Investigation of the Cellular Immunopathogenesis and Cereal Toxicity of Coeliac Disease

A thesis submitted for the degree of Doctor of Medicine in the University of London

Dr Richard Patrick Sturgess

Coeliac Research Unit
Division of Pharmacology
United Medical and Dental School of Guy's and St Thomas' Hospitals
London SE1 7EH

Bachelor of Science  1981
Bachelor of Surgery   1984
Bachelor of Medicine  1984

University of London

1995
ABSTRACT

Coeliac disease is characterised by small intestinal enteropathy resulting from the ingestion of certain dietary cereals by genetically predisposed individuals. Immunological factors are thought to be involved in the pathogenesis of the intestinal lesion but the precise mechanisms remain unknown. The exact nature of the active moiety within coeliac toxic cereals has not been defined. The aims of this thesis were to investigate (i) small intestinal cellular adhesion mechanisms involved in coeliac disease (ii) γδ T cell populations within the intestinal mucosa of patients with coeliac disease and dermatitis herpetiformis and (iii) cereal toxicity in coeliac disease by in vivo challenge with synthetic gliadin oligopeptides.

Immunohistochemistry and organ culture was used to study the expression of intercellular adhesion molecule-1 (ICAM-1) within the small intestinal mucosa. Differential upregulation of ICAM-1 was demonstrated in the intestine of coeliac patients, with marked increases within the lamina propria but not the epithelium. Increased expression occurred rapidly, following gluten challenge in treated coeliac patients. The cytokines interferon-γ and tumour necrosis factor-α induced increased expression of ICAM-1 within the lamina propria of cultured jejunal biopsies from normal patients. These studies suggest that the lamina propria is a major site of immune activation in coeliac disease. T lymphocytes expressing the γδ form of the T cell receptor were found to be increased in the intestinal mucosa in both coeliac disease and dermatitis herpetiformis. The increased numbers of γδ+T cells correlated with morphometric indices of enteropathy. Local gluten challenge in the rectal mucosa of coeliac patients showed that the early lymphocyte response did not involve γδ+ T cells.

Three peptides corresponding to amino acids 3-21, 31-49 and 202-220 of A-gliadin were synthesised. Four treated coeliac patients underwent acute in vivo challenges with each of the oligopeptides. Coeliac toxicity was confined, with the exception of one patient, to the oligopeptide corresponding to amino-acids 31-49 of A-gliadin.
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CHAPTER 1: INTRODUCTION

Historical Aspects

Aretaeus the Cappadocian made probably the first extant description of coeliac disease in the first century A.D. (Dowd et al. 1974). He recorded, however, a disease that only affected adults and it was not until 1888 that Samuel Gee, in his classic paper, described what is clearly recognised as coeliac disease today (Gee 1888). He described a condition that caused diarrhoea, emaciation and cachexia in people of all ages. Although the role of cereal foodstuffs in the disease was not recognised at the time, he did state “the allowance of farinaceous foods must be small” and “that if the patient can be cured at all, it must be by means of diet”. Initial dietary treatment included the use of a diet consisting of bananas alone by Hass in 1924 (Hass 1924). It was not until the 1950's that Dicke described the successful use of a gluten-free diet, which has remained the definitive treatment of the condition to the present day (Dicke et al. 1953).

The earliest description of the pathological changes associated with coeliac disease was made in 1947 by Schein, who described the histological changes seen at autopsy in a teenage child (Schein 1947). He noted that “the villi were broad based, squat, bulbous and plump”. These findings were later confirmed by Paulley who published his findings of the histology of the jejunal mucosa obtained operatively at laparotomy (Paulley 1954). It had previously been thought that the abnormal changes seen at autopsy were post-mortem artifacts (Thaysen 1932). In the 1950's the technique of peroral jejunal biopsy was developed. Doniach and Shiner reported the histological appearances of small bowel biopsies from 23 patients with steatorrhoea which all exhibited villous atrophy compared to 10 controls which did not (Doniach et al. 1957).

Clinical Features

Coeliac disease is associated with villous atrophy of the proximal small intestine. The clinical features of the condition result from impairment of the normal physiological function of the proximal bowel, namely digestion and absorption of nutrients. The
Introduction

condition usually presents during infancy or the middle adult years (Swinson et al. 1980). The common presentation in infants is with diarrhoea and failure to thrive. In adults the presentation may be more varied with constitutional, gastrointestinal and metabolic disorders (Cooke et al. 1984). Malaise, weakness and diarrhoea are common, although many patients come to accept their altered bowel habit as “normal”. Symptoms may be very mild and a significant proportion of patients are detected by chance, with for example, non-responsive iron-deficiency anaemia. Some individuals may be of short stature and thin, but the majority are normal on examination.

Haematological and biochemical indices of malabsorption may be abnormal, as may immunological assays such as anti-gliadin antibody titres (see below), but the diagnosis is made by small intestinal biopsy. Confirmation of improvement after treatment with a gluten-free diet is essential, although the necessity for subsequent gluten challenge has recently been questioned (Meeuwisse 1970; Walker-Smith et al. 1990). Patients should continue on a gluten-free diet for life, as there is evidence that such treatment reduces the risk of the major long-term complication of the condition, the development of malignancy (Holmes et al. 1989).

Dermatitis Herpetiformis

Dermatitis herpetiformis (DH) is characterised by a symmetrical, intensely itchy, bullous rash that responds to treatment with dapsone. The condition may occur at any age but the majority of patients present in early adulthood. The common site for involvement is the extensor surfaces of the elbows and knees, and the buttocks. The diagnosis is made by the immunofluorescent demonstration of IgA, either in granular or linear granular deposits in the dermis, usually the papillae (Fry et al. 1974).

In 1966 an enteropathy was demonstrated in nine of twelve patients with DH (Marks et al. 1966). Shortly afterwards, studies by Fry and colleagues established similarities between DH and coeliac disease such as low serum IgM, evidence of splenic atrophy and haematinic deficiencies. The rash and enteropathy in DH were shown to be gluten-dependent indicating that this was not merely the association of
two conditions, but that common pathogenetic mechanisms were likely to be involved (Fry et al. 1967; Fry et al. 1968a; Fry et al. 1968b). The intestinal mucosal pathology associated with dermatitis herpetiformis will be discussed in greater detail later in this chapter and in Chapter 3.

**Enteropathy Associated T cell Lymphoma**

It was first suggested in 1962 that the small bowel lymphoma that was associated with steatorrhoea, was a complication of coeliac disease (Gough et al. 1962). Subsequently, it was shown that there was an increased prevalence of intestinal lymphoma in coeliac patients (Harris et al. 1967). Initially the tumour was classified as malignant histiocytosis of the intestine based on morphological, immunological and histochemical criteria. Isaacson and colleagues studied DNA extracted from four patients tumours and demonstrated re-arrangement of the T cell receptor β-chain, confirming the T cell origin of these tumours (Isaacson et al. 1985). Recent work has shown that the distribution of T cell phenotypes in the non-lymphomatous intestinal mucosa of patients with these tumours is identical to that from non-affected coeliac patients, supporting the hypothesis that the lymphoma is a complication of coeliac disease (Spencer et al. 1989b).

In an important study from Birmingham, Holmes and colleagues followed a cohort of 210 patients previously diagnosed as suffering from coeliac disease (Holmes et al. 1989). The cohort was divided into three groups, one consisting of patients who had been on a strict gluten-free diet for at least five years (n=108), a second group who appeared to be on a normal diet (n=46) and a third group who were on a reduced gluten diet (n=56). In the cohort as a whole, there was an increased incidence of carcinomas of the mouth, pharynx and oesophagus, and non-Hodkin's lymphoma. The excess risk was confined to those on a normal or reduced gluten diet and there was no increased risk amongst those patients who had been on a strict gluten-free diet for at least five years. These results support the hypothesis that the lymphoma arises as a complication
of coeliac disease, and emphasises the importance of patients adhering to a strict gluten-free diet for life.

**Epidemiology and Genetics**

The exact incidence of coeliac disease is not known, as undoubtedly the condition is underdiagnosed because of the wide variation in symptoms, with some patients having very mild, or no symptoms at all. Nevertheless, there are clear geographical and racial variations. Environmental factors clearly play a role in the incidence of coeliac disease since the disease only occurs if gluten forms part of the diet and is therefore most prevalent in Europe, North America and Australasia where wheat is a staple food. Genetic factors are also active since the condition either does not occur, or is very rare, in blacks living in North America, Europe or South Africa, nor does it appear to have been documented in the Chinese or Japanese. Coeliac disease has been reported in the children of Asians living in the UK (Nelson et al. 1973); prior to this there had been few reports of the condition in Asians. The disease is thus encountered over a wide geographical area with the Europeans having the highest prevalence. There is considerable variation in the prevalence of the condition within the European populations themselves. Around the Galway region in the West of Ireland, the childhood incidence has been estimated as 1 in 300 (Mylotte et al. 1973). In the UK overall approximately 1 in 2000 are thought to be affected (Carter et al. 1959) but there is variation in studies of individual areas. In the Lothian region of Scotland an incidence of 1 in 1776 was found (Logan et al. 1981) but in the Derby area 1 in 874 has been described (Arthur et al. 1981).

An examination of the incidence of childhood coeliac disease in County Galway has demonstrated a decreasing incidence which correlated with a later introduction of gluten into the diet of infants and to an increase in breast-feeding (Stevens et al. 1987). In contrast, a review of childhood coeliac disease in Birmingham, UK between the years 1960-1985 found that the age of onset of coeliac disease was delayed by the later introduction of gluten and an increase in breast-feeding but that the overall prevalence
of the disease remained unchanged (Kelly et al. 1989). Similar reports from other countries suggest that the disease is presenting later in childhood with the later introduction of cereals, rather than declining in frequency (Maki et al. 1988). Two recent reports from Scandanavia indicate a rising incidence, with an increase from 1:1000 to 1:300 live born infants in Sweden (Ascher et al. 1991; Maki et al. 1990). The authors concluded that the most probable reasons for these findings was an increased diagnostic awareness of the physicians concerned, but that this could not account for all the observed changes.

It is clear that coeliac disease is more common in families that already contain one affected individual. A study from Ireland of 28 families gave a sibling prevalence of between 8.5 and 14% (Mylotte et al. 1974) although a thorough study from Sweden estimated the sibling prevalence to be lower at 3% (Stenhammar et al. 1982). Reports of discordant monozygotic twins have been made in the literature (Polanco et al. 1981; Walker-Smith 1973), but at least two of these cases have subsequently been reported as showing the typical changes of coeliac disease at a later biopsy (Salazar de Sousa et al. 1987).

**Immunogenetics**

**HLA STUDIES**

It has been recognised for some time that coeliac disease occurs more frequently in individuals having certain human leucocyte antigens (HLA). HLA molecules, encoded within the human major histocompatibility complex, are membrane-bound glycoproteins that exhibit considerable polymorphism and are encoded by genes on the short arm of chromosome 6. Initial identification of HLA types has been performed serologically but with the advent of molecular biology techniques allowing sequencing of the polymorphic regions of HLA molecules, it is obvious that these molecules are considerably more polymorphic than had been recognised by serology.
The HLA gene complex harbours three main families: class I, class II and class III genes. The class III and related genes encode for components of the complement system (C2, C4, factor B) and also for heat shock protein 70, tumour necrosis factor α and β, steroid 21-hydroxylase and a peptide transporter thought to be involved in the transport of antigenic peptides from the cytosol to the endoplasmic reticulum (Kleijmeer et al. 1992). This region also includes a large group of yet unclassified genes which may prove to have important immunoregulatory functions.

A major feature of the HLA complex is the tight linkage between alleles of different loci. Thus the genes encoding for HLA-A1, -B8, -DR3, and -DQw2 are often inherited together on the same chromosome constituting a particular extended haplotype. These linkage relationships have made it difficult to identify the exact locus within the HLA complex that is responsible for disease susceptibility.

The HLA class I genes encode an α-chain, of three domains, that together with β2-microglobulin, forms a molecule that is expressed on all nucleated cells. Class I molecules present endogenously derived peptide to the antigen receptor of T cells. Class I molecules bind CD8 accessory molecules, so that CD8+ve T cells are class I restricted and are therefore predominantly involved in the recognition of endogenous peptides.

The HLA class II genes are located in the centromeric end of the HLA complex on chromosome 6. Expressed genes are divided into three major subregions termed HLA-DR, -DQ and -DP. Each of these subregions contain at least one A and one B gene that encode for an α- and a β-chain respectively. These chains form a heterodimer that is expressed primarily on B lymphocytes, macrophages, dendritic cells, haemopoietic progenitor cells and activated T cells. They can also be induced and upregulated on various cell types including intestinal epithelial cells by cytokines such as interferon-γ (Sollid et al. 1987; Volk et al. 1989). In contrast to class I molecules, class II bind the CD4 accessory molecule and thus CD4+ve T cells are predominantly class II restricted and are involved in the presentation of exogenously derived antigens.
The DR loci are located at the telomeric end of the class II region. DRA is a monomorphic gene encoding all the DR α-chains. DRB1 is the most polymorphic gene determining most of the primary DR specificities, with 59 alleles currently recognised (Marsh et al. 1992). DRB2 is similar to DRB1 but is incapable of expression and is therefore termed a pseudogene. DRB3 is polymorphic and 4 alleles are described. DRB4 is monomorphic whilst 4 alleles are recognised at the DRB5 locus. Each haplotype does not contain more than three DRB loci i.e. DRB1, DRB2 and either DRB3, DRB4 or DRB5, or none of the final three.

The HLA-DQ subregion consists of two polymorphic genes, DQA1 and DQB1, which encode for an α- and a β-chain, the combined product being the DQ antigen. DQA2 and DQB2 are pseudogenes. Thirteen alleles are described at the DQA1 locus and 19 at DQB1 (Marsh et al. 1992). Since both of the expressed DQ genes are polymorphic, four different potential DQ antigens might be produced. The two pairs of genes inherited direct from the parents, each pair on a single chromosome i.e. in cis arrangement, could each produce a different expressed antigen. Alternatively, the paternal DQA1 product could combine with maternal DQB1 product, and vice versa, to give a further two different possible antigens, each α- and β-chain encoded from separate chromosomes, i.e. in trans arrangement. Such trans complementation does occur and may have importance in the aetiology of coeliac disease (Lundin et al. 1990).

The HLA-DP subregion contains the genes for the DP antigen, DPA1 and DPB1, and two pseudogenes, DPA2 and DPB2. Eight alleles are recognised at the DPA1 locus and 32 at the DPB1 locus (Marsh et al. 1992).

X-ray crystallographic studies of HLA class I molecules reveal that the membrane-distal domains form a peptide binding groove composed of a floor of eight anti-parallel β-strands and walls of two α-helices (Bjorkman et al. 1987). Based on these studies, a model has been proposed for the structure of class II molecules that is analogous to the structure of class I (Brown et al. 1988). The polymorphism of class II molecules is produced by variations in the amino-acid residues in the membrane-distal domains. Most of these are directed towards the antigen binding groove with some
assumed to point towards the T cell receptor. The crystallographic studies on class I molecules have demonstrated that amino-acid polymorphisms alters pockets within the peptide binding groove which provides a structural explanation for allelic specificities in peptide binding (Garrett et al. 1989).

**HLA Studies in Coeliac Disease**

Two independent reports appeared in 1972 of an association between HLA-B8 and coeliac disease with 88% of patients but less than 30% of controls carrying the marker (Falchuk et al. 1972; Stokes et al. 1972). A stronger association was demonstrated between HLA-DR3 and the condition, 96% of patients compared to 27% of controls (Keuning et al. 1976). It was concluded that the major association was with DR3, that with B8 being due to linkage disequilibrium. HLA-DR7 has also been reported to be associated with coeliac disease (Albert et al. 1978) but predominantly in the Southern European populations, where most patients are either DR3 or DR5/DR7 heterozygotes, compared to Northern European populations where most patients are DR3 positive (Mearin et al. 1983). DR3 appears to confer susceptibility with almost any other DR type, whereas DR7 only confers susceptibility in conjunction with DR3 or DR5 (Mearin et al. 1983).

An association with DQ2, that was stronger than that with DR3, was reported in 1983 (Tosi et al. 1983). DQw2 was found to be in linkage disequilibrium with DR3 and DR7. In an important study, Sollid and colleagues reported that coeliac disease in 99% of patients was primarily associated with a particular pair of DQA1 and DQB1 genes either positioned in cis or trans arrangements (Sollid et al. 1989a). The DQA1 gene of the DR5, DQ7 haplotype is identical to the DQA1 gene of the DR3, DQ2 haplotype, and the DQB1 gene of the DR7, DQw2 haplotype differs only by a single amino-acid in the second domain from the DQB1 gene of DR3, DQ2 haplotype. Thus an almost identical DQ2 molecule could be expressed by DR5, DQ7/DR7, DQ2 heterozygotes as that expressed by DR3, DQ2 individuals. They extended their studies showing that three alloreactive T cell clones could recognise the DQA1*0501,
DQB1*0201 heterodimer whether encoded in cis or trans arrangements, which provided further evidence for the role of this particular molecule in the aetiology of the condition (Lundin et al. 1990).

The presence of the DQA1*0501, DQB1*0201 heterodimer provides a very strong association with coeliac disease but it clearly does not confer disease since almost a quarter of the normal population carry these genes and approximately 5% of coeliac patients carry DR4, and are DR3 and DR7 negative (Solli et al. 1989b). Thus the possible associations of coeliac disease with other gene loci assumes importance.

In 1986 a DP restriction fragment length polymorphism was reported to be associated with the condition (Howell et al. 1986). An increase of the DPB1*0402 and DPB1*0301 alleles among Italian (Bugawan et al. 1989) and an increase in the DPB1*0101 and DPB1*0301 alleles among American patients has been reported (Howell et al. 1988). A study of British and Irish families concluded that a DP polymorphism provided an independent risk factor for coeliac disease (Caffrey et al. 1990). However, linkage between DP and DQ was not examined and the absence of DR3 positive control subjects makes interpretation of this data difficult. The results of other studies provide conflicting evidence and suggest that whilst coeliac disease is associated with certain DP polymorphisms, the association is not independent and due to linkage disequilibrium (Bolsover et al. 1991; Hall et al. 1990; Rosenberg et al. 1989). This conclusion is supported by the fact that the DP polymorphisms reported to be associated with the condition vary in the different ethnic populations studied.

The question of whether a unique DR, DQ or DP structural variant is associated with coeliac disease arises. Sequence analysis of DRB, DQA, DQB, DPA and DPB genes from coeliac patients encoding the membrane distal domains did not reveal any disease specific sequences suggesting that patients carry normal allelic variants of HLA molecules (Kagnoff et al. 1989).

Strong evidence exists that the DQA1*0501, DQB1*0201 heterodimer confers susceptibility to coeliac disease but how does this molecule mediate its effect? HLA molecules influence the T cell repertoire (see below) and it is possible that possession
of the DQA1*0501, DQB1*0201 encoded molecule affects T cell repertoire selection either within the thymus or extra-thymically, perhaps in the gut. Gliadin could act as a superantigen activating intestinal T cells expressing particular variable region segments. However, this does not seem likely since superantigens bind to the outer surface of class II molecules and coeliac disease susceptibility depends on both a DQα and a DQβ chain. It is unlikely that a superantigen could bind to the outer surface of both chains. Neither does it seem likely that increased expression of this heterodimer could be responsible for the association with coeliac disease since expression of DQ molecules is not increased in the intestine in the condition (Marley et al. 1987). The most likely explanation seems to be that the DQA1*0501, DQB1*0201 heterodimer preferentially binds a particular fragment of gliadin and presents it to specific T cells within the intestine, initiating a locally destructive immune response. The exact nature of the coeliac toxic fragment is of obvious importance to this hypothesis.
Cereal Protein Chemistry

Coeliac disease is activated by dietary exposure to cereals: wheat gluten and a similar group of cereal proteins in rye, barley and possibly oats. Despite considerable investigation the exact nature of the toxic moiety has yet to be established.

CLASSIFICATION OF CEREALS

The cereals belong to the family Gramineae (grasses) of the angiosperms or flowering plants (Fig 1.1). They have been extremely successfully cultivated as basic foodstuffs and are a major source of dietary protein and carbohydrate throughout the world.

The sub-family Pooideae contains two tribes, the Triticeae, within which are the major temperate cereals wheat, rye and barley, and the Panicoideae, within which are the major tropical cereals including maize. Rice is not closely related to the other cereals but may be classified in the subfamily Pooideae, within the tribe Oryzeae.

The taxonomic relationships of the Gramineae are important as they reflect to a considerable degree, the chemical structure of their seed storage proteins and therefore their potential toxicity in coeliac disease.
Wheat grains can be separated into the outer husk or bran, the germ or semolina and the endosperm or flour (Fig 1.2), which usually represents approximately 70% of the total grain weight. Gluten is the cohesive mass that is produced when a ball of dough has been exhaustively worked and washed in tap water (Fig 1.2). The main constituents of wheat flour are starch (70%), protein (7-15%), lipids (1-2%) and water (14%). Wheat endosperm proteins may be categorised into four main classes according to their solubility: albumins, which are soluble in water; globulins which are soluble in salt solutions, but insoluble in water; gliadins, which are soluble in ethanol and glutenins, which are insoluble in neutral aqueous and saline solutions, and ethanol, but are partially soluble in dilute acid or alkali (Osborne 1907) (Fig 1.3).

The ethanol soluble fractions of cereal proteins were termed the prolamins by Osborne, reflecting their high content of proline and amide nitrogen (now known to be derived from glutamine). The prolamins from different species have been given trivial names, including gliadins from wheat, secalins from rye, hordeins from barley, avenins from oats and zeins from maize. The ethanol insoluble fractions were collectively termed glutelins, which include glutenins from wheat.
Introduction

Many of the proteins present in the glutelin fraction of cereals are closely related to the ethanol soluble prolams but are not soluble in ethanol because they form high molecular mass aggregates stablised by intermolecular disulphide bonds. Due to the similarities between these two classes of cereal proteins, it is now usual to consider the glutelins as prolams as well.

The Prolamins

Wheat prolams will be considered predominantly. The gliadins are a group of monomeric storage proteins that comprise, in wheat, approximately 50% of endosperm protein by weight. The gliadin fraction of any particular wheat variety consists of between 30–50 different proteins (Mecham et al. 1978), which may be classified into four groups on the basis of their electrophoretic mobility, namely α-, β-, γ- and ω-, in order of decreasing mobility (Woychik et al. 1961). Whole gliadin comprises approximately 30% each of α-, β- and γ- and 10% of ω-gliadin. Their molecular weights rise from 30KDa to 70KDa from α- through ω-gliadin. The α-, β- and γ-gliadins contain intra-chain disulphide bonds, whereas ω-gliadin contains no cysteine and therefore no disulphide bonds.

The glutenins consist of alcohol soluble monomers which form insoluble polymers when stabilised by inter-chain disulphide bonds. The subunits are classified according to electrophorectic mobility as low molecular weight (LMW) or high molecular weight (HMW).

Since the application of molecular cloning techniques with the deduction of amino-acid sequences from cloned cDNA's, extensive sequence data has become available on all the major prolams. This has allowed an alternative classification of the prolams (fig1.4).
Three major groups of prolams are recognised, the sulphur-rich (S-rich), sulphur-poor (S-poor) and high-molecular weight (HMW). The HMW and S-poor groups consist of the HMW glutenin subunits and \( \omega \)-gliadins respectively. The S-rich group contains three subgroups, the \( \gamma \)-type (\( \gamma \)-gliadins), the \( \alpha \)-type (\( \alpha \)- and \( \beta \)-gliadins) and aggregated-type (with intermolecular bonds). The latter consists predominantly of LMW glutenin subunits, but there is evidence that some \( \alpha \)-type, \( \gamma \)-type and possibly some S-poor subunits, are also present.

Barley and rye also appear to contain S-rich, S-poor and HMW prolams, but neither species contains \( \alpha \)-type S-rich prolams. The major S-rich prolams of barley, B hordeins, are aggregated-type, but some \( \gamma \)-type are also present.

**Amino-Acid Sequences**

There is great variation in the amino-acid sequences and composition of the prolams. There are, however, two common features, they all consist of at least two distinct regions termed domains and one of these domains contains a repetitive sequence of amino-acids.

The S-rich prolams consist of an amino-terminal repetitive domain containing single or interspersed repeats which vary in length and consensus sequence, but which are all rich in proline and glutamine, and a carboxy-terminal non-repetitive domain, containing most of the cysteines, methionines and charged residues. All groups have repeat motifs containing the tetrapeptide PQQP, which is related to the penta-(PQQPY) and octapeptide (PQQPFPQQ) motifs of C hordein. There is no homology, however,
between the S-rich and S-poor prolamins compared to those of the HMW prolamins (Tatham et al. 1990).

The amino-acid sequence of the S-poor prolamins is not yet known but a proposed structure has been deduced by directly sequencing enzymatic digests and from partial cDNA’s encoding the C-terminus. It consists almost entirely of repeats, with only short unique sequences at either terminus. Comparisons with α-gliadins and α-secalins indicate similarities with C hordein.

The HMW subunits of wheat consist of a central repetitive domain of tandem or interspersed repeats, which are particularly rich in glycine with flanking shorter non-repetitive domains. Less is known about the HMW subunits of barley and rye. The repetitive motifs of the HMW subunits are not homologous with those of the S-rich and S-poor prolamins; there are homologies in the non-repetitive domains of the HMW subunits and the S-rich prolamins.

Structure of Prolamins

The primary structure, the amino-acid sequence of the prolamins has been discussed above. Secondary structure refers to the regular local arrangement of the polypeptide backbone which is stabilised by hydrogen bonds between peptide, amide and carbonyl groups. Several types of secondary structure exist including α-helices, β-sheets and β-turns. The spectroscopic techniques of optical rotatory dispersion and circular dichroism are sensitive to the polypeptide backbone conformation and have been used to analyse the prolamins. The latter method involves the use of circularly polarised light, which consists of two components, right and left circularly polarised light. An asymmetric or chiral medium will absorb light of these two components to different extents. The change in extinction of the light when it passes through a medium containing asymmetrical carbon atoms can be used as an indication of the type of asymmetry present in the molecules. Because β-turns do not have a dominant type of spectrum, but exhibit a range of spectral types that are weaker than those of α-helix or β-sheet, their spectra are difficult to determine in the presence of these other types of structure. Predictive methods, such as the probabilistic method of Chou and Fasman,
have also been applied to the analysis of prolamin peptide secondary structure (Chou et al. 1978). These methods estimate the preference of each amino acid for adopting a structural type based on a number of proteins of known structure. On average predictive methods are correct in 60% of cases, and prolamins, because of their repeated sequences, may be incorrectly predicted. These methods, however, can be a useful adjunct to other methods such as CD spectroscopy.

The repetitive domains of the S-rich prolamins are predicted to form β-turns, which has been confirmed by spectroscopic methods for both α-type and γ-type gliadins (Tatham et al. 1990). Regular β-turns may be able to form a helical structure in the γ-gliadins and γ-secalins. The non-repetitive domains of the S-rich and HMW prolamins appear mostly to be globular with tightly-folded conformations consisting of α-helices and β-sheets. There is also evidence of β-turns in the carboxy-terminal non-repetitive domain of A-gliadin, particularly between residues 211-217.

The Prolamins of Oats, Rice and Maize

Oats and rice differ from other cereals in that prolamins account for only approximately 5-15% of the total seed proteins. Oat prolamin, avenin, consists of about 12 fractions, one of which was subjected to Edman degradation demonstrating a single major sequence starting with a triple threonine sequence (Bietz 1983). Related amino-terminal sequences have since been reported in other purified fractions (Egorev 1988; Pernollet et al. 1989). A unique amino-terminal sequence of 20 residues had homology with γ-type gliadins (Burgess et al. 1986).

Electrophoretic analysis of rice prolamins show a major fraction of between 11 and 14kD with minor bands of lower mass (Bietz 1983). Amino-acid sequences deduced from cDNA's revealed proteins of 145 residues (Kim et al. 1988). They did not contain repeat sequences and had no significant homology with prolamins from other species.

The zeins of maize may be electrophoretically split into four groups. The α-zeins contain a repetitive domain consisting of 20 residue blocks, which are degenerate in sequence such that it is impossible to recognise a consensus motif. They have no
obvious relationship to the repetitive motifs present in wheat, barley and rye prolams. The β-zeins do not contain repetitive sequences, but they do have conserved regions related to those present in S-rich and HMW prolams of wheat, barley and rye. The γ- and δ-zeins do not appear to have repetitive sequence homologies that are related those in wheat, barley and rye prolams. The toxicity of cereals in coeliac disease will discussed in Chapter 4.

Pathogenesis of Coeliac Disease

ENZYME DEFECT HYPOTHESIS

This was first proposed by Frazer and colleagues in the 1950's (Frazer 1956). They suggested that a primary deficiency of an intestinal peptidase could lead to the accumulation of an intrinsically toxic peptide resulting in enterocyte damage and villous atrophy. Support for this hypothesis came from the finding that a peptic-tryptic digest of gluten (Frazer's Fraction III) remained toxic to coeliac patients, whereas pre-incubation of gluten with an extract of porcine intestinal mucosal homogenate, or with crude papain, abolished toxicity (Frazer et al. 1959). A further study demonstrated that a specific toxic fraction of gliadin was less efficiently hydrolysed by the mucosa of coeliac children in remission than by controls, suggestive of a primary deficiency (Cornell et al. 1973). However, this hypothesis has not been validated; several studies have shown that gluten digestion by coeliac mucosa is normal, and whilst activities of various intestinal peptidases are deficient in untreated coeliac disease, their activity reverts to normal on treatment (Bailey et al. 1989; Douglas et al. 1970).

The finding, described above, that digestion by purely proteolytic enzymes fails to detoxify gluten, led to the hypothesis that a non-protein component of gluten might be responsible for toxicity (Phelan et al. 1975). It was shown that some carbohydrases could detoxify gliadin and it was therefore suggested that a carbohydrase deficiency could underlie coeliac disease. These, however, results have not been confirmed and it has been shown that A-gliadin, an amino-acid sequenced α-gliadin, contains no carbohydrate moieties and is known to be toxic (Bernadin et al. 1976).
Introduction

It has recently been demonstrated that there is a persistent, abnormal increase in jejunal transglutaminase activity, suggestive of a role for this enzyme in disease pathogenesis (Bruce et al. 1985). If transglutaminase could bind gluten peptides to the enterocyte membrane, it would provide a mechanism whereby anti-gluten mediated immune reactions could be targeted against the enterocyte. However, animal studies suggest that enterocytes contain less than 1% of total jejunal transglutaminase activity which seems to make this particular mechanism unlikely.

There is no convincing evidence of a major perturbation in intestinal enzyme function that primarily leads to coeliac disease; many of the demonstratable abnormalities would appear to be secondary to the intestinal lesion.

THE LECTIN HYPOTHESIS

Lectins are glycoproteins that are widely distributed throughout the plant kingdom. They bind sugar moieties, and because they are polyvalent will precipitate glycoconjugates and agglutinate cells. They preferentially agglutinate tumour cells, transformed cells and trypsinsed normal cells, as well as human foetal intestinal epithelial cells and also select undifferentiated crypt cells from the rat intestine (Shoham et al. 1970). The effect on any particular cell is attributed to the specificity that the lectin has for carbohydrate moieties in the cell membrane. Douglas described a carbohydrate component of gluten that bound to a crude preparation from coeliac intestinal mucosa much more effectively than to a similar preparation from normal intestine although others have been unable to confirm this observation (Douglas 1976). This led to the suggestion that gluten, or a subfraction, would bind to altered cell membrane glycoproteins and act as a enterotoxic lectin (Weiser et al. 1976).

Auricchio et al demonstrated binding of A-gliadin and other peptides derived from toxic prolamin to foetal rat intestine (Auricchio et al. 1984), although other workers have been unable to confirm this finding with a variety of mammalian cells including coeliac enterocytes (Colyer et al. 1986; Colyer et al. 1987). There is no doubt that cereal derived lectins can damage mammalian intestine since it has been shown that
wheat germ agglutinin (WGA) infused into isolated rat jejunum produced extensive microvillous damage and thus a potential toxic role for WGA remains to be determined (Lorenzsonn et al. 1982) However, there is no evidence that gliadin thus bound is directly toxic.

MUCOSAL PERMEABILITY DEFECT HYPOTHESIS

This hypothesis argues that a primary defect in intestinal mucosal permeability underlies coeliac disease. In patients with coeliac disease there is enhanced permeability to larger molecules, while that to smaller molecules is decreased when using orally administered sugars as markers (Menzies et al. 1979). This finding reverts to normal when a gluten-free diet is instituted. Studies by Bjarnason and colleagues, using $^{51}$Cr-EDTA as a marker, found a persistent defect in intestinal permeability in 79% of coeliac patients who were in remission after treatment with a gluten-free diet for more than six months and up to twenty three years (Bjarnason et al. 1985; Bjarneson et al. 1983). These workers argued that enhanced entry of gluten peptides through the coeliac intestinal epithelium might initiate enterocyte damage and activate the cascade of immunological events that characterise coeliac disease. However, such alterations in intestinal permeability are not specific to coeliac disease and so their persistence in the treated individual may merely represent continuing subtle damage. If they were a primary abnormality, it would remain to be explained why gluten itself is toxic to certain individuals.

ADENOVIRUS 12

There is clearly an important genetic susceptibility to coeliac disease that is most closely associated with the genes that encode a particular HLA-DQ heterodimer (Sollid et al. 1989b). However, the discordance for the disease among monozygotic twins (Polanco et al. 1981) and the fact that less than 1% of people with the documented HLA susceptibility markers develop the disease suggest that enviromental factors other than gluten are involved in the aetiology of the condition.
In a study published in 1984, Kagnoff and colleagues undertook a database search of known protein structures, looking for homologies with A-gliadin (Kagnoff et al. 1984). A sliding segment to detect eight or more amino-acid identities over twenty residues detected only eleven proteins with some homology and only two with a tetrapeptide identity. The region of homology between A-gliadin and the early region E1b protein of human adenovirus 12 appeared important. The region included eight identities over a span of twelve amino-acid residues and had an identical penta-peptide identity (Fig 1.5).

**Fig 1.5**

<table>
<thead>
<tr>
<th>Ad12 E1b</th>
<th>384</th>
<th>395</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Arg-Arg-Gly-Met-Phe-Arg-Pro-Ser-Gln-Cys-As</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| 206 | 217 |
| Leu-Gly-Gln-Gly-Ser-Phe-Arg-Pro-Ser-Gln-Gln-As |
| A-Gliadin |

Human adenovirus 12 (Ad12) may be isolated from the intestine and is one of several human adenoviruses that can cause malignant transformation in infected mammalian cells. The transforming region of the viral genome consists of two genes termed early regions E1A and E1B, which encode non-structural proteins that are involved in the initiation of replication. Cross-reactivity was shown between antisera raised against Ad12 transformed baby rat kidney cells that were expressing the 54 kD E1b protein and A-gliadin, a 119 cyanogen bromide fragment of A-gliadin containing the region of homology and a synthetic heptapeptide from within the region of homology (Kagnoff et al. 1984). It was suggested that an encounter of the immune system with a protein produced during intestinal viral infection may be important in the pathogenesis of coeliac disease because of immunological cross-reactivity between the viral protein and dietary gliadin.
Kagnoff and colleagues went on to show that coeliac patients had significantly raised titres of neutralising antibody against the coat proteins of Ad12 (Kagnoff et al. 1987). These antibodies were not directed against the 54kD E1b protein which is synthesised in infected cells but is not incorporated into virus particles. There was therefore no evidence of an immune response directed against the 54kD E1b protein which shares the region of homology with A-gliadin.

Support for the involvement of Ad12 in the pathogenesis of coeliac disease has come from studies of the peripheral cellular immune response of coeliac patients to synthetic peptides from the region of homology between the E1b protein and A-gliadin (Karagiannis et al. 1987; Mantzaris et al. 1990). The same group undertook an in vivo challenge study, using a synthetic dodecapeptide (residues 206-217 of A-gliadin) from the region of homology and found some, but not clear cut, evidence of toxicity in the two coeliac patients studied compared to two control subjects (Mantzaris et al. 1991).

No evidence exists however, of a specific immune response against the 54kD E1b protein of Ad12 in coeliac patients (Dieleman et al. 1991; Howdle et al. 1989). Similarly there is no evidence of significant, persistent infection with Ad12, by Southern blot or polymerase chain reaction analysis using oligonucleotide primers specific for the E1B gene of Ad12, in the small intestinal mucosa of patients with coeliac disease. Although an initially attractive hypothesis, the verdict on the possible involvement of Ad12 in the pathogenesis of coeliac disease must remain that of “unproven”. 
The Mucosal Immune System and Coeliac Disease

Many factors suggest the involvement of immunological mechanisms in the pathogenesis of coeliac disease and the characteristic intestinal lesion. Gliadin shock, the response of patients to corticosteroids, the infiltrate of intraepithelial lymphocytes and plasma cells in the lamina propria of jejunal mucosa, hyposplenism, the development of lymphomas, an increased incidence of auto-immune diseases and the presence of anti-gliadin antibodies all suggest involvement of the immune system. It is useful to review briefly the the normal intestinal mucosal immune system before considering the immune mechanisms that may be functional in coeliac disease.

THE NORMAL INTESTINAL MUCOSAL IMMUNE SYSTEM

The gastrointestinal immune system provides protection from the environment at a particularly vulnerable site, where only a single epithelial layer separates the gut lumen from the tissues. The bowel contains large numbers of immunocytes which are present both in organised aggregates, such as Peyer’s Patches, and as single cells in the epithelium and lamina propria. Changes occur in the mucosal immune system in disease, suggesting that immune mediated reactions may be involved in the pathogenesis of certain conditions. It seems likely that there are complex interactions in the local immuno-epithelial microenvironment that can lead to structural change and damage to the intestine.

Organised Lymphoid Tissue–Peyer’s Patches

Throughout the intestine they are aggregates of well defined, organised lymphoid tissue called Peyer’s patches (PP). PP, similar to other secondary lymphoid tissue, have a well defined zonation. A follicle centre is usually surrounded by a thin mantle of small lymphocytes, which in turn merges into the dome. The dome area contains plasma cells, dendritic cells, macrophages and B cells which infiltrate the overlying epithelium (Spencer et al. 1986). The specialised epithelium adjacent to the PP, follicle-associated epithelium (FAE), does not form into crypts or villi and consists of cuboidal epithelial cells with few goblet cells. The FAE also contains specialised cells
with short, irregular microfolds on their luminal surface and are thus termed M cells
(Owen et al. 1974). M cells do not express HLA class II antigens and are therefore
unlikely to be involved in classical antigen presentation (Bjerke et al. 1988). They do
contain, however, numerous tubules, vesicles and vacuoles which probably function as
a translocation system allowing direct sampling of antigen from the gut lumen by
pinocytosis (Bye et al. 1984). T and B cells, plasma cells, dendritic cells and
macrophages populate the sub-epithelial area suggesting this area is
important in antigen recognition (Parrott et al. 1974).

Lamina Propria

The lamina propria is infiltrated by large numbers of immuno-competent cells.
Lymphocytes are present in large numbers of which almost half are B cells and plasma
cells. IgA plasma cells constitute approximately 30% of the total mononuclear cells and
almost 80% of the total plasma cells, accounting for the dominance of IgA
immunoglobulin isotype secretion in the gut. IgM (20%) and IgG (5%) secreting
plasma cells make up the remainder with almost no IgD or IgE secreting cells being
present. Most of the IgA secretion is dimeric and is bound via J chain/secretory
component interaction before being actively transported into the lumen of the gut
(Brandtzaeg et al. 1987; Brandtzaeg et al. 1989; Brandtzaeg et al. 1988).

T cells comprise almost half the lymphocyte population of the lamina propria, of
which approximately two-thirds are CD4+ (helper/inducer) and one-third CD8+
(suppressor/cytotoxic) (Janossy et al. 1980; Selby et al. 1983b). In humans, most of
the lamina propria T cells do not express the activation markers HLA-DR and
interleukin-2 receptor (IL2-R) (Hirata et al. 1986; Selby et al. 1983a). The majority of
the HLA-DR+ cells in the lamina propria, are small stellate cells with the characteristics
of dendritic cells, although the distinction between dendritic and macrophage cell types
in the intestine is not clear (Selby et al. 1983a). These cells are likely to be antigen
presenting cells.
Epithelium

The lymphocyte population in the epithelium of the intestine, the intraepithelial lymphocytes (IEL), unlike the lamina propria consists entirely of T cells. A further distinction from the lamina propria, is the striking predominance of CD8+ cells among the IEL, such that approximately 90% of the IEL carry this marker (Cerf-Bensussan et al. 1987; Janossy et al. 1980; Selby et al. 1983b). Equal proportions of IEL express different isoforms of the surface antigen CD45RA/RO, which suggests some of them may be antigen-primed memory cells (Brandtzaeg et al. 1989). Most IEL are negative for markers of recent activation such as HLA-DR and IL2-R (Malizia et al. 1985; Selby et al. 1983a), nor do they respond well to mitogens although their proliferation can be increased via the CD2-sheep red blood cell receptor (Ebert 1989).

The origin of the IEL is controversial. The IEL probably come from the lamina propria, since increased numbers of IEL is accompanied by increased lymphocyte density in the lamina propria (Monk et al. 1988). IEL are normally found just above the basal lamina, and appear to be crossing it in either direction (Marsh 1975). Clearly, there are mechanisms controlling the movement of IEL into and out of the epithelium, since if it were a random event, the ratio of T cell subsets would reflect that found in the lamina propria, which is does not. This subject and that of IEL expressing the y/δ form of the T cell receptor will be expanded upon in Chapters 4 and 5.

Humoral Immune System in Coeliac Disease

Local Intestinal Humoral Immunity

Specific

One of the major immunopathological features of coeliac disease is an increase in the number of immunoglobulin producing cells in the lamina propria, of all three major classes (Baklien et al. 1974; Jenkins et al. 1989; Scott et al. 1984a; Scott et al. 1980). In untreated disease, the average increase of IgA, IgM and IgG producing cells is, respectively, 2.4, 4.6 and 6.5 times. In treated disease, the numbers of Ig-producing cells lay between those in with untreated disease and controls, at least in adults (Baklien
et al. 1977). In children, there is some evidence that Ig-producing cell numbers may normalise after institution of a gluten-free diet (Scott et al. 1980). Secretory component expression is enhanced, in agreement with in vitro studies which have demonstrated cytokine mediated upregulation of secretory component, but IgA and IgM epithelial external transport is normal (Baklien et al. 1977; Brandtzaeg et al. 1989; Kvale et al. 1988).

An immunohistochemical study of jejunal mucosa from adult coeliacs found a larger percentage of IgG-producing cells (5.7%) were producing gluten specific antibodies compared to IgA-producing cells (1.6%), suggesting a locally imbalanced response to gluten (Brandtzaeg et al. 1976). Increased production of gliadin-specific antibodies has been found in the medium of jejunal biopsy cultures from coeliac patients compared to controls (Ciclitira et al. 1986a) and also from studies of isolated intestinal lymphocytes maintained in culture (Ciclitira et al. 1989; Crabtree et al. 1989).

IgG1 is the major IgG subclass expressed by Ig-producing cells in the jejunal mucosa in coeliac disease, whatever the dietary status, but IgG2-producing cells are significantly increased in untreated compared to treated patients and therefore may reflect better the local mucosal immune response (Rognum et al. 1989). It is unlikely, however, that IgG2 antibodies contribute to the development of the mucosal lesion since this subclass shows little complement-activating capacity or Fc receptor binding.

The increased numbers of IgA-producing cells found in coeliac disease is mainly due to increases in the IgA2 subclass, of which there was a high frequency of J chain expression (Kett et al. 1990). These facts and the increased epithelial expression of secretory component (Baklien et al. 1977) suggest that there is an upregulated state of secretory immunity in coeliac disease. Kett and colleagues suggested that since the antimicrobial effect IgA2 may be greater than that of IgA1, the immunoregulatory shift favouring IgA2 production in coeliac disease may contribute to antimicrobial protection of the intestinal mucosa.

Increased concentrations of IgA antibodies, with a high frequency of J chain expression, have been found in jejunal aspirates from coeliac patients (Labrooy et al.
1986; Volta et al. 1988). The antibody titres were related to the severity of the mucosal lesion and declined more slowly than those from the serum following initiation of a gluten-free diet. Recently, Ferguson's group have been studying local humoral responses by means of intestinal lavage with polyethylene glycol solutions (O'Mahony et al. 1991; O'Mahony et al. 1990a). Untreated coeliac patients had higher IgA and IgM anti-gliadin titres than treated and control patients, both in jejunal aspirates and lavage solutions. The raised IgM anti-gliadin titres persisted in the treated patients. This highlighted a dissociation between the mucosal and systemic immune response in these patients. Increased titres of IgA and IgG anti-gliadin antibodies have been detected in salivary fluid, although they don't appear to correlate with titres in jejunal aspirates (al-Bayaty et al. 1989; O'Mahony et al. 1991).

Non-Specific

Early studies attempted to demonstrate complement activation in the intestinal lesion but conclusive evidence was not produced (Gallagher et al. 1989; Scott et al. 1977; Shiner et al. 1972), perhaps because of methodological problems (Baklein et al. 1974). The application of monoclonal antibodies specific for C3b/C3c (early activation) and terminal complement complex (TCC, late activation), in immunohistological studies have produced valuable data (Halstensen et al. 1992). Subepithelial deposition of TCC was observed in 93% of untreated coeliac patients compared to 10% of controls and was significantly correlated with the degree of villous atrophy. Gluten challenge increased subepithelial TCC and produced additional C3b deposition, suggesting recent complement activation. The authors concluded that Ig-mediated subepithelial complement activation, following the ingestion of gluten, might lead to epithelial damage.

Systemic Humoral Immunity

Immunoglobulins

Abnormal serum levels of immunoglobulins is a well described phenomenon in untreated coeliac disease. Raised IgA titres are commonly found in children and adults (Asquith et al. 1969; Kenrick et al. 1970), which tend to drop after gluten withdrawal
(Baklien et al. 1977). IgG is normal in most patients whilst a low serum IgM is commonly, but not universally, reported.

**Specific**

Serum antibodies to various food antigens have been found in coeliac patients (Ferguson et al. 1972), but reactions against gluten or its derivatives is the most constant finding (Scott et al. 1984b; Unsworth et al. 1981). High titres of serum IgA and IgG anti-gliadin antibodies are present in untreated coeliac patients (O'Farrelly et al. 1983; Savilahti et al. 1983; Volta et al. 1983). In treated coeliacs IgA anti-gliadin levels are similar to controls, but IgG anti-gliadin levels persist at an intermediate level between untreated patients and controls, even when there has been complete gluten exclusion and mucosal healing (Savilahti et al. 1983; Unsworth et al. 1981). It has been proposed that IgA anti-gliadin titres can be used to monitor dietary compliance, however, in patients maintained on a low gluten, as opposed to gluten-free, diet, serum IgA anti-gliadin titres were within normal limits (Montgomery et al. 1988).

**Non-Specific**

Evidence exists for the presence of circulating immune complexes and hypocomplementaemia in coeliac disease (Doe et al. 1975; Mowbray et al. 1973). The only antigen definitely shown to be associated with such immune complexes is a 90-kDa glycoprotein which is a constituent of both intestine and skin (Maury et al. 1986). There does not seem to be consistent evidence of a role for circulating immune complexes in the pathogenesis of the mucosal lesion.

**Diagnosis of Coeliac Disease**

**Anti-Gliadin Antibodies**

There is an extensive literature on anti-gliadin antibodies in the diagnosis and in screening for coeliac disease, which has recently been reviewed (Troncone et al. 1991). The sensitivity of the assays are very high although they don't achieve 100%. IgG anti-gliadin antibodies are more sensitive but less specific than IgA antibodies. Age is an important factor as the sensitivity of the test declines with increasing age. Studies from
Sweden found raised titres in 100% of untreated children, but only 67-78% of adults (Kilander et al. 1985; Stenhammar et al. 1984).

**Anti-Reticulin and Anti-Endomysial Antibodies**

The presence of serum antibody to reticulin has been demonstrated in coeliac patients and proposed as a diagnostic test (Seah et al. 1973). However subsequent studies have shown that the test is of little diagnostic value with low sensitivities reported compared to anti-gliadin antibodies (Lazzari et al. 1984; Monteiro et al. 1986), although the specificity is high (Maki et al. 1984; Unsworth et al. 1983).

Very high sensitivity and specificity has been reported for IgA anti-endomysial antibodies in coeliac patients which may prove to be useful in the diagnosis and screening for the condition (Chorzelski et al. 1984; Hallstrom 1989; Unsworth et al. 1994).

**Humoral Mechanisms in the Pathogenesis of Coeliac Disease**

Humoral mechanisms appear to have a role, although cellular immunological mechanisms probably are more fundamental to the pathogenesis of coeliac disease. The dominant mucosal humoral response is a typically enhanced secretory IgA and IgM production. Overstimulation of the B cell system leads to an aberrant, enhanced local and systemic IgG response to both gluten and other dietary antigens (Brandtzaeg et al. 1989; Scott et al. 1985). The local IgG response could lead to antibody-dependant cytotoxic mucosal damage. Complement activation has been demonstrated immunohistologically (Halstensen et al. 1992), and the timing of histological relapse following gluten challenge supports the involvement of antibody-dependant cytotoxic mechanisms in coeliac disease (Bramble et al. 1985). The secondary nature of humoral mechanisms in coeliac disease, however, is emphasised by the report of the condition occurring in a patient with common variable hypogammaglobulinaemia (Webster et al. 1981).
Introduction

CELLULAR IMMUNE SYSTEM IN COELIAC DISEASE

Mucosal T Cells in Coeliac Disease

Intra-Epithelial Lymphocytes

The quantification of IEL has been an inappropriate area of controversy. IEL have been quantified in terms of their density per 100 enterocytes (Ferguson 1977; Ferguson et al. 1971), their density per millimetre of epithelium (Holmes et al. 1974), or their absolute number per unit volume of epithelium (Corazza et al. 1984; Marsh 1980). Different methods have led to different conclusions. The density of IEL per 100 enterocytes is increased in untreated coeliac disease and to a lesser degree in treated coeliac disease compared to controls. In contrast, the absolute number of IEL is decreased as surface epithelial volume is decreased, in the established coeliac lesion.

There is no doubt, however, that in the early "infiltrative" coeliac lesions, where there are little or no changes in the mucosal architecture, that both the density and the absolute numbers of IEL are increased, and that IEL numbers are responsive to gluten within the intestine either as part of the diet or in experimental challenges (Leigh et al. 1985). In the more advanced lesions, the IEL become larger, increase their mitotic rate and show blast transformation (Marsh 1980; Marsh 1982).

Initial studies suggested that the phenotypic characteristics of the small intestinal IEL in coeliac patients were essentially similar to those of normal subjects (Selby et al. 1983b). It has subsequently been shown, using multiple label immunostaining, that the proportion of IEL expressing CD45RO, a marker of antigen priming, was found to be increased from 50% in controls to 75% in untreated coeliac patients (Halstensen et al. 1990). This tended to revert to normal in the treated coeliac patients. Studies have also revealed that there is an expanded population of CD3+, CD4-, CD8-, so called "double negative", IEL in coeliac patients (Spencer et al. 1989b). An expansion of the IEL population expressing the γδ TCR, which might represent this expanded population of CD3+, CD4-, CD8- IEL, within the intestine of coeliac patients had been suggested in preliminary studies (Spencer et al. 1989a).
Introduction

The function of IEL remain poorly understood. Infiltration of the epithelium by IEL is a marker of a cell-mediated response (Ferguson 1987), but IEL do not appear to be inherently destructive to surrounding enterocytes since the enterocytes that line the small intestinal crypts in the established coeliac lesion, which are infiltrated by increased numbers of IEL, show no evidence of damage (Marsh et al. 1986). The role of IEL in coeliac disease will be discussed in greater detail in Chapter 3.

Lamina Propria Lymphocytes

The IEL are involved in the coeliac lesion, although their role is unclear. What of the other major mucosal population of lymphocytes, the lamina propria lymphocytes? Studies suggest that in coeliac disease there is little or no difference in the ratio of CD4+ to CD8+ T cells in the lamina propria of the intestinal mucosa to that of controls (Freedman et al. 1987; Griffiths et al. 1988; Lancaster-Smith et al. 1975; Selby et al. 1983b). There does not appear to be a major difference in the absolute numbers of lamina propria T cells either, apart from from the study of Griffiths and colleagues who reported a fifty-fold increase in the numbers of lamina propria T cells in coeliac patients compared to controls, although it was not clear, how the lamina propria T cells were quantified (Griffiths et al. 1988). Limited evidence also exists that the lamina propria T cells are activated in coeliac disease (Griffiths et al. 1988; Malizia et al. 1985).

Non-Specific Cellular Mechanisms

Eosinophils and Mast Cells

It is known that the eosinophil population of the intestinal mucosa is expanded in coeliac disease (Marsh et al. 1985). Eosinophil granule proteins, such as eosinophil cationic protein (ECP) and major basic protein (MBP) may be cytotoxic to a variety of cells. Two groups have recently expanded studies in this area, demonstrating release of MBP and expression of interleukin-5 mRNA by intestinal eosinophils in coeliac patients (Colombel et al. 1992; Desreumaux et al. 1992; Talley et al. 1992). Receptors for IgA have been reported on human eosinophils and IgA is a potent signal for eosinophil degranulation. It was suggested that the formation of IgA-gliadin complexes in the intestinal mucosa could lead to eosinophil degranulation with local release of
cytotoxic basic proteins with resultant tissue damage (Colombel et al. 1992). Using the an alternative approach of balloon-isolated jejunal perfusion experiments, Lavo and colleagues have reported increased concentrations of granule constituents from eosinophils, mast cells and basophils in coeliac patients (Lavo et al. 1989).

In the small intestinal mucosa, mast cell populations are markedly increased in untreated coeliac and tend towards normal following gluten restriction (Marsh et al. 1985; Strobel et al. 1983). Gluten challenge, both in the jejunal and rectal mucosa is associated with an apparent rapid decrease in mast cell numbers (Horvath et al. 1989; Loft et al. 1989), which is presumed to be due to mast cell degranulation and hence loss of recognisable cytoplasmic granules used in quantitation.

The eosinophil and mast cell both contain a variety of potentially tissue-damaging substances. The studies described above suggest they may be involved in the pathogenesis of the coeliac intestinal mucosal lesion, particularly in the early phases. Although their role has been discussed under the heading of non-specific cellular mechanisms it is possible that there is a major component to their activity that is dependent on antigen-specific recognition of gliadin either by mucosal immunoglobulin or T cells.

Cellular Mechanisms in the Pathogenesis of the Coeliac Intestinal Lesion

In consideration of the immune cellular mechanisms that might contribute to the pathogenesis of the coeliac intestinal lesion it is useful to examine experimental studies of in vitro enteropathy before the in vivo situation is addressed.

Experimental Studies

The intestinal mucosal lesion of coeliac disease resembles that of experimental and human graft-versus-host disease (GVHD) in many ways. The villous flattening and crypt hypertrophy, increased rate of crypt cell production and migration, the presence of CD4+ and CD8+ surface and crypt IEL, many demonstrating blast-transformation and a raised proliferative rate, the expression of MHC class II antigens by enterocytes and reductions in disaccharidase enzyme activity per cell, all bear similarities to the mucosal lesion in coeliac disease. It has been suggested that coeliac disease might
actually represent a graft-versus-host reaction initiated by gluten, or one of its fractions, combining with a mucosal immunocyte which is subsequently recognised by the host as foreign (Neild 1981).

The intestinal graft-versus-host reaction is driven by MHC class II incompatibilities, and T cells play a major role (Ferguson et al. 1975; Ferguson et al. 1973; MacDonald et al. 1977). CD4+ T cells appear to be more important than CD8+ T cells in the development of enteropathy. Experimental murine GVHD is only seen if unseparated T cells are given from a donor differing at the class II loci and in experiments with complete MHC mismatches, purified CD4+ T cells are more effective than CD8+ cells in inducing enteropathy (Guy-Grand et al. 1986; Mowat et al. 1986).

The pathogenesis of experimental GVHD, therefore, appears to be mediated by T cells, with the CD4+ subset predominating. Cytotoxic lymphokine production by CD4+ cells is probably the main mechanism of damage (Guy-Grand et al. 1986). It is unrelated to cytotoxic T cells, cytotoxic antibodies, or natural killer cells (Borland et al. 1983; Ferguson 1987; Guy-Grand et al. 1986; Mowat et al. 1986).

Immune rejection of intestinal allografts also occur as a result of cell mediated reactions. A useful model to study this process is provided by the heterotopic transplantation of foetal mouse intestine under the kidney capsule, which ensures that the graft is both antigen and bacteria free (Ferguson et al. 1972). The histological features of intestinal allograft rejection include marked mononuclear cell infiltration of the lamina propria, crypt hypertrophy, villous atrophy and epithelial cell damage, associated with increased crypt epithelial cell proliferation (MacDonald et al. 1976; MacDonald et al. 1977). These experimental studies demonstrate that local cell-mediated responses within the intestinal mucosa can produce morphological changes that are characteristic of coeliac disease.

MacDonald and colleagues have extended these experimental studies into human tissue by developing a model of cultured human foetal intestine (MacDonald et al. 1988). Explants of foetal small intestine can be maintained in culture for extended periods and the T cells within the explants activated by culture with the lectin pokeweed
mitogen (PWM) or anti-CD3. Activation of T cells, and macrophages was demonstrated by increased expression of CD25, the IL-2 receptor. The activated T cells in the lamina propria were CD4+ and few activated CD8+ IEL were seen. The explant supernatants cultured with PWM were found to contain increased, albeit low, levels of the T cell products IL-2 and interferon-γ. Marked effects were seen on the morphology of the explants with villous flattening, crypt hypertrophy and crypt cell hyperplasia. The addition of cyclosporin A, which inhibits T cell responses, prevented these effects. Interestingly, the addition of IL-2 and IFN-γ to the explants did not produce any enteropathic change.

This model, examining the effects of T cell activation on foetal intestine, closely resembles the intestinal lesion of coeliac disease. Unlike coeliac disease, however, there was no epithelial cell damage in the in vitro system which is an important difference and cautions against a complete extrapolation from one system to another. The foetal model uses immature gut that is sterile, has not been exposed to dietary antigens and is activated polyclonally rather than by antigen-specific activation, as is presumed although little evidence exists, in the coeliac lesion.

These studies show that epithelial cell damage is not necessary for the development of a flat mucosa. Crypt cell hyperproliferation is an important early event in the development of morphological change and occurs before any obvious villous atrophy, certainly in vitro (Ferreira et al. 1990). Is there any evidence for this in vivo?

The Intestinal Mucosal Lesion of Gluten Sensitivity

The classic intestinal histological lesion of coeliac disease, the flat mucosa or villous atrophy, is well recognised. It has been argued, by Marsh in particular, that terms such as villous atrophy are inadequate to describe fully, the potential range of mucosal abnormalities that may be present in gluten sensitive patients (Marsh 1992).

In vitro models described above have been used to demonstrate experimental villous effacement and crypt hypertrophy. These studies established that activation of T lymphocytes can mediate morphological changes within the mucosa and induce infiltration of the small intestinal epithelium by lymphocytes. It is of note that the
less severe GVHD reactions closely resemble mucosal lesions seen in patients with dermatitis herpetiformis and relatives of patients with coeliac disease, suggesting that there is a continuum of potential mucosal change within the intestine that may be mediated immunologically.

The careful studies of Marsh and his colleagues using techniques of computer-aided image analysis and graduated gluten challenge have been of great importance in this area. He has proposed that a range of dynamically interrelated mucosal lesions exist within the spectrum of intestinal gluten-sensitivity (Marsh 1992) (Fig. 1.6.)

Fig 1.6

![Diagram of mucosal lesions](image)

Pre-Infiltrative (Type 0)  Infiltrative (Type 1)  Hyperplastic (Type 2)  Destructive (Type 3)

Marsh has characterised five histological lesions of the jejunal mucosa as a result of gluten-sensitivity:

**Type 1.** The infiltrative lesion – this consists of normal mucosal architecture, with a marked infiltration of non-mitotic IELs. This is the classic lesion of DH (Fry et al. 1972), occurring in approximately 40% of DH patients and is gluten-dependent as shown by the decline in lymphocytic infiltrate following gluten-restriction (Marsh 1989). In a study of relatives of patients with coeliac disease from the northwest of England, 25% of first degree relatives of coeliac patients demonstrated such lesions. (Marsh et al. 1990). This lesion was not associated with clinical symptoms, malabsorption or increased intestinal permeability as assessed by $^{51}$Cr-EDTA. In addition, oral gluten challenge may induce a similar lymphoid infiltrate, in a dose-dependent fashion (Freedman et al. 1987; Leigh et al. 1985).
Type 2. The hyperplastic lesion – this lesion is similar to the type 1 lesion with the additional development of crypt hyperplasia, and is found in approximately 20% of DH patients (Marsh 1989) and is also inducible by gluten challenge (Leigh et al. 1985). The importance of this lesion to the understanding of the pathogenesis of the mucosal lesion is that it presumably represents the early response of the mucosal architecture to immune activation, namely crypt hypertrophy. The demonstration of this lesion supports the in vitro evidence that crypt hypertrophy is an early event in the morphological changes seen in the coeliac lesion.

Type 3. The destructive lesion—the classic established coeliac disease lesion, with loss of villous architecture and infiltration of the lamina propria by lymphocytes and plasma cells in addition to crypt hyperplasia and an increase in the numbers of IELs. This changes are not exclusive to coeliac disease or DH, but may occur in tropical sprue, giardiasis, infantile food sensitivities and experimental GVHD disease and allograft rejection (Ferguson 1987; Marsh 1988). The type 3 lesion occurs in approximately 40% of DH patients and 10-20% of first degree coeliac relatives. This lesion is found in all symptomatic patients, but in most DH patients and almost half the coeliac relatives, such lesions produce no obvious symptoms and therefore, remain subclinical or latent.

Type 0. The pre-infiltrative lesion—in most series of DH patients a small proportion of patients, 5-10%, although presumed gluten-sensitised by virtue of granular IgA deposits at the dermo-epidermal junction, have biopsies that appear indistinguishable from normal (Fry et al. 1972; Gawkrodger et al. 1991; O'Mahony et al. 1990b). Marsh has termed this the Type 0, or pre-infiltrative lesion. It has recently been reported that the characteristic coeliac like intestinal antibody pattern, high concentrations of IgM antigliadin antibody/other IgM class antibodies/IgA antigliadin antibody, in specimens of intestinal fluid and lavage specimens occurs in DH patients without detectable enteropathy (O'Mahony et al. 1990b).

Type 4. In addition to the lesions represented in Fig 1.6, Marsh also described a hypoplastic/atrophic lesion. This represented the atrophic, unresponsive
jejunal lesion found most commonly in association with the onset of enteropathy-associated lymphoma.

It is not clear whether the pre-infiltrative, or the type 1 and 2 lesion always progress remain static or regress. One possibility is that the type 0 and 1 lesions represent a predisposition to coeliac disease and that an additional environmental trigger is required for the development of clinically overt disease.

Summary

The pathogenesis of coeliac disease appears to involve an aberrant, autodestructive response of the intestinal mucosal immune system to certain components of ingested cereals. This thesis attempts to investigate this proposal further by investigation of aspects of the cellular mechanisms involved in the mucosal lesion and of the precise nature of cereal toxicity in coeliac disease. The involvement of adhesion molecules, T cells expressing the γδ receptor and the coeliac toxicity of synthetic gliadin oligopeptides have been studied. These studies are described in the following three chapters.
CHAPTER 2: INTERCELLULAR ADHESION MOLECULE-1 AND COELIAC DISEASE

Introduction

The circulatory and migratory properties of white blood cells have evolved to allow efficient surveillance of tissues for infectious pathogens and rapid accumulation at sites of injury and infection. Localisation of cells can be driven by the interplay between interactions with cell surface and matrix molecules and chemoattractant gradients that direct cell migration. To patrol the body effectively, the cells of the immune system must both circulate as nonadherent cells within the vascular system and migrate as adherent cells through tissues. In the presence of foreign antigen they must be able to congregate in lymphoid organs, cross endothelial and basement membrane barriers to aggregate at sites of infection and inflammation and adhere to cells bearing foreign antigen. Rapid transition between adherent and non-adherent states is of key importance to the dual immune functions of immune surveillance and responsiveness.

It has recently become apparent that homologous families of adhesion molecules have crucial functions in the cell-cell interactions of the immune system (Makgoba et al. 1989; Springer et al. 1987a).

ADHESION RECEPTOR FAMILIES

The Selectins

Selectins are a family of surface molecules possessing three different structural motifs. The term selectin was proposed to highlight the amino-terminal lectin-like domain and to indicate the selective function of these molecules. A standard nomenclature designates each family member according to the cell type upon which it was originally identified: L-selectin (Lymphocytes), E-selectin (Endothelium), P-selectin (Platelets). Selectins possess a single N-terminal (extracellular), lectin domain of 117-120 amino acids. This is followed by an epidermal growth factor motif of 34-40
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amino acids and a varying number of short consensus repeat homology units as found in the complement binding proteins.

L-selectin is expressed on all circulating leucocytes, except for a population of memory lymphocytes. P-selectin is stored preformed in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets. It is rapidly mobilised to the plasma membrane to bind neutrophils and monocytes in response to acute inflammatory mediators. E-selectin is induced on vascular endothelial cells by cytokines including IL-1, lipopolysaccharide or TNF. De novo mRNA and protein synthesis is required for such induction which occurs 2-8 hours after stimulation.

Selectins mediate the attachment or tethering of flowing leucocytes to the walls of the vasculature through labile adhesions that permit leucocytes to roll in the direction of flow. Selectins appear to be regulated by selective loss from the cell surface: L-selectin is lost within minutes of leucocyte activation. This contrasts with integrins, which are increased on the neutrophil surface by mobilisation from granule compartments within minutes of stimulation and then remain permanently upregulated. Although both selectins and integrins can regulate neutrophil adhesion to endothelium, when selectins mediate adhesion, integrins are still required for subsequent transendothelial migration.

All the selectins appear to recognise a sialylated carbohydrate determinant on their counter-receptors. E-selectin and P-selectin recognise carbohydrate structures that are distinct but closely related to the tetra saccharide sialyl Lewis^x and its isomer sialyl Lewis^a. The carbohydrate ligand for L-selectin is related to sialyl Lewis^a and sialyl Lewis^x and contains sialic acid and sulphate.

The Integrins

Integrins are a family of cell surface heterodimers that participate in diverse cell-cell and cell-extracellular matrix interactions. The family shows strong conservation of a basic structural plan. Each integrin contains a non-covalently associated larger α and smaller β subunit with characteristic structural motifs. The integrin family is loosely organised into three subfamilies based on three distinct β subunits: β1 (VLA proteins,
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CD29), \( \beta 2 \) (leucocyte integrins, CD18) and \( \beta 3 \) (cytoadhesins, CD61), each of which associates with own complement of a subunits.

The \( \beta 2 \) subfamily, known as the leucocyte integrins because their expression is limited to white blood cells, consist of the \( \beta \) chain, CD18, paired with CD11a (LFA-1), CD11b (Mac-1) or CD11c (p150, 95). The importance of the leucocyte integrins to immune function is demonstrated by the condition known as leucocyte adhesion deficiency, in which they are deficient due to mutations in the common \( \beta 2 \) subunit. Patients suffer frequent and fatal infections, unless the condition is corrected by bone marrow transplantation. LFA-1 has been shown to be involved and required for a wide variety of cell to cell interactions including T cell mediated killing, T helper and B cell responses, natural cell killing, antibody-dependent cellular cytotoxicity by monocytes, granulocytes and adherence of leucocytes to endothelial cells, fibroblasts and epithelial cells (Dustin et al. 1989; Larson et al. 1990).

The \( \beta 1 \) integrin subfamily includes receptors that bind to extracellular matrix components including fibronectin, laminin and collagen. They are expressed on many non-haemopoetic and leucocyte cell types. The \( \beta 1 \) family have been designated VLA (very late activation) as two, VLA-1 and VLA-2, appear on lymphocytes 2-4 weeks after antigen stimulation in vitro. Some VLA molecules however, are basally expressed on leucocytes and non-haematopoetic cells. Induction of VLA-1, -2, -3, and -5 after leucocytes cross the endothelial barrier appears to be of importance controlling leucocyte localisation in inflammation. VLA-4 is unusual as it functions both as a matrix and a cell receptor. It binds to a domain of fibronectin distinct from the binding site of VLA-5 when functioning as a matrix receptor, and to VCAM-1, a member of the immunoglobulin supergene family when functioning as a cell receptor. VCAM-1 is induced by inflammatory mediators on endothelium in a similar fashion to ICAM-1. In congenital deficiency of the \( \beta 2 \) integrins, which does not affect VLA-4, lymphocytes continue to migrate across the endothelium at inflammatory sites which is presumably related to the expression of VLA-4 by lymphocytes and not neutrophils. There is
apparent functional redundancy between LFA-1 and VLA-4 in T cell mediated cell killing and in homotypic adhesion.

The α-subunit of integrins contain approximately 1100 amino-acid residues and the β-subunit, 750 residues. Highly conserved areas in the β-subunit, when associated with an α-subunit, may form a ligand binding pocket. The α-subunits have three or four putative divalent cation-binding motifs and require Ca^{2+} or Mg^{2+} for function, as demonstrated by the requirement for Mg^{2+} in T cell adhesion and in the binding of purified LFA-1 to ICAM-1.

THE IMMUNOGLOBULIN SUPERFAMILY

The immunoglobulin superfamily is a functionally diverse family of molecules many of which are expressed on the cell surface. Members of the superfamily share the immunoglobulin domain, composed of 70-110 amino-acids arranged in a sandwich of two sheets of anti-parallel β-strands, usually held together by a disulphide bond (Dustin et al. 1988b). They are evolutionarily related and hence structurally related. The immunoglobulins and the TCR, which are specialised for antigen recognition, are the only known members of this family that undergo somatic diversification. Adhesive functions of members of the family appear to evolutionarily predate antigen recognition functions. The intercellular adhesion molecules (ICAMs), CD4, CD8, and VCAM-1 are all members of the family.

ICAM-1

ICAM-1 was first inferred to be a ligand for LFA-1 on the basis of monoclonal antibody inhibition of LFA-1-dependent adhesion and was soon proven to be a ligand by studies with purified protein (Dustin et al. 1986a; Makgoba et al. 1988; Rothlein et al. 1986).

Structure

ICAM-1 was found to be a member of the immunoglobulin supergene family, with 5 Ig domains. The binding site for LFA-1 lies within the two most membrane distal Ig domains (Springer 1990). The molecular weight of ICAM-1 varies between 90
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and 114000, depending on glycosylation, which varies between cell types expressing the molecule (Dustin et al. 1986b).

Tissue Distribution

Immunohistological studies found ICAM-1 to be expressed on only a few cells, in a pattern correlating with MHC class II expression. Vascular endothelium of both small and large vessels demonstrated expression in most tissues studied (Dustin et al. 1986b). The most intense staining of vascular endothelium was seen in the interfollicular areas of lymph nodes, tonsils and Peyer's patches. In the thymus, staining was predominantly on dendritic cells and the thymic epithelium. In peripheral lymphoid tissue, the germinal centres were intensely stained, with predominantly dendritic and B cells positive. In the intestine macrophage-like cells were stained in the lamina propria and Peyer's patches. The intestinal epithelial cells were not stained, neither were hepatocytes or biliary epithelial cells. Tonsillar epithelium, however, consistently stained. Analysis of peripheral blood mononuclear cells by flow cytometry revealed a striking absence on all lymphoid cells, granulocytes with only the monocytic cells demonstrating weak expression of ICAM-1 (Wawryk et al. 1989).

Induction of Expression

The relatively restricted expression of ICAM-1 contrasts with the expression of LFA-1 by all leucocytes. Regulation of ICAM expression allows control of the spectrum of cells to which activated leucocytes can adhere. In most cases ICAM-1 expression is regulated by cytokine receptors, often coupled to mechanisms for altering gene expression (Dustin et al. 1991). Inflammatory mediators including lipopolysaccharide, interferon-γ (IFN-γ), interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) cause strong induction of ICAM-1 in a wide variety of cells and tissues (Dustin et al. 1988a; Dustin et al. 1988c; Springer et al. 1987b).

Function

Due to its importance as a ligand for LFA-1, ICAM-1 has a fundamental role in many cellular immunological interactions, including antigen-specific T cell recognition and lysis of certain target cells (Dustin et al. 1986b; Makgoba et al. 1989) Antibody to
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ICAM-1 inhibits antigen-specific T cell proliferation and co-transfection of ICAM-1 and HLA-DR into murine L cells greatly augmented their capacity to function as human antigen-presenting cells (Altmann et al. 1989).

Evidence exists of synergism between the LFA-1–ICAM-1 adhesion mechanism and the TCR, as TCR cross-linking dramatically increases LFA-1 dependent adhesion (Dustin et al. 1989) A model has been proposed for the cooperation between the TCR and the LFA-1–ICAM-1 adhesion mechanism. On contact with cells bearing specific antigen, TCR ligation generates intracellular signals which lead to energy-dependent conversion of LFA-1 to a high avidity state, favouring LFA-1–ICAM-1 dependent adhesion. Antigen specificity is maintained because the stimulus to convert LFA-1 to a high avidity state is controlled by the TCR (Dustin et al. 1991).

Expression in Inflammatory Conditions

In contrast to LFA-1, which is restricted to leucocytes, ICAM-1 can be expressed on a wide variety of cells and its induction in inflammatory states is an important means of regulating the LFA-1–ICAM-1 adhesion mechanism and thereby, presumably inflammatory responses. In vivo histological studies are of particular use in this area and have demonstrated induced or augmented expression on a variety of cell types at the site of inflammatory responses. Thus increased ICAM-1 expression has been shown in inflammatory dermatoses, graft-versus-host disease, autoimmune thyroiditis and liver-graft rejection (Adams et al. 1989; Makgoba et al. 1989; Weetman et al. 1989). Increased expression is presumably due to local pro-inflammatory cytokine production, as discussed above.

ICAM-2

A second LFA-1 ligand, ICAM-2, differing in tissue distribution from ICAM-1, was originally defined functionally by the ability of LFA-1 antibodies, but not ICAM-1 antibodies, to inhibit certain cell adhesion assays. ICAM-2 has two Ig-like domains that are most homologous to the two N-terminal domains of ICAM-1 (Staunton et al. 1989). ICAM-2 has a peptide backbone of Mr 31,000, consistent with the size predicted from the cDNA (de Fougerolles et al. 1991). ICAM-2 had a broad
distribution on hematopoietic cell lines and little expression on other cell lines, the sole exception being cultured endothelial cells which possess high levels of ICAM-2. Resting lymphocytes and monocytes expressed ICAM-2, while neutrophils did not. Staining of tissue sections with anti-ICAM-2 monoclonal antibodies confirmed their strong reactivity to vascular endothelium, but demonstrated a lack of ICAM-2 expression on other tissues. In contrast to ICAM-1 there was little or no induction of ICAM-2 expression on lymphocytes or cultured endothelium upon stimulation with inflammatory mediators.

**ICAM-1 EXPRESSION IN THE INTESTINE**

At the time of this study no work had been published specifically examining the expression of ICAM-1 in the diseased intestine. In view of the strong evidence for pivotal immune mechanisms in the pathogenesis of coeliac disease and the importance of ICAM-1 to certain fundamental immune functions, it was decided to examine the expression of ICAM-1 in the intestinal mucosa of patients with coeliac disease.

**Aims**

1) To examine the expression of ICAM-1 in the small bowel of normal patients, treated and untreated coeliac patients.

2) To study the effect of gluten challenge on the kinetics of expression of ICAM-1 in the small bowel of treated coeliac patients.

3) To study the mechanisms of control of ICAM-1 expression in the small bowel through organ culture of the small bowel with the cytokines TNF-α and IFN-γ.
Methods

JEJUNAL STUDIES

Quinton Hydraulic Capsule Biopsy Procedure

The Quinton hydraulic capsule allows multiple small bowel biopsies to be taken without removing the tube. The biopsies are relatively untraumatised compared to endoscopic biopsies, facilitating optimum orientation. The biopsy capsule is diagrammatically represented below (Fig 2.1) A syringe attached to the specimen return tube is used to apply suction, thus drawing a section of mucosa into the capsule through the biopsy aperture. The pump is activated and isotonic fluid is driven down the high pressure tube, causing the biopsy knife to close and a shunt to open between the high pressure and specimen return tubes. The fluid then washes the biopsy back to the exterior via the specimen return tube.

![Diagram of Quinton Hydraulic Capsule Biopsy Procedure](image)

The patient was fasted overnight prior to the procedure. Informed consent was obtained and the procedure explained. Intravenous access was secured and a short acting benzodiazepine, usually midazolam (2.5–5mg) was given to sedate the patient. Following sedation, intravenous metoclopramide (10mg) was given to facilitate passage of the biopsy tube through the pylorus. The patient then swallowed the tube, which
with the aid of fluoroscopy, was positioned in the proximal jejunum, in the region of
the ligament of Treitz.

Biopsies

Immediately upon recovery, from the Quinton hydraulic capsule, the biopsies
were carefully orientated epithelial surface upwards on aluminium foil, coated in
Optimal Cutting Temperature Compound (OCT) and snap frozen in thawing isopentane
suspended over liquid nitrogen. Biopsies were then stored in liquid nitrogen until
further processing.

Cryostat sections were cut at 5μm on a Bright Open–Top Cryostat with an
advanced Robomatic Motor Drive. Prior to staining, the slides were wrapped in
aluminium foil and stored at -70° C.

Biopsies were also fixed in formal saline, for wax section processing, to be used
in morphometric analysis.

Patients

Peroral jejunal biopsies were taken from treated (n=10) and untreated (n=10)
coeliac patients as part of their diagnostic management. The diagnosis was based on the
presence of total or severe villous atrophy with crypt hyperplasia together with both
clinical and morphometric improvement in a repeat jejunal biopsy following at least six
months treatment with a gluten free diet. The treated patients all had normal or near
normal villous architecture and had all been on a gluten free diet for at least a year. The
controls (n=10), in whom jejunal morphology was normal, had a jejunal biopsy for
diagnostic purposes and were all subsequently diagnosed as suffering from irritable
bowel syndrome.

Jejunal Challenge Studies

Patients and Challenge

A further three patients with treated coeliac disease had a Quinton hydraulic
multiple biopsy tube positioned in the jejunum, as described above. A biopsy was taken
prior to an oral gluten challenge and then at hourly intervals for 6 hours after the
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challenge. The challenge consisted of 10g of commercial wheat gluten suspended in 150ml tap water.

ORGAN CULTURE STUDIES

Patients

Eight patients, who were subsequently found to have normal jejunal histology and to be suffering from irritable bowel syndrome, underwent a jejunal biopsy as part of their routine diagnostic management, to exclude coeliac disease. Multiple jejunal biopsies were taken as described previously.

Culture Technique

The technique used was that described by Browning and Trier (Browning et al. 1969). Freshly taken jejunal biopsies were placed villous side uppermost on stainless steel grids resting on the central well of an organ culture dish. The biopsies were handled at all times with fine watch-makers forceps to avoid damaging the villous surface (Fig 2.2).

![Fig 2.2](image)

The tissue culture medium, the details of which are supplied, containing the cytokines described below, which had been made up freshly, was added to the central well such that the medium just made contact with the inferior surface of the grid and was then drawn up by capillary action through the grid. The outer well of the dish was filled with sterile saline and the dish covered. The dish was immediately placed in an incubator at 37°C, in 95% O₂, 5% CO₂ and cultured for 24 hours. One biopsy from each patient was frozen prior to culture as a control for normal ICAM-1 expression in the jejunal mucosa.
Cytokines

Human recombinant IFN-γ (Boehringer Mannheim, Lewes, U.K.) and TNF-α (Ernst Boehringer Institute für Arzneimittel-Forschung, Vienna, Austria.) were added to the culture medium, immediately before culture, both individually and mixed in equal proportions at concentrations of 10, 100 and 1000 IU/ml. A single biopsy was cultured with each dose of cytokine. Biopsies cultured with medium alone or phytohaemagglutinin (10mg/ml), a T cell mitogen, served as negative and positive controls respectively. Following 24 hrs culture the biopsies were removed, orientated, embedded in OCT, snap frozen in thawing isopentane over liquid nitrogen and stored at -70°C until they were processed.

IMMUNOHISTOCHEMISTRY

A streptavidin–biotin method was used throughout, for all studies (Hsu et al. 1981). The technique utilises the high affinity of streptavidin for biotin, which has four potential binding sites on the streptavidin molecule. A biotinylated antibody acts as a link between the primary monoclonal antibody and a preformed streptavidin–biotin–peroxidase complex. The peroxidase then acts on a chromogenic substrate to detect primary monoclonal binding.

Streptavidin–Biotin Technique

Pre-mounted frozen sections at -70°C.
Air dry for approximately one hour.
Fix in acetone for 5 minutes.
Gently wash in water, followed by wash in Tris-Buffered-Saline (TBS) for 15 minutes.
Incubate in 50μl of primary antibody, at appropriate dilution, for one hour at room temperature.
Wash in TBS for 15 minutes.
Make up ABC Complex 1:100.
Incubate in biotinylated rabbit anti-mouse immunoglobulin (1:300) for 30 minutes
at room temperature.
Wash in TBS for 15 minutes.
Incubate in ABC Complex for 30 minutes at room temperature.
Wash in TBS for 15 minutes.
Wash in TRIS buffer.
Incubate in 3,3-diaminobenzidine solution for 15 minutes.
Wash well in water for 3-5 minutes.
Counterstain in Mayers Haematoxylin for 3 minutes.
Wash in tap water.
Dehydrate, clear and mount.

Monoclonal Antibodies

The monoclonal antibodies employed for the first layer were: 84H10, against ICAM-1 (Makgoba et al. 1988). Omission of this primary antibody with and without the addition of MAC 181 (Freedman et al. 1988), a monoclonal antibody against gliadin, served as control against non-specific antibody binding and endogenous peroxidase activity.

Quantification

The pattern of distribution and number of cells stained with the antibodies were assessed independently and blindly by two of us (myself and either Dr James Macartney or Dr Jo Spencer). Staining with 84H10, against ICAM-1, was graded with an arbitrary scale: nil = 0, few = 1, moderate = 2, most = 3, and the mean of the two assessments taken. Villous height: crypt depth ratio and epithelial surface cell height were determined on the gluten challenge specimens. The results were compared by use of the Mann-Whitney U test.

Morphometry

Morphometric measurements were performed on 5μm wax mounted sections, that had been fixed in formal saline. Using a Leitz Microscope with an eyepiece micrometer, the sections were examined under the x10 objective. On each section, measurements
were made of the height of ten separate villi together with the depth of the adjoining crypt and the villus–height/crypt–depth ratio calculated and the mean of the ten ratios taken. Under the 40x objective, the heights of ten separate enterocytes, approximately in the middle third of the villi were measured, recorded and the mean value and standard deviation taken. The results were compared to the prechallenge biopsies by use of Student's t Test.
Results

JEJUNAL BIOPSIES

The results for ICAM-1 expression are demonstrated in the following photomicrographs and summarised in the accompanying tables.

Enterocytes

There was no expression of ICAM-1 by enterocytes in normal, treated or untreated coeliac patients. This was a consistent finding in every biopsy examined (Fig 2.3).

Intraepithelial Lymphocytes

There was no expression of ICAM-1 by IEL in control, treated or untreated coeliac patients. This was a consistent finding in every biopsy examined (Fig 2.3).

Lamina Propria

Superficial:

There was significantly greater expression of ICAM-1 in the superficial, immediately sub-epithelial compartment of the lamina propria of the jejunal mucosa from patients with untreated coeliac disease compared to treated coeliac and control patients (P<0.01) (Table 2.1, Fig 2.3). Expression was seen predominantly on larger cells with the morphology of macrophages but also on smaller lymphocyte-like cells. There was also expression on extracellular matrix elements. Overall few cells of the superficial lamina propria in the control or treated coeliac patients expressed ICAM-1 other than endothelial cells (see below), neither was there any obvious matrix expression. There was a trend towards greater ICAM-1 expression in the superficial lamina propria of the treated coeliac patients compared to controls, but this was not significant.

Deep:

In the jejunal mucosa of all three groups there was moderate expression of ICAM-1 within the deeper lamina propria (Table 2.1, Fig 2.3). This was seen, as in the superficial lamina propria, on both macrophage and lymphocyte-like cells. Unlike the
superficial compartment, however, there was no significant difference between the groups. Particularly heavy expression of ICAM-1 was seen on all cells in lymphoid aggregates.

Endothelium

Vascular endothelium expressed ICAM-1 in all biopsies in all three patient groups. The intensity of staining was variable and tended towards greater intensity in the untreated coeliacs but was present in all cases (Fig 2.3).
2.3 (a) Untreated Coeliac Disease (high power)

2.3 (b) Untreated Coeliac Disease (low power)
2.3 (c) Treated Coeliac Disease

2.3 (d) Control Patient
### TABLE 2.1 ICAM-1 EXPRESSION IN THE JEJUNAL MUCOSA

#### 2.1 (a) Untreated Coeliac Disease

<table>
<thead>
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#### 2.1 (b) Treated Coeliac Disease

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<tbody>
<tr>
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<td>0</td>
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</tr>
<tr>
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#### 2.1 (c) Control Patients

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<td>2</td>
<td>1</td>
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CHALLENGE JEJUNAL BIOPSIES

Pre-challenge

The biopsies prior to challenge demonstrated the pattern of ICAM-1 expression that had previously been found for treated coeliac patients with few or less cells in the superficial lamina propria positive for ICAM-1 (Fig 2.4a, Table 2.2). Likewise the deeper lamina propria showed moderate cellular staining and there was consistent endothelial positivity. There was no ICAM-1 expression in the epithelial compartment.

Post-challenge

Technical failures with biopsy processing are designated in the tables as NA. Two of the three patients showed increased ICAM-1 expression in the superficial lamina propria by two hours post biopsy and by three hours all the patients had increased expression (Figs 2.4b and c, Table 2.2a). This maximised at four hours with most cells expressing ICAM-1 in a similar pattern to that found in untreated coeliac disease with the most marked staining in the immediate subepithelial zone (Fig 2.4c and d). These changes were sustained at six hours.

There were no obvious changes in the deeper lamina propria and at no time was any epithelial expression of ICAM-1 observed, either on the enterocytes or the IEL. The intensity of endothelial staining appeared increased, but all endothelium had been positive in the pre-challenge biopsies (Table 2.2b)

Morphometric Changes

Morphological damage following challenge was manifest in all patients by falls in villous height: crypt depth ratio, which reached significance at three hours (P<0.05) and epithelial surface cell height, which failed to reach significance (Table 2.2c).
FIG. 2.4 ICAM-1 EXPRESSION IN THE JEJUNAL MUCOSA FOLLOWING GLUTEN CHALLENGE IN TREATED COELIAC PATIENTS

2.4 (a) Prior to Challenge

2.4 (b) 2 Hours Post Challenge
FIG. 2.4 ICAM-1 EXPRESSION IN THE JEJUNAL MUCOSA FOLLOWING GLUTEN CHALLENGE IN TREATED COELIAC PATIENTS

2.4 (c) 3 Hours Post Challenge
FIG. 2.4 ICAM-1 EXPRESSION IN THE JEJUNAL MUCOSA FOLLOWING GLUTEN CHALLENGE IN TREATED COELIAC PATIENTS

2.4 (d) 4 Hours Post Challenge (high power)

2.4 (e) 4 Hours Post Challenge (medium power)
Table 2.2 ICAM-1 Expression in the Jejunal Mucosa After Gluten Challenge

2.2 (a) Superficial Lamina Propria

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<th>Hours Post Challenge</th>
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<td>1</td>
<td>2</td>
<td>3</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>Patient 3</td>
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<td>2</td>
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2.2 (b) Deep Lamina Propria

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<th>Hours Post Challenge</th>
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2.2 (c) Villous Height to Crypt Depth Ratio and Enterocyte Height

<table>
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<tr>
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<tr>
<td>VH/CD</td>
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<td>2.0±.5</td>
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<tr>
<td>Ent. Ht</td>
<td>27±1</td>
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<td>20±1</td>
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<tr>
<td>Ent. Ht</td>
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<td><strong>Patient 3</strong></td>
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<tr>
<td>VH/CD</td>
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<td>1.9±.3</td>
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<td>31±2</td>
<td>NA</td>
<td>26±3</td>
</tr>
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</table>
ORGAN CULTURE STUDIES
Initial Biopsies

These biopsies showed the pattern of ICAM-1 expression that was previously found with control patients. There was no ICAM-1 expression in the epithelial compartment and predominantly weak expression on the superficial lamina propria cells of the initial biopsies. In this group of patients however, the deeper lamina propria also showed predominantly weak expression. There was consistent endothelial positivity and there was no ICAM-1 expression in the epithelial compartment.

Control Biopsies

Some biopsies were unsuitable for assessment after culture and are designated NA. The biopsies cultured in medium alone did not show any consistent differences in ICAM-1 expression compared with the initial biopsies (Fig 2.5a, Table 2.3). In three patients there was an increase, in one patient a decrease and in four patients there was no change. There was no obvious histological evidence of tissue damage due to the process of organ culture, apart from minor degeneration of the serosal aspect of the biopsy immediately adjacent to the supporting grid.

Superficial Lamina Propria

When staining was detected, all cell types appeared to be expressing ICAM-1 together with some matrix positivity.

IFN-γ

Expression of ICAM-1 was increased following culture with IFN-γ in seven of the eight patients (Fig 2.5b, Table 2.3). In all but one of these cases the increase was by one point on the assessment scale. There was no obvious dose response effect.

TNF-α

Expression of ICAM-1 was increased following culture with TNF-α in five of the eight patients (Fig 2.5c, Table 2.3). In two of these cases the increase was by two points and in three cases, by one point on the assessment scale. Likewise, there was no obvious dose response effect.
TNF-α and IFN-γ

Expression of ICAM-1 was increased following culture with TNF-α and IFN-γ together in five of the eight patients (Table 2.3). In one of these cases the increase was by two points and in four cases, by one point on the assessment scale. Again, there was no obvious dose response effect.

PHA

Expression of ICAM-1 was increased following culture with PHA in six of the eight patients (Table 2.3). In one of these cases the increase was by three points, in one case by two points and in four cases, by one point on the assessment scale.

Deep Lamina Propria

There were no obvious changes in ICAM-1 expression by the cells in the deeper lamina propria although assessment of this area was made less accurate because of the mild degeneration discussed above.

Endothelium

Endothelial expression of ICAM-1 was strong on all biopsies. There appeared to be a trend towards increased intensity of endothelial expression of ICAM-1 following culture with the cytokines (Fig 2.3).

Epithelium

There was no epithelial compartment expression of ICAM-1 in any of the cultured biopsies (Fig 2.3).
FIG. 2.5 ICAM-1 EXPRESSION ON CULTURED JEJUNAL BIOPSIES PATIENT 1

2.5 (a) Biopsy Cultured in Medium Alone

2.5 (b) Biopsy Cultured IFN-γ – 100IU/ml
2.5 (c) Biopsy Cultured in TNF-α – 10IU/ml
TABLE 2.3 ICAM-1 EXPRESSION ON CULTURED JEJUNAL BIOPSIES

-LAMINA PROPIA

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>1</th>
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Discussion

Increased Expression of ICAM-1 in the Lamina Propria

The major finding of this study was a clearly demonstrated upregulation of ICAM-1 expression in jejunal biopsies from patients with untreated coeliac disease. This finding is in keeping with other immunohistological studies that have demonstrated augmented ICAM-1 expression on a variety of cell types at the site of inflammation. There are, however, several interesting features to the pattern of increased ICAM-1 expression.

The site of increased ICAM-1 expression was the superficial lamina propria, particularly the immediately subepithelial region. Increased ICAM-1 expression is an important means of regulating the activity of LFA-1–ICAM-1 adhesion pathway, which appears to be of considerable importance to many fundamental immunological processes including antigen-specific T cell recognition, antigen-specific T cell proliferation and target cell lysis (Dustin et al. 1986b; Makgoba et al. 1989). This suggests that the superficial, subepithelial microenvironment of the lamina propria is a major site of immunological activity in coeliac disease, certainly of processes involving the LFA-1–ICAM-1 adhesion pathway. This is supported by the demonstration of subepithelial deposition of recently activated complement in untreated coeliac patients which significantly correlated with the degree of villous atrophy (Halstensen et al. 1992).

Subsequent to the work undertaken for this thesis, Smart and colleagues also studied ICAM-1 and LFA-1 expression in control patients, untreated and treated coeliac patients with seven patients in each group (Smart et al. 1991). They also demonstrated an increase in subepithelial lamina propria ICAM-1 expression in the coeliac patients although to a less marked extent. ICAM-1 expression was found on macrophages and also on the extracellular matrix. The reasons for the less marked increase in ICAM-1 expression are not immediately clear; a different primary antibody and the less sensitive technique of immunofluorescence might contribute.
Macrophage- and lymphocyte-like cells together with matrix elements were seen to be expressing ICAM-1. To confirm definitively which cell types were expressing ICAM-1, double label immunostaining would have been necessary. Subsequent studies of colonic inflammatory bowel disease and of a foetal human intestinal model have confirmed ICAM-1 expression by these cell types in the inflammed or activated state (Dogan et al. 1993; Malizia et al. 1991).

Lack of ICAM-1 Expression by Enterocytes.

One of the classic features of the intestinal mucosa in coeliac disease is enterocyte damage with microvillus irregularities, mitochondrial swelling, dilatation of the endoplasmic reticulum and lysosomal rupture. The "haemolytic" model of mucosal change suggests that this is a primary event and crypt hypertrophy occurs as a purely secondary response to loss of enterocytes from the villus tip, although Marsh has argued persuasively that this is not the case. It has been suggested that the IEL maybe directly cytotoxic to the immediately adjacent enterocytes or that the enterocytes might be functioning as accessory antigen presenting cells to the IEL, supported by the finding of increased enterocyte expression of HLA-class II antigens in coeliac intestinal mucosa (Arnaud-Battandier et al. 1986). Direct cytotoxicity and antigen presentation have both been shown to be ICAM-1–LFA-1 dependent processes. (Makgoba et al. 1989). Previous immunohistological studies had demonstrated augmented ICAM-1 expression on a variety of cell types at the sites of inflammation in the skin, thyroid and liver (Adams et al. 1989; Singer et al. 1989; Weetman et al. 1989). These studies suggested that augmented expression of ICAM-1 might render the epithelial cells affected more susceptible to direct cytotoxic damage or facilitate antigen presentation by such cells.

The lack of expression of ICAM-1 by the small intestinal epithelium in the non-inflamed state, demonstrated by this study, is consistent with Dustin and colleagues report on the tissue distribution of ICAM-1 (Dustin et al. 1986b). The lack of expression of ICAM-1, however, in untreated coeliac disease and thus inflamed intestinal epithelium, is a novel finding that is not consistent with previous studies in
ICAM-1 and Coeliac Disease

other tissues and diseases, as described above. Such lack of expression of ICAM-1 by the intestinal epithelium in inflammatory states has been confirmed by others in coeliac disease (Smart et al. 1991), in H. pylori. associated gastritis (Sheynius and Engstrand. 1991), in colonic inflammatory bowel disease (Malizia et al. 1991) and in an immunologically activated foetal human intestinal model (Dogan et al. 1993). Colonic epithelial cells, in one study of human graft versus host disease, did show some ICAM-1 positivity on the luminal membrane (Norton et al. 1992). The authors, however questioned the significance of this finding.

Lack of ICAM-1 Expression by IEL

ICAM-1 is known to be expressed on effector leucocytes as well as target cells (Wawryk et al. 1989), and thus if the IEL were utilising the ICAM-1–LFA-1 adhesion mechanism, expression of ICAM-1 might be expected. In all the patient groups studied, there was no expression of ICAM-1 by the IEL. This finding together with the lack of expression by enterocytes of ICAM-1, allows the conclusion to be drawn that the ICAM-1–LFA-1 adhesion mechanism does not function, to any significant extent, in the epithelial compartment of the small intestine either in the normal state or in coeliac disease. It is not possible to fully exclude low density expression of ICAM-1 within the epithelial compartment below the level of sensitivity of immunohistology. If this were so, however, there is clearly a major difference in terms of level of expression such that the above conclusion is still valid. The lack of intestinal epithelial ICAM-1 expression in other studies, using different immunohistological techniques, suggests that lack of expression is not artefactual.

The study of Smart and colleagues examined the expression of LFA-1 as well as ICAM-1(Smart et al. 1991). Only 30-60% of the IEL expressed detectable LFA-1, most weakly. There was a slight overall increase in LFA-1 expression by IEL, mainly by the CD4+ subset. This data supports the above conclusion. Likewise, IEL in colonic inflammatory bowel disease (Malizia et al. 1991) and in an immunologically activated foetal human intestinal model (Dogan et al. 1993).have been shown not to express ICAM-1.
ICAM-1 and Coeliac Disease

The lack of expression of ICAM-1 in the epithelial compartment argues strongly against a role for enterocytes presenting gliadin-derived peptides, to IEL, at least by the classical pathway, which is supported by the arguments of Brandtzaeg (Brandtzaeg et al. 1992). There is evidence that IEL may be activated by non-classical pathways such as the CD2–LFA-3 interaction, perhaps using alternative accessory molecules (Ebert 1989). This will be discussed in greater detail in the following chapter.

Constant Expression of ICAM-1 by Intestinal Endothelium

Descriptive changes of the intestinal mucosal vasculature in coeliac disease have included hypertrophy of endothelial cells, margination, adherence and extravasation of inflammatory cells including lymphocytes, neutrophils, eosinophils and basophils, together with plasma protein (Loft et al. 1989; Marsh et al. 1985). Mucosal infiltration by leucocytes is one of the cardinal histological features of coeliac disease and infiltrating cells presumably enter from the vasculature. Induction of ICAM-1 on endothelium may increase cell-cell interactions and leucocyte extravasation at inflammatory sites.

This study demonstrated a constitutively high expression of ICAM-1 on vascular endothelium of the small intestinal mucosa in both the normal and diseased bowel. The intensity of staining appeared to be greater in the untreated coeliacs compared to treated and normal controls. This is a subjective assessment, however, and it should be treated with caution. The studies of ICAM-1 expression in the inflamed intestine quoted above, all describe high levels of expression on the vascular endothelium both in normal and inflamed mucosa. It is of note that Norton et al quantified the level of ICAM-1 expression by microscopic densitometry and did not find any increase in expression in the diseased colonic mucosa (Norton et al. 1992).

High constitutive levels of endothelial ICAM-1 may reflect the large number of leucocytes trafficking through the intestinal mucosa. This data provides no definite evidence for increased endothelial expression of ICAM-1 which may merely reflect the limitations of the technique of immunohistology. In vitro evidence exists of increased ICAM-1 expression by endothelium following immune activation and it is possible, if
not probable, that this occurs in vivo and might be a factor in the development of the vascular changes associated with coeliac disease, described above.

**GLUTEN CHALLENGE**

The antigen which stimulates what we presume to be immune mediated damage in coeliac disease, is partially characterised, can be administered relatively safely and biopsies of damaged tissue can be obtained relatively easily. Thus gluten challenge in coeliac disease is a well established, valuable technique for studying the generation and progression of the intestinal lesion. In addition, dissection of the individual pathogenetic elements involved in the lesion, which may be obscured in its established form, can be undertaken. Gluten challenge can also be used as a model for studying the effects of immune activation in the intestinal mucosa, with relevance to other intestinal diseases.

The major disadvantage of gluten challenge is its invasive nature, particularly when undertaken in the hyper-acute form as in this study, with the necessity of maintaining a Quinton tube in position for 6 hours. Few patients are able to agree to undergo such a study and thus, patient numbers are inevitably small, with associated individual variability. Each patient, however, acts as their own control with sequential biopsies being taken and therefore valuable information can be obtained even with small numbers of individual patients.

**Kinetics of in vivo upregulation of ICAM-1 expression**

This study demonstrated for the first time the kinetics of in vivo upregulation of ICAM-1 in both the intestine and other tissues. Initial studies characterising the biology of ICAM-1 had suggested that rapid upregulation of ICAM-1 could occur in fibroblast cell lines (Dustin et al. 1986b). A $t_{1/2}$ of 2hr and 3.75hrs for IL-1- and IFN-γ-stimulated upregulation, respectively, was shown and studies with mRNA and protein synthesis inhibitors suggested that increased expression was dependant on de novo protein synthesis. Similarly, cytokine-stimulated increased ICAM-1 expression has been shown within 2hrs on human dermal microvascular endothelial cells (Detmar et al.)
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1992) and within 4hrs on intestinal epithelial cells lines (Kvale et al. 1992). In addition to de novo ICAM-1 protein expression, certain cells, notable the monocyte series, possess intracellular stores of ICAM-1 that can be mobilised rapidly on stimulation (Dustin et al. 1991). These in vitro studies demonstrate the capacity for stimulated cells to rapidly upregulate ICAM-1 expression and support the in vivo findings in coeliac disease following gluten challenge.

ICAM-1 expression and mucosal damage

A considerable literature has been published regarding gluten challenge, investigating both the pathogenesis of the coeliac intestinal lesion and cereal toxicity in the disease. This will be discussed further in Chapter 4. Controversy is present within the literature, much of which exists because different challenge protocols and methods of assessment have been used. Despite this, certain conclusions can be drawn.

Light microscopic histological change, in terms of both villous structure and cellular infiltrates, occur within 4 hours post challenge and perhaps earlier (Anand et al. 1981; Bramble et al. 1985; Ferguson 1974; Freedman et al. 1987). Ultrastructural and biochemical change can occur within 2-21/2hrs (Bailey et al. 1989; Shmerling et al. 1970). Ciclitira and colleagues reported increased epithelial HLA-DR expression within 1-2 hours suggestive of immune activation in the intestinal mucosa. A series of studies using an isolated perfused jejunal segment technique has demonstrated increased secretion of eosinophilic cationic protein, histamine and prostaglandin E2 at 20 to 60 minutes after gliadin challenge (Lavo et al. 1990a; Lavo et al. 1989). The same workers showed increased leakage of proteins across the jejunal mucosa within 60 minutes of gliadin challenge, suggesting significant damage to the mucosa at that time (Lavo et al. 1990b)

The demonstration of increased ICAM-1 expression following gluten challenge in treated coeliac patients within 3 hours is thus compatible with the studies described above. The similar time course of expression and mucosal damage, suggests that ICAM-1 may have a role in the pathogenesis of the intestinal coeliac lesion.
ORGAN CULTURE STUDIES AND ICAM-1 EXPRESSION

The studies on untreated coeliac patients and treated coeliac patients undergoing gluten challenge demonstrated marked upregulation of ICAM-1 in the superficial lamina propria of the small intestine. What is controlling such changes in expression? A variety of cytokines are capable of increasing ICAM-1 expression and local release of cytokines following antigen-specific immune activation seems likely.

The third part of this study was designed to address the questions of control of ICAM-1 expression in the inflamed intestine, maintained in organ culture. IFN-γ and TNF-α were studied, as these cytokines had been shown to increase ICAM-1 expression on cell lines and were likely to stimulate expression. Lack of expression of ICAM-1 by the intestinal epithelium was an unexpected finding and begged the question whether such lack of expression was due to local control of cytokine expression in the subepithelial microenvironment or that the intestinal epithelial cells did not have the capability to express ICAM-1, at least as differentiated cells in the intact intestine.

Increased ICAM-1 expression was consistently seen in the superficial lamina propria with IFN-γ, although responses to TNF-α and both cytokines when combined were less consistent between patients, but definitely present within some individuals. We could not demonstrate any dose-response relationship, or synergism between the actions of IFN-γ and TNF-α. It is probable that the sensitivity of the system is insufficient to detect such an effect, if it existed.

No expression, however, was induced on the intestinal epithelium despite strong expression in the immediate subepithelial lamina propria. Thus the pattern of in vitro cytokine-induced ICAM-1 expression, with total lack of epithelial expression, is the same as that seen in the intact, inflamed state, either in the established coeliac lesion or following acute gluten challenge. This supports the hypothesis that these cytokines have a role in controlling the expression of ICAM-1 in the inflamed intestine. Furthermore lack of expression of ICAM-1 by the intestinal epithelium is more likely to be due to an inability of the intact epithelium to express ICAM-1, either because of a
constitutive lack of expression or the presence of an inhibitory factor, rather than lack of cytokine stimulation since the epithelium of cultured biopsies would have been bathed in the cytokine-containing medium.

Evidence for local intestinal cytokine production in coeliac disease, potentially responsible for upregulation of ICAM-1 expression, has been forthcoming from in situ hybridisation studies demonstrating cytokine mRNA species both in untreated patients and treated patients undergoing acute gliadin challenge (discussed further in Chapter 4) (Kontakou et al. 1995a; Kontakou et al. 1995b; Kontakou et al. 1994). The lamina propria of untreated coeliac patients contained a significantly increased number of IFNγ, IL-2, IL-6 and TNF-α producing cells compared with controls; there was no significant difference between the coeliac patients treated with a gluten-free diet and controls. Four coeliac patients in remission who had been challenged with gliadin demonstrated significantly increased numbers of cytokine mRNA-expressing cells 4 hours after challenge in the lamina propria.

Controversy has arisen concerning the expression of ICAM-1 by intestinal epithelial cells as a result of studies of ICAM-1 expression by intestinal cell lines (Kaiserlian et al. 1991; Kvale et al. 1992). Both of these studies provided convincing evidence of ICAM-1 expression on CaCo-2, HT-29 and T84 cell lines, which with the exception of CaCo-2 cells in the study of Kaiselian et al, could be upregulated by incubation with IFN-γ, TNF-α, IL-1 and IL-6. One group demonstrated synergism between the actions of IFN-γ and TNF-α and an inhibitory effect of IL-4 on the actions of IFN-γ (Kvale et al. 1992). What are the possible reasons for the apparent discrepancy in results for ICAM-1 expression on intact intestinal epithelium and cultured epithelial cell lines?

The most obvious explanation is that intestinal adenocarcinoma epithelial cell lines, whilst displaying many features of mature differentiated intestinal cells, differ in their expression of ICAM-1. Supporting this is the clear immunohistological demonstration of ICAM-1 expression by colonic epithelial cells from colonic tumour specimens (Dippold et al. 1993). It is possible that minute amounts of ICAM-1, below
the level of immunohistological detection, are being expressed by intact epithelium. This seems unlikely in view of the consistent finding of lack of epithelial expression in the different studies described above, with their differing primary antibodies and immunohistological techniques. ICAM-1 is glycosylated to different degrees by different tissues and it is possible that unique glycosylation occurs in the intestinal epithelium. There is no positive evidence for this effect.

Kvale and colleagues argue that a rapid increase in epithelial ICAM-1 expression might be significant only in the initial phase of mucosal activation (Kvale et al. 1992). The changing profile and kinetics of local cytokine release in the chronic state, would lead to downregulation of epithelial ICAM-1 expression. The results presented on epithelial ICAM-1 expression following both in vitro organ culture over 24 hours and in vivo gluten challenge at 3 hours, with a complete lack of epithelial expression despite marked lamina propria expression, would appear to refute this argument. The discrepancy in results for ICAM-1 expression on intact intestinal epithelium and cultured epithelial cell lines emphasises the value of organ culture as a technique, where the complex interacting microenviroments of the intestinal mucosa are maintained as closely intact as possible. It also overcomes the potential danger of extrapolating data obtained using transformed cell lines, to the in vivo situation.

ICAM-1 AND THE PATHOGENESIS OF THE COELIAC LESION

The data presented show that intestinal ICAM-1 expression is upregulated in untreated coeliac disease and that similar upregulation can rapidly be induced by gluten challenge or by culture of normal intestine with proinflammatory cytokines. Such upregulated ICAM-1 expression is localised into the superficial, immediately subepithelial lamina propria and is absent from the epithelial compartment.

Increased ICAM-1 expression in patients with untreated coeliac disease associates ICAM-1 expression with the presence of the characteristic mucosal lesion of coeliac disease. Rapid upregulation of ICAM-1 expression in patients with treated coeliac disease following gluten challenge together with the development of mucosal damage,
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suggests that ICAM-1 expression is associated with the pathogenesis of such mucosal damage. The functional properties of the ICAM-1–LFA-1 molecular interaction have been discussed in the introduction to this chapter but localised induction of ICAM-1 is one of the important means of regulating the ICAM-1–LFA-1 interaction (Springer 1990). Whatever the extent of its role in the pathogenesis of the intestinal lesion of coeliac disease, the interaction appears to function within the lamina propria compartment of the intestinal mucosa. This is in keeping with the considerable body of evidence that the lamina propria is the major site of immune response within the coeliac mucosa. This includes granulocyte activation and infiltration (Desreumaux et al. 1992; Marsh et al. 1985), complement deposition (Halstensen et al. 1992), activation and expression of CD25 by T cells and macrophages (Griffiths et al. 1988; Halstensen et al. 1993) and expression of cytokine mRNA (Kontakou et al. 1994).

How might increased expression of ICAM-1 contribute to the pathogenesis of the intestinal lesion of coeliac disease. The evidence for the involvement and importance of T cell mechanisms has been discussed in Chapter 1. T cell recognition and activation by antigen requires intracellular processing of antigen and binding of short peptide fragments of antigen to a, subsequently cell surface expressed, HLA molecule. There is a pivotal role for the ICAM-1–LFA-1 interaction in activation of resting T and B cells by cell membrane presenting antigen and ICAM-1 expression is a virtual requirement of the professional antigen presenting cell (Wawryk et al. 1989). Increased ICAM-1 expression in the immediately sub-epithelial lamina propria could enhance, the presentation of gliadin-derived coeliac-toxic peptides after they had crossed the epithelium. This would lead to the localised activation of antigen specific helper T cells, either to produce pro-inflammatory cytokines, potentially damaging tissues, stimulating epithelial proliferation, activating macrophages, eosinophils and mast cells, and amplifying ICAM-1 expression itself, or to stimulate B cell proliferation and immunoglobulin production. Enhanced expression on T and B cells would facilitate further transient interactions and locomotions, enhancing the immune response to ingested gliadin proteins, with associated tissue damage.
ICAM-1 and Coeliac Disease

Due to the constitutively high levels of ICAM-1 expression on endothelial cells both in the normal and inflamed intestinal mucosa and thus not being able to detect increased levels of expression immunohistologically, no useful data can be presented on the component of endothelial ICAM-1 expression to the mucosal lesion. Clearly this is an important mechanism since binding of leucocytes to the intestinal endothelium and thereby the recruitment of such cells into the lamina propria, would be a first step in localisation of circulating cells into the inflamed mucosa.

The ICAM Family

In 1992 a third ICAM was characterised (de Fougerolles et al. 1992). It was demonstrated that some T and B lymphocyte cell lines bound to the LFA-1 chiefly through a pathway independent of ICAM-1 and ICAM-2. This ligand, designated ICAM-3, based on its functional relatedness to ICAM-1 and -2, is a highly glycosylated protein of 124,000 Mr with five Ig-like domains that are homologous with those of ICAM-1 (52% identity) and ICAM-2 (37% identity). ICAM-3 expression was restricted to the haemopoetic system and is reciprocal in its expression to ICAM-1 in that it was present on resting cells and its level fell as a result of cell activation (Cordell et al. 1994).

A high degree of functional similarity and redundancy exist in the LFA-1 counter receptors: what are the possible reasons? The multiplicity of ICAMs may allow finer regulation of the adhesion pathway since there are significant differences in tissue distribution and inducibility. ICAM-1 is highly inducible in immune and inflammatory reactions on many cell types, as shown here for coeliac disease, whereas ICAM-2 is constitutively expressed on endothelium. These results point towards ICAM-1 being the major ligand for LFA-1 during inflammatory or immune responses, while ICAM-2 is of more relative importance in the unstimulated resting state or early on during a response before ICAM-1 expression is increased. Thus these molecules may regulate leucocyte circulation in disease and health respectively. Since ICAM-3 is well expressed on all leucocytes and absent from non-haemopoetic cells, it may play an
important role in initiating immune responses (de Fougerolles et al. 1994) and thus may have some importance in the initial immune response contributing to the coeliac lesion, before ICAM-1 is upregulated.

NON-ICAM-1 ADHESION MECHANISMS IN THE INTESTINE

The majority of work has concentrated on in vitro studies of endothelial expression of adhesion molecules, predominantly in inflammatory bowel disease. Increased expression of VLA-1 has been reported on the basolateral aspects of crypt cells and de novo expression on the surface epithelium of the small and large bowel of patients with colitis and Crohn’s disease (MacDonald et al. 1990). It was suggested that this was an adaptive response to prevent epithelial cell loss as a result of inflammation in the underlying lamina propria. The same group studied the induction of adhesion molecules in their well described foetal model of enteropathy (Dogan et al. 1993). After T cell activation in fetal intestinal explants, the expression of ICAM-1 and VCAM-1 was increased on most endothelial cells, leucocytes, and stromal cells in the lamina propria but not the epithelium. In contrast, the induction of E-selectin was rapid but transient, despite the continuing presence of activated T cells and macrophages. The authors suggest that other factors might be required to prevent the down-regulation of E-selectin to maintain the sustained expression sometimes observed disease states.

The mechanisms controlling homing of leucocytes to the gut are of obvious importance to the pathogenesis of the coeliac intestinal lesion. No data has been published regarding the homing of lymphocytes in coeliac disease specifically. Gut homing lymphocytes display the α4β7 integrin which appears to function, at least in mice, via an interaction with the mucosal vascular addressin, MAdCAM-1 (Springer 1994). This molecule is expressed by mucosal venules, potentially directing lymphocyte traffic into Peyer’s Patches and the lamina propria. The human homologue of MAdCAM-1, that is currently unknown will probably be of critical importance to mucosal traffic of gut memory lymphocytes and thus to coeliac disease.
Infiltration of the epithelium by IEL is one of the cardinal features of the intestinal lesion of coeliac disease, yet the mechanisms controlling the migration of such cells, in health and disease is not defined. The HML-1 antibody, which binds to an antigen highly expressed by IEL, is now known to recognise the αEβ7 integrin (Cerf-Bensussan et al. 1992). This suggests that the αEβ7 integrin may help mediate localisation of IEL to the epithelium (Springer 1994). There is some evidence that αEβ7 may function as an alternative accessory molecule for IEL allowing activation via CD3-TCR pathways (Sarnacki et al. 1992). Further definition of these mechanisms and their study in coeliac disease would be of great interest. A possible mechanism could involve immune activation in the lamina propria with subsequent cytokine-stimulated increase in expression of the αEβ7, as yet unknown, counter-ligand leading to increased migration of lymphocytes to the epithelium.

**Conclusions**

ICAM-1 expression is markedly increased in the lamina propria but not the epithelium of the small intestine of patients with untreated coeliac disease. Gluten challenge in treated coeliac patients rapidly induced ICAM-1 expression in a similar pattern to that seen in untreated patients. ICAM-1 expression can be induced within the lamina propria of intestinal biopsies from normal individuals following culture with IFN-γ and TNF-α. These data suggest that upregulated expression of ICAM-1 has functional importance in the development of the intestinal lesion of coeliac disease and provides further evidence that the lamina propria is a major site of immune activation within the small intestine of coeliac patients.
CHAPTER 3: \(\gamma/\delta\) T CELLS IN COELIAC DISEASE AND DERMATITIS HERPETIFORMIS

Introduction

The evidence that cellular mechanisms, and in particular T cells, are critically involved in the pathogenesis of coeliac disease has been discussed. Activation of mucosal T cells following the ingestion of toxic cereal proteins presumably leads to pathogenic events in the mucosa culminating in the development of the characteristic intestinal mucosal lesion of coeliac disease. The previous chapter concerned pathogenetic mechanisms occurring in the lamina propria. This chapter will consider the involvement of \(\gamma/\delta\) TCR expressing lymphocytes within the intestinal epithelium of patients with coeliac disease and dermatitis herpetiformis. The nature of the TCR-HLA-peptide interaction will be discussed initially.

THE T CELL RECEPTOR

Unlike B cells, T cells are unable to recognise antigen unless it is “presented” by certain specialised cells, antigen presenting cells. The TCR recognises antigen that is bound to cell surface HLA molecules. The TCR is composed of two protein chains, either an \(\alpha\) and \(\beta\) chain, or a \(\gamma\) and \(\delta\) chain. These chains display immense polymorphism enabling the recognition of a large range of antigen. This polymorphism is generated by rearrangement of TCR genes. Essentially each protein chain is produced by the re-arrangement of variable (V), diversity (D), joining (J) and constant (C) region genes in an analogous manner to the re-arrangement of immunoglobulin genes. Multiple loci exist at each of these V, D, J and C region genes creating a large repertoire of possible re-arranged TCRs. In man 50 \(V_\beta\), 2 \(D_\beta\), 13 \(J_\beta\) and 2 \(C_\beta\) loci have been described and allelic polymorphisms occur at some of these loci. In addition to the possible recombination sequences, additional diversity is created by the random insertion or deletion of nucleotides at the sites of re-arrangement–junctional diversity so that an almost infinite number of TCR sequences can potentially be produced.
The protein structure produced by the rearranged TCR signal forms a similar conformation to immunoglobulin (Davis et al. 1988). The variable domain of immunoglobulin is also composed of a rearrangement of variable, diversity and joining sequences. The sequence of the rearranged TCR signals indicate that three variable sequence regions correspond in position to the immunoglobulin hypervariable antigen binding loops, the complementarity-determining regions (CDR). The TCR CDR1 and 2 are derived from germ-line variable region genes, whilst the TCR CDR3 region is located at the V (D) J junctional sites. The CDR3 region therefore also contains the sites where junctional diversity occurs. The interaction between TCR and HLA-antigen peptide predicts that the CDR 1 and 2 loops overlie the polymorphic residues of the HLA α-helices and hence there derivation from germ-line sequences ensures HLA recognition. The hypervariable CDR3 loop overlies the peptide antigen bound within the HLA cleft, so that the variability obtained by rearrangement and random junctional diversity will allow the recognition of a wide range of possible antigens (reviewed by Tighe et al. 1995).

In view of the wide range of potential TCR sequences generated during rearrangement, some form of selection is required to ensure HLA compatibility. Selection for functional TCR molecules occurs during thymic ontogeny when developing T-cells come into contact with the HLA antigens on thymic epithelium. The TCR sequences that are able to recognise an individual's HLA system are positively selected. This positive selection requires the presence of the T-cell accessory molecules CD4 and CD8, which are co-expressed at this stage of development. Those sequences that are unable to recognise the individual's HLA molecules do not undergo any further maturation and are lost from the repertoire. A second stage of selection is then required to delete those sequences which react strongly to self peptides-HLA ligands. These thymic processes of positive and negative selection ensure the development of a T-cell repertoire that allows efficient antigen recognition in the context of an individual's antigen presentation system without auto-reactivity (Adorini 1990).
The almost infinite polymorphic potential among expressed TCR genes is necessary to allow for the recognition of all possible antigenic sequences encountered during life. This polymorphism contrasts with the much more limited polymorphism seen among an individual’s HLA antigen presenting system.

The TCR proteins are expressed on the cell surface of T-cells in association with the CD3 protein complex. This complex is composed of five non-polymorphic polypeptides, termed gamma, delta, epsilon, zeta and eta. The CD3 complex acts as the primary signalling pathway through the action of tyrosine kinase and phospholipase C on its intracellular structure. The CD3 complex is formed intra-cellularly in association with the TCR protein chains and prior to transport to the cell surface.

Antigen processing

Class I HLA polypeptides are synthesised and transported into the endoplasmic reticulum where they are complexed with β2-microglobulin. These complexes are unstable in the absence of bound peptide but form a stable trimolecular structure with peptide. The stable complex is transported via the golgi apparatus to the cell surface for presentation to T cells.

Class II molecules are similarly transported into the endoplasmic reticulum where they associate into a stable complex with a third polypeptide, known as the invariant chain (Ii). The invariant chain binds to the peptide binding cleft preventing the binding of peptides that would bind to class I molecules. The invariant chain has targeting signals that direct it and the associated class II molecule through the golgi apparatus to the endosome compartment. The low pH of the endosome facilitates dissociation of the invariant chain, leaving the class II molecule free to bind antigenic peptides derived from the proteolysis of exogenous proteins.

INTRAEPITHELIAL LYMPHOCYTES

The IEL, as discussed in Chapter 1, consist almost entirely of T cells. A further distinction from the lamina propria lymphocytes, is the striking predominance of CD8+ cells among the IEL, such that approximately 90% of the IEL carry this marker (Cerf-
Bensussan et al. 1987; Janossy et al. 1980; Selby et al. 1983b). The remainder are either CD4+ or CD4-CD8-,"double negative" (Spencer et al. 1989b). The CD8+ IEL also express CD5 but at reduced levels (Malizia et al. 1985). Similarly, IEL demonstrate reduced expression of LFA-1 (Smart et al. 1991).

Mechanisms of IEL homing are generally poorly understood, but clearly they are of considerable importance to the selective accumulation of certain subgroups of mucosal lymphocytes in the epithelial compartment. The HML-1 antibody, which binds to an antigen highly expressed by IEL (Cerf-Bensussan et al. 1987), is now known to recognise the αEβ7 integrin. This suggests that the αEβ7 integrin may help mediate localisation of of IEL to the epithelium (Springer 1994). Data presented in the previous chapter has suggested that the ICAM-1–LFA-1 interaction is unlikely to function in the epithelial compartment. The state of activation of lymphocytes might affect their ability to preferentially migrate into the epithelial compartment; activated lymphocytes appear more mobile (Wilkinson 1986) and possess stronger adhesive qualities than resting T cells (Shimizu et al. 1990).

Activation and Proliferation

In order to respond to antigen, lymphocytes need to be activated via the CD3-TCR complex by antigen presented on MHC-encoded molecules. Classical antigen presenting cells, generally belong to the macrophage-mononuclear cell lineage which are not normally present within the intestinal epithelium. Since all enterocytes express HLA class I antigens and villous enterocytes also express HLA-DR, -DP and possibly DQ molecules, a role for the enterocyte in presenting antigen to IEL has been proposed (Bland et al. 1986; Mayer et al. 1987). These in vitro experiments however, used isolated rat or human enterocytes to present soluble protein antigens, such as ovalbumin, to previously sensitised peripheral T cells. The lack of ICAM-1 expression in the epithelial compartment argues further against activation of IEL by the classical route. Thus role of the enterocyte in vivo, in respect of antigen presentation remains to be defined.
Specific binding of the CD3-TCR complex leads to T cell proliferation. In vitro studies suggest that IEL have a relatively weak response to alloantigens, mitogens and anti-CD3 antibody (Ebert 1989). They do respond vigorously, however, to anti-CD2 antibodies, however, which activate T cells throught the CD2-LFA-3 pathway. Other studies have emphasised the role of accessory signals in the activation of T cells via the CD3-TCR pathway and suggest that the nature of such accessory signals determine whether triggering of this pathway results in activation or tolerance (Geppert et al. 1990). There is, for example, some evidence that the integrin αβ7 may function as an alternative accessory molecule for IEL allowing activation via CD3-TCR pathways (Sarnacki et al. 1992). The nature of accessory signals available to IEL in vivo are not known but they may be of considerable importance in determining the response of IEL following binding of antigen.

Function of IEL

The functions of IEL are not clearly defined. Defence against foreign organisms at the epithelial barrier seems likely and could be mediated either through direct cytotoxic mechanisms or through the local secretion of cytokines.

Animal studies provide evidence that IEL are cytotoxic cells with the identification of granzymes and perforins in murine IEL (Guy-Grand et al. 1991). Cytotoxicity by both αβ and γδ TCR+ IEL against target cells has been demonstrated in rodents (Goodman et al. 1989; Viney et al. 1990b). IEL are markedly increased in numbers during parasitic infestation with Giardia lamblia (Ferguson 1977) and spontaneous cytotoxicity has been observed against the parasite (Kanwar et al. 1986). Studies suggest that IEL are spontaneously cytotoxic against virally infected cells (Carman et al. 1986) and may therefore be able to protect the mucosa by eliminating infected epithelial cells. Knowledge of the cytotoxic properties of human IEL are limited by the relative paucity of relevant studies. They do not appear to be spontaneously cytotoxic against malignant cell lines (Cerf-Bensussan et al. 1985) but in vivo their cytotoxic activity may be increased under certain circumstances (Ruthlein et al. 1992). Lymphokine-activated
killer and allogeneic cytolytic activity but not antibody-dependent cellular cytotoxicity has been observed (Roberts et al. 1993).

There is little direct evidence for the secretion of cytokines by IEL and such a role for these cells is not proven, certainly in humans. Secretion of a soluble factor with all the characteristics of IFN-\(\gamma\), by rat IEL has been observed (Cerf-Bensussan et al. 1984). It has been suggested that in situations where excessive antigenic stimulation leads to an accumulation of IEL (Arnaud-Battandier et al. 1986; Ciclitira et al. 1986b; Guy-Grand et al. 1986), IEL synthesising large amounts of IFN-\(\gamma\), induce increased expression of HLA class II molecules. There is no direct evidence that the IEL are responsible for such IFN-\(\gamma\) secretion which may be produced by the adjacent lamina propria lymphocytes. Evidence against IEL being major cytokine producers is provided by a study of experimental graft-versus host disease where cytokine secretion by CD8+ cells was poor (Guy-Grand et al. 1986). In addition, recent studies using radioactive in situ hybridisation to detect and localise mRNA expression of cytokines in human jejunal mucosa detected little or no expression of cytokine mRNA by IEL (Kontakou et al. 1995a; Kontakou et al. 1994). Immunohistochemical studies, however, have shown expression of IFN-\(\gamma\) by IEL (Al-Dawoud et al. 1992). The differences in results may reflect the difficulties of localising a secreted product by immunohistochemistry.

The \(\gamma\delta\) TCR and \(\gamma\delta\) TCR POSITIVE INTRAEPITHELIAL LYMPHOCYTES

The \(\alpha/\beta\) TCR had been well characterised before the isolation of the a third gene which, similarly to TCR \(\alpha\) and \(\beta\) chains, was specifically rearranged in T cells and the biochemical characterisation of the corresponding cell surface glycoprotein together with another polymorphic chain, the \(\delta\) chain. The structural homologies between the \(\alpha/\beta\) and \(\gamma/\delta\) chains together with its obligatory association with the CD3 signal transduction complex suggested that \(\gamma/\delta\) chains formed another class of recognition structure (Lefranc et al. 1990). The lymphocytes that are \(\gamma/\delta\) TCR+ can be subdivided into several groups depending their stage of appearance during ontogeny, the sets of \(\gamma\) and \(\delta\) genes they use and the extent of VDJ junctional diversity. Most \(\gamma/\delta\) T cells do not
express either CD4 or CD8, suggesting they may recognise antigen in its native form or presented by non-classical MHC molecules. The \( \gamma \delta \) T cells vary in their tissue distribution and interest arose when evidence was presented suggesting that intestinal IEL of mice preferentially use the \( \gamma \delta \) TCR (Bonneville et al. 1988; Goodman et al. 1988). Confusion occurred regarding the degree of \( \gamma \delta \) TCR usage probably because of the dietary and microbiological status of the experimental animals, since in germ free or weanling mice, IEL were mostly \( \gamma \delta \) TCR+, compared to normal mice where the frequency of \( \alpha \beta \) TCR usage rose (Viney et al. 1990a).

The human differentiation pathway of \( \gamma \delta \) TCR+ IEL has not been established, but in mice there is reasonable evidence that thymus independent differentiation may occur, possibly under the influence of the intestinal microenvironment. Human in vitro studies have demonstrated that immature T cell precursors can differentiate into \( \gamma \delta \) TCR+ cells in the presence of various cytokines and \( \gamma \delta \) TCR+ cells have been found in a patient with complete thymic aplasia (Cerf-Bensussan et al. 1991). Human \( \gamma \delta \) TCR+IEL are usually double negative for CD4 and CD8, with approximately 30% expressing CD8, some of which only express the \( \alpha \)-chain of the CD8 molecule. This phenotype is a characteristic of thymus-independent mouse IEL, as is lack of expression of CD5, which is also demonstrated by human \( \gamma \delta \) TCR+IEL (Jarry et al. 1990).

The availability of different monoclonal antibodies directed at epitopes encoded by certain \( V_\gamma \) or \( V_\delta \) gene segments has allowed further analysis of the repertoire of human \( \gamma \delta \) T cells. Monoclonal antibody BB3 (Ciccone et al. 1988) recognises an epitope encoded by the \( V_82 \) gene and monoclonal \( \delta \)TCS-1 (Wu et al. 1988) recognises an epitope dependent on the \( V_81-J_81 \) combination. The subset identified by the \( \delta \)TCSi predominates in the thymus and peripheral blood at birth and then persists as a relatively constant proportion of peripheral T cells. The subset of \( \gamma \delta \) TCR+ cells recognised by the BB3 antibody constitute a small proportion of \( \gamma \delta \) TCR+ cells in the thymus and cord blood but rapidly expand after birth and accounts for the major population \( \gamma \delta \) TCR+ cells in the peripheral blood of adults. The distribution of
γδ T Cells in Gluten Sensitive Enteropathy

Intestinal γδ TCR+IEL subsets appears more variable than in the peripheral blood but there appears to be a preferential accumulation of cell using the Vδ1 gene segment (Halstensen et al. 1990; Jarry et al. 1990; Spencer et al. 1989a). The mechanisms underlying the differential expansion of γδ TCR+ cells in different tissues is unknown.

The antigens recognised and functions of γδ T cells remains unknown. Janeway and colleagues have suggested that they perform an “autologous surveillance” role by removing damaged epithelial cells possibly by recognition of heat shock or “stress” proteins, which are evolutionarily highly conserved (Janeway 1988). Evidence also exists of recognition of peptide presented by both MHC class I and II molecules, although the proportion of γδ T cell clones able to perform this function appears to be relatively low (Spits et al. 1990). γδ T cell clones also display high levels of in vitro cytolytic activity against various cell lines.

γδ TCR Positive Intraepithelial Lymphocytes and Coeliac Disease

Patients with coeliac disease have a significantly increased number of jejunal epithelial γδ+IEL (Halstensen et al. 1989; Savilhati et al. 1990; Spencer et al. 1989a). The role of these cells in the pathogenesis of coeliac disease was unclear, but removal of damaged intestinal epithelial cells or direct cytotoxic damage to the epithelium were possibilities. The increase appeared to be constant despite the dietary status of patient and therefore irrespective of the degree of enteropathy. This suggested that γδ+IEL might have a fundamental role in the aetiology of coeliac disease.

DERMATITIS HERPETIFORMIS–INTESTINAL IMMUNOPATHOLOGY

In 1966 Marks and colleagues described an identical intestinal lesion to that found in coeliac disease, in 9 out of 12 patients with dermatitis herpetiformis (Marks et al. 1966). At that time only 60-70% of patients with dermatitis herpetiformis appeared to have mucosal abnormalities (Fry et al. 1967; Shuster et al. 1968). Shortly afterwards, however, a study demonstrated that 21 of 22 patients with dermatitis herpetiformis had intestinal changes, if multiple biopsies were taken and suggested that the previously described lower incidence of intestinal change, was due to single biopsies being
assessed (Brown et al. 1971). This idea was supported by the description of patchy change within the intestine of patients with dermatitis herpetiformis, together with a somewhat milder degree of villous shortening (Scott et al. 1976). Fry and colleagues subsequently showed that the frequency of histologically abnormal jejunal mucosa increased to 93% if the diagnostic criteria included an increased IEL count (Fry et al. 1972).

The HLA association between dermatitis herpetiformis and coeliac disease appears to be virtually identical. In a study from this laboratory, HLA-DQ2 was present in 100% of patients with dermatitis herpetiformis versus 40% of control subjects (Hall et al. 1991a). It is likely that HLA class II genes directly influence the immune responses leading to mucosal damage in both diseases. Other genetic influences probably are involved and may explain, at least in part, the susceptibility to develop a rash in certain gluten-sensitive individuals.

The degree of enteropathy in patients with dermatitis herpetiformis varies from sub-total villous atrophy through to normal villous architecture with an increased density of IEL, the Type I lesion as described by Marsh (Fry et al. 1972; Marsh 1992). Detailed computerised image analysis has shown that these are not two separate entities but represent a continuum of change (Marsh 1988). Where a particular patient lies within that continuum may depend on the amount of gluten in their diet since patients with apparently normal villous architecture may develop further enteropathy following dietary gluten loading (Chorzelski et al. 1988; Ferguson et al. 1987; Weinstein 1974).

There remains however, a group of patients with dermatitis herpetiformis who are free of gastrointestinal lesions. Approximately 90% of patients with dermatitis herpetiformis have granular IgA and 10% have linear IgA deposits at the dermoepidermal junction. Whilst almost all patients with granular IgA deposits have an associated enteropathy, the frequency of enteropathy in patients who have linear IgA deposition is greatly reduced to between 10-20% (Ciclitira et al. 1986a). The patients with linear IgA deposition do not show any association with the extended haplotype, HLA-A1, -B8, -DR3, DQ2, (Hall et al. 1991b). These findings suggest that linear IgA
Cells in Gluten Sensitive Enteropathy associated dermatitis herpetiformis is pathophysiologically a distinct entity. If patients with linear IgA disease are excluded then all dermatitis herpetiformis patients demonstrate gastrointestinal involvement.

The immunological abnormalities described so far within the intestine in patients with dermatitis herpetiformis appear to be almost identical to those found in coeliac patients. Increased numbers of lymphocytes are found in the lamina propria, of which a large number are B cells and plasma cells. IgA producing cells predominate, although IgG and IgM producing cells are also increased (Jenkins et al. 1986; Lancaster-Smith et al. 1977). Lamina propria T cells are increased in numbers and there has been a single report of a relative increase of such cells in dermatitis herpetiformis compared to coeliac patients (Jenkins et al. 1989). The ratio of CD4+/CD8+ T cells is the same in both conditions (Griffiths et al. 1988). Increased epithelial expression of HLA class II antigens and secretory component is also similar (Scott et al. 1981). In those patients with little evidence of enteropathy subtle markers of gluten sensitivity have been shown. Intestinal humoral immunity was examined in patients with dermatitis herpetiformis and normal jejunal histology on a gluten-containing diet (O'Mahony et al. 1990b). The pattern of secretory immune responses in the dermatitis herpetiformis patients was similar to that in untreated coeliacs but serum levels of antigliadin antibodies did not show such similarities. The contribution of γδ+IEL to the intestinal lesion of dermatitis herpetiformis had not been studied prior to the thesis being undertaken.

THE RECTAL MUCOSA IN COELIAC DISEASE

Colonic histopathological changes in coeliac disease were first studied by Rubin and colleagues in the early 1960's (Flick et al. 1962). The first study detailed biopsy appearances of 212 patients of whom 13 had coeliac disease (Flick et al. 1962). Of the 13, 3 were assessed as normal, 7 as equivocal and 3 as abnormal. A further publication added more patients to the series, 14 had normal biopsies, 7 equivocal and 2 abnormal (Dobbins et al. 1964). The authors speculated whether the findings might reflect an
abnormal reaction of the rectal mucosa to gluten in a parallel mechanism to that found in the small intestine.

Two more recent retrospective studies have also detailed non-specific changes in the rectal mucosa in symptomatic coeliac patients (Breen et al. 1987; DuBois et al. 1989). The main limitation with these studies is that they were retrospective, dealt only with symptomatic patients and it was difficult to determine whether the observed changes were due to some manifestation of coeliac disease or to some other associated disease process such as ulcerative colitis.

There are well documented case reports of coeliac disease occurring in association with ulcerative colitis (Kitis et al. 1980; Kumar et al. 1979). Evidence has also emerged that this occurs more than by chance with the demonstration of a familial association between the two conditions (Mayberry et al. 1986; Shah et al. 1990). It is not clear, however, whether the proctitis observed in coeliac disease is gluten associated or whether it is at the mild end of a spectrum of disease that continues through to ulcerative colitis.

Recent studies by Loft, Marsh and colleagues have addressed these issues (Loft et al. 1990; Loft et al. 1989). Using a computerised image analysis of the rectal mucosa in patients with coeliac disease, the lamina propria in untreated coeliacs was found to contain increased mononuclear cells, which reverted to normal on withdrawal of gluten. Rectal gluten challenge with Frazer's Fraction III induced an acute inflammatory response 1-2 hours post challenge with swelling and increased microvascular permeability probably brought about by mast cell degranulation. A late-phase swelling at 48-72 hours was preceded by an influx of neutrophils and basophils. At 4-8 hours there was a marked rise in IEL. A prospective study using rectal gluten challenge confirmed the previous findings and demonstrated that a predefined rise in the IEL numbers had a useful predictive diagnostic sensitivity and specificity of greater than 90%. The production of rectal mucosal inflammatory changes following gluten challenge in coeliac patients suggests strongly that the proctitis seen in coeliac patients is an entity separate from ulcerative colitis and is not on a continuum of mucosal
**γδ T Cells in Gluten Sensitive Enteropathy**

Inflammation varying from mild to overt ulcerative colitis. The role of γδ IEL had not been studied previously in the rectal mucosa and Dr Marsh made the tissue taken during their series of rectal gluten challenges available for this study.

**Aims**

1) To study the utilisation of the γδ TCR by small intestinal IEL in coeliac disease and dermatitis herpetiformis.

2) To relate γδ IEL density to the degree of enteropathic change.

3) To study utilisation of the γδ TCR by rectal IEL before and after local gluten challenge with FFIII.
Methods

JEJUNAL STUDIES

Patients

Biopsies were obtained from patients with coeliac disease on a normal diet (n=9, mean age 43 years, range 24-80), with coeliac disease on a gluten free diet (n=10, mean age 44, range 23-65), patients with dermatitis herpetiformis (n=10, mean age 47, range 29-63) and controls (n=9, mean age 41, range 23-72), who were subsequently found to be suffering from irritable bowel syndrome. The patients with dermatitis herpetiformis were all on a normal diet. The diagnosis of dermatitis herpetiformis had previously been made clinically, histologically and by the demonstration of granular IgA deposits on immunofluorescence study of a biopsy of unaffected skin. All the patients were on dapsone by the time of the study. The treated coeliac patients had all been on a gluten free diet for at least one year.

Biopsies

All the biopsy specimens were obtained using a Quinton multiple biopsy instrument from the subjects as part of their diagnostic management. The biopsies were immediately orientated, embedded in OCT, snap frozen in thawing isopentane over liquid nitrogen and stored in liquid nitrogen until they were processed. Cryostat sections were cut at 5μm on a Bright Open-Top Cryostat.

Immunohistochemistry

A streptavidin-biotin immunoperoxidase method was employed as described in Chapter 2. The following primary monoclonal antibodies were used: CD3 (Dako, High Wycombe, Bucks, UK), a pan T cell marker, TCRδ1 (T Cell Sciences, Cambridge, MA, USA), which recognises all γδ TCR expressing T cells, δTCS-1 (T Cell Sciences, Cambridge, MA, USA) recognises an epitope dependent on the Vδ1-Jδ1 and BB3 (a kind gift of Dr A Moretta) which recognises an epitope encoded by the Vδ2 gene. Omission of the primary antibody with and without the addition of MAC 181
γδ T Cells in Gluten Sensitive Enteropathy

(Freedman et al. 1988), a monoclonal antibody against gliadin, served as control against non-specific antibody binding and endogenous peroxidase activity.

Quantitation

The density of cells expressing CD3 or TCRδ1 in the epithelium was determined by counting the number of stained cells as a percentage of the total cells in the epithelium, both IEL and enterocytes, since in frozen sections it is not always possible to determine whether a negative cell is epithelial or lymphoid. Over 1000 cells were counted for each antibody, in each patient. Counts were expressed as IEL per 100 epithelial cells. Percentage γδ TCR usage within the epithelial T cell population was calculated from the densities of CD3 and TCRδ1 positive IEL.

Morphometric measurements were performed using an eyepiece micrometer; for each section at least 5 measurements of villus height/crypt depth ratio and enterocyte height were made and the mean calculated.

Statistics

Differences between the densities of stained cells in the patient groups were compared with the Mann Whitney U Test. Correlations between cell densities and morphometric data was determined using the Kendall Rank Correlation test.

RECTAL

Patients

Seven patients with treated coeliac disease were studied (5 women and 2 men; mean age 40 years, range 24–68), all of whom had been on and responded well to a gluten free diet for more than one year. The disease control group comprised of 4 patients (3 men and 1 woman) suffering from iron deficiency anaemia(1), irritable bowel syndrome(1), hepatic secondary carcinoma(1) and scleroderma(1). All patients gave written consent and the study was approved by the Ethical Committee for Salford District (North-Western Regional Health Authority).
Gluten Challenge

The gluten challenge consisted of 2g of Frazer’s Fraction III, a physiological peptic-tryptic digest of gluten, which was dissolved in sterile water and instilled into the lower rectum via a small catheter. Sigmoidoscopic biopsies were obtained prior to and at 1, 2, 4, 6, 12, 24 and 48 hours after the challenge.

Biopsies

The biopsies were immediately orientated, and laid flat on a small cube of kidney, embedded in OCT, snap frozen in thawing isopentane over liquid nitrogen and stored in liquid nitrogen until they were processed. Cryostat sections were cut at 5μm on a Bright Open-Top Cryostat.

Quantitation

The density of cells expressing CD3 or TCRδ1 in the epithelium was determined by counting the number of stained cells as a percentage of the total cells in the epithelium, both IEL and enterocytes, since in frozen sections it is not always possible to determine whether a negative cell is epithelial or lymphoid. Over 500 cells were counted for each antibody, in each patient. Counts were expressed as IEL per 100 epithelial cells.

Statistics

The epithelial densities of stained cells during challenge were compared by repeated measures analysis of variance. Bonferroni post hoc analysis was used to analyse time points of significant change. The Mann-Whitney U test was used to compare the pre-challenge cell densities between the coeliac and control groups. Statistical significance was set at P<0.05 and results expressed as means ± standard error of the mean.
Results

JEJUNAL STUDIES

The data is summarised in Tables 3.1 and 3.2 and represented with histological examples and graphically in figures. The density of CD3+IEL was significantly increased in the untreated coeliac patients compared to treated coeliacs (P<0.0005) and compared to controls (P<0.0005). There was a significant increase in CD3+IEL in the dermatitis herpetiformis patients compared to controls (P<0.0005), but no difference between the dermatitis herpetiformis patients and untreated coeliacs (Fig 3.2).

The density of δ1+IEL was significantly increased in the untreated coeliac patients compared to treated coeliacs (P<0.05) and compared to controls (P<0.0005). There was a significant increase in δ1+IEL in the dermatitis herpetiformis patients compared to controls (P<0.0005), but no difference between the dermatitis herpetiformis patients and untreated coeliacs (Fig 3.2).

The density of δTCS1+IEL was significantly increased in the untreated coeliac patients compared to treated coeliacs (P<0.05) and compared to controls (P<0.0005). There was a significant increase in δTCS1+IEL in the dermatitis herpetiformis patients compared to controls (P<0.0005), but no difference between the dermatitis herpetiformis patients and untreated coeliacs (Fig 3.3). There were no significant differences in the expression of BB3+IEL between the different patient groups (Fig 3.3).

There was no major difference in the fractional usage of the γδ TCR by CD3+IEL between among the coeliac patients and those with dermatitis herpetiformis (Fig 3.4). There was a marked difference between these patients and the controls. A significant correlation was found between CD3+IEL counts and δ1+IEL counts, for all patients and also for the coeliac and dermatitis herpetiformis group (τ=0.75, P<0.0001, τ=0.64, P<0.0001 respectively, Fig 3.5). Similarly, a significant correlation was found between CD3+IEL counts and δTCS1+IEL counts, for all patients and also for the coeliac and dermatitis herpetiformis group (τ=0.75, P<0.0001, τ=0.59, P<0.0001.
respectively, Fig 3.5). No correlation was found between CD3+IEL counts and BB3+IEL counts in either patient group.

The untreated coeliac patients all demonstrated marked mucosal damage with villous atrophy (Table 3.2). Only one of the treated patients had a VH/CD greater than 3 with the rest showing varying degrees of damage. The dermatitis herpetiformis patients demonstrated mucosal damage in between the two coeliac groups.

Correlation between IEL density and morphometric indices was examined for all patients and those with coeliac disease and dermatitis herpetiformis as a group. There were significant correlations in the total patient group between CD3+IEL counts and villus height-crypt depth ratio ($\tau=-0.57, P<0.0001$) and enterocyte height ($\tau=-0.36, P<0.005$); between $\delta1$+IEL counts and villus height to crypt depth ratio ($\tau=-0.57, P<0.0001$) and enterocyte height ($\tau=-0.39, P<0.001$); between $\delta$TCS1+IEL counts and villus height to crypt depth ratio ($\tau=-0.59, P<0.0001$) and enterocyte height ($\tau=-0.38, P<0.005$). No significant correlation was found between BB3+IEL counts and villus height to crypt depth ratio and enterocyte height (Figs 3.6 and 3.7).

There were significant correlations in the coeliac disease and dermatitis herpetiformis group between CD3+IEL counts and villus height-crypt depth ratio ($\tau=-0.37, P<0.01$) and enterocyte height ($\tau=-0.35, P<0.05$); between $\delta1$+IEL counts and villus height to crypt depth ratio ($\tau=-0.37, P<0.01$) and enterocyte height ($\tau=-0.39, P<0.005$); between $\delta$TCS1+IEL counts and villus height to crypt depth ratio ($\tau=-0.32, P<0.05$) and enterocyte height ($\tau=-0.4, P<0.005$). No significant correlation was found between BB3+IEL counts and villus height to crypt depth ratio and enterocyte height (Figs 3.6 and 3.7).

RECTAL

The data is summarised in Table 3.3 and represented graphically in figures. In the control group, adequate tissue for analysis was available only for one patient at the 24hr time point. There was no significant difference in CD3+ IEL counts between coeliac and control patients prior to challenge. Similarly there was no significant
difference in $\gamma\delta^+$ IEL counts between coeliac and control patients prior to challenge, although generally the mean $\gamma\delta^+$ IEL count was higher in the coeliac group both prior to and throughout the challenge.

A marked and significant rise in CD3+ IEL occurred after challenge, in the coeliac patients, peaking at 6hr and returning to normal by 48hr ($P<0.05$) (Figs 3.8). There were no significant changes in the $\gamma\delta$TCR+ IEL (0hr-1.2±0.2, 6hr-1.3±0.2; NS). There were no significant changes following challenge in the controls (Figs 3.9).
### TABLE 3.1 CD3+, δ1+, δTCS1+ AND BB3+ CELLS IN THE JEJUNAL MUCOSA

<table>
<thead>
<tr>
<th>PATIENT GROUP</th>
<th>CD3+</th>
<th>δ1+</th>
<th>δTCS1+</th>
<th>BB3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatitis Herpetiformis</td>
<td>39.9±2.8</td>
<td>14.6±1.8</td>
<td>14.4±2.0</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Coeliac–Normal Diet</td>
<td>43.4±1.6</td>
<td>13.5±1.4</td>
<td>11.9±1.4</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Coeliac–Gluten Free Diet</td>
<td>26.7±1.2</td>
<td>8.3±0.9</td>
<td>7.5±0.9</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Controls</td>
<td>15.3±1.1</td>
<td>0.7±0.1</td>
<td>0.5±0.1</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

### TABLE 3.2 MORPHOMETRIC VARIABLES IN JEJUNAL MUCOSA – VILLOUS HEIGHT TO CRYPT DEPTH RATIO AND ENTEROCYTE HEIGHT (μM)

<table>
<thead>
<tr>
<th>PATIENT GROUP</th>
<th>VILLOUS/CRYPT RATIO</th>
<th>ENTEROCYTE HEIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatitis Herpetiformis</td>
<td>1.4±0.1</td>
<td>33±2</td>
</tr>
<tr>
<td>Coeliac–Normal Diet</td>
<td>1.0±0.2</td>
<td>31±3</td>
</tr>
<tr>
<td>Coeliac–Gluten Free Diet</td>
<td>2.2±0.2</td>
<td>37±1</td>
</tr>
<tr>
<td>Controls</td>
<td>3.4±0.1</td>
<td>41±1</td>
</tr>
</tbody>
</table>
3.1 (a) Untreated coeliac disease

3.1 (b) Treated coeliac disease
3.1 (c) Dermatitis herpetiformis

3.1 (d) Control patient
Fig 3.2 Plot of CD3 and δ1+ intraepithelial lymphocyte counts in all patient groups.
Fig 3.3 Plot of γδTCS1 and BB3+ intraepithelial lymphocyte counts in all patient groups.
Fig 3.4 Plot of percentage δ1+IEL/CD3+IEL in all patient groups

- DH
- CD/ND
- CD/GFD
- Controls
Figure 3.5: Plot of $\delta 1$, $\delta$TCS1, and BB3+ versus CD3+ intraepithelial lymphocyte counts in all patient groups.

- Top graph: $\delta 1$ IEL/100 epithelial cells vs. CD3+ IEL/100 epithelial cells. Correlation coefficient $\tau = 0.75$, $P < 0.0001$.
- Bottom graph: $\delta$TCS1 and BB3+ IEL/100 epithelial cells vs. CD3+ IEL/100 epithelial cells. $\delta$TCS1: correlation coefficient $\tau = 0.75$, $P < 0.0001$. BB3: non-significant (NS).
**Fig 3.6** Plot of CD3 and δ1+ intra-epithelial lymphocytes versus villous height to crypt depth ratio and enterocyte height

IEL/100
Epithelial Cells

\[ \tau = -0.57, \ P < 0.0001 \]

\[ \tau = -0.39, \ P < 0.001 \]

\[ \tau = -0.57, \ P < 0.0001 \]

\[ \tau = -0.36, \ P < 0.005 \]
**Figure 3.7** Plot of δTCS1 and BB3+ Intraepithelial Lymphocytes Versus Villous Height to Crypt Depth Ratio and Enterocyte Height

IEL/100 Epithelial Cells

- DH
- CD/ND
- CD/GFD
- Controls

\[ \tau = -0.59, P < 0.0001 \]

\[ \tau = -0.38, P < 0.005 \]

\[ \tau = 0.05, P - NS \]

\[ \tau = 0.05, P - NS \]
**TABLE 3.3 CD3+ AND γδ+ INTRAEPITHELIAL LYMPHOCYTES IN THE RECTAL MUCOSA FOLLOWING LOCAL GLUTEN CHALLENGE**

Time in hours post challenge and cell counts per 100 epithelial cells.

<table>
<thead>
<tr>
<th>TIME</th>
<th>CD3+ CELLS</th>
<th>γδ+ CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COELIAC</td>
<td>CONTROLS</td>
</tr>
<tr>
<td>0</td>
<td>12.2±2.0</td>
<td>8.0±0.7</td>
</tr>
<tr>
<td>1</td>
<td>10.2±1.7</td>
<td>9.0±1.5</td>
</tr>
<tr>
<td>2</td>
<td>18.0±2.3</td>
<td>7.8±0.8</td>
</tr>
<tr>
<td>4</td>
<td>19.1±1.4</td>
<td>7.6±0.0</td>
</tr>
<tr>
<td>6</td>
<td>26.9±3.6</td>
<td>9.8±0.7</td>
</tr>
<tr>
<td>12</td>
<td>18.5±2.9</td>
<td>8.8±1.1</td>
</tr>
<tr>
<td>24</td>
<td>19.1±0.9</td>
<td>9.3±—</td>
</tr>
<tr>
<td>48</td>
<td>11.9±2.2</td>
<td>8.3±0.7</td>
</tr>
</tbody>
</table>
FIG 3.8 PLOT OF CD3+ INTRA-EPITHELIAL LYMPHOCYTE COUNTS IN THE RECTAL MUCOSA FOLLOWING GLUTEN CHALLENGE
**Fig 3.9** Plot of $\gamma^\delta+$ intra-epithelial lymphocyte counts in the rectal mucosa following gluten challenge.
Discussion

A previous report had shown initially an increase in CD3+CD4-CD8-, so called "double negative", IEL which was soon followed by the clear demonstration of increased numbers of γδ+ IEL in coeliac disease once specific monoclonal antibodies were available for immunohistochemical studies (Halstensen et al. 1989; Savilhati et al. 1990; Spencer et al. 1989a; Spencer et al. 1989b). The data presented in this chapter confirmed the well described increased small intestinal IEL density in treated and untreated coeliac disease, together with dermatitis herpetiformis, compared to controls.

Likewise, increased numbers of γδ+ IEL were found in all three groups of patients compared to controls. The increase in γδ+ IEL was of a similar order to that described by Spencer and colleagues using the same method of enumeration, although none of our control patients had occasional high level of γδ TCR usage as seen in their study (Spencer et al. 1991). Other studies have also failed to demonstrate this phenomenon (Trejdosiewicz et al. 1991). Increased numbers of γδ T cells were found in all of our dermatitis herpetiformis patients, which is in agreement with the work of Marsh and colleagues who, using computerised image analysis, found abnormalities in the jejunal biopsies from all patients with dermatitis herpetiformis on a normal diet (Marsh 1989).

The great majority of the γδ+ IEL appeared to be recognised by the δTCS1 antibody, suggesting usage of the Vδ1-Jδ1 gene product, which supports the findings of previous studies (Spencer et al. 1989a; Trejdosiewicz et al. 1991). Using double immunostaining and antibody 3/62, which recognises the Vδ1 gene product irrespective of the chain junction region, Trejdosiewicz and colleagues found virtually exclusive usage of Vδ1 TCR by IEL, not only in the small intestinal mucosa but also in the gastric and colonic mucosa. Recently, V-region specific PCR analysis of mRNA prepared from intestinal biopsies confirmed predominant expression of Vδ1 within the small intestine of patients with coeliac disease (Bucht et al. 1995). Other Vδ transcripts could be detected however, of which Vδ2 and Vδ5 were commonly expressed. The
predominance of Vδ1+Vδ2-Vγ9-IEL in the entire gastrointestinal mucosa argues that gastrointestinal γδ+ IEL were a distinct population from the peripheral γδ+ T cells, which were mainly of the Vδ1-Vδ2+Vγ9+ phenotype.

Investigation of lamina propria γδ+ T cells was not an aim of this study, since the majority of workers have found them to be present in very low numbers if at all (Halstensen et al. 1989; Spencer et al. 1989a; Trejdosiewicz et al. 1991). In agreement with this, although not quantified, few γδ+ T cells were seen in the lamina propria of the intestinal biopsies from any patient. Savilahti and colleagues, however, have quantified lamina propria γδ+ T cells and found a constant increase, in coeliac patients (Savilahti et al. 1990). All the published work, including data from this thesis, demonstrates an increased utilisation of the γδ TCR by IEL in coeliac disease and dermatitis herpetiformis. There is disagreement, however, with regard to whether the epithelial density of γδ+ IEL remains constant following treatment with a gluten-free diet and recovery from enteropathy.

Halstensen and colleagues using multiple-label immunofluorescence found comparably increased (20-23%) γδ TCR usage by IEL in untreated and treated coeliac patients, some of whom had no histological evidence of villous damage (Halstensen et al. 1989). Absolute epithelial densities, presumably because an immunofluorescence technique was used making epithelial cell assessment difficult, were not commented on. Since one can assume that epithelial density of CD3+ IEL would decrease following treatment, then if the fractional usage of the γδ TCR was maintained, the epithelial density of γδ+ IEL would presumably have decreased. Trejdosiewicz and colleagues found similar γδ TCR usage by IEL in untreated and treated coeliac patients (Trejdosiewicz et al. 1991). Spencer and colleagues, using peroxidase-immunostaining, found similar increased γδ TCR usage by IEL in untreated and treated coeliac disease, but in two of the four treated patients, low epithelial CD3+ IEL densities were associated with low epithelial γδ+ IEL densities, although the degree of villous damage was not commented on (Spencer et al. 1991). This led the authors to
propose that if the IEL density of a coeliac patient decreases, then the γδ IEL density also decreases.

Savilahti and colleagues, in contrast to the studies described above, have reported constant increases in γδ IEL density (assessed per mm of epithelium) with increased fractional usage of the γδ TCR in treated patients compared to untreated coeliac patients (Savilhati et al. 1990). A study from Cerf-Bensussan's laboratory, suggested that epithelial densities of α/β+IEL but not γδ+IEL correlated with the grade of villous atrophy (Kutlu et al. 1993). Fractional usage of the γδ TCR was not reported, but epithelial densities of γδ+IEL remained high despite lack of obvious villous damage or dietary status. Examination of the scatter diagram published, however, shows that the majority of coeliac patients with normal mucosa or only partial villous atrophy had relatively low γδ+IEL densities. In addition, the patients described in the publication as group A, who were children with total villous atrophy had low γδ+IEL densities compared to the the study of Spencer et al and the data presented in this thesis (Spencer et al. 1991). These unusually low γδ+IEL densities in a group of patients with severe mucosal damage might skew the data such that a relationship with mucosal damage was not obvious. A further criticism of this study is that the method of assessment of mucosal damage was by subjective grading rather than by quantitative morphometric analysis of continuous variables, reducing the potential to detect an effect.

The novel finding presented in this thesis is that whilst γδ+IEL numbers are clearly and substantially elevated in all coeliac patients, compared to controls, there is a significant difference between γδ+IEL numbers in treated and untreated coeliacs. The fractional usage remains reasonably constant as demonstrated by the significant correlation presented graphically in Fig 3.5, in agreement with the studies discussed above (Halstensen et al. 1989; Spencer et al. 1989a; Trejdosiewicz et al. 1991). Although double-label immunostaining was not used and thus the figures for fractional usage of the γδ TCR by CD3+ IEL can be criticised, it seems relatively unlikely that there are major inaccuracies.
Significant correlations between γδ+IEL numbers and morphometric indices of enteropathy in the jejunal epithelium were found in all patients and also when the gluten-sensitive patients were examined as a group. Similar correlations were found, as expected, between indices of enteropathy and CD3+ IEL numbers. This suggests that γδ+IEL may form a component part of the epithelial cellular mucosal immune response to gluten in sensitive patients and argues against the hypothesis that γδ+IEL have a fundamental role in the aetiology of gluten-sensitive enteropathy. Early reports had suggested that a rise in γδ+IEL was specific for coeliac disease (Savilhati et al. 1990). If this was correct, γδ+IEL would appear to have a fundamental role in the aetiopathogenesis of the condition. However, it has subsequently been shown that in patients with cows milk sensitive enteropathy/post-enteritis syndrome there are also increased numbers of γδ+IEL in the small intestine (Spencer et al. 1991). It is possible that similar but poorly understood mechanisms are operating in both conditions.

The relationship between epithelial γδ+IEL density and the development of enteropathy is complex. Although γδ+IEL density correlates overall with the degree of enteropathy, there are patients with gluten-sensitive enteropathy and raised γδ T cell numbers that have little evidence of enteropathy. The question remains as to what is the cause of the unequivocal rise in γδ+IEL in coeliac disease and dermatitis herpetiformis. It is known that small amounts of gliadin may contaminate nominally gluten-free foods (Ciclitira et al. 1985). It is possible that despite being on a gluten free diet, patients ingest sufficient gluten to activate mucosal immune responses, but which are not severe enough to produce villous damage, as determined by conventional techniques. This may be a factor, but it is unlikely to be the major reason.

The relation between the genetic factors associated with coeliac disease and γδ+IEL distribution in both coeliac patients and their healthy first-degree relatives has been studied (Holm et al. 1992). The density of γδ+IEL was significantly associated with the HLA markers for coeliac disease susceptibility, DQA1*0501 DQB1*0201. In addition, a dose effect of these coeliac susceptibility DQA and DQB genes on γδ+IEL numbers was demonstrated. The mechanisms underlying these associations is
unknown but they do not necessarily reflect direct genetic influences on γδ+IEL selection or numbers. It is possible that the recognised genetic susceptibilities for coeliac disease predispose the individual to the condition and other currently unknown factors, genetic or enviromental, are necessary for full clinical expression. Partial mucosal immune activation may take place, including increased numbers of γδ+IEL, in absence of such currently unknown factors.

The relevance of γδ+IEL to the pathogenesis of coeliac disease would be easier to understand, if the function of these cells was known. It has been hypothesised that γδ+IEL are responsible for epithelial surveillance and removal of damaged stressed cells, possibly by recognition of heat-shock protein derived peptides, thereby maintaining epithelial integrity (Janeway 1988). This hypothesis fails to explain, however, the lack of increase in γδ+IEL in other intestinal conditions where epithelial damage occurs, albeit in a less selective manner than coeliac disease, such as H. pylori-associated gastritis and Crohn’s disease (Trejdosiewicz et al. 1991). Several alternative hypotheses have been suggested as possible roles for γδ+IEL. These have come from murine studies, however, which are probably not directly comparable to the human situation because of the much greater usage of γδ+IEL in the normal murine gut. One study has shown that alloreactive γδ+IEL are eliminated in the thymus but persist in the intestinal epithelium, in an anergic form. If this anergy were lost then it is possible that they might mediate cytotoxic damage to the intestinal mucosa (Barrett et al. 1993). A further study has shown that transfer of antigen-specific γδ+IEL from a tolerant mouse to the systemic circulation of a second tolerant mouse breaks oral tolerance to the antigen allowing an immune response to be mounted towards the antigen (Fujihashi et al. 1992). Possibly the function of the γδ+IEL in the normal gut is to allow the local immune system to escape systemic immune tolerance, so allowing it to address orally encountered antigens. This may occur through interactions between the IEL and CD4+ LPL.

Halstensen and colleagues, used three-colour immunofluorescence staining for expression of the nuclear proliferation marker Ki-67 and the IL-2 receptor, in an
attempt to identify any dominating subset of activated T cells in intestinal biopsies from coeliac patients (Halstensen et al. 1993). Both α/β+ CD8+ and γδ+ mucosal T cells proliferated in the epithelium in coeliac patients in contrast to subepithelial T cells with only 1.5% expressing the Ki-67 marker. By contrast, CD25 was almost exclusively expressed on CD4+ T cells in the lamina propria, and was significantly increased in both treated and untreated coeliac patients. The marked dichotomy in the expression of the activation markers is unusual. The IEL positive for Ki67 may represent a direct antigen driven proliferation but alternatively, gluten specific CD4+ LPL may produce cytokines capable of producing not only epithelial damage but also local proliferation of IEL, both α/β+ and γδ+. IL-2 driven proliferation of IEL would favour a polyclonal expansion which is supported by our data discussed below, suggesting that no selection of a particular Vβ gene occurs during exposure to gliadin (Hall et al. 1993).

Studies of cloned IEL are likely to be of considerable value in answering questions regarding their role in coeliac disease. IEL cultures were produced from small intestinal biopsies of coeliac patients and T cell clones generated by the limiting dilution method (Rust et al. 1992). As expected increased numbers of Vδ1+IEL were detected from the coeliac patients. One hundred and seven clones were produced, of which 47 were γδTCR+. Phenotypic and biochemical analysis showed the clones to be heterogenous and that the increase in γδ+IEL found in coeliac patients was not the result of a monoclonal expansion. The majority of the γδTCR+ clones generated were functional in vitro, as shown by their ability to lyse target cell lines, suggesting that they might have cytolytic effector functions in vivo. Similar results were obtained in a further study, with no expansion of any one particular clone (De Libero et al. 1993).

Workers from Oslo have isolated antigen specific T cells from the peripheral blood of coeliac patients (Gjertsen et al. 1994a). A clone was tested against an overlapping series of peptides from α-gliadin amino acid sequence 1-58. The minimal peptide recognized by the T-cell clone corresponded to residues 31-47 of α-gliadin, with the strongest response to a peptide corresponding to residues 31-49. Responses were blocked by an anti-HLA-DQ monoclonal antibody indicating that the clone was
γδ T Cells in Gluten Sensitive Enteropathy

HLA-DQ restricted. The relationship of these findings to the study of cereal toxicity in coeliac disease will be discussed in the next chapter. The same group had also produced αβ T cell clones from the jejunal mucosa of treated coeliac patients (Lundin et al. 1993). Gliadin-sensitive T cells were isolated from jejunal biopsies following overnight culture with FFIII. Cloning of these cell lines enabled the production of several gliadin-sensitive T cell clones. These clones were all sensitive to FFIII and were HLA-DQ restricted. The characterisation of the individual peptide epitopes has not yet been reported but the limited number of clones demonstrated no common Vβ or Vα genes and no common VDJβ or VJa sequence motifs. The same group has subsequently reported that although a large proportion of the gluten specific T cell clones derived from peripheral blood were restricted by DQ molecules, several were restricted instead by either DR or DP molecules (Gjertsen et al. 1994b).

The TCR repertoire of IEL has been suggested to be oligoclonal (Balk et al. 1991; Blumberg et al. 1993). A preliminary study form our laboratory has provided alternative data. Jejunal biopsies from coeliac patients and controls were used to study the TCR repertoire (Hall et al. 1993). Epithelial and lamina propria cellular preparations were produced and mRNA was subjected to reverse transcription. cDNA concentrations were corrected for TCRβ DNA concentration prior to any attempts at polymerase chain reaction (PCR) quantification because of differences in T cells numbers between biopsies specimens. PCR amplification was performed using 20 sets of Vβ primers. mRNA for all Vβ genes was present within both the jejunal epithelial and lamina propria compartments, suggesting no selection of a particular Vβ gene occurs during exposure to gliadin. These results do not support the theory of expansion of a single gliadin specific T cell clone and argue against gliadin acting as a superantigen. The major limitation of this study however, is that the VDJ region of the TCR was not examined and therefore it is possible that a common CDR3 motif is responsible for the recognition of a specific gliadin peptide.
Savilahti and colleagues have reported on jejunal γδ+IEL numbers in patients with dermatitis herpetiformis, both treated and on a normal diet. Patients on a normal diet all demonstrated marked enteropathy, either subtotal or severe partial villous atrophy, similarly to the patients studied for this thesis. Patients with dermatitis herpetiformis had constantly increased γδ+IEL numbers, regardless of the state of the intestinal mucosa or diet; fractional usage of the γδ TCR was not commented on (Savilahti et al. 1992). Similarly, the patients with dermatitis herpetiformis studied for this thesis all had significant mucosal changes as did those studied by Marsh (Marsh 1989). This is contrast to a study of 82 patients with dermatitis herpetiformis from Edinburgh, 51 of whom were taking a normal diet of which 18 had no evidence of villous atrophy (Gawkrodger et al. 1991).

There seems to be few or no qualitative differences between the enteropathy of coeliac disease and that of dermatitis herpetiformis. The continuing challenge for investigators is to determine why some gluten sensitive individuals develop the characteristic skin lesions.

RECTAL STUDIES

The data presented demonstrates T cell infiltration of the rectal mucosa in treated coeliac patients following a local gluten challenge, supporting previous observations by computerised-image analysis of 1μm toluidine blue stained sections (Loft et al. 1990; Loft et al. 1989). Consistent with the previous study, we found no significant difference in CD3+ IEL counts (i.e. total T cell count) between coeliac and control patients before challenge. The CD3+ IEL counts were in agreement with data published previously (Austin et al. 1988).

A study from Dr Marsh’s group of 65 patients with coeliac disease, did find a significant difference in CD3+ IEL numbers between the coeliac patients and controls, in contrast to their previous studies and this one (Ensari et al. 1993b). The reason suggested for this discrepancy, was that immunohistological measurements were
"better" than histological analysis because of the difficulty in assessing whether a particular cell is a lymphocyte or not, using histological methods. This does not explain the difference in results with the data presented here. Certainly in the coeliac patients there was a non-significant trend towards higher CD3+ IEL counts and it is possible that when a much larger group of patients were studied this trend would be demonstrated to be a real effect, as the study of Ensari suggests. No significant difference was detected in γδ+ IEL numbers between pre-challenge coeliac patients and controls. In the larger study discussed above, a significant difference was detected and similarly, the trend towards higher γδ+ IEL counts seen in the data presented might become significant if a larger number of patients were studied.

After challenge, however, a rapid and significant rise occurred in CD3+ IEL in the coeliac patients, which was not matched by a rise in γδ+ IEL counts. This suggests that α/β+ IEL and not the γδ+ IEL are involved in the early mucosal response to local gluten challenge. The criticism of low patient numbers failing to detect a subtle effect is less tenable in this situation, since there was clearly a significant response in CD3+IEL numbers in the coeliac patients following FFIII. This finding obviously does not preclude a role for γδ+IEL in the pathogenesis of coeliac disease since it is clear that jejunal γδ+IEL numbers are increased in coeliac disease. The rate of infiltration of the mucosa by γδ+IEL, however, would appear to be delayed compared to that of α/β+IEL. It is possible, or even probable, that these cells are involved in the long term established coeliac lesion, given the evidence of their cytotoxic capabilities.

The data confirms and extends earlier work demonstrating that the rectal mucosa is abnormal in coeliac disease. It demonstrates the capability of the sensitised coeliac rectal mucosa to respond briskly to a luminal antigen challenge by the epithelial infiltration of CD3+ lymphocytes. Presumably the lack of coeliac toxic gluten peptides in the rectal lumen, because of proximal digestion and absorption, restricts the potential expression of colonic gluten sensitivity to a relative minority of coeliac patients. The rectal mucosa in coeliac patients is not usually exposed to coeliac toxic peptides and thus the infiltration by T cells following local gluten challenge is strong evidence of
sensitised cells that are circulating, at least in the mesenteric territory. This is further evidence for T cell mechanisms playing a fundamental role in the pathogenesis of coeliac disease.

The role played by IEL, however, is difficult to interpret. In Loft’s analysis of the same rectal gluten challenges, the IEL responses occurred after the initial events seen in the lamina propria, suggesting that they do not have a major role in initiating the mucosal lesion (Loft et al. 1989). This carefully timed study showed that lymphocytes initially appeared in vessels at 2 hr, emigrated into the lamina propria at 4-6 hr, and peaked in the epithelium some 6-8 hr post-challenge. The IEL response reached a maximum subsequent to the early-phase lamina propria response, and receded before the late-phase inflammatory response. Whatever the role of IEL in coeliac disease, the twofold increase in IEL 6 hr post-challenge did seem to be a good marker of gluten sensitivity. Further studies from Dr Marsh’s group have examined the time course of mucosal adhesion molecule expression following local rectal gluten challenge (Ensari et al. 1993a). Early rises within 4 hours were demonstrated for both E-selectin and VCAM in the lamina propria. ICAM-1 did not change significantly, perhaps due to a high level of constitutive expression in the rectal mucosa. This study provides further evidence of the importance of immunopathological events in the lamina propria of the intestine in coeliac disease.

FUTURE DIRECTIONS

The demonstration of increased numbers of γδ+IEL in the intestinal mucosa of gluten sensitive patients has been an exciting discovery in the subject. Their role in the aetiopathogenesis of the disease however, still remains to be clearly defined. Useful information is most likely to come from further functional studies of cloned γδTCR+ lymphocytes. In particular, the antigens they respond to, how such antigens are presented and by which cells, are questions to which studies should be directed. In dermatitis herpetiformis, since the intestinal lesion appears to be essentially identical to that of coeliac patients, it would seem sensible to direct studies at understanding the
CONCLUSIONS

γδ+IEL are found in increased numbers within the intestinal mucosa of patients with coeliac disease and dermatitis herpetiformis. In distinction from some other studies, a correlation between γδ+IEL numbers and enteropathy was found, suggesting that they form a component part of the epithelial mucosal immune response to gluten in sensitive patients. The relationship between γδ+IEL numbers and enteropathy is complex however, since they do not seem to be involved in the early response to gluten challenge, at least in the rectal mucosa and in some individuals raised γδ+IEL numbers are found in the absence of obvious enteropathy. It is possible that γδ+IEL subserve more than one function in the aetiopathogenesis of coeliac disease; there is strong evidence from functional studies that such cells are capable of cytotoxicity but other functions remain to be defined.
CHAPTER 4: GLIADIN PEPTIDE TOXICITY IN COELIAC DISEASE

Introduction

Perhaps the most important environmental factor associated with coeliac disease is the ingestion of toxic cereal products. The interaction between, what we presume to be a relatively short, cereal peptide and the host leads to the eventual clinical expression of the condition. Definition of the toxic peptide is thus of considerable importance to understanding the pathogenesis of coeliac disease.

METHODS OF ASSESSMENT OF TOXICITY

It is helpful before a detailed consideration of cereal toxicity in coeliac disease to examine the methods that have, and are being used in the assessment of cereal toxicity.

In Vivo Challenge

Coeliac disease is characterised by the development of enteropathy following the ingestion of cereals. In vivo challenge studies approximate most closely to the clinical situation and are thus the ultimate test of coeliac toxicity for any cereal, or fraction thereof. The exact technique may vary from an oral challenge with clinical assessment or measurement of faecal fat excretion, as was used by the Dutch workers in their early studies, through to intraduodenal infusion of the cereal fraction followed by sequential intestinal biopsy and histological assessment of toxicity.

The clear disadvantage of this method of assessment is the invasive nature of the assay with its attendant potential risks of morbidity. Subject numbers in any study tend to be low, as relatively few patients are willing to participate in such studies. Reproducibility of response is difficult to assess for similar reasons and also because of the marked variability between individuals in their response to gluten and its fractions. If cereal peptides are being used, production of sufficient quantities of pure peptides may be prohibitively expensive. For these reasons workers have strived to develop less invasive and simpler methods of assessment of cereal toxicity in coeliac disease.
Intestinal Organ Culture

This technique has been employed to assess coeliac toxicity by a variety of groups but initially by Strober and colleagues (Falchuk et al. 1974). Jejunal biopsies from patients with untreated coeliac disease showed improvements in enzyme levels and morphology when cultured in the absence of gliadin, but not when the prolamin was present. No damaging effects were seen, however, when the biopsies from treated patients or controls were cultured with gliadin.

This system has been used to demonstrate toxicity of smaller gliadin peptides (see below). The technique is relatively demanding and still requires multiple intestinal biopsies. The patients however are not antigenically challenged and much smaller quantities of peptide can be used; it is probably the best alternative to in vivo challenge. Controversy exists over whether biopsies from treated patients show damage when exposed to gliadin, since some workers have demonstrated such damage (Howdle et al. 1981). In view of the difference in effect, some caution should be used when interpreting such assay.

Immunological Assays

In view of the potential role of the immune system in the pathogenesis of coeliac disease, some workers have developed assays based on immune activation of the easily accessible peripheral blood leucocytes of coeliac patients.

Lymphocyte Stimulation and Proliferation Assays

When T lymphocytes are exposed to a specific antigen they are stimulated to undergo cell division. This mitogenic response is associated with de novo synthesis of DNA. The degree of lymphocyte activation can therefore be assayed by the incorporation of a radiolabelled nucleotide by newly synthesised DNA. Using such an assay, a positive response was obtained when lymphocytes from coeliac patients were cultured with a subfraction of Frazer's Fraction III (Sikora et al. 1976). No response was seen following culture with control dietary antigens or with control patients. This assay therefore might form the basis of a test of cereal toxicity.
Indirect Cytokine Production Assays

Indirect measurement of cytokine production can be used as an assay of cell mediated immunity. The assays are based on the inhibition of migration of peripheral blood leucocytes following the exposure of lymphocytes to appropriate antigen. Inhibition of migration is thought to consequent upon the release of cytokines by activated T cells. Positive results have been obtained using this technique with leucocytes from coeliac patients and gluten fractions (Bullen et al. 1978). It was suggested that these effects might not be T cell mediated but related to the production of cytophilic antibodies (Simpson et al. 1983). This was not confirmed by other workers, who in addition suggested that differences in antigen preparation in different laboratories might be the basis for variations in results (Guan et al. 1987).

The basic technique has been refined for testing cereal fractions both by using an indirect assay (O'Farrelly et al. 1982), by using purified T cells rather than total peripheral blood mononuclear cells (Penttila et al. 1991), and by the use of a macrophage procoagulant activity assay (Devery et al. 1990). The indirect assay was found to be more sensitive than the direct assay and the use of purified T cells demonstrated greater migration inhibition than with total mononuclear cells. The latter workers were unable to demonstrate responses to extracts of rye, barley or oats, suggesting that extraction of the prolamins altered the immunogenicity of the potential antigens or that these prolamins were not coeliac toxic. Although there remains some doubt regarding the coeliac toxicity of oats, as will be discussed later, there is good evidence to show that rye and barley are toxic and thus the suitability of these assays to detect coeliac toxicity must be thrown into some question.

Animal Tissue Assays

There is no reliable animal model for coeliac disease. The cereal related enteropathy that has been described in Irish Red Setter dogs is of great interest and continues to be investigated (Batt et al. 1987; Hall et al. 1990a; Hall et al. 1991). Its exact relevance to coeliac disease however, is not clear.
Cultured foetal chick gut, disruption of rat liver lysosomes and culture of foetal rat gut have been used as substrates for cereal toxicity assays (Auricchio et al. 1991; Cornell et al. 1993; Mothes et al. 1985). The results obtained will be discussed below but the major problem with these assays and studies is their exact relevance to human disease.

TOXICITY OF CEREAL PROTEINS

Early Studies on Wheat

In the years immediately before, during and after the Second World War, studies by Dicke and colleagues in Holland, established that wheat contained a factor that was harmful to coeliac patients. During the famine conditions following the German occupation, he noted that the coeliac patients, deprived of cereals, improved, only to deteriorate after the Allies dropped Swedish white bread into Holland in a relief operation. In the post-war years Dicke treated coeliac patients with a wheat free diet and also challenged patients with different cereals to determine their potential toxicity (Dicke et al. 1953b).

It was established, by a series of feeding studies in which a young child was given different fractions of wheat flour in turn and clinical symptoms used to assess their toxicity, that the gliadin fraction of wheat protein was toxic, (Kamer et al. 1953). Gluten was found to be toxic as well as gliadin; the glutenin fraction caused a mild reaction which may have been due to contamination with gliadin. An alternative, later study however, concluded that both gliadin and glutenin fractions were toxic (Sheldon 1955).

Toxicity of Non-Wheat Cereals

Cereals belong to the Gramineae family and are therefore all taxonomically related (Fig 1.1). Prolamins, other than gliadin from wheat, have also been found to exacerbate coeliac disease. Rye is most closely related to wheat, followed by barley and then oats; rice and maize are more distantly related.
Gliadin Peptide Toxicity in Coeliac Disease

The toxicity of rye and oats, in addition to wheat, was reported by Dicke and colleagues, using faecal fat excretion as a measure of toxicity in feeding experiments (Dicke et al. 1953a; Dicke et al. 1953b). They also concluded that rice flour and maize starch were not toxic. The question of the toxicity of oats is complex, since studies have yielded conflicting results. Two reports suggested that they were not toxic (Dissanayake et al. 1974b; Rubin et al. 1962), but a further, using xylose absorption as a test of mucosal damage, suggested that they were (Baker et al. 1976). The latter study also demonstrated the toxicity of barley, which has been confirmed by feeding studies with histological evaluation of jejunal biopsies (Anand et al. 1981b). A recent study has found oat cereals not to be immunogenic to coeliac patients, providing further conflicting evidence (Srinivasan et al. 1995).

Enzymatic Digestion of Wheat Protein

Fig 4.1 Gluten

Frazer and colleagues developed a method of fractionating gluten by enzymatic digestion, in an attempt to investigate the nature of the toxic fraction of wheat (Fig 4.1) (Frazer 1956; Frazer et al. 1959). The starting material was commercially prepared gluten, which was sequentially subjected to peptic-tryptic digestion, thus mimicking digestion in the upper intestine. Six different fractions were produced and fed to coeliac patients, using faecal fat measurements to assess their toxicity. All except fraction VI were found to be toxic, although this fraction was only tested in a single subject. Frazer's Fraction III (FIII), which unlike gluten or gliadin is readily soluble in water...
and salt solutions, has been used extensively as a toxic fraction in the investigation of coeliac disease.

Further fractionation of FFIII, using ultrafiltration, produced three subfractions; A, B, and C (Dissanayake et al. 1974a). Fractions B and C produced histological changes and marked reduction in intestinal disaccharidase activity in feeding studies. Fraction A, which consisted of amino-acids and oligopeptides of molecular weight less than 1kD, did not. Fraction B contained a heterogeneous mixture of peptides around molecular weight 8kD.

Anand separated fraction B into B1, B2, and B3. Feeding experiments demonstrated that fraction B1 was non-toxic, whereas B2 and B3 caused harmful effects (Anand et al. 1977). Gliadin digests isolated by gel chromatography were found to contain products between molecular weights 4-10kD (Jos et al. 1978).

Deamidation using hydrochloric acid changes glutamine residues to glutamic acid and renders gliadin non-toxic, implicating glutamine as component of toxic fractions (van der Kamer et al. 1953). Crude, but not pure, papain treatment detoxifies gluten, at least by the criteria of faecal fat excretion (Messer et al. 1964). It was suggested that contamination of crude papain with some other deamidase was responsible for the detoxification. The authors suggested that the toxic moiety might be a peptide with an N-terminal glutamine residue.

Toxicity of Gliadin Fractions

Alpha-gliadin, used in a duodenal infusion challenge study caused toxic changes in serial jejunal biopsies (Hekkens et al. 1970). The α-gliadin appeared to be 80% pure using electrophoresis. All four gliadin subfractions were subsequently tested in two coeliac patients (Ciclitira et al. 1984). Aluminium lactate-starch gel electrophoresis was used to assess purity of the fractions. Visual inspection of the gel confirmed that the fractions were pure, although the β-gliadin fraction may have been contaminated with 5% of α- and γ-gliadins. Toxic changes were demonstrated in both patients with all four subfractions. These in vivo challenge studies have been confirmed by organ culture studies (Howdle et al. 1984).
Gliadin Peptide Toxicity in Coeliac Disease

Testing of Gliadin Peptides of Known Sequence

The natural progression in the investigation of cereal toxicity was to study smaller gliadin fragments of known sequence.

In Vitro Studies

Wieser separated a peptic-tryptic digest of whole gliadin from the cultivar Kolibri, using ultrafiltration and high performance liquid chromatography (HPLC). The pure peptide fractions yielded at each step of the separation, the B314 series, have been subsequently used in a series of studies and it is useful to examine their sequence in some detail (Fig 4.2) (Wieser et al. 1992; Wieser et al. 1984; Wieser et al. 1983; Wieser et al. 1986).

\[ \begin{array}{c}
\text{\( \alpha \)-Gliadin} \\
\text{I II III IV V}
\end{array} \]

The peptide B3142 corresponds to residues 3 to 55 of the A-gliadin sequence published (Kasarda et al. 1984). The sequence of this peptide however differs from A-gliadin with the substitution of a proline residue at position 31 instead of the leucine found in A-gliadin. Two further peptides in this series, B3143 and B3144 have an additional leucine and thus correspond to residues 3-56 of A-gliadin. B3143 is a slightly different \( \alpha \)-gliadin peptide, the proline at position 36 in A-gliadin being replaced by a glutamine residue. This substitution is important because the sequence QQQP then becomes QQQQ. These B314 peptides displayed some evidence of toxicity in both leucocyte migration inhibition assays and organ culture of jejunal biopsies from coeliac patients, with B3142 displaying toxicity in the greatest number of tests. Chymotryptic cuts, CNI and CNII from peptide B3142 corresponding to residues 3-24, and 25-55 of \( \alpha \)-gliadin. In vitro testing suggested that both peptides may be toxic.
De Ritis and colleagues used cyanogen bromide, which cleaves peptides at methionine residues, to produce three peptides from A-gliadin, CNI (residues 1-127), CNII (residues 128-246), and CNIII (residues 247-266) (de Ritis et al. 1988). Further peptides were derived from CNI by chymotryptic digestion: XT(1-30), XT(31-55), XT(1-55), and XT(56-68). Fragments CNI (1-127), CNII (128-246), XT(1-30), XT(31-55), and XT(1-55) were found to be toxic using an organ culture assay. Analysis of the toxic peptides showed them to contain the two tetrapeptide motifs PSQQ and QQQP (single letter amino acid code, Appendix III). These tetrapeptides were absent from those A-gliadin fragments found to be non-toxic. Peptides XT(1-30) and XT(31-55) are obviously structurally very closely related to peptides CTI and CTII discussed above (Wieser et al. 1986). The motifs PSQQ and QQQP occur six times within the A-gliadin molecule, and four times within Domain I and are shown in bold in Fig 4.3, with the adenovirus 12 homology underlined.

Ion exchange chromatography has been used to separate peptides from an enzymatic digest of gliadin (Cornell et al. 1974). The fraction 9 appeared toxic using organ culture assays. Fraction 9 was further purified using reverse phase HPLC to yield a pure peptide of sequence RPQQPYQPQPQ. Fraction 9 and this peptide were both active in screening tests with rat liver lysosomes (Cornell et al. 1993).

Auricchio used organ culture of foetal rat intestine and treated and untreated coeliac mucosa to test a number of gliadin peptides which have been reported in abstract form (Auricchio et al. 1991). Wheat gliadin peptides but not peptides from non-toxic cereals, inhibited the development of 17 day old foetal rat intestine and improvement in of jejunal mucosa in biopsies from patients with untreated coeliac disease. Using organ culture and foetal rat intestine toxicity models, peptides encompassing amino-acids 1-30 and 31-55 of A-gliadin were coeliac active. Amino-acid sequence 31-55 was further broken down into 31-45, which incorporates QQQP, and 44-55, which incorporates PSQQ. The former, but not the latter peptide was found to be active in these systems. The largest sequence common to all the active peptides in these sequences was QQQP.
Gliadin Peptide Toxicity in Coeliac Disease

Ellis and colleagues have used the approach of producing monoclonal antibodies to sequenced gliadin peptides and then using such antibodies to probe toxic and non-toxic cereals (Ellis et al. 1993b). Monoclonal antibodies were raised against a sequenced 54-amino-acid peptide of α-gliadin, peptide B3144 (Wieser et al. 1992). Two of eight of the antibodies produced, bound specifically to coeliac toxic prolamins, of which antibody WC2 had very low titres to the non-toxic cereals. The antibodies were screened by ELISA against three amino-acid-sequenced peptides of α-gliadin with single amino-acid differences, B3142, B3143 and B3144. These peptides differ from a previously published A-gliadin sequence, with the substitution of a leucine residue by a proline residue at position 31 (Fig 4.2). Peptide B3143 also has a glutamine instead of
a proline at position 36. This substitution almost abolished the reactivity of WC2 with B3143, suggesting that this antibody binds in the region of residue 36, a proline, where there may be an antigenic β-reverse turn. This proline residue forms part of a tetrapeptide motif, QQQP, which is thought to be present in all coeliac-active peptides and to play a central role in the formation of a potentially antigenic β-reverse turn (Tatham et al. 1990). The idea that WC2 binds in the region of residue 36 of A-gliadin is supported by strong binding of the antibody to CT2, residues 25-55 of A-gliadin, but weak binding to CT1. The motif QQQP is also present in CT1, but does not form part of a predicted β-reverse turn. This data suggested that a peptide around the region of position 36 of A-gliadin might be of importance to coeliac toxicity.

Similarly, antibodies were raised to the dodecapeptide of A-gliadin, which shares amino acid homologies with the E1b protein of adenovirus 12 (Ellis et al. 1992). Five monoclonal antibodies were tested against whole wheat gliadin and its alpha, beta, gamma, and omega subfractions, and the prolamins of rye, barley, oats, maize, millet, rice, and sorghum. Four of the five antibodies cross reacted with one or more of the coeliac non-toxic cereals--maize, millet, sorghum, and rice. The monoclonal antibody that did not cross react with these non-toxic cereals, did not recognize Frazer's fraction III, which is known to be toxic. The results suggest that the A-gliadin dodecapeptide shares a region of homology with cereals that do not exacerbate coeliac disease and therefore did not support the hypothesis that prior infection with adenovirus 12 might be a precipitating factor in coeliac disease.

Antigenicity, T-cell Receptor and HLA Class II Molecular Binding.

Many workers have focussed on the potential toxicity of small peptides produced by the action of both physiological enzymes and chemical cleavage. An alternative approach, in view of the possible involvement of the HLA class II and T cell receptor molecular interaction in the pathogenesis of coeliac disease, examines the potential binding of gliadin peptides to the HLA class II and T cell receptor molecules.
Protein Structure

Beta-reverse turns are secondary structural features of proteins that introduce bends into the primary amino acid chain. They are polar and likely to occupy outer positions in the protein structure. These may be predicted to function as antigenic sites and adjacent and overlapping turns have been shown to form compact loops which extend from the surfaces of some proteins and enhance their interaction with antibodies (Dyson et al. 1988). This surface location could promote interaction with the mucosal surface or with antigen presenting cells in patients with coeliac disease. It has additionally been pointed out that β-turns will usually form in short synthetic peptides, thus facilitating the raising of monoclonal antibodies that are reactive with conformational epitopes which are present in the native protein. Tatham and colleagues studied the structures of four peptides corresponding to parts of α-gliadin using structure prediction and circular dichroic spectroscopy (Tatham et al. 1990). Three of the peptides corresponded to parts of the coeliac-activating N-terminal region (residues 3-55, 3-19 and 39-45) and contained the tetrapeptide motifs PSQQ and QQQP. The fourth consists of the final seven residues of the region of homology with Ad12 and contains PSQQ (Kagnoff et al. 1984). These studies showed that beta-reverse turns were the predominant structural feature in all the peptides examined.

T-Cell Epitope Common Sequence Patterns.

A common sequence pattern has been suggested from analysis of known cytotoxic and helper T-cell epitopes (Rothbard et al. 1988). An algorithm was developed that was used successfully to predict other T-cell epitopes. Devery and colleagues have used the algorithm to identify a potential common epitope sequence in domain III of α-gliadin and examined the toxicity of an analogous synthetic peptide using an in vitro method; the results will be discussed below (Devery et al. 1991).

Amphipathic Structures

Amphipathic structures are protein structures with separated hydrophobic and hydrophillic surfaces, displaying periodicity in hydrophobic residues. It has been suggested that protein sites functioning as T cell antigenic epitopes can form stable
amphipathic structures (de Lisi et al. 1985). Since T-cells do not recognise soluble antigen, but rather antigen complexed with HLA molecules on the antigen presenting cell, the antigen may be stabilised by the hydrophobic structures on the surface of the antigen presenting cell. It was suggested that within the secondary structure of an antigenic site, there would be one face comprising hydrophobic residues. On the opposite side of an amphipathic structure, the hydrophilic polar residues would allow binding to the the T-cell receptor in a manner that would confer specificity to the interaction.

Similarly to the T cell epitope common sequence prediction, Devery and colleagues identified four sequences in gliadin that might form an amphipathic structure and examined the toxicity of analogous synthetic peptides using an in vitro method—the results will be discussed below (Devery et al. 1991).

**HLA Molecular Binding**

The close association of coeliac disease with certain HLA class II heterodimers (Hall et al. 1990b; Sollid et al. 1989), and the role of these molecules in presenting antigen to T cells as part of the mechanism for identifying foreign antigen and generating an immune response, makes it likely that a toxic gliadin peptide would bind to an HLA molecule. Evidence suggests that peptides bound to HLA class II molecules vary between 12-24 amino acids in length (Chicz et al. 1992; Hunt et al. 1992). No data has been published that has specifically examined the binding of gliadin peptides to HLA molecules, although as will be discussed below, preliminary evidence is now becoming available.

**Testing of Synthetic Gliadin Peptides**

**In Vitro Studies**

Jewell and colleagues have used indirect migration inhibition and proliferation assays to study the potential activity of synthetic peptides derived from the region of homology between A-gliadin and the E1b protein of adenovirus 12. Significant dose related responses were found to a synthetic A-gliadin peptide in treated coeliac patients but not in disease controls, although untreated patients had similar migration indices to
normal controls (Karagiannis et al. 1987). A subsequent study examined the effects of
the homologous viral peptide and both the viral peptide and the A-gliadin peptide
simultaneously (Mantzaris et al. 1990). Significant responses were again seen in the
treated coeliac group compared to controls, for both peptides. The investigators
concluded that in coeliac patients there was cross reactivity at the T cell level between
synthetic peptides derived from the E1b protein of Ad12 and A-gliadin.

Devery selected a panel of 8 peptides from α- and γ-gliadin, termed A-H, for use
in in vitro tests (Devery et al. 1991). The parameters used were, presence of PSQQ
motif (because of previous in vitro studies), length of 5-15 amino acid residues
(because of the predicted length of T cell epitopes), conformation to the amphipathic
helix algorithm, and conformation to the algorithm of Rothbard and Taylor. Peptides
were synthesised and their ability to initiate a cell-mediated immune response was
assessed using an indirect leucocyte migration inhibition assay, and a macrophage
procoagulant assay. The activities to the peptides were compared to the activities of
FFIII. Peptides A and B, both with homologies to domain I of α-gliadin, were found to
be active in these systems, peptide B being the most active. Activities were greater in
the treated coeliac group. Both contain the PSQQ motif, peptide A contains the QQQP
motif. Peptide C which contains the PSQQ motif, and peptide G which contains the
QQQP motif were inactive. Peptide C shares a nine amino-acid homology,
GSFRPSQQN, with the A-gliadin peptide having homology with Ad12. Peptides
selected on the basis of either the Rothbard/Taylor algorithm or the amphipathic helix
algorithm showed no activity. This argues against the use of epitope predictive methods
in the assessment of potential cereal coeliac toxicity, although one must take into
account the assay used, as discussed above.

Workers from Oslo, have recently isolated and cloned T cells from a coeliac
patient (Gjertsen et al. 1994; Lundin et al. 1993). A panel of synthetic α-gliadin
oligopeptides from domain I was prepared and tested against the T cell clones. A T-cell
clone from a coeliac patient recognised a synthetic α-gliadin peptide, when presented by
the cis- or trans-encoded coeliac disease associated DQ(alpha 1*0501, beta 1*0201)
heterodimer. The greatest response occurred to a peptide corresponding to residues 31-49 and the minimal peptide recognized by the T-cell clone corresponded to residues 31-47 of α-gliadin. Their findings demonstrated that the CD-associated DQ(α1*0501, β1*0201) heterodimer may serve as an antigen-presenting molecule to T cells for certain gliadin peptides and implicated residues 31-49 of α-gliadin as a coeliac toxic moiety.

In Vivo Studies

There had been only one in vivo challenge study to date involving a synthetic gliadin peptide of known amino acid sequence, before the work for this thesis was undertaken. Mantzaris and colleagues demonstrated non significant histological abnormalities in the jejunal mucosae of two treated coeliac patients after intraduodenal infusion of 100 mg of a synthetic peptide corresponding to amino acid residues 206-217 of A-gliadin, the region of adenovirus 12 homology. The results of this study will be discussed in greater detail below.

The prolamins of wheat, rye, barley and probably oats are toxic to coeliac patients. In vivo testing showed all four subfractions of gliadin to be toxic and in vitro studies of gliadin peptides have implicated the N-terminal domain of α-gliadin as toxic. The peptide motifs PSQQ and QQP appear to have particular importance as the longest sequences common to the toxic peptides. In addition, some evidence exists for toxicity of gliadin peptides homologous to adenovirus 12. There was clearly a need for further in vivo toxicity studies of gliadin peptides but these have been hampered by the difficulties of obtaining sufficient pure peptide. The availability of peptides produced by automated, solid phase synthesis in relatively large quantities has allowed such studies to be undertaken.
Aims

1) To select, using the published data on gliadin peptide toxicity, a set of gliadin-derived oligopeptides likely to be coeliac toxic and to synthesise such peptides.

2) To use the oligopeptides, following confirmation of sequence and purity, in a series of in vivo challenge experiments in patients with treated coeliac disease to assess their potential toxicity.
Gliadin Peptide Toxicity in Coeliac Disease

Methods

SELECTION OF GLIADIN PEPTIDES

Three oligopeptides were selected, termed A, B and C, from the A-gliadin sequence and are represented in Fig 4.4 (Kasarda et al. 1984) The data of Gjertsen and colleagues, with whom we collaborated, suggested that a 19mer gliadin oligopeptide was the most effective at activating HLA-DQ restricted, gliadin-specific T cells from a coeliac patient (Gjertsen et al. 1994). Thus oligopeptides of 19 amino-acids in length were selected.

Peptide A was designed to be closely related to peptides that have demonstrated in vitro coeliac activity (Auricchio et al. 1991; de Ritis et al. 1988; Wieser et al. 1986) and in addition, corresponded to the peptide that had shown to be recognised by a T cell clone (Gjertsen et al. 1994). The data of Ellis from this laboratory had shown a monoclonal antibody raised against an active peptide, residues 3-56 of α-gliadin, cross-reacts with only toxic cereal prolams and appeared to be restricted by the proline residue at position 36, which is incorporated by peptide A (Ellis et al. 1993a).

Peptide B was selected because of its homology with the E1b protein of adenovirus 12 (Kagnoff et al. 1984). In addition, there is a PSQQ motif and a probable β-reverse turn centred on the proline residue 213 (Tatham et al. 1990), together with in
vitro and in vivo evidence of coeliac activity (Karagiannis et al. 1987; Mantzaris et al. 1991; Mantzaris et al. 1990).

Peptide C was selected on the basis of the motifs PSQQ and QQQP (de Ritis et al. 1988), the presence of β-reverse turns (Tatham et al. 1990) and partial identity with peptides displaying in vitro toxicity (de Ritis et al. 1988; Devery et al. 1991; Wieser et al. 1986). The peptide-gliadin amino-acid homologies are shown in Table 4.1

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>SEQUENCE</th>
<th>α-GLIADIN HOMOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>LGQQQPFPPQOPYFQPQPFPF</td>
<td>31-49</td>
</tr>
<tr>
<td>Gjertsen et al</td>
<td>LGQQQPFPPQOPYFQPQ</td>
<td>31-47</td>
</tr>
<tr>
<td>Wieser et al</td>
<td>VQQQFDGQFPQFPPQOPYFQPQPFPFSQQNP</td>
<td>25-55</td>
</tr>
<tr>
<td>De Ritis et al</td>
<td>LGQQQPFPPQOPYFQPQPFPFSQQNY</td>
<td>31-55</td>
</tr>
<tr>
<td>Auricchio et al</td>
<td>LGQQQPFPPQOPY</td>
<td>31-43</td>
</tr>
<tr>
<td>B</td>
<td>QQYPLGQGSFRPSQQNPQA</td>
<td>202-220</td>
</tr>
<tr>
<td>Karagiannis et al</td>
<td>LRRGMFRPSQCN</td>
<td>*384-395</td>
</tr>
<tr>
<td>(*)Ad12 homology</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LGQGSFRPSQQN</td>
<td>206-217</td>
</tr>
<tr>
<td>Mantazaris et al</td>
<td>LGQGSFRPSQQN</td>
<td>206-217</td>
</tr>
<tr>
<td>C</td>
<td>VPVPQLQPQNPSSQQPSEQ</td>
<td>3-21</td>
</tr>
<tr>
<td>Wieser et al</td>
<td>VPVPQLQPQNPSSQQPSEQVPL</td>
<td>3-24</td>
</tr>
<tr>
<td>De Ritis et al</td>
<td>VRVPVQLQPQNPSSQQPSEQVPLQVQQGF</td>
<td>1-30</td>
</tr>
<tr>
<td>Devery et al</td>
<td>CPQLQPQNPSSQQPSEQ</td>
<td>5-22</td>
</tr>
</tbody>
</table>

STUDY DESIGN

Four subjects agreed to undergo a series of challenges (details below). Each subject underwent a gliadin challenge of 1000mg of unfractionated gliadin followed by 200mg of each of the oligopeptides in random order, with at least one week between each procedure to allow for recovery of the jejunal mucosa.
CHALLENGE PROCEDURE

A standard endoscopic biliary cannula was taped to the Quinton biopsy tube, with the distal tip of the cannula lying 10 cm proximal to the suction port of the biopsy tube. The biopsy tube was positioned in the jejunum, as described previously. Intravenous access was secured with a large bore cannula; intravenous fluids together with adrenaline and hydrocortisone were immediately available in case of significant reactions to the gliadin or peptides.

For each challenge, the peptides were dissolved in 50 ml of 0.5% bovine serum albumin in distilled water, to prevent significant adsorption by delivery syringe and cannula. Gliadin, 1000mg, was dissolved in 250ml of 0.5% bovine serum albumin. The dissolved peptide was infused into the distal duodenum over a period of two hours by syringe-driver pump (Sage Instruments, Cambridge, Mass., USA.). Biopsies were taken prior to and at hourly intervals for a period of six hours after starting the infusion.

SUBJECTS

We studied four, unrelated caucasian individuals with treated celiac disease (Table 4.2). The diagnosis of coeliac disease was made according to previous ESPGAN criteria (Meeuwisse 1970). All patients gave written informed consent to the study, which was approved by the local ethics committee.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Years on Gluten Free Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>63</td>
<td>M</td>
<td>24</td>
</tr>
<tr>
<td>II</td>
<td>76</td>
<td>F</td>
<td>23</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>F</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td>29</td>
<td>F</td>
<td>4</td>
</tr>
</tbody>
</table>

TABLE 4.2  PATIENT CHARACTERISTICS-GLIADIN PEPTIDE CHALLENGE
PREPARATION OF GLIADIN AND GLIADIN PEPTIDES

Gliadin

These had been prepared previously (Ciclitira et al. 1984), by established methods (Patey et al. 1973). Pure strain Kolibri wheat flour, provided by Plant Breeding International was used. The flour was defatted with butanol, extracted with ethanol, dialysed against acetic acid and lyophilised.

Synthesis of Gliadin Peptides

The peptides were kindly synthesised by my colleague Dr Paul Day, working in collaboration with Dr Gerard Evan and members of the peptide synthesis laboratory of The Imperial Cancer Research Fund, London.

Syntheses were performed using a solid phase peptide synthesiser (Model 431 A, Applied Biosystems Inc, CA, USA) on a preloaded Wang resin using 9-fluorenylmethylxy-carbonyl for temporary α-amino group protection (Carpino et al. 1972). Each amino acid residue was coupled as a hydroxy benzotriazole active ester, automatically formed immediately prior to use from the protected amino acid. Cleavage from the resin, together with deprotection of the side chains, and terminals, of the resultant peptides, was achieved using 20 mls of a mixture containing 95% (v/v) trifluoroacetic acid, 2.5% (v/v) ethanedithiol and 2.5% (v/v) water at 20°C for 2 hours. Cleavage of peptides from the resin, was followed by lyophilisation.

Analysis and Confirmation of Sequence.

The peptides were analysed using high performance liquid chromatography (HPLC), plasma desorption mass spectrometry (BioIon Spectrometer, Applied Biosystems Inc, CA, USA), and then sequenced using automated phenyl-thiohydantoin gas phase sequencing (ABI 475, Applied Biosystems Inc, CA, USA). They were then purified on a gel exclusion column (Sephadex G15, Pharmacia LKB Biotechnology, Uppsala, Sweden) The fractions were collected using a UV-1 single path monitor (Pharmacia LKB Biotechnology, Uppsala, Sweden) and FRAC-100 fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). The resulting peptide solutions were frozen using liquid nitrogen and double lyophilised.
HISTOLOGICAL ANALYSES

Tissue Preparation

Immediately upon recovery from the Quinton tube, the biopsies were carefully orientated epithelial surface upwards on filter paper and fixed in formal saline. In addition, biopsies taken prior to challenge and at four hours after starting the infusion, were snap frozen for immuno-histochemistry. Cryostat sections were cut at 5μm. Prior to staining, the slides were wrapped in aluminium foil and stored at -70°C.

Immunohistochemistry

A streptavidin-biotin immunoperoxidase method was employed as described in Chapter 2. Anti-CD3 (Dako, High Wycombe, Bucks, UK), a pan T cell marker was used as the primary monoclonal antibody.

Morphometric Analyses

Morphometric measurements were performed on 5μm wax mounted sections as described in Chapter 2. The density of cells expressing CD3 in the epithelium was determined as described in Chapter 3.

Statistical Analysis

Mean changes in villous height to crypt depth ratio (VH/CD) and enterocyte height were analysed by repeated measures analysis of variance. Bonferroni post hoc analysis was used to analyse time points of significant change both within the grouped data and individuals. Individuals' CD3+ IEL counts prior to and four hours after the start of the challenge was by the Mann-Whitney U Test. Significance was set at P<0.05.
Results

PEPTIDE SYNTHESIS

HPLC of all three peptides produced a single, narrow peak, indicative of synthesis of a single species (Fig 4.5). This was confirmed by mass spectrometry (Fig 4.6). Automated sequencing confirmed the desired sequence. Several synthetic cycles were required for each peptide to produce sufficient peptide for multiple feeding studies. Confirmatory analyses were performed for each synthetic cycle.
FIG 4.5  HPLC ANALYSES PEPTIDES A, B AND C

Fig 4.5 (a) Peptide A

Fig 4.5 (b) Peptide B
Fig 4.5 HPLC Analysis Peptides A, B and C (cont’d)

Fig 4.5 (c) Peptide C

![HPLC Analysis Peptide C](image-url)
FIG 4.6 MASS SPECTROMETRY ANALYSES PEPTIDES A, B AND C

Fig 4.6 (a) Peptide A

Fig 4.6 (b) Peptide B
FIG 4.6 MASS SPECTROMETRY ANALYSES PEPTIDES A, B AND C (CONT'D)

Fig 4.6 (c) Peptide C
GLIADIN AND PEPTIDE CHALLENGE

None of the patients reported any ill effects from the challenges. The mean data is summarised in Table 4.3 and individual data at four hours in Figure 4.7. Full data for the individuals with each peptide and gliadin is shown in subsequent graphs and tables (*=P<0.5).

Subject I

This patient demonstrated changes in response to both gliadin and peptide A. The changes in response to gliadin returned to normal by six hours both for the VH/CD and enterocyte height. The changes in VH/CD in response to peptide A, did not normalise by the end of the challenge although the less marked changes in enterocyte height did. Significant increases occurred in the CD3+ IEL counts at four hours to both gliadin and peptide A. No mucosal changes followed challenge with peptide B or C.

Subject II

This patient demonstrated changes in response to both gliadin and peptide A. The changes in response to gliadin returned to normal by six hours both for the VH/CD and enterocyte height. The changes in VH/CD in response to peptide A, did normalise by the end of the challenge and the changes in enterocyte height trended back to normal, although remaining significantly different. Significant increases occurred in the CD3+ IEL counts at four hours to both gliadin and peptide A. No mucosal changes followed challenge with peptide B or C.

Subject III

This patient demonstrated changes in response to both gliadin, peptide A and peptide B. The changes in response to gliadin remained abnormal after six hours for the VH/CD but not enterocyte height. The changes in VH/CD in response to peptide A, did normalise by the end of the challenge, as did the less marked changes in enterocyte height. Significant increases occurred in the CD3+ IEL counts at four hours to both gliadin and peptide A. Challenge with peptide B produced changes in both VH/CD ratio
and enterocyte height but no change in CD3+ IEL counts. No mucosal changes followed challenge with peptide C.

**Subject IV**

This patient demonstrated changes in response to both gliadin and peptide A which were less marked than in the other patients. There was no change in the VH/CD in response to gliadin, although there were significant changes in enterocyte height. The changes in response to peptide A normalised by the end of the challenge. Significant increases occurred in the CD3+ IEL counts at fours hours to both gliadin and peptide A. No mucosal changes followed challenge with peptide B or C.
### TABLE 4.3 MUCOSAL CHANGES FOLLOWING GLIADIN AND PEPTIDE CHALLENGES

#### VILLOUS HEIGHT TO CRYPT DEPTH RATIO

<table>
<thead>
<tr>
<th>Time</th>
<th>Gliadin</th>
<th>Peptide A</th>
<th>Peptide B</th>
<th>Peptide C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.1±0.2</td>
<td>3.3±0.2</td>
<td>3.3±0.1</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>1</td>
<td>2.9±0.2</td>
<td>2.9±0.2</td>
<td>3.0±0.1</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>2</td>
<td>2.5±0.2</td>
<td>2.7±0.3</td>
<td>3.1±0.1</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>3</td>
<td>2.4±0.2</td>
<td>2.3±0.3</td>
<td>3.0±0.1</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>4</td>
<td>2.3±0.3*</td>
<td>2.2±0.2*</td>
<td>3.0±0.2</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>5</td>
<td>2.3±0.3*</td>
<td>2.7±0.2</td>
<td>3.0±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>6</td>
<td>2.9±0.3</td>
<td>2.9±0.1</td>
<td>3.1±0.1</td>
<td>3.0±0.1</td>
</tr>
</tbody>
</table>

#### ENTEROCYTE HEIGHT (μM)

<table>
<thead>
<tr>
<th>Time</th>
<th>Gliadin</th>
<th>Peptide A</th>
<th>Peptide B</th>
<th>Peptide C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38±1</td>
<td>37±1</td>
<td>36±1</td>
<td>36±1</td>
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<tr>
<td>1</td>
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<td>33±1*</td>
<td>36±1</td>
<td>35±1</td>
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<tr>
<td>2</td>
<td>32±1</td>
<td>33±1*</td>
<td>35±1</td>
<td>34±1</td>
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<td>3</td>
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<td>33±2*</td>
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<tr>
<td>4</td>
<td>28±2*</td>
<td>32±1*</td>
<td>34±2</td>
<td>35±1</td>
</tr>
<tr>
<td>5</td>
<td>30±3*</td>
<td>34±1</td>
<td>35±1</td>
<td>36±1</td>
</tr>
<tr>
<td>6</td>
<td>37±1</td>
<td>36±1</td>
<td>36±1</td>
<td>35±1</td>
</tr>
</tbody>
</table>

#### CD3+ IEL COUNTS

<table>
<thead>
<tr>
<th>Time</th>
<th>Gliadin</th>
<th>Peptide A</th>
<th>Peptide B</th>
<th>Peptide C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25±2</td>
<td>23±3</td>
<td>23±1</td>
<td>23±1</td>
</tr>
<tr>
<td>4</td>
<td>36±3*</td>
<td>33±4*</td>
<td>24±2</td>
<td>23±1</td>
</tr>
</tbody>
</table>
FIG 4.7 Gliadin and Peptide Challenge—Enteropathic Change at Four Hours—All Patients

- Patient I
- Patient II
- Patient III
- Patient IV

Villous Height to Crypt Depth Ratio  * P<0.05
Enterocyte Height (μm)
CD3+ IEL/100 Epithelial Cells
Fig 4.8 Gliadin and Peptide Challenge—Patient I

Villous Height to Crypt Depth Ratio

Gliadin 1000mg

A 200mg

B 200mg

C 200mg

Hours Post Challenge
FIG 4.9  GLIADIN AND PEPTIDE CHALLENGE PATIENT I

ENTEROCYTE HEIGHT

Gliadin 1000mg

A 200mg

B 200mg

C 200mg

Enterocye Height (mm)

0 1 2 3 4 5 6

0 10 20 30 40 50

0 1 2 3 4 5 6

0 10 20 30 40 50

Hours Post Challenge
FIG 4.10 Gliadin and Peptide Challenge—Patient II

Villous Height to Crypt Depth Ratio

Gliadin 1000mg

A 200mg

B 200mg

C 200mg

Hours Post Challenge
FIG 4.11 Gliadin and Peptide Challenge Patient II

Enterocyte Height

Gliadin 1000mg

A 200mg

B 200mg

C 200mg

Hours Post Challenge
FIG 4.12 Gliadin and Peptide Challenge—Patient III

Villous Height to Crypt Depth Ratio

Gliadin 1000mg

A 200mg

B 200mg

C 200mg

Hours Post Challenge
FIG 4.13 GLIADIN AND PEPTIDE CHALLENGE PATIENT III

ENTEROCYTE HEIGHT

Gliadin 1000mg

A 200mg

B 200mg

C 200mg

Hours Post Challenge
FIG 4.14 GLIADIN AND PEPTIDE CHALLENGE—PATIENT IV

**VILLOUS HEIGHT TO CRYPT DEPTH RATIO**

- Gliadin 1000mg
- A 200mg
- B 200mg
- C 200mg

Hours Post Challenge
Gliadin Peptide Toxicity in Coeliac Disease

FIG 4.15 GLIADIN AND PEPTIDE CHALLENGE PATIENT IV

ENTEROCYTE HEIGHT

![Graphs showing enterocyte height over time for different challenges.](image)

- Gliadin 1000mg
- A 200mg
- B 200mg
- C 200mg

Hours Post Challenge
FIG 4.16 GLIADIN AND PEPTIDE CHALLENGE CD3+ IEL COUNTS

- ALL PATIENTS

<table>
<thead>
<tr>
<th>Patient I</th>
<th>Patient II</th>
<th>Patient III</th>
<th>Patient IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Gliadin</td>
<td>Peptide A</td>
<td>Peptide B</td>
<td>Peptide C</td>
</tr>
</tbody>
</table>

Hours Post Challenge
### Table 4.4 Morphometric Variables Following Gliadin and Peptide A Challenge

<table>
<thead>
<tr>
<th>Time</th>
<th>Gliadin – 10000mg</th>
<th>Peptide A – 200mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Patients</td>
</tr>
<tr>
<td></td>
<td>Villous Height/Crypt Depth</td>
<td>Enterocyte Height (μm)</td>
</tr>
<tr>
<td></td>
<td>I     II   III  IV</td>
<td>I    II   III  IV</td>
</tr>
<tr>
<td>0</td>
<td>3.3±0.2 3.0±0.3 2.9±0.2 3.2±0.2</td>
<td>40±2 40±2 37±1 38±1</td>
</tr>
<tr>
<td>1</td>
<td>3.1±0.2 2.8±0.2 2.4±0.3 3.3±0.2</td>
<td>37±1 33±2 36±1 35±1</td>
</tr>
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<td>32±5 32±2 33±1 33±1</td>
</tr>
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<td>30±1 27±2 32±1 34±2</td>
</tr>
<tr>
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<td>25±2 30±2 24±4 34±2</td>
</tr>
<tr>
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<td>27±2 27±1 29±2 38±1</td>
</tr>
<tr>
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<td>38±2 37±1 33±1 39±1</td>
</tr>
</tbody>
</table>
### Table 4.5 Morphometric Variables Following Peptides B and C Challenge

#### Peptide B – 200MG

<table>
<thead>
<tr>
<th>Time</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
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</tr>
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</tr>
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<td>2.6±0.2</td>
<td>3.2±0.2</td>
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<td>38±1</td>
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<td>37±2</td>
<td>38±1</td>
<td>36±1</td>
</tr>
</tbody>
</table>

#### Peptide C – 200MG

<table>
<thead>
<tr>
<th>Time</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
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<td>34±2</td>
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<td>35±2</td>
<td>36±1</td>
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**Table 4.6 CD3+ Intraepithelial Lymphocyte Counts Following Gliadin and Peptide Challenges**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gliadin 1000mg</th>
<th>Peptide A 200mg</th>
<th>Peptide B 200mg</th>
<th>Peptide C 200mg</th>
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<tbody>
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<td>0 hrs</td>
<td>4 hrs</td>
<td>0 hrs</td>
<td>4 hrs</td>
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<tr>
<td>I</td>
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<td>II</td>
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<td>25.2±2.4</td>
<td>36.3±1.6</td>
</tr>
<tr>
<td>III</td>
<td>25.9±3.0</td>
<td>41.2±3.4</td>
<td>25.9±2.7</td>
<td>42.3±3.1</td>
</tr>
<tr>
<td>IV</td>
<td>29.4±2.6</td>
<td>38.7±4.1</td>
<td>22.3±2.7</td>
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</table>
Gliadin Peptide Toxicity in Coeliac Disease

Discussion

Acute challenge as a method of assessing cereal toxicity in coeliac disease is a major undertaking, both for the patients involved and the investigators. In view of the lack of a totally reliable in vitro assay, it remains the ultimate test of toxicity and because of the small quantities available, it is the most applicable form of challenge when studying oligopeptides. There are however, several problems with the technique, not the least of which is the invasive nature of the procedure with the necessity of leaving the Quinton tube in place for six hours. The number of patients who will agree to undergo such a procedure is limited. There are potential errors in sampling, as biopsies are taken from a field where one assumes there is a uniform change, but this is not necessarily so. This source of error is not encountered with the organ culture system. In addition, there is considerable variation in any individual’s response to challenge, both in terms of the degree and time course of response. This may in part be determined by the degree of immune activation within the individual’s mucosa, perhaps reflecting the length of time on a gluten-free diet. Finally, to test the toxicity of any one gliadin fraction or peptide, one has to be certain of purity with no contamination by other potentially toxic elements.

The study was designed to minimise as far as possible these factors. Each patient underwent challenge with all three peptides, thus reducing the problem of intersubject variability and also acting as intrasubject controls. Each patient underwent a gliadin challenge at sufficient dosage to make intestinal damage likely, thus acting as both a positive control, and since the experimental subjects had been on a gluten-free diet for at least two years, potentially “repriming” the intestinal immune system to gliadin antigens, thus hopefully maximising any toxic response.

The amount of peptide to use that was both safe but also likely to cause significant intestinal damage, assuming it to be toxic, was not clear. As discussed above, there had previously been only one other in vivo challenge study, which had used 100mg of a dodecapeptide, producing equivocal evidence of damage and no deleterious clinical
Gliadin Peptide Toxicity in Coeliac Disease

effects. Previous studies had demonstrated significant intestinal damage when using 1000mg of unfractionated gliadin. A rough calculation of molar equivalence against A-gliadin suggested 70mg of peptide A to be equivalent to 1000mg of A-gliadin. We chose to use 200mg of each of the test peptides, as potentially having at least as much activity as 1000mg of A-gliadin and unfractionated gliadin, if not more and therefore maximising the chances of detecting toxicity.

Assessment of damage following challenge is obviously of importance for the validity of the technique. A variety of different methods exist for the assessment of morphometric change. One study has compared the different methods for the quantitative assessment of jejunal mucosal architecture (Corazza et al. 1985). Three methods, standard linear measurements, stereology and computer aided microscopy, were compared by assessing the same fifty biopsies. Good correlation between the results were obtained by each, with no overlap between controls and those with untreated coeliac disease. Although stereological methods were favoured, their results demonstrated that simple linear morphometry is a reliable method of assessing biopsies. The significance of infiltration of the intestinal epithelium by IEL in coeliac disease remains undecided, but it is one of most consistently reported, early features following gluten challenge, whether absolute numbers or epithelial density of IEL is assessed and does not suffer from problems of tissue orientation (Marsh 1992).

The identities of our peptides were confirmed by mass spectrometry, HPLC and amino-acid sequencing. The peptides were purified by gel exclusion chromatography and dialysed to remove reagents remaining after synthesis. The method of synthesis makes contamination by other related, potentially toxic peptides unlikely. Similarly, any observed responses were unlikely to be due to chemical toxicity, as such effects would have been seen with the other peptides.

Gliadin and Peptide Toxicity

Previous findings of a significant reduction in VH:CD ratio and enterocyte surface-cell height together with an increase in IEL count within four hours of duodenal
infusion of 1g of gliadin in patients with treated coeliac disease were confirmed (Ciclitira et al. 1984).

Significant histological changes occurred after instillation of 200mg of peptide A, corresponding to amino acids 31-49 of A-gliadin. Changes in the CD3+ IEL counts in particular, were equal or greater than those demonstrated using unfractionated gliadin, in all four subjects, and is evidence of in vivo, immunological activation within the intestinal mucosa, by peptide A. These results provide the first in vivo evidence of toxicity for this peptide and its region of homology within α-gliadin. They are supported by the substantial in vitro evidence of toxicity for peptides encompassing this region, discussed above. Additional, recent evidence for the coeliac toxicity of peptide A is presented below.

Peptide B was selected because of its homology with the E1b protein of adenovirus 12 (Kagnoff et al. 1984), and the presence of the PSQQ motif and a probable β-reverse turn centred on the proline residue 213. The lack of histological change caused by this peptide in three of the four patients studied, argues against the proposed role for adenovirus 12 in the aetiology of coeliac disease. One patient, however, developed significant morphometric changes in response to peptide B, although there was no response by the IEL. We have subsequently shown some increase in cytokine mRNA expression in biopsies from this patient after challenge with peptide B, discussed below (Kontakou et al. 1995b). These changes may represent coeliac toxicity to this fraction, which did not occur in the other three patients studied and raises the possibility that there may be a toxic epitope within this peptide to which the sensitivity of individual coeliac patients varies.

A previous study, which did not include gliadin control infusions, showed minor histological abnormalities in the jejunal mucosae of two treated coeliac patients, after infusion of 100mg of peptide corresponding to amino acid residues 206-217 of A-gliadin (Mantzaris et al. 1991). Some, but not significant, histological abnormalities occurred in the jejunal mucosae of two treated coeliac patients, after infusion of 100mg of synthetic dodecapeptide corresponding to amino acid residues 206-217 of A-gliadin.
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The authors reported for one patient no change in the IEL count throughout the study, but from two hours onwards a significant increase in the number of mononuclear cells in the lamina propria. After 4 hours this patient's biopsy showed patchy change in the mucosal architecture. The CV ratio dropped from 1:3 to 1:2/1:3. At 24 hours the lamina propria was congested and infiltrated by polymorphonuclear cells. In the second subject the IEL counts were normal up to six hours. There was, however, a progressive increase in the number of mononuclear cells, which did not reach significance. At 24 hours there was a significant increase in lamina propria mononuclear cells. No change was seen in the CV ratio. Focal changes could be seen in the electron microscopy of the coeliac patients following challenge, appearing at 2 hours and peaking at 24 hours. Measurement of disaccharidases activities in the biopsies revealed no changes up to 6 hours, but at 24 hours both patients' biopsies demonstrated significant changes. The authors postulated that the quantity of peptide used was suboptimal and therefore marked changes were not seen. The lamina propria inflammatory cell infiltrate seen in these two patients could be argued to represent early toxic change. The earliest changes seen in most other studies, however, have been increases in the IEL count. No significant change was seen in this study. Similarly whilst a decrease in brush border enzyme levels has been used as an indication of coeliac toxicity of prolamin fractions (Falchuk et al. 1978), this has been associated with changes in the villous structure (Bailey et al. 1989). Organ culture studies have subsequently confirmed lack of toxicity of peptide B, discussed below (Shidrawi et al. 1995a) Controversy therefore persists regarding the adenovirus hypothesis and the aetiology of coeliac disease. On balance, I believe the evidence is against the hypothesis. It is possible, that the relevant homology within A-gliadin is coeliac toxic, perhaps to a lesser degree or only in certain individuals. Such toxicity, of course, may be entirely incidental to the homology with the E1b protein of Ad12.

Peptide C was selected on the basis of the motifs PSQQ and QQQP (de Ritis et al. 1988), the presence of β-reverse turns (Tatham et al. 1990) and partial identity with peptides displaying in vitro toxicity (de Ritis et al. 1988; Devery et al. 1991; Wieser et
Gliadin Peptide Toxicity in Coeliac Disease

al. 1986). No significant changes were seen in any of the four patients following infusion with peptide C. These results conflict with those of others who have shown in vitro toxicity of related peptides. The C-terminal motif, VPL, represents the only difference between the peptide of Wieser and peptide C. It is possible that the omission of this motif has diminished the toxicity of peptide C. However, there was in vitro evidence of cellular immunity in peripheral blood to a peptide corresponding to amino acids 5-22 of α-gliadin, which also lacks the motif VPL (Devery et al. 1991). The present study is therefore the first in vivo demonstration of lack of coeliac activity of the far N-terminal region of A-gliadin.

All the peptides contained the putatively coeliac active tetrapeptide motifs, QQQP and/or PSQQ, and all contain potentially antigenic β-reverse turns, both of which have been proposed as models for peptide toxicity in coeliac disease (de Ritis et al. 1988; Tatham et al. 1990). Recent evidence, however suggests that HLA class II molecules bind peptides in an extended conformation and thus secondary peptide structure may be of lesser importance (Brown et al. 1993). This is consistent with this thesis, which suggests that such structures do not themselves confer coeliac toxicity to gliadin peptides, since they are predicted to be present in peptides B and C (Tatham et al. 1990) and peptide A is truncated so that the PSQQ motif is omitted. This in vivo data is supported by in vitro studies, published in abstract, demonstrating activity of synthetic peptides corresponding to residues 31-43, but not 44-55 of α-gliadin, thereby omitting the PSQQ motif (Auricchio et al. 1991).

Marsh has published in abstract form, in vivo challenge data using similar synthetic peptides homologous to residues 31-43, 44-55 and 56-68 of α-gliadin (Marsh et al. 1995). Each peptide was given to two patients, although the dose is not clear from the abstract. Toxicity was assessed by morphometric assessment, cellular infiltration, brush border enzyme analysis and serum IL-1 and IL-2 levels. In addition magnetic resonance and CD spectroscopy analysis of the peptides was performed. Peptides 31-43 and 44-55 were coeliac toxic but peptide 56-68 was not. Interestingly, structure analysis did not reveal any particular conformation, suggesting that neither β-turns nor
\( \alpha \)-helices were present or necessary for coeliac activity, which is in keeping with both the results presented here and the data regarding peptide-HLA binding. Marsh's study supports the results of this thesis, confirming in vivo toxicity for the region encompassed by peptide A and presents evidence for toxicity of peptide 44-55, that overlaps with peptide A, but has a separate PSQQP motif present. The significance of this is not clear and full details of this study are awaited.

Development of enteropathy

Controversy has arisen in the literature regarding acute gluten challenge, perhaps due to difficulties in the interpretation and comparison of studies because of the differing subfractions of wheat used, differing doses and whether the cereal fraction is administered as a bolus, intermittent boluses, or as continuous dietary intake.

Rapid effects following gluten challenge, as discussed in Chapter 2, have been described previously for markers of immune activation, inflammation and brush border enzymes and there seems little doubt that early, that is within 1-3 hours, mucosal changes occur. Descriptions of morphological change in the villous structure are more variable.

Early studies by Shiner and colleagues showed electron microscopic changes within 2 hours of a duodenal gluten challenge, but relatively mild light microscopic change after 48 hours (Shiner 1973; Shmerling et al. 1970). Similar conclusions were drawn by Doe and colleagues (Doe et al. 1975). Lancaster-Smith reported cellular infiltration of the epithelium and lamina propria at 24-48 hours post oral gluten challenge (Lancaster-Smith et al. 1975). No mention was made of morphological changes.

Anand and colleagues found evidence of histological damage, together with enzymatic changes, within 3-4 hours following instillation of an ultrafiltrate of FFIII (Anand et al. 1981a). One patient developed severe partial villous atrophy, although the other individual studied had less marked changes. Ciclitira et al, when testing gliadin fractions for toxicity, reported severe damage within 4 hours quantitated as reductions in the VH/CD to 1.0 or less, together with reduction in enterocyte height and IEL
infiltration of the jejunal mucosa (Ciclitira et al. 1984). In a study of ten treated patients, challenged with FFIII, evidence of mucosal damage manifest by falls in brush border enzymes and villous cell population counts but without changes in villous height or crypt depth, was demonstrated within 3-4 hours (Bramble et al. 1985). Freedman studied five treated coeliac patients after an oral gluten challenge. In four of these five infiltration by IEL, together with deterioration in the villus architecture, occurred within two hours of having started the challenge, although the changes were relatively mild at that point with progression over the period of the challenge (Freedman et al. 1987).

Work from Marsh’s laboratory, using FFIII in the jejunum and rectum has provided important information on the development of the mucosal lesion of coeliac disease, as discussed previously (Leigh et al. 1985; Loft et al. 1989; Marsh et al. 1992). In the jejunum, oral challenges of FFIII up to 1.5gm produced a dose dependent infiltration of IEL but no changes in mucosal architecture, with a maximal response by 12 hours for the surface IEL, the earliest time point studied. Significant architectural change occurred at doses above 3gm, initially in crypt epithelial volumes but subsequently in surface epithelial volume, increasing until at a challenge of 12gm of FFIII, villous flattening was seen at 12 hours post challenge. This careful study, with computerised image analysis of mucosal morphometry, has thus demonstrated that the jejunal epithelium has the capacity to flatten within 12 hours of challenge, if sufficient antigen is present. Since this was the earliest time point studied, no comment can be made regarding earlier change. In the rectal studies, however, biopsies were taken earlier during the challenges. Lamina propria swelling was seen 1-2 h after challenge with a rapid fall in mast cells together with granule discharge suggested their involvement in this response. A marked rise in epithelial lymphocytes began 4 hours, but volumes of surface and crypt epithelium remained constant throughout.

The results presented in this thesis, are consistent with previous studies that have demonstrated early change. In those individual challenges, where enteropathic changes developed, such changes were often manifest within 2 hours, although progressive and maximal at four hours and beyond. Occasionally some recovery appeared by the end of
the challenge. The magnitude of change in terms of the VH/CD ratio was not as marked as in some other studies, with maximal changes reducing VH/CD ratio to the order of 2, which is far from the classical flat lesion described (Anand et al. 1981a; Ciclitira et al. 1984). The reasons for this are not clear, but may reflect variations in individual sensitivity. Changes in enterocyte height and IEL infiltration were similar to changes described in other studies (Ciclitira et al. 1984; Freedman et al. 1987; Marsh et al. 1992).

MacDonald's foetal model explains enteropathy in terms of crypt epithelial proliferation, probably secondary to cytokine stimulation, in the absence of epithelial damage (MacDonald et al. 1988). Similarly, Marsh's studies emphasise the importance of crypt epithelial volume enlargement prior to changes in villous epithelial volume (although the latter does not necessarily reflect villous height) in the progressive development of enteropathy (Marsh et al. 1992). Enterocyte damage, however, is a principal feature of the established coeliac lesion. There is, in addition, good evidence from a variety of workers that villous enterocyte damage is an early feature, that is within 2 hours, following gluten challenge (Bailey et al. 1989; Shiner 1973). Bramble and colleagues have also demonstrated a reduction in villous epithelial cell numbers following challenge, that reached significance at 4 hours (Bramble et al. 1985). These data suggest that epithelial cell changes, whatever, the mechanism, play a role in the development of the enteropathy of coeliac disease.

Despite evidence that villous epithelial damage and cell loss occurs in coeliac disease, it is difficult to explain completely the rapidity in change, and in particular, the evidence that some recovery occurs within the time span of the challenge, by changes in rates of villous-crypt proliferation. An alternative mechanism that might be operating, involves villous subepithelial myofibroblasts. Madara and colleagues, using a rat enteropathy model and quantitative morphometric analysis have shown that contraction of the villus occurs as an immediate response to injury (Moore et al. 1989). Villous shortening of 32% was demonstrated within sixty minutes of injury. The subepithelial myofibroblasts form a three dimensional network that subtends from the basement
membrane and criss-crosses within the lamina propria. The fact that villous shortening is energy dependent and that condensation of microfilaments within the myofibroblasts occurs simultaneously with shortening, suggests that such cells are mediating shortening of the villus. It was suggested that contraction of the subepithelial myofibroblast network played a supporting role in epithelial restitution by effectively reducing the size of the injured surface area to be re-epithelialised. Human studies are limited but there is some evidence that inflammatory cytokines can mediate myofibroblast contraction (Valentich et al. 1994). There is no evidence that such mechanisms operate in coeliac disease, but the hypothesis provides an attractive explanation for some of the changes seen in response to gliadin peptide mediated damage and is worthy of further investigation.

Further Studies of Gliadin Peptides A, B and C

Subsequent to the work undertaken for this thesis several studies have been undertaken in Professor Ciclitira's laboratory, using the oligopeptides A, B and C. In situ hybridisation was used to investigate the presence of mRNA coding for the cytokines, IFNγ, TNFα, IL2 and IL-6 in the intestinal biopsies taken during the in vivo challenges described (Kontakou et al. 1995b). Four hours after gliadin challenge, the number of cells in the lamina propria expressing mRNA for IFNγ, TNFα, IL2 and IL-6 increased significantly in all four patients. Six hours after the challenge, the number of positive cells of some cytokines, such as TNFα in patients III and IV and IL2 in patients I and III returned to pre-challenge levels.

After challenge with peptide A, patients III and IV showed a significant increase in the number of cells expressing mRNA for IFNγ, TNFα, IL2 and IL6, but patients 1 and 2 showed a significant increase in IFNγ and IL6 mRNA-expressing cells only. Challenge with peptide B resulted in increased numbers of TNFα mRNA-expressing cells in the lamina propria of patient 3 only, but caused no changes in the other patients. No response was observed in any of the patients with peptide C.

Increased cytokine expression in the lamina propria of treated coeliac patients, 4 hours after challenge with gliadin and peptide A, coincided with significant histological
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changes in the small intestinal mucosa of these individuals, as demonstrated by the results presented in this chapter. An association between histological changes and cytokine expression was observed with in one individual challenged with peptide B. This peptide caused significant changes in VH/CD ratio and enterocyte height, but not increased IEL counts in patient III. The same patient demonstrated increased numbers of cells expressing TNFα in response to peptide B. No changes in cytokine expression were observed in any of the patients following challenge with peptide C, confirming our previous findings of no histological change and no increase in intraepithelial lymphocyte counts in these same patients after challenge with this peptide.

Presumably, gliadin oligopeptide induced activation of immunocompetent cells in the small intestinal lamina propria leads to increased synthesis of cytokines, such as IFNγ and IL2 by activated T-cells and TNFα and IL6 by activated macrophages. Previous studies have shown significantly greater protein and mRNA levels of IL6, IL2 and TNFα cells in the lamina propria of untreated coeliac patients compared with treated patients and controls, but very low levels in the epithelium of all three groups (Kontakou et al. 1995a; Przemioslo et al. 1994).

This work provides information regarding both oligopeptide toxicity and local immunopathogenetic mechanisms in coeliac disease (discussed in Chapter 2). Increased cytokine mRNA expression following challenge with peptide A clearly provides further evidence that this oligopeptide is coeliac toxic and likewise lack of increased expression following challenge with peptide C suggests that this peptide is not coeliac activating. The slight increase in cytokine mRNA expression following challenge with peptide B, in patient III, who also demonstrated some histological changes is intriguing and suggests that this peptide is genuinely coeliac activating, albeit to a lesser extent and perhaps only in particular individuals.

These gliadin oligopeptides have been used in organ culture coeliac toxicity studies (Shidrawi et al. 1995a). A significant reduction in enterocyte height was observed in treated and untreated patients, compared to controls, following culture with both FFIII and peptide A. No significant changes in the mean enterocyte height
occurred following culture with peptides B or C. Changes to peptides B, C and a control protein occurred in a few different individuals, both coeliac and control patients to the same frequency, suggesting this was a non-specific effect. The changes in biopsies from treated patients is in agreement with other workers using morphometric parameters for assessing toxicity, rather than changes in brush border enzymes (Howdle et al. 1981). This study strongly supports and complements the in vivo challenge study undertaken and presented in this thesis, providing further evidence that peptide A is coeliac toxic. Since a different and larger group of patients were studied to those in this thesis, it answers the criticism of low numbers that were involved in the in vivo challenge, which is inevitable. In particular it argues against the idea that the toxicity of peptide A might be restricted just to those four individual patients studied, with alternative peptides toxic in other patients. This clearly demonstrates the strength of the the organ culture system, allowing relatively large numbers of patients to be assessed.

The same group has extended this work by investigating the binding of the three oligopeptides (A, B and C) and FFIII to HLA class II molecules (Shidrawi et al. 1995b). Coeliac disease has a strong association with HLA-DQ(α1*0501, β1*0201). Binding and presentation of gliadin peptides by such an HLA-DQ molecule might be an important step in the pathogenesis of the coeliac lesion. A lymphoblastoid B cell line derived from the peripheral blood of a coeliac patient, homozygous for HLA-DQ, was used in a competitive binding assay with FFIII, peptides A, B and C. Significant inhibition of binding, indicative of active binding of the test peptides, occurred with FFIII and peptide A but not with peptides B, C or ovalalbumin, used as a control protein. These results support the hypothesis that binding of gliadin peptides to HLA-DQ molecules is important in the pathogenesis of coeliac disease, emphasise the importance of peptide A as a coeliac activating peptide and provide further evidence of the molecular mechanisms involved in coeliac disease.
Gliadin Peptide Toxicity in Coeliac Disease

Future Directions

The obvious path to follow would be to define further the known regions of coeliac toxicity within α-gliadin, in particular the homology surrounding peptide A. In view of the problems of in vivo challenge, initially this would best be accomplished with a panel of oligopeptides using intestinal organ culture as the assay system. Gliadin sensitive T cell lines and clones from coeliac patients would offer an alternative screening assay for gliadin peptides. Gliadin reactivity, however, does not imply coeliac toxicity and thus direct tests of toxicity are important. Likewise, peptides that are toxic in organ culture need in vivo verification of toxicity.

The challenge of producing non-coeliac toxic flour with the baking qualities of wheat flour is formidable because of the genetic complexities of cereal storage proteins but the definition of coeliac toxic motifs is an initial step. The production of monoclonal assays that are specific for coeliac toxic sequences is a more easily attainable goal in refining the so called "gluten-free diet". Clearly if there were more than one toxic sequence, which seems highly likely, then a panel of monoclonal assays would need to be developed to screen foodstuffs.

Finally, coeliac disease is virtually unique as an immunologically mediated disease, that is extremely tightly HLA-linked with a closely characterised peptide antigen. Further definition of the toxic sequence is of importance, as coeliac disease can act as a human model for HLA-peptide-TCR interactions, which has potential importance for other less easily managed HLA-related disease.

Conclusion

Three peptides, 19 amino-acids long and homologous to residues 3-21, 31-49 and 202-220 of A-gliadin, were selected as being potentially coeliac toxic. These peptides were synthesised in sufficient quantities and purity for subsequent use during a series of controlled, in vivo challenge experiments in a group of treated coeliac patients. This study has demonstrated for the first time, in vivo toxicity of the synthetic peptide corresponding to residues 31-49 of A-gliadin.
CHAPTER 5: FINAL SUMMARY

This thesis has investigated the cellular immunopathogenesis and cereal toxicity of coeliac disease. Evidence has been presented and discussed suggesting that upregulated expression of ICAM-1 is of importance in the development of the mucosal lesion of coeliac disease and that such upregulation may be cytokine driven. The lack of expression of ICAM-1 by the intestinal epithelium emphasises the importance of the lamina propria as probably the major site of immune activation within the intestinal mucosa of coeliac patients. The significance of T cell mechanisms in the pathogenesis of coeliac disease is suggested by the tight HLA class II linkage and the recent cloning of gliadin-reactive T cells. The ICAM-1–LFA-1 molecular interaction is of considerable importance to most T cell functions and thus the evidence from this thesis supports the hypothesis that T cell activation within the lamina propria is fundamental to the coeliac lesion. The ICAM-1–LFA-1 interaction, however, facilitates other non-T cell immune interactions which may have considerable importance, particularly in the generation of enterocyte damage and the acute mucosal lesion after gluten challenge. The immediately subepithelial localisation of upregulated ICAM-1 expression suggests that immune events, including presentation of antigen and generation of effector responses, occur within this intestinal microenvironment.

Increased numbers of intraepithelial lymphocytes relative to the numbers of enterocytes is recognised as a hallmark of the coeliac intestinal lesion, yet their functional role in the pathogenesis of the condition remains unclear. γδ+IEL are found in increased numbers in the intestinal mucosa of patients who are gluten sensitive. Evidence has been presented of a correlation between γδ+IEL numbers and enteropathy which together with evidence from functional studies demonstrating that γδ+IEL are capable of cytotoxicity, suggests that they form a component part of the epithelial mucosal immune response to gluten, perhaps as enterocytolytic cells. Direct evidence for this role, however is lacking. Certainly they do not seem to be involved in the early response to gluten challenge.
The recognition that certain cereals were toxic to coeliac patients, revolutionised the treatment of the condition and initiated a search for the precise toxic factor within the toxic cereals and in particular, wheat. The availability of automated peptide synthesisers has allowed the synthesis of chemically pure gliadin peptides in sufficient quantity to use in in vivo challenge experiments. This thesis has presented evidence for the toxicity of a synthetic peptide homologous to residues 31-49 of A-gliadin. Definition of cereal toxicity at a molecular level is now possible and is a major step in dissecting the precise events in the pathogenesis of the condition.

Many questions remain to be answered regarding coeliac disease but a key one is why some individuals with apparently the same genetic susceptibilities develop coeliac disease and some do not. Healthy individuals can mount an immune response to gliadin, as evidenced by the presence of gliadin-antibodies in the serum and is likely that, in these individuals, gliadin-specific T cells are present in the intestine of these individuals. Clearly, individuals with a normal intestine are tolerant to gliadin; the development of enteropathy presumably reflects abrogation of this tolerance rather than the presence of gliadin-specific T cells in the intestinal mucosa. It is possible that other, as yet unidentified, genetic susceptibilities exist for coeliac disease. Abrogation of tolerance and unidentified genetic susceptibilities are relevant to the question of variation in the expression of gluten sensitivity which varies through truly latent coeliac patients to asymptomatic mild enteropathy to symptomatic patients with severe enteropathy. Coeliac disease continues to pose questions for investigators.
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APPENDIX I:

Ringer Tyrode Solution

Sodium chloride 40g
Potassium chloride 1g
Calcium chloride dihydrate 1.27g
Magnesium chloride hexahydrate 1.07g
D-glucose 5g
Sodium hydrogen carbonate 5g
Distilled water 5l

Jejunal Biopsy Organ Culture Medium

Trowell’s T8 1.2ml
NCTC 135 medium 0.4ml
L-glutamine 200mM 0.04ml
Hepes buffer 1M 0.02ml
Penicillin 5000IU/ml and streptomycin 5000IU/ml
0.04ml
Foetal calf serum 0.3ml

TRIS–Buffer

Tris (hydroxymethyl) methylamine

0.1 M TRIS 12g / 1000 mls
0.1 M HCl 10 mls / 1000 mls

1000 mls of 0.1 M Tris + 800 mls of 0.1 M HCl

Adjust to pH 7.6

TRIS–Buffered–Saline (TBS)

Tris - buffer diluted 1:10 with 0.9 % sodium chloride

9 g sodium chloride in 1000 mls distilled water + 100 mls Tris buffer
DAB

3.3' Diaminobenzidinetetrahydrochloride

1% Hydrogen peroxide—1 ml 6% hydrogen peroxide in 5 mls distilled water

Tris buffer—10 mls

1% Hydrogen peroxide—3 drops

DAB—7.5 mgs

Use immediately

ABC–Complex 1%

Streptavidin 10 μl

Biotinylated horseradish peroxidase 10 μl

TBS 10 mls
APPENDIX II: CONDUCT OF THE STUDIES

Location

The clinical studies involving gluten challenge, sequenced peptide challenge and jejunal biopsies were undertaken by myself under the supervision of Dr PJ Ciclitira in the Gastrointestinal Unit of St Thomas’ Hospital. The rectal gluten challenges were undertaken by Dr D Loft, working under the supervision of Dr M. Marsh in the University Department of Medicine, Hope Hospital, Salford, Manchester.

Collaboration and Help with the Studies

The studies described in this thesis were performed personally by myself, under the supervision of Dr PJ Ciclitira. Mr J. Nelufer and Dr M. Kontakou assisted with the immunohistochemical staining. Dr J. McCartney and Dr J. Spencer assisted with the assessment of the stained biopsies. Dr P. Day prepared the solid phase synthesised peptides used in the challenge studies at the peptide synthesis laboratory of The Imperial Cancer Research Fund, London.

Consent and Ethical Approval

All the patients undergoing intestinal biopsies with or without gluten challenge gave their written informed consent to the procedures. Approval for these studies was first obtained from the Ethical Committee of St Thomas’ Hospital.
APPENDIX III: PERSONAL COMPUTATION AND STATISTICAL ADVICE

A Macintosh SE30 was used throughout. This thesis was written using Word 4 (Microsoft Ltd). Production of graphs and figures was accomplished using a combination of MacDraw II (Claris Ltd) and Cricket Graph (Cricket Software Ltd). Statistical analysis was performed using Statview SE+Graphics and SuperAnova (Abacus Concepts Ltd).

In the planning of the studies reported in this thesis and for subsequent presentation and analyses of data, advice was sought from the statisticians in the Department of Public Health Medicine at the UMDS.
APPENDIX IV: SINGLE LETTER AMINO-ACID CODE

A Alanine
C Cysteine
D Aspartate
E Glutamate
F Phenylalanine
G Glycine
H Histidine
I Isoleucine
K Lysine
L Leucine
M Methionine
N Asparagine
P Proline
Q Glutamine
R Arginine
S Serine
T Threonine
V Valine
W Tryptophan
Y Tyrosine
APPENDIX V: PUBLICATIONS RELATING TO THE THESIS


Kontakou, M, Sturgess, RP, Przemioslo, RT, Limb, GA, Nelufer, JM & Ciclitira, PJ.
Detection of interferon-γ mRNA in the mucosa of patients with coeliac disease by in situ hybridisation.
Gut 1994; 35: 1037-1041

Ellis, HJ, Doyle, A, Weiser, H, Sturgess, RP, Day, P & Ciclitira, PJ
Measurement of gluten using a monoclonal antibody to a sequenced peptide of α-gliadin from the coeliac activating domain I.
Journal of Biochemical and Biophysical Methods 1994; 28: 77-82

Kontakou, M, Przemioslo, RT, Sturgess, RP, Limb GA & Ciclitira, PJ.
Expression of tumour necrosis factor-α, IL-6 and IL-2 mRNA in the jejunum of patients with coeliac disease

Kontakou, M, Przemioslo, RT, Sturgess, RP, Limb, GA, Ellis, HJ, Day, P & Ciclitira, PJ.
Cytokine mRNA expression in the mucosa of treated coeliac disease patients after wheat peptide challenge
Gut 1995 37: 52-57
Appendices

**CORRIGENDA**

Pg 2  L9  "gliadin" for "giadin"
Pg 14 L9  "malabsorption" for "malabsortion"
Pg 25 L16  "therefore" for "therefore"
Pg 27 L17  "peptide" for "petide"
Pg 29 L26  "results, however" for "however, results"
Pg 30 L8  "perturbation" for "peturbation"
Pg 37 L25  insert "of"
Pg 47 L8  "morphological" for "morphologiacal"
Pg 47 L12  "these" for "this"
Pg 51 L2  "its α" for "a"
Pg 52 L14  "evolutionary" for "evolutionarily"
Pg 52 L17  "evolutionary" for "evolutionarily"
Pg 77 L26  "extracellular" for "extracellular"
Pg 79 L26  "These" for "This"
Pg 80 L26  "These" for "This"
Pg 89 L11  "unknown" for "unknown"
Pg 93 L27  "the role" for "role"
Pg 94 L4  "through" for "throught"
Pg 94 L10  "is" for "are"
Pg 94 L25  "is" for "are"
Pg 95 L24  "their" for "its"
Pg 96 L1  "or when" for "or"
Pg 96 L24  "δTCS-1" for "δTCSi"
Pg 97 L2  "cells" for "cells"
Pg 97 L18  "the patient" for "patient"
Pg 107 L4  "occurred" for "occured"
Pg 126 L20  "biopsy" for "biopsies"
Pg 127 L10  "is in" for "in"
Pg 142 L10  "that" for "tha"
Pg 146 L15  "incorporated" for "incorporated"
Pg 156 L2  "ill" for "il"
Pg 178 L24  "at 4 hours" for "4 hours"
Pg 180 L5  "restitution" for "restitution"
Pg 182 L23  "ovalbumin" for "ovalalbumin"
Pg 183 L11  "complexities" for "complexicities"
Pg 185 L12  "it is" for "is"
Pg 185 L20  "asymptomatic" for "asymptomatic"