DERMATITIS HERPETIFORMIS:

CLINICAL AND IMMUNOLOGICAL ASPECTS

A thesis submitted to the University of Glasgow for the degree of MD in the Faculty of Medicine

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ABSTRACT

Although many workers have demonstrated that the rash of dermatitis herpetiformis responds to withdrawal of gluten from the diet, there are many who remain sceptical. The role of a gluten free diet in the management of patients with dermatitis herpetiformis was re-examined and its beneficial effects confirmed. Furthermore, a gluten free diet was shown to protect patients with dermatitis herpetiformis from developing lymphoma but the diet had to be strict and the protective effect only became apparent once the diet had been adhered to for five years.

Relatives of three patients (with a family history of dermatitis herpetiformis/coeliac disease) were screened in order to detect the clinical carrier rate for the genes for dermatitis herpetiformis and coeliac disease within these families. The carrier rate in the three families studied varied from 23% to 57%. This study demonstrated that many so-called "unaffected" relatives of patients with dermatitis herpetiformis may have silent or latent coeliac disease. Screening by means of serology alone was shown to be unreliable.

The pathogenic mechanisms leading to the development of the rash and enteropathy are poorly understood. An infiltrate of T cells, composed mainly of activated CD4 positive memory cells, was shown to be present in the dermis of dermatitis herpetiformis lesional skin. Moreover, these T cells demonstrated over-representation of specific TCR Vβ subsets, namely Vβ2, Vβ5.2/5.3 and Vβ5.3. These findings would indicate that these T cells recognise a specific, but as yet unknown, antigen; the antigen is unlikely to be gluten because intradermal injection of Frazer's fraction III failed to elicit the rash of dermatitis herpetiformis in patients.

An infiltrate of T cells is also found in the small intestinal mucosa of patients with dermatitis herpetiformis and coeliac disease. It has been proposed that these T cells belong predominantly to the TH1 group of cells and it has been suggested that IFN-γ, which is produced by these cells, plays a key role in the production of the small intestinal pathology. However, this hypothesis has not been supported by others. Reverse transcriptase-polymerase chain reaction and immunostaining demonstrated no statistically significant differences in the detection of mRNA for IFN-γ and IL-2 or their proteins when biopsies from patients with dermatitis herpetiformis who had villous atrophy were compared with a control group of patients.

Finally, possible mechanisms, whereby ingestion of gluten in susceptible individuals leads to the rash of dermatitis herpetiformis, are discussed.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification to this or any other university or other institute of learning.

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DEDICATION

To Simon, my husband

ABBREVIATIONS

AEA	anti-endomysial antibody
AGA	anti-gliadin antibody
ARA	anti-reticulin antibody
CD	coeliac disease
CD1a	Langerhans' cell
CD3	pan T cell
CD4	helper T cell
CD8	suppressor/cytotoxic T cell
CD25	interleukin-2 receptor
CD45RO	memory T cell
cDNA	copy deoxyribonucleic acid
D2	second part of the duodenum
DH	dermatitis herpetiformis
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity response
EM	erythema multiforme
FFIII	Frazer's fraction III
GFD	gluten free diet
GM-CSF	granulocyte macrophage - colony stimulating factor
GSE	gluten sensitive enteropathy
GvHD	graft versus host disease
HLA	human leucocyte antigen
IDDM	insulin dependent diabetes mellitus
IEL	intra-epithelial lymphocyte

ABBREVIATIONS continued

IFN-γ	interferon-γ
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-2	interleukin-2
IL-4	interleukin-4
IL-8	interleukin-8
kD	kilodalton
LCA	leucocyte common antigen
LE	lupus erythematosus
mRNA	messenger ribonucleic acid
NIDDM	non insulin dependent diabetes mellitus
OCT	optimal cutting temperature
PVA	partial villous atrophy
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
SECH	surface epithelial cell height
SLE	systemic lupus erythematosus
SEM	standard error of the mean
SRR	standard registration ratio
SVA	severe villous atrophy
TCR	T cell receptor

ABBREVIATIONS continued

TH1	T helper cell - type 1
TH2	T helper cell - type 2
tTG	tissue transglutaminase
VH:CD	villous height:crypt depth ratio

CHAPTER ONE

INTRODUCTION

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INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

The term "dermatitis herpetiformis" was first proposed by Dühring in 1884 [Dühring, 1884]. He used this name for a group of skin diseases which had, up until that juncture, either been considered to be unusual variants of "eczema, herpes, or pemphigus" or, in some cases as undescribed diseases. In fact, Dühring first recognised the condition as early as 1871, but it was only by observing additional cases that he became confident enough to suggest that these diseases, despite the fact that some cases were dissimilar, were indeed the same disease. Dühring included Hebra's "impetigo herpetiformis" [Hebra, 1872], which we recognise today as pustular psoriasis, under his classification of dermatitis herpetiformis .

Dühring found that the disease was rare. All of his patients, with one exception, were adults, including both sexes in about equal proportion. He stated that the disease was chronic and characterised by exacerbations or relapses. Most cases persisted for years. He found that all body sites could be affected, including both flexor and extensor surfaces, face, scalp, elbows, knees, palms and soles.

The disease was summarised by Dühring as follows:

1. *The existence of a distinct, well-defined, rare, serious, inflammatory disease of the skin, manifestly of an herpetic nature, characterised by systemic disturbance, a great variety of primary lesions, by severe itching and burning, and by a disposition to appear in*

repeated successive outbreaks.

2. That the disease is capable of exhibiting itself in many forms, all having a tendency to run into or to succeed one another irregularly in the natural course of the process.

3. The principal varieties are the erythematous, papular, vesicular, bullous and pustular, which may occur singly or in various combinations.

4. That it is a remarkably protean disease.

5. That the pustular variety is the same manifestation as the disease described by Hebra under the name "impetigo herpetiformis", this being the only form hitherto described.

6. That the several other and equally important forms are worthy of special remark.

7. That the term "dermatitis herpetiformis" is sufficiently comprehensive and appropriate to include all varieties of the process.

8. That it may occur in both sexes, and in women independent of pregnancy.

9. That it usually pursues a chronic, variable course, often lasting years, and is exceedingly rebellious to treatment.

In 1888, Brocq published his rather lengthy report on the history of bullous diseases and

commented on Dühring's newly described DH [Brocq, 1888]. Brocq, however, was of the opinion that impetigo herpetiformis should **not** be classified with DH:

"*Que l'impétigo herpétiformis d'Hebra nous paraît constituer une entité morbide à part, et ne saurait, d'après nous, rentrer dans la dermatite herpétiforme de Dühring.*"

Following Dühring's description in 1884, DH was lumped together with bullous pemphigoid and pemphigus. There was much confusion and debate with regard to the separation of these diseases. Auspitz had already described intraepithelial separation in pemphigus in 1881, which he called *acantholysis*, but this was forgotten and had to be "rediscovered" [Auspitz, 1881]. Civatte redescribed acantholysis and in 1943 he demonstrated that pemphigus could be differentiated histologically from DH and erythema multiforme (EM) - he demonstrated the intra-epidermal nature of the blister in pemphigus and the sub-epidermal nature of the blister in DH and EM [Civatte, 1943]. He emphasised the importance of biopsying blistering rashes to differentiate pemphigus from the sub-epidermal blistering lesions. This was again emphasised in Lever's histopathological study of pemphigus published in 1951 [Lever, 1951].

The next important milestone came in 1966 when Marks and Shuster demonstrated the presence of enteropathy similar to that found in idiopathic steatorrhoea in nine out of twelve DH patients [Marks *et al*, 1966]. A high prevalence of similar small intestinal changes in DH patients was again reported by van Tongeren et al in 1967 [van Tongeren *et al*, 1967] and later in the same year by Fry et al [Fry *et al*, 1967]. Fry also suggested that the small intestinal changes could be secondary to gluten intolerance [Fry *et al*, 1967] and this was confirmed a year later [Fry *et al*, 1968]. It was also suggested in 1968 that the

skin lesions were gluten dependent [Fry *et al*, 1968] although this suggestion was rejected by others [Shuster *et al*, 1968; Weinstein *et al*, 1971]. Later studies confirmed Fry's original suggestion that the rash is indeed gluten dependent [Fry *et al*, 1973; Heading *et al*, 1976; Harrington & Read, 1977; Reunala *et al*, 1977; Katz *et al*, 1980; Frödin *et al*, 1981; Ljunghall & Tjernlund, 1983; Gawkrodger *et al*, 1984].

The late 1960s saw the advent of immunofluorescence, pioneered by Cormane. He was able to demonstrate the presence of immunoglobulin at the dermo-epidermal junction in patients with DH [Cormane, 1967]. This finding was investigated further by van der Meer who found that this immunoglobulin was in fact immunoglobulin A (IgA) which formed a "striking and consistent granular pattern" at the dermo-epidermal junction [van der Meer, 1969]. Later it was recognised that there were two patterns of IgA deposition: granular deposits in the tips of the dermal papillae and a linear pattern along the dermo-epidermal junction. More recently, the linear pattern has been subdivided into linear granular and linear homogeneous. The linear granular pattern is seen in DH but the linear homogeneous pattern has been recognised to be pathognomonic for a further immunobullous disorder which is now known as linear IgA disease. The presence of granular deposits of IgA within the dermal papillae or along the dermo-epidermal junction is now recognised as a pre-requisite for the diagnosis of DH.

1.2 CLINICAL OVERVIEW

1.2.1 Introduction

DH is an uncommon immunobullous disorder characterised by a chronic, symmetrical eruption consisting of urticated lesions, papules and blisters which typically occur on the extensor aspects of the limbs and dorsal aspect of the trunk, particularly over the scapulae

(Figure 1.1). Severe and persistent itch is a dominant feature. Its onset may occur at any age but it usually presents during the third decade. In adults, it is slightly more common in males with a male:female ratio of 3:2 compared with a male:female ratio of 1:2 in children [Ermacora *et al*, 1986].

1.2.2 Prevalence and geographical distribution

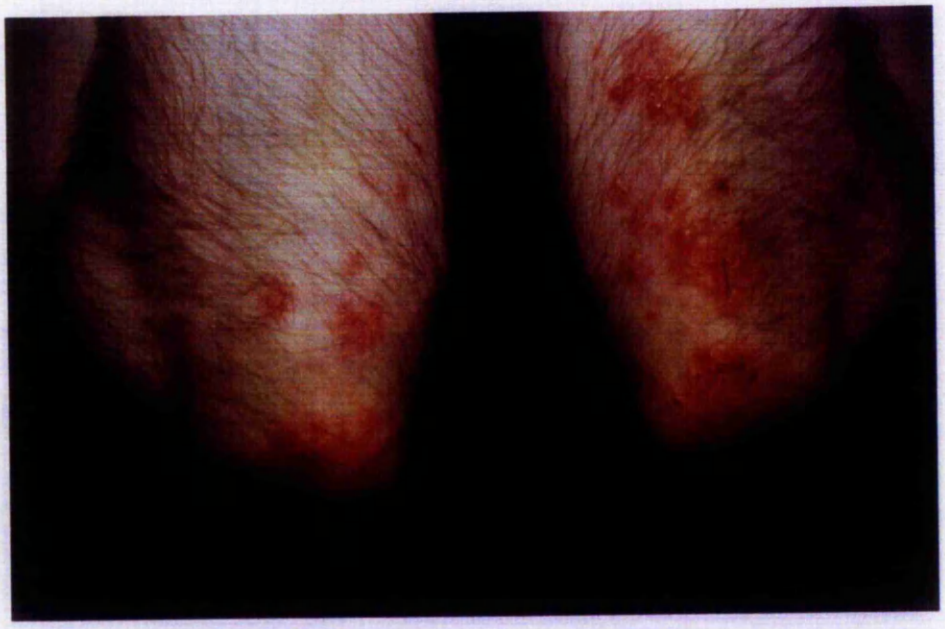
Few studies have looked at the prevalence of DH. Those which have been performed suggest that in Britain it occurs in approximately 12 per 100 000 population [Gawkrodger *et al*, 1984] which is similar to the prevalence found in Finland [Reunala & Lokki, 1978] but lower than that found in Sweden [Christensen *et al*, 1973; Mobacken *et al*, 1984]. There have been even fewer studies which have looked at the geographical distribution of DH; it shares the same geographical distribution as CD and so it is found in European countries and those countries to which Europeans have emigrated.

1.2.3 Direct immunofluorescence of skin

The clinical diagnosis of DH is confirmed by biopsying uninvolved skin to detect the presence of IgA. The majority of patients with DH have granular deposits of IgA within the dermal papillae; approximately 10% of patients have a linear granular pattern along the dermo-epidermal junction (Figure 1.2). The IgA is deposited throughout the skin but it is important that the biopsy for direct immunofluorescence is obtained from uninvolved skin as the IgA may be destroyed in perilesional skin by the inflammatory process thus resulting in a falsely negative result.

FIGURE 1.1: Dermatitis herpetiformis lesions on

(a) the extensor aspect of the elbows



(b) the extensor aspect of the knees

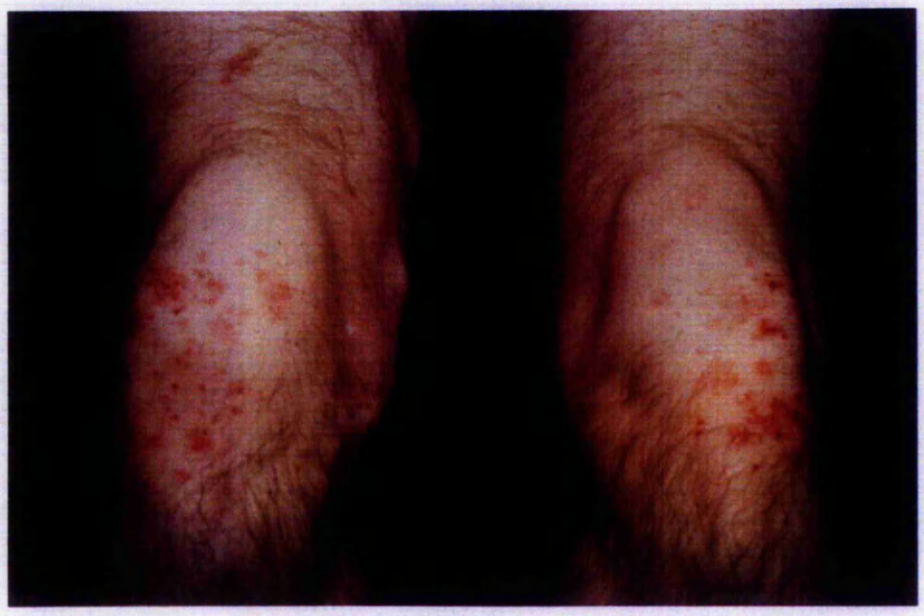
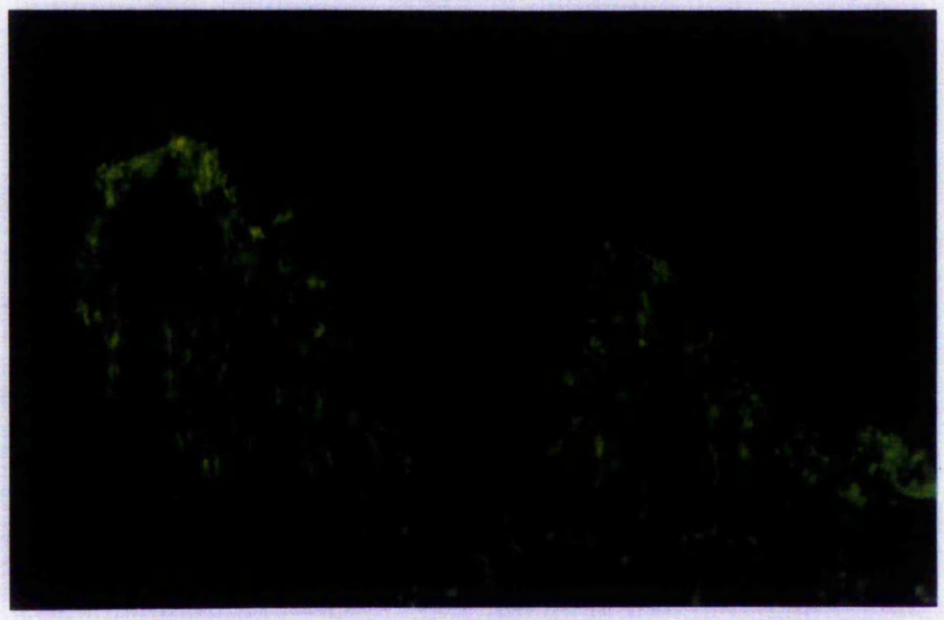


FIGURE 1.2: Direct immunofluorescence of dermatitis herpetiformis skin demonstrating

(a) Granular deposits of IgA within the dermal papillae



(b) Linear granular deposition of IgA along the dermo-epidermal junction

1.2.4 Histology

Biopsy of lesional skin in DH demonstrates the presence of subepidermal blistering, microabscesses containing neutrophils in the adjacent dermal papillae and a perivascular infiltrate of mononuclear cells in the dermis (Figure 1.3). It is not necessary to perform a lesional biopsy to confirm the diagnosis as this is achieved by performing direct IF; indeed, histology cannot reliably differentiate between DH and two other immunobullous disorders, linear IgA disease and bullous pemphigoid [Blenkinsopp *et al*, 1983].

1.2.5 Gluten sensitive enteropathy and malabsorption

As already mentioned in the historical section, DH is associated with gluten sensitive enteropathy and it is likely that all patients have intestinal involvement. Two thirds of patients with DH have abnormal small intestinal mucosal architecture following a single small intestinal biopsy [Marks *et al*, 1966; Fry *et al*, 1967] but 95% of patients can be shown to have architectural changes following multiple biopsies [Brow *et al*, 1971]. Thus, the mucosal changes may be patchy. However, even in patients with normal mucosal architecture, there is evidence of gluten sensitive enteropathy as demonstrated by the presence of raised intra-epithelial lymphocyte counts [Fry *et al*, 1974] and induction of architectural change following gluten challenge [Weinstein *et al*, 1971]. More recently, it has been shown that all patients with DH have raised numbers of intra-epithelial cells bearing the γ/δ T cell receptor [Savilahti *et al*, 1992].

The morphological changes of the small intestine in DH are indistinguishable from those seen in coeliac disease. It is now recognised that there is a spectrum of changes and these can be broadly categorised into the following groups [Marsh, 1992]:

1. infiltrative lesions

2. hyperplastic lesions

3. destructive (flat) lesions

Infiltrative lesions are characterised by:

- normal mucosal architecture
- infiltrate of lymphocytes in epithelium

Hyperplastic lesions are characterised by:

- normal villi
- enlarged crypts
- infiltrate of lymphocytes in the epithelium

Destructive (flat) lesions are characterised by:

- flat surface epithelium
- hypertrophic crypts
- infiltrate of lymphocytes in the epithelium
- lamina propria swelling

In addition to the changes listed above, as the severity of mucosal damage progresses, the columnar epithelial cells with their well developed brush border become progressively more cuboidal with attenuation of their brush borders. Increased cellularity of the lamina propria occurs and the infiltrate is composed mainly of lymphocytes and plasma cells.

The majority of the cells found in the epithelium, as in the normal intestine, are T lymphocytes of the CD8+ (suppressor/cytotoxic) subset. This is in contrast with the lamina

propria where most of the cells are T lymphocytes of the CD4+ (helper) subset.

All mucosal changes are reversible following withdrawal of gluten from the diet with the exception of the raised number of lymphocytes bearing the γ/δ T cell receptor.

Most patients with DH have no gastrointestinal symptoms. Less than 10% have symptoms of gluten sensitive enteropathy including bloating, diarrhoea and malabsorption [Fry *et al*, 1967; Fraser *et al*, 1967; Shuster *et al*, 1968; Brow *et al*, 1971]. Although most DH patients are asymptomatic, laboratory studies documenting abnormal intestinal function have been reported including abnormal D-xylose, iron, folate, glucose, water and bicarbonate absorption [Fry *et al*, 1967; Fraser *et al*, 1967; Shuster *et al*, 1968; Brow *et al*, 1971].

Thus, although the majority of DH patients have changes of their small intestinal mucosa in keeping with gluten sensitive enteropathy, these changes tend to be milder than those observed in coeliac disease. Consequently, only a minority of DH patients have gastro-intestinal symptoms and significant malabsorption. Gluten intolerance in patients with DH is manifested by the development of the rash.

1.2.6 HLA associations

The discovery that patients with DH had GSE led several investigators to initiate studies to determine if the HLA associations present in patients with isolated GSE were also present in patients with DH. Initial studies revealed that 58% of DH patients expressed the HLA class I antigen B8 compared with 20-30% of controls [Katz *et al*, 1972; White *et al*, 1973]. However, when only patients with granular deposits of IgA were studied, it was found that

70-90% expressed HLA B8 [Kárpáti *et al*, 1986; Sachs *et al*, 1986; Sachs *et al*, 1988; Hall *et al*, 1989].

Further investigations revealed stronger associations with the HLA class II antigens, HLA-DR3 and HLA-DQw2: 90-95% of patients with DH were found to express HLA-DR3 compared with 23% of normal controls; 95-100% were found to express HLA-DQw2 compared with 40% of controls [Solheim *et al*, 1976; Thomsen *et al*, 1976; Strober, 1980; Park *et al*, 1983; Sachs *et al*, 1986; Hall *et al*, 1989].

Molecular analysis of the HLA-DR and HLA-DQ genes has shown the strongest association of DH is with the HLA-DQ alleles DQ B1*0201 and DQ A1*0501 in *cis* or *trans* position (DQ2): almost 90% of DH patients carry the genes encoding this heterodimer whilst most of the remainder carry the genes for the heterodimer DQ A1*03 B1*0302 (DQ8) [Fronek *et al*, 1991; Otley *et al*, 1991; Spurkland A *et al*, 1997].

The HLA associations for DH and GSE are essentially similar. However, not all individuals expressing these genes develop DH or GSE and so other factors such as additional genes and environmental factors must be operating to contribute towards the development of these disorders.

1.2.7 Autoimmune disease associations

Patients with DH are at risk of developing autoimmune diseases. The commonest autoimmune disease to occur in association with DH is thyroid disease. Thyroid antibodies were shown to be present in 48% of patients with DH [Weetman *et al*, 1988] with overt

thyroid disease occurring in 5-34% [Davies *et al*, 1978; Gawkrodger *et al*, 1984; Cunningham *et al*, 1985; Weetman *et al*, 1988].

The next most frequent autoimmune disease to occur in patients with DH is pernicious anaemia. As many as 30% of a cohort of DH patients were found to have gastric parietal cell antibodies with 3% having pernicious anaemia [Fry, 1992]. This incidence of pernicious anaemia was similar to another study [Gawkrodger *et al*, 1984].

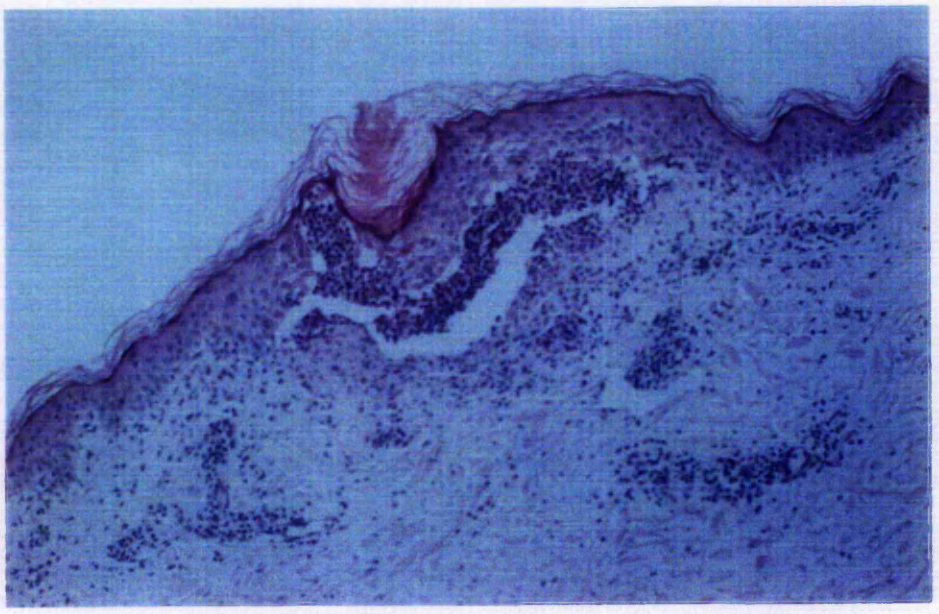
DH patients are also at risk of developing diabetes mellitus [Holt *et al*, 1980]. Eight (4%) patients out of a cohort of 212 patients attending St Mary's Hospital dedicated DH clinic had diabetes mellitus [unpublished observations].

Other autoimmune diseases which have been reported to occur in association with DH include rheumatoid arthritis, lupus erythematosus, primary biliary cirrhosis, autoimmune chronic active hepatitis and vitiligo [Kaplan *et al*, 1992].

1.2.8 Lymphoma

Patients with DH, like patients with CD, are at risk of developing lymphoma and these lymphomas can be intestinal or extra-intestinal [Andersson *et al*, 1971; Månsson, 1971; Goodwin & Fry, 1973; Tönder *et al*, 1976; Silk *et al*, 1977; Gould & Howell, 1977; Freeman *et al*, 1977; Leonard *et al*, 1983]. A previous retrospective study of 109 DH patients reported that DH patients taking a normal diet appeared to have a greater risk of developing lymphoma than those adhering to a GFD although the results were not statistically significant [Leonard *et al*, 1983].

Figure 1.3: **Histology of DH skin demonstrating subepidermal blistering, collections of neutrophils within and just below the sudepidermal blister and a perivascular infiltrate of mononuclear cells in the dermis (x100).**



1.2.9 Treatment

DH can be treated with drugs (dapsone, sulphamethoxypyridazine or sulphapyridine) or with a GFD or with a combination of drugs and GFD.

Today, two sulphonamides, sulphamethoxypyridazine and sulphapyridine, are used to treat DH. Sulphapyridine was the first drug used to treat DH successfully [Costello, 1940]. It was one of the first antibiotics available and was given to patients with DH because it was believed that their rash was an allergic manifestation to a bacterial infection. Sulphonamides have many adverse effects including nausea, lethargy, depression, rashes, hepatitis and bone marrow suppression; pneumonitis has been described with sulphamethoxypyridazine [McFadden *et al*, 1989].

Dapsone, which is a sulphone, was first used in 1950 and is extremely effective in controlling the skin lesions of DH [Esteves and Brandao, 1950]. Indeed, prior to the advent of immunofluorescence, a trial of treatment with dapsone was used as a diagnostic test. Dapsone has many adverse effects which are common and include headache, lethargy and depression. Patients taking dapsone will develop some evidence of haemolysis, the degree of which can be monitored by measuring the reticulocyte count. Severe haemolytic anaemia may occur as an idiosyncratic response or in patients with glucose-6-phosphate dehydrogenase deficiency and this deficiency must be checked for in at-risk patients before commencing dapsone.

All patients taking 100mg of dapsone daily will have some degree of methaemoglobinaemia and sulphaemoglobinaemia. Peak methaemoglobin concentrations occur by the second week of treatment, and can exacerbate ischaemic heart disease in

susceptible individuals. Other occasional adverse effects include hepatitis, agranulocytosis and neuropathy which may be sensory, motor or both. Neuropathy can occur at any stage after commencing dapsone and is usually reversible.

The mechanism whereby dapsone and the sulphonamides exert their effect is unknown. It has been shown that they can prevent migration of neutrophils to extravascular sites by interfering with adhesion molecules, the leukocyte integrins [Booth *et al*, 1992]. It is likely that additional, hitherto unidentified, properties also play a role.

Neither dapsone nor the sulphonamides have any effect on the enteropathy of DH. Both the rash and the enteropathy of DH respond to withdrawal of gluten from the diet and this should be the treatment of choice. Patients who adhere to a strict GFD should achieve complete control of their rash just over two years after commencing the diet. Until control of the rash has been achieved by the diet, dapsone or one of the sulphonamides will be required in addition. Patients who adhere to their diet strictly should be able to start reducing their medication after approximately 6 months [Fry *et al*, 1973]. The less strict the diet, then the longer will be the period until complete control is achieved.

Although several studies have confirmed the efficacy of a GFD in treating the rash of DH, this is not universally accepted by dermatologists and gastroenterologists. Consequently, there are many dermatologists who fail to advocate the use of a GFD in the treatment of these patients.

1.3 Aims of study

In general terms, the work of this thesis aimed to improve the management of patients with DH; to assess the incidence of **clinical** expression of the gene for CD and DH within families; and to elucidate the pathogenic mechanisms involved in the development of the skin and small intestinal lesions.

The specific aims were:

1. To re-evaluate the efficacy of a GFD in the treatment of DH and to assess the long term benefits of the diet, particularly with respect to the development of lymphoma.

2. To study so called "unaffected" members of families with more than one member with CD or DH in order to determine the incidence of those expressing the gene(s) for CD/DH within these families.

3. To determine whether the skin lesions of DH are due to T cell mediated responses to antigen.

4. To determine whether intestinal T cells in DH patients belong to the TH1 or TH2 sub-groups of T cells.

CHAPTER TWO

CLINICAL ASPECTS

CHAPTER TWO

CLINICAL ASPECTS

1. GLUTEN-FREE DIET IN THE MANAGEMENT OF PATIENT WITH DERMATITIS HERPETIFORMIS

2.1.1 Introduction

It has always been controversial as to whether the rash of DH truly responds to withdrawal of gluten from the diet. Van Tongeren [Van Tongeren *et al*, 1967] reported a patient with DH whose rash improved when he was treated with a gluten free diet (GFD) but worsened when gluten was re-introduced into the diet. Fry *et al* [1968] proposed that the rash of DH was dependent on the presence of gluten in the diet and later reported clearance of the rash in three out of seven patients who had been taking a GFD for one year [Fry *et al*, 1969]. Some early investigators were unable to confirm that the DH rash was gluten dependent but their findings were based on studies in which the GFD was given to the patients for short periods of time [Shuster *et al*, 1968; Weinstein *et al*, 1971]. Subsequent investigators were able to confirm that the rash of DH was indeed dependent on the presence of gluten in the diet [Fry *et al*, 1973; Heading *et al*, 1976; Harrington & Read, 1977; Reunala *et al*, 1977; Frödin *et al*, 1981; Fry *et al*, 1982; Ljunghall and Tjernlund, 1983; Gawkrodger *et al*, 1984]. Of these latter studies, the largest was performed by Reunala and his colleagues [Reunala *et al*, 1977]: they were able to show that 23 out of 81 patients taking a GFD for 6 – 36 months were able to discontinue their medication completely a mean of 24 months after commencing the GFD. Fry reported similar findings in 1982: of 42 patients taking a GFD, 30 were eventually able to discontinue their medication after a mean of 29 months after commencing the diet [Fry *et al*, 1982].

Despite the published literature supporting the benefits of a GFD in treating DH patients, there continue to be many dermatologists and gastroenterologists who remain sceptical. Therefore, I performed this retrospective study of a large cohort of DH patients to confirm finally the overwhelming benefits of a GFD in the treatment of the rash of DH.

2.1.2 Methods

Patients

Two hundred and twelve patients (128 male, 84 female; average age at presentation 42 years, range 9-83 years) with DH were seen in the dedicated clinic at St Mary's Hospital during the period 1967-92. Many of the patients had been followed up by a single physician since 1967 (Professor Lionel Fry). The number of patients attending this clinic had steadily grown since its inception. The casesheets for these patients had always been kept in the Dermatology Department: meticulous notes had been made of the clinical signs and symptoms; results of investigations; treatment modalities including drug doses; response of the rash to withdrawal of gluten from the diet. A dietitian had always been in attendance at the clinic and had also recorded dietary strictness in the patients' casesheets. The DH patients in this study had all been under the care of Professor Lionel Fry and/or Dr Jonathan Leonard.

The casesheet for each patient was available for me to see and to extract the relevant information. The diagnosis in all cases had been confirmed by demonstration of IgA deposits in the upper dermis. Treatment with a GFD had been discussed with all patients.

Classification of strictness of GFD

I discussed the strictness of each patient's diet with the consultants in charge of the

patients (Professor Lionel Fry and Dr Jonathan Leonard) and with the DH clinic's dietitian (Samantha Sargant). Following these discussions, I classified patients who were taking a GFD into the following categories listed below.

Group I	strict - when patients could not be faulted
Group II	partial with occasional lapses - when patients deviated more than twice per year
Group III	partial with frequent lapses - when patients deviated more than twice per year, but no more than once per week.

Patients whose dietary lapses occurred more than once per week were considered to be taking a normal diet.

Small intestinal biopsy

When possible, patients underwent a small intestinal biopsy prior to commencing their GFD.

Skin biopsies

Skin biopsies, to detect the presence of IgA in patients whose rash was controlled by GFD alone, were repeated at intervals.

Time to reduce and stop medication after commencing a GFD

The time taken to reduce and eventually stop the sulphones or sulphonamides, required to suppress the rash of DH, was calculated in patients who were successful in controlling

their rash by diet alone.

Statistics

The Mann-Whitney *U*-test was applied to determine significant differences in the times taken to reduce and stop medication between groups I, II and III.

2.1.3 Results

Dietary status

An analysis of patients according to diet is shown in Table 2.1. The GFD was tried by 172 (81%) of the patients, of whom 133 (63%) continued to take the diet. Seventy-eight of the patients taking a GFD achieved control of their rash by diet alone, and 55 continued to require medication in addition to their GFD for complete control. Sixty-nine patients opted to continue with a normal diet and medication; an additional 10 patients entered remission, i.e. they were taking a normal diet and required no medication.

Reasons for patients not taking or discontinuing GFD

Of the 79 patients taking a normal diet, 39 had tried a GFD and then stopped it. Patients who tried the diet and then discontinued it did so for the following reasons: (i) they found that the diet interfered with their family life; (ii) it was socially limiting; (iii) their work made it difficult to adhere to the diet; (iv) some found the diet was too difficult to adhere to, and these patients were usually poorly motivated; (v) two entered spontaneous remission. The reasons for the 40 patients not taking a GFD were: (i) they simply did not wish to try this treatment; (ii) they considered it would restrict their social life; (iii) they thought it would interfere with their work, e.g. patients who had to travel a great deal.

Spontaneous remission of skin lesions

Of the 77 patients taking a normal diet, eight entered spontaneous remission, giving a remission rate of 10%. These patients retained IgA in their skin, but were able to take a normal diet without recurrence of skin lesions. A further two patients went into remission; one of these had been taking a strict GFD, and remission was discovered fortuitously when his rash did not recur whilst he was participating in a gluten challenge study [Leonard *et al*, 1983]. The remission rate in patients taking a strict GFD is unknown, as this could only be determined by stopping their diet. The remaining patient whose disease remitted had been taking a partial GFD. He was the only patient not taking a strict GFD who lost the IgA from his skin, and this was demonstrated in two separate biopsies.

Strictness of diet

Of the 133 patients taking a GFD, 44 (33%) were thought to be strictly adhering to the diet (group I), and could not be faulted by the dietitian. Of the remaining 89 patients, 22 were taking a GFD with occasional lapses (group II), 62 were taking a GFD with frequent lapses (group III), and in five the strictness of the diet was unknown (Table 2.2).

Small intestinal pathology at presentation

Small intestinal biopsies were performed in 166 patients at presentation and prior to starting a GFD. One hundred and sixteen (70%) of these patients had villous atrophy, 65 (39%) had partial villous atrophy, and 51 (31%) total villous atrophy. In the group whose disease was controlled by strict GFD alone (group I), 10 of 35 (29%) patients had a normal villous architecture of their small intestinal biopsy prior to commencing a GFD (biopsies not performed in six.)

Loss of IgA from the skin

Loss of IgA from the skin was observed in 10 of 41 (24%) patients taking a strict GFD. The average time after commencing a GFD until loss of IgA was detected was 13 years (range 5 - 24 years); the average duration of control of the rash by GFD alone until loss of IgA was 10 years (range 3 - 16 years). Of the remaining patients taking a strict GFD, a reduction in the amount of IgA in the skin was observed in 11.

Time taken to reduce and stop medication after commencing GFD

The results are shown in Table 2. 3.

Patients who were taking a strict GFD (group I), and who were eventually able to discontinue medication, reduced their medication by 50% after a mean of 13 months, and were able to discontinue medication after a mean of 28 months from commencement of the diet. Patients who erred from the diet no more than twice per year (group II), and who were eventually able to discontinue medication, reduced their medication by 50% after a mean of 14 months, and were able to stop their medication a mean of 44 months from commencement of the diet. Patients who frequently strayed from the diet (group III), but who were eventually successful in stopping medication, took a mean of 21 months to reduce medication by 50%, and 62 months to stop medication from the time of commencement of the diet. The time taken to discontinue medication in those who adhered strictly to their GFD (group I) was significantly shorter than in those who strayed frequently from their diet (group III) ($P < 0.02$, Mann-Whitney U test). The differences between groups I and II, and also between groups II and III, were in line with the general trend that the stricter the GFD the less time was required to cease medication, although these differences failed to reach statistical significance.

2.1.4 Discussion

This large series of patients confirms the observation that the rash of DH is gluten-dependent, and that it takes many months for total resolution to occur. Treatment with a GFD abrogates the need for medication associated with a high frequency of side-effects [McFadden *et al*, 1989]. Patients who adhere strictly to their diets should be able to start reducing the dose of their medication a few months after commencing the diet and stop approximately after 28 months. The less strict the diet, the longer it takes before patients can reduce and discontinue their drugs. Indeed, some patients who continue to have small amounts of gluten in their diet may not be able to stop their medication completely. Other advantages of a GFD are that patients often experience a feeling of well-being after commencing the diet, and malabsorption is corrected due to resolution of the enteropathy. The disadvantages of the diet are that it is difficult to adhere to, and patients must be motivated, gluten-free products are expensive (although many are available on prescription in the U.K.) and many patients may find that the diet interferes with family life, work or social activities. Thus although a GFD is the treatment of choice, there will be patients for whom the diet is not suitable. Once committed to a GFD this treatment will be lifelong as remission is uncommon.

Three patients who had been taking a "strict" GFD were unable to stop their medication. One was thought to have been taking his strict GFD for 4 years, but was unable to reduce the dose of his medication. Of the other two patients, one had been taking a strict GFD for 4 years and was successful in reducing her daily dapsone requirements by over a half. The third patient was an insulin-dependent diabetic who was diagnosed as having coeliac disease in childhood and had been taking a GFD since then. These patients were taking GFDs when they presented to the DH clinic, and their small intestinal biopsies were

normal, confirming that they were adhering to their diets. It is known that a GFD containing proprietary "gluten-free" products is not completely devoid of gluten [Ciclitira *et al*, 1984], and perhaps there was a sufficient amount of gluten present in these patients' diets to trigger their skin lesions.

A further four patients had been diagnosed as having coeliac disease in childhood. Patients who had been diagnosed as having coeliac disease in childhood, and who had been taking a strict GFD, should not develop the skin lesions of DH later in life. These four patients were not taking a GFD when they presented to the DH clinic, and villous atrophy was present on their small intestinal biopsies.

The incidence of spontaneous remission of the rash in patients taking a normal diet in this study is similar to that reported in other series [Alexander *et al*, 1975]. The reason for remission is unknown. There is evidence to suggest that T cells may be implicated in the pathogenesis of the skin lesions in DH, although the antigen-specificity of these T cells has not been identified [Nestor *et al*, 1987]. However, it is likely that the antigen is a component of the skin. The development of anergy of these T cells is one possible mechanism whereby remission could occur.

It is thought that the presence of IgA in the skin is necessary for the production of skin lesions, but it is equally possible that it is an epiphenomenon. It has been suggested that neutrophils, which are a characteristic feature of DH lesions, bind to the IgA, with subsequent activation and release of enzymes [Graeber *et al*, 1993]. In the cohort of patients studied here, 10 of 41 (24%) patients taking a strict GFD lost the IgA from their skin after periods ranging from 3 to 16 years following complete control of the rash by diet

alone. It is difficult to ascertain with accuracy how quickly the IgA was lost in these patients. In an earlier report, IgA was present in patients whose rash had been controlled by a GFD for periods ranging from 6 months to 7 years [Fry *et al*, 1978]. In a subsequent study, three patients who had been taking a GFD for a minimum of 7 years were found to have lost the IgA from their skin [Leonard *et al*, 1983]. This same study demonstrated recurrence of the IgA and the rash following gluten challenge [Leonard *et al*, 1983]. It is thought that the IgA in the skin originates from the small intestine, because of the presence of "J" chains in the skin [Unsworth *et al*, 1982]. The stimulus for IgA production and its subsequent deposition in the skin is likely to be gluten. It might therefore be asked why it takes so long for the IgA to disappear from the skin. It has been demonstrated that IgA is difficult to elute from the skin [Egelrud *et al*, 1985; Meyer *et al*, 1987], indicating that it is tightly bound, and this may explain why it may take many years for the IgA to gradually disappear.

Loss of IgA was observed in a single patient who was not taking a strict GFD. This patient was one of the oldest to attend the DH clinic. He was 69 years old when his rash began, and 80 when he achieved remission. At the time of writing, he was taking a normal diet. It is possible that the loss of IgA from this patient's skin was related to his age. DH tends to be less severe in elderly patients, and this may be a result of altered immune function in the elderly.

In conclusion, a GFD is the most appropriate treatment for patients with DH. The success of a GFD in the management of patients with DH is aided by the contribution of a dietitian who has a special interest in coeliac disease. In addition to reducing the need for drugs with a high incidence of side effects, treatment with a GFD produces resolution of

the associated enteropathy and prevents malabsorption.

TABLE 2.1: Analysis of patients according to diet

DIETARY STATUS	NUMBER OF PATIENTS
GFD alone	78
GFD and medication	55
Normal diet and medication	69
Remission (normal diet, no medication)	10
TOTAL	**212**

TABLE 2.2: Analysis of patients according to strictness of gluten free diet

Strictness of diet	Diet alone	Diet & medication
Strict (group I)	41	3
Partial GFD (group II)*	9	13
Partial GFD (group III)+	26	36
Strictness unknown	2	3
TOTAL	**78**	**55**

* err no more than twice per year
+ err more than twice per year, but no more than once per week

TABLE 2.3: Time taken in months to reduce and stop medication from commencement of gluten free diet in patients whose rash was controlled by diet alone

Strictness of diet	Mean time (months) to reduce medication by 50% ± SEM	Mean time (months) to stop medication ± SEM
Group I (strict GFD)	13 ± 3 (range 1 – 66) n = 25	28 ± 4** (range 4 – 88) n = 30
Group II (partial GFD)*	14 ± 4 (range 3 – 24) n = 5	44 ± 15 (range 9 – 106) n = 7
Group III (partial GFD)+	21 ± 6 (range 6 – 92) n = 14	62 ± 13** (range 6 – 223) n = 19

n	number of patients in analysis (data was not available for all patients)
SEM	standard error of the mean
**	significant difference at $P < 0.02$ (Mann-Whitney *U*-test) between groups I and III
*	err no more than twice per year
+	err more than twice per year, but no more than once per week

CHAPTER TWO

CLINICAL ASPECTS

2. A GLUTEN FREE DIET PROTECTS PATIENTS WITH DERMATITIS HERPETIFORMIS FROM DEVELOPING LYMPHOMA

2.2.1 Introduction

It is known that patients with DH, like patients with CD, have an increased risk of developing lymphoma when compared with the general population [Reunala *et al*, 1982; Reunala *et al*, 1983; Leonard *et al*, 1983; Sigurgeirsson *et al*, 1984]. It has previously been suggested that if patients with CD adhered to a strict GFD for 5 years or longer, then their risk of developing lymphoma would be no greater than that of the general population provided that they continued to take the diet [Holmes *et al*, 1989]. Although there have been no other detailed studies, anecdotal evidence supports this view [McCarthy *et al*, 1990].

A GFD has been used in the management of patients with DH at St. Mary's Hospital, Paddington since 1967 and patients have been kept under regular review. The cohort of patients attending St Mary's Hospital was studied in conjunction with a comparable group of Finnish patients and the resulting data were analysed with a view to determining whether a GFD protects patients with DH from the development of lymphoma.

2.2.2 Methods

Patients

487 (275M:212F) patients were included in the study: 206 had been followed up in St.

Mary's dermatitis herpetiformis clinic from 1969 until May 1993; 281 patients had been followed up at Tampere University Hospital, Finland from 1976 until May 1993.

The diagnosis of DH had been confirmed in both the British and Finnish cohorts by the demonstration of IgA in the dermal papillae of uninvolved skin. For the purpose of this study, the point of entry was taken as being the date of first attendance.

Information was gathered from patients themselves, from casesheets (see section 2.1.2) and from the family doctor. Where death had occurred, the date, autopsy result and death certificate were obtained for patients seen at St. Mary's Hospital. Deaths and cancers in the Finnish group were checked in the Finnish Health and Cancer Registry for 1993. Patients who had defaulted from the clinic in the UK were traced from the National Health Service Central Register at Southport. Twenty patients from St Mary's Hospital could not be traced and were considered to have been at risk from the point of entry until their last outpatient attendance. Data were available on all the Finnish patients. Information pertaining to the English patients was collected by Dr Helen Lewis (Dermatology Registrar) and myself. The English patients were under the care of Professor Lionel Fry and/or Dr Jonathan Leonard. Information on the Finnish patients was collected by Professor Timo Reunala – the majority of these patients had had small intestinal biopsies performed by Professor Reunala's colleague, Dr Peka Collin.

Dietary Groups

Patients were classified into groups depending on the degree of gluten ingestion:

Group I This group included patients who adhered to a strict GFD for five or more

consecutive years. This five year limit was selected prior to analysis of the data and was the cut off point chosen in a similar study carried out in patients with CD [Holmes *et al*, 1989].

Group II This group was designated the partial GFD group and included those who attempted to adhere to a GFD but assessment by a dietitian revealed ingestion of gluten, although the degree of ingestion was less than the equivalent of 1-2 pieces of bread weekly.

Group III This was the normal diet group. This included those patients who had no record of taking a GFD, those who had attempted a GFD but dietary evaluation revealed only sporadic adherence to GFD, those in whom the strictness of gluten exclusion was uncertain and those who had received a GFD for less than five years.

Statistical analysis

Statistical analysis was performed by a professional statistician, John Fry. He analysed the data using the "man years at risk" life-table method [Hill, 1972] where the observed number of deaths or cancer registrations is compared with that expected from the general population rates. The rates are broken down by sex and 5-year age group. The ratio of the observed to the expected number of registrations is described as the standardised registration ratio (SRR). Patients commenced on a strict GFD were assumed to contribute man years to the normal diet group until 5 years after starting the diet. If the diet was discontinued, the patient contributed man years to the GFD group for a further 5 years following which they were assigned to the normal diet group.

To examine the significance of the SRRs the observed number of registrations (O) was treated as having a Poisson distribution with mean and variance equal to the expected number of registrations (E). To test if the Groups I and II were behaving as Group III, the expected number of registrations for each of these groups was multiplied by the SRR/100 for group III to give the estimated number of registrations if Groups I and II behaved as Group III (E2). The probability of observing less than or equal to the observed number of registrations using E2 as the Poisson parameter was then calculated.

Death rates are based on 5-year death rates made available by the World Health Organisation. Registration rates are based on the 1981 figures from the Office of Population and Censuses survey for England and Wales and for the 1977-1981 figures from the Finnish Cancer Registry.

Histopathology Review

Original paraffin sections of 7 out of 8 tumours diagnosed as lymphoma were available. David Evans, Professor of Pathology at St Mary's Hospital, reviewed this material and performed immunoperoxidase studies with antibodies to leucocyte common antigen (LCA), B cell antigen CD20 (L26) and T cell antigens CD3 and CD45RO (UCHL1).

2.2.3 Results

The cohort was followed up for a total of 4918 man years: for Group I there were 1153.7 man years; Group II had 389.4 man years; and Group III had 3375.3 man years.

Eight (1.6%) patients with DH developed lymphoma (SRR 3810). The mean time from entry into the study until diagnosis of lymphoma was 78 (range 2 - 240) months. Clinical

details and classification are given in Table 2.4. This includes a patient from the Finnish group (number 4) who underwent resection of a small bowel reticulum cell sarcoma 11 years before presentation of DH [Reunala *et al*, 1982]. After a further three years, whilst taking a partial GFD, a histiocytic diffuse-type lymphoma involving the ileum, colon and stomach was identified at laparotomy.

All lymphomas occurred in Group III and no lymphoma developed in patients who had adhered to a strict GFD for 5 years or more. The difference between Group III and Group I reached statistical significance (E2=3.084; *P*=0.0457). No lymphoma developed in Group II but there was no statistical difference compared with group III (E2=0.998; *P*=0.369).

Twenty two malignancies other than lymphoma were identified (Table 2.5). In Group I a patient developed carcinoma of the stomach and a second patient developed carcinoma of the prostate. In Group II, one patient developed carcinoma of the colon and a further patient had carcinoma of the bronchus diagnosed. The remaining patients with malignancy were in Group III. The overall incidence of malignancy was marginally greater than the expected incidence of 14.44 but this difference did not reach statistical significance.

Three patients developed haematological malignancies other than lymphoma. A male patient, aged 67, developed acute lymphoblastic leukaemia 2 months prior to diagnosis of DH. A female patient had DH confirmed at age of 48 years. Although she gave a history of a typical rash from the time myeloma was diagnosed 4 years previously. These patients were not included in the analysis as the presentation of malignancy preceded the diagnosis of DH. Finally, a male patient, controlled on dapsone and a normal diet following a diagnosis of DH aged 69 years, developed myeloma 5 years later. The difference

between Group I and Group III for all lymphomas and myeloma reached statistical significance (E2 3.458; *P*=0.0365; SRR 3100).

2.2.4 Discussion

This study has confirmed that there is an increased incidence of lymphoma in patients with DH. Furthermore, it has demonstrated that patients with DH who adhere to a strict GFD for 5 years or longer, have no increased risk of developing a lymphoma compared with the general population. This finding supports the protective role of a GFD against the development of lymphoma. Three lymphomas occurred in patients treated with a GFD, but presentation was within 5 years of commencing the diet. An explanation for this may be that the relatively short time on the diet was insufficient to reverse the effect of an oncogenic stimulus which may have operated for many years. Alternatively, the lymphoma may have already developed before introduction of the gluten free diet, and taken some years for clinical manifestations to occur. Interestingly, no lymphoma developed in patients taking a partial GFD. However, there was no statistical difference in the incidence of lymphoma in this group compared with patients taking a normal diet. It is possible that a reduction in gluten intake reduces the risk of developing lymphoma, but the number of man years contributed by the partial GFD group was small and may not have allowed demonstration of such an effect.

A protective role for a GFD against the development of lymphoma in CD has also been suggested [Holmes *et al*, 1989; Collin *et al*, 1994]. In coeliac disease, the majority of cases of lymphomas, originally termed malignant histiocytosis of the intestine, are thought to be of T cell origin, and the term "enteropathy associated T-cell Lymphoma" (EATL) is now used [Isaacson *et al*, 1985]. Furthermore, it has been suggested that in some patients

adult onset coeliac disease may be due to a low grade lymphoma of intra-epithelial lymphocytes [Wright *et al*, 1991]. However, the site of presentation of lymphoma is not confined to the intestine [Fowler & Thomas, 1976].

Although the majority of lymphomas reported in dermatitis herpetiformis have been classified as histiocytic tumours, the occurrence of Hodgkin's disease and lymphomas of B cell origin have also been described [Reunala *et al*, 1982; Sigurgeirsson *et al*, 1994; Fowler & Thomas,1976; Gawkrodger *et al*, 1982; Buckley *et al*, 1983; Månsson, 1971]. Interestingly, lymphomas represented in this study were of a mixed nature, in keeping with a recent study from Sweden [Sigurgeirsson *et al*, 1994], and include those of T and B cell lineage, and Hodgkin's disease.

An increased risk for developing carcinomas of the mouth, pharynx, oesophagus and small bowel has been shown in coeliac disease [Holmes *et al*, 1989; Swinson *et al*, 1983], however no increase of these malignancies has been shown in larger follow up series of patients with dermatitis herpetiformis [Sigurgeirsson *et al*, 1994; Swerdlow *et al*, 1993]. As reported here, carcinomas have been reported in patients with dermatitis herpetiformis, but the frequency and site of these malignancies is no different to that seen in the general population.

The aetiology of lymphoma in patients with coeliac disease or dermatitis herpetiformis is unknown but several mechanisms may be involved. Both diseases are associated with a wide range of autoimmune disorders [Gaspari *et al*, 1990] and splenic atrophy and it may be postulated that an underlying immunological disturbance in the presence of gluten may predispose to the development of lymphoma. The increased risk of developing lymphoma

in the gastrointestinal tract may be due to polyclonal stimulation of lymphocytes by gluten giving rise to malignant transformation. Such a malignant clone could arise in proliferating T or B cells, and it is possible that myeloma, also reported in these cohorts, may have arisen by this mechanism. More general theoretical mechanisms include carcinogens gaining access through damaged intestinal mucosa and mucosal detoxifying enzyme depletion allowing accumulation of potentially damaging substances.

The majority of patients with dermatitis herpetiformis do not have symptoms from their gluten sensitive enteropathy and the eruption can be controlled by drugs. However, it now appears that gluten withdrawal is protective against the development of lymphoma and this is a further compelling argument for encouraging patients to adhere to a GFD.

TABLE 2.4: Details and morphological type of lymphoma occurring in patients with dermatitis herpetiformis

*	Second lymphoma presentation
+	Patients from Finland
NA	not available
Null	non-B, non-T
LN	lymph node
bm	bone marrow

TABLE 2.4

Patient no.	Sex	Age of presentation with DH	Diet	Age of Presentation with lymphoma	Site of lymphoma	Original classification of lymphoma	Phenotype & revised classification
1	M	65	normal	78	axillary LN-stage IV	centroblastic	B cell plasmacytoid
2	M	46	normal	46	bm	immunoblastic	T cell large cell
3	M	54	normal	63	small intestine	histiocytic	B cell plasmacytoid
4	M	47	partial GFD	50* +	widespread-ileum, colon, stomach	diffuse-type histiocytic	NA
5	M	28	strict GFD for 4 years	37 +	colon	histiocytic	T cell large cell
6	F	35	strict GFD for 2 years	42 +	inguinal LNs	lymphocytic-diffuse	B cell
7	F	64	strict GFD for 4 years	68 +	mesenteric & cervical LNs	histiocytic	Null
8	M	67	normal	70	cervical LN, bm	Hodgkin's	mixed cellularity

TABLE 2.5: Comparison of observed and expected numbers of malignancies

Site of malignancy	Observed number	Expected number	SRR	*P*
bronchus	7	4.834	144.8	NS
stomach	3	1.671	179.5	NS
colon	2	1.410	141.8	NS
prostate	3	1.760	170.5	NS
breast	2	1.946	102.8	NS
skin	1	1.781	56.1	-
rectum	1	1.062	94.2	-
thyroid	1	0.180	555.6	-
pancreas	1	0.746	134.0	-
myeloma	1	0.155	645.1	-

CHAPTER TWO

CLINICAL ASPECTS

3. IDENTIFICATION OF CLINICAL CARRIERS FOR THE GENE(S) FOR DERMATITIS HERPETIFORMIS AND COELIAC DISEASE WITHIN FAMILIES

2.3.1 Introduction

There is compelling evidence which suggests that dermatitis herpetiformis and coeliac disease have a genetic basis which is shared. Both conditions are associated with HLA DR3 and DQ2 [Yunis, 1992], and in particular with the DQ alleles A1*0501 and B1*0201 [Sollid *et al*, 1989; Hall *et al*, 1991]. In addition, both have been shown to cluster in families: one study, performed on a cohort of patients living in Utah, reported that 6.5% of DH patients had first degree relatives with DH [Meyer & Zone, 1987] whilst a further study, based on Finnish patients, reported that 4.4% of DH patients had affected first degree relatives with DH and 6.1% with CD [Reunala, 1996]. Furthermore, there have been several reports of DH and CD occurring within monozygotic twins: some twin pairs were found to be concordant for DH whilst in other twins, one had DH and the other CD [Jepsen & Ullman, 1980; Kósnai *et al*, 1985; Green *et al*, 1986; Anstey *et al*, 1991; Reunala *et al*, 1991]. Thus, genetic factors appear to be important in the aetiology of DH and CD.

It is also known that patients diagnosed as having coeliac disease only represent the tip of the iceberg as those with milder forms are likely to remain undiagnosed as they are either asymptomatic or have non-specific or atypical symptoms. Serological screening of population groups has highlighted this fact [Catassi *et al*, 1994; Unsworth & Brown, 1994]. Consequently, it is possible that previous studies may have underestimated the

occurrence of CD within relatives of patients with DH, as only those with overt disease would have been identified. Two previous studies have demonstrated that if relatives of patients with DH have small intestinal biopsies, then as many as 40% will have evidence of gluten sensitive enteropathy [Marks *et al*, 1970; Reunala *et al*, 1976].

The aim of this study, therefore, was to screen relatives of DH patients in three families in order to identify those with silent or latent coeliac disease. Families in which there was at least one additional member with DH or CD, over and above the index case, were chosen. Serology, small intestinal biopsy and evaluation of intraepithelial T cells expressing the γ/δ cell receptor were used in the screening process. This study formed part of a larger project in which it was planned to look for gene linkage within these families.

2.3.2 Methods

Subjects

Ethical permission for this study was granted by St Mary's Hospital Local Ethics Committee.

Three families were identified and invited to participate via the index cases who attended the DH clinic at St Mary's Hospital. Consent was obtained from each individual family member. Family members were interviewed by myself and asked specifically about gastrointestinal and skin symptoms. I examined the skin of those patients volunteering skin symptoms. I took blood for serology from all those participating. Those family members who were identified as being potential carriers of the gene(s) and aged 16 years and over were offered duodenal biopsy, irrespective of their antibody results.

Small intestinal biopsy

Those who consented to small intestinal biopsy had biopsies taken from the second part of the duodenum via endoscopy. The small intestinal biopsies were performed by Dr Huw Thomas, Consultant Gastroenterologist at St Mary's Hospital. Each participating individual had two sets of duodenal biopsies taken: one for routine histology and one which was snap frozen in OCT (Tissue Tek©) and stored at -70°C until processed - this latter set was used for evaluating intra-epithelial γ/δ T cells.

Serology

As a proportion of individuals with CD exhibit IgA deficiency, all those participating had their IgA levels measured. In addition, ARA and AEA were measured and in order to ensure that the serology results were reproducible, a random selection of samples were sent to Helsinki, Finland where AGA and AEA estimations were performed in a blind manner by Dr Erkki Savilahti.

Morphometric analysis of small intestinal biopsies

Villous height: crypt depth ratios (VH:CD) and surface enterocyte cell heights (SECH) were measured by myself. I took care to avoid analysis of sections cut tangentially and to avoid analysis of mucosa overlying Brunner's glands [Shidrawi *et al*, 1994]. The normal range for VH:CD was considered to be 3-5 whilst the normal range for SECH was considered to be 29-34 μm [Shidrawi *et al*, 1994].

Intra-epithelial lymphocyte cell counts

I expressed the intra-epithelial lymphocytes per 100 epithelial cells. I performed counts on three sections and took the mean of these. I regarded values greater than 30 lymphocytes

cells per 100 epithelial cells as being abnormal [Fry *et al*, 1974].

Intra-epithellal cells expressing the γ/δ T cell receptor

These were performed by Dr. Savilahti, Helsinki, as previously described [Savilahti *et al*, 1990]. Positively staining cells were expressed per mm of surface epithelium. Values less than 7 cells per mm were regarded as normal [Savilahti *et al*, 1997].

2.3.3 Results

Seventy one individuals from 3 families participated and this included 8 who were already known to have DH (5) or CD (3) (Figures 2.4, 2.5, 2.6). Therefore, 63 "unaffected" individuals were interviewed, examined and had blood samples taken for serology. None of the "unaffected" relatives gave a history of significant gastrointestinal symptoms nor did they exhibit signs of malabsorption. After excluding those who had married into the family, 48 were identified as being potential carriers, including 5 children. Forty three were offered duodenal biopsy and 23 accepted. A further patient, who was in fact the wife of one of the DH index cases (Figure 2.6: subject II.10), was found to be positive for AGA-IgA, ARA and AEA and she too was offered duodenal biopsy. Therefore, a total of 24 individuals underwent endoscopy and duodenal biopsy at St Mary's Hospital, Paddington. An additional patient (Figure 2.4: subject II.9), on learning that she was anti-endomysial antibody positive had an endoscopy arranged at her local hospital in Scotland by her General Practitioner.

Serology

No individual had IgA deficiency. Six individuals were identified as having positive serology: 4 from family 1 (subjects II.5, II.9, II.11, III.1); 1 from family 2 (subject III.1); and

1 from family 3 who was the wife of our index case (subject II.10). The serology results are shown in detail in Tables 2.6, 2.7 and 2.8.

Villous height: crypt depth ratios, surface epithelial cell heights and intra-epithelial lymphocyte counts

Family 1 (Figure 2.4 and Table 2.6)

One of the 4 participants with positive serology in this family (subject II.11, ↑ AGA-IgA) declined endoscopy. Of the remaining 3, subject II.5 (↑ AGA-IgA) had a raised IEL count; subject II.9 (EAE +ve), who had her duodenal biopsy performed at her local hospital, had normal villous architecture but raised IELs (formalin fixed sections were available for me to analyse); and subject III.1 (weak +ve ARA) had normal villous architecture and normal IELs. An additional family member (III.10), who had negative serology, was found to have a mildly raised IEL count (VH:CD not performed as all sections contained Brunner's glands in the sub-mucosa).

Family 2 (Figure 2.5 and Table 2.7)

In this family, one individual (III.1) was found to have circulating AEA and subsequent small intestinal biopsy demonstrated reduced VH:CD, reduced SECH and elevated IELs. One further individual (II.4), whose serology was negative, was also found to have a reduced VH:CD in the presence of normal SECH and a normal IEL count.

Family 3 (Figure 2.6 and Table 2.8)

In this family, one participating member (II.10), the wife of our index case, was found to have raised levels of AGA-IgA and to have circulating ARA and AEA. Small intestinal

biopsy confirmed the presence of a reduced VH:CD, reduced SECH and elevated numbers of IELs.

Intra-epithelial cells expressing the γ/δ T cell receptors

Family 1 (Figure 2.4 and Table 2.6)

Subjects II.5, III.1 and III.16 were found to have raised levels of intra-epithelial T cells expressing the γ/δ receptor. Subject III.10, who had a raised IEL count, had normal levels of γ/δ T cells. γ/δ T cell estimations were not performed in subject II.9.

Family 2 (Figure 2.5 and Table 2.7)

Subject III.1 who had positive serology, reduced VH:CD, reduced SECH and a raised IEL count in keeping with gluten sensitive enteropathy, was found to have elevated numbers of T cell expressing the γ/δ receptor. One further participant (II.4), whose serology was negative but who had a reduced VH:CD suggestive of mild GSE, had elevated numbers of γ/δ T cells.

Family 3 (Figure 2.6 and Table 2.8)

Subject I.3, whose serology was negative and whose VH:CD, SECH and IEL count were normal, had elevated numbers of T cell expressing the γ/δ receptor. Subject II.10, who had positive serology, a reduced VH:CD, reduced SECH and an elevated IEL count, was found to have elevated numbers of γ/δ T cells.

Summary of results

Family 1. (Figure 2.4) Thirty three individuals participated of whom 7 were considered to be carriers for the gene(s) for DH/CD and this included 3 previously identified as having

DH (2) or CD (1). Of the additional 4 individuals identified during the study, 3 were found to have an elevated γ/δ T cell count (II.5, III.1 and III.16) and in two (III.1 and III.16) this was the only abnormality detected indicating that they had latent coeliac disease. Participant II.5 had a raised IEL count accompanying an elevated γ/δ T cell count. Unfortunately, subject II.9 did not have γ/δ T cell evaluations performed as her biopsy was performed in Scotland; in view of the fact that her IEL count was greatly elevated in the presence of a positive AEA, we felt it very likely that she was a carrier.

Subject III.10 had an isolated borderline elevated IEL count and in the absence of any further supportive features, it seemed unlikely that he was a carrier. I was unable to explain this isolated finding.

Looking at the family tree for Family One (Figure 2.4), if it is assumed that the gene(s) is inherited in an autosomal dominant fashion, then it seems likely that family members II.1 and II.3 were also silent carriers.

Thus, of the 33 individuals investigated, 7 appeared to be definite carriers with a possible further 2 by deduction. After excluding those who had married into the family, (i.e. 7 individuals), the carrier rate for this family appeared to be 7/26 or 27%; or, if we also consider subjects II.1 and II.3 as carriers, then the rate becomes 9/26 or 35%.

Family 2. (Figure 2.5) Eight individuals participated and 4, including the father and son who were already known to have DH, were found to be affected. After excluding the wife of the propositus, the carrier rate was 4/7 or 57%.

Family 3. (Figure 2.6) Thirty individuals participated and 5 were found to be affected. This included subject I.3, who was aged 75 years at the time of the study and who was found to have elevated numbers of IELs expressing the γ/δ T cell receptor indicating that she had latent CD or was a gene carrier. The wife of the propositus was also demonstrated as having CD (II.10).

Thus, after excluding those who had married into the family and subjects III.3-6, III.9 and III.11-15 as I believed them to be true negatives as NO evidence of CD was detected in their parents, the carrier rate for this family was 3/13 or 23%.

2.3.4 Discussion

This study, which was part of a larger study which aimed to look for gene linkage for DH/CD within families, has confirmed that so called healthy relatives of patients with DH have a significant risk of silent or latent coeliac disease. It has also demonstrated that screening families of DH patients by means of serological testing alone is not wholly reliable in excluding CD. CD can only be absolutely excluded following morphometric evaluation of small intestinal mucosa, enumeration of IEL counts and of IELs bearing the γ/δ T cell receptor.

Serological testing for CD using AGA, ARA and AEA can be useful in screening large populations and can pinpoint those individuals who warrant further investigation by means of small intestinal biopsy. Indeed, recent reports, in which populations have been screened using these antibodies, have highlighted the fact that there are many individuals with hitherto undiagnosed CD [Catassi *et al*, 1994; Unsworth & Brown, 1994]. AGA (class IgA), which is detected using an enzyme-linked immunosorbent assay (ELISA), has a high

sensitivity in the diagnosis of CD but a low specificity as it may be present in normal individuals [Savilahti *et al*, 1983]. ARA and AEA are both IgA antibodies. The first publications on ARA appeared in 1971 and it was noted that it had a lower sensitivity but higher specificity than AGA-IgA in the detection of CD [Seah *et al*, 1971; Seah *et al*, 1971]. Later, a further antibody was discovered, AEA, and it was shown to have a sensitivity and specificity similar to ARA, with the latter approaching 100% in DH and CD [Beutner *et al*, 1986; Chorzelski *et al*, 1984]. ARA and AEA are more likely to be positive in individuals with more severe forms of villous atrophy and negative in patients with milder forms [Leonard *et al*, 1985; Beutner *et al*, 1986; Reunala *et al*, 1987; Hällstrom, 1989]. Thus, given that the presence of ARA and AEA is dependent on the severity of the villous atrophy, screening with these antibodies alone would fail to detect those with mild enteropathy or those with latent CD.

It has recently been demonstrated that patients with CD and DH have increased numbers of intra-epithelial cells bearing γ/δ T cell receptors [Spencer *et al*, 1989; Halstensen *et al*, 1989; Savilahti *et al*, 1990; Savilahti *et al*, 1992] and that these cells remain elevated even when patients adhere to a gluten free diet [Savilahti *et al*, 1992]. Moreover, it has been shown that patients with elevated numbers of γ/δ T cells and normal villous architecture are at risk of developing CD i.e. they have latent CD [Mäki *et al*, 1991], or of developing DH [Savilahti *et al*, 1992]. It is not known if T cells bearing the γ/δ receptor are involved in the pathogenesis of the enteropathy of CD or whether they simply indicate a genetic susceptibility to develop CD and DH. It has been demonstrated that elevated numbers of intra-epithelial γ/δ T cells are associated with HLA DQA and DQB, genetic markers for CD and DH, and furthermore, the greater number of these gene markers present within an individual, then the more likely they are to have elevated γ/δ T cell counts

[Holm *et al*, 1992].

In conclusion, the families studied here support a genetic basis for DH and CD and in particular, an autosomal dominant mode of inheritance, which is in agreement with others [Reunala, 1996]. Finally, genetic studies, which aim to look for gene linkage, should not rely solely on serology but should also perform small intestinal biopsies, IEL and IEL γ/δ T cell counts wherever possible.

TABLES 2.6, 2.7 AND 2.8: serology; morphometric analysis of small intestinal biopsies including villous height: crypt depth ratios and surface enterocyte cell heights; intra-epithelial lymphocyte counts; and counts of T cells bearing the γ/δ receptor.

Key to tables:

IELs	intra-epithelial lymphocyte counts
ND	not done
AGA-IgA	anti-gliadin antibodies, class IgA
AEA	anti-endomysial antibodies
DH	dermatitis herpetiformis
CD	coeliac disease

TABLE 2.6: Family one

SUBJECT	SEROLOGY	D2 BIOPSY			
		VH:CD	SECH	IELs	γ/δ T CELLS
II.1	negative	ND	ND	ND	ND
II.2	negative	ND	ND	ND	ND
II.3	negative	ND	ND	ND	ND
II.4	negative	ND	ND	ND	ND
II.5	**AGA-IgA (↑)**	4.2	35	**47 (↑)**	**15 (↑)**
II.6	negative	ND	ND	ND	ND
II.7	negative	ND	ND	ND	ND
II.9	**AEA +ve**	3.8	29	**49 (↑)**	ND
II.10	negative	ND	ND	ND	ND
II.11	**AGA-IgA (↑)**	ND	ND	ND	ND
III.1	**ARA weak +ve**	3.9	30	14	**15.8 (↑)**
III.2	negative	ND	ND	ND	ND
III.3	negative	3.5	34	14	0.6
III.4	negative	4.9	35	18	2.7
III.5	**KNOWN DH**				
III.6	negative	ND	ND	ND	ND
III.7	**KNOWN DH**				
III.8	**KNOWN CD**				
III.9	negative	ND	ND	ND	ND
III.10	negative	ND	31	**33 (↑)**	1.5
III.11	negative	3.1	31	7	0
III.12	negative	ND	ND	ND	ND
III.13	negative	ND	ND	ND	ND
III.14	negative	5.6	29	26	0
III.16	negative	5.1	34	16	**33.3 (↑)**
IV.1	negative	ND	ND	ND	ND
IV.2	negative	ND	ND	ND	ND
IV.3	negative	ND	ND	ND	ND
IV.4	negative	ND	ND	ND	ND
IV.5	negative	ND	ND	ND	ND
IV.6	negative	ND	ND	ND	ND
IV.7	negative	6.2	33	14	1.4
IV.8	negative	2.9	31	11	2

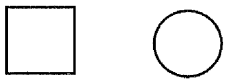
TABLE 2.7: Family two

SUBJECT	SEROLOGY	D2 BIOPSY			
		VH:CD	SECH	IELs	γ/δ T CELLS
I.1	negative	ND	ND	ND	ND
II.1	negative	3.8	31	11	6
II.2	**KNOWN DH**				
II.3	negative	ND	ND	ND	ND
II.4	negative	**2.5 (↓)**	31	22	**8.3 (↑)**
III.1	**AEA +ve**	**1.8 (↓)**	**23 (↓)**	**44 (↑)**	**12 (↑)**
III.2	**KNOWN DH**				
III.3	negative	3.5	36	28	2.5

TABLE 2.8: Family three

SUBJECT	SEROLOGY	D2 BIOPSY			
		VH:CD	SECH	IELs	γ/δ T CELLS
I.3	negative	3.3	32	12	**23 (↑)**
I.4	negative	ND	ND	ND	ND
II.2	**KNOWN CD**				
II.3	negative	ND	ND	ND	ND
II.4	negative	3.3	32	8	ND
II.6	negative	3.9	38	11	5
II.7	negative	ND	ND	ND	ND
II.9	**KNOWN DH**				
II.10	**AGA-IgA ↑ ARA +ve AEA +ve**	**0.3 (↓)**	**20 (↓)**	↑↑↑	**7 (↑)**
II.11	**KNOWN CD**				
II.12	negative	ND	ND	ND	ND
II.13	negative	3.7	34	16	0
II.14	negative	ND	ND	ND	ND
II.15	negative	3.7	31	11	5
II.16	negative	ND	ND	ND	ND
II.17	negative	3.7	36	10	1
III.1	negative	ND	ND	ND	ND
III.2	negative	ND	ND	ND	ND
III.3	negative	ND	ND	ND	ND
III.4	negative	ND	ND	ND	ND
III.5	negative	ND	ND	ND	ND
III.6	negative	ND	ND	ND	ND
III.7	negative	3.6	34	16	1
III.8	negative	4.2	32	8	0
III.9	negative	ND	ND	ND	ND
III.11	negative	ND	ND	ND	ND
III.12	negative	4.6	33	18	3
III.13	negative	ND	ND	ND	ND
III.14	negative	ND	ND	ND	ND
III.15	negative	ND	ND	ND	ND

Key to Figures 2.1, 2.2 and 2.3.



serology only obtained;
no intestinal biopsy performed

small intestinal biopsy normal

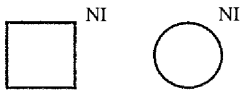
reduced villous height : crypt depth ratio (VH : CD)
(normal range : 3-5)

reduced surface enterocyte cell height (SECH)
(normal range : 29-34 μm)

raised intra-epithelial lymphocyte count (IEL)
(normal range : < 30 per 100 epithelial cells)

raised numbers of intra-epithelial cells γ/δ T cell receptor
(normal range : < 7 per mm)

known to have either
dermatitis herpetiformis (DH) or
coeliac disease (CD) prior to study



not investigated

FIGURE 2.1: Family one

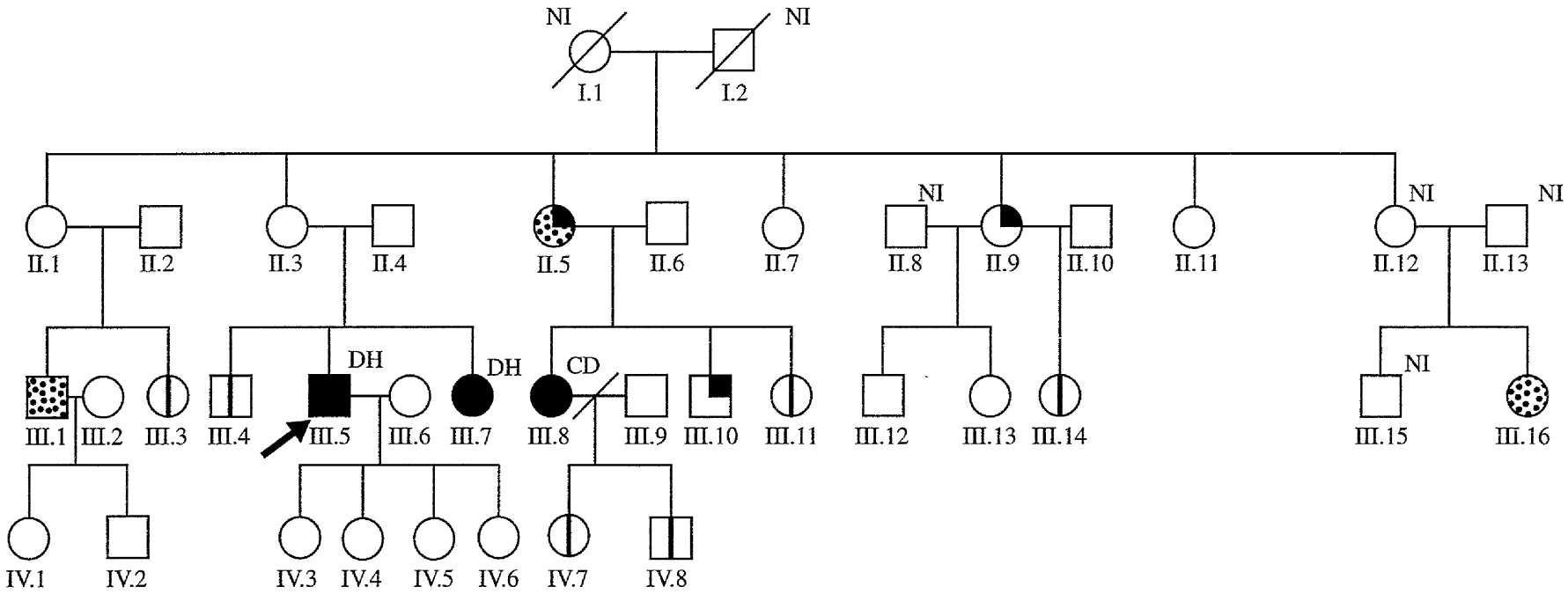

NI
NI
I.1
I.2
NI
NI
NI
II.1
II.2
II.3
II.4
II.5
II.6
II.7
II.8
II.9
II.10
II.11
II.12
II.13
DH
DH
CD
NI
III.1
III.2
III.3
III.4
III.5
III.6
III.7
III.8
III.9
III.10
III.11
III.12
III.13
III.14
III.15
III.16
IV.1
IV.2
IV.3
IV.4
IV.5
IV.6
IV.7
IV.8

FIGURE 2.2: Family two

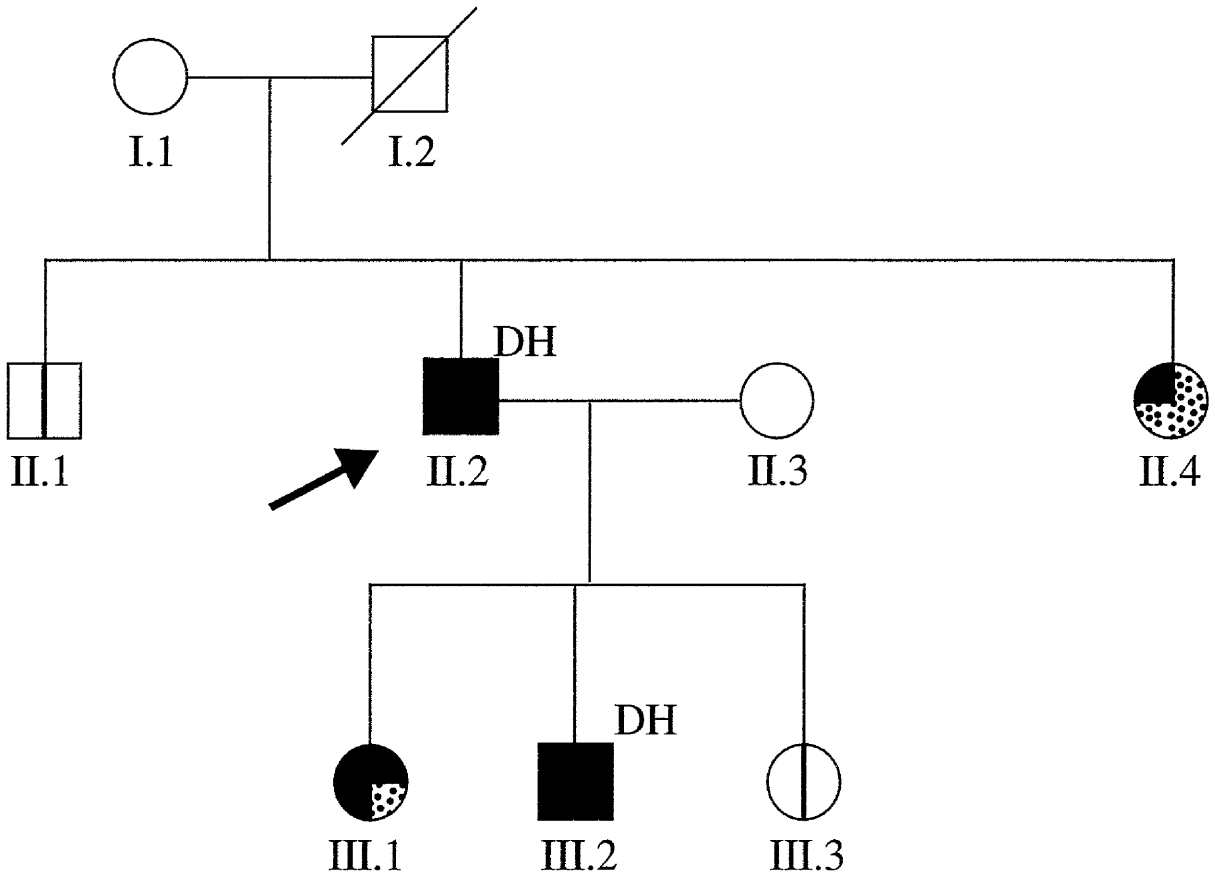
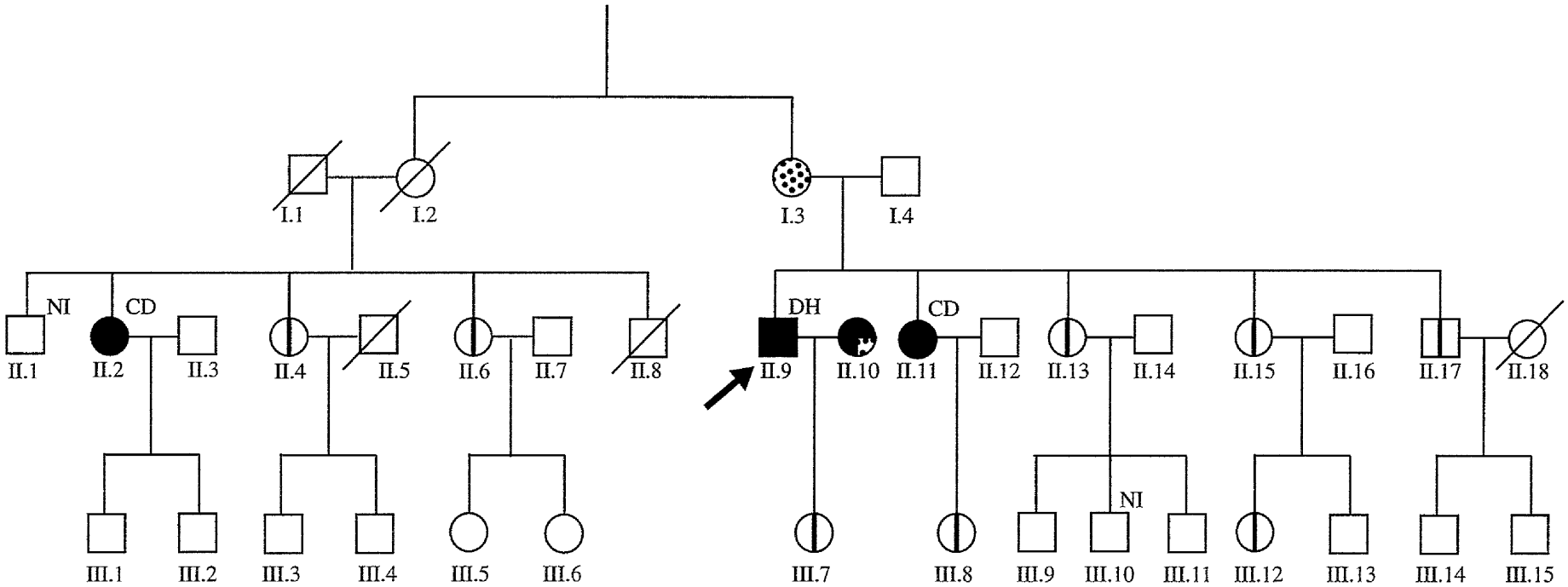

I.1
I.2
DH
II.1
II.2
II.3
II.4
DH
III.1
III.2
III.3

FIGURE 2.3: Family three


I.1
I.2
I.3
I.4
NI
CD
DH
CD
NI
II.1
II.2
II.3
II.4
II.5
II.6
II.7
II.8
II.9
II.10
II.11
II.12
II.13
II.14
II.15
II.16
II.17
II.18
III.1
III.2
III.3
III.4
III.5
III.6
III.7
III.8
III.9
III.10
III.11
III.12
III.13
III.14
III.15

CHAPTER THREE

LABORATORY STUDIES

CHAPTER 3

LABORATORY STUDIES

1. SKIN TESTING WITH GLUTEN IN PATIENTS WITH DERMATITIS HERPETIFORMIS

3.1.1 Introduction

Patients with dermatitis herpetiformis (DH) have an enteropathy which is indistinguishable from gluten sensitive enteropathy (GSE). T cell mediated mechanisms have been implicated in the pathogenesis of GSE because of the similarity between the histological features of GSE and graft versus host disease (GvHD) [Nield, 1981] and the characteristic finding of elevated numbers of intra-epithelial lymphocytes (IELs) in the small intestine of patients with CD and DH which decrease during a gluten free diet (GFD). Furthermore, normal foetal small bowel, exposed *in vitro* to T cell activators, develops villous atrophy and crypt hyperplasia [MacDonald, 1988]. Moreover T cell responses have been demonstrated both in the peripheral blood [Sikora *et al*, 1976; O'Farrelly *et al*, 1982] and more recently in the small intestinal mucosa [Lundin *et al*, 1993] of patients with coeliac disease. It has been postulated that GSE is due to a breakdown of the normal immunological mechanisms which ensure oral tolerance to gluten [Mowat, 1987].

Previous studies of intradermal skin testing in CD and DH [Anand *et al*, 1977; Logan & Ferguson, 1978; Marks & Young, 1978] using the saline soluble enzyme digest of gluten "Frazer's fraction III" [Frazer *et al*, 1959] provoked "Arthus" type reactions at 6 hours in patients and also in many control subjects, but delayed type hypersensitivity (DTH) responses were not seen clinically. The absence of DTH responses was surprising if the

postulated T cell pathogenesis of GSE is correct. Many of the patients in these previous studies who gave "Arthus" like reactions had been on a strict GFD for many months and so presumably gluten antibody negative arguing against a local immune complex type reaction. Unfortunately, antibody studies were not conducted in the above mentioned studies.

Recent rectal challenge studies in CD using FFIII as antigen [Loft *et al*, 1990] revealed that CD patients showed increased IELs on biopsy as early as 6 hours whilst the majority of disease controls did not. It could be postulated that intradermal testing with FFIII might lead to a sub-clinical T cell influx into the skin in gluten sensitive patients. Furthermore, FFIII has been shown to be a potent activator of complement via the alternative pathway *in vitro* and it has been proposed that this property may be involved in the pathogenesis of GSE [Unsworth *et al*, 1993]. This led me to perform the present pilot study of intradermal testing with FFIII: biopsies were taken at 6 hours and 48 hours and processed immunohistologically for T cell infiltration. I chose patients so as to include those with and without anti-gluten antibody.

3.1.2 Methods

Patients and controls

Ethical permission for this study was granted by St. Mary's Hospital Local Ethics Committee. Three normal volunteers (2 males, 1 female) and six patients (4 males, 2 females) with DH were enrolled in the study. Three of the DH patients were controlled by gluten free diet (GFD) alone and had been taking their diets for a mean of 8 years (range 6 - 11 years): gut biopsies in two of these patients were normal and not available in the third. The other three DH patients were taking normal diet and required dapsone for

disease control: gut biopsies were abnormal in these patients with one having partial villous atrophy and two severe villous atrophy. I asked the patients who required dapsone for disease control to discontinue their dapsone 48 hours before attending for their intradermal injections and asked them not to restart it until after the biopsies had been taken.

Preparation of Frazer's fraction III

Frazer's fraction III was prepared by my co-worker Dr Joe Unsworth (Consultant Immunologist, Regional Department of Immunology, Southmead Hospital, Bristol) as previously described [Frazer *et al,* 1959]. Wheat gluten (SIGMA Chemicals Ltd) was dissolved in sterile phosphate buffered saline pH 7.2 at 10mg/ml (phenol was omitted). Microbiological assessment prior to use showed the preparation to be free of bacterial and fungal contaminants. The preparations were frozen at -20° until used. Confidence that the FFIII prepared was of good quality was based on the fact that it bound *in vitro* to reticulin in rat tissue sections and the fact that patients with active but not treated coeliac disease gave high titres in the FFIII ELISA assay [Unsworth *et al*, 1985].

Intradermal skin testing and skin biopsies

I injected 50μl of phosphate buffered saline (PBS) control or FFIII intradermally into the flexor surface of each forearm. Two of the patients with DH with enteropathy had an additional site on one forearm injected with FFIII. I marked the sites of injection and monitored these at regular intervals for the first 8 hours and then at 48 hours: patients were asked to note any skin changes at the injection sites between 8 hours and 48 hours. I took 4mm punch biopsies from the PBS and FFIII injected sites at 48 hours. In addition, I took biopsies in two patients from FFIII injected sites at 6 hours. The skin biopsies were

snap frozen and stored in liquid nitrogen. Later, I cut 6μm sections using a cryostat (Slee, London).

I used a battery of common recall antigens (Multitest CMI, Pasteur Mérieux) consisting of streptococcus, old tuberculin, candida albicans, trichophyton mentagrophytes and proteus mirabilis antigens and a control of glycerin and applied this battery intradermallly by multipuncture to the flexor aspect of the forearms. I assessed these clinically at 48 hours.

ELISA assays

An ELISA for anti-FFIII and anti-gliadin antibodies was performed as described previously by my co-worker Dr Joe Unsworth [Unsworth *et al*, 1985].

Immunoperoxidase staining

I performed immunostaining on the skin sections using the avidin-biotin peroxidase technique: I used a Vectastain Elite ABC kit (Vector Laboratories, Peterborough) and Becton Dickinson (USA) monoclonal antibodies to stain for CD3 (pan T cell), CD4 (helper T cell) and CD8 (suppressor/cytotoxic T cell). Briefly, I air dried the sections, fixed them in acetone and then blocked them using normal serum. I quenched endogenous peroxidase with 0.3% H_2O_2 in methanol. I then incubated the sections with primary mouse monoclonal antibodies followed by biotinylated horse anti-mouse secondary antibodies. Following this, I incubated the sections with the avidin-biotin peroxidase complex and developed the colour reaction with the substrate 3-amino-9-ethyl-carbazole (AEC) tablets (Sigma, USA). Finally, I counterstained the sections with haematoxylin and mounted them in glycergel (Dako).

Immunofluorescent staining

A neoepitope exposed in human terminal complement complex, and recognised by a mouse monoclonal antibody (Dakopatts: Dako-C5b-9, ae11), was used to detect complement activation product by indirect immunofluorescence with a fluorescein labelled rabbit anti-mouse Ig reagent (Dakopatts) in the second layer. This was performed by my colleague Dr Joe Unsworth.

Quantitation of T cells

My colleague, Dr Barbara Baker coded the slides containing the skin sections. I then counted positively staining cells in the upper dermis in a *blind* manner in 4-6 sequential high power fields (x400) in two sections. Statistical analysis was not performed due to the small number of subjects and controls.

3.1.3 Results

Anti-gliadin antibodies

The three controls had anti-gliadin antibodies (AGA) within the normal range (optical density values: IgA < 0.3, IgG < 0.75). The three DH patients who were taking a normal diet had elevated levels of IgA AGA whereas the three DH patients taking a gluten free diet had IgA AGA within the normal range (Table 3.1).

Clinical assessment

Intradermal injection of FFIII produced a wheal and flare reaction within a few minutes of being injected in all 6 patients and in the 3 controls - this persisted for up to 6 hours. Injection of PBS as control did not cause these early lesions in either the patients or controls. No skin changes were reported to have occurred in either group between 8 and

48 hours. Patients and controls reacted to one or more of the recall antigens present in the battery and there was no difference between the groups (Table 3.2).

Histological assessment/lymphocyte counts

No increase in the number of T lymphocytes was observed in the biopsies taken from FFIII injected sites at 6 hours or 48 hours compared with the PBS injected lesions. The wheal and flare reaction had subsided by the time of biopsy in the two patients who had biopsies taken at 6 hours. The T lymphocyte counts are shown in Table 3.3.

Detection of terminal complement component (TCC)

TCC was absent in the biopsies taken at PBS and FFIII injected sites in two of the controls. However, TCC was found to be present in biopsies taken at PBS and FFIII (6 hours and 48 hours) in each of two of the DH patients tested.

3.1.4 Discussion

GSE is thought to be due to T cell mediated mechanisms [Nield, 1981; MacDonald, 1988]. It has been postulated that there is a breakdown of oral tolerance mechanisms in CD and DH [Mowat, 1987]. Therefore, it might be expected that intradermal injection of FFIII might lead to an influx of T cells sensitised to FFIII to the skin with subsequent development of a delayed hypersensitivity response. This study failed to demonstrate this. Cell mediated immunity appeared to be intact in the subjects studied since they did mount responses clinically to common recall antigens.

It is possible that failure to elicit a response following intradermal injection of FFIII was due to too low a concentration of antigen being used. This is unlikely since our laboratory

has previously shown that both peripheral blood and gut T lymphocytes from DH patients respond to the antigen at 1mg/ml *in vivo* [Baker *et al*, 1995]. Another possibility is that the antigen given was not in a form suitable to allow processing and presentation to T cells by antigen presenting cells in the skin. A third explanation is that the antigen may have cleared from the site of injection before an immune response could develop; again this is improbable as FFIII is a large complex molecule which would not be expected to clear quickly from the skin.

The wheal and flare response obtained in both patients and controls is intriguing and has been noted before [Logan & Ferguson, 1978; Marks & Young, 1978]. It has previously been hypothesized that this represents an Arthus response (type III hypersensitivity) [Anand *et al*, 1977]. However, this seems unlikely as in the group of patients studied here, even those with undetectable anti-gliadin antibody demonstrated this phenomenon. Moreover, the response occurred in normal individuals with trivial levels of antibody. An alternative hypothesis is that injection of FFIII intradermally leads to activation of complement with a resultant wheal and flare reaction. It has recently been demonstrated that FFIII is indeed a strong activator of complement via the alternative pathway [Unsworth *et al*, 1993] and this property of FFIII may be involved in the pathogenesis of GSE. The absence of TCC in the biopsies taken from the controls injected with FFIII and the failure of this study to demonstrate increased amounts in DH skin after FFIII injection detract from the hypothesis.

T lymphocytes are found in significantly increased numbers in lesional DH skin compared with non-lesional DH skin [Nestor *et al*, 1987; Garioch *et al*, 1994] and I have found that many are activated [Garioch *et al*, 1994]. The contribution of these T cells in the

pathogenesis of DH skin lesions is unknown. It seems unlikely that these T cells are specific for FFIII as intradermal injection of FFIII failed to result in an increase in the number of T cells in the skin or the production of delayed hypersensitivity. Furthermore, previous experiments in our laboratory have failed to demonstrate proliferation of T cells from DH lesional skin to FFIII [Baker *et al*, 1995]. If these T cells are involved in the pathogenesis of DH skin lesions, it is more probable that they are specific for an unidentified antigen present in the skin.

Thus, if GSE is T cell mediated, then failure to provoke DTH by intradermal skin testing of patients known to be gluten sensitive suggests that the T cells concerned are mucosally associated and unable to migrate to the skin.

TABLE 3.1: Optical density measurements of anti-gliadin antibodies in dermatitis herpetiformis patients

Patient	IgG	IgA	Gut biopsy	Diet
P1	<0.75*	0.36 (↑)	SVA	normal
P2	<0.75*	0.35 (↑)	PVA	normal
P3	1.7 (↑)	1.1 (↑)	SVA	normal
P4	1.15 (↑)	<0.1*	not done	GFD
P5	<0.75*	<0.1*	normal	GFD
P6	<0.75*	<0.1*	normal	GFD

*undetectable (i.e. sub-normal) levels of IgG and IgA antibody.
Normal range: IgG < 0.75; IgA < 0.1.

P	patient
SVA	severe villous atrophy
PVA	partial villous atrophy
GFD	gluten free diet

TABLE 3.2: Clinical response to a battery of recall antigens in controls and dermatitis herpetiformis (DH) patients at 48 hours (+ = positive response; - = negative response)

	Controls			DH patients					
ANTIGEN	**C1**	**C2**	**C3**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Tetanus toxoid	+	+	+	+	+	+	-	+	+
Diptheria toxoid	-	-	+	+	+	-	+	-	+
Strep Grp C	-	-	-	-	+	-	+	+	-
Tuberculin	+	+	-	+	+	-	+	+	+
Candida albicans	+	+	-	-	-	-	-	+	+
T. mentagrophytes	-	-	-	+	-	-	+	-	+
Proteus mirabilis	-	+	+	-	-	-	+	+	+
Glycerin (control)	-	-	-	-	-	-	-	-	-

C control
P patient

TABLE 3.3: Mean number of lymphocytes per high power field (x400) ± standard error of the mean

	CD3	CD4	CD8
DH patients (*n* = 6)			
PBS biopsies (48 h)	17 ± 4	7 ± 1	14 ± 4
FFIII biopsies (48 h)	18 ± 6	13 ± 7	10 ± 5
DH patients (*n* = 2)			
FFIII biopsies (6 h)	12 ± 2	10 ± 7	5 ± 1
Controls (*n* = 3)			
PBS biopsies (48 h)	19 ± 16	11 ± 9	4 ± 2
FFIII biopsies (48 h)	24 ± 7	12 ± 2	7 ± 3

n number of patients
PBS phosphate buffered saline
DH dermatitis herpetiformis
FFIII Frazer's fraction III
CD3 pan T cell
CD4 helper T cell
CD8 suppressor/cytotoxic T cell

Note: Statistical analysis was not performed due to small number of controls and subjects.

CHAPTER 3

LABORATORY STUDIES

2. T LYMPHOCYTES IN LESIONAL SKIN OF PATIENTS WITH DERMATITIS HERPETIFORMIS

3.2.1 Introduction

It has previously been postulated that DH is an immune complex disorder because it is often associated with raised levels of immune complexes composed of IgA [Mowbray *et al*, 1973; Zone *et al*, 1980; Vainio *et al*, 1983; Hall & Lawley, 1985]. However, this hypothesis fails to explain: 1) the absence of skin lesions in uninvolved skin although IgA is present; 2) why disease activity is not related to the level of circulating immune complexes [Mowbray *et al*, 1973]; and 3) why it takes two years for the rash of DH to clear after patients have been commenced on a GFD [Fry *et al*, 1973; Fry *et al*, 1982]. It is possible, therefore, that other immunological mechanisms are involved in the pathogenesis of DH. Perivascular cellular infiltrates composed mainly of T lymphocytes were shown to be characteristic of developing lesions induced by application of potassium iodide patch tests in patients with DH [Reitamo *et al*, 1981]. Recently it has been shown that certain cytokines may be involved in the development of skin lesions of DH [Graeber *et al*, 1993].

The aim of this study was to quantitate the T cells in involved and uninvolved skin taken from patients with DH and to investigate their distribution and state of activation. The distribution of CD1a positive dendritic cells was also studied.

3.2.2 Methods

Patients

Ten patients with DH controlled by sulphones and/or sulphonamides were asked to stop their medication and I took two 4mm punch biopsies 2-4 days later: one from involved skin and the other from uninvolved skin. Seven of the "involved" biopsies were from clinically urticated lesions and 3 were from small vesicles: 3 of the urticated lesions showed subepidermal blisters microscopically. I then snap froze the skin biopsies in OCT (Tissue Tek©) and stored them in liquid nitrogen. Later, I cut 6μm sections using a cryostat (Slee, London) and stored them at -80°C until staining.

Immunoperoxidase staining

I used the avidin-biotin peroxidase technique (as described in section 3.1.2) and monoclonai antibodies to stain for CD3, CD4, CD8, CD25, CD1a and CD45RO. All monoclonal antibodies were obtained from Becton-Dickinson (Oxford, UK) with the exception of OKT6 specific for CD1a (Orthodiagnostics Systems Ltd., High Wycombe, Bucks). I stained control sections in an identical manner except for omission of the primary monoclonal antibody.

Double labelled immunofluorescent staining

I used a 5 layered method to stain simultaneously for CD3 and HLA-DR in involved skin [Baker *et al*, 1984]. Briefly, I air dried the sections and fixed them in acetone:chloroform (50:50). I initially incubated the sections with mouse monoclonal antibody to CD3 (Becton-Dickinson, Oxford, UK) followed by incubation with biotin labelled horse-anti-mouse antibody and then streptavidin-texas red (Amersham, UK). I then incubated the sections with the monoclonal rat-anti-DR antibody (Dako, High Wycombe, Bucks) and finally with

rabbit-anti-rat-FITC (Sigma, Poole, Dorset). I mounted the slides in 10% phosphate buffered saline in glycerol with 2.5% 1,4-diazabicyclo(2.2.2.)octane (DABCO) (Sigma, Poole, Dorset) added to prevent quenching of the FITC.

Quantification

I counted positively stained cells in 4-6 consecutive high power fields (x400) in 2 sections and expressed these per high power field. By comparing control sections with stained sections, I excluded neutrophils, which may have stained non specifically with the avidin-biotin complex.

Statistics

I used the Mann Whitney U test to compare the T cell counts in involved skin with those in uninvolved skin.

3.2.3 Results

T lymphocyte counts

I found that the number of CD3 positive lymphocytes was significantly increased in the upper dermis of involved compared with uninvolved skin ($p < 0.0005$) (Table 3.4). Clumps of T lymphocytes were in a perivascular distribution in the upper dermis but were not observed within the epidermis nor in the dermal papillae whereas neutrophils were largely confined to the tips of the dermal papillae. The majority of these T cells were CD4 positive and they exceeded the number of CD8 positive T cells by a ratio of 4:1. There was also a significant increase in the number of CD45RO positive memory T cells, in involved compared with uninvolved skin ($p < 0.0005$). The total number of CD45RO positive cells was greater than the total number of CD3 positive cells, presumably due to staining of

CD45RO on small numbers of neutrophils, monocytes and macrophages which also had a perivascular distribution. Staining with CD25 was not detected in either involved or uninvolved skin.

Double labelling of CD3 positive cells showed that approximately 20-40% also expressed HLA-DR (Figure 3.1).

CD1 positive cells

I observed that CD1a positive cells appeared to be present in the epidermis of both involved and uninvolved skin in similar numbers (numbers not counted). However, CD1a positive cells were also present in the dermis of involved skin in 9 out of the 10 patients: these cells were admixed with the perivascular collections of T lymphocytes (again, numbers not counted) (Figure 3.2). I found no CD1a positive cells to be present in the dermis of uninvolved skin.

3.2.4 Discussion

This study has shown that there is a significant increase in the number of T lymphocytes in the upper dermis of involved compared with uninvolved skin in DH. These lymphocytes were arranged around the blood vessels in the upper dermis and were composed mainly of CD4 positive memory T cells. Furthermore, a proportion expressed HLA-DR antigens but, surprisingly, were CD25 antigen negative. The differential expression of CD25 and HLA-DR expression may be due to differences in kinetics of expression of these two activation molecules. The CD25 monoclonal antibody used in this study stained T cells of chronic plaque psoriasis using the same method excluding the possibility that it was not functioning properly. In addition, CD1a positive Langerhans' cells were found in close

association with the T lymphocytes in involved skin suggesting interaction between the two cell types. A previous study of mononuclear cell infiltrates in bullous diseases also found a significantly increased number of T cells in the dermis of involved compared with uninvolved DH skin [Nestor *et al*, 1987]. However, this previous study did not examine markers of T cell activation and, in contrast with my study, found that Langerhans'/dendritic cells were absent from the dermis of involved DH skin. This variation in results could be due to the fact that a two layered method of immunostaining was used in contrast with the more sensitive three layered method I used.

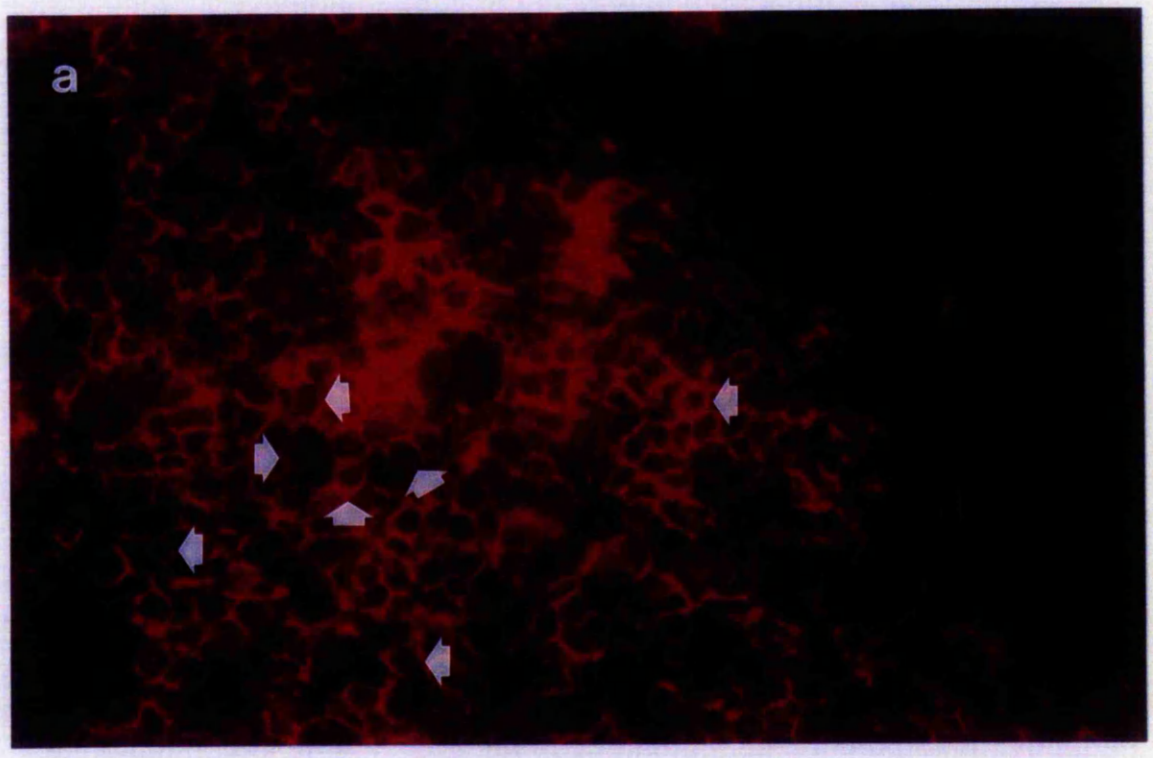
The role of these activated T cells in the pathogenesis of DH skin lesions is open to speculation. One possibility is that they enter the skin early and, via the secretion of cytokines such as interferon-γ, tumour necrosis factor-α or interleukin-2, cause neutrophils and eosinophils to leave the blood vessels, enter the dermis and become activated. A study [Graeber *et al*, 1993] performed in our department demonstrated interleukin-8 (IL-8) on basal epidermal cells and granulocyte macrophage-colony stimulating factor (GM-CSF) on dendritic cells at the dermo-epidermal junction of involved skin in DH. It has been proposed that the neutrophils migrate towards the tips of the dermal papillae because of the presence of the chemo-attractant IL-8 on basal epidermal cells and bind to the IgA once activated by GM-CSF expressed by dendritic cells at the dermo-epidermal junction [Graeber *et al*, 1993]. However, it is difficult to determine whether T lymphocytes enter the dermis prior to neutrophils in DH. Unfortunately, there are no suitable models available which would allow time course studies to be performed. Although the application of potassium iodide patch tests in patients with DH produces lesions which are clinically and histologically indistinguishable from DH [Reitamo *et al*, 1981], the mechanisms involved must differ from those involved in spontaneous evolution of lesions as IL-8 has not been

detected in lesions induced by application of potassium iodide patch tests (unpublished observations).

The close association of T cells and Langerhans' cells and the expression of HLA-DR antigens by a proportion of the T cells suggest that a cell mediated immune response is taking place in the DH skin lesion. However, the antigen specificity of the T cells in the involved skin of DH is unknown. Preliminary experiments in our laboratory have failed, so far, to demonstrate proliferation of these T cells to Frazer's fraction III (the partial peptic tryptic digest of gluten which is antigenic) (unpublished observations). It is possible that these T cells are specific for a component of the skin.

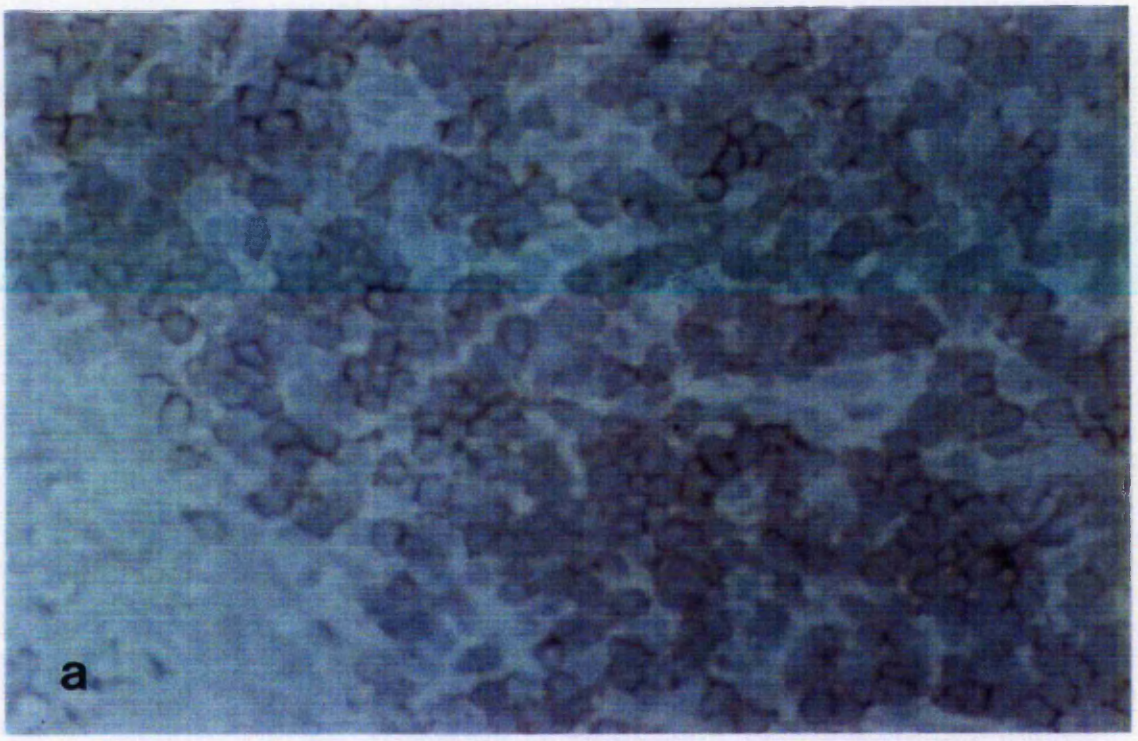
In conclusion, T lymphocytes may play a role in the pathogenesis of the skin lesions in DH via production of cytokines. Further work is required to elucidate the antigen specificity of these cells.

FIGURE 3.1: Double-labelled immunofluorescent staining of the upper dermis in lesional dermatitis herpetiformis skin for (a) CD3, and (b) HLA-DR membrane staining. The arrows indicate some examples of double=labelled T lymphocytes (x400).


a


b

FIGURE 3.2: Immunoperoxidase staining of the upper dermis in iesional dermatitis herpetiformis skin for (a) CD3, and (b) CD1a membrane expression (x400).


a

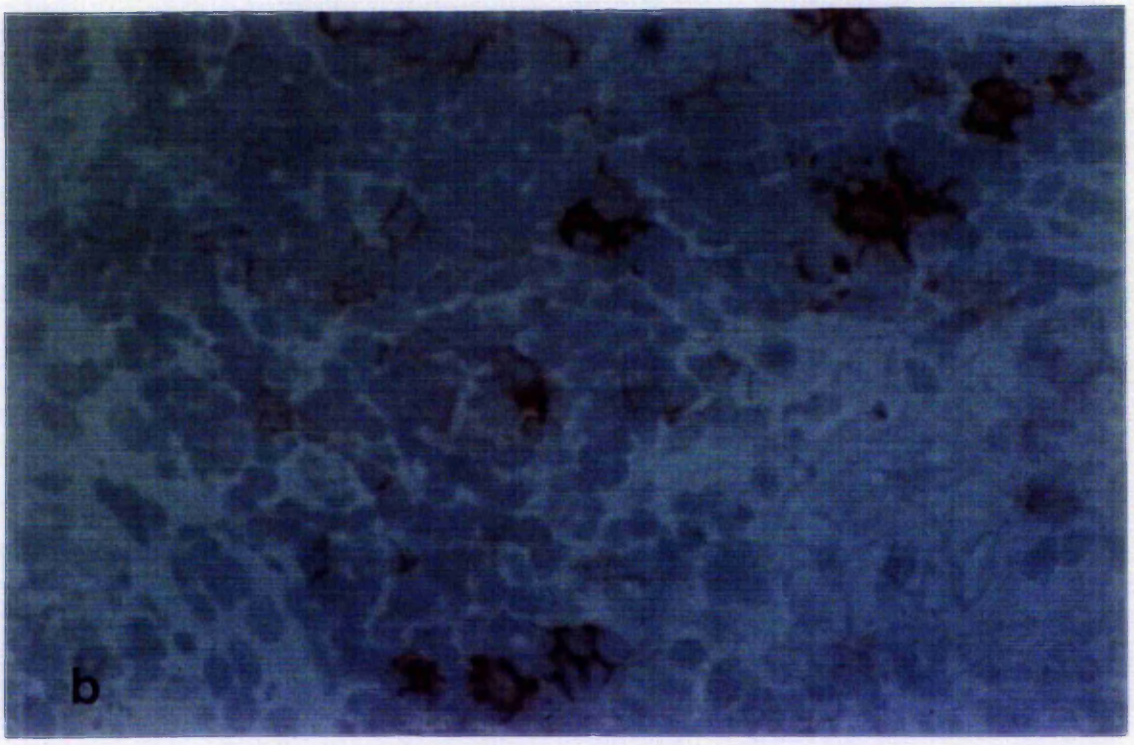

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TABLE 3.4: Mean lymphocyte counts in the upper dermis per high power field (x400)

	Uninvolved (n = 10)	Involved (n = 10)
CD3	6 (SEM ± 1)	41 (SEM ± 12)*
CD4	5 (SEM ± 1)	39 (SEM ± 13)**
CD8	4 (SEM ± 1)	10 (SEM ± 2)***
CD45RO	7 (SEM ± 1)	53 (SEM ± 15)*
CD4:CD8	1:1	4:1

*P < 0.0005 compared with uninvolved
**P < 0.002 compared with uninvolved
***P < 0.05 compared with uninvolved
SEM standard error of the mean

Note: Numbers of CD1a positive cells not evaluated.

CHAPTER 3

LABORATORY STUDIES

3. RESTRICTION OF T CELL RECEPTOR Vβ EXPRESSION IN DERMATITIS HERPETIFORMIS SKIN

3.3.1 Introduction

It has previously been hypothesised that the skin lesions of DH are due to T cell mediated responses directed against an as yet unidentified autoantigen [Baker *et al*, 1995]. Restricted T-cell receptor Vβ gene expression would support the hypothesis that recognition of a specific antigen(s) or superantigen is important in the pathogenesis of DH skin lesions. Restricted expression of T-cell receptor Vβ genes has been demonstrated in other disorders such as rheumatoid arthritis [Miltenberg *et al*, 1990; Sottini *et al*, 1991] and sarcoidosis [Moller *et al*, 1988]. Previous work from our department demonstrated restriction of the T-cell receptor Vβ gene expression in patients with psoriasis [Lewis *et al*, 1993]. The aim of this study, therefore, was to examine the expression of T-cell receptor Vβ genes in the skin of patients with DH using monoclonal antibodies and an immunoperoxidase technique.

3.3.2 Methods

Patients and samples

Approval for this study was granted by St Mary's Hospital Local Ethical Committee. Patient consent was also obtained prior to skin biopsies and blood samples being taken.

Ten patients (mean age 54 years, range 27-71) with DH controlled by dapsone and/or

sulphonamides were enrolled in the study. Of these patients, 8 were taking a normal diet and 2 a strict gluten free diet (GFD) but had not yet managed to control their rash with diet alone. I asked patients to discontinue their medication and I took two 4-mm punch biopsies 7 days later: one from a blister and the other from uninvolved skin, a few centimetres from the involved site. I then snap froze the skin biopsies in OCT (Tissue Tek®) and stored them in liquid nitrogen. Later, I cut 6 μm sections using a cryostat (Slee, London), and stored the sections at -80°C until staining.

Peripheral blood lymphocytes (PBL) were isolated from 20 ml of heparinised blood by Ficoll-Hypaque density gradient centrifugation by my colleague, Dr Barbara Baker.

Antibodies

I used monoclonal antibodies which had been shown to recognise specifically a variable region epitope on the β chain of the following Vβ families: Vβ2, Vβ8 (both kindly donated by Professor Boylston, University of Leeds), Vβ3 (a gift kindly donated by Dr. M. Owen, ICRF [Viney *et al*, 1992]), Vβ5.1, Vβ5.2/5.3, Vβ5.3, Vβ6.7, Vβ12.1, Vβ13.1/13.3 (T-Cell Sciences Inc., Cambridge, MA, U.S.A.), Vβ2, Vβ9 and Vβ17 (Immunotech, Marseille, France). Other antibodies used were pan αβ (T cell Sciences Inc., Cambridge, MA, U.S.A.) and CD3 (Becton Dickinson, Oxford, U.K.). All primary antibodies were used neat with the exception of Vβ9 and Vβ17 which were used at dilutions of 1:10 and 1:5 respectively.

Immunoperoxidase staining

I used the avidin-biotin peroxidase technique, as described in section 3.1.2, to stain the skin sections. Control sections included substitution of the primary antibody with a mouse

IgG isotype control antibody (Vector laboratories, Peterborough, UK) and omission of the primary antibody.

Quantification

The slides were coded by my colleague Dr Barbara Baker following which I counted positively stained cells in four to six consecutive high power fields (x400) in two sections in a blind manner.

Flow cytometric analysis

Flow cytometric analysis was performed by Dr Barbara Baker on PBL obtained from the DH patients at the time of biopsy. PBL were separately incubated with each anti-TCR Vβ antibody (all used neat with the exception of pan αβ, Vβ9 and Vβ13.1/13.3 antibodies which were used at a dilution of 1:2) and followed by FITC-labelled sheep anti-mouse IgG antibody (Sigma) diluted 1:10 and double stained for CD3 expression with phycoerythrin-conjugated Leu 4 antibody (Becton-Dickinson), and then analysed in an EPICS flow cytometer (Coulter Electronics Ltd.). FITC-labelled goat anti-mouse IgM (Sigma) diluted 1:100 was used with the Immunotech Vβ2 antibody.

Statistics

The results are expressed as mean ± SEM: expression of Vβ families in lesional skin and blood was expressed as a mean percentage of CD3+ T-lymphocytes. I used the Mann Whitney *U*-test to compare the T-cell counts in involved skin with those in uninvolved skin. In addition, I compared Vβ TCR expression in the skin with peripheral blood using Wilcoxon's paired rank sum test.

3.3.3 Results

Expression of V_β families in involved skin compared with PBLs

The number of CD3 positive lymphocytes was significantly increased in the upper dermis of involved skin (89 ± 15 per high power field) compared with uninvolved skin (17 ± 3 per high power field) ($P < 0.005$). No CD3 positive lymphocytes were observed in the epidermis.

T lymphocytes expressing each Vβ family in the dermis of involved skin and PBLs are represented as mean values ± standard error of the mean (SEM) for the group as a whole in Figure 3.3; Wilcoxon's paired rank sum test was used to analyse the data. Similar to other comparable studies [eg Menssen *et al*, 1995], for each individual patient, over-representation of specific Vβ receptors was considered significant if the number of T cells expressing the Vβ receptor in the skin was at least double the number present in the PBLs. Thus, I found that 6 out of 10 patients had significant over-representation of Vβ2 (20.1 (mean) ± 4.5 (SEM) in skin; 5.6 (mean) ± 0.8 (SEM) in blood, $P < 0.02$); 8 out of 10 patients had over-representation of Vβ5.2/5.3 (4.9 ± 0.5 in skin; 2 ± 0.2 in blood, $P < 0.01$); and 7 out of 10 patients had over-representation of Vβ5.3 (2.6 ± 0.6 in skin; 0.8 ± 0.1 in blood, $P < 0.05$). Much larger numbers of cells expressing TCR Vβ2 were seen in comparison with TCR Vβ5.2/5.3 and Vβ5.3 (Figures 3.3 and 3.4).

Furthermore, 5 out of 10 patients demonstrated cytoplasmic staining with anti-Vβ5.3 antibody in involved but not uninvolved skin. These cells were scattered throughout the dermis and some were present in the vesicles. Two of these patients demonstrated similar staining with anti-Vβ6.7 antibody. I did not count these cells when evaluating the sections because they did not exhibit the expected membrane staining as observed in other

positively staining cells.

In an attempt to confirm the increased Vβ2 expression in DH skin shown by A. Boylston's antibody, I emlpoyed a second Vβ2 antibody (Immunotech, Marseille, France). Similar results were obtained with the two antibodies for the PBLs although the Immunotech antibody stained approximately 1% less cells. However, I found that the Immunotech antibody gave high background staining on tissue sections which precluded enumeration of positively staining cells.

I was unable to interpret staining with the Vβ antibodies in uninvolved skin as only small numbers of T cells were found.

3.3.4 Discussion

This study has shown over-representation of specific TCR Vβ subsets, namely Vβ2, Vβ5.2/5.3, Vβ5.3 in DH lesional skin compared with blood. Large numbers of T-cells expressing Vβ2 were observed in DH lesional skin (Figs. 3.3 and 3.4) compared with Vβ5.2/5.3 and 5.3. Indeed, in one patient, 50% of the infiltrating T-cells expressed Vβ2. My colleague, Dr Helen Lewis, has previously also shown preferential usage of TCR Vβ2 in psoriasis by immunohistochemistry [Lewis *et al*, 1993], a finding which has been confirmed by Leung et al [1995]. It could be hypothesised that T cells bearing TCR Vβ2 "home in" to the skin in inflammatory dermatoses. However, Leung et al [1995], using the same Vβ2 antibody and a similar immunohistochemical technique as I did, demonstrated that expression of TCR Vβ2 was not significantly increased in atopic eczema nor irritant dermatitis. Nor was TCR Vβ2 expression found to be increased in lichen planus by PCR

[Dunn *et al*, 1993]. With regard to the TCR repertoire in normal skin, conflicting results have been obtained: one study demonstrated overexpression of TCR Vβ2 in normal skin by PCR although this was found to be polyclonal by sequence analysis [Menssen *et al*, 1995]; another study demonstrated increased expression of TCR Vβ1, Vβ7, Vβ14 and Vβ16 by PCR [Dunn *et al*, 1993]; and more recently, TCR Vβ3 and Vβ14 were shown to be significantly overexpressed in normal breast skin compared to PBLs [Ahangari *et al*, 1996].

DH is strongly associated with HLA A1, B8, DR3, DQ2 with over 95% of DH patients expressing the DQ2 antigen [Yunis Juan & Ahmed, 1991]. There is now good evidence from twin and family studies that the human TCR repertoire is primarily genetically determined [Loveridge *et al*, 1991]. Moreover, family studies support HLA class I and class II antigens as having a profound effect on the selection of T cells expressing particular Vβ segments [Gulwani-Akolkar *et al*, 1991]. It has been proposed that HLA associations with disease may be explained by the effects of HLA antigens on determination of the TCR V segment frequencies, thereby biasing the individual toward the development of autoimmunity [Gulwani-Akolkar *et al*, 1991]. It is therefore perhaps not surprising that I found over representation of certain Vβ families in DH lesional skin.

As over 90% of patients with DH have evidence of a GSE [Fry *et al*, 1972; Fry *et al*, 1974]. It is highly probable that the presence of GSE is necessary for the development skin lesions in these patients. It has previously been proposed that damage to the small bowel by gluten, exposes an antigenic determinant in the small intestine which is similar to a corresponding epitope in the skin [Baker *et al*, 1995]. T-cells recognising this antigen would therefore migrate from the small intestine to the skin. One candidate antigen could

be reticulin; patients with DH have anti-reticulin antibodies [Seah *et al*, 1971] and furthermore the titre of antibody correlates with the degree of intestinal damage [Hällström *et al*, 1985] (see discussion and hypothesis).

An intriguing finding from this study was the presence of cytoplasmic staining of Vβ5.3 in 5 out of 10 patients and of Vβ6.7 in 2 out of 10 patients. It is known that specific recognition of antigen by T-cells is followed by internalisation of the T-cell receptor [Krangel, 1987]. It is possible, therefore, that these cells represented recently activated T-cells in which surface re-expression of the TCR had not yet occurred. Unfortunately, I have been unable to prove this.

To date, there have been no published studies on TCR Vβ expression in the small intestine of patients with DH. Troncone et al [1995] have demonstrated over-representation of TCR Vβ8 by immunohistochemistry in the lamina propria of untreated coeliac patients compared with controls. These findings, they concluded, supported the role of either an antigen or superantigen in the pathogenesis of coeliac disease. It remains to be shown whether there is over-expression of specific TCR Vβ(s) in the small intestine of DH patients.

In summary, this study has shown bias of usage of TCR Vβ receptors which implicates the involvement of an antigen or superantigen in the pathogenesis of DH skin lesions.

FIGURE 3.3: Expression of Vβ families in the lesional skin and blood of 10 patients with dermatitis herpetiformis, expressed as a mean percentage of CD3+ T-lymphocytes. * indicates Vβ families significantly elevated in the skin compared with peripheral blood lymphocytes using Wilcoxon's paired rank sum test ($P < 0.05$).

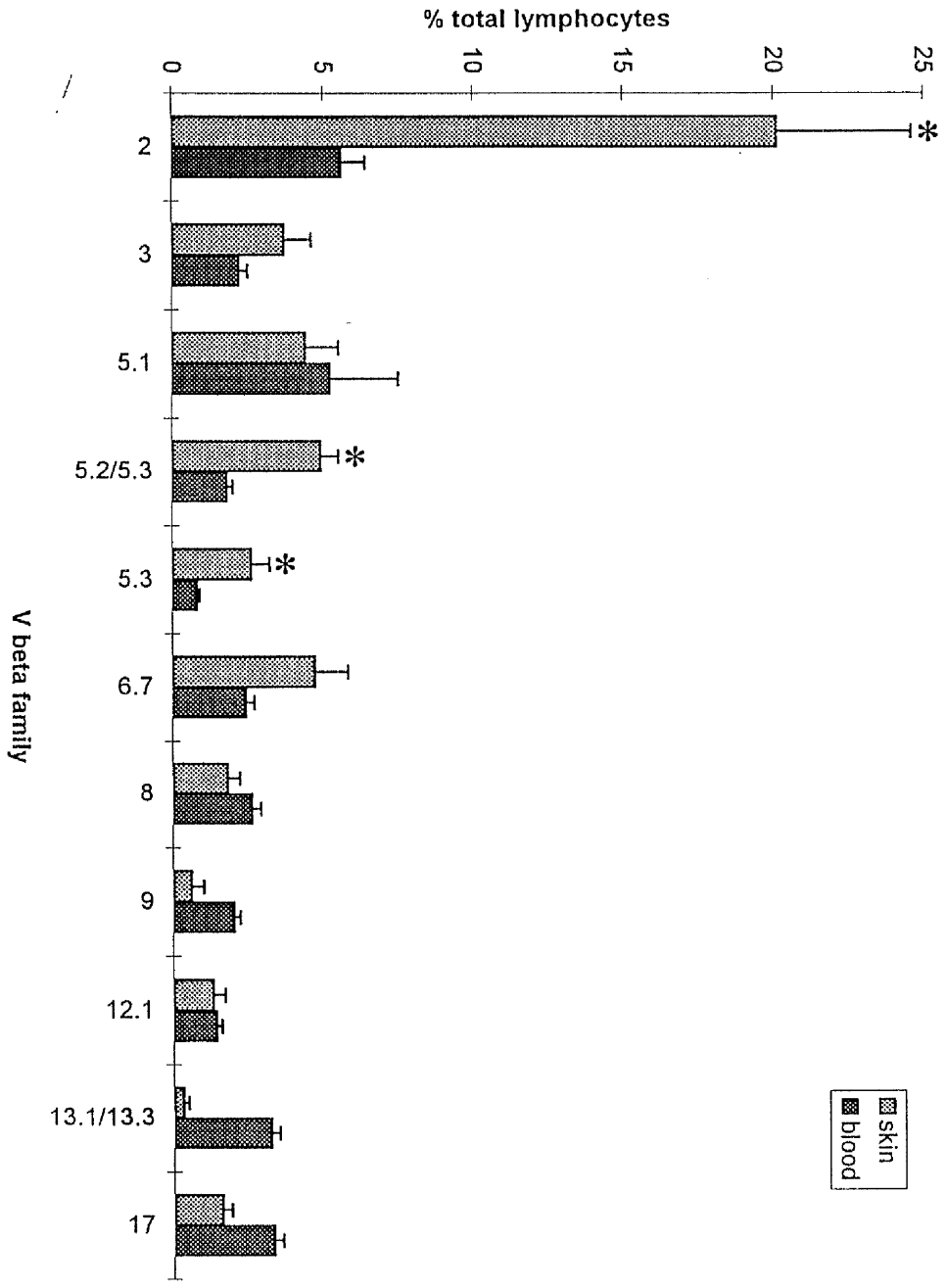
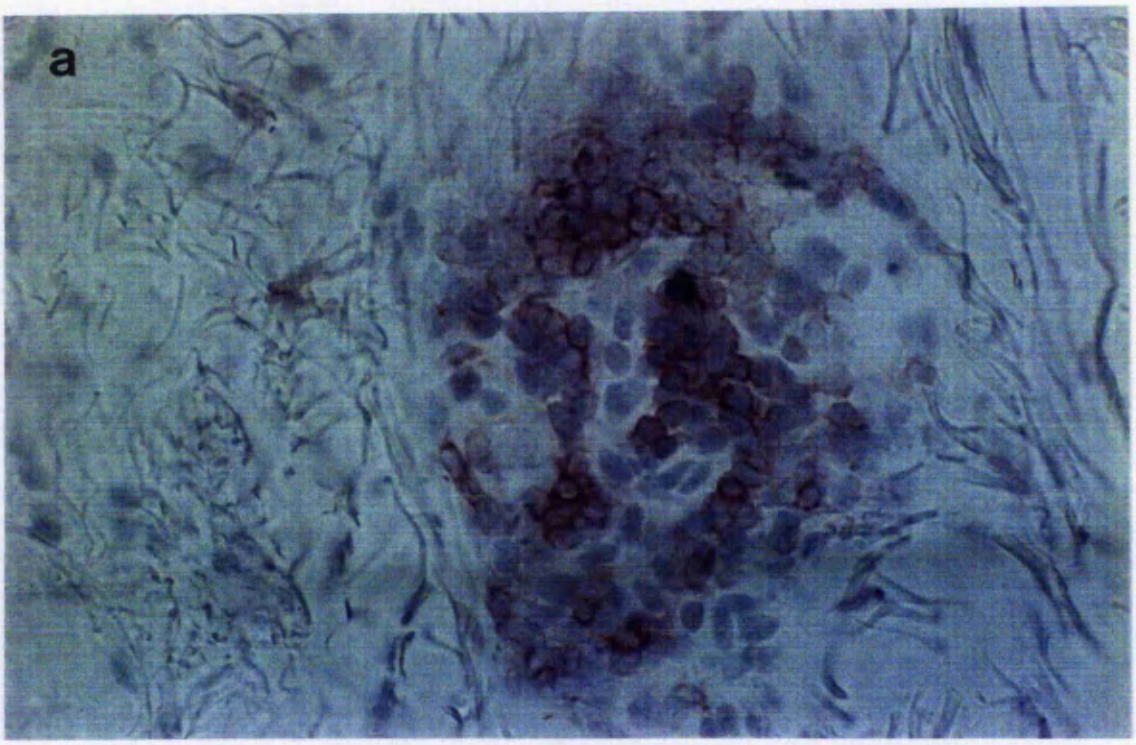
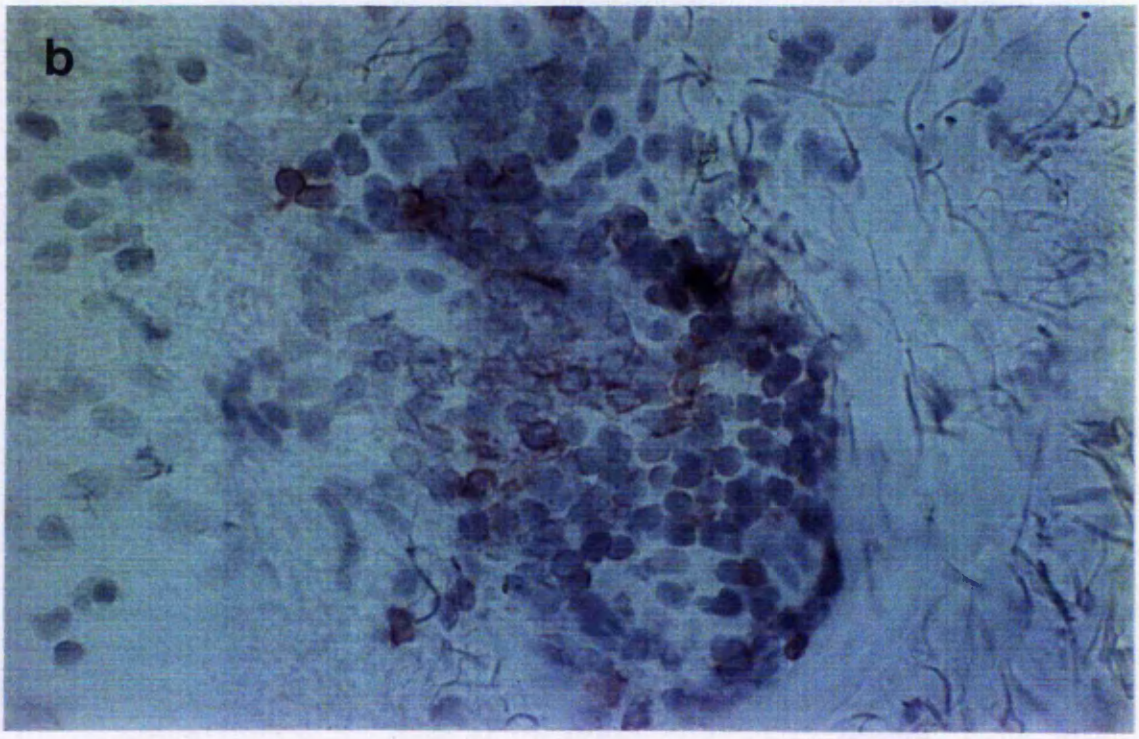

% total lymphocytes
0
5
10
15
20
25
2
3
5.1
5.2/5.3
5.3
6.7
8
9
12.1
13.1/13.3
17
V beta family
skin
blood

FIGURE 3.4: Immunoperoxidase staining of the upper dermis in lesional dermatitis herpetiformis skin from a representative patient for (a) CD3 and (b) TCR Vβ2 expression (x400).


a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CHAPTER 3

LABORATORY STUDIES

4. CYTOKINES IN THE SMALL INTESTINE OF DH PATIENTS USING IMMUNOHISTOCHEMISTRY AND THE POLYMERASE CHAIN REACTION

3.4.1 Introduction

The mechanisms whereby gluten causes damage to the small intestine in CD and DH is unknown. T cell mediated mechanisms have been implicated in the pathogenesis because of the similarity between the histological features of GSE and graft versus host disease (GvHD) [Neild, 1981] and the characteristic finding of elevated numbers of intra-epithelial lymphocytes (IELs) in the small intestine of patients with CD and DH which decrease during a gluten free diet (GFD). Furthermore, normal foetal small bowel, exposed *in vitro* to T cell activators, develops villous atrophy and crypt hyperplasia [MacDonald & Spencer, 1988]. These changes in small intestinal morphology in GSE may be induced by gluten specific T cells via the production of cytokines: antibodies to interferon-γ (IFN-γ) abolished the development of small intestinal GvHD [Mowat, 1989]. Previous studies have examined the T cell cytokine IFN-γ in CD with conflicting results [Al-Dawoud *et al*, 1992; Kontakou *et al*, 1994]: no similar studies have been published on DH small intestine to date.

The aim of this study was, therefore, to examine the expression of the cytokine IFN-γ using RT-PCR to detect cytokine specific mRNA and immunostaining to enumerate cytokine positive cells in DH gut versus normal gut mucosa. In addition, mRNA and protein for the T cell growth factors, interleukin-2 (IL-2) and interleukin-4 (IL-4) were studied.

3.4.2 Patients and methods

Patients and biopsy specimens

Ethical approval was given by St Mary's Hospital Local Ethics Committee; patient consent was obtained prior to biopsies being taken. Peroral biopsies were taken from the second part of the duodenum at endoscopy from patients with DH taking a normal diet (n=6, mean age=57 years, range 51-68) and from controls (n=6, mean age=46 years, range=26-71) who were being investigated for abdominal pain or dyspepsia. Small intestinal biopsies from the controls were all shown to be normal. Biopsies from the DH patients all demonstrated villous atrophy (3 patients had severe villous atrophy and 3 partial villous atrophy), crypt cell hyperplasia, increased intra-epithelial lymphocyte counts and reduced villous height/crypt depth ratios compared with the controls. I was present whilst patients and controls underwent endoscopy so as to collect the specimens which I snap froze in liquid nitrogen. I used a dissecting microscope to orientate specimens, which I planned to use for immunostaining, on liver with the villi uppermost before snap freezing; and, immediately prior to cutting, I embedded them in OCT (Tissue Tek©); I then cut 5μm sections using a cryostat (Slee, London).

RNA extraction and cDNA synthesis

Total RNA was extracted from homogenised whole gut using TRIzol reagent, a mixture of phenol and guanidine isothiocyanate, according to manufacturers instructions (Gibco BRL, Paisley, Scotland). cDNA was then synthesized by incubation of 4 μg RNA with 200U MMLV reverse transcriptase (Gibco BRL), 20 ng oligo$(dT)_{12\text{-}18}$ primer (Pharmacia Botech, St Albans, Herts) and 10 mM of each 4 dNTP (Pharmacia) in the presence of 0.1M DTT (Gibco BRL) in a 20 μl reaction volume for 1 hr at 42°C. The reaction was stopped by incubating at 95°C for 3 mins, followed by cooling on ice.

PCR

0.5 μl cDNA was amplified in each PCR reaction in a 25μl volume. The final concentration of the reaction mixture was 0.625U Taq polymerase (Gibco BRL), 0.2mM each dNTP and 75 ng of each 5' and 3' cytokine-specific or β-actin primer (Oswel DNA service, Edinburgh) in PCR buffer containing 1mM $MgCl_2$ and 0.05% W1 detergent. The reaction mixture was overlaid with mineral oil and amplified by 30 (IL-4) or 35 (IL-2, IFN-γ, β-actin) cycles in a DNA thermocycler (Perkin-Elmer, Applied Biosystems, Warrington, Cheshire). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min (IL-2, IFN-γ, β-actin) or 65°C for 2 mins (IL-4) and extension at 72°C for 1 min. The temperature was maintained at 72°C for 5 mins after the final cycle to allow completion of synthesis of amplified products.

The PCR products were separated from the primers by electrophoresis on a 1.5% agarose gel containing 10 μg/ml ethidium bromide, and visualised under UV light. They were then transferred onto Hybond-N nylon membrane (Amersham, Bucks, UK), hybridised at 55°C overnight with ^{32}P-labelled cytokine-specific probes and visualised by autoradiography. Densitometer readings of the cytokine-specific bands were normalised to those of β-actin before statistical analysis. One of the control cDNA samples did not give a signal for β-actin and was not, therefore, studied further.

The sequence of the primer pairs and probes specific for IL-2, IFN-γ and β-actin used in the study were taken from Yamamura et al [1992].

The RNA extraction, cDNA synthesis and PCR were performed by Mr Senur Bokth (Laboratory Assistant) under the supervision of Dr Barbara Baker.

Staining technique

I used the avidin-biotin peroxidase technique, as described in section 3.1.2, and monoclonal antibodies against CD3 (Becton Dickinson, Oxford, UK), IFN-γ (Sera-Lab, Sussex, UK), IL-2 and IL-4 (Genzyme, Kent, UK). I initially incubated the sections with the cytokine antibodies diluted 1:10 at 4°C overnight followed by incubation at room temperature with biotinylated horse-anti-mouse secondary antibodies.

Negative controls consisted of omission of the primary antibody or its substitution with mouse IgG (Vector Laboratories, Peterborough, UK) used at a dilution of 1:10. For positive controls, I stained tuberculin-purified protein derivative-induced delayed hypersensitivity type reactions biopsied at 48 hours: these showed the presence of dermal cells which stained positively with antibodies to IFN-γ, IL-2 and IL-4.

Enumeration of positively stained cells

I counted the number of intra-epithelial lymphocytes expressing CD3 per 1000 epithelial cells. For quantification of positively stained cells in the lamina propria I used an eye piece graticule under x400 magnification. The eye piece graticule was divided into 100 x $1mm^2$ squares: I counted at least 500 hundred of these squares in consecutive areas in 2-4 sections (within and below the villi going into the deeper lamina propria to the level of the crypts) and I expressed the results as the mean number of positive cells per unit area ie $500mm^2$.

All the counting was performed by myself but in order to confirm reproducibility, a sample of sections chosen at random were counted by my colleague Dr Barbara Baker.

Statistical analysis

I used the Mann-Whitney U test to compare the immunostaining and PCR densitometer values obtained from the DH and normal groups.

3.4.3 Results

Control mucosa

Expression of IFN-γ-specific mRNA was readily detected in all the normal gut biopsies, but levels were variable (Figure 3.5). In addition, a mean of 69 (12% of CD3+) cells in the lamina propria were shown to be IFN-γ-positive by immunostaining (Table 3.5). In contrast, IL-2 mRNA was barely detectable in 3 of the normal biopsies but strongly expressed in the other two although only one of the replicates gave a positive result in each case (Figure 3.5). This was associated with a lower frequency of IL-2 compared to IFN-γ-positive cells in the lamina propria by immunostaining (Table 3.5). However, unlike IL-2 and IFN-γ, the PCR and immunostaining findings for IL-4 did not correlate. Thus a mean of 117 lamina propria cells stained for IL-4 but IL-4 mRNA was undetectable in all but one of the normal gut biopsies (Table 3.5, Figure 3.5). Many of the cells expressing IL-4 protein were probably mast cells with the cytokine stored in their cytoplasmic granules. The lack of mRNA for IL-4 would suggest that these cells were not actively synthesising IL-4 at the time of the small intestinal biopsy.

Dermatitis herpetiformis mucosa

Strong expression of IFN-γ-, and to a lesser extent, IL-2-specific mRNA was observed in all of the DH guts with some variability of the latter (Figure 3.5). These levels were not, however, significantly raised compared to the normal gut biopsies. Immunostaining demonstrated comparable numbers of cells expressing IFN-γ and IL-2 in individual

patients (Table 3.5, Figure 3.6). Although the absolute number and proportion of IL-2-positive cells were increased in DH versus control mucosa, these differences did not reach statistical significance (p=0.07). Furthermore, the number of IFN-γ- and IL-4-positive cells were similar in the two groups. IL-4 mRNA was, however, only weakly expressed in 2 out of the 6 DH gut biopsies (Figure 3.5).

Cells expressing IFN-γ and IL-2 were morphologically similar to T cells. Although some cells expressing IL-4 were also T cell-like, many which contained granules in the cytoplasm were probably mast cells (Figure 3.6). IL-4 positive cells were not therefore expressed as a frequency of CD3 positive T cells.

In the epithelium, cells staining positive for IFN-γ and IL-4 were either infrequently present or absent in DH and normal guts. Staining of epithelial cells by the anti-IL-2 (DH and normal) and anti-IFN-γ (DH) antibodies made evaluation of positively stained intra-epithelial cells inaccurate.

3.4.4 Discussion

This study has shown no significant differences in mRNA or protein for IFN-γ, IL-2 or IL-4, in the small intestine of patients with DH compared with control biopsies. However, approximately twice as many cells expressing IL-2 protein were present in the DH guts compared with controls but this did not quite reach statistical significance (p=0.07).

No increase in the absolute number of T cells expressing protein for IFN-γ was detected in the lamina propria of DH patients when compared with controls which is in agreement with a previous immunohistochemical study of IFN-γ in CD [Al-Dawoud *et al*, 1992].

However, this same study [Al-Dawoud *et al*, 1992] found a decrease in the proportion of cells expressing IFN-γ in CD when they expressed these cells as a percentage of the total number of cells expressing CD45 which would have included T cells, B cells, monocytes, macrophages and granulocytes. In contrast, another study of CD patients found a significant increase in the number of cells expressing mRNA for IFN-γ in the lamina propria when compared with that of controls [Kontakou *et al*, 1994]. However, staining for IFN-γ by immunohistochemistry was not performed and it is possible that the IFN-γ mRNA was not translated into protein.

IFN-γ has been implicated in the pathogenesis of the gut changes seen in CD because in a mouse model of GvHD, in which the morphological changes are similar to GSE [Neild, 1981], antibodies to IFN-γ abolished the development of intestinal GvHD [Mowat, 1989]. IFN-γ may have a direct effect on intestinal tissues: it has been shown to have the ability to modify the barrier function of the intestinal epithelial cells resulting in altered permeability [Adams *et al*, 1993; Madara & Stafford, 1989] and can influence ion and electrolyte secretion [Holmgren *et al*, 1989]. Alternatively, the changes may be due to the ability of IFN-γ to activate other immune effector cells which in turn may have direct effects on the tissues or act via production of further inflammatory mediators. It is possible that the small intestinal changes observed in DH and CD may be due to altered receptors for IFN-γ or due to an abnormal end organ response to this cytokine, but it does not appear to be due to an increase in the quantity of this cytokine.

Other cytokines have been implicated in the aetiology of the morphological changes of GSE. A recent study in CD reported an increase in the total number of macrophages in the lamina propria expressing the cytokines IL-6 and tumour necrosis factor α [Przemioslo *et*

al, 1994] and the authors concluded that these cytokines may be involved in the pathogenesis of GSE. However, it is more likely that these cytokines are part of the inflammatory process and not the causal agents. Another study found increased numbers of cells expressing IL-4 by immunohistochemistry in DH small intestinal biopsies and it was proposed that TH2 T helper cells were involved in the aetiology of the small intestinal changes [Hall *et al*, 1994]. My study does not support this hypothesis. Indeed, I found a poor correlation between expression of mRNA and protein for IL-4.

Strong epithelial staining with antibody to IL-2 was observed in both control and DH patients and to a lesser extent with IFN-γ antibody in the DH patients. This made assessment of positively staining intra-epithelial lymphocytes difficult. Epithelial cells have been shown to possess receptors to IL-2 and IFN-γ which are functional [Madara & Stafford, 1989; Ciacci *et al*, 1993]. The staining of epithelial cells with IL-2 and IFN-γ antibodies could have been due to binding of these cytokines, secreted by intraepithelial or subepithelial lymphocytes, to receptors.

In conclusion, if cytokines are responsible for producing the morphological changes observed in the small intestine of DH patients, it is likely that a complex interaction of several cytokines is involved. Further work is required to elucidate the involvement of cytokines and thus advance our knowledge of the pathogenic mechanisms involved in this disorder.

FIGURE 3.5: Cytokine-specific mRNA in DH and normal gut mucosa. After electrophoresis, PCR products for IFN-γ, IL-2, IL-4 and β actin were probed with a radiolabelled oligonucleotide internal for each primer pair. From the left hand side, duplicate samples were run for the six DH patients and the six controls. CON = PHA-stimulated PBL control.

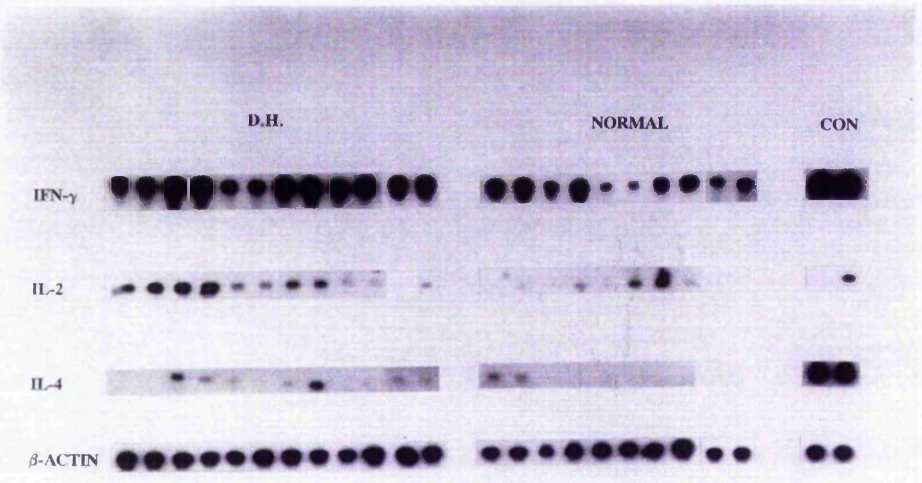
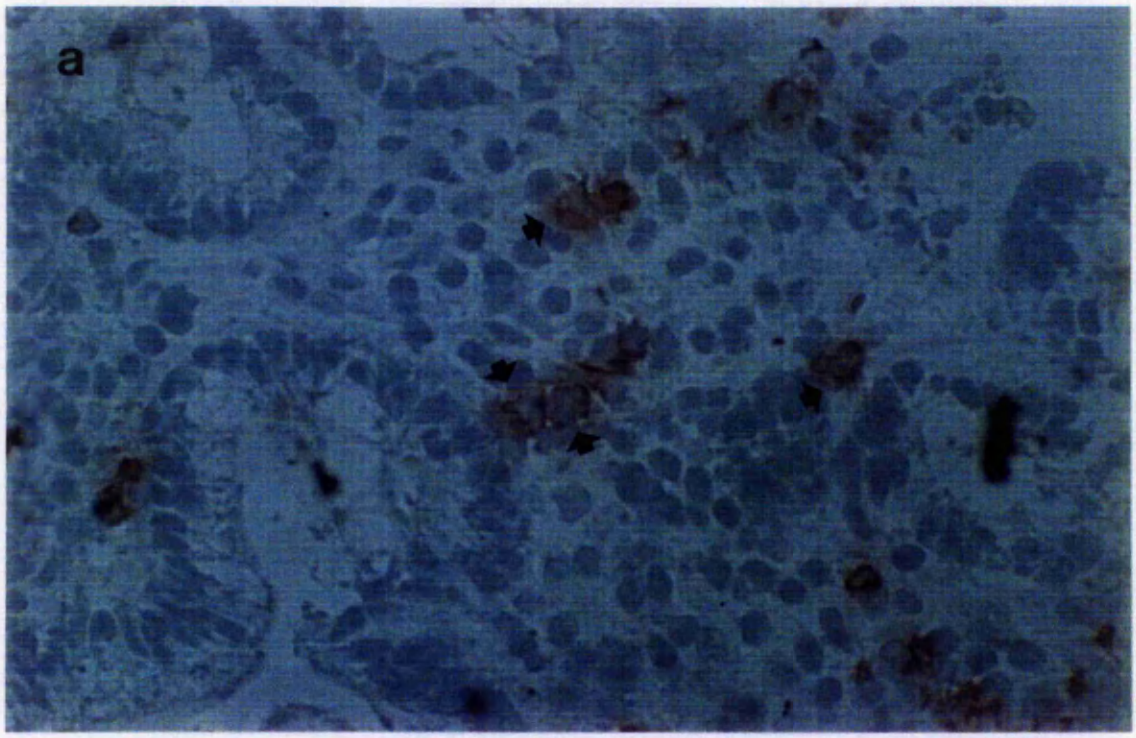
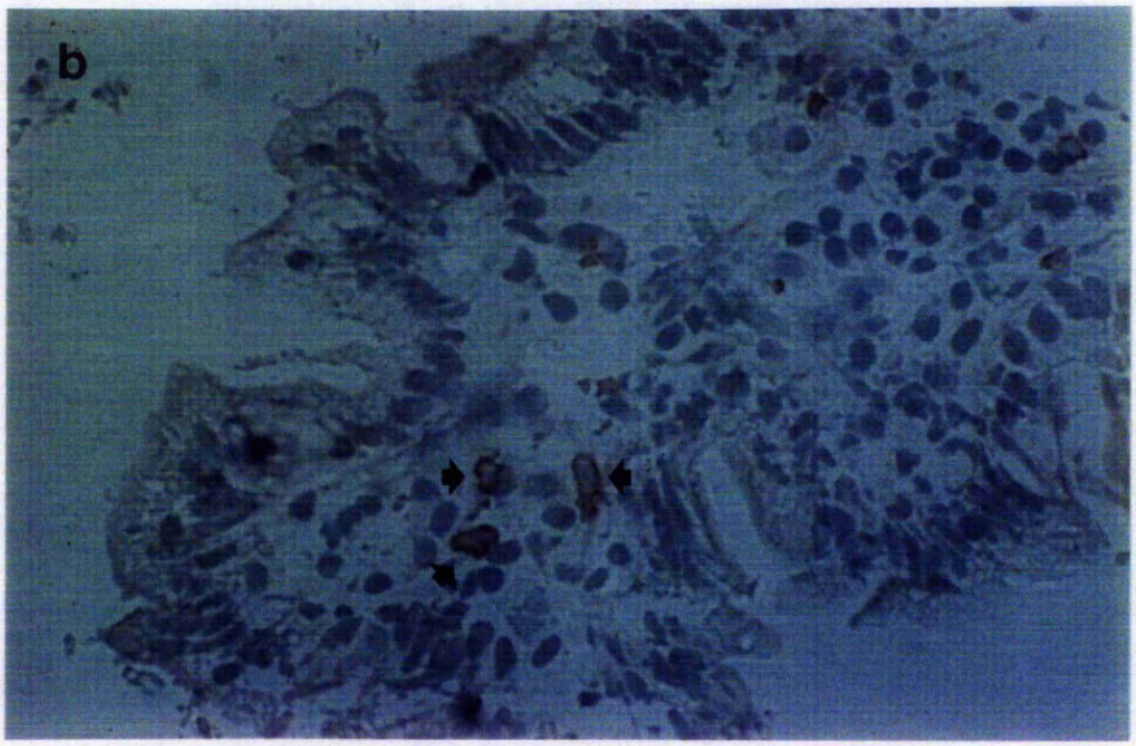


FIGURE 3.6: Immunoperoxidase staining of dermatitis herpetiformis lamina propria taken from a patient with partial villous atrophy demonstrating cells staining positively for (a) IFN-γ, (b) IL-2 and (c) IL-4 (x400). The arrowheads indicate some examples of positively staining cells.


a


b

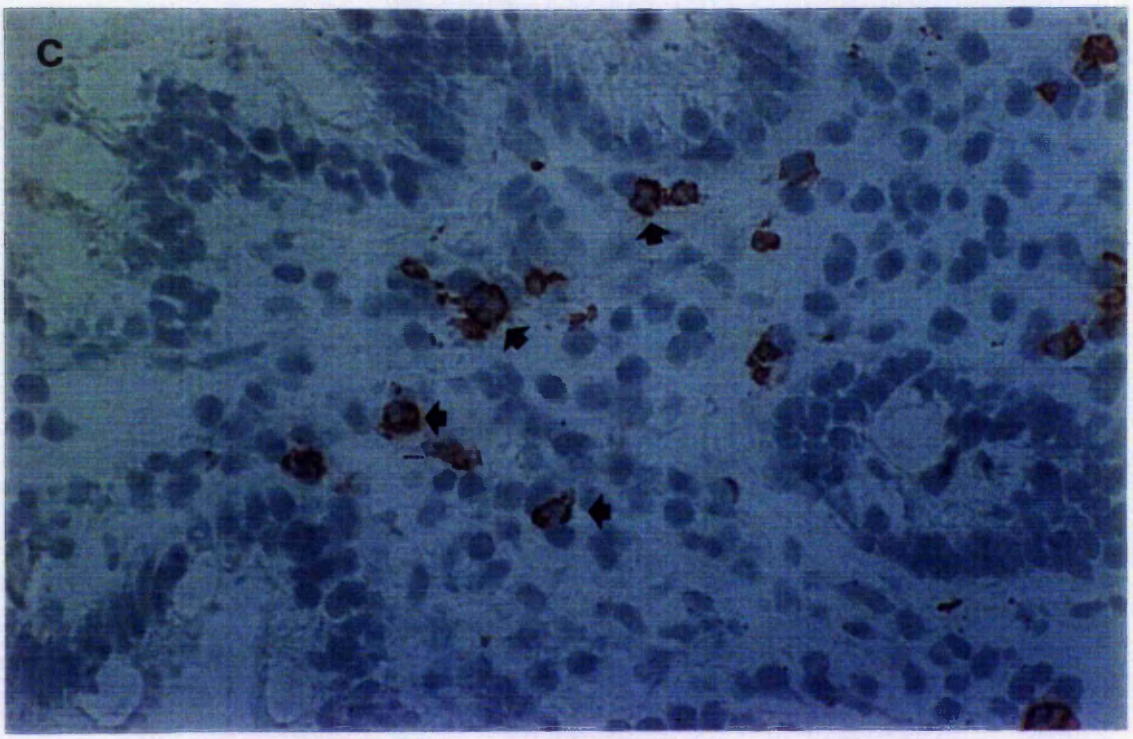

C

TABLE 3.5: Counts of intraepithelial and lamina propria CD3 positive T cells, and cells expressing cytokines IFN-γ, IL-2 and IL-4 in lamina propria

		IEL**	LP***	IFN-γ^{+}	IL-2^{++}	IL-4^{+++}
Controls	*n* = 6	82 ± 12	535 ± 78	69 ± 16	45 ± 14	117 ± 14
Range		(30 - 105)	(248 – 782)	(22 – 135)	(25 – 113)	(82 – 149)
%				(12% ± 1)	(8% ± 1)	
DH guts	*n* = 6	413 ± 53*	585 ± 61	79 ± 8	81 ± 9	125 ± 12
Range		(192 – 576)	(357 – 744)	(53 – 113)	(47 – 107)	(86 – 154)
%				(15% ± 4)	(15% ± 3)	

* $P < 0.005$, Mann Whitney *U* test
** T cells counted per 1000 epithelial cells
*** T cells counted per unit area of lamina propria
$^{+}$ Cells positive for IFN-γ expressed per unit area
$^{++}$ Cells positive for IL-2 expressed per unit area
$^{+++}$ Cells positive for IL-4 expressed per unit area
n number of patients
% positively staining cells expressed as a percentage of CD3 positive cells

CHAPTER FOUR

DISCUSSION AND HYPOTHESIS

CHAPTER 4

DISCUSSION AND HYPOTHESIS

4.1 Discussion

The term dermatitis herpetiformis encompasses both the skin and the small intestinal changes observed in this condition. The rash and the enteropathy of DH are both triggered by the storage proteins found in the cereals wheat, barley and rye. Oats have also been incriminated but their involvement in the aetiology of dermatitis herpetiformis, and coeliac disease, is controversial. Indeed, a recent study suggested that oats were not toxic in CD [Janatuinen *et al*, 1995] and my own group demonstrated that moderate amounts of *pure* oats could be added to the diets of DH patients without deleterious effects to the skin or intestine [Hardman *et al*, 1997]. Cereals contain many storage proteins but it is the alcohol-soluble fractions, known as prolamins, that are responsible for triggering the rash and enteropathy of DH. In wheat, the prolamins are known as gliadins. Although we know that ingestion of gliadin, in susceptible individuals, leads to the development of DH, the mechanisms involved remain elusive.

In addition to involvement of gliadin as the trigger, pathogenic mechanisms of DH would have to take into account the additional clues provided by research since the late 1960s including the following findings: the presence of IgA in the dermal papillae of the skin; genetic susceptibility to developing DH; the presence of circulating ARA and AEA; and the knowledge that there are activated T cells in the skin.

The skin and small intestinal changes of DH appear to be inextricably linked, and in my

view, it is very likely that the development of the rash is dependent on the presence of the enteropathy. Therefore, it seems probable that common pathogenic mechanisms exist for both organs. With regard to CD, two main theories prevail in the literature and these are T cell mediated mechanisms and autoimmune processes. Let us consider each of these in turn for CD.

Possible mechanisms whereby gliadin induces the enteropathy of coeliac disease

i) GSE is due to T cell mediated mechanisms

There is much circumstantial evidence to support the hypothesis that the small intestinal changes of coeliac disease are mediated by T cell mechanisms.

In untreated coeliac disease, the number of T cells within the epithelium is increased. The vast majority of IELs are CD3+ T cells and 70-90% express CD8+ with many expressing CD45RO (memory cells) in addition. Most IELs express the conventional T cell receptor α/β; oligoclonality of these cells has been demonstrated in normal small intestine indicating that these cells are specific for a limited number of antigens [Balk *et al*, 1991; Blumberg *et al*, 1993]. A minority of IELs express the γ/δ T cell receptor. The function of T cells expressing the γ/δ TCR is unknown but their numbers are increased in the epithelium of CD (and DH) [Spencer *et al*, 1989; Halstensen *et al*, 1989; Savllahti *et al*, 1990; Savilahti *et al*, 1992]. In contrast with T cells expressing the α/β TCR, IELs expressing the γ/δ TCR remain elevated after withdrawal of gluten from the diet [Savilahti *et al*, 1992]. Furthermore, the presence of elevated numbers of IELs expressing the γ/δ TCR, in otherwise normal individuals, is a marker for those with latent CD or DH [Mäki *et al*,1991; Savilahti *et al*, 1992] or for those with a genetic susceptibility to develop CD [Holm *et al*,

1992].

Increased numbers of T lymphocytes are also observed in the lamina propria of untreated coeliac disease. These are predominantly CD4+ CD45RO+ indicating that these are antigen-primed or memory cells. The α/β TCR is the predominant TCR and upregulation of expression of CD25 (interleukin 2 receptor) has been shown to occur [Halstensen & Brandtzaeg, 1993]. Polyclonal T cell lines and T cell clones recognising gliadin have been established from coeliac mucosa [Lundin *et al*, 1993]. These T cells were CD4+ and recognised gliadin when presented by antigen presenting cells expressing the CD-associated DQ (A1*0501, B1*0201) heterodimer. It has been proposed that *in vivo* these T cells interact with antigen and produce cytokines which have damaging effects on the mucosa.

Other studies support the involvement of cytokines produced by activated T cells. Addition of the T cell activators pokeweed mitogen and anti-CD3 to organ cultures of foetal small bowel resulted in an increased proliferation of epithelial cells, crypt hyperplasia and villous atrophy; IL-2 and γ-IFN were detected in supernatants of damaged explants [MacDonald & Spencer, 1988]. Furthermore, antibodies to γ-IFN abolished the development of intestinal graft versus host disease [Mowat, 1989]. These studies would tend to support involvement of TH1 T cells ie those which produce IL-2 and γ-IFN. γ-IFN has been implicated in the pathogenesis of coeliac disease because of the evidence outlined above and also because increased levels of γ-IFN were demonstrated in CD small intestinal mucosa compared with normal mucosa [Kontakou *et al*, 1994]. However, if this hypothesis is correct, then other factors must be operating as addition of γ-IFN to organ cultures did not result in villous atrophy nor crypt hyperplasia [MacDonald & Spencer,

1988]. My own work, outlined in Chapter 3.4, did not show a significant increase in γ-IFN in DH small intestine compared with controls and my findings were in agreement with others [Al-Dawoud *et al*, 1992].

Recent work has further contributed towards our understanding of the pathogenesis of CD. The main target antigen for AEA has been identified as tissue transglutaminase (tTG) [Dieterich *et al*, 1997]. TTG is a calcium dependent enzyme which catalyses the cross-linking of proteins resulting in the formation of ε-(γ-glutamyl)-lysine bonds. It is normally localised within the cytoplasm but can be released during wounding and is thought to stabilise the extra-cellular matrix in granulation tissue. Dieterich and his colleagues were able to demonstrate that gliadin was an excellent substrate for tTG and was quantitatively cross-linked by it [Dieterich *et al*, 1997]. Subsequently, an elegant set of experiments showed that tTG enhanced the recognition of gliadin by T cells derived from the small intestine by ordered and specific deamidation of gliadin resulting in an epitope that bound efficiently to HLA-DQ2 on antigen presenting cells [Molberg *et al*, 1998]. This effect was limited to T cells derived from the small intestine as it could not be replicated using peripheral blood [Molberg *et al*, 1998].

Despite the plethora of evidence supporting T cell mediated mechanisms in the development of the enteropathy of CD, it is not known if T cell activity is indeed the initial event or secondary to other mechanisms.

ii) GSE is due to autoimmune mechanisms

There is substantial evidence to suggest that CD may be an autoimmune disease. All the elements essential for an autoimmune process are present in CD: the trigger (gliadin),

susceptibility MHC class II genes and the autoantigen.

CD is strongly associated with the HLA molecules B8, DR3, DQ2 and DQ8 encoded by the major histocompatibility complex. Approximately 95% of CD patients carry the heterodimer DQA1*0501 DQB1*0201 (DQ2) in *cis* or *trans* [Sollid *et al*, 1989; Hall *et al*, 1991]; most of the remaining CD patients instead carry genes encoding the DQ A1*03 DQB*0302 heterodimer also known as DQ8 [Spurkland *et al*, 1992; Mantovani *et al*, 1993; Tighe *et al*, 1993; Sollid *et al*, 1993]. The HLA class II molecules DQ2 and DQ8 on antigen presenting cells could present self-peptides.

Patients with CD produce antibodies directed against reticulin (ARA) and endomysium (AEA) and these antibodies have a high sensitivity and specificity for CD. Furthermore, the levels of these antibodies diminish and eventually disappear when gliadin has been withdrawn from the diet. Gliadin has been shown to drive the production of AEA in organ cultures of small intestine [Picarelli *et al*, 1996]. These findings raise the possibility that ARA and AEA, or the antigen recognised, are involved directly in the pathogenesis of CD. ARA and AEA have been shown to be directed against fibroblast derived extra-cellular matrix proteins termed coeliac disease autoantigen proteins [Martinnen & Mäki, 1993; Mäki *et al*, 1991; Mäki, 1995]. Autoantibodies recognise antigenic determinants or epitopes which are highly conserved in evolution and it has been demonstrated that T cells specific for these cryptic epitopes may escape thymic education and become activated and autoagressive when the epitopes are presented in high concentrations [Quarantino *et al*, 1996]. These cryptic epitopes may become visible to the immune system by damage to the intestinal mucosa caused by gliadin and/or binding of gliadin to self antigens.

The recent discovery that the predominant, if not sole, target antigen for AEA is tTG and that the preferred substrate for tTG is gliadin, [Dieterich *et al*, 1997] has given further credence to the auto-immune hypothesis of CD. Dieterich and his coworkers hypothesised that damage to the small intestine, perhaps by toxic fractions of gliadin, triggers the release of tTG from its normally intracytosolic compartment; this in turn leads to subsequent cross-linking of gliadin resulting in gliadin-gliadin and gliadin-tTG complexes thus creating antigenic neotopes; these neotopes could then initiate an immune response directed against gliadin and tTG in genetically susceptible individuals [Dieterich *et al*, 1997].

Thus presentation of antigenic neoepitopes by antigen presenting cells expressing DQA1*0501 DQB1*0201 to the T cells, primes and triggers T cells - continued ingestion of the gliadin sustains the autoimmune response. However, in contrast with other autoimmune diseases, the immune processes cease and the pathological changes reverse once the trigger, gliadin, has been withdrawn.

Let us now consider the above hypotheses in relation to the pathogenesis of DH skin lesions.

i) T cell mediated mechanisms

Like CD, dietary gliadin is the trigger for the rash and intestinal changes observed in DH. I have been able to show unequivocally that the rash of DH resolves following withdrawal of gluten from the diet although it takes many months for the skin to heal completely. Eventually, so long as patients adhere to a strict GFD, the amount of IgA within the skin declines and eventually disappears.

My work has shown the presence of large numbers of activated CD4+ T cells, many of which were memory cells, in the dermis of DH lesional skin. Their close apposition with Langerhans' cells would suggest that a cell mediated immune response was taking place. Furthermore, I was able to demonstrate restricted T cell Vβ gene expression suggesting that these T cells were recognising a limited number of antigens. However, intradermal injection of Frazer's fraction III failed to reproduce the skin lesions of DH clinically nor did I observe an influx of T cells histologically. In addition, my colleague Dr Baker has been unable to isolate T cells recognising gliadin as an antigen from DH lesional skin [Baker *et al*, 1995]. Previous studies have failed to identify the presence of gliadin in the skin of DH patients [Eterman *et al*, 1977; Pehamberger *et al*, 1979]. Therefore, although my work would support the involvement of T cell mediated mechanisms in the pathogenesis of DH skin lesions, it seems likely that the T cells are recognising an antigen other than gliadin.

ii) Autoimmune hypothesis

The elements essential for an autoimmune process are also present in DH: the trigger (gliadin), susceptibility MHC class II genes and the autoantigen. Let us consider the possible autoantigen.

Levels of ARA and AEA decline and eventually disappear in DH patients who have excluded gluten from their diets. The high specificity of these antibodies for DH suggests that they, or their antigen, may be important in the pathogenesis of the skin lesions. In Chapter 3.2, I proposed the possibility that the activated T cells in DH lesional skin could be reacting to a component of the skin. Previous work by Unsworth and his colleagues has shown that reticulin is present in human skin and that it is most abundant in the dermal papillae and immediately below the basement membrane zone [Unsworth *et al*, 1981]. The

sites of abundant reticulin correspond to the localisation of IgA in the skin and this gives support to the proposal made by Seah and his coworkers that that the IgA may in fact be binding to reticulin fibres [Seah *et al*, 1972].

As mentioned above, in respect to CD , tTG has been identified as being the target antigen for AEA and this could be relevant to the pathogenesis of the rash of DH. In the skin, tTG is found along the dermo-epidermal junction, in the micro-fibrillar apparatus of the papillary dermis and surrounding capillaries and in differentiating epidermis – it appears to play an important role in cross-linking the papillary dermis and the dermo-epidermal junction [Raghunath *et al*, 1996]. Thus, with the exception of the dermal capillaries and differentiating epidermis, tTG is present at the same sites as IgA in the skin of DH patients.

Therefore, there would appear to be two potential autoantigens in DH: reticulin and tTG. In susceptible individuals, gliadin could damage reticulin in the small intestine or it could crosslink with tTG to form neoepitopes. This would lead to priming and triggering of relevant T cells some of which would enter into the general circulation and eventually find their way to the skin where they would interact with antigen, produce cytokines which would initiate the cascade of events leading to production of DH skin lesions.

4.2 Hypothesis

Taking existing evidence into account, I favour the following hypothesis.

Ingestion of toxic fractions of gliadin, in genetically susceptible individuals, leads to damage of the small intestine. This results in exposure of self antigens, which are normally

hidden from the immune system, and renders them immunogenic by damaging them or by adhering to them. A possible candidate antigen could be reticulin because ARA is a feature of DH and has a high specificity in the diagnosis of DH. Furthermore, like patients with CD, DH patients are at risk of developing splenic atrophy and a major component of the spleen is reticulin. In light of recent work, a second possible antigen could be tTG which would be released following gliadin induced damage: tTG-gliadin and gliadin-gliadin complexes would form leading to the production of neoepitopes. The development of neoepitopes in turn leads to eventual activation and proliferation of autoreactive T cells.

These autoreactive T cells then enter the general circulation and some eventually find their way to the skin where they react with reticulin or with tTG. This results in the production of cytokines by these T cells and this in turn leads to the production of IL-8 by the epidermal cells and GM-CSF by dendritic cells at the basement membrane [Graeber *et al*, 1993]. Neutrophils enter the dermis slightly later than the T cells; the presence of IL-8 on the epidermal cells causes them to migrate towards the epidermis. IgA, present in the tips of the papillae, attaches to receptors on the neutrophils causing activation of the neutrophils which then produce cytokines and inflammatory mediators eventually leading to destruction and damage in the region of the basement membrane resulting in subepidermal blistering.

Continued ingestion of gliadin leads to continued production of a population of T cells primed against reticulin or tTG whilst withdrawal of gliadin from the diet results in a gradual decline in the numbers of primed T cells. This could explain why it takes so long for the rash to clear once exposure to gliadin has ceased and why re-ingestion leads to rapid activation and proliferation of the T cells accounting for recurrence of the rash within a few

weeks.

4.3 Final discussion

Genetic factors appear to be important in the aetiology of DH and CD as these conditions tend to cluster in families and my work has shown that many so-called "unaffected" family members may in fact have silent or latent CD (Chapter 2.3). Although there is a strong association with the HLA molecules DQA1*0501 DQB1*0201 and DQ A1*03 DQB*0302, other non HLA factors must be operating as not all individuals with these HLA types develop CD or DH. A number of possible gene linkage sites has been reported [Zhong *et al*, 1996; Houlston *et al*, 1997; Breco *et al*, 1998]. One of the pitfalls in looking for gene linkage for CD and DH is that it is insufficient to exclude CD in "normal" individuals on the basis of serology alone – my work has shown that CD can only be absolutely excluded by small intestinal biopsy. The discovery of the candidate gene(s) for CD and DH should lead to a better understanding of the pathogenesis of these conditions and should explain why only a small percentage of patients with CD develop the rash of DH.

Finally, my work has confirmed the efficacy of a GFD in the treatment of DH, not only because it allows healing of the skin and small intestine to occur, but also because it reduces the risk of these patients developing lymphoma.

REFERENCES

Adams RB, Planchon SM and Roche JK. IFN-γ modulation of epithelial barrier function. *J Immunol* 1993;**150**:2356-2363.

Ahangari G, Berg A, Jeddi-Tehrani M, Halapi E, Hammar H and Wigzell H. RT-PCR based analysis of T-cell receptor β variable region gene usage in normal human breast skin resident T lymphocytes (SRT). *Scan J Immunol* 1996;**44**:330-334.

Al-Dawoud A, Nakshabendi I, Foulis A and Mowat A McI. Immunohistochemical analysis of mucosal gamma-interferon production in coeliac disease. *Gut* 1992;**33**:1482-1486.

Alexander JO'D. In: *Dermatitis Herpetiformis. Major Problems in Dermatology* (Rook AJ, ed). Philadelphia: WB Saunders, 1975;28-9.

Anand BS, Truelove SC and Offord RE. Skin test for coeliac disease using a subfraction of gluten. *Lancet* 1977;**i**:118-20.

Andersson H, Dotevall G and Mobacken H. Malignant mesenteric lymphoma in a patient with dermatitis herpetiformis, hypochlohydria and small bowel abnormalities. *Scand J Gastroenterol* 1971;**6**:397-399.

Anstey A, Wilkinson JD and Walshe MM. Dermatitis herpetiformis in monozygous twins - concordance for dermatitis herpetiformis and gluten-sensitive enteropathy. *Clin Exp Dermatol* 1991;**16**:51-52.

Auspitz H. *System der Hautkrankheiten.* Vienna:Braumüller, 1881:143-62.

Baker BS, Swain AF, Fry L and Valdimarsson H. Epidermal T lymphocytes and HLA-DR expression in psoriasis. *Br J Dermatol* 1984;**110**:555-564.

Baker BS, Garioch JJ, Bokth S, Leonard JN and Fry L. Absence of gluten-specific T lymphocytes in the skin of patients with dermatitis herpetiformis. *J Autoimmunity* 1995;**8**:75-82.

Baker BS, Garioch JJ, Bokth S, Thomas H, Walker M, Leonard JN and Fry L. Lack of proliferative response by gluten-specific T cells in the blood and gut of patients with dermatitis herpetiformis. *J Autoimmunity* 1995;**8**:561-574.

Balk SP, Ebert EC, Blumenthal RL, McDermott FV, Wuchherpfennig KW, Landau SB and Blumberg RS. Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. *Science* 1991;**253**:1411-1415.

Beutner EH, Chorzelski TP, Kumar V, Leonard JN and Krasny S. Sensitivity and specificity of IgA class anti-endomysial antibodies for dermatitis herpetiformis and findings relevant to their pathogenic significance. *J Am Acad Dermatol* 1986;**15**:464-473.

Blenkinsopp WK, Fry L, Haffenden GP and Leonard JN. Histology of linear IgA disease, dermatitis herpetiformis and bullous pemphigoid. *Am J Dermatopath* 1983;**5**:547-554.

Blumberg RS, Yockey CE, Gross GG, Ebert EC and Balk SP. Human intestinal intraepithelial lymphocytes are derived from a limited number of T cell clones that utilize multiple Vβ T cell receptor genes. *J Immunol* 1993;**150**:5144-5153.

Booth SA, Moody CE, Dahl MV, Herron MJ and Nelson RD. Dapsone suppresses integrin-mediated neutrophil adherence function. *J Invest Dermatol* 1992;**98**:135-140.

Brocq L. De la dermatite herpétiforme de Duhring. *Ann Dermatol Syphiligr 2nd ser* 1888;**9**:1-20, 65-83, 133-157, 209-227, 305-327, 433-454, 493-518.

Brow J, Parker F, Weinstein W and Rubin CE. The small intestinal mucosa in dermatitis herpetiformis: severity and distribution of small intestinal lesion and associated malabsorption. *Gastroenterology* 1971;**60**:355-361.

Buckley DB, English J, Molloy W, Doyle CT and Whelton MJ. Dermatitis herpetiformis: a review of 119 cases. *Clin Exp Dermatol* 1983;**8**:221-248.

Catassi C, Rätsch I-M, Fabiani E, Rossini M, Bordicchia F, Candela F, Coppa GV and Giorgi PL. Coeliac disease in the year 2000: exploring the iceberg. *Lancet* 1994;**343**:200-203.

Chorzelski TP, Beutner EH, Sulej J, Tchorzewska H, Jablonska S, Kumar V and Kapuscinska A. IgA anti-endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. *Br J Dermatol* 1984;**111**:395-402.

Christensen OB, Hindsén M and Svensson Å. Natural history of dermatitis herpetiformis in Southern Sweden. *Dermatologica* 1986;**173**;271-277.

Ciacci C, Mahida YR, Dignass A, Koizumi M and Podolsky DK. Functional interleukin-2

receptors on intestinal epithelial cells. *J Clin Invest* 1993;**92**:527-532.

Ciclitira PJ, Ellis HJ and Fagg NLK. Evaluation of a gluten free product containing wheat gliadin in patients with coeliac disease. *Br Med J* 1984;**289**:83.

Civatte A. Diagnostique histopathologique de la dermatite polymorphe douloureuse ou maladie de Duhring-Brocq. *Ann Dermatol Syphiligr* 8th series 1943;**3**:1-30.

Collin P, Reunala T, Pukkala E, Laippala P, Keyrilämen O and Pasternack A. Coeliac disease - associated disorders and survival. *Gut* 1994;**35**:1215-1218.

Cormane RH. Immunofluorescent studies of the skin in lupus erythematosus and other diseases. *Path Europ* 1967;**2**:170-80.

Costello M. Dermatitis herpetiformis treated with sulphapyridine. *Arch Dermatol Syph* 1940;**41**:134.

Cunningham MJ and Zone JJ. Thyroid abnormalities in dermatitis herpetiformis: prevalence of clinical thyroid disease and thyroid autoantibodies. *Ann Intern Med* 1985;**102**:194-196.

Davies MG, Marks R and Nuki G. Dermatitis herpetiformis - a skin manifestation of a generalised disturbance in immunity. *Q J Med* 1978;**186**:221-248.

Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO and Schuppan D.

Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature Medicine* 1997;**3**:797-801.

Dühring L A. Dermatitis herpetiformis. *JAMA* 1884;**3**:225-9.

Dunn DA, Gadenne A-S, Simha S, Lerner EA, Bigby M and Bleicher PA. T-cell receptor Vβ expression in normal human skin. *Proc Natl Acad Sci* 1993;**90**:1267-1271.

Egelrud T and Bäck O. Dermatitis herpetiformis: biochemical properties of the granular deposits of IgA in papillary dermis. Characterisation of SDS-soluble IgA-like material and potentially antigen-binding IgA fragments released by pepsin. *J Invest Dermatol* 1985;**84**:239-245.

Ermacora E, Prampolini L, Tribbia G, Pezzoli G, Gelmetti C, Cucchi G, Tettamanti A, Giunta A and Gianotti F. Long term follow up of dermatitis herpetiformis in children. *J Am Acad Dermatol* 1986;**15**:24-30.

Esteves J and Brandao FN. Au sujet de l'action des sulfamides et des sulphones dans la maladie de Dühring. *Trab Soc Port Dermatol* 1950;**8**:209.

Eterman KP, Nefkens MJJ and van der Meer JB. Failure to detect antigens associated with the immune aggregates in the skin in dermatitis herpetiformis. *Arch Dermatol Res* 1977;**260**:247-52.

Fowler J and Thomas DJB. Lymphoma in dermatitis herpetiformis. *Br J Med* 1976;**2**:757.

Fraser NG, Murray D and Alexander JO. Structure and function of the small intestine in dermatitis herpetiformis. *Br J Dermatol* 1967;**79**:509-518.

Frazer AC, Fletcher RF, Ross CAC, Shaw B, Sammons HG and Schneider R. Gluten induced enteropathy. The effect of partially digested gluten. *Lancet* 1959;**ii**:252-5.

Freeman HJ, Weinstein WM, Shritka TK, Piercy JRA and Wensel RH. Primary abdominal lymphoma. Presenting manifestation of coeliac sprue or complicating dermatitis herpetiformis. *Am J Med* 1977;**63**:385-394.

Frödin T, Gotthard R, Hed J, Molin L, Norrby K and Walan A. Gluten-free diet for dermatitis herpetiformis: the long term effect on cutaneous, immunological and jejunal manifestations. *Acta Derm Venereol (Stockh)* 1981;**61**:405-411.

Fronek Z, Cheung MM, Hanbury AM and Kagnoff MF. Molecular analysis of HLA DP and DQ genes associated with dermatitis herpetiformis. *J Invest Dermatol* 1991;**97**:799-802.

Fry L, Keir P, McMinn RMH, Cowan JD and Hoffbrand AV. Small-intestinal structure and function and haematological changes in dermatitis herpetiformis. *Lancet* 1967;**ii**:729-734.

Fry L, McMinn RMH, Cowan JD and Hoffbrand AV. Effect of gluten-free diet on dermatological, intestinal and haematological manifestations of dermatitis herpetiformis. *Lancet* 1968;**i**:557-561.

Fry L, Seah PP, McMinn RMH and Hoffbrand AV. Lymphocytic infiltration of epithelium in

diagnosis of gluten-sensitive enteropathy. *BMJ* 1972;**iii**:371-374.

Fry L, Seah PP, Riches DJ and Hoffbrand AV. Clearance of skin lesions in dermatitis herpetiformis after gluten withdrawal. *Lancet* 1973;**i**:288-291.

Fry L, Seah PP, Harper PG, Hoffbrand AV and McMinn RMH. The small intestine in dermatitis herpetiformis. *J Clin Pathol* 1974;**27**:817-824.

Fry L, Haffenden G, Wojnarowska F, Thompson BR and Seah PP. IgA and C3 complement in the uninvolved skin in dermatitis herpetiformis after gluten withdrawal. *Br J Dermatol* 1978;**99**:31-37.

Fry L, Leonard JN, Swain F, Tucker WFG, Haffenden G, Ring N and McMinn RMH. Long term follow up of dermatitis herpetiformis with and without dietary gluten withdrawal. *Br J Dermatol* 1982;**107**:631-640.

Fry L. In: *Coeliac Disease* (Marsh MN, ed). Blackwell Scientific Publications 1992:93.

Garioch JJ, Baker BS, Leonard JN and Fry L. T lymphocytes in lesional skin of patients with dermatitis herpetiformis. *Br J Dermatol* 1994;**131**:822-826.

Garioch JJ, Unsworth DJ, Baker BS, Leonard JN and Fry L. Failure of intradermal skin testing with gluten to produce delayed hypersensitivity reactions in patients with dermatitis herpetiformis. *Br J Dermatol* 1995;**132**:698-702.

Garioch JJ, Baker BS, Teare J, Allison MED, Thomas HJW, Leonard JN and Fry L. An immunohistochemical study of interferon-γ, interleukin-2 and interleukin-4 in the small intestine of patients with dermatitis herpetiformis. (abstract) *J Invest Dermatol* 1995;**105**:515.

Gaspari AA, Huang C, Davey RJ, Bondy C, Lawley TJ and Katz SI. Prevalence of thyroid abnormalities in patients with dermatitis herpetiformis and in control subjects with HLA-B8/DR3. *Am J Med* 1990;**88**:145-150.

Gawkrodger DJ and Barnetson RStC. Dermatitis herpetiformis and lymphoma. *Lancet* 1982;**2**:987.

Gawkrodger DJ, Blackwell JN, Gilmore HM, Rifkind EA, Heading RC and Barnetson RStC. Dermatitis herpetiformis: diagnosis, diet and demography. *Gut* 1984;**25**:151-157.

Goodwin P and Fry L. Reticulum cell sarcoma complicating dermatitis herpetiformis. *Proc R Soc Med* 1973;**66**:625-626.

Gould DJ and Howell R. Dermatitis herpetiformis and reticulum cell sarcoma, a rare complication. *Br J Dermatol* 1977;**96**:561-562.

Graeber M, Baker BS, Garioch JJ, Valdimarsson H, Leonard JN and Fry L. The role of cytokines in the generation of skin lesions in dermatitis herpetiformis. *Br J Dermatol* 1993;**129**:530-532.

Greco L, Corazza G, Babron M-C, Clot F, Fulchignoni-Lataud M-C, Percopo S, Zavattari P, Bouguerra F, Dib C, Tosi R, Troncone R, Ventura A, Matavoni W, Magazzù G, Gatti R, Lazzari R, Giunta A, Perri F, Iacono G, Cardi E, de Virgiliis S, Cataldo F, De Angelis G, Musumeci S, Ferrari R, Balli F, Bardella M-T, Volta U, Catassi C, Torre G, Eliaou J-F, Serre J-L and Clerget-Darpoux F. Genome search in celiac disease. *Am J Hum Genet* 1998;**62**:669-675.

Green ST, Natarajan S, Connor JM and Forrest JAH. Monozygotic twins concordant for duodenojejunal villous atrophy and dermatitis herpetiformis. *Gut* 1986;**27**:970-971.

Gulwani-Akolkar B, Posnett DN, Janson CH, Grunewald J, Wigzell H, Akolkar P, Gregerson PK and Silver J. T-cell receptor V-segment frequencies in peripheral blood T cells correlate with human leukocyte antigen type. *J Exp Med* 1991;**174**:1139-1146.

Hall MA, Lanchbury JSS, Bolsover WK, Welsh KI and Ciclitira PJ. HLA association with dermatitis herpetiformis is accounted for by a *cis* or *trans*associated DQ heterodimer. *Gut* 1991;**32**:487-490.

Hall RP and Lawley TJ. Characterisation of circulating and cutaneous IgA immune complexes in patients with dermatitis herpetiformis. *J Immunol* 1985;**135**:1760-1765.

Hall RP, Sanders ME, Duquesnoy RJ, Katz SI and Shaw S. Alterations in HLA-DP and HLA-DQ antigen frequency in patients with dermatitis herpetiformis. *J Invest Dermatol* 1989;**93**:501-505.

Hall RP, Wilson JAP and Streilein RD. Cytokine patterns in the small bowel of patients with dermatitis herpetiformis (abstract). *J Invest Dermatol* 1994;**102**:631.

Hällström O and Reunala T. IgA class reticulin antibodies in dermatitis herpetiformis: a good indicator of jejunal damage. *Acta Derm Venereol (Stockh)* 1985;**65**:330-332.

Hällström O. Comparison of IgA-class reticulin and endomysium antibodies in coeliac disease and dermatitis herpetiformis. *Gut* 1989;**30**:1225-1232.

Halstensen TS, Scott H and Brandtzaeg T. Intraepithelial T cells of TCR γ/δ+CD8- and Vδ1/Jδ1+ phenotypes are increased in coeliac disease. *Scand J Immunol* 1989;**30**:665-672.

Halstensen TS and Brandtzaeg P. Activated T lymphocytes in the celiac lesion: non-proliferative activation (CD25) of CD4+ α/β cells in the lamina propria but proliferation (Ki-67) of α/β and γ/δ cells in the epithelium. *Eur J Immunol* 1993;**23**:505-510.

Hardman CM, Garioch JJ, Leonard JN, Thomas HJW, Walker MM, Lortan JE, Lister A and Fry L. Absence of toxicity of oats in patients with dermatitis herpetiformis. *N Engl J Med* 1997;**337**:1884-1887.

Harrington CI and Read NW. Dermatitis herpetiformis: effect of a gluten-free diet on skin IgA and jejunal structure and function. *Br J Dermatol* 1977;**i**:872-875.

Heading RC, Paterson WD, McLelland DBL, Barnetson RStC and Murray M. Clinical

response of dermatitis herpetiformis skin lesions to a gluten-free diet. *Br J Dermatol* 1976;**94**:509-514.

Hebra F. Herpes impetiginiformis. *Lancet* 1872;**1**:399-400.

Hill ID. Computing man years at risk. *Br J Prev Soc Med* 1972;**26**:132-134.

Holm K, Mäki M, Savilahti E, Lipsanen V, Laippala P and Koskimies S. Intra-epithelial γ/δ T-cell-receptor lymphocytes and genetic susceptibility to coeliac disease. *Lancet* 1992;**339**:1500-1503.

Holmes GKT, Prior P, Lane MR, Pope D and Allan RN. Malignancy in coeliac disease - effect of a gluten free diet. *Gut* 1989;**30**:333-338.

Holmgren J, Fryklund J and Larsson H. Gamma-interferon-mediated down-regulation of electrolyte secretion by intestinal epithelial cells: a local immune mechanism? *Scand J Immunol* 1989;**30**:499-503.

Holt S and Blackwell JN. Dermatitis herpetiformis and diabetes mellitus. *Postgrad Med J* 1980;**56**:15-17.

Houlston RS, Tomlinson IPM, Ford D, Seal S, Marossy AM, Ferguson A, Holmes GKT, Hosie KB, Howdle PD, Jewell DP, Godkin A, Kerr GD, Kumar P, Logan RFA, Love AHG, Johnston S, Marsh MN, Mitton S, O'Donoghue D, Roberts A, Walker-Smith JA and Stratton MF. Linkage analysis of candidate regions for coeliac disease genes. *Hum Mol*

Genet 1997;**6**:1335-1339.

Isaacson PG, O'Connor NTJ, Spencer J, Bevan DH, Connolly CE, Kirkham N, Pollock DJ, Wainscoat JS, Stein H and Mason DY. Malignant histiocytosis of the intestine: a T-cell lymphoma. *Lancet* 1985;**ii**:688-691.

Janatuinen E, Pikkareinen P, Kemppainen T, Kosma V-M, Jarvinen R, Uusitupa M and Julkune R. A comparison of diets with and without oats in celiac disease. *N Engl J Med* 1995;**333**:1033-1037.

Jepsen LV and Ullman S. Dermatitis herpetiformis and gluten-sensitive enteropathy in monozygotic twins. *Acta Derm Venereol (Stockh)* 1980;**60**:353-355.

Kaplan RP and Callen JP. Dermatitis herpetiformis: autoimmune disease associations. In: AR Ahmed, S Jablonska, eds. *Clinics in Dermatology* 1991;**9**:347-360.

Kárpáti S, Kósnai I, Verkasalo M, Kuitunen P, Simon Z, Koskimies S, Reunala T, Gyódi E and Török E. HLA antigens, jejunal morphology and associated diseases in children with dermatitis herpetiformis. *Acta Pediatr Scand* 1986;**75**:297-301.

Katz SI, Falchuk ZM, Dahl MV, Rogentine GN and Strober W. HL-A8: a genetic link between dermatitis herpetiformis and gluten-sensitive enteropathy. *J Clin Invest* 1972;**51**:2977-2980.

Katz SI. Dermatitis herpetiformis: the skin and the gut. *Ann Intern Med* 1980;**93**:857-874.

Kelly J, O'Farrelly C, O'Mahony C, Weir DG and Feighery C. Immunoperoxidase demonstration of the cellular composition of the normal and coeliac small bowel. *Clin Exp Immunol* 1987;**68**:177-188.

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

Krangel MS. Endocytosis and recycling of the T3-T cell receptor complex. *J Exp Med* 1987;**165**:1141-1159.

Leonard JN, Haffenden GP, Tucker W, Unsworth J, Swain F, McMinn R, Holborow J and Fry L. Gluten challenge in dermatitis herpetiformis. *N Engl J Med* 1983;**308**:816-819.

Leonard JN, Tucker WFG, Fry JS, Coulter CAE, Boylston AW, McMinn RMH, Haffenden GP, Swain AF and Fry L. Increased incidence of malignancy in dermatitis herpetiformis. *Br Med J* 1983;**i**:16-18.

Leonard JN, Chorzelski TP, Beutner EH, Sulej J, Griffiths CEM, Kumar VJ and Fry L. IgA antiendomysial antibody detection in the serum of patients with dermatitis herpetiformis following gluten challenge. *Arch Dermatol Res* 1985;**277**:349-351.

Leung DYM, Travers JB, Giorno R, DA Norris, R Skinner, J Aelion, Kazemi LV, Kim MH, Trumble AE, Kotb M and Schievert PM. Evidence for a streptococcal superantigen - driven process in acute guttate psoriasis. *J Clin Invest* 1995;**96**:2106-2112.

Lever WF. Pemphigus - A histopathologic study. *Arch Dermatol Syphil* 1951;**64**:727-753.

Lewis HM, Baker BS, Bokth S, Powles AV, Garioch JJ, Valdimarsson H and Fry L. Restricted T-cell receptor Vβ gene usage in the skin of patients with gutttate and chronic plaque psoriasis. *Br J Dermatol* 1993;**129**:514-520.

Ljunghall K, Lööf L and Forsum U. T lymphocyte subsets in the duodenal epithelium in dermatitis herpetiformis. *Acta Dermatovener (Stockholm)* 1982;**62**:485-489.

Ljunghall K and Tjernlund U. Dermatitis herpetiformis: effect of gluten-restricted and gluten-free diet on dapsone requirement and on IgA and C3 deposits in uninvolved skin. *Acta Derm Venereol (Stockh)* 1983;**63**:129-136.

Loft DE, Marsh MN and Crowe PT. Rectal gluten challenge and diagnosis of coeliac disease. *Lancet* 1990;**335**:1293-95.

Logan RFA and Ferguson A. Skin tests for coeliac disease. *Lancet* 1978;**ii**:1042-1043.

Loveridge JA, Rosenberg WMC, Kirkwood TBL and Bell JI. The genetic contribution to human T-cell receptor repertoire. *Immunology* 1991;**74**:246-250.

Lundin KEA, Scott H, Hansen T, Paulsen G, Halstensen TS, Fausa O, Thorsby E and Sollid LM. Gliadin specific, HLA-DQ(α1*0501,β1*0201) restricted T cells isolated from the small intestinal mucosa of coeliac disease patients. *J Exp Med* 1993;**178**:187-96.

MacDonald TT and Spencer J. Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. *J Exp Med* 1988;**167**:1341-1349.

McCarthy CF. Coeliac Disease. *Ir J Coll Phys Surg* 1990;**19**:45-47.

McFadden JP, Leonard JN, Powles AV, Rutman AJ and Fry L. Sulphamethoxypyridazine for dermatitis herpetiformis, linear IgA disease and cicatricial pemphigoid. *Br J Dermatol* 1989;**121**:759-762.

Madara JL and Stafford J. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* 1989;**83**:724-727.

Mäki M, Holm K, Collin P and Savilahti E. Increase in γ/δ T cell receptor bearing lymphocytes in normal small bowel mucosa in latent coeliac disease. *Gut* 1991;**32**:1412-1414.

Mäki M, Hällström O and Martinnen A. Reaction of human non-collagenous polypeptides with coeliac disease autoantibodies. *Lancet* 1991;**338**:724-725.

Mäki M The humoral immune system in coeliac disease. in:Howdle PD, ed. Coeliac disease. *Bailliere's Clin Gastroenterol* 1995;**9**:231-249.

Månsson T. Malignant disease in dermatitis herpetiformis. *Acta Derm Venereol (Stockh)* 1971;**51**:379-382.

Mantovani V, Corazza GR, Bragliani M, Frisoni M, Zaniboni MG and Gasbarrini G. Asp57-negative HLA DQ beta chain and DQA1*0501 allele are essential for the onset of DQw2-positive and DQw2-negative coeliac disease. *Clin Exp Immunol* 1993;**91**:153-156.

Marks J, Shuster S and Watson AJ. Small-bowel changes in dermatitis herpetiformis. *Lancet* 1966;**ii**:1280-1282.

Marks J, Birkett D, Shuster S and Roberts DF. Small intestinal mucosal abnormalities in relatives of patients with dermatitis herpetiformis. *Gut* 1970;**11**:493-497.

Marks J and Young S. Skin tests for coeliac disease. *Lancet* 1978;**ii**:1303.

Marsh MN. In: *Coeliac Disease* (Marsh MN, ed). Blackwell Scientific Publications 1992;136-191.

Martinnen A and Mäki M. Purification of fibroblast-derived celiac disease autoantigen molecules. *Pediatr Res* 1993;**34**:420-423.

Mazzola G, Berrino M, Bersanti M, D'Alfonso S, Cappello N, Bottaro A, Curtoni ES, Fusco P, Vallati M, Bundino S, Barbera C and Amorosa A. Immunoglobulin and HLA-DP genes contribute to the susceptibility to juvenile dermatitis herpetiformis. *Eur J Immunogenet* 1992;**19**:129-139.

Menssen A, Trommler P, Vollmer S, Schendel D, Albert E, Gürtler L, Riethmüller G and

Prinz JC. Evidence for an antigen-specific cellular response in skin lesions of patients with psoriasis vulgaris. *J Immunol* 1995;**155**:4078-4083.

Meyer LJ and Zone JJ. Familial incidence of dermatitis herpetiformis. *J Am Acad Dermatol* 1987;**17**:643-647.

Meyer L, Carioto L and Zone JJ. Dermatitis herpetiformis: extraction of intact IgA from granular deposits in dermal papillae. *J Invest Dermatol* 1987;**88**:559-563.

Miltenberg AMM, van Laar JM, Daha MR, de Vries RRP, van den Elsen PJ and Breedveld FC. Dominant T-cell receptor β-chain gene rearrangements indicate clonal expansion in the rheumatoid joint. *Scand J Immunol* 1990;**31**:121-125.

Mobacken H, Kastrup W and Nilsson LA. Incidence and prevalence of dermatitis herpetiformis in Western Sweden. *Acta Derm Venereol (Stockh)* 1984;**64**:400-404.

Molberg Ø, McAdam SN, Körner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Norén O, Roepstorff P, Lundin KEA, Sjöström H and Sollid LM. Tissue transglutaminase selectively modifies gliadin peptides that are recognised by gut-derived T cells in celiac disease. *Nature Medicine* 1998;**4**:713-717.

Moller DR, Konishi K, Kirby M, Balbi B and Crystal RG. Bias toward use of a specific T-cell receptor β-chain variable in a subgroup of individuals with sarcoidosis. *J Clin Invest* 1988;**82**:1183-1191.

Mowat AMcI. The regulation of immune responses to dietary protein antigens. *Immunology Today* 1987;**8**:93-98.

Mowat AMcI. Antibodies to IFN-γ prevent immunologically mediated intestinal damage in murine graft-versus-host reaction. *Immunology* 1989;**68**:18-23.

Mowbray JF, Hoffbrand AV, Holborow EJ, Seah PP and Fry L. Circulating immune complexes in dermatitis herpetiformis. *Lancet* 1973;**i**:400-402.

Nestor MS, Cochran AJ and Ahmed AR. Mononuclear cell infiltrates in bullous disease. *J Invest Dermatol* 1987;**88**:172-5.

Nield GH. Coeliac disease; a graft versus host like reaction to the small bowel wall? *Lancet* 1981;**i**:811-12.

O'Farrelly C, Feighery C, Greally JF and Weir DG. Cellular response to alpha-gliadin in untreated coeliac disease. *Gut* 1982;**23**:83-7.

Otley CC, Wenstrup RJ and Hall RP. DNA sequence analysis and restriction fragment length polymorphism (RFLP) typing of the HLA-DQw2 alleles associated with dermatitis herpetiformis. *J Invest Dermatol* 1991;**97**:318-322.

Park MS, Terasaki PI, Ahmed AR and Zone J. The 90% incidence of HLA antigen (Te 24) in dermatitis herpetiformis. *Tissue Antigens* 1983;**22**:263-266.

Pehamberger H, Gschnait F, Menzel J and Holubar K. Failure to detect gliadin or gliadin-binding sites in the skin of patients with dermatitis herpetiformis: immunofluorescence, organ culture and autoradiographic studies. *J Invest Dermatol* 1979;**73**:174-5.

Picarelli A, Maiuri L, Frate A, Greco M, Auricchio S and Londie M. Production of anti-endomysial antibodies after *in-vitro* gliadin challenge of small intestine biopsy samples from patients with coeliac disease. *Lancet* 1996;**348**:1065-67.

Przemioslo RT, Kontakou M, Nobili V and Ciclitira PJ. Raised pro-inflammatory cytokines interleukin 6 and tumour necrosis factor α in coeliac disease mucosa detected by immunohistochemistry. *Gut* 1994;**35**:1398-1403.

Quarantino S, Feldmann M, Dayan CM, Acuto O and Londei M. Human self-reactive T cell clones expressing identical T cell receptor chains differ in their ability to recognise a cryptic self-epitope. *J Exp Med* 1996;**183**:349-58.

Raghunath M, Höpfner B, Aeschlimann D, Lüthi U, Meuli M, Altermatt S, Gobet R, Bruckner-Tuderman L and Steinmann B. Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyte autografts. Anchoring fibrils are a target for tissue transglutaminase. *J Clin Invest* 1996;**98**:1174-1184.

Reitamo S, Reunala T, Konttinen YT, Saksela O and Salo OP. Inflammatory cells, IgA, C3, fibrin and fibronectin in skin lesions in dermatitis herpetiformis. *Br J Dermatol* 1981;**105**:167-177.

Reunala T, Salo OP, Tiilikainen A, Selroos O and Kuitunen P. Family studies in dermatitis herpetiformis. *Ann Clin Res* 1976;**8**:254-261.

Reunala T, Blomqvist K, Tarpila S, Halme H and Kangas K. Gluten-free diet in dermatitis herpetiformis. *Br J Dermatol* 1977;**97**:473-480.

Reunala T and Lokki J. Dermatitis herpetiformis in Finland. *Acta Derm Venereol (Stockh)* 1978;**58**:505-510.

Reunala T, Helin H, Kuokkanen K and Hakala T. Lymphoma in dermatitis herpetiformis: report on four cases. *Acta Derm Venereol (Stockh)* 1982;**62**:343-346.

Reunala T, Hakala T and Helin H. Dermatitis herpetiformis, lymphoma and gluten-free diet. *J Am Acad Dermatol* 1983;**10**:526-527.

Reunala T, Chorzelski TP, Viander M, Sulej J, Vainio E, Kumar V and Beutner EH. IgA anti-endomysial antibodies in dermatitis herpetiformis: correlation with jejunal morphology, gluten free diet and anti-gliadin antibodies. *Br J Dermatol* 1987;**117**:185-191.

Reunala T, Koskimies S and Ilonen J. Dermatitis herpetiformis in identical twins [abstract]. *J Invest Dermatol* 1991;**96**:1023.

Reunala T. Incidence of familial dermatitis herpetiformis. *Br J Dermatol* 1996;**134**:394-398.

Sachs JA, Leonard JN, Awad J, McCloskey D, Festenstein H, Hitman GA and Fry L. A

comparative serological and molecular study of linear IgA disease and dermatitis herpetiformis. *Br J Dermatol* 1988;**118**:759-764.

Savilahti E, Perkkiö M, Kalimo K, Viander M, Vainio E and Reunala T. IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet* 1983;**i**:320-322.

Savilahti E, Arato A and Verkasalo M. Intestinal γ/δ receptor-bearing T lymphocytes in celiac disease and inflammatory bowel diseases in children. Constant increase in celiac disease. *Pediatr Res* 1990;**28**:579-581.

Savilahti E, Reunala T and Mäki M. Increase of lymphocytes bearing the γ/δ T cell receptor in the jejunum of patients with dermatitis herpetiformis. *Gut* 1992;**33**:206-211.

Savilahti E, Örmälä T, Arato A, Hacsek G, Holm K, Klemola T, Nemeth A, Mäki M and Reunala T. Density of γ/δ positive T cells in the jejunal epithelium of patients with coeliac disease and dermatitis herpetiformis is increased with age. *Clin Exp Immunol* 1997;**109**:464-467.

Seah PP, Fry L, Hoffbrand AV and Holborow EJ. Tissue antibodies in dermatitis herpetiformis and adult coeliac disease. *Lancet* 1971;**i**:834-836.

Seah PP, Fry L, Rossiter MA, Hoffbrand AV and Holborow EJ. Antireticulin antibodies in childhood coeliac disease. *Lancet* 1971;**ii**:681-2.

Seah PP, Fry L, Stewart JS, Chapman BL, Hoffbrand AV and Holborow EJ. Immunoglobulins in the skin in dermatitis herpetiformis and coeliac disease. *Lancet* 1972;**i**:611-14.

Selby WS, Janossy G and Jewell DP. Immunohistological characterisation of intraepithelial lymphocytes of the human gastrointestinal tract. *Gut* 1981;**22**:169-176.

Selby WS, Janossy G, Goldstein G and Jewell DP. T lymphocyte subsets in human intestinal mucosa: the distribution and relationship to MHC-derived antigens. *Clin Exp Immunol* 1981;**44**:453-458.

Selby WS, Janossy G, Bofill M and Jewell DP. Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease. *Clin Exp Immunol* 1983;**52**:219-228.

Shidrawi RG, Przemioslo R, Davies DR, Tighe MR and Ciclitira PJ. Pitfalls in diagnosing coeliac disease. *J Clin Pathol* 1994;**47**:693-94.

Shuster S, Watson AJ and Marks J. Coeliac syndrome in dermatitis herpetiformis. *Lancet* 1968;**i**:1101-1106.

Sigurgeirsson B, Agnarsson BA and Lindelöf B. Risk of lymphoma in patients with dermatitis herpetiformis. *Br Med J* 1994;**308**:13-15.

Sikora K, Anand BS, Truelove SC, Ciclitira PJ and Offord RE. Stimulation of lymphocytes

from patients with coeliac disease by a subfraction of gluten. *Lancet* 1976;**ii**:389-91.

Silk DBA, Mowat NAG, Riddell RH and Kirby JD. Intestinal lymphoma complicating dermatitis herpetiformis. *Br J Dermatol* 1977;**96**:555-560.

Solheim BG, Ek J, Thune PO, Baklien K, Bratlie A, Rankin B, Thoresen AB and Thorsby E. HLA antigens in dermatitis herpetiformis and coeliac disease. *Tissue Antigens* 1976;**7**:57-59.

Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F and Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ α/β heterodimer. *J Exp Med* 1989;**169**:345-350.

Sollid LM and Thorsby E. HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* 1993;**105**:910-922.

Sottini A, Imberti L, Gorla R, Cattaneo R and Primi D. Restricted expression of the T-cell receptor Vβ but not Vα genes in rheumatoid arthritis. *Eur J Immunol* 1991;**21**:461-466.

Spencer J, Isaacson PG, Diss TC and MacDonald TT. Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor gamma/delta heterodimer in human intestinal intraepithelial lymphocytes. *Eur J Immunol* 1989;**19**:1335-1338.

Spurkland A, Sollid LM, Polanco I, Vartdal F and Thorsby E. HLA-DR and –DQ genotypes of celiac disease patients serologically typed to be non-DR3 or non-DR5/7.

Hum Immunol 1992;**35**:188-92.

Spurkland A, Ingvarsson G, Falk ES, Knutsen I, Sollid LM and Thorsby E. Dermatitis herpetiformis and celiac disease are both primarily associated with the HLA-DQ (α1*0501, β1*02) or the HLA-DQ (α1*03, β1*0302) heterodimers. *Tissue Antigens* 1997;**49**:29-34.

Strober W. Immunogenetic factors: In Katz SI (moderator). Dermatitis herpetiformis: the skin and the gut. *Ann Intern Med* 1980;**93**:857-874.

Swerdlow AJ, Whittaker S, Carpenter LM and English JSC. Mortality and cancer incidence in patients with dermatitis herpetiformis: a cohort study. *Br J Dermatol* 1993;**129**:140-144.

Swinson CM, Slavin G, Coles EC and Booth CC. Coeliac disease and malignancy. *Lancet* 1983;**i**:111-115.

Thomsen M, Platz P, Marks J, Ryder LP, Shuster S, Svejgaard A and Young SH. Association of LD-8a and LD-12a with dermatitis herpetiformis. *Tissue Antigens* 1976;**7**:60-62.

Tighe MR, Hall MA, Ashkenazi A, Siegler E, Lanchbury JS and Ciclitira PJ. Celiac disease among Ashkenazi Jews from Israel. A study of the HLA class II alleles and their associations with disease susceptibility. *Hum Immunol* 1993;**38**:270-276.

Tönder M, Sörlie D and Keaney M. Adult coeliac disease. A case with ulceration, dermatitis herpetiformis and reticulosarcoma. *Scand J Gastroenterol* 1976;**11**:107-111.

Troncone R, De Berardinis P, Mazzarella G, Gianfrani C, Maiuri L and Auricchio S. T cell Receptor (TCR) Vβ usage in small intestinal biopsies of untreated coeliac patients. *J Pediatr Gastroenterol Nutr* 1995;**20**:474.

Unsworth DJ, Johnson GD, Haffenden G, Fry L and Holborow EJ. Binding of wheat gliadin *in vitro* to reticulin in normal and dermatitis herpeiformis skin. *J Invest Dermatol* 1981:**76**:88-93.

Unsworth DJ, Payne AW, Leonard JN and Fry L. IgA in dermatitis herpetiformis skin is dimeric. *Lancet* 1982;**i**:478-480.

Unsworth DJ, Walker-Smith JA, McCarthy D and Holborrow EJ. Studies on the significance of the R1 anti-reticulin antibody associated with gluten sensitivity. *Int Arch Allergy Appl Immunol* 1985;**76**:47-51.

Unsworth DJ, Leonard JN, Hobday DM, Griffiths CEM, Powles AV, Haffenden GP and Fry L. Gliadins bind to reticulin in a lectin-like manner. *Arch Dermatol Res* 1987;**279**:232-5.

Unsworth DJ, Würzner R, Brown DL and Lachmann PJ. Extracts of wheat gluten activate complement via the alternative pathway. *Clin Exp Immunol* 1993;**94**:539-543.

Unsworth DJ and Brown DL. Serological screening suggests that adult coeliac disease is underdiagnosed in the UK and increases the incidence by up to 12%. *Gut* 1994;**35**:61-64.

Vainio E, Kalimo K, Reunala T, Viander M and Palosuo T. Circulating IgA- and IgG-class

anti-gliadin antibodies in dermatitis herpetiformis detected by enzyme-linked immunosorbent assay. *Arch Dermatol Res* 1983;**275**:15-18.

van der Meer JB. Granular deposits of immunoglobulins in the skin of patients with dermatitis herpetiformis. An immunofluorescent study. *Br J Dermatol* 1969;**81**:493-503.

van Tongeren JH, van der Staak and Schillings PHM. Small-bowel changes in dermatitis herpetiformis. *Lancet* 1967;**i**:218.

Viney JL, Prosser HM, Hewitt CRA Lamb JR and Owen MJ. Generation of monoclonal antibodies against a human TCR β chain expressed in transgenic mice. *Hybridoma* 1992;**11**:701-713.

Weetman AP, Burrin JM, Mackay D, Leonard JN, Griffiths CEM and Fry L. The prevalence of thyroid autoantibodies in dermatitis herpetiformis. *Br J Dermatol* 1988;**118**:377-383.

Weinstein WM, Brow JR, Parker F and Rubin CE. The small intestinal mucosa in dermatitis herpetiformis. *Gastroenterology* 1971;**60**:362-369.

White AG, Barnetson RStC, Da Costa JAG and McLelland BDL. The incidence of HL-A antigens in dermatitis herpetiformis. *Br J Dermatol* 1973;**89**:133-136.

Wright DH, Jones DB, Clark H, Mead GM, Hodges E and Howell WM. Is adult-onset coeliac disease due to a low-grade lymphoma of intra-epithelial T lymphocytes? *Lancet* 1991;**337**:1373-1374.

Yamamura M, Wang X-H, Ohmen JD, Uyemura K, Rea TH, Bloom BR and Modlin RL. Cytokine patterns of immunologically mediated tissue damage. *J Immunol.* 1992; **149**: 1470-1475.

Yunis J and Ahmed AR. Immunogenetics of dermatitis herpetiformis. In: AR Ahmed, S Jablonska, eds. *Clinics in Dermatology* 1991;**9**:341-346.

Zhong F, McCombs CC, Olson JM, Elston RC, Stevens FM, McCarthy and Michalski JP. An autosomal screen for genes that predispose to celiac disease in the western counties of Ireland. *Nature Genetics* 1996;**14**:329-333.

Zone JJ, LaSalle BA and Provost TT. Circulating immune complexes of IgA type in dermatitis herpetiformis. *J Invest Dermatol* 1980;**75**:152-155.