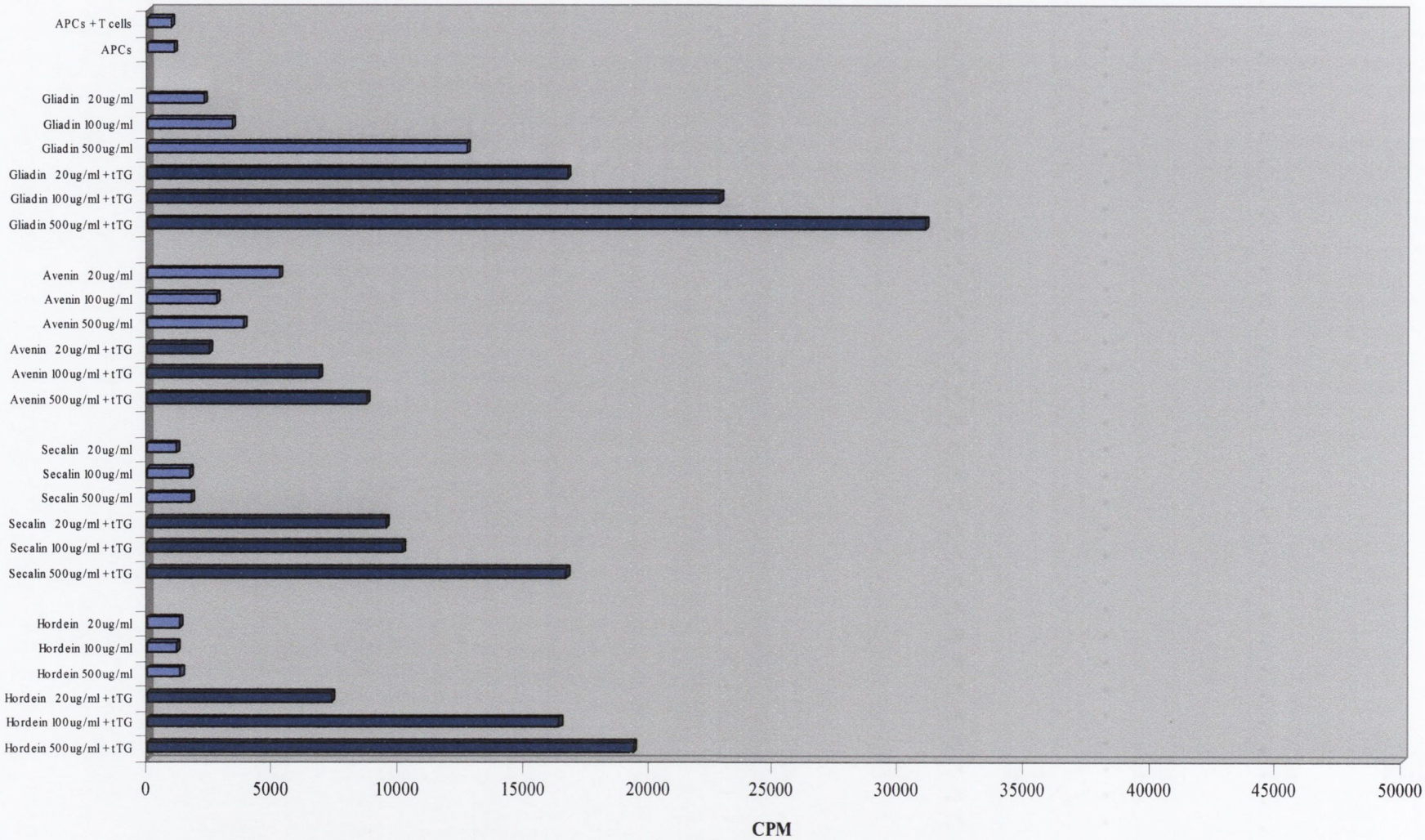


Figure 4.18 Proliferative response of T cell line 437.1.4 to prolamin proteins

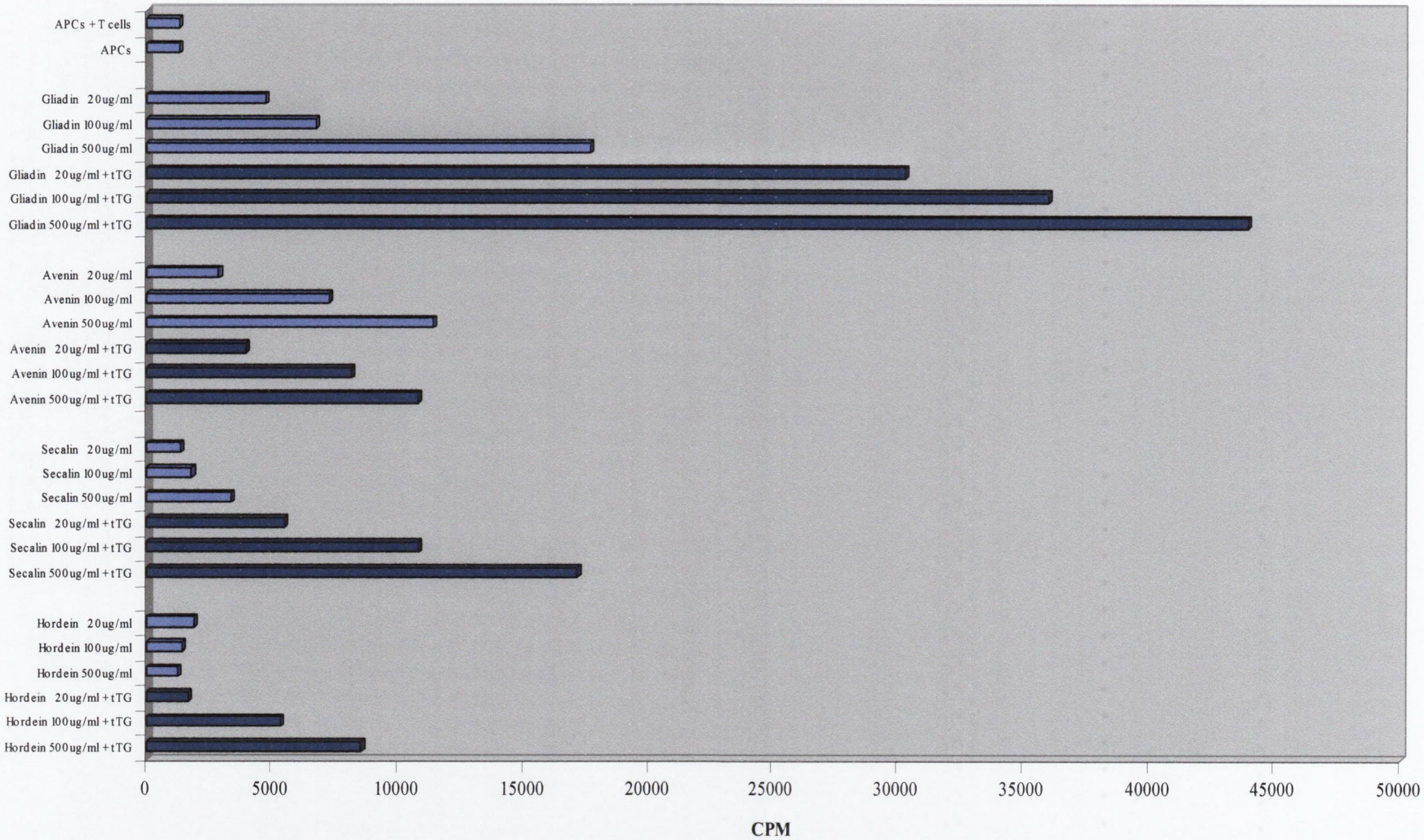


Figure 4.19 Proliferative response of T cell line 432.2.1 to prolamin proteins

4.4 DISCUSSION

In this study we used two different methods to generate intestinal TCLs from coeliac tissue. We were unsuccessful in establishing lines from protocol 1 so our initial step was to test the potency of the reagents used. Experiments with PBMCs revealed that our stocks of PHA, IL-2 and human serum could sustain cell growth and proliferation *in vitro*. It has been reported that T cells cultured in IL-2 are susceptible to activation induced cell death (Lenardo 1991). We therefore omitted IL-2 on days 3, 4 and 5 of the stimulation cycle in an effort to reduce apoptosis. This change in the protocol had no effect on T cell viability or proliferation. Moreover, increasing the concentration of PHA did not augment T cell proliferation. Protocol 2, however, yielded rapidly expanding T cell lines. There were many differences between the two methods, which may have contributed to the growth of the cells. Each one will be dealt with below.

4.4.1 Mitomycin C versus irradiation

Mitomycin C was used in protocol 1 to inhibit the proliferation of PBMC feeder cells. The mitomycins are a group of antibiotics isolated from *Streptomyces caespitosus*. Of this group, mitomycin C has received the most attention. Mitomycin C is an alkylating agent which crosslinks DNA and inhibits its synthesis (Tomasz 1995; Verweij and Pinedo 1990). It has been used successfully to inhibit the proliferation of stimulator or feeder cells in the growth of cell lines (Macpherson and Bryden 1971). Mitomycin C treated fibroblast feeders were shown to be just as effective as X-irradiated feeders in stimulating cell growth (Macpherson and Bryden 1971).

In our hands mitomycin C treated feeders did not support T cell proliferation. We postulated that residual mitomycin C on the PBMCs was preventing the growth of the intestinal T cells. One group reported washing mitomycin C treated feeder cells and incubating them for 3-7 days at 37°C before adding them to a cell line (Butcher *et al.* 1988). This incubation was to permit the metabolism and breakdown of unreacted mitomycin C. We did not include this step as part of our protocol. Instead we increased the number of PBMC washes from three to six. Extra washing of PBMCs did not enhance T cell proliferation.

Despite our observations, many groups have successfully used mitomycin C treated feeder cells to expand T cell lines and clones (Kawakami *et al.* 1995; Redelman and Bussett 1983; Scibienski *et al.* 1987; Smith *et al.* 1990). One of these studies reported long-term T cell line growth, dismissing the possibility that small amounts of unmetabolised mitomycin C could accumulate over time and inhibit T cell growth (Redelman and Bussett 1983). We altered protocol 1, in two cases, by γ-irradiating feeder cells rather than treating them with mitomycin C. There was no improvement in T cell growth, leading us to believe that some other factor was responsible for the lack of proliferation. For convenience and because a γ-irradiator became available we later irradiated all feeder cells (protocol 2).

4.4.2 Presence of a mycoplasma inhibitor

Mycoplasma infection may have been responsible for the lack of T cell growth observed when protocol 1 was used. Stable cells lines in continuous culture are frequently contaminated with mycoplasma bacteria. Their size (0.3-0.8μm diameter) enables them pass through filters, which block the passage of other bacteria. They are also difficult to detect in cell cultures by light microscopy (Rottem and Barile 1993; Stanbridge 1971). Mycoplasma infection in primary cell lines may originate from the original tissue. Another source of contamination in cell lines is infection by previously contaminated cell cultures (Rottem and Barile 1993). Mycoplasma infection can have many different effects on cell cultures. It can affect cell function and metabolism, cell morphology and induce chromosomal abnormalities. Moreover mycoplasma infection can inhibit the proliferation of cells in culture (Rottem and Barile 1993; Stanbridge 1971).

Non-fermenting mycoplasmas inhibit cell proliferation by depletion of the amino acid arginine from the medium (Claesson *et al.* 1990). *Mycoplasma arginini* produces an enzyme called arginine deiminase, which converts arginine into citrulline (Gong *et al.* 1999). It was reported that *M. arginini* and cell free medium after the growth of this organism inhibited the stimulation of lymphocytes by allogeneic cells or mitogens (Callewaert *et al.* 1975). The organism also inhibited the development of cytotoxic T cell lines (Claesson *et al.* 1990). Another study reported that arginine deiminase inhibited the growth of human T cells and T lymphoblast cell lines in a dose dependent manner (Komada *et al.* 1997). The morphology of the cells indicated that they were

undergoing apoptosis. The enzyme also suppressed IL-2 production and IL-2R expression in T cells stimulated by mitogens (Komada *et al.* 1997).

Mycoplasma infection can be prevented by the addition of antibiotics to cell culture medium (Rottem and Barile 1993). In our study T cell lines could be established in medium containing the mycoplasma inhibitor, Plasmocin. Plasmocin contains two bactericidal components: one, which acts on the protein synthesis machinery by interfering with ribosome translation and the other acts on DNA replication by interfering with the replication fork.

4.4.3 Concentration of 2-Mercaptoethanol

2-ME is known to have beneficial effects on the growth of mammalian cells cultured *in vitro*. In lymphocyte cultures, 2-ME enhances viability, antibody formation and proliferation of cells (Ishii *et al.* 1981). The favourable effects of 2-ME may be due to its antioxidant properties and/or to its ability to enhance the uptake of cystine by lymphocytes.

2-ME as an antioxidant

When phagocytic leukocytes (neutrophils, monocytes and macrophages) interact with certain pro-inflammatory mediators or bacterial products they produce reactive oxygen species (ROS) (Forman and Torres 2001). Oxidative stress occurs when there is an imbalance between oxidant exposure and antioxidant protection (Forman and Torres 2001). Severe oxidative stress can threaten cell function and viability (Hildeman *et al.* 2003). Naturally occurring cytosolic antioxidants protect cells intracellularly against the continuous production of ROS (Conner and Grisham 1996). In cell culture systems antioxidants, such as 2-ME, may protect cells against external oxidative damage by reducing harmful ROS.

T cells cultured under high oxygen tensions or with the oxidant copper:phenanthroline proliferate poorly in response to Con A. 2-ME reverses the inhibitory effects of these treatments and restores proliferation to Con A (Noelle and Lawrence 1980). In cell culture systems oxidative products produced by macrophages or neutrophils may inhibit T cell proliferation. One study found that macrophage aided T cell activation can be enhanced by 2-ME (Noelle and Lawrence 1980). A more recent study found a

correlation between T cell hyporesponsiveness *in vitro* and the concentration of ROS produced by neutrophils (Cemerski *et al.* 2002). The induction of T cell hyporesponsiveness could be blocked by addition of the antioxidants N-acetyl cysteine, Mn (II-I)tetrakis(4-benzoic acid)porphyrin chloride and catalase to the culture medium. Defective activation of phospholipase C following TCR ligation was thought to be responsible for the hyporesponsiveness of these cells (Cemerski *et al.* 2002).

Cysteine uptake

Cysteine is the limiting substance for the synthesis of glutathione, which is essential for cell cycle progression (Messina and Lawrence 1989). T lymphocytes have a strong transport activity for cysteine but the transport activity for cystine is 10 fold lower (Gmünder *et al.* 1991; Ishii *et al.* 1987). It is known that lamina propria T cells proliferate poorly in thiol deficient cystine containing culture medium (Sido *et al.* 2000). However, when 2-ME is added to the medium proliferation of lamina propria T cells is restored (Sido *et al.* 2000). 2-ME reacts with cystine to form a cysteine-2-ME mixed disulphide, which is rapidly transported into the cell via a membrane transporter. Upon internalisation the mixed disulfide is reduced liberating cysteine and reduced 2-ME initiating a cyclic process (Ishii *et al.* 1981).

Concentration of 2-ME in cell culture medium

In most cases 2-ME is effective at concentrations from 10 to 100μM (Ishii *et al.* 1981). In protocol 2 a concentration of 100μM 2-ME was used. At this concentration 2-ME protected T cells against oxidative damage and also increased cysteine uptake. However the concentration of 2-ME used in protocol 1 was 100 fold higher (10,000μM). An excess of 2-ME may have been responsible for the lack of T cell growth observed with protocol 1. A recent study found that while thiol deprivation causes apoptosis, so too does thiol excess (Kovar *et al.* 2002). In this study concentrations higher than 300μM 2-ME induced apoptosis in the B cell lymphoma 38C13, the T cell lymphoma EL4 as well as Raji and Hela cell lines. Apoptosis induction by thiol excess was associated with a decrease in the anti-apoptotic protein Bcl-2 (Kovar *et al.* 2002).

4.4.4 IL-15

IL-15 was added to the cell culture medium in protocol 2 to protect against T cell apoptosis. IL-15 is expressed in a variety of different cells and tissues including

monocytes and macrophages (Doherty *et al.* 1996), placenta (Grabstein *et al.* 1994), skeletal muscle (Quinn *et al.* 1995) and skin (Mohamadzadeh *et al.* 1995). It is not produced by T or B cells. IL-15 induces proliferation and activates cytotoxic activity of NK cells (Carson *et al.* 1994; Leclercq *et al.* 1996). It also promotes the growth and differentiation of B cells (Armitage *et al.* 1995). IL-15 and IL-2 use the same IL-2R β and γc-chain for binding and signal transduction. However IL-15 uses a specific IL-15Rα chain that is structurally similar to IL-2Rα but does not bind IL-2 (Tagaya *et al.* 1996).

One of the first studies investigating its anti-apoptotic properties found that IL-15 could inhibit the induction of apoptosis in PBMCs that were pre-activated with PHA and IL-2 (Lorenz *et al.* 1997). IL-15 also suppressed experimentally induced apoptosis in B cells and human T lymphoblasts treated with anti-FAS antibody (Lenardo 1991). The anti-apoptotic effect of IL-15 extends beyond the lymphatic system. Cells from the liver, spleen and thymus of mice challenged with an anti-FAS antibody were rescued from apoptosis by an IL-15-IgG2b fusion protein (Bulfone-Paus *et al.* 1997).

IL-2 renders activated T cells susceptible to apoptosis via TCR re-engagement (Lenardo 1991). In contrast to IL-2, IL-15 protects against TCR-induced death signalling. *In vitro* $CD4^+$ lymphocytes treated with IL-15 were resistant to apoptosis induced by antigen stimulation (Dooms *et al.* 1998). IL-15 also kept antigen experienced T cells in a quiescent condition for a prolonged period in the absence of TCR triggering (Dooms *et al.* 1998). In addition, IL-15 treated cells that were treated with anti-CD3 antibody remained viable in a growth-arrested state, while IL-2 treated cells were susceptible to anti-CD3 induced cell death (Dooms *et al.* 1998).

Addition of IL-15 to the culture medium in protocol 2 may have protected against TCR or PHA induced T cell apoptosis. It may also have improved cell viability between stimulation periods. It is possible that activation induced cell death contributed to the poor T cell viability and growth observed with protocol 1.

4.4.5 T cell lines generated using protocol 2

Rapidly expanding T cell lines were obtained using protocol 2. On average, 97% of the cells were positive for CD3. TCLs 1 and 2 were mainly $CD4^+$ $TCR\alpha\beta^+$, while in lines 3 and 4 almost half the cells were $CD8^+$ $TCR\alpha\beta^+$ and half $CD4^+$ $TCR\alpha\beta^+$. Using flow cytometry we could distinguish apoptotic PBMC feeder cells from viable T cells by their smaller size and poor expression of T cell surface markers.

4.4.6 Cytokine production in response to prolamin stimulation

T cell line 1

Two independent groups reported that TCCs generated from coeliac intestinal mucosa, produce IFN-γ in response to gliadin stimulation (Nilsen *et al.* 1995; Troncone *et al.* 1998). Using ELISA, we found that TCL 1 secreted IFN-γ not only in response to gliadin but also to secalin, hordein and avenin (Figure 4.7). Levels of IFN-γ protein exceeding 4ng/ml were detected following T cell culture with gliadin, which were comparable to levels reported by Troncone *et al.* (1998). Considerable IFN-γ production was also detected in response to secalin and hordein after 72h culture. Quantities exceeded those for gliadin at this time point. Considering the data from both time points, avenin was the poorest stimulator of IFN-γ.

We found that the optimum time for IL-2 protein detection in line 1 was 24h. High levels of IL-2, produced in response to secalin, were detected at 24 hours. There was a lesser response to gliadin and avenin at this time point. IL-2 secretion in response to hordein was close to baseline levels. Nilsen *et al.* could not detect IL-2 in TCC supernatants following 48h stimulation with gliadin (Nilsen *et al.* 1995). This was most likely due to consumption of the growth factor by the TCCs. This group did however detect low quantities of IL-2 in supernatants after 6 hours culture. They also detected IL-2 mRNA in 3 of 4 TCC tested after 4-8 hours of gluten stimulation (Nilsen *et al.* 1995).

Prolamin treatment with tTG did not enhance IFN-γ production to a great degree in TCL 1. There were more pronounced increases in IL-2 following prolamin deamidation. At 24h, tTG treatment of gliadin resulted in a marked increase in IL-2 production. Increases were also noted with tTG treatment of hordein and secalin. IL-2 responses to tTG alone were marginal and could not account for the enhanced IL-2 production observed. tTG

treatment of avenin appeared to have little effect on IL-2 production. These IL-2 results correlate with the proliferation data for TCL 1. tTG treatment appeared to enhance the proliferative response to secalin, hordein and gliadin but did not augment the response to avenin.

T cell line 2

Despite strong proliferative responses, TCL 2 secreted very low levels of IFN-γ and IL-2 in response to the four prolamins (Figure 4.10). Gliadin was the only prolamin to induce an IFN-γ response, though levels were significantly lower than those observed for TCL 1. Similarly IL-2 was only detected following culture with tTG-gliadin and tTG-secalin. The high background levels of IL-2 may be a reflection on the activation status of the cells. FACS analysis of T cell line 2 at time of the assay, revealed a population of activated cells at the upper end of the FSC axis (Figure 4.8).

Due to the high percentage of cells expressing CD25 in line 2 (Figure 4.8 (A) and (B)), it would be tempting to speculate that a population of $CD4^+CD25^+$ Treg cells proliferated in response to the prolamins and suppressed Th1 responses. However these Treg cells are anergic and do not proliferate in response to antigen stimulation (Roncarolo and Levings 2000). A number of alternative explanations may account for the lack of IFN-γ and IL-2 in the supernatants. (1) It is possible that the time points chosen were not optimal for cytokine detection. IFN-γ and IL-2 may not have been secreted or may have already been consumed at the time of analysis. (2) Conversely, the T cells proliferating in response to prolamin stimulation may have been of the Th2 phenotype. This is unlikely, however, as the cytokine profile in the coeliac mucosa is of a Th1 or a Th0 phenotype. Troncone *et al.* and Nilsen *et al.* found that their coeliac TCCs secreted Th2 cytokines in addition to IFN-γ when cultured with gliadin, giving the cells a Th0 profile (Nilsen *et al.* 1995; Troncone *et al.* 1998). However, in the Italian study one clone produced IL-4 only (Troncone *et al.* 1998). Following further expansion with IL-2 and PHA the clone also produced IFN-γ in response to gliadin. (3) It is possible therefore that the procedure used for expansion of the cells based on the use of IL-2 and PHA may affect the nature of cytokines produced.

T cell line 3

TCL 3 did not proliferate or produce IFN-γ in response to prolamin stimulation. IL-2 was detected in the cell supernatants of this line following culture with gliadin, tTG-

gliadin and tTG-hordein. In TCL 3, almost half of the T cell population were $CD8^+$. These $CD8^+$ may have been cytotoxic or may have been suppressing the activation of prolamin reactive $CD4^+$ cells (see below). The IL-2 production indicates that not all cells were suppressed. Perhaps, IL-2 was a more sensitive marker of T cell activation than the proliferation assay.

T cell line 4

In TCL 4 there was no proliferation, IFN-γ or IL-2 response to the prolamins. This line may not be gliadin or prolamin reactive. In line 4 as in line 3 there was no spontaneous IL-2 production (Figure 4.13 and Figure 4.16) whereas spontaneous production was noted in the proliferating T cell lines (lines 1 and 2) (Figure 4.7 and Figure 4.10). It is possible that the rIL-2 added during the propagation of the TCLs might have downregulated their own production of this cytokine as was previously suggested (Nilsen *et al.* 1995). This may account for the lack of proliferation and cytokine production observed in this TCL. The T cells in lines 3 and 4 were viable at the time of assay, proliferated and produced IL-2 and IFN-γ in response to PHA. Troncone *et al.* found that all TCCs isolated from the coeliac mucosa produced high levels of IFN-γ in most cases with IL-4 on stimulation with anti-CD3 and PMA regardless of gliadin reactivity (Troncone *et al.* 1998). As previously discussed, the suppressive effect of $CD8^+$ T cells in line 4 may also account for the lack of proliferation and cytokine production observed.

4.4.7 T cell proliferation in response to prolamin stimulation

TCL 3 and 4 did not proliferate in response to prolamin stimulation. These lines may not have been gliadin or prolamin reactive. Alternatively Treg or cytotoxic cells may have suppressed T cell activation. Two different cell types may have mediated this suppression.

$CD4^+CD25^+$ Treg cells

Treg cells in the periphery are generated from mature T cell populations under certain conditions of antigenic stimulation. These cells are $CD4^+CD25^+$ and specific not only for self antigens but also for dietary and foreign antigens (Bluestone and Abbas 2003; Taams *et al.* 2002). $CD4^+$ T cells can be induced to become anergic and suppressive when stimulated by antigen presented by non-professional APCs. They mediate

suppression of $CD4^+CD25^-$ cells by cell-to-cell contact or by the secretion of immunosuppressive cytokines such as TGF-β and IL-4 (Bluestone and Abbas 2003). Treg cells can be induced *in vitro* by culturing mature $CD4^+$ T cells with antigen or polyclonal activators in the presence of immunosuppressive cytokines (Roncarolo and Levings 2000). In our culture system, IL-15 may have contributed to the expansion of these cells. Recent evidence suggests that this cytokine is critical for stimulating proliferation of Treg cells *in vitro* (Roncarolo and Levings 2000).

$CD8^+$ suppressor/cytotoxic cells

Almost half of the T cells in line 3 and 4 were $CD8^+$. The coeliac TCLs and TCCs isolated by other groups were $CD8^-$. These groups did not use IL-15 to establish their T cell lines. IL-15 is a well-known growth factor for $CD8^+$ cells (Judge *et al.* 2002; Schluns *et al.* 2002; Weng *et al.* 2002). It is possible that the cytokine promoted the expansion of $CD8^+$ cells in our cultures. T cell lines 3 and 4 were in culture 1 week longer than lines 1 and 2, which may account for the higher percentage of $CD8^+$ cells in these lines. Patient 4 was diagnosed with EATL, this condition may have also contributed to the rapid expansion of $CD8^+$ cells in line 4.

It has been shown that IELs and LPLs isolated from the human normal intestine exhibit significant cytotoxicity, which is essentially abolished when $CD8^+$ cells are removed from these populations (Melgar *et al.* 2002). Cells become cytotoxic following anti-CD3 stimulation and perforin/granzyme exocytosis appears to be the major pathway in anti-CD3 dependent killing (Melgar *et al.* 2002). It is possible that the $CD8^+$ T cells in lines 3 and 4 became cytotoxic following gliadin/prolamin stimulation. Until recently it has been thought that $CD8^+$ cells were not gliadin reactive. However a recent report found $CD8^+$ lymphocytes from coeliac patients recognised the gliadin-derived peptide, A-gliadin 123-132 (Gianfrani *et al.* 2003). PBMCs and mucosal T cells from coeliac patients secreted significant amounts of IFN-γ in response to the HLA-A*0201 restricted peptide. PBMCs cultured with the peptide demonstrated cytolytic activity (Gianfrani *et al.* 2003).

It is unlikely, however, that gliadin or prolamin stimulation induced cytotoxicity. The APCs and the T cells were not MHC class I matched. Also we didn't detect any IFN-γ secretion in response to prolamin stimulation. Besides, it has been reported that only a small fraction of IELs and LPLs are responsible for anti-CD3 dependent cytotoxic killing

(Melgar *et al.* 2002). IELs and LPLs also mediate killing by FAS-FASL interaction (Melgar *et al.* 2002). The $CD8^+$ cells in our lines may have been killing $CD4^+$ Th cells by FAS/FASL mediated apoptosis.

The $CD8^+$ T cells may have also been suppressing $CD4^+$ reactions. A population of $CD8^+CD28^-$ Treg or T suppressor cells have been described (Cortesini *et al.* 2001). These cells suppress reactive $CD4^+$ Th cell proliferation by blocking the upregulation of co-stimulatory molecules on APCs, required to induce Th cell activity. APCs exposed to $CD8^+CD28^-$ Treg cells are impared in the CD40 pathway and do not upregulate B7 molecules (Cortesini *et al.* 2001; Roncarolo and Levings 2000).

In TCL 3 and 4 there was strong proliferation and cytokine secretion following T cell culture with PHA. It has been reported that APCs and co-stimulatory molecules are necessary for PHA induced T cell activation (Leivestad *et al.* 1988; Majumdar *et al.* 1990). It could be argued that $CD8^+$ cells could also inhibit PHA induced T cell activation by FAS/FASL apoptosis of APCs or by inhibiting upregulation of co-stimulatory molecules on these cells. However it has been shown that there is only a requirement for APCs when low concentrations of the mitogen are used (Roosnek *et al.* 1985). We used 10μg/ml of PHA in our proliferation assays. At this concentration the lectin is able to induce cell clustering in the absence of accessory cells (Roosnek *et al.* 1985).

Prolamin reactive cell lines

TCL 1 responded poorly to gliadin when PBMCs from a HLA-DQ2 homozygous patient were used as APCs. Lundin *et al.* (1993) also noted that T cells did not respond well when gliadin was presented by PBMCs and thought that it may be due to the low expression of HLA-DQ on these cells. We used the EBV transformed B cell line, VAVY, as APCs in subsequent proliferation assays. This cell line is homozygous HLA-DQA1*0501, DQB1*0201 and is known to bind gliadin peptides (Chirdo *et al.* 2002). The proliferative response of TCL 1 to gliadin was significantly enhanced when VAVY cells were used as APCs.

The cellular responses to gliadin have been well characterised and a number of T cell epitopes defined in the wheat prolamin (see section 4.1). However there is no information on T cell responses to secalin, hordein or avenin in coeliac patients. In this

study we found that two T cell lines generated in our lab and three T cell lines derived from Norwegian coeliac patients proliferated in response to all four prolamins. This is not surprising considering the sequence similarity between the proteins. The cereals from which the prolamins are derived i.e. wheat, barley, rye and oats all belong to the subfamily Pooideae (Shewry *et al.* 1992b). It has been shown that all four prolamins share considerable sequence similarity (Chestnut *et al.* 1989).

In the cell lines derived from Irish coeliac patients (TCL 1 and 2), the T cell responses to untreated secalin, hordein and avenin were comparable to gliadin. In the Norwegian lines (TCLs 422.1.3, 437.1.4, 432.2.1) responses to gliadin and avenin were slightly higher than those to secalin or hordein. The dose-response effect was more obvious in the Norwegian lines. The prolamin digests used in all five proliferation assays were from the same source. It is possible that our lines were more sensitive to prolamin stimulation. Perhaps if lower concentrations of protein were used, a more obvious dose-response effect would have been seen. The sensitivity or specificity of the TCL may depend on the method of expansion used. The Norwegian coeliac biopsies were cultured for the first 24 hours with a different source of PT gliadin to the Irish biopsies.

tTG treatment enhanced T cell recognition of gliadin. A marked increase in proliferation was noted in the Norwegian cell lines. This was not as pronounced in the Irish T cell lines. Experiments with our lines were carried out with guinea pig tTG whereas human recombinant tTG was used in assays conducted with the Norwegian lines. However, this does not account for the discrepancy. Most of the earlier work on gliadin modification by tTG, was done with a guinea pig preparation (Molberg *et al.* 1998; Quarsten *et al.* 1999; van de Wal *et al.* 1998a). Workers showed that guinea pig tTG deamidated glutamine residues in gliadin peptides allowing them to bind more efficiently to HLA-DQ2. This greatly improved T cell recognition of gliadin. It has been shown that gliadin is a good substrate of several TGs including guinea pig tTG (Skovbjerg *et al.* 2002). A number of contaminating proteins have been found in guinea pig tTG preparations, which may mean that the concentration of tTG in our assays was lower than that in the Norwegian assays.

tTG also enhanced T cell recognition of secalin and hordein in the majority of cases, indicating that these prolamins contain tTG target sequences. In the Norwegian cell lines, proliferative responses to these tTG-modified prolamins were considerably lower

than responses to tTG-gliadin. In T cell line 1 and 2 responses to tTG-modified gliadin, secalin and hordein were comparable.

For the most part tTG did not augment T cell recognition of avenin. In TCLs 1 and 2 and in line 432.2.1, tTG did not enhance T cell recognition of avenin whatsoever. In the two remaining cell lines, proliferative responses to tTG-avenin were slightly higher than responses to the untreated prolamin. Avenin differs from the other three prolamins in that it contains significantly lower percentages of proline residues. Glutamine content in gliadins, hordeins, and secalins ranges from 35-37% and proline from 17-23%. Avenins contain a similar percentage of glutamine (34%) but half the amount of proline (10%) (Wieser 1994; Wieser *et al.* 1983). Vader *et al.* (2002a) found that the spacing between glutamine and proline in gluten plays an essential role in the specificity of deamidation by tTG. This group designed algorithms to predict novel T cell stimulatory peptides in gluten. These algorithms also identified many similar peptides in hordeins and secalins but not in avenins. Another study found that many variants of the sequence motif PQPQLPY are high affinity substrates of tTG and are found in wheat, barley and rye derived proteins. Whereas the closest homologues of this sequence found in rice, corn or oat proteins were much poorer substrates of tTG (Piper *et al.* 2002).

4.4.8 Conclusion

To summarise, we found that many different factors contribute to the successful growth of intestinal T cell lines *in vitro*. The method of expansion may affect the sensitivity, specificity and/or cytokine secretion of the line. The use of IL-15 during cell line expansion may promote the growth of cytotoxic or suppressor cells which may curb Th cell activation. We report here that not only gliadin but also secalin, hordein and avenin stimulate proliferation and cytokine secretion in HLA-DQ2 restricted T cell lines from coeliac patients. In this study tTG enhanced T cell recognition of gliadin, secalin and hordein in the majority of cases. These preliminary results suggest that tTG does not modify avenin to the same extent. This may be due to a lack of tTG target sequences in avenin and may explain why coeliac patients can tolerate oats *in vivo*. However, we need to test the reactivity of a larger number of T cell lines before meaningful statistics can be carried out. In the future we also hope to generate T cell clones, which will help pinpoint the T cell epitopes in secalin, hordein and avenin.

CHAPTER 5

General Discussion

5.1 Review of results

Coeliac disease develops because of intolerance to cereal prolamin proteins. For 50 years, research has focussed on the wheat prolamin, gliadin. We now have a good understanding of gliadin mediated T cell responses in the intestine, which are central to disease pathogenesis. Researchers have isolated the immunodominant gliadin epitopes, and have found that the enzyme tTG deamidates gliadin peptides introducing negative charges, which favour binding to HLA-DQ2 and –DQ8 molecules (Arentz-Hansen *et al.* 2000a; Molberg *et al.* 1998; Shan *et al.* 2002; van de Wal *et al.* 1998a). However, our knowledge of immune responses to barley, rye and oats in coeliac patients is somewhat limited, particularly *in vitro*. The focus of this thesis was to develop *in vitro* assays to assess the immunogenicity of the prolamin protein avenin. Additional studies were also carried out with hordein and secalin.

In chapter two we investigated brush border lactase reduction as a marker for testing potentially harmful prolamins. In our organ culture system, gliadin or gluten did not reduce enterocyte lactase expression in coeliac biopsies. In a recent publication, Maiuri *et al.* (2003) reported that gliadin does not induce enterocyte apoptosis in treated coeliac biopsies. Since reduced lactase expression is a consequence of enterocyte apoptosis (Moss *et al.* 1996) it is not surprising that we did not detect enzyme changes in our biopsies, the majority of which were from treated patients. In our study marked T cell activation did not alter lactase expression either. It was only when TNF-α was added to the culture system for 48 hours that a reduction in lactase expression was noted.

Workers have speculated as to whether enterocyte damage *in vivo* is due to direct toxicity of gliadin or whether it is the result of pro-inflammatory cytokines produced in the underlying mucosa. The issue of toxicity versus immunogenicity will be discussed below (section 5.2). Regardless of how it occurs, our results suggest that reduction in enterocyte lactase expression is a slow process. We therefore concluded that it is not suitable as a marker of cereal induced disease activity *in vitro*.

In chapter two, we also investigated mucosal EMA production as a possible assay for testing prolamins. Earlier studies reported that EMA production in coeliac biopsies is a sensitive marker of gliadin immunogenicity (Picarelli *et al.* 1999; Picarelli *et al.* 1996). However other groups have questioned its reliability (Biagi *et al.* 2000; Vogelsang *et al.*

1999). We did find mucosal production of EMA, however, it was not gliadin dependant. We also deduced from our results that mucosal EMA production *in vitro* is not a function of the length of the GFD nor is it dependant on the inflammatory infiltrate in the mucosa. We concluded that mucosal EMA production was not a good marker of prolamin immunogenicity.

In chapter three, we cultured biopsies with gliadin and avenin and measured IFN-γ and IL-2 as evidence of cereal induced T cell activation. This model reflects the immune hypothesis, which is currently used to explain the pathogenesis of coeliac disease. In this model gliadin peptides, which are resistant to enzyme processing, are transported across the epithelium. The peptides are deamidated by tTG, which is present at the brush border and in the subepithelial region in the active lesion. The low pH in the proximal small intestine favours tTG-mediated deamidation of the peptides. Binding affinity for HLA-DQ2 and –DQ8 molecules is increased. Gliadin is presented to $CD4^+$ T cells in the lamina propria, which secrete predominantly Th1 cytokines on activation (Sollid 2002).

We presented results where gliadin induced a significant increase in IFN-γ and IL-2 mRNA and protein, which was specific for coeliac patients. Avenin, in contrast, failed to stimulate a significant IFN-γ or IL-2 response in the patients studied. These results advocate the safety of oats in the coeliac diet and concur with several clinical trials, which reported that oats caused no adverse clinical effects or immune activation (Hoffenberg *et al.* 2000; Janatuinen *et al.* 2002; Janatuinen *et al.* 2000; Janatuinen *et al.* 1995; Srinivasan *et al.* 1996). Our findings dispute the theory that oats is safe because of its low prolamin content.

The fourth chapter describes the expansion of intestinal TCLs. TCLs and TCCs generated from coeliac intestinal tissue are gliadin reactive and HLA-DQ restricted (Lundin *et al.* 1993). An important breakthrough was the discovery that deamidation by tTG enhances TCL / TCC recognition of gliadin (Molberg *et al.* 1998). We investigated responses to prolamins gliadin, secalin, hordein and avenin in T cell lines isolated from Norwegian and Irish coeliac patients. We observed considerable proliferation and IFN-γ and IL-2 production to all four PT digested prolamins. This is not surprising considering wheat, barley, rye and oats all belong to the subfamily Pooideae and their prolamins share considerable sequence homology (Shewry *et al.* 1992b).

Why, however, did we see a T cell response to avenin in the TCLs and not the organ culture system? The reason may be due to enzyme digestion. T cell epitopes appear to cluster around regions in gliadin that are rich in proline (Arentz-Hansen *et al.* 2002). Most mammalian peptidases cannot cleave peptide bonds located next to this amino acid (Arentz-Hansen *et al.* 2002). Recently a 33mer gliadin peptide was isolated which is resistant to pancreatic, gastric and brush border enzyme digestion (Shan *et al.* 2002). This peptide is a potent stimulator of TCCs and reacts with tTG with great affinity. Avenin contains a lower percentage of proline than gliadin, secalin and hordein, which most likely renders it more susceptible to enzymatic digestion. In our organ culture study, it is possible that brush border enzymes facilitated the breakdown of potentially toxic sequences in avenin. In the TCL assay no such digestion occurred and immunogenic sequences triggered T cell activation. Several homologues of the 33mer have been identified in hordeins and secalins. However no homologues have been identified in avenin, rice or maize (Shan *et al.* 2002).

We found that tTG enhanced T cell recognition of gliadin, secalin and hordein indicating that all three prolamins contain tTG target sequences. Of significance was that tTG did not enhance T cell recognition of avenin to the same extent. Vader *et al.* (2002a) found that the spacing between proline and glutamine plays an important role in the specificity of tTG. The authors suggested that due to its low content of proline, tTG non-selectively deamidates avenin (Vader *et al.* 2002a). Thus potent T cell stimulatory epitopes are not generated in this prolamin.

Sollid *et al.* proposed that anti-tTG antibodies are produced when tTG specific B cells take up tTG-gliadin complexes, present them to gliadin reactive T cells, which in turn aid antibody production by tTG specific B cells (Sollid *et al.* 1997). The absence of EMAs in coeliac patients consuming oats (Srinivasan *et al.* 1996) (Størsrud *et al.* 2003) would indicate that avenin does not complex with tTG *in vivo*. In the future it would be interesting to analyse tTG treated avenin peptides by mass spectrometry to determine the pattern of deamidation. Testing overlapping avenin peptides against a panel of TCCs would allow us to define T cell epitopes in avenin.

5.2 Identifying 'toxic' gliadin sequences

Toxicity versus immunogenicity

Much of the research in coeliac disease has concentrated on identifying the elements in gliadin that are responsible for disease progression. The complex nature of gluten has made this task difficult. Another problem has been the number of different assays that have been used to test for 'toxicity' over the years (Table 5.1) (McAdam and Sollid 2000). From these assays two different types of peptide have emerged as being harmful; (i) toxic peptides aa31-49 and aa31-55 of A gliadin which reduce enterocyte height in organ culture assays and (ii) immunogenic peptides α-2 (aa62-75), α-9 (aa57-68) and a 33mer (aa57-89 of α2 gliadin) which induce activation of HLA-DQ2 restricted T cell clones and aa216-217 gliadin which activates HLA-DQ8 restricted clones. Interestingly the toxic peptides do not correspond to the immunogenic sequences and vice versa. McAdam *et al.* (2000) have been unable to demonstrate T cell reactivity to the toxic peptides. In their organ culture system using enterocyte height as a measure of toxicity, de Ritis *et al.* (1988) found that a peptide corresponding to the immunodominant region was non-toxic.

A recent publication by Maiuri *et al.* (2003) demonstrated that the toxic peptide aa31-43 triggers a T cell independent response in the coeliac mucosa. The group compared the effects of peptide 31-43 with immunodominant peptides α-2 and α-9 in an organ culture system. They found that only the toxic peptides induced IL-15, CD83 (marker of mature dendritic cells), COX-2 and CD25 on $CD3^-$ cells and enterocyte apoptosis. 31-43 did not activate CD25, CD69 or IFN-γ in T cells (Maiuri *et al.* 2003). This non-immunodominant peptide also induced p38 MAP kinase activation in $CD3^-$ cells while α-2 and α-9 did not. Changes were specific for coeliac patients and were not observed in control patients. Interestingly, three hours incubation with 31-43 followed by 21 hours with α-2 or α-9 increased CD25 expression on $CD3^+$ cells in treated and untreated coeliac biopsies and caused substantial enterocyte apoptosis in biopsies from untreated patients. Incubation with α-2 or α-9 alone did not cause substantial changes in the biopsy. Therefore prior incubation with the non-immunodominant peptide favoured T cell activation by the immunodominant gliadin peptides (Maiuri *et al.* 2003).

There have been reports of other peptides, which activate the innate immune system. A gliadin peptide, which causes direct stimulation of TNF-α, IL-10 and NO production in

Table 5.1 Toxic and immunogenic gliadin and glutenin peptides and the *in vitro* systems used to test them

Sequence	Method used	Reference
3-24 α gliadin	Organ culture - reduced enterocyte height	(Weiser *et al.* 1983)
25-55 α gliadin	Organ culture - reduced enterocyte height	(Weiser *et al.* 1983)
1-127 A gliadin	Organ culture - reduced enterocyte height	(De Ritis *et al.* 1988)
128-246 A gliadin	Organ culture - reduced enterocyte height	(De Ritis *et al.* 1988)
1-30 A gliadin	Organ culture - reduced enterocyte height	(De Ritis *et al.* 1988)
31-55 A gliadin	Organ culture - reduced enterocyte height	(De Ritis *et al.* 1988)
31-49 A gliadin	Organ culture - reduced enterocyte height	(Shidrawi *et al.* 1995)
31-43 A gliadin	Organ culture - reduced enterocyte height	(Maiuri *et al.* 1996b)
31-55 A gliadin	Organ culture - reduced enterocyte height	(Maiuri *et al.* 1996b)
44-55 A gliadin	Organ culture - reduced enterocyte height	(Maiuri *et al.* 1996b)
31-43 A gliadin	Caused agglutination of K652 (S) cells	(De Vincenzi *et al.* 1994)
44-55 A gliadin	Caused agglutination of K652 (S) cells	(De Vincenzi *et al.* 1994)
31-43 A gliadin	Inhibited growth of CaCo-2 cells	(Giovannini *et al.* 1997)
75-86 A gliadin	Inhibited an increase in disaccharidase activity in foetal chick intestine	(Cornell and Mothes 1993)
31-43 α gliadin	Induced EMA production in coeliac intestinal biopsies	(Picarelli *et al.* 1999)
31-45 A gliadin	Altered villous height : crypt depth, reduced enterocyte height, increased IELs in vivo	(Sturgess *et al.* 1994)
57-68 α 9 gliadin	Stimulated HLA-DQ2 restricted T cell clones (deamidated by tTG)	(Arentz-Hansen *et al.* 2000a)
62-75 α 2 gliadin	Stimulated HLA-DQ2 restricted T cell clones (deamidated by tTG)	(Arentz-Hansen *et al.* 2000a)
134-153 γ gliadin	Stimulated HLA-DQ2 restricted T cell clones (deamidated by tTG)	(Molberg *et al.* 1998)
206-217 gliadin	Stimulated HLA-DQ8 restricted T cell clones (deamidated by tTG)	(van de Wal *et al.* 1998a)
724-734 glutenin	Stimulated HLA-DQ8 restricted T cell clones	(van de Wal *et al.* 1998b)
57-73 A gliadin	IFN-γ production by peripheral blood (deamidated by tTG)	(Anderson *et al.* 2000)
57-89 α 2 gliadin	Stimulated HLA-DQ2 restricted T cell clones (deamidated by tTG)	(Shan *et al.* 2002)

peritoneal macrophages, has been isolated (Tuckova *et al.* 2002). More of these peptides are expected to emerge. It would be interesting to see if avenin contains sequences that could potentially trigger the innate immune system. This raises the question: do sequences that trigger the innate immune system determine the toxicity of a prolamin *in vivo*?

Epitope spreading

The number of gluten stimulatory epitopes is now larger than first imagined. Vader *et al.* estimated that over 50 stimulatory peptides exist in gluten (Vader *et al.* 2002b). The group reported that only half of the paediatric coeliac patients in their study responded to the immunodominant peptides α-2 and α-9 (Vader *et al.* 2002b). Also, T cell clones from paediatric patients but not adults responded to a glutenin peptide sequence. This peptide was the only one to activate clones from the youngest patient studied. Interestingly some of the responses to peptides were deamidation independent. Responses to non-deamidated peptides were less common in adult patients (Vader *et al.* 2002b). Authors postulated that the following sequence of events occurs; in the early stages of coeliac disease responses are directed against a wide range of gluten peptides from glutenin and gliadin molecules. Epitope spreading may occur due to the cross reactivity between glutenin and gliadin. In later stages of the disease increasing tissue injury leads to release of tTG which in turn deamidates gluten peptides. The immune response is then polarised towards peptides capable of strong binding affinity to DQ2 molecules and of potent T cell stimulation (Vader *et al.* 2002b).

5.3 The future of coeliac disease: Therapy

One method of controlling coeliac disease might be to create non-toxic wheat strains. However, this task is made difficult by the fact that gliadin and glutenin genes are located at multiple loci on different chromosomes. Also, patients respond to a number of different epitopes in gliadin. Frisoni *et al.* (1995) tested fraction III from lines of wheat that were deficient in alpha and beta gliadins (Gli-A2), and partially deficient in alpha, beta, gamma, and omega gliadins (Gli-A2/Gli-D1). These lines had no effect on enterocyte height when tested in an organ culture system. However, Gli-A2 and Gli-A2/Gli-D1 still contain some gliadins and high concentrations of these lines may be toxic to coeliac patients (Frisoni *et al.* 1995). An alternative method may be to transfer the genes responsible for bread-making qualities i.e. the HMW glutenins to other grains.

This approach is only feasible if glutenins do not contain antigenic sequences. Molberg *et al.* (2003) found that tTG modified HMW glutenin induced proliferation in a number of TCLs from coeliac patients. These results would indicate that cereals containing HMW glutenin would not be safe for coeliac consumption.

It may be possible to re-induce tolerance in patients with coeliac disease. Mice, immunised with gliadin through the intravenous or intranasal route, showed reduced proliferation and IFN-γ and IL-2 production in lymph node cells when they where later challenged with gliadin (Rossi *et al.* 1999). Nasal immunisation is thought to trigger a local inflammatory response, which stimulates IL-4 and TGF-β production in draining lymph nodes. These immunosuppressive T cells migrate to the Peyer's patches where they induce tolerance in the intestine (Weiner 1997). Gliadin challenge in the nasal mucosa of coeliac patients caused an antigen specific increase in lymphoid cells and granulocytes. The number of $CD3^+$ and $CD25^+$ cells were also increased in patients compared to controls (Torre *et al.* 2002). This would suggest that gliadin is being recognised in the nasal mucosa and therefore this immunisation via this route may trigger a Th3 response in draining lymph nodes. It has already been shown that intranasal immunisation of mice with purified α-gliadin reduces lymph node T cell proliferation and IFN-γ production to whole gliadin (Maurano *et al.* 2001). Our increasing knowledge of T cell epitopes in gliadin and epitope spreading may allow use to develop potent tolerogens in the future.

Another approach is peptidase therapy, which would digest immunodominant gliadin peptides. Shan *et al.* (2002) discovered that their 33mer gliadin peptide is susceptible to digestion by a prolyl endopeptidase (PEP) from *Flavobacterium meningosepticum*. Workers showed that breakdown of the peptide with PEP abolished its ability to stimulate T cells (Shan *et al.* 2002). This PEP targets Pro-Xaa-Pro sequences and therefore has the potential to target other T cell stimulatory epitopes not found in this 33mer region. Koning and Vader argued, however, that enzymatic degradation of gluten might be difficult since it is present in a food matrix with many other compounds. This also complicates detection of degraded gluten peptides (Koning and Vader 2003).

It may also be possible to control disease progression by targeting the innate immune system. Maiuri *et al.* (2003) have suggested IL-15 and the MAP kinase pathway as potential therapeutic targets. Inhibiting these two components would control the innate

response, which would in turn curtail the adaptive response to gliadin (Maiuri *et al.* 2003). It is interesting that the innate response to gliadin is only found in coeliac patients and not HLA-DQ2-positive controls. Genes of the innate immune system may become the focus of therapeutic strategies in the future (Schuppan *et al.* 2003).

Another potential target for therapy is tTG. Manipulating the acidity of APC endosomes in the small intestine could control tTG reactions (Sollid 2002). In this way only deamidation reactions are affected and not transamidation reactions, which are related to many important cellular functions. However the finding of deamidation independent epitopes in gliadin may mean that this approach is unsuitable (Sollid 2002).

Directing therapy towards gliadin reactive T cells may also prevent disease. Apoptosis of gliadin specific T cells may be achieved in two ways (i) using soluble dimers of HLA-peptide complexes, which induce apoptosis by T cells by inappropriate stimulation (Sollid 2002) (ii) using genetically engineered APCs which present the dominant gliadin epitope and also express FAS L (Martucci and Corazza 2002). Preventing the binding of gliadin peptides to HLA-DQ2 and DQ8 molecules and blocking the CD28/B7 co-stimulation pathway are just some of the many alternative strategies that have been suggested (Martucci and Corazza 2002; Sollid 2002).

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Appendices

APPENDIX 1

Three and one letter abbreviations of the amino acids

	Symbol	Meaning
Ala	A	Alanine
Cys	C	Cysteine
Asp	D	Aspartic Acid
Glu	E	Glutamic Acid
Phe	F	Phenylalanine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Lys	K	Lysine
Leu	L	Leucine
Met	M	Methionine
Asn	N	Asparagine
Pro	P	Proline
Gln	Q	Glutamine
Arg	R	Arginine
Ser	S	Seronine
Thr	T	Threonine
Val	V	Valine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Asx	B	Asp or Asn
Glx	Z	Glu or Gln
Xaa		Unknown or other

APPENDIX II

Reagents for RNA isolation

Denaturing solution

4M guanidium thiocyanate

25mM sodium citrate, pH 7

0.1M 2-ME

0.5% N-lauroylsarcosine

A stock solution was prepared by dissolving 250g guanidium thiocyanate in a solution of 293ml H_2O, 17.6ml of 0.75M sodium citrate and 26.4ml of 10% N-lauroylsarcosine at 60-65°C with stirring. A working solution was prepared by adding 7µl of 2-ME per 1ml of stock solution.

Chloroform/isoamyl alcohol

49:1 (volume/volume) choloroform:isoamyl alcohol

Reagents for ELISAs

PBS (10X stock solution)

137mM NaCl

2.7mM KCl

8.1mM Na_2HPO_4

1.5mM KH_2PO_4

P-nitro-phenylphosphate

1mg/ml p-nitro-phenylphosphate in 10% diethanol amine buffer containing 0.5 mM $MgCl_2$ pH 9.8

Block buffer

1% Bovine serum albumin

5% Sucrose in PBS with 0.05% NaN_3

Reagent diluent

0.1% BSA

0.05% Tween 20 in Tris-buffered Saline (20mM Trizma base, 150mM NaCl)

pH 7.3

APPENDIX III

Flow cytometer settings used in chapter 2

Parameter	Detector	Voltage	Amp Gain	Mode
P1	FSC	E00	1.35	Lin
P2	SSC	391	1.00	Lin
P3	FL1	547	1.00	Log
P4	FL2	564	1.00	Log
P5	FL3	643	1.00	Log
P6	FL3-A		1.00	Lin
P7	FL3-W		1.00	Lin

Threshold:	Compensation
	FL1 - 0.9% FL2
Parameter: FSC	FL2 - 23.0% FL1
	FL2 - 0.0% FL3
Value: 52	FL3 - 25.0% FL2

Flow cytometer settings used in chapter 4

Parameter	Detector	Voltage	Amp Gain	Mode
P1	FSC	E00	1.35	Lin
P2	SSC	391	1.00	Lin
P3	FL1	485	1.00	Log
P4	FL2	551	1.00	Log
P5	FL3	643	1.00	Log
P6	FL3-A		1.00	Lin
P7	FL3-W		1.00	Lin

Threshold:	Compensation
	FL1 - 0.9% FL2
Parameter: FSC	FL2 - 23.0% FL1
	FL2 - 0.0% FL3
Value: 52	FL3 - 25.0% FL2

APPENDIX IV

The guidelines adhered to when designing TaqMan primers and probes

Primers

The GC content should be 20-80%

The melting temperature (Tm) should be 58-60°C

There should be a maximum of 2Gs and/or Cs at the 3' end

There should be less than 2°C difference in Tm between the two primers

The 3' end of the primer should be as close to the probe as possible without overlapping it

Probes

The GC content should be 20-80%

There should be less than four contiguous Gs

The Tm should be 10°C greater than the primer Tm and should be 65-67°C

There should be no G on the 5' end

There must be more Gs than Cs on the probe strand