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An Investigation Of The Humoral Immune Response In Patients With Gluten Ataxia

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A thesis submitted in partial fulfilment of the requirements of

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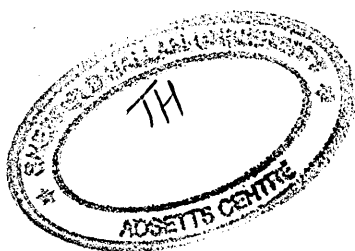
ABSTRACT

It is now well established that gluten sensitivity comprises a spectrum of disorders, which affect different target organs. The small-intestine (coeliac disease), skin (dermatitis herpetiformis) and peripheral and central nervous systems are most frequently affected. These manifestations may occur alone or in combination with one another.

Neurological complications affect approximately 6-10% of patients with gluten sensitivity with ataxia being the most frequent disorder seen in these patients. It has recently been established that previously undiagnosed gluten sensitivity may present solely with ataxia (gluten ataxia) and this disease entity may account for a large number of patients with sporadic idiopathic ataxia. Preliminary findings suggest an immune pathogenesis for gluten ataxia, in common with other manifestations of gluten sensitivity. The studies in this thesis are concerned with investigation of the humoral immune response in the pathogenesis of gluten ataxia.

Assessment using enzyme-linked immunosorbent assay, immunohistochemistry and western blotting has shown the presence of elevated levels of circulating gluten sensitivity associated antibodies (anti-gliadin and anti-tissue transglutaminase antibodies) in patients with gluten ataxia. Studies have also shown the cross-reaction of anti-gliadin antibodies with epitopes on cerebellar Purkinje cells. In addition, patients with gluten ataxia possess circulating antibodies directed against cerebellar Purkinje cells, which are distinct from anti-gliadin antibodies although the target antigen remains unknown. Finally, studies have also shown that patients with gluten ataxia have elevated levels of circulating antibodies directed against glutamic acid decarboxylase.

These studies suggest a role for the humoral immune response in gluten ataxia. However, preliminary evidence is also suggestive of a T-cell mediated response and the relative contributions of each in the pathogenesis of gluten ataxia remains to be elucidated.

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ABBREVIATIONS

ABC – Avidin biotin complex

AEA – Anti-endomysial antibody

AGA – Anti-gliadin antibody

AJA – Anti-jejunal antibody

ALP – Alkaline phosphatase

APC – Antigen presenting cell

APS – Ammonium persulphate

ARA – Anti-reticulin antibody

ATG2A – Anti-tissue transglutaminase antibody

BCA - Bicinchoninic acid

BSA – Bovine serum albumin

CANTAB - Cambridge Automated Neuropsychological Test Battery

CD – Coeliac disease

CNS – Central nervous system

COX – Cyclo-oxygenase

CSF – Cerebrospinal fluid

%CV – Percentage coefficient of variation

DAB - 3,3'-Diaminobenzidine

DC – Dendritic cell

d.H$_2$O – Distilled water

DH – Dermatitis Herpetiformis

DRPLA – Dentatorubral pallidoluysian atrophy

EATL – Enteropathy associated intestinal T-cell lymphoma

ECM – Extracellular matrix

ELISA – Enzyme-linked immunosorbent assay

ESPGAN – European Society of Paediatric Gastroenterology and Nutrition

GA – Gluten ataxia

GABA - γ aminobutyric acid

GAD – Glutamic acid decarboxylase

GFD – Gluten-free diet

GI – Gastrointestinal

HLA – Human leukocyte antigen

HRP – Horseradish peroxidase

IDDM – Insulin dependent diabetes mellitus

IEL – Intraepithelial lymphocyte

IFN - Interferon

Ig – Immunoglobulin

IHC - Immunohistochemistry

IL – Interleukin

kDa – Kilodalton

KGF – Keratinocyte growth factor

M – Molar

mg - Milligram

MIC – Major histocompatibility class I chain related gene

MIF – Migration inhibition factor

mins – Minutes

ml - Millilitre

mM - Millimolar

MMP – Matrix metalloproteinase

MRI – Magnetic resonance imaging

mRNA – Messenger ribonucleic acid

MS – Multiple sclerosis

MSA-C - Cerebellar variant of multiple sytem atrophy

ng - Nanogram

nm - Nanometre

***p*-NPP** - *p*-nitrophenylphosphate

OD – Optical density

PBS – Phosphate buffered saline

PBST – PBS-Tween

PCD – Paraneoplastic cerebellar degeneration

RIA – Radioimmunoassay

RMM – Relative molecular mass

rpm – Revolutions per minute

RT – Room temperature

SCA – Spinocerebellar ataxia

SD – Standard deviation

SDS - Sodium dodecylsulphate

SDS-PAGE - Sodium dodecylsulphate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SPS – Stiff-person syndrome

TBS – Tris buffered saline

TCR – T-cell receptor

TEMED - N, N, N', N' – tetramethylethylenediamine

TG2 – Tissue transglutaminase

TGF – Transforming growth factor

TIMP – Tissue inhibitor of matrix metalloproteinase

TMB - 3,3'5,5'-tetramethylbenzidine

TNF – Tumour necrosis factor

v/v – Volume/volume

w/v – Weight/volume

Yrs - Years

μg – Microgram

μl - Microlitre

μm - Micrometre

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Ataxia

Ataxia is a term used to describe abnormalities in the co-ordination and accuracy of execution of voluntary movements. It may manifest at any age and has a number of causes. Ataxia results from disruption of the normal functioning of the cerebellum.

1.2 The Cerebellum

The cerebellum (little brain, *Latin*) is located at the base of the brain and constitutes approximately 10% of the brain's total volume (Kandel *et al.,* 1991).

1.2.1 The function of the cerebellum

As early as 1809, it was noted that cerebellar disruption resulted in disturbance of posture and voluntary movements (Middleton & Strick, 1998). It is now known that the cerebellum plays an important role in the control and timing of voluntary movement (Fonnum & Lock, 2000). Generally, the cerebellum has a regulatory, stabilising role, co-ordinating different muscle groups so that actual movements precisely match the intended action. It continually receives information about *intended* movement from other parts of the brain (internal feedback) as well as information about *actual* movement from peripheral sources (external feedback). Using this information, the cerebellum compares the intended action with what is actually happening and makes any necessary adjustments, through modulation of motor tracts, to ensure the intended action is executed successfully (Kandel *et al.,* 1991).

1.2.2 The functional structure of the cerebellum

The cerebellum is composed of an outer layer of grey matter (the cerebellar cortex), internal white matter and three pairs of deep nuclei (fastigial, interposed and dentate) situated in its core. The cerebellum is divided into three functional areas: the vestibular cerebellum, the spinocerebellum and cerebrocerebellum, each containing neurones that are arranged in a highly regular, repetitive pattern. Each of these areas acts on inputs from different areas of the brain as well as also sending outputs to distinct areas (Kandel *et al.,* 1991). The cerebellar cortex is pivotal to cerebellar function in receiving, processing and outputting information. The cortex itself is composed of three layers: the molecular layer, the Purkinje cell layer and the granular layer (figure 1.1) and contains five types of neurones: stellate, basket, Purkinje, Golgi and granule cells (figure 1.2). There are two different input systems into the cerebellar cortex, both

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Figure 1.1 Haemotoxylin and eosin stain of a section of the human cerebellar cortex
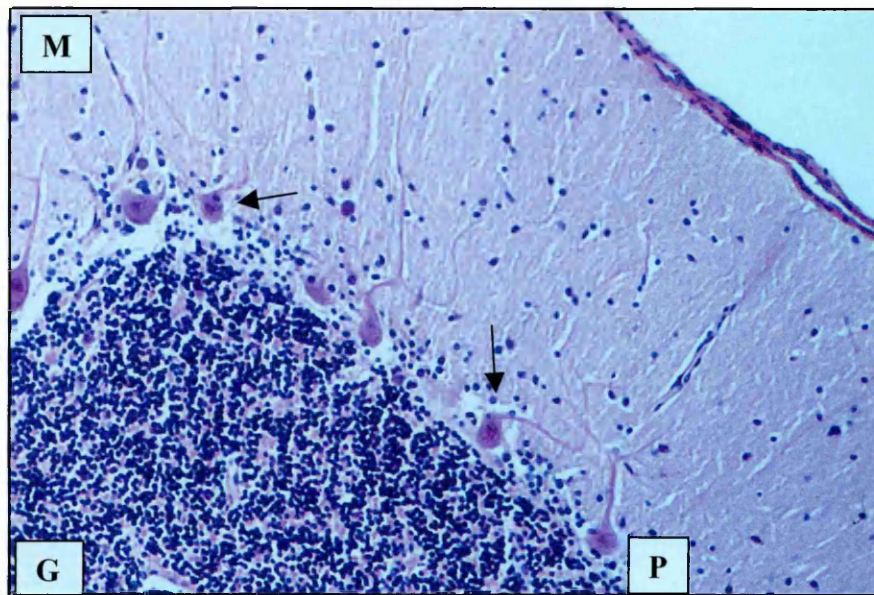
A paraffin-embedded section of post-mortem human cerebellar tissue stained with haemotoxylin and eosin. The molecular (M), granular (G) and Purkinje cell (P) layers of the cerebellar cortex are clearly visible. Purkinje cells are indicated by arrows. *Photograph courtesy of Dr. M. Hadjivassiliou.*

Figure 1.2 The structure of the cerebellar cortex
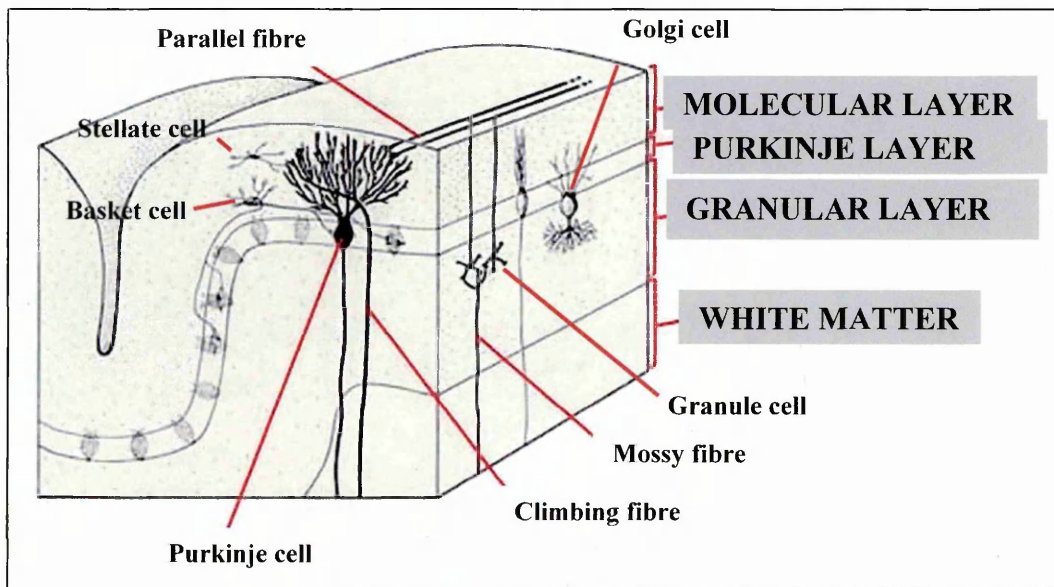
Taken from www.Purkinjeworld.com

having the end-result of exciting Purkinje cells, which are the sole output of the cerebellar cortex (Carpenter, 1996; Kandel *et al.,* 1991).

1.2.2.1 Cerebellar Purkinje cells

Purkinje cells are one of the largest neurones of the central nervous system (CNS) with a cell body diameter of 50-80μm. They are unmyelinated, inhibitory neurones and use γ-aminobutyric acid (GABA) as their neurotransmitter. During development, Purkinje cells form a multi-cellular layer between the molecular and granular layers of the cerebellar cortex. In early post-natal life, the Purkinje layer undergoes numerous changes and forms a unicellular structure consisting of a side by side arrangement of Purkinje cell bodies in the Purkinje cell layer with thick dendrites extending into the molecular layer. There are approximately 7-14 x10^6 Purkinje cells in the adult human cerebellum, the numbers of which remain relatively constant throughout life (Kandel *et al.,* 1991). However, some apoptotic death does occur and, as with all neurones, some degree of age-related loss is expected in individuals over 70 years of age (Goldowitz & Hamre, 1998).

1.2.2.2 Cerebellar connections: pathways to and from the cerebellar cortex

The major afferent input of the cerebellar cortex comes from mossy fibres that excite Purkinje cells indirectly. Upon entering the cerebellar cortex, mossy fibres excite local populations of granule cells within the granular layer. Ascending to the molecular layer the granule cell axons make connections with nearby Purkinje cells then, within the molecular layer, they split into two thin fibres (parallel fibres) that run in opposite directions intersecting with the Purkinje cell dendrites (figure 1.2). Each granule cell receives input from many mossy fibres and, in turn, contacts a large number of Purkinje cells making this input system very diffuse and non-specific. This is in contrast to the second afferent input system of the cerebellar cortex. The second input into the cerebellar cortex is provided by climbing fibres, which excite Purkinje cells directly. Climbing fibres enter the cortex and wrap around Purkinje cells forming numerous synaptic contacts, predominantly on the Purkinje cell dendrites (figure 1.2). These contacts are highly excitatory, each climbing fibre contacts only 1-10 Purkinje cells and each Purkinje cell receives input from only one climbing fibre. Inhibitory controls over these input systems are provided by Golgi cells, which inhibit granule cells, and basket and stellate cells, which inhibit Purkinje cells. Purkinje cells are the sole output of the cerebellar cortex and are directly connected to the deep cerebellar nuclei, which in turn

feed out to other regions of the brain (Carpenter, 1996; Kandel *et al.,* 1991). These connections are summarised in figure 1.3.

1.3 Symptoms Of Ataxia

Damage to the cerebellum results in characteristic signs and symptoms, related to which of the three functional areas is affected. Symptoms vary between individuals and according to the type of ataxia. Common symptoms include abnormal jerky eye-movements (nystagmus), difficulties of postural co-ordination (eg. difficulties in standing upright, seating) and problems with abnormal gait (eg. unsteadiness when walking and the need to walk with the feet placed widely apart), attributable to damage of the vestibulocerebellum. Damage to other cerebellar areas also causes more generalised co-ordination problems. Frequently, difficulty co-ordinating all kinds of movement is experienced (asynergia), often in association with a loss of muscle tone (hypotonia). Consequently, individuals with ataxia often 'overshoot' movements (dysmetria), for example reaching out too far to pick up an object from a table. The overshoot is corrected by an overshoot in the opposite direction and a cycle is set up resulting in an action tremor around the desired position (intention tremor). Other symptoms include difficulties in executing rapidly alternating tasks with the limbs and difficulty co-ordinating speech resulting in slurring (dysarthria) (Carpenter, 1996; Kandel *et al.,* 1991).

1.4 Causes Of Ataxia

Ataxic conditions may be inherited, acquired or be of unknown cause.

Inherited ataxias comprise a wide group of disorders, which have a collective prevalence of approximately 6-7 individuals per 100,000 population (Albin, 2003). They are divided into autosomal dominant and autosomal recessive ataxias depending on their mode of inheritance with each class able to be further subdivided according to the mechanism of genetic mutation (Albin, 2003). One major group of dominantly inherited ataxias is the spinocerebellar ataxias (SCAs), which are assigned different numbers according to the specific associated gene or chromosomal locus (Mariotti & Di Donato, 2001). The SCAs are caused by primarily tri, but also tetra or penta, nucleotide repeats in either coding or non-coding regions of specific genes, which results in the formation of aberrant proteins with 'toxic' functions and consequent neuronal cell death

Figure 1.3 A summary of the inputs/outputs and actions of the three functional areas of the cerebellum

INPUTS

CEREBELLUM

OUTPUTS

ACTIONS

MEDULLA

SPINAL CORD

VISUAL & AUDITORY SYSTEMS

CEREBRAL CORTEX

VESTIBULO-CEREBELLUM

SPINO-CEREBELLUM

CEREBRO-CEREBELLUM

VESTIBULAR NUCLEI (in medulla)

INTERPOSED NUCLEUS

FASTIGIAL NUCLEUS

DENTATE NUCLEUS

LATERAL DESCENDING SYSTEMS

MEDIAL DESCENDING SYSTEMS

MOTOR & PRE-MOTOR CORTICES

BALANCE & EYE MOVEMENT

MOTOR EXECUTION

MOTOR PLANNING & INITIATION

(Carpenter, 1996; Kandel *et al.*, 1991)

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and degeneration (Albin, 2003; Mariotti & Di Donato, 2001). The affected proteins and associated locus have now been discovered for some, but not all, SCAs (Mariotti & Di Donato, 2001). Other dominantly inherited ataxias are dentatorubral pallidoluysian atrophy (DRPLA), which is also caused by a trinucleotide repeat, and the episodic ataxias (Albin, 2003). There are four types of episodic ataxias, all of which are the result of mutations in genes encoding ion channel proteins. Types 1 and 2 result in dysfunction of potassium and calcium channels respectively and the genes affected in type 3 and 4 have yet to be isolated (Albin, 2003). The recessive ataxias are also a heterogeneous group, which commonly result from a loss of function of various gene products (Albin, 2003). The most common of this group is Friedreich's ataxia, caused by a trinucleotide repeat in the frataxin gene, but other recessive ataxias include ataxia associated with a primary vitamin E deficiency and ataxia-telangiectasia (Albin, 2003; Mariotti & Di Donato, 2001).

Acquired ataxias may arise as a secondary result of several different conditions. These include alcoholism, vitamin deficiencies, malignancy (paraneoplastic syndromes) and other neurological disorders (e.g. multiple sclerosis, MS) (Mariotti & Di Donato, 2001).

A further, significant proportion of ataxia cases occur sporadically and the cause remains unknown (idiopathic ataxia). A subgroup of these patients have been reported to possess circulating antibodies directed against glutamic acid decarboxylase (GAD) and it has been suggested that, in these cases, the ataxia may have an immune-mediated pathogenesis. The association between ataxia and circulating anti-GAD antibodies is described in more detail in section 6.1.2.4. It is also known that ataxia is the most common neurological manifestation of gluten sensitivity and that recent research has suggested that a significant number of cases of sporadic idiopathic ataxia may be due to previously undetected gluten sensitivity (gluten ataxia) (Hadjivassiliou *et al.*, 1996; 1998). Identification of a cause is important, as these cases of ataxia may be potentially treatable or preventable if the aetiology can be determined.

1.5 The modern epidemic of gluten sensitivity

Grain has only been a significant part of mans' diet since the agricultural revolution, a consequential event of the climate change during the transition from the Paleolithic to the Neolithic age, approximately ten thousand years ago (Diamond, 2002; Greco, 1997;

Lev-Yadun et al., 2000). Efficient agricultural systems are believed to have started within the 'Fertile Crescent' (a wide area including southern Turkey, Palestine, Lebanon and northern Iraq) (Diamond, 2002; Greco, 1997; Lev-Yadun et al., 2000). Advantages including the physical properties of grain (easy storage, plentiful harvests) and the sense of wellbeing induced after ingestion of wheat (from exorphins, partially digested peptides that exert morphine-like effects) resulted in its growth in popularity as a food source, despite an initial decline in the health and stature amongst peoples of the first farming communities (Diamond, 2002; Fukudome & Yoshikawa, 1992, 1993; Greco, 1997; Zioudrou et al., 1979). Thereafter, agricultural practices were readily accepted and spread rapidly throughout the Mediterranean region before finally reaching Northern and Western Europe approximately five thousand years ago (Greco, 1997).

During this period, grains themselves also underwent extensive artificial selection in order to adapt to the changing demands of agriculture and large-scale cultivation. These changes resulted in the eventual production of *Triticum Aestivum*, the progenitor of all modern wheat species, of which there are over twenty thousand. Todays' wheat contains extremely large quantities of gluten (the storage protein of wheat) as a response to the current demands for a product that is well adapted for baking and easily handled by modern agricultural machinery (Greco, 1997). The inclusion of grains in mans' diet, therefore, occurred extremely late on in evolutionary terms and many populations have consumed them for only several thousand years. As may be expected, not everybody adapted to these dramatic changes over such a short time period and a proportion of humans could not recognise gluten as a 'tolerable' protein and developed gluten sensitivity. Although strictly referring to the toxic proteins of wheat, the term 'gluten' used in the context of gluten sensitivity within this thesis also encompasses the analogous toxic proteins, hordeins and secalins, found in barley and rye respectively.

It is now known that gluten sensitivity is a complex, immune-mediated condition with a strong genetic association. Onset is triggered by the combination of genetic susceptibility and the interactions with various environmental factors. Gluten sensitivity has a highly variable clinical spectrum (figure 1.4). The first described manifestation of gluten sensitivity was that affecting the small intestine (coeliac disease, CD) and with many symptoms related to the gastrointestinal (GI) tract or malabsorption, it was long assumed that the gut was the sole target organ. However, it is now well-established that this is not always the case and that the condition may manifest as

different diseases in areas other than the small intestine, most commonly the skin (dermatitis herpetiformis, DH) and the nervous system. These manifestations share certain aetiological genetic and environmental factors and may occur alone or in combination with one another, with differing severity, in the same individual.

Research into gluten sensitivity has advanced considerably in recent years and, reflecting the wide clinical spectrum, gluten sensitivity is defined as *'a state of heightened immunological responsiveness to ingested gluten proteins in genetically predisposed individuals'* (Marsh, 1995).

Figure 1.4 The spectrum of gluten sensitivity

Neurological Dysfunction
(peripheral and central nervous systems)
Symptoms include: Ataxia, peripheral neuropathy, epilepsy, migraine

Coeliac Disease
(small intestine)
Symptoms include:
Diarrhoea/constipation, abdominal pain & bloating, fatigue, weight-loss, vomiting, loss of appetite

Dermatitis Herpetiformis
(skin)
Symptoms: Itchy vesicular rash, predominantly on elbows, buttocks, knees

Gluten sensitivity has a wide-ranging clinical spectrum. Target organs are diverse and the condition commonly affects the intestine, skin and nervous system. These manifestations may occur alone or in combination with one another. Overlapping areas demonstrate co-existing manifestations previously reported in literature (not to scale).

1.6 Epidemiology of gluten sensitivity

Early studies suggested that CD was an uncommon condition affecting approximately 1 in 1000 people or less within Europe (Feighery, 1999; Guandalini & Gupta, 2002). However, it soon became apparent that clinically recognised cases of CD were greatly outnumbered by undiagnosed cases of asymptomatic or more atypical forms of CD and the concept of the CD 'iceberg' was proposed (figure 1.5).

Figure 1.5 The coeliac disease 'iceberg'
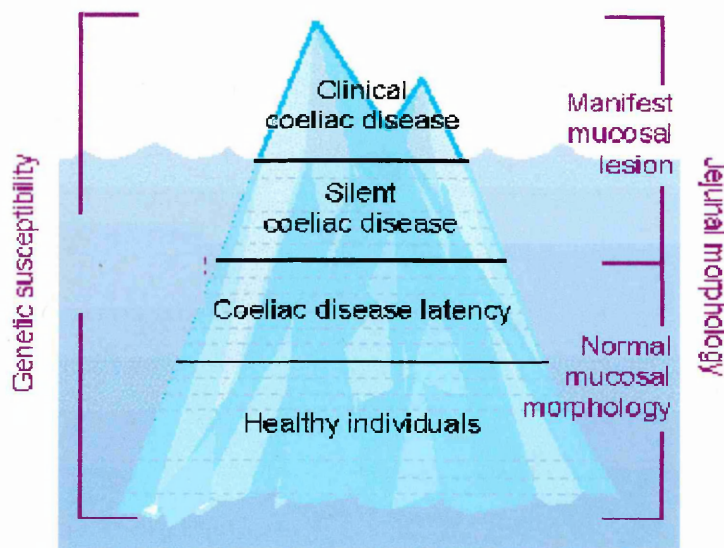
At present, clinically diagnosed cases of CD (the tip of the iceberg) are vastly outnumbered by unrecognised asymptomatic or atypical cases. Severe mucosal damage is not a prerequisite for diagnosis of CD although genetic susceptibility is a common factor. In addition, a large number of otherwise healthy individuals who are genetically susceptible to CD may be considered as potentially gluten sensitive.
Adapted from Maki & Collin, 1997.

Large population screening studies have confirmed a much higher prevalence of CD (table 1.1), many suggesting that up to 1% of the population may be affected. Data suggests that for every clinically diagnosed case, another 3-14 times more cases may be detected by screening (Bramwell Cook *et al.*, 2000; Catassi *et al.*, 1996, 1997; Csizmadia *et al.*, 1999; Johnston *et al.*, 1998).

Table 1.1 Reported prevalence of coeliac disease determined by screening studies

Area	Population screened	n	Method of screening*	Biopsy proven (Y/N)	Prevalence	Reference
United Kingdom	(A) General population	1823	(IgA) AGA, AEA, ARA	Y	1:122	Johnston et al., 1998
	(A) General population	1200	(IgA/IgG) AGA, AEA	Y	1:100	Sanders et al., 2002
	(A) General population	7550	AEA	N	1:83	West et al., 2002
Netherlands	(A) General population	1440	AEA, ATG2A, HLA	N	1:227	Schweizer et al., 2002
Finland	(A) General population	1070	(IgA) AEA	Y	1:130	Kohlo et al., 1998
	(C) School children	3654	(IgA) AEA, ATG2A, HLA	Y	1:99	Mäki et al., 2002
Sweden	(A) General population		AGA		1:256	Grodzinsky et al., 1992
Italy	(C) School children	6315	(IgA/IgG) AGA, (IgA) AEA	Y	1:193	Catassi et al., 1997
	(A) General population	3483	(IgA) AEA	Y	1:175	Volta et al., 2001
Sardinia	(C) School children	1607	(IgA/IgG) AGA, (IgA) AEA	Y	1:94	Meloni et al., 1999a
New Zealand	(A) General population	1064	AEA	Y	1:82	Bramwell Cook et al., 2000
Argentina	(A) General population	2000	(IgA/IgG) AGA, (IgA) AEA	Y	1:167	Gomez et al., 2001
Brazil	(C & A) Out-patients	1030	(IgA/IgG) ATG2A, AEA	N	1:52	Galeano et al., 2002
USA	(A) Blood donors	2000	(IgA/IgG) AGA, AEA	N	1:250	Not et al., 1998
	(A) General population	4126	(IgA/IgG) AGA, AEA	N	1:133	Fasano et al., 2003
Iran	(A) Blood donors	2000	(IgA) AGA, (IgA) AEA	Y	1:166	Shahbazkhani et al., 2003

A- Adult, C – Child population. AGA – Anti-gliadin, ARA – Anti-reticulin, ATG2A – Anti-tissue transglutaminase, AEA – Anti-endomysial antibodies. n denotes the number of individuals within the study. *Further details of antibody screening methods are described in sections 1.9 and 3.1.

If genetically susceptible individuals with extra-intestinal manifestations and serological markers of gluten sensitivity but histologically normal intestinal mucosa are also included, the prevalence of gluten sensitivity as a whole is likely to be even greater than 1% of the population. Additionally, a large number of genetically susceptible but otherwise healthy individuals may also be considered at a potential risk (figure 1.5).

Gluten sensitivity tends to be most prevalent in areas where bread and pasta were historically adopted as the major source of carbohydrate. Therefore, it is primarily a disease of European Caucasians and their descendants in countries such as the USA, Australia and New Zealand (Sollid, 2000). Most non-Caucasian populations have a lower prevalence and the condition generally remains rare in sub-Saharan African, Japanese and Chinese populations, even after migration to areas of higher prevalence (Cole & Kagnoff, 1985). However, gluten sensitivity has been reported in populations including those of Asian Indians (Kaur *et al.,* 2002) and Arabs (Neuhausen *et al.,* 2002) and the highest reported prevalence of 1 in 20 was found amongst North African Saharawi people (Catassi *et al.,* 1999). Recent screening studies in such areas suggest that the incidence may, in fact, be similar to many European countries and the USA (Shahbazkhani *et al.,* 2003).

CD has been reported to be more common in women than men (Gomez *et al.,* 2001; Hin *et al.,* 1999) but other studies have suggested that both are affected equally (Bramwell Cook *et al.,* 2000; Johnston *et al.,* 1998; Not *et al.,* 1998). The sex distribution of gluten sensitivity as a whole is not known although neurological manifestations appear to affect both sexes evenly (Hadjivassiliou *et al.,* 2002a; 2003a).

1.7 Genetic susceptibility to gluten sensitivity

A strong genetic component to the aetiology of gluten sensitivity is suggested by the facts that gluten sensitivity has long been noted to run in families, first-degree relatives of sufferers have a 10-15% risk of suffering themselves and there is a high concordance of disease among monozygotic twins of 76% (Greco *et al.,* 2002). An extremely strong association of CD with certain human leukocyte antigen (HLA) class II genes is now well established. Gluten sensitive individuals with DH and gluten ataxia share the same HLA profile as patients with CD (Hadjivassiliou *et al.,* 2003a; Wills *et al.,* 2002). This suggests that gluten sensitivity is associated with a particular susceptible HLA genotype, regardless of the clinical manifestations, which may be a reliable indicator for

the condition. However, the fact that these genes are expressed by approximately 15-30% of the population (Catassi *et al.,* 2002; Torinsson Nalual *et al.,* 2001), yet only a small minority develops gluten sensitivity despite being exposed to similar environmental influences, is evidence for the involvement of other additional susceptibility genes. It is believed that HLA genes account for less than 40% of the heritability of CD (Bevan *et al.,* 1999). The role of additional susceptibility genes in the aetiology of other manifestations of gluten sensitivity has not been investigated.

1.7.1 HLA genes

The HLA class I and class II genes are located on the short arm of chromosome 6 and are inherited together as a haplotype. The HLA system is highly polymorphic, each of the genes possessing multiple alleles. One haplotype is inherited from each parent and the HLA class I and II alleles are expressed co-dominantly (Staines *et al.,* 1994). Initially, gluten sensitivity was associated with HLA class I molecule, B8 (Falchuk *et al.,* 1972; Stokes *et al.,* 1972) but stronger associations were later found with the HLA class II molecules, DR3 and DQ2 (Keuning *et al.,* 1976; Solheim *et al.,* 1976; Tosi *et al.,* 1983).

It is now known that 90-99% of individuals with gluten sensitivity express the DQ2 molecule, the percentage varying somewhat depending on the geographical region (Catassi *et al.,* 2002; Ciclitira & Moodie, 2003; Guandalini & Gupta, 2002; Hadjivassiliou *et al.,* 2003a; Sollid *et al.,* 1989; Wills *et al.,* 2002).

HLA class II molecules are composed of two polypeptide chains, designated α and β. The α-chain and β-chain of the DQ2 molecule are encoded by the DQA1*05 and DQB1*02 alleles respectively. Depending on the genetic make-up of an individual, the DQ2 molecule is, most commonly, encoded in *cis* (where both alleles are on the same chromosome) or in *trans* (where the alleles are located on different chromosomes) (figure 1.6) (Sollid *et al.,* 1989). There is some evidence that encoding of the DQ2 molecule in *trans* confers an increased risk of CD, suggesting that another gene present on the inherited haplotypes may affect the disease risk (Fernandez-Arquero *et al.,* 1995; Mazzilli *et al.,* 1992). Several studies have also identified a gene dosage effect of the DQB1*02 allele whereby individuals who have inherited more than one copy of the allele are also at an increased risk for disease (Lopez-Vazquez *et al.,* 2002; Ploski *et al.,* 1993; Sollid, 2000).

Figure 1.6 Genetic encoding of the HLA class II DQ2 heterodimer
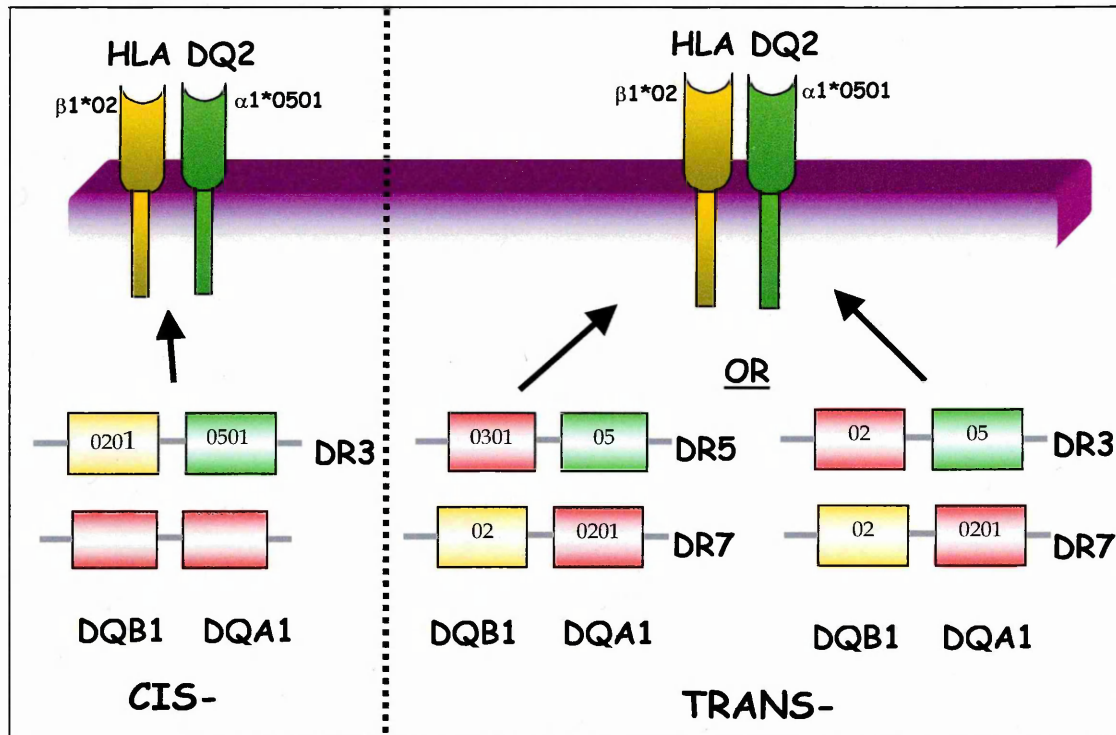
Individuals who are either DR3-DQ2 or DR5/DR7 or DR3/DR7 heterozygous can express the same DQ2 molecule. The heterodimer is either encoded in *cis-* (alleles present on the same chromosome) in DR3-DQ2 individuals or in *trans-* (alleles present on opposite chromosomes) in DR5/DR7 and DR3/DR7 heterozygous individuals. It is rare that the DQ2 heterodimer is encoded by any other than these haplotypes. *Adapted from Sollid, 2002.*

Depending on the population studied, 2-10% of patients with gluten sensitivity do not express the DQ2 heterodimer (Ciclitira & Moodie, 2003; Guandalini & Gupta 2002; Hadjivassiliou *et al.*, 2003a; Sollid, 2000; Wills *et al.*, 2002). It is now known that virtually all of these individuals instead express the DQ8 molecule (encoded by DQA1*03 and DQB1*0302 alleles).

It is accepted that DQ2 and DQ8 HLA class II molecules confer the primary genetic susceptibility to gluten sensitivity. As mentioned, the relative proportions of DQ2 and DQ8-expressing patients appear somewhat dependent on region. For example, in Chile, expression of the DQ8 heterodimer appears more prevalent (Pérez-Bravo *et al.*, 1999), whereas in Northern and Southern Europe 98% and 92% of sufferers respectively express DQ2 (Parnell & Ciclitira, 1999).

Research has focused on the possible effects of other HLA genes that may be inherited on the same haplotypes as the DQ2 or DQ8 alleles. Several specific, sometimes unique, haplotypes containing the DQ2 gene have been found to be expressed more frequently in patients with CD depending on the geographical location. Specific haplotypes have been associated with Asian Indian (Kaur *et al.*, 2002), Sardinian (B18-DR3-DQ2) (Congia *et al.*, 1992) and Turkish (A25-B8-DR18-DQ2) populations (Erkan *et al.*, 1999). Other work has suggested that certain haplotypes may be more associated with atypical forms of CD rather than a typical presentation with GI symptoms (Lopez-Vazquez *et al.*, 2002). Expression of other HLA genes carried on certain haplotypes, such as the DR heterodimer, DR53, which is capable of binding to gliadin peptides with high affinity (Clot *et al.*, 1999), and the DP allele (DPB1*0101) are also reported to increase disease risk and severity (Clot *et al.*, 1999; Polvi *et al.*, 1997).

1.7.2 Non-HLA genes

Despite extensive investigation and concentrated research, to date, no non-HLA genes have been definitively identified as contributing to the risk of gluten sensitivity. This implies that any associated non-HLA genes have only a minor influence on disease susceptibility and that, apart from the HLA class II genes, there is no genetic factor which exerts a significant effect (Ciclitira & Moodie, 2003; Sollid, 2000). Research into this area has mostly been carried out using genome-wide scans to search for disease-associated regions and candidate-gene targeting of specific genes of interest. The results from these studies have so far been mixed and often contradictory.

Genome-wide scans have been carried out on Irish (Zhong *et al.,* 1996), British (Houlston *et al.,* 1997), Italian (Greco *et al.,* 1998), Finnish (Woolley *et al.,* 2002) and Scandinavian (Torinsson Nalual *et al.,* 2001) populations. Regions on chromosomes 4p, 5q, 7q31, 8p, 11q, 11q2, 11p11, 15q26 and 22 were highlighted as of interest on one or more of these studies and are interesting in that they contain genes with various immunological products. Many subsequent linkage studies have been carried out, looking directly at these candidate regions to identify specific genes. Some associations with the CTLA-4 (Djilali-Saiah *et al.,* 1998; Mora *et al.,* 2003; Popat *et al.,* 2002), tumour necrosis factor (TNF) (de la Concha *et al.,* 2000) genes and major histocompatibility class I chain related gene (MIC)-A (Fernandez *et al.,* 2002; Lopez-Vazquez *et al.,* 2002; Rueda *et al.,* 2003) have been reported although not confirmed. Lie and co-workers suggested that a gene in the region D6S2223 on chromosome 6 predisposes individuals to both coeliac disease and insulin-dependent diabetes mellitus (IDDM) (Lie *et al.,* 1999). However, a recent, extremely large, collaborative European study (Louka *et al.,* 2003) failed to identify any additional non-HLA risk-modifying factors on the common DR3-DQ2 susceptibility haplotype, which notably contains the TNF and MIC-A genes as well as the D6S2223 region.

1.8 Environmental aetiological factors of gluten sensitivity

The most critical environmental factor is exposure to gluten and other similar cereal proteins. The major constituents of grains are the outer husk (bran), the germ and the endosperm, which are separated from one another during milling. The storage proteins, which are the toxic agent in gluten sensitivity, are contained within the endosperm (Ciclitira & Moodie, 2003). Their function is to act as stores of the amino acids that are necessary during germination (Molberg *et al.,* 2003). They contain high levels of nitrogen, which in turn relates to the high content of glutamine and proline residues found in the proteins (up to 50% of the total content) (Vader *et al.,* 2002) although other amino acids, such as glutamic acid and aspartic acid are unusually rare (Sollid, 2002). For this reason, the storage proteins are also known as the prolamines. Prolamines from wheat (gluten), rye (secalins) and barley (hordeins) all have a relative molecular mass (RMM) of 30-74kDa (Duggan, 1997).

Gluten is a sticky substance, made up of a complex mixture of many different gliadin and glutenin polypeptides (figure 1.7). All varieties of wheat carry several, linked gluten-encoding genes located in clusters on several different chromosomes (Molberg *et*

al., 2003). Gliadins are monomers (~250-300 residues in length, 20-40kDa) and are of numerous types (Molberg *et al.*, 2003; Sollid, 2002). The gliadins are sub-divided into α,β,γ and ω type gliadins based on their N-terminal sequence and size and mobility on gel electrophoresis (Molberg *et al.*, 2003). Each variety of wheat may contain several different variants of each of these gliadins. Glutenins form large polymeric structures within gluten and are subdivided into high-RMM (~650-800 residues in length, 70-120kDa) and low-RMM (~270-330 residues in length, 30-75kDa) sub-types (Molberg *et al.*, 2003; Sollid, 2002). As with the gliadins many variants exist and several different types may be present in each variety of wheat.

Figure 1.7 The components of gluten

WHEAT PROTEIN

GLOBULINS
(Saline soluble)

GLUTEN
(Ethanol soluble)

ALBUMINS
(Water soluble)

Gliadins
(α,β,γ,ω)

Glutenins

Adapted from Parnell & Ciclitira, 1999.
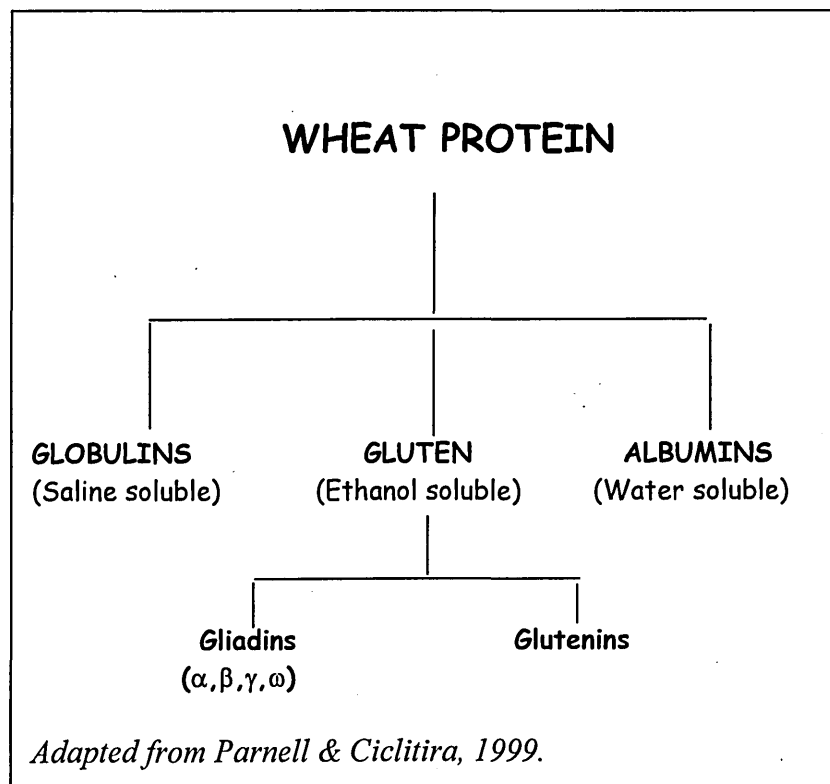
1.9 Diagnosis of gluten sensitivity

Specific guidelines for the diagnosis of CD are in place and certain elements, such as determination of mucosal damage and presence of circulating markers of gluten sensitivity form a central part of the diagnosis of other manifestations of gluten sensitivity too.

The cornerstone of CD diagnosis remains the detection of characteristic mucosal changes that result from the immunological response to gluten by examination of a biopsy taken from the small intestine (Feighery, 1999; Guandalini & Gupta, 2002). The most widely adopted diagnostic criteria for CD are those originally proposed, in 1970, by the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) (Meeuwisse, 1970). Briefly, the guidelines state that a diagnosis of CD should fulfil the following points;

o   The presence of compatible symptoms and the demonstration of a structurally abnormal small intestinal mucosa on a gluten-containing diet (1st intestinal biopsy)

o   A clear, clinical response to a gluten-free diet (GFD)

o   Documentation of unequivocal improvement of the villous structure after following a GFD for approximately 6 months (2nd intestinal biopsy)

o   Deterioration of mucosa and reappearance of symptoms during gluten challenge (3rd intestinal biopsy)

These conditions were revised in 1990 (Walker-Smith *et al.*, 1990). As a result, gluten challenge with a third biopsy is not routinely performed and, in some cases, a complete response to a GFD with a decrease in one or more serological markers, rather than a second biopsy, is sufficient for diagnosis (Ciclitira & Moodie, 2003). The development of reliable assays to detect serological markers (specific antibodies that are produced as a result of the immune response against gluten) of gluten sensitivity has led to major advances in the diagnosis of the condition. These assays have proved particularly useful in detecting genetically susceptible patients with atypical, silent or extra-intestinal manifestations of gluten sensitivity, who may have a histologically normal intestinal mucosa. Currently, anti-gliadin, anti-endomysial and anti-tissue transglutaminase antibody assays are most frequently used for detection of gluten sensitivity. Antibody profile varies among patients and some have no detectable serological markers (Rostami *et al*, 1998), therefore, it is advisable to test for as wide a range of antibodies and isotypes as possible. Further details on serological assays for gluten sensitivity are given in section 3.1.

1.10 Coeliac Disease

'Classic' overt gluten sensitivity, affecting the GI tract, CD, was first described by Samuel Gee more than a century ago in 1888 in his report entitled 'On the Coeliac Affection' (Ciclitira & Moodie, 2003). However, the disease was mentioned as early as the second century AD, by the clinician Aretaeus the Cappadocian, the name coeliac originating from his description in which he used the word 'κοιλιακη' (abdominal, *greek*) (Dowd *et al.*, 1974). The link between CD and dietary factors was first recognised in 1924 with the description by Sidney Haas of a novel treatment in the form of a banana diet, which excluded bread, crackers, potatoes and cereals (Haas, 1924). During the Second World War, a Dutch paediatrician named W.K. Dicke noted that children with CD recovered dramatically during this period of cereal and bread scarcity but relapsed quickly following their re-introduction. Dicke went on to prove in 1950 that the toxicity was caused by gluten (Dicke, 1950).

1.10.1 Symptoms of coeliac disease

CD was historically considered a disease of childhood. Infants and toddlers presented before 2 years of age with classic symptoms including irritability, chronic diarrhoea, failure to thrive, vomiting, abdominal distension, muscle wasting and retarded growth (Challacombe, 1994; Ciclitira & Moodie, 2003; Feighery, 1999; Guandalini & Gupta, 2002). However, an increasing number of cases are being diagnosed in older children and adults in the absence of typical GI symptoms (Bottaro *et al.*, 1999; Feighery, 1999; Visakorpi & Mäki, 1994). It is currently believed that almost 50% of newly diagnosed patients present without GI symptoms but instead with non-specific, often vague symptoms frequently not immediately suggestive of CD or with other, seemingly unrelated conditions (Guandalini & Gupta, 2002).

1.10.2 Treatment of coeliac disease

At present, the only treatment for gluten sensitivity is complete, life-long elimination of wheat, barley and rye from the diet. Although well tolerated in both *in vivo* and *in vitro* studies (Janatuinen *et al.*, 2000, 2002; Kilmartin *et al.*, 2002, 2003; Lundin *et al.*, 2002), elimination of oats too is generally advised (Parnell & Ciclitira, 1999). A GFD provides a dramatic and rapid relief of clinical symptoms followed by improvements in intestinal function and return to histologically normal mucosa for the vast majority of patients (Ciclitira & Moodie, 2003; Parnell & Ciclitira, 1999). The risks of most

conditions and complications associated with untreated CD also improve or disappear after commencement of a GFD. Most commonly, these include risks of nutritional deficiencies, low bone mineral density (Bai *et al.,* 1997; Kemppainen *et al.,* 1999; Mustalahti *et al.,* 1999) and, most importantly, the increased risk of malignancy (particularly enteropathy associated intestinal T-cell lymphoma, EATL) (Holmes *et al.,* 1989; Howdle *et al.,* 2003). Rarely, cases of CD may be non-responsive to a GFD (refractory CD).

1.10.3 The association of coeliac disease with autoimmune diseases

Patients with CD appear to have a greater than expected chance of developing certain autoimmune conditions (table 1.2). It is known that patients with established CD are 3-5 times more likely to develop an associated autoimmune condition, particularly if diagnosed with gluten sensitivity at a later age (Sategna Guidetti *et al.,* 2001; Ventura *et al.,* 1999). The association is thought to reflect a shared genetic background or immune-mediated mechanism between diseases (Feighery, 1999; Maki & Collin, 1997). A GFD does not seem to eliminate the risk, possibly indicating that early ingestion of gluten may somehow modify the immunological response (Sategna Guidetti *et al.,* 2001).

Table 1.2 Prevalence of associated autoimmune diseases among patients with coeliac disease

CONDITION	PERCENTAGE OF SUFFERERS WHO HAVE OR WILL DEVELOP CD	REFERENCE
IDDM	4%	Sategna Guidetti et al., 2001; Ventura et al., 1999
Autoimmune thyroid disorders	14%	Sategna Guidetti et al., 2001
Psoriasis	3%	Sategna Guidetti et al., 2001
Autoimmune hepatitis	1.1%	Ventura et al., 1999

1.10.4 The immune-mediated pathogenesis of coeliac disease

It is now known that CD is the result of an immune response, mounted against gluten, within the small intestine. It is widely believed that this immune response requires a precipitating factor for its full initiation in genetically susceptible individuals on a gluten-containing diet. The exact nature of this factor remains unclear but candidate factors include:

o Abnormal metabolism of gluten (Biagi *et al.*, 1999a) or a shift to deamidation of gluten in the intestine (Arentz-Hansen *et al.*, 2000; Sjostrom *et al.*, 1998) causing a lack of or subsequent loss of oral tolerance to gluten.

o An episode of intestinal hyperpermeability either through infection or altered expression of permeability mediators (eg. zonulin) (Clemente *et al.*, 2003; Fasano *et al.*, 2000).

o Cross-reaction of immune responses against infectious agents with gluten (Nieuwenhuizen *et al.*, 2003).

o Increased dietary gluten (Hernell *et al.*, 2001)

o Selective initiation of an innate immune response against gluten (Maiuri *et al.*, 2003; Schuppan *et al.*, 2003)

Once initiated, the immune process leads to inflammation, loss of intestinal structure and eventual loss of function. A histological image of a CD lesion, showing the disease associated hallmarks of crypt hyperplasia and mucosal flattening (villous atrophy), is shown in figure 1.8. Importantly, these histological and functional changes occur along a continuum (figure 1.9) ranging from a histologically normal mucosa (preinfiltrative) to irreversible hypoplastic atrophic lesions (Marsh, 1995).

The mounting of an intestinal immune response against gluten requires the entry of intact peptides into the tissue for recognition by the immune system. Recent *in vitro* experiments have shown the existence of a highly stable 33-mer α-gliadin peptide, produced by digestion by gastric and pancreatic enzymes, which is able to resist subsequent digestion by small intestinal brush-border enzymes, thus passing into the tissue intact (Shan *et al.*, 2002).

The immune response against gluten is thought to involve an early inflammatory response, activated by regions of gliadin distinct to those recognised by T-cells, followed by an adaptive gluten specific predominantly T-cell mediated response (Maiuri

Figure 1.8 Histological images of a) Normal mucosa and b) A severe coeliac disease associated lesion
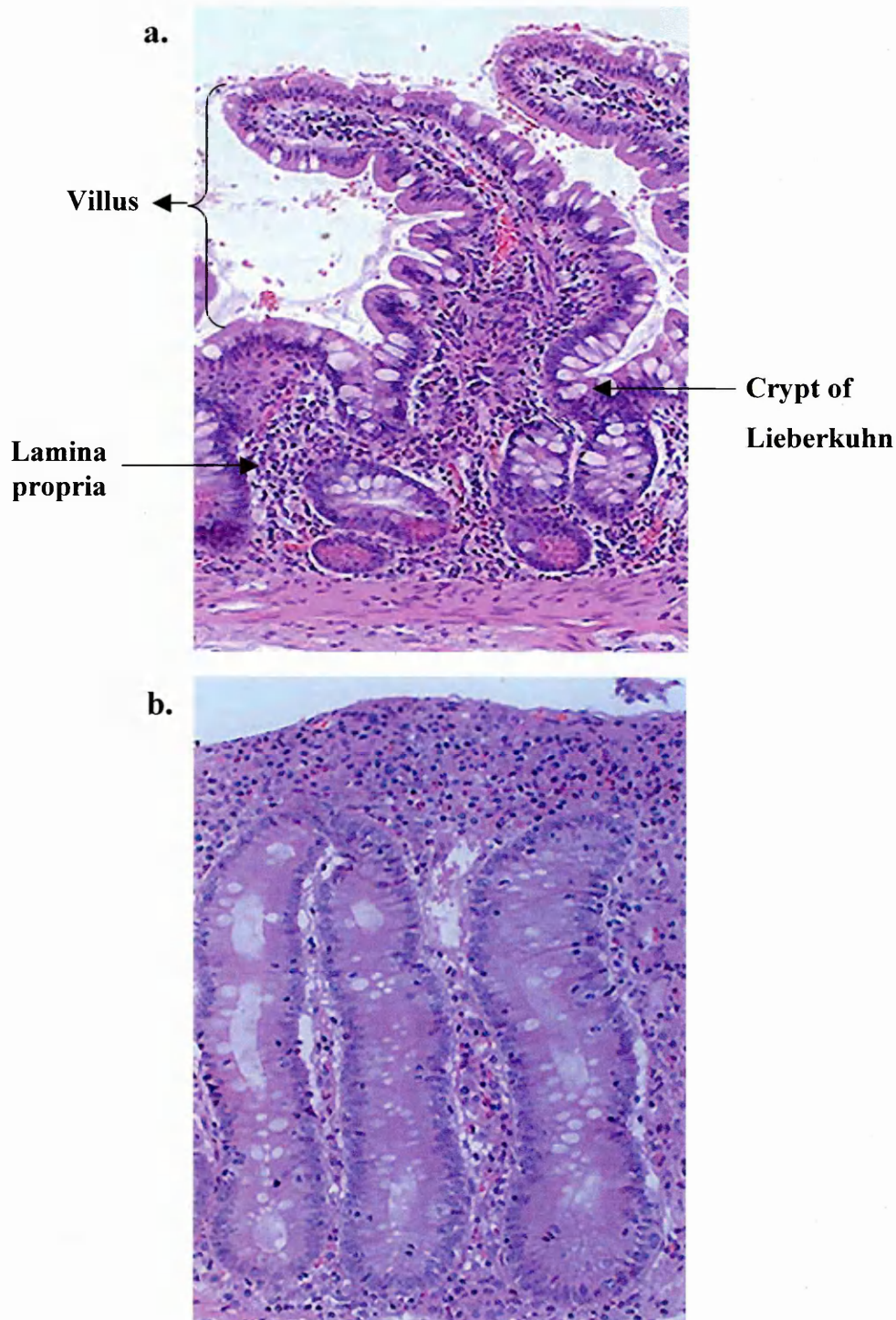
A haemotoxylin and eosin stain of a paraffin-embedded section of a duodenal biopsy. Complete flattening of the mucosa (villous atrophy) and infiltration of T-cells (stained dark blue) into the lamina propria is evident in the coeliac disease-associated lesion (b.).

Adapted from Sollid, 2002.

Figure 1.9 The Marsh Classification of coeliac disease associated mucosal lesions

| Type 0 | Type 1 | Type 2 | Type 3 | Type 4 |
| "PRE-" INFILTRATIVE" | "INFILTRATIVE" | "INFILTRATIVE - HYPERPLASTIC" | "FLAT DESTRUCTIVE" | "ATROPHIC - HYPOPLASTIC" |
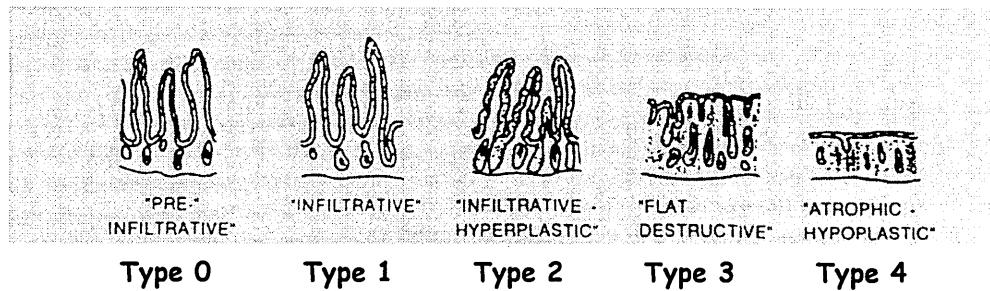
Type 0 (preinfiltrative) lesions have been reported in some gluten sensitive individuals and are associated with a normal mucosa in conjunction with genetic susceptibility and, in some cases, increased gluten sensitivity associated circulating antibodies.

The Type 1 lesion is associated with the infiltration of lymphocytes into the villous epithelium resulting in increased numbers of intraepithelial lymphocytes (IELs) in an otherwise normal mucosa. A Type 2, infiltrative-hyperplastic lesion, has the additional characteristic of hypertrophic crypts. The flat-destructive lesion (Type 3) is the classic 'text-book' lesion with crypt hyperplasia and villous atrophy both evident. This lesion is divided into three sub-types (3a, b and c) depending on the degree of villous atrophy (mild, partial or complete respectively). Type 4, atrophic-hypoplastic, lesions are rarely encountered and most frequently associated with cases of refractory CD or EATL.

Taken from Marsh, 1995.

et al., 1996, 2003; Tuckova *et al.,* 2002). This early response results in a rapid overexpression of HLA-DR on epithelial cells and lamina propria macrophages. Other events include the release of interleukin (IL) -15 (mainly from the macrophage/dendritic type CD68^{+} cells of the lamina propria) and upregulation of cyclo-oxygenase (COX) -2 from macrophages and monocytes as well as increased numbers of activated (CD83^{+}/CD25^{+}) antigen-presenting macrophages, monocytes and dendritic cells (Maiuri *et al.,* 1996, 2003). T-cell migration, observed to occur 12-24 hours after gliadin challenge *in vitro*, then follows. Importantly, activated (CD25^{+}) CD4^{+} T-cells were observed to accumlate in the lamina propria whilst only CD8^{+} T-cells invaded the epithelium, a process which did not require their activation (Maiuri *et al.,* 1996).

Following these early responses, it is thought that lamina propria $\alpha\beta$ CD4^{+} T-cells recognise gluten antigens, presented by HLA class II DQ2 or DQ8 molecules located on specific antigen-presenting cells activated by the innate response, and mount an adaptive immune response (figure 1.10). DQ2 or DQ8 restricted, gluten-reactive CD4^{+} lamina propria T-cells have been isolated and propagated from the intestine of patients with CD but not healthy individuals (Arentz-Hansen *et al.,* 2000; Halstensen *et al.,* 1993; Lundin *et al.,* 1993; Molberg *et al.,* 1997; Nilsen *et al.,* 1995).

1.10.4.1 Presentation of gluten to T-cells by DQ2 and DQ8 molecules

In its native state, gluten does not appear to have the appropriate properties required for successful processing and presentation by DQ2 and DQ8 molecules. Both DQ2 and DQ8 molecules share a preference for binding negatively charged peptides at certain positions of their binding clefts (Godkin *et al.,* 1997; Johansen *et al.,* 1996; Kwok *et al.,* 1996; Vartdal *et al.,* 1996; van de Wal *et al.,* 1996) (figure 1.11). Native gluten peptides have very few negatively charged residues. It is now known that gluten is deamidated in the intestine by the enzyme tissue transglutaminase (TG2) (section 1.10.4.6), thereby converting its abundant glutamine residues to negatively charged glutamic acid residues. This results in the generation of peptides with a greater binding affinity to DQ2 and DQ8 molecules (Molberg *et al.,* 1998; van de Wal *et al.,* 1998). Importantly, proline residues within gluten also facilitate processing and presentation by DQ2 and DQ8 molecules by acting as an anchor residue at the P6 pocket of the DQ2 binding cleft (figure 1.11) or by inducing a structure conducive to binding (Sollid, 2002; Vartdal *et al.,* 1996; van de Wal *et al.,* 1996).

Figure 1.10 Proteins involved in the interaction between T-cells and antigen-presenting cells
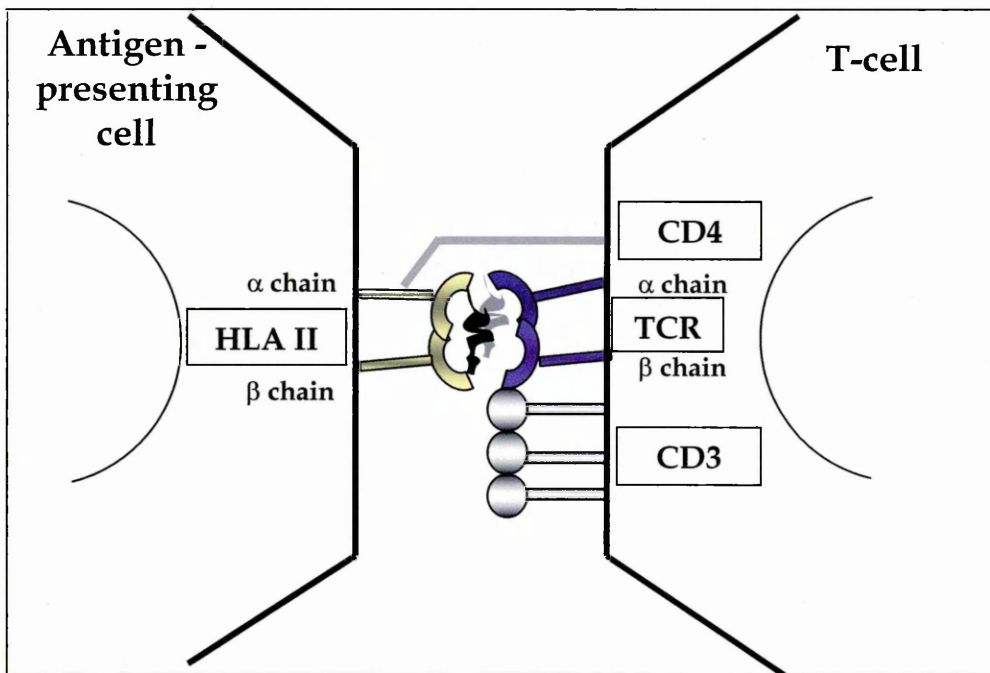
The T-cell receptor (TCR) (composed of an α and β chain) interacts with a peptide presented in an HLA class II molecule (also composed of an α and β chain) on the surface of the antigen-presenting cell. The CD4 molecule stabilises this interaction, allowing a signal to be initiated by the CD3 molecule that eventually activates genes in the T-cell nucleus.

- peptide

Adapted from Staines et al., 1994.

Figure 1.11 The peptide binding cleft of DQ2 indicating the preferred/not preferred

binding positions of peptide glutamic acid and proline residues
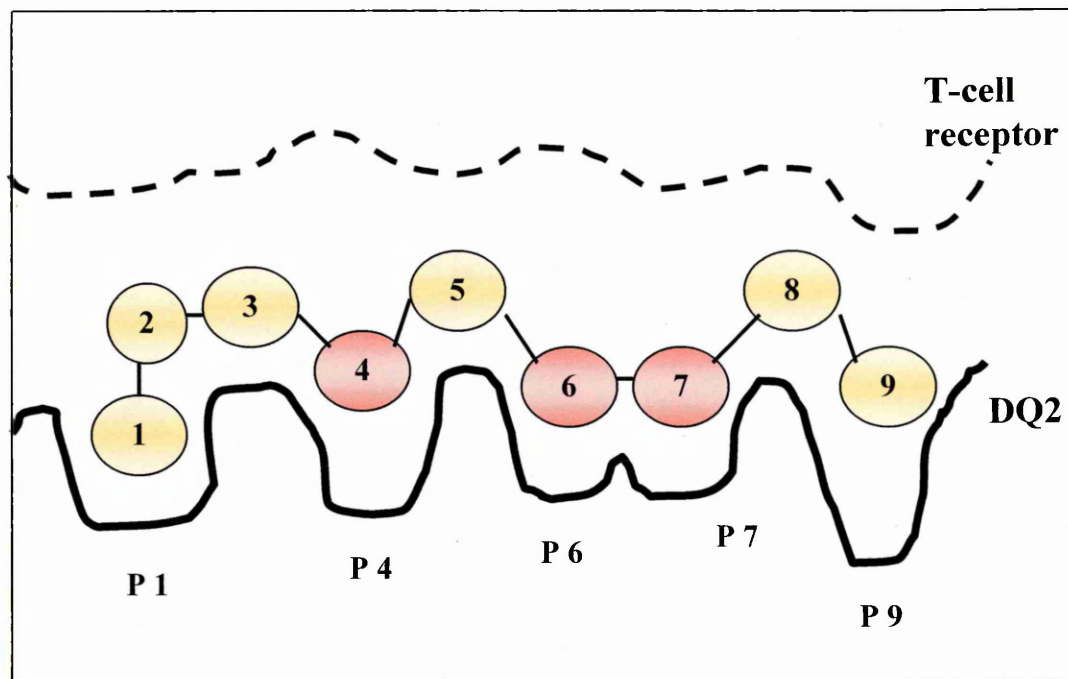
Indicates a DQ2 anchor position where the presence of negatively charged residues within the peptide increases binding affinity.

DQ2 POCKET	Preferred residue
# 4	Glutamic acid
# 6	Glutamic acid or proline
#7	Glutamic acid

Adapted from Sollid, 2000; 2002

1.10.4.2 Gluten T-cell epitopes

Mapping of the gluten molecule to identify sites of potential T-cell epitopes has demonstrated that the actual number of distinct epitopes is limited although there may be as many as fifty or more within gluten, hordein and secalin proteins (Vader *et al.*, 2002). Increasingly, data is available on the precise regions and sequences of T-cell reactive gluten epitopes.

Epitopes recognised by both HLA DQ2 and HLA DQ8-restricted CD4^{+} T-cells have been identified within α-gliadin, γ-gliadin and glutenin proteins (Arentz-Hansen *et al.*, 2002; Molberg *et al.*, 2003; Vader *et al.*, 2002; van de Wal *et al.*, 1998, 1999).

Deamidation by TG2 and the position of proline residues are also important factors in the creation of recognisable T-cell gluten epitopes. For most identified T-cell epitopes deamidation enhances their antigenic activity. Many are only rarely recognised in their native state and some not at all (Arentz-Hansen *et al.*, 2002; Molberg *et al.*, 2003; Vader *et al.*, 2002). Further details of identified gluten T-cell epitopes are given in table 1.3.

Some T-cell epitopes are more immunodominant than others. For example, reactivity to DQ2 and DQ8 presented α-gliadin epitopes is also found in most, if not all, adult CD patients, whereas reactivity to the DQ2 presented γ-gliadin epitope is only found in a minority (Arentz-Hansen *et al.*, 2000; Sollid, 2000). It is not certain how frequently the glutenin epitope is recognised although, in one study, none of five children with CD reacted to the peptide (Sollid, 2000; van de Wal *et al.*, 1999).

Differences are also apparent in the range of T-cell epitopes recognised by different subgroups of patients with CD. Children with CD respond to a wider range of gluten epitopes, many of which are recognised in the native form without the need for deamidation by TG2. In adults, the immune response appears more focused with only a few immunodominant epitopes involved, which require deamidation for recognition (Vader *et al.*, 2002). It is possible that recognition of a wide range of gluten epitopes is characteristic of early (potential/latent) CD in general, rather than age.

Table 1.3 Gluten epitopes, identified to date, recognised by lamina propria CD4^{+} T-cells from patients with coeliac disease

PROTEIN	HLA restriction	CORE SEQUENCE	REFERENCE
α-gliadin	DQ2	PFPQPQLPY	Arentz-Hansen *et al.*, 2002
α-gliadin	DQ2	PQPQLPYPQ	
α-gliadin	DQ2	PYPQPQLPY	
α-gliadin	DQ8	SGQGSFQPSQQ	van de Wal *et al.*, 1998
γ-gliadin	DQ2	PQQSFPQQQ	Arentz-Hansen *et al.*, 2002
γ-gliadin	DQ2	IIQPQQPAQ	
γ-gliadin	DQ2	FPQQPQQPYPQQP	
γ-gliadin	DQ2	FSQPQQQFPQPQ	
γ-gliadin	DQ2	LQPQQPFPQQPQQP YPQQPQ	
Glutenin	DQ2	PFSQQQQSPF	Vader *et al.*, 2002
Glutenin	DQ2	PFSQQQQQ	
Glutenin	DQ8	QQGYYPTS	van de Wal *et al.*, 1999

Glutamine residues targeted by TG2 are shown in red. Glutamine residues where deamidation is necessary for T-cell recognition are shown in green.

Indeed, it has been suggested that deamidation may be important in later stages of the disease but that initially, the immune reaction may be initiated by T-cells recognising unmodified epitopes (Arentz-Hansen *et al.*, 2002).

1.10.4.3 Cytokine profile of gluten specific intestinal T-cells

In CD, there is increased cytokine production by activated $\alpha\beta$ CD4$^+$ lamina propria T-cells (Lahat *et al.,* 1999). The cytokine profile is dominated by interferon (IFN)-γ and there is a large increase in both the number of IFN-γ producing cells and the amount of IFN-γ production (Kontakou *et al.,* 1994; Lahat *et al.,* 1999; Nilsen *et al.,* 1995). In addition, messenger ribonucleic acid (mRNA) of IFN-γ, IL-2, TNF-β, TNF-α, IL-10, IL-1β and transforming growth factor (TGF) -β have been found in CD biopsy samples (Lahat *et al.,* 1999). IL-2 and TNF-β mRNA are not normally present in the mucosa, suggesting a potential role in the disease pathogenesis for these cytokines (Lahat *et al.,* 1999). Finally, the presence of mature IL-18, a crucial cytokine in maintaining the Th1 response, is associated with CD mucosa but not controls (Salvati *et al.,* 2002). Patients with active CD have increased numbers of T-cells expressing both Th1 and Th2 cytokines, suggestive of Th0 activation (Lahat *et al.,* 1999). The cytokines TNF-α and migration inhibition factor (MIF) have both been detected in lamina propria T-cells from patients with CD (O'Keefe *et al.,* 2001). In addition, stimulated mucosal T-cells from patients with active CD have been shown to secrete high levels of IFN-γ as well as IL-4, IL-5, IL-6, IL-10, TNF and TGF-β, in a Th0 pattern (Nilsen *et al.,* 1995; Przemioslo *et al.,* 1994). Others secreted high levels of IFN-γ along with TNF but not IL-4 or IL-5, a Th1 pattern (Nilsen *et al.,* 1995).

Intraepithelial lymphocytes (IELs), too, from patients with CD have also been shown to express markedly increased levels of TNF-α compared to controls (O'Keefe *et al.,* 2001).

1.10.4.4 Production of coeliac disease associated antibodies

Several disease-associated antibodies have been identified in patients with CD. Jejunal secretion rates of immunoglobulin (Ig) A (both monomeric and polymeric) and IgM are increased by two-fold and five-fold respectively, which parallels the observed increase in B-cells in the lamina propria (Colombel *et al.,* 1990). Antigenic epitopes (which may be different from the recognised T-cell epitopes) are bound to specific B-cells, which then receive help from activated lamina propria CD4$^+$ T-cells, through their production of cytokines such as IL-2, IL-4 and IL-5. In this way, the B-cells are stimulated to become antibody-producing cells.

1.10.4.4.1 Anti-gliadin antibodies

Anti-gliadin antibodies are primarily of mucosal origin and are readily measured in the intestinal fluid of untreated patients with CD (Volta *et al.*, 1990), although they may also be produced in the lymph nodes, possibly as a result of gliadin leakage into the periphery (Marzari *et al.*, 2001; Sollid *et al.*, 1997).

B-cell epitopes have been identified in all four of the major gliadin subtypes (α, β, γ and ω) and patients normally produce antibodies against multiple gliadin proteins (Levenson *et al.*, 1985). The antigenic gliadin epitopes identified so far are somewhat different to those known to be recognised by T-cells (Krupicova *et al.*, 1999) (table 1.4). Both linear and conformational epitopes have been described, indicating the secondary structure is of some importance (Alfonso *et al.*, 1998). Some evidence suggests that β-turn motifs may be an important secondary structure for antibody recognition and two identified epitopes are contained within regions of gliadin that form β-turns (Alfonso *et al.*, 1998; Osman *et al.*, 2000).

TG2-mediated deamidation of gluten appears important for antibody recognition and has been shown to increase the *in vitro* recognition of gliadin peptides by circulating anti-gliadin antibodies (Aleanzi *et al.*, 2001; Osman *et al.*, 2000). However, *in vivo* B-cells recognising deamidated gliadin peptides have not been found to date (Aleanzi *et al.*, 2001). Some anti-gliadin antibodies cross-react with enterocytes and regions of the ubiquitous calcium-binding protein, calreticulin, and it is likely that the two proteins share common epitopes (Krupickova *et al.*, 1999; Tuckova *et al.*, 1997).

1.10.4.4.2 Tissue autoantibodies

Untreated CD is frequently associated with the production of autoantibodies directed against certain extracellular matrix (ECM) structures (Maki & Collin, 1997). Tissue autoantibodies in CD are strictly gluten driven and are regarded as target organ-related, predominantly IgA-class autoantibodies (Karpati *et al.*, 1990; Maki, 1995). These antibodies may be detected using a variety of tissue substrates and, according to the tissue antigen used and the observed antibody binding pattern, the autoantibodies are classified as anti-reticulin (ARA), anti-endomysial (AEA) or anti-jejunal (AJA) antibodies (Maki & Collin, 1997). In 1997 it was discovered that the main target antigen of AEA is TG2 (Dieterich *et al.*, 1997). Very recently, Korponay-Szabo and co-workers observed that antibodies directed against TG2 are also responsible for

Table 1.4 Gluten epitopes, identified to date, recognised by anti-gliadin antibodies from patients with coeliac disease

PROTEIN	SEQUENCE	REFERENCE
α-gliadin	QXQPFP (X=P, Q, L)	Osman et al., 2000
α-gliadin	WQIPEQ	
α-gliadin	QGXFQP (X=F, S)	
α-gliadin	PQQLPQ	
γ-gliadin	QPQQPF	
α-gliadin	QEQVPLVQQQQF	Krupickova et al., 1999
α-gliadin	QILQQILQQQLI	
α-gliadin	WQIPEQSQCQAI	
α-gliadin	QTLPAMCNVY	
α- gliadin	AMCNVYIPPYCT	

ARA, AEA and AJA tissue binding patterns of serum samples from patients with CD, indicating that these tissue autoantibodies are identical (Korponay-Szabo et al., 2000, 2003a). Anti-TG2 antibodies are produced in the intestine (Marzari et al., 2001; Sollid et al., 1997) and levels are correlated with the degree of enteropathy (Tursi et al., 2003). Their presence in serum is believed to be a result of spillover into the blood from the intestine (Marzari et al., 2001). The proposed mechanism of anti-TG2 antibody production is shown in figure 1.12. As TG2-specific T-cells are unlikely to be present, the necessary T-cell help may be provided by gliadin-specific T-cells, which recognise gliadin peptides cross-linked to TG2 bound to TG2 specific-B-cells (Marsh, 1997; Sollid et al., 1997). Anti-TG2 antibodies recognise both conformational and linear epitopes (Molberg et al., 2000; Seissler et al., 2001; Sulkanen et al., 1998). Two major epitopes appear to be located within the N- (a.a. 1-281) and C-terminals (a.a. 473-687) of the protein, each of which were recognised by approximately 70% of test sera (Seissler et al., 2001). However, B-cell epitopes are spread across all regions of the protein with most patients possessing antibodies against more than one and, as a result,

four major anti-TG2 antibody binding patterns of sera from untreated patients with CD have been defined,

I - Antibodies binding to N-terminus, middle region and C-terminus

II - Antibodies binding to N-terminus and C-terminus

III - Antibodies binding to N-terminus only

IV - Antibodies binding to C-terminus only

(Seissler et al., 2001).

The production of anti-gliadin and anti-TG2 antibodies is shown in figure 1.12.

1.10.4.4.3 Other autoantibodies associated with coeliac disease

Untreated CD is also associated with the presence of IgG and IgA-class antibodies directed against actin, a protein especially abundant in microvilli (Clemente et al., 2000; Korponay-Szabo et al., 2003). They appear to be associated with more severe degrees of villous atrophy and may be a result of unmasking of epitopes following mucosal damage (Clemente et al., 2000). Remarkably, these antibodies have been detected in 90% of the adult and 60% of the childhood cases of CD that were investigated. The antibodies appear to be gluten driven and become undetectable within approximately 5 months on a GFD (Clemente et al., 2000). Other autoantibodies include a novel 55-kDa nuclear antigen, recognised by IgA antibodies of approximately half of examined CD patients in a recent study, which shares epitopes with wheat proteins and is distinct from both tissue transglutaminase and calreticulin (Natter et al., 2001). A recent proteomic study has also identified autoantibodies in the sera of patients with CD directed against ATP synthase beta chain and enolase alpha species (Stulik et al., 2003). The results of such studies are sugestive of the existence of additional, not yet discovered, autoantigens associated with CD.

1.10.4.5 Effector mechanisms of mucosal damage

It is still not entirely clear how, once released, the cytokines interact to cause mucosal damage although it seems reasonable to assume the involvement of multiple effector mechanisms. IFN-γ is able to directly induce expression of TG2 and HLA II molecules and IL-2 may promote T-cell proliferation within the intestine (Kim et al., 2002).

Figure 1.12 The production of coeliac disease associated antibodies
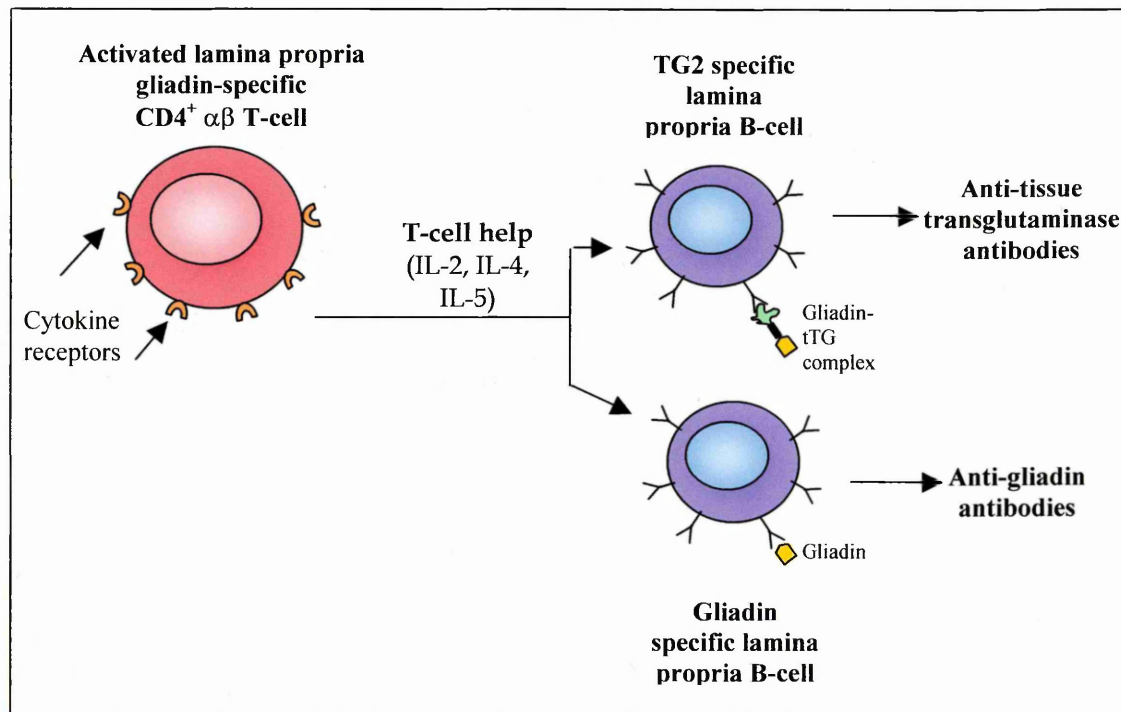
Activated T-cells release cytokines that are able to trigger nearby B-cells with bound antigen on their surface to produce anti-gliadin or TG2 antibodies. As the existence of TG2 specific T-cells is unlikely, activated gliadin specific T-cells are likely to provide the necessary help to TG2 specific B-cells by responding to gliadin peptides cross-linked with TG2 on their surface.

Crypt hyperplasia is attributed to increased epithelial cell proliferation. It is possible that IFN-γ and TNF-α are able to mediate this hyperplasia via their direct mitogenic effect on epithelial cells (MacDonald et al., 1999). Alternatively, cytokines released from lamina propria T-cells may increase the production of keratinocyte growth factor (KGF) by myofibroblasts within the lamina propria. These cells control epithelial renewal via KGF interaction with KGF-receptors on epithelial cells (MacDonald et al., 1999; Salvati et al., 2001). Increased KGF mRNA is found in CD mucosa (Salvati et al., 2001). Thus cytokines released from lamina propria T-cells may mediate increased epithelial proliferation and subsequent crypt hyperplasia indirectly (Sollid, 2000).

Villous atrophy is believed to result primarily from an increased rate of epithelial cell apoptosis (Ciccocioppo et al., 2000; Moss et al., 1996). An increased number of apoptotic epithelial cells are seen in CD mucosa, many expressing the Fas receptor (Ciccocioppo et al., 2002).

In addition, there is an increase in the number of $\alpha\beta$ CD8$^+$ IELs expressing the Fas ligand and perforin, the levels of which were found to positively correlate with the degree of enterocyte apoptosis (Ciccocioppo et al., 2000; di Sabatino et al., 2001). In addition, activated intraepithelial $\alpha\beta$ CD8$^+$ T-cells have lytic potential and produce IFN-γ, IL-2, IL-8 and TNF-α (Sollid, 2000). Together with TNF produced by lamina propria CD4$^+$ T-cells, these high levels of TNF may result in disruption of ECM production and degradation. Active CD is associated with an increased degradation of ECM, which may contribute to villous atrophy (Sollid, 2000). Normally, ECM production by myofibroblasts is balanced by ECM degradation mediated by matrix metalloproteinases (MMPs). MMP actions are tightly controlled and mediated by release of several endogenous MMP inhibitors, known as tissue inhibitors of matrix metalloproteinases (TIMPs). High levels of TNF are likely to cause excess MMP production resulting in the net degradation of ECM causing villous atrophy (MacDonald et al., 1999). This is also supported by the observation that the ratio of cells expressing TIMP-1 mRNA to those expressing MMP-1 and MMP-3 is decreased in active coeliac disease (Daum et al., 1999).

It is also possible that IELs, particularly $\gamma\delta$ T-cells, may contribute to epithelial cell destruction by recognising MIC molecules expressed on their surface, which are

induced by stress (Groh *et al.*, 1998). These observations are suggestive of a direct role for IELs in the increase of epithelial cell apoptosis, which leads to villous atrophy.

Finally, a recent study reported the increased expression of Bak protein in CD mucosa, a member of the Bcl-2 family of proteins, which acts as an endogenous promotor of apoptosis in enterocytes. Immunoreactivity was primarily localised to the epithelium and it was hypothesised that IFN-γ confers increased susceptibility of enterocytes to undergo apoptosis through the upregulation of this protein (Chernavsky *et al.*, 2002).

The role of antibodies in effecting mucosal damage is unclear. Anti-gliadin antibodies may cross-react with calreticulin in enterocytes thus exerting a pathogenic effect (Tuckova *et al.*, 1997). It has been hypothesised that anti-gliadin antibodies may cause intestinal damage by antibody-dependent cell-mediated cytotoxic reactions or by activation of the complement system (Saalman *et al.*, 1998; Troncone & Ferguson, 1991; Unsworth *et al.*, 1987). It has also been suggested that anti-gliadin antibodies may aid the gluten T-cell response by binding gliadin peptides and transporting them to the appropriate HLA class II expressing, antigen-presenting cells for presentation by DQ2 molecules to T-cells (Osman *et al.*, 2000). IgA anti-TG2 antibodies have been shown to be capable of inhibiting epithelial cell differentiation and increasing epithelial cell proliferation *in vitro* by disturbing the TGF-β mediated cross-talk between fibroblasts and epithelial cells necessary for these functions (Halttunen & Maki, 1999), although other studies have suggested that anti-TG2 antibodies from patients with CD are not able to fully block the enzymatic activity of TG2 (Dieterich *et al.*, 2003). Finally, anti-actin antibodies may have the ability to disrupt the cytoskeleton, thereby affecting the activity of genes such as those coding for fibronectin, collagenase, TIMP and TGF-β proteins, which would result in significant structural mucosal damage (Clemente *et al.*, 2000).

Figure 1.13 summarises the main events in the pathogenesis of CD.

Key events in the pathogenesis of coeliac disease. Gluten is digested and regions that are resistant to processing by luminal and brush border enzymes are transported into the lamina propria either through epithelial cells or between them, once mucosal integrity is impaired especially during infection or after mechanical or chemical injury. Gluten epitopes are processed and presented by DQ2 or DQ8 molecules on the surface of antigen-presenting cells, particularly dendritic cells but also epithelial cells and B-cells. The enzyme, TG2, is released from endothelial cells, fibroblasts and inflammatory cells of the lamina propria and is upregulated during periods of infection and/or stress. In the lamina propria, TG2 encounters gluten and deamidates gluten residues or crosslinks itself to gluten peptides resulting in a potentiation of uptake and presentation by antigen presenting cells and enhanced recognition by CD4$^+$ lamina propria T-cells. The activation of these T-cells results in the release of cytokines with a Th1 and Th0 profile, which act by various means to bring about characteristic villous atrophy and crypt hyperplasia as well as production of disease-associated antibodies. Finally, activated CD8$^+$ intraepithelial lymphocytes and $\gamma\delta$ T-cells in the epithelium also contribute to crypt hyperplasia by increasing apoptosis of epithelial cells.

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APC – Antigen-presenting cell   DC – Dendritic cell

Figure 1.13 Summary of the molecular basis of coeliac disease
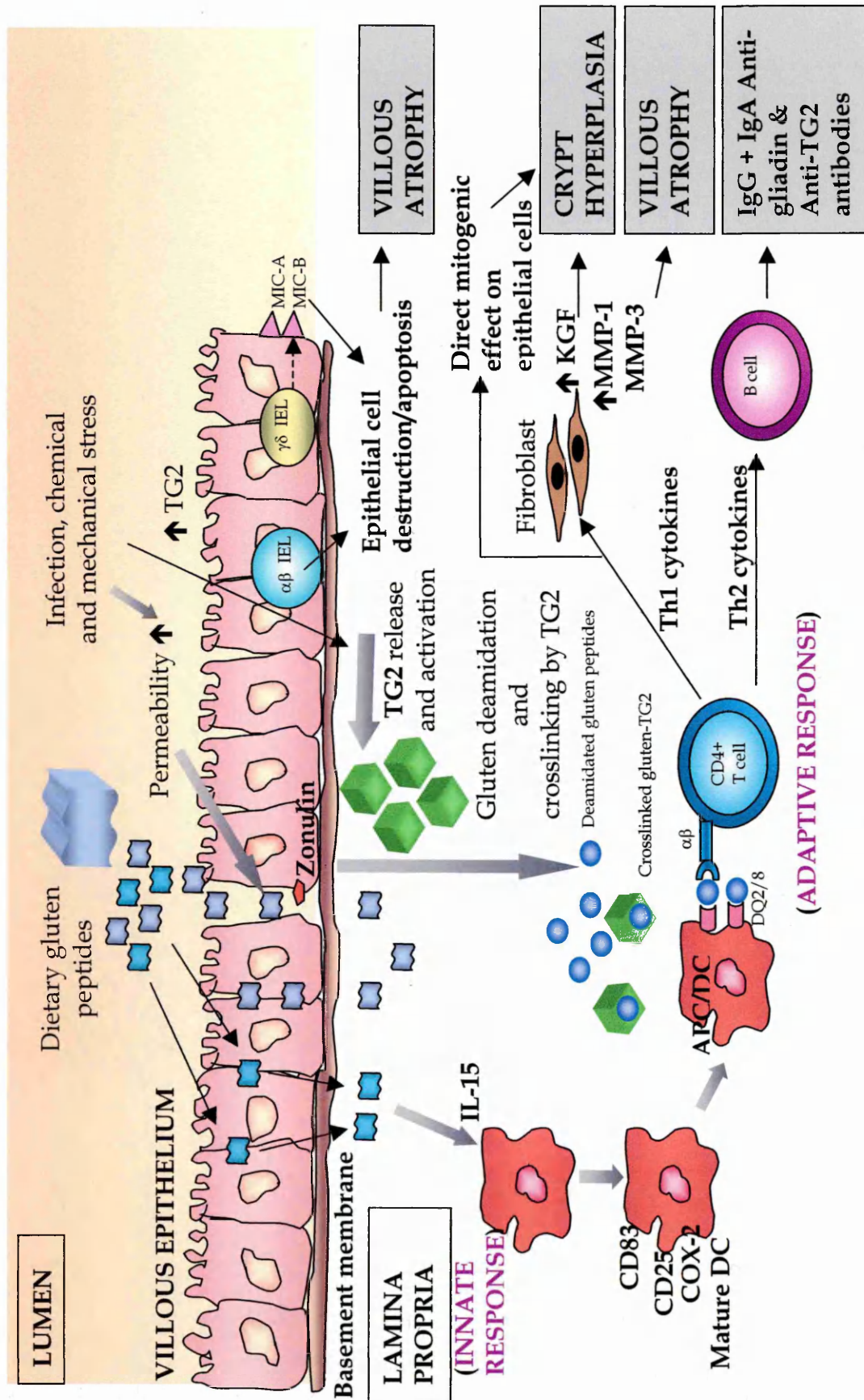
1.10.4.6 The role of tissue transglutaminase in coeliac disease

TG2 is one of nine members of the transglutaminase family, the first member of which was described more than 40 years ago (Fesus & Piacentini, 2002). TG2 is primarily an intracellular (cytoplasmic or nuclear) enzyme but also acts extracellularly when it is membrane-bound (Lorand & Graham, 2003). TG2 is a ubiquitous enzyme, thought to be involved in processes as diverse as cytoskeleton modification, ECM organisation, wound healing, angiogenesis, apoptosis and cell signalling (Fesus & Piacentini, 2002; Lorand & Graham, 2003). TG2 carries out these functions primarily via several types of transamidation reactions or deamidation of specific polypeptide bound glutamine residues. The favoured reactions are transamidations, mainly protein crosslinking by means of formation of an isopeptide bond (Lorand & Graham, 2003) (figure 1.14) and they have a quicker reaction rate than the deamidation reaction (Fleckenstein et al, 2002). However, under these circumstances some deamidation occurs, even in the presence of excess appropriate transamidation substrates (Fleckenstein et al, 2002). The rate of deamidation is dramatically increased when transamidation substrates are scarce or in situations of low pH (Fleckenstein et al., 2002) (figure 1.14). TG2 is responsible for the deamidation of gluten within the intestine of patients with CD. TG2 expression is increased in the intestine of patients with CD compared to controls (Bruce et al., 1985; Molberg et al., 1998), although the exact location of gluten deamidation by TG2 is not known.

In the active intestinal lesion, TG2 is mainly present extracellularly in the subepithelial region but is also expressed by mature epithelial cells and at the epithelial brush border (Fleckenstein et al., 2002; Molberg et al., 1998; Molberg et al., 2000). TG2 is also expressed by activated macrophages and monocytes as well as in maturing dendritic cells (Akimov & Belkin, 2001; LeNaour et al., 2001). The low pH of the intestine (~6.6) favours marked TG2-mediated deamidation over protein cross-linking (Evans et al., 1988; Fleckenstein et al., 2002). Therefore, gluten could undergo significant deamidation by TG2, extracellularly, in the subepithelial space or at the brush border. Alternatively, macrophages, dendritic cells or TG2-specific B-cells could be involved directly in the deamidation of gluten if membrane-bound TG2 is endocytosed in these cells (Sollid, 2002).

Figure 1.14 Actions of tissue transglutaminase
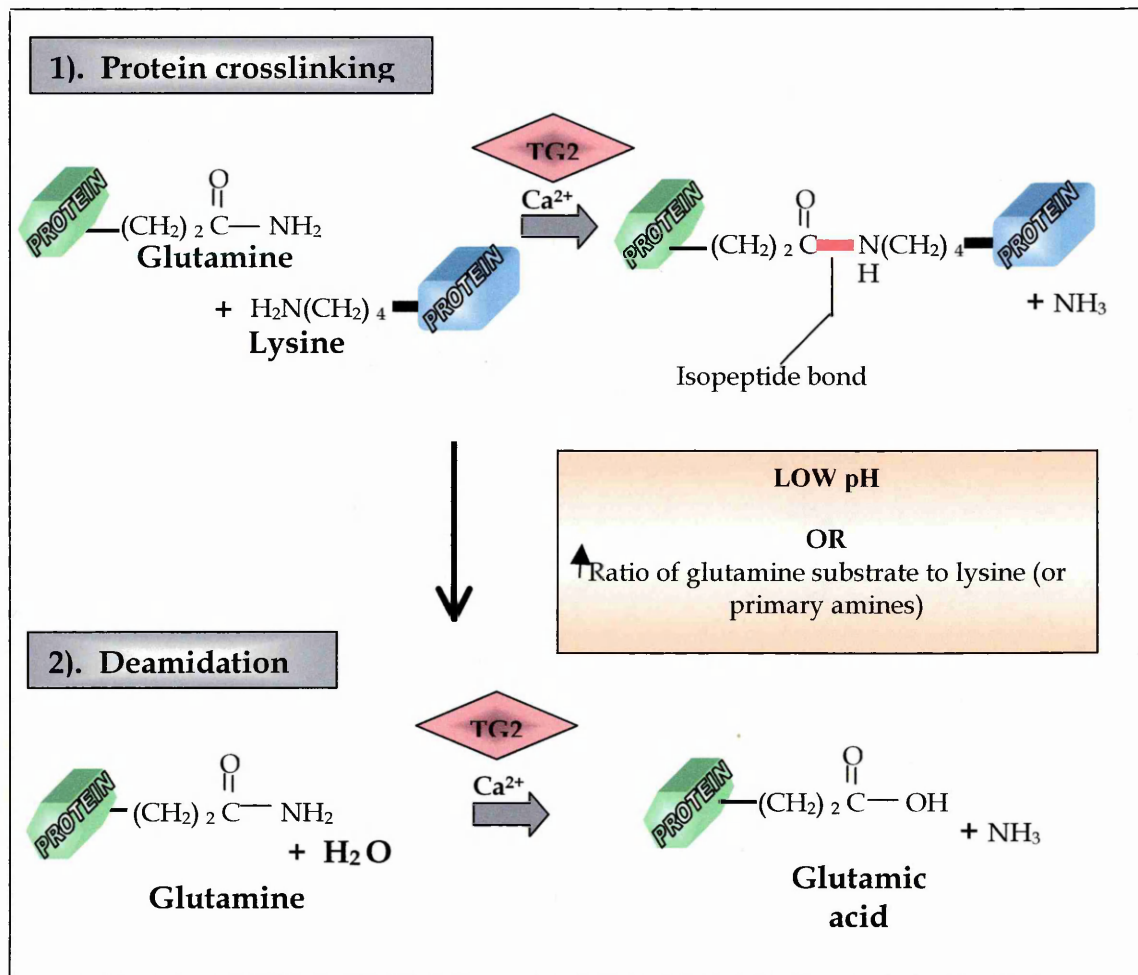
TG2 exerts most of its biological effects through catalysis of several different transamidation reactions (protein crosslinking being the preferred of these reactions) or by a deamidation reaction. Under normal circumstances, the predominant action of calcium-activated TG2 is the catalysis of an acyl transfer reaction between a polypeptide bound glutamine residue and the primary amino group of a polypeptide bound lysine residue resulting in the formation of an isopeptide bond between or within proteins (1). However, in conditions of low pH or when transamidation substrates are scarce, the calcium-activated TG2 catalysed deamidation reaction predominates. In this case, TG2 uses water as a substrate to simply convert a polypeptide bound glutamine residue to a glutamic acid residue (2).

1.11 Dermatitis Herpetiformis

DH is a blistering skin disease, characterised by an itchy rash, which is associated with granular IgA deposits in the basement membrane. Detection of these IgA deposits confirms diagnosis (Collin & Maki, 1994; Wills *et al.,* 2002). There is strong evidence to suggest that DH is related to gluten ingestion and one identity of the spectrum of gluten sensitivity, a manifestation that primarily affects an area distinct from the small intestine. Individuals with DH express the same gluten sensitivity associated genetic profile as individuals with CD (section 1.7) (Reunala, 1998) and both CD and DH may exist in the same family (Reunala, 1996). Patients express serological markers of gluten sensitivity (section 1.9) (Reunala & Hallstrom, 1990). The majority of individuals with DH also show evidence of the gluten related changes in the small intestine that are recognised features of CD, even though the skin is the major affected site in these patients (Collin & Maki, 1994; Wills *et al.,* 2002). GI symptoms are often minimal and skin involvement can occur even without histological evidence of intestinal involvement. DH provided the first evidence that the effects of gluten sensitivity need not be confined solely to the small intestine. Finally, it has been shown that patients with DH also benefit from a GFD. The rash responds to a GFD although skin changes may take up to two years to fully resolve, during which time the drug dapsone is frequently used for relief of symptoms.

1.12 Neurological Disorders associated with gluten sensitivity

A variety of neurological disorders have been reported in association with gluten sensitivity. Many of these observations are based on patients with initial GI symptoms, in whom neurological dysfunction occurred after varying time-periods following their diagnosis with CD. Data from clinical follow-up of adult patients diagnosed with CD suggests that subsequent, otherwise unexplained, neurological dysfunction is a complication in between 6-10% cases (Holmes, 1997).

However, in recent years it has become apparent that neurological manifestations of gluten sensitivity need not always be associated with established CD and that neurological disorders may present prior to the onset of GI symptoms. Alternatively, neurological disorders may be the sole presenting feature of gluten sensitivity in individuals with asymptomatic CD or in genetically susceptible individuals with

serological markers of gluten sensitivity but no histological evidence of GI involvement (Hadjivassiliou *et al.*, 1998).

Early hypotheses, on the link between gluten sensitivity and neurological dysfunction, centred on the possibility of vitamin and trace element deficiencies, resulting from malabsorption, causing neurological disturbances. However, CD now tends to be diagnosed before malabsorption reaches such a degree and such deficiencies are rarely reported. Malabsorption does not explain the associated neurological complications and research is now focused on other possible mechanisms such as altered autoimmunity (Pengiran Tengah *et al.*, 2002).

1.12.1 Neurological complications associated with established coeliac disease

A review of all reports of neurological disorders associated with established CD published between the years 1964 and 2000 revealed that ataxia (with or without myoclonus) and peripheral neuropathy are the most common neurological manifestations occurring in patients with CD (table 1.5) (Hadjivassiliou *et al.*, 2002a).

Other neurological complications that have frequently been reported in association with established CD include epilepsy, dementia and psychiatric disturbances (see below).

1.12.1.1 Ataxia

In 1966, a pivotal paper published by Cooke and Smith was the first to systematically detail the clinical and neuropathological characteristics of a group of sixteen patients with CD that subsequently developed neurological dysfunction. The predominant abnormality was ataxia. All patients had gait ataxia and some also had limb ataxia. In addition, evidence of severe peripheral neuropathy was seen in ten of the patients. Dick and co-workers reported a case of ataxia associated with previously diagnosed CD in a female patient (Dick & Abraham, 1995). Reports of ataxia with myoclonus associated with established CD are rare. Bhatia and co-workers described four cases of progressive myoclonic ataxia in which the neurological syndrome presented up to twenty-five years after the development of CD or DH. The majority of these cases were phenotypically similar, dominated by action myoclonus associated with mild ataxia and

occasional seizures. In the other cases, myoclonus was a later feature of a predominantly ataxic condition (Bhatia *et al.,* 1995).

1.12.1.2 Peripheral neuropathy

Peripheral neuropathies associated with established CD are usually of axonal or demyelinating type (Kaplan *et al.,* 1988). As Cook and Smith reported, peripheral neuropathy may occur concomitantly with ataxia and it is frequently of late-onset (Cooke & Smith, 1966). A recent study of 26 patients with treated CD found that 23.1% showed findings of axonal neuropathy upon examination, indicating that patients even without overt malabsorption are still at risk from neurological complications (Luostarinen *et al.,* 2003).

Table 1.5 Summary of common neurological complications associated with established coeliac disease

Patient Information:	
Total number of patients	83
Male to female ratio	44 : 39
Mean age (yrs)	48
Neurological Diagnosis:	Number
Ataxia (with or without myoclonus)	38
Peripheral Neuropathy	29

Data collected from a review of single or multiple case reports from 1964-2000. *Adapted from Hadjivassiliou et al., 2002a.*

1.12.1.3 Dementia

Dementia associated with previously diagnosed CD frequently occurs in the presence of other neurological symptoms. Two patients, previously described with myoclonic ataxia, also developed dementia (Bhatia *et al.*, 1995). Collin and co-workers described five patients, all under 60 years of age, with dementia associated with CD (Collin *et al.*, 1991). Two of the patients also suffered from progressive ataxia. The remaining three were found to have brain atrophy upon examination by computerised tomography of the brain.

1.12.1.4 Epilepsy

The association between epilepsy and established CD is well documented. It is believed that epilepsy affects approximately 5% of adult CD patients, the majority of whom suffer from complex partial attacks (Chapman *et al.*, 1978). This same association does not appear to exist in children. A large study of children with CD found that the incidence of epilepsy did not differ from that of the general paediatric population (Vascotto & Fois, 1997). A well-defined subgroup of patients has epilepsy associated with calcifications located in the occipital or parietal cortical and subcortical regions. In one study, cerebral calcifications were found in five out of twelve (42%) patients with CD and epilepsy (Gobbi *et al.*, 1992*)*. Interestingly, this syndrome of epilepsy associated with calcifications has mostly been reported in Italian patients. The association was not found in an Irish study (Cronin *et al.*, 1998) and cases appear to be rare in the UK (Hadjivassiliou *et al.*, 2002a).

1.12.1.5 Psychiatric disturbances

The association between CD and psychiatric disturbances has long been recognised although it remains unclear whether depression is more common or severe amongst patients with CD compared to the general population (Holmes, 1997). A study of adults with CD attending a UK clinic found that 10% suffered from depression (Holmes, 1997) and it is clear that depression remains a real risk in patients with CD.

Previous research has suggested possible associations between CD and both autism and schizophrenia (Coleman, 1997; Dohan, 1970; Reichelt & Landmark, 1995). However, in both cases, other studies have not supported this view (Holmes, 1997; Pavone *et al.*, 1997) and the associations remain controversial.

1.12.1.6 Effect of treatment

Many of the studies discussed above report that a GFD is unable to affect the progression of these neurological complications despite resolution of intestinal histology and normalisation of serological markers (Bhatia *et al.*, 1995; Collin *et al.*, 1991; Cooke & Smith, 1966). Vitamin and nutritional deficiencies were not reported and supplementation proved mostly ineffective, with various exceptions. Mauro and co-workers described one case of a female patient with a cerebellar syndrome associated with CD who was subsequently found to have a vitamin E deficiency and responded well to supplements (Mauro *et al.*, 1991). Epilepsy with cerebral calcifications tends to be associated with low serum levels of folate and elimination of gluten with or without folate supplementation can be of benefit in these patients (Gobbi *et al.*, 1992; Lea *et al.*, 1995).

1.12.2 Neurological complications associated with undiagnosed gluten sensitivity

In 1996, a large study investigated the prevalence of IgG and IgA anti-gliadin antibodies as a marker of gluten sensitivity, in two groups of patients attending the Neurology clinic at The Royal Hallamshire Hospital, Sheffield UK. The first group (53 patients) had a variety of neurological disorders of unknown cause and the second group (94 patients) had neurological disorders of known aetiology (including stroke, multiple sclerosis and Parkinson's disease). Circulating anti-gliadin antibodies were found at a much higher prevalence among patients with neurological disorders of unknown aetiology (57%) than among either patients with neurological disorders of known aetiology (5%) or healthy control patients (12%). Subsequent duodenal biopsies from 26 of these anti-gliadin antibody positive patients with neurological disorders of unknown cause revealed histological evidence of CD in 35%, non-specific duodenitis in 38% and normal mucosa in the remaining patients (Hadjivassiliou *et al.*, 1996). Since that date, a total of 131 patients with gluten sensitivity presenting with neurological disorders of unknown aetiology have been identified (table 1.6) (Hadjivassiliou *et al.*, 2002a). Interestingly, in these patients too, ataxia and peripheral neuropathy are the most common manifestations.

Since then, several studies have been carried out by different research groups in an attempt to determine the proportion of neurological disorders of unknown cause, which may actually be due to undetected gluten sensitivity. Disorders, of otherwise unknown

Table 1.6 Presenting neurological conditions of patients attending the gluten sensitivity/neurology clinic at The Royal Hallamshire Hospital, Sheffield UK

Total number of patients :	131
NEUROLOGICAL CONDITION	**NUMBER OF PATIENTS**
Ataxia	56 (four with myoclonus)
Sensorimotor axonal neuropathy	26
Mononeuropathy multiplex	15
Motor neuropathy	10 (three motor neurone disease-like)
Small fibre neuropathy	4
Mixed demyelinating/axonal neuropathy	2
Myopathies	8
Episodic headache and white matter abnormalities	19
Stiff-person syndrome	4
Neuromyotonia	1

Adapted from Hadjivassiliou et al., 2002a.

cause, that have been reported in association with previously undetected gluten sensitivity include ataxia (discussed further below), neuropathy, epilepsy and headache. Recently, Volta and co-workers found neurological disorders (including epilepsy, ataxia and peripheral neuropathy) in 8% of 160 patients with CD. In all but two cases, the neurological disorder had preceded the CD diagnosis (Volta *et al.*, 2002). A recent study of adult patients with neuropathy found that 2.5% had undetected CD, most with no GI symptoms (Chin *et al.*, 2003). Gobbi and co-workers found a high incidence of undiagnosed CD in adult patients with unexplained cerebral calcifications and epilepsy (24/31) (Gobbi *et al.*, 1992). A study of patients presenting with episodic headaches and otherwise unexplained white matter abnormalities on magnetic resonance imaging (MRI) found previously unrecognised gluten sensitivity in all ten patients examined. A GFD provided symptomatic response in nine of these patients (Hadjivassiliou *et al.*,

2001). Another recent study of 90 patients with idiopathic migraine found that 4.4% had previously undiagnosed CD compared to 0.4% of a control group (Gabrielli *et al.*, 2003).

With the possible exception of certain types of epilepsy (Labate *et al.*, 2001), occult gluten sensitivity does not appear to be a major cause of idiopathic neurological dysfunction in children (Lahat *et al.*, 2000), which is consistent with published descriptions of predominantly adult patients with later onset of neurological symptoms.

1.12.3 Gluten Ataxia

Of the 25 patients with idiopathic ataxia included in the initial study by Hadjivassiliou and co-workers (1996), 68% were found to have circulating IgG and/or IgA anti-gliadin antibodies. The term gluten ataxia was subsequently proposed to describe cases of apparently idiopathic ataxia actually due to previously unknown gluten sensitivity (Hadjivassiliou *et al.*, 1998).

Following the original studies by Hadjivassiliou and co-workers, describing the condition of gluten ataxia, a number of studies have been carried out involving the screening of adult patients with idiopathic ataxia for undiagnosed gluten sensitivity. These studies are summarised in table 1.7. Results are somewhat variable, however a general trend of a greater than expected incidence of undiagnosed gluten sensitivity among patients with idiopathic sporadic ataxia is evident. This notion is not without controversy. One study concluded that there is no association between idiopathic sporadic ataxia and gluten sensitivity (Combarros *et al.*, 2000). However, without comparative information on the general prevalence of gluten sensitivity in that population, it is difficult to draw such conclusions. In their study of 32 patients, Abele and co-workers concluded that the observed association was not strong enough to justify recommendation of a GFD to such patients as significance was not reached (Abele *et al.*, 2003). However, the general association is evident in their results and may prove significant if a greater number of patients were examined. In all studies, malabsorption and vitamin deficiencies were excluded as possible causes of ataxia. Consistent with other published studies of ataxia associated with established CD, sporadic ataxia was often found with concomitant peripheral neuropathy, which was not always clinically evident (Hadjivassiliou *et al.*, 2003a; Luostarinen *et al.*, 2001; Pellechia *et al.*, 1999a).

Table 1.7 A summary of studies investigating the prevalence of gluten sensitivity amongst adult patients with ataxia

Study Details	Anti-gliadin antibody positive/Total number tested (percentage positive)		
	Sporadic ataxia	Familial ataxia	Healthy controls
Hadjivassiliou *et al.*, 2003a (UK)	59/143 (41%)	8/51 (14%)	149/1200 (12%)
Pellechia *et al.*, 1999a (Italy)	3/24 (13%)	0/23 (0%)	-
Combarros *et al.*, 2000 (Spain)	0/32 (0%)	-	-
Luostarinen *et al.*, 2001 (Finland)	44 (17%)	-	(2%)
Burk *et al.*, 2001a (Germany)	12/104 (12%)	-	(5%)
Bushara *et al.*, 2001 (USA)	7/26 (27%)	9/24 (37%)	-
Lim *et al.*, 2001 (Ireland)	3/7 (43%)	-	-
Abele *et al.*, (Germany)	13/98 (13%)	1/15 (6%)	(5%)
Abele *et al.*, 2003 (Germany)	6/32 (19%)	63 (8-15%)	6/73 (8%)

(-) indicates data was not available.

Isolated case reports include an early report of a 56-year old man with a cerebellar syndrome that was the presenting and predominant feature of CD (Finelli *et al.*, 1980), a description of a 31-year old woman presenting with gait disorder, myoclonus and abnormal eye-movement (Hanagasi *et al.*, 2001) and a 37-year old woman with a 12-year history of progressive dysarthria and ataxia (Sander *et al.*, 2003), which were also both associated with a subsequent diagnosis of gluten sensitivity.

1.12.3.1 Effect of treatment

A recent large-scale study of the effect of a GFD in patients with gluten ataxia found significant improvement in ataxia amongst patients following the diet as compared to those who did not (Hadjivassiliou *et al.*, 2003c). Importantly, this study demonstrates gluten ataxia as a treatable cause of sporadic ataxia. Previously, in much smaller studies, a GFD had been reported to be of some benefit for patients with gluten ataxia, mainly for individuals with less severe symptoms where diagnosis was prompt (Hadjivassiliou *et al.*, 1996; Pellechia *et al.*, 1999b). A small study of intravenous Ig therapy in four ataxia patients with circulating anti-gliadin antibodies, but without histological evidence of CD, also suggested some benefit with improvement of symptoms seen in all patients after several weeks (Burk *et al.*, 2001b). Intravenous Ig was also reported to have a beneficial effect in the female patient recently described in a case report by Sander (Sander *et al.*, 2003).

1.12.3.2 Evidence that gluten ataxia is immune-mediated

The pathogenesis of gluten ataxia remains obscure. In agreement with other published studies, patients with gluten ataxia attending the neurology/gluten sensitivity clinic in Sheffield, which form the study base for the work in this thesis, do not exhibit any vitamin or nutritional deficiencies and prove negative for other possible causes of sporadic ataxia (Hadjivassiliou *et al.*, 1998). This finding makes malabsorption or vitamin deficiency an unlikely cause of gluten ataxia.

Preliminary evidence however, is suggestive of an immunological mechanism resulting in cerebellar damage and associated symptoms (Hadjivassiliou *et al.*, 1998). Immune involvement is central in gluten sensitivity and the notion of an immune-based pathogenesis of gluten ataxia is supported by the immunological pathogenesis of other manifestations of the condition, such as in CD and DH and also the association between CD and other autoimmune diseases (section 1.10.3). Other researchers have suggested

an inflammatory process as the likely cause of a neurological syndrome, involving the brainstem and cerebellum, in a patient with established CD (Ghezzi *et al.*, 1997). Interestingly, in the series of 160 CD patients examined by Volta, 61% of the patients with neurological dysfunction were found to have central nervous system anti-neuronal antibodies compared to 5% of patients with CD only and 0% of healthy controls (Volta *et al.*, 2002).

Research from our group strongly suggests the involvement of both T-cell mediated and humoral immune responses. Hadjivassiliou and co-workers previously reported the presence of perivascular cuffing and lymphocytic infiltration of cerebellar tissue by $CD4^+$ and $CD8^+$ positive T-cells and lesser numbers of B-cells and macrophages in two cases of gluten ataxia studied at post-mortem (Hadjivassiliou *et al.*, 1998). Later work revealed that the chemokine IP-10, a T-cell chemoattractant, was upregulated in the cerebrospinal fluid (CSF) of ten patients with gluten ataxia compared to a headache control group (Hadjivassiliou *et al.*, 2003b). This evidence is strongly supportive of the involvement of a cell-mediated response and other recent work from our group has demonstrated a role for a humoral immune response in gluten ataxia too (Hadjivassiliou *et al.*, 2002b).

1.13   Aims of this thesis

Gluten ataxia appears to account for a large number of cases of sporadic idiopathic ataxia and therefore may represent a potentially treatable cause of ataxia. Preliminary evidence is suggestive of an immune-mediated pathogenesis, in common with other manifestations of gluten sensitivity such as CD and DH. Although much research has been carried out into the immunological mechanisms of CD, the mechanisms of neural damage, occurring within the CNS of patients with gluten ataxia, remains unknown.

The aim of this thesis is to investigate the role of the humoral immune response in gluten ataxia. This knowledge is important as elucidation of the underlying immunological mechanisms may give an insight into any relationship or similarities between gluten ataxia and other manifestations of gluten sensitivity. Secondly, this knowledge may provide means for the detection of gluten sensitive patients likely to develop gluten ataxia enabling treatment to be initiated at the earliest opportunity. Broadly, to achieve this aim the following objectives were set,

- Determination, by enzyme-linked immunosorbent assay (ELISA), of the profile of serum antibody markers of gluten sensitivity expressed by patients with gluten ataxia and comparison with patients with CD without neurological complications, familial ataxia, sporadic ataxia (non-gluten) and healthy controls (chapter 3).

- Investigation of the specificity of serum antibodies from patients with gluten ataxia to cerebellar tissue, using immunohistochemistry (IHC) and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) with western blotting (chapters 4 & 5).

- Investigation of any association of gluten ataxia with circulating anti-GAD antibodies, using ELISA and SDS-PAGE with western blotting (chapter 6)

CHAPTER TWO

MATERIALS AND METHODS

2.1 Selection And Monitoring Of Patients

2.1.1 Differential diagnosis of patients with ataxia

From January 1994, all patients with the diagnosis of cerebellar degeneration attending the ataxia clinic, based within the Department of Neurology at the Royal Hallamshire Hospital, Sheffield (n=224), were prospectively screened and followed-up at 6 monthly intervals. Patients were questioned about family history of neurodegenerative disease, alcohol consumption and drug ingestion. Patients with a history of alcohol abuse, prolonged use of the medication phenytoin (an anticonvulsant) or laboratory evidence of multiple sclerosis, vitamin E deficiency, paraneoplastic cerebellar degeneration (PCD) or viral cerebellitis were excluded. All patients underwent genetic testing for SCA (1, 2, 3, 6 and 7) and Friedreich's ataxia, screening for circulating anti-gliadin antibodies and a detailed neurological examination. These were recorded as the baseline assessments (Hadjivassiliou *et al.*, 2003a). Based on these assessments, patients were divided into three groups:

- Group 1 consisted of patients with a familial history of ataxia and/or positive genetic testing for SCA or Friedreich's ataxia (n=59).
- Group 2 consisted of patients with sporadic idiopathic cerebellar ataxia without clinical evidence of cerebellar variant of multiple sytem atrophy (MSA-C) (n=132).
- Group 3 consisted of patients with clinically probable MSA-C (n=33).

Patients with sporadic idiopathic cerebellar ataxia (Group 2, above) who were positive for circulating anti-gliadin antibodies (IgG, IgA or both) were identified. Other causes of ataxia in these patients were excluded by further investigation including estimation of serum vitamin E and B_{12} levels, thyroid and liver function tests, lipid profile and autoimmune profile. Additional tests were performed, when clinically indicated (Hadjivassiliou *et al.*, 1998). Therefore, patients positive for circulating anti-gliadin antibodies (IgG, IgA or both) and not excluded by any of the above criteria were diagnosed with gluten ataxia (n=54) (Hadjivassiliou *et al.*, 2003a). All gluten ataxia patients then underwent HLA genotype testing and duodenal biopsy to establish whether an enteropathy was also present. A GFD was recommended for all gluten ataxia patients regardless of the presence or absence of enteropathy. Detailed dietary advice by a dietician, with expertise in gluten-sensitivity, was given to all patients and reviewed at follow-up visits. If requested by the patient, a GFD was commenced following the baseline assessment of neurological dysfunction. Dietary compliance was

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monitored through measurement of serum anti-gliadin antibody levels at each follow-up visit.

2.1.1.1 Serum collection and storage

Serum samples were taken on the initial visit and at all follow-up visits from all 224 patients. Serum taken from the initial visit to the ataxia clinic was recorded as the baseline sample. Following collection from -70^0C storage at the Royal Hallamshire Hospital, serum was separated into aliquots of 500µl, catalogued and stored long-term at -70^0C. Working aliquots of 20-100µl were stored at -20^0C. Repeated freeze-thawing of samples was avoided as much as practically possible although, due to the limited amounts of some samples, samples were sometimes subjected to several freeze-thaw cycles.

2.1.1.2 Anti-gliadin antibody measurement

Serum samples were screened, initially, by the Immunology Department of the Northern General Hospital, Sheffield. Screening was originally by an in-house ELISA method, which was subsequently replaced with a commercially available ELISA (Hycor Biomedical Ltd, UK).

2.1.1.3 HLA typing

HLA typing was performed at the National Blood Service, Sheffield.

2.1.1.4 Duodenal biopsy collection and storage

Duodenal biopsies were taken by Dr. D. Sanders in the Gastroenterology Clinic at the Royal Hallamshire Hospital, Sheffield. Briefly, four biopsies were taken from the distal duodenum using biopsy forceps, through a conventional forward viewing endoscope (Key-Med, UK). The presence of gluten-sensitive enteropathy was established by histological examination looking for characteristic changes associated with CD (crypt hyperplasia, villous atrophy and an increase in intraepithelial lymphocytes), which was carried out within the Histopathology Department at the Royal Hallamshire Hospital, Sheffield. Patients with gluten ataxia, in the presence or absence of overt mucosal damage, were included in subsequent studies. Biopsies were wrapped in foil, placed in an airtight container and stored at -70^0C.

2.1.1.5 Cerebrospinal fluid collection and storage

CSF samples were taken from a number of gluten ataxia patients, when clinically indicated during the diagnostic process, although this was not done routinely. CSF samples were stored at -70^0C.

2.1.2 Monitoring Of Gluten Ataxia Patients

In common with other patients attending the ataxia clinic, patients with gluten ataxia were followed-up every six months by appointments at the clinic. Follow-up visits involved collection of serum, review by a dietician and clinical assessment of ataxia as outlined below. In order to minimise inter-observer variability and patient bias, clinical assessment was undertaken by one consultant neurologist (Dr. M. Hadjivassiliou), without knowledge of baseline results or current anti-gliadin antibody titre.

2.1.2.1 Clinical assessment of ataxia

The ataxia assessment tests used were based on the standard neurological examination which had been validated in patients with progressive ataxia and were, as far as possible, objective (Notemans et al., 1994). Briefly, the tests, as described in a recent paper by Hadjivassiliou and co-workers (2003c), were as follows:

1. Computerised finger nose test

This was an adaptation of the motor screening test from the Cambridge Automated Neuropsychological Test Battery (CANTAB) (Sahakian & Owen, 1992). The patient was positioned at arm's length from a touch-sensitive screen. The patient was then asked to place their right index finger on the tip of their nose and to touch, as quickly and as accurately as possible, the centre of a flashing cross that repeatedly appeared nine times in different positions on the screen. The patient was instructed to place their finger back on the tip of their nose until the image reappeared on the screen (at equal time intervals). The mean response latency in milliseconds was recorded by the computer. The test was then repeated using the left index finger.

2. Grooved pegboard test

This test required the patient to insert pegs into a board consisting of 25 holes with randomly positioned locating slots (Lafayette Instruments, USA). The pegs, which have a key along one side, must be rotated to match the slot position before they can be inserted. The subject was asked to insert the pegs, using only one hand as quickly, as

possible. The time taken to fill all the holes was recorded and the task was then repeated using the other hand.

3. Tapping test

This test uses a device consisting of two manually operated counters fixed 35cm apart on a wooden platform. The patient was positioned with the device positioned at arm's length and asked to press the two counters alternately, with their right index finger, as quickly as possible for 30secs. The total count from the two counters after 30secs was recorded. The task was repeated using the left hand and, with the device placed on the floor, the patient using each foot in turn.

4. Quantitative Romberg's test

The patient was asked to stand with feet together and close their eyes. They were instructed to try and keep their eyes closed and their feet together for as long as possible. The time from start to first corrective foot movement and/or opening the eyes was recorded. Because of pronounced 'floor' and 'ceiling' effects in this test (ie. some patients were unable to attempt the test, others could stand indefinitely), changes in performance during the study were recorded as 'better' or 'worse or same'.

5. Subjective global clinical impression

The patient was asked to mark on a visual analogue scale their impression of their symptoms of imbalance over the last month. No change was marked at the centre of the line, better towards the right and worse towards the left end of the line. The distance from the left end of the line to the mark made by the patient was recorded as a proportion of the total length of the line.

2.1.3 Patients with gluten ataxia included in studies within this thesis

Study protocols for patients with gluten ataxia were approved by the South Sheffield Research Ethics Committee. Patients who refused or abandoned a GFD within a month of starting were included in the gluten ataxia (untreated) group of any studies. Patients with CD without neurological complications, familial ataxia (Group 1 as described in section 2.1.1), sporadic idiopathic ataxia (non-gluten ataxia) (Group 2 as described in section 2.1.1), other neurological disorders and normal controls were included, when relevant, as control groups. Serum samples were collected (as detailed in section 2.1.1.1) from patients attending Neurology or Gastroenterology clinics at the Royal

Hallamshire Hospital, Sheffield or obtained from the National Blood Service, Sheffield. All patients gave written informed consent before inclusion in any study.

Detailed clinical characteristics of the randomly selected subset of untreated gluten ataxia patients included in the studies contained within this thesis are given in table 2.1.

Table 2.1 Clinical characteristics of untreated gluten ataxia patients included in the studies within this thesis

No.	SEX	AGE (YRS)	Duration of ataxia (YRS)	Gait/limb ataxia (Y/N)	Normal MRI (Y/N)	GI symptoms (Y/N)	Duodenal biopsy indicative of CD-associated changes* (Y/N)	Anti-gliadin antibody status** (IgG or IgA)	HLA type
1	M	66	22	Y	N	N	N	IgG	DQ2
2	M	48	6	Y	N	N	N	IgG	DQ1
3	F	67	10	Y	N	N	N	IgG	DQ2
4	M	74	5	Y	N	N	N	IgG	DQ2
5	F	76	5	Y	Y	N	N	IgG	DQ2
6	M	70	10	Y	N	Y	N	IgG + IgA	DQ1
7	F	52	10	Y	N	Y	Y	IgG + IgA	DQ2
8	F	77	6	Y	N	N	N	IgG	DQ2
9	M	55	45	Y	N	N	N	IgG	DQ1
10	M	65	9	Y	N	N	N	IgG	DQ2
11	F	68	5	Y	Y	N	N	IgG	DQ2
12	M	57	30	Y	N	N	N	IgG	DQ2
13	M	49	30	Y	N	N	N	IgG + IgA	DQ8
14	M	73	7	Y	N	Y	Y	IgG	DQ2
15	F	61	9	Y	N	N	Y	IgG +IgA	DQ2
16	F	60	3	Y	Y	N	N	IgG	DQ2
17	F	80	16	Y	N	N	N	IgG	DQ2

* - CD-associated changes defined as crypt hyperplasia, villous atrophy or increase in intraepithelial lymphocytes
** - Results as determined by the Immunology department of the Northern General Hospital, Sheffield UK

Table 2.1 cont'd Clinical characteristics of untreated gluten ataxia patients included in the studies within this thesis

No.	SEX	AGE (YRS)	Duration of ataxia (YRS)	Gait/limb ataxia (Y/N)	Normal MRI (Y/N)	GI symptoms (Y/N)	Duodenal biopsy indicative of CD-associated changes* (Y/N)	Anti-gliadin antibody status**	HLA type
18	M	45	15	Y	N	N	N	IgG	DQ1
19	M	59	3	Y	N	N	Y	IgG + IgA	DQ2
20	M	38	8	Y	N	N	N	IgG	DQ1
21	F	79	5	Y	N	N	N	IgG	DQ2
22	M	72	20	Y	N	N	N	IgG	-
23	M	70	6	Y	N	N	N	IgG + IgA	DQ2
24	F	73	4	Y	N	Y	N	IgG + IgA	DQ2
25	M	71	12	Y	N	N	N	IgG	DQ2
26	M	83	16	Y	N	N	N	IgG	DQ2
27	F	83	29	Y	N	N	N	IgG	DQ2
28	M	78	3	Y	N	N	Y	IgG	DQ2
29	M	56	16	Y	N	N	N	IgG + IgA	DQ1
30	M	70	14	Y	N	Y	Y	IgG + IgA	DQ2
31	F	59	4	Y	N	N	N	IgG	DQ2
32	M	72	11	Y	Y	N	N	IgG	DQ1
33	F	79	5	Y	N	N	N	IgG	DQ2
34	M	58	8	Y	N	Y	Y	IgG	DQ2

* - CD-associated changes defined as crypt hyperplasia, villous atrophy or increase in intraepithelial lymphocytes
** - Results as determined by the Immunology department of the Northern General Hospital, Sheffield UK

Table 2.2 Summary of clinical characteristics of patients with untreated gluten ataxia

Clinical characteristic	
Male: female patient numbers	21 : 13
Average age (yrs) (Range, yrs)	66 (38 - 83)
Average duration of ataxia (yrs) (Range, yrs)	12 (3 – 45)
GI symptoms (%)	18
Evidence of CD associated changes on biopsy (%)	21
HLA genotype (%):	
DQ2	76
DQ8	3
DQ1	21

Yrs - Years

2.2 Enzyme-Linked Immunosorbent Assay

2.2.1 Principles of the technique

ELISA is an extremely versatile technique, which has replaced radioimmunoassay (RIA) in many applications. The ELISA technique is simple and results are obtained quickly. Virtually any antigen or antibody may be assayed using ELISA. For example, ELISA is used in clinical laboratories routinely to screen for hormones, infectious diseases and anti-viral antibodies (Wilson & Walker, 1995), as well as for antibodies associated with disease processes, such as CD.

The majority of laboratories, particularly clinical, use widely available commercial ELISA kits to screen for antibodies of interest. These give the reassurance of thorough validation, excellent reproducibility and reliability as well as provision of standardised controls and calibrants. These properties are particularly useful in situations where large numbers of samples need to be rapidly and accurately screened and aid comparison of results between different laboratories.

The basic principle of ELISA makes use of the sensitive and specific interaction that occurs between antigen and antibody. Either the antigen or antibody is bound to a solid-phase support (coating). Most frequently, the solid-phase is a disposable 96-well polystyrene microtitre plate, which allows large numbers of samples to be assayed simultaneously. Antigen or antibody adsorbs to the surface via formation of hydrophobic bonds with the plastic matrix. In this thesis, all ELISAs involve detection of antibodies and therefore antigen-coated plates are used. The antigen is usually applied in a 'coating buffer', which minimises non-specific binding to the plate by other reagents at later stages in the assay. Typical 'coating buffers' include carbonate, tris-hydrochloride and phosphate-buffered saline (PBS) based solutions. Coating of the solid-phase is affected by a variety of conditions including temperature, pH, incubation time and concentration of antigen or antibody in the coating mixture. Coating may be enhanced by certain factors but care must be taken to optimise conditions whilst avoiding deleterious results. For example, too dense a coating may result in impairment of subsequent reagent binding due to steric inhibition (Crowther, 1995). Following coating of the solid-phase, samples containing an unknown amount of the antibody of interest are added and incubated for a specified length of time. During this period, the antibodies bind to the antigen coated on the plate. Most often this sample is serum but

ELISAs designed for other test fluids such as urine or CSF are available. Unbound antibody within the sample is washed off. Following a series of steps, which vary depending on the ELISA type used, binding of the antibody of interest to the solid-phase is visualised using an enzyme (usually horseradish peroxidase (HRP) or alkaline phosphatase (ALP)) conjugated secondary antibody that is specific for the primary antibody bound to the plate. Addition of a suitable substrate results in the formation of a coloured, soluble product. Commonly used substrates include p-nitrophenylphosphate (p-NPP), converted to yellow p-nitrophenol by ALP and o-phenylenediamine or 3,3'5,5'-tetramethylbenzidine (TMB), which are converted to green and blue products respectively by peroxidase enzymes. Finally, the reaction is usually stopped by addition of a strong (molar concentration) acid or base to quickly denature the enzyme and stop activity to allow results to be read in a spectrophotometer at an appropriate wavelength. Samples of known concentrations of antibody, from which a calibration curve is prepared to allow unknown sample antibody levels to be calculated, are included in all assays. This indirect ELISA procedure is the method used in this thesis and is summarised in figure 2.1.

2.2.2 General ELISA methods

Measurements of anti-gliadin IgG or anti-gliadin IgA antibodies were carried out using an in-house ELISA method or by AUTOSTAT™II Anti-Gliadin IgG or AUTOSTAT™II Anti-Gliadin IgA ELISA kits respectively, both from Hycor Biomedical Ltd, UK. Anti-TG2 IgA serological antibodies were assayed using Tissue Transglutaminase IgA ELISA Kits from Genesis Diagnostics, UK. Measurements of anti-glutamic acid decarboxylase (GAD) IgG antibodies were carried out using Isletest™-GAD IgG ELISA kits from Biomerica, US (distributed by IDS, UK).

2.2.2.1 ELISA kit storage

All kits were stored at 4^0C until used. After use, any remaining components were resealed, stored at 4^0C and used within 3 months of opening. The expiry date on all kits was checked prior to use. Kits were not used after the expiry date. Reagents were not interchanged between kits with different lot numbers nor were any reagents from different manufacturers used. All kits were allowed to reach room temperature (RT) (18-25^{0}C) for a minimum of 30min before use.

Figure 2.1 The indirect ELISA method for determination of antibody levels

Specific antigens adsorbed to solid phase (96-well plate).

Sample containing a mixture of antibodies, including antibody of interest is added. The plate is incubated and then washed.

Only the specific antibody of interest is bound.

Enzyme labelled secondary antibody directed against Fc portion of antibody of interest is added. The plate is incubated and then washed.

Secondary antibody, binds to the bound antibody of interest

Enzyme substrate added and plate incubated.

Measured enzyme activity is directly proportional to the amount of antibody in the test sample.

2.2.2.2 Sample preparation

Serum samples were collected and stored as in section 2.1.1.1. Any samples showing signs of haemolysis were not used as this would affect assay results. If necessary, samples were defrosted prior to use. All samples were allowed to reach room temperature and vortexed before use. CSF samples were collected and stored as in section 2.1.1.5. Again, samples were allowed to reach room temperature and vortexed before use.

2.2.2.3 Assay Plan

All ELISA kits were supplied with 96-well microplates, coated with the appropriate antigen, as the solid phase of the assay. The sample ELISA template shown in figure 2.2 was used as the plan for each assay. All samples standards and assay controls were assayed in duplicate. As shown in figure 2.2, wells A1 and A2 of the plate were filled with sample diluent only, to act as a blank. Wells H11 and H12 were filled with a repeat sample of either standard 1 or positive control. This allowed intra-assay variability to be calculated and to control for any effects of time taken to load the plate. Using this template, up to 40 samples could be assayed on each plate supplied with the anti-gliadin IgG and IgA kits, 38 samples could be assayed on each plate from the anti-TG2 IgA kits and 41 samples could be assayed on each plate from the anti-GAD IgA kits.

Figure 2.2 96-well microplate loading template used in ELISA experiments

STD = Assay standard, **POS** = Positive control, **NEG** = Negative control.

Test samples were assayed in duplicate in all remaining wells.

2.2.2.4 Assay controls

After each assay, validity was ensured by confirming that the values of the positive and negative control serum samples, provided with the kit, fell within the range specified by the manufacturers. If the values were not within this range, the plate was considered invalid and the results not used. In addition, to maintain assay reproducibility, it was ensured that the percentage coefficient of variation (%CV) of the duplicate absorbances for the standards, controls and samples was <20%. Any sample with a %CV of >20% was re-assayed. The cut-off figure of 20% was selected in order to allow the maximum numbers of samples to be recorded as valid whilst eliminating sample readings that were not close enough to guarantee an accurate value. In practice, most samples had a %CV of <10%.

2.2.3   Anti-gliadin antibody ELISAs

2.2.3.1   In-house anti-gliadin ELISA

- 96-well microtitre plate (Costar, UK)
- PBS (pH 7.4)

  1.54M Sodium Chloride

  0.13M Potassium Phosphate (K_2HPO_4)

  0.02M Potassium Phosphate (KH_2PO_4)

- Carbonate Buffer (pH 9.6):

  0.015M Sodium Carbonate

  0.035M Sodium Hydrogen Carbonate

- Wash Buffer:

  0.5% Tween20 in PBS

- Blocking solution:

  5% bovine serum albumin (BSA) (made using a 30% IgG-free BSA solution) in PBS

- Crude gliadin (1mg/ml):

  Crushed crude gliadin in carbonate buffer

- HRP-conjugated goat anti-human IgG (affinity purified, γ-chain specific)
- HRP-conjugated goat anti-human IgA (affinity purified, α-chain specific)
- TMB substrate (0.1mg/ml):

  1mg TMB tablet in 0.05M Phosphate-citrate buffer with 0.03%

  sodium perborate (pH5)

- Stop solution (0.1M H_2SO_4)

All reagents from Sigma, UK unless stated.

The crude gliadin solution was prepared by crushing the required amount of crude gliadin into a fine powder using a pestle and mortar and adding to the corresponding volume of carbonate buffer. The resulting solution was stirred on a magnetic stirrer whilst being heated gently until the gliadin had dissolved. It was ensured that the temperature of the solution did not rise above 60^0C to avoid denaturation of the gliadin. The 96-well microtitre plate was coated with the gliadin solution by adding 100μl to each well and incubation overnight at 4^0C. After incubation, the gliadin solution was decanted and the plate washed three times in wash buffer. During each wash, 200μl of wash buffer was added to each well, the plate was inverted and then excess buffer removed by tapping the plate several times on a paper towel. 200μl blocking solution was added to each well and the plate was incubated at RT for 60min. Serum samples were diluted in PBS and vortexed to ensure thorough mixing. The blocking solution was discarded from the wells and the plate washed three times in wash solution. 100μl of diluted sample or blank was added to the appropriate wells and the plate incubated at RT for 60min. Meanwhile, either goat anti-human IgG or IgA HRP-conjugates were diluted in PBS. The plate was washed three times in wash buffer. 100μl of the appropriate conjugated-antibody solution was added to each well and the plate incubated at RT for 60min. The working TMB substrate was prepared and used within 30min. The plate was washed three times in wash buffer. 100μl working TMB substrate solution was added to each well and the plate incubated at RT for 20mins. Following incubation, 50μl of stop solution was added to each well. The plate was read at 450nm within 15min on a Wallac 1420 plate reader (Version 2.00, PerkinElmer Life Sciences, USA). Data were reported as mean OD values.

2.2.3.2　Commercial anti-gliadin IgG ELISA

The plate and all reagents were supplied with the kit,

- 96-well microplate coated with purified gliadin
- Mouse anti-human IgG HRP-conjugate
- Human anti-gliadin standards calibrated to arbitrary units;
  Standard 1 (5U/ml), Standard 2 (20U/ml), Standard 3 (50U/ml),
  Standard 4 (100U/ml)
- Human anti-gliadin positive control serum
- Human anti-gliadin negative control serum
- Sample diluent buffer
- TMB substrate solution

- Wash buffer concentrate
- Stop solution (0.25M H_2SO_4)

Assays were performed according to the manufacturers' instructions. All reagents and test samples were brought to RT. Meanwhile, 50ml of wash buffer concentrate was measured and diluted to one litre with d.H_2O. All serum samples and positive/negative assay controls were diluted 1:100 with sample diluent by adding 5µl sample or controls to 495µl sample diluent and vortexed to ensure thorough mixing. CSF samples were used undiluted. The protective covering was then removed from the gliadin-coated microtitre plate and sufficient wells selected to accommodate patient samples, standards and assay controls, all of which were tested in duplicate. 100µl of standards, assay controls or samples were pipetted into the wells, according to a pre-determined plate plan based on the template shown in figure 2.2, and the plate incubated at RT for 30min. Following incubation, the well contents were discarded and the plate washed three times with diluted wash buffer using a multi-channel pipette. During each wash, 200µl of wash buffer was added to the wells and then emptied, the plate was inverted and tapped several times on a dry paper towel to remove excess wash buffer. 100µl of HRP-conjugated mouse anti-human IgG was added to each well. The plate was incubated for 15min at RT, the well contents discarded and the plate washed a further three times. 100µl of TMB substrate was added to each well and the plate incubated for 15min at RT. After this time, 50µl of stop solution was added to each well and the plate tapped gently to ensure uniform colour distribution. The plate was read at 450nm, within 15min, on a Wallac 1420 plate reader (Version 2.00, PerkinElmer Life Sciences, USA).

2.2.3.3 Commercial anti-gliadin IgA ELISA

Assays were performed according to the manufacturers' instructions. The materials and methods for the anti-gliadin IgA ELISA were as for the anti-gliadin IgG ELISA except the HRP-conjugated mouse anti-human IgG reagent was replaced with HRP-conjugated mouse anti-human IgA reagent.

2.2.4 Commercial anti-tissue transglutaminase IgA ELISA

The plate and all reagents were supplied with the kit,
- 96-well microplate coated with calcium-activated guinea pig tissue transglutaminase

- Sample diluent concentrate (150mM Tris-buffered saline (TBS), pH 7.2)
- HRP–conjugated rabbit anti-human IgA
- Human serum IgA anti-tissue transglutaminase standards in 10mM TBS, calibrated to arbitrary units

  Standard 1 (0U/ml), Standard 2 (5U/ml), Standard 3 (10U/ml)

  Standard 4 (25U/ml), Standard 5 (50U/ml), Standard 6 (100U/ml)
- Human IgA anti-tissue transglutaminase positive control serum in 10mM TBS
- Human IgA anti-tissue transglutaminase negative control serum in 10mM TBS
- TMB substrate (aqueous solution of TMB and hydrogen peroxide)
- Stop solution (0.25M H_2SO_4)

Assays were performed according to the manufacturers' instructions. All reagents and test samples were brought to RT. Meanwhile, working strength wash and sample diluent buffers were prepared by diluting each concentrate 1:15 with d.H_2O. All serum samples and positive/negative assay controls were diluted 1:100 with sample diluent by adding 5μl sample or controls to 495μl sample diluent and vortexed to ensure thorough mixing. Sufficient wells to accommodate patient samples, standards and assay controls, all of which were tested in duplicate, were assembled. 100μl of standards, assay controls or samples were pipetted into the wells according to a pre-determined plate plan based on figure 2.2. The plate was incubated at RT for 30min. After 30min, the well contents were discarded and the plate washed three times. During each wash 200μl of wash buffer was added to each well, the plate inverted and excess buffer removed by tapping the plate on a paper towel several times. 100μl of HRP-conjugated anti-human IgA reagent was added to each well and the plate incubated for a further 30min at RT. The well contents were then discarded and the plate washed four times with wash buffer. 100μl of TMB substrate was added to each well and the plate incubated for 10min at RT after which 100μl of stop solution was added to each well. The plate was read at 450nm within 10min on a Wallac 1420 plate reader (Version 2.00, PerkinElmer Life Sciences, USA).

2.2.5 Commercial anti-glutamic acid decarboxylase IgG ELISA

The plate and all reagents were supplied with the kit,
- 96-well microplate coated with purified porcine GAD antigen
- Isletest Sample diluent concentrate

- Isletest Conjugate diluent concentrate
- ALP-conjugated goat anti-human IgG concentrate
- Human serum IgG anti-GAD standards calibrated to arbitrary units (Ready to use)
- Human serum IgG anti-GAD negative control (Ready to use)
- Human serum IgG anti-GAD positive control (Ready to use)
- Isletest Substrate solution (p-NPP) (Ready to use)
- Isletest Washing buffer concentrate
- Isletest Stopping solution (1N NaOH) (Ready to use)

Assays were performed according to the manufacturers' instructions. All reagents and test samples were brought to RT. Meanwhile, reagents were prepared. Working strength wash and sample diluent buffers were prepared by diluting the concentrates 1:24 or 1:4 respectively with d.H_2O, taking care to rinse out any crystals of concentrate. The ALP- goat anti-human IgG conjugate was prepared by transferring a 5ml aliquot of the supplied conjugate diluent into each of the two bottles containing 1ml of the enzyme conjugate concentrate and mixing well. All serum samples were diluted 1:100 with working sample diluent by adding 10μl serum to 1ml sample diluent and vortexed to ensure thorough mixing. Sufficient wells to accommodate patient samples, standards and assay controls, all of which were assayed in duplicate, were assembled. 100μl of standards, assay controls or diluted serum samples were dispensed into the appropriate wells according to a pre-determined plate plan based on figure 2.1. The plate was incubated for 60min at RT. After the incubation, the well contents were discarded and the plate washed three times. During each wash 200μl of wash buffer was added to each well, discarded from the wells and excess buffer removed by tapping the plate on a paper towel several times. 100μl of reconstituted goat anti-human IgG ALP- conjugate was added to all wells. The plate was incubated in the dark for 60min at RT. Following the incubation, well contents were discarded and the plate washed a further three times with wash buffer. 100μl of substrate solution was added to all wells and the plate was incubated in the dark for 30min at RT. At the end of 30min, 50μl of the stop solution was added to each well. The plate was read at 405nm on a Wallac 1420 plate reader (Version 2.00, PerkinElmer Life Sciences, USA) within 10min of addition of the stop solution.

2.2.6 Calculation of antibody levels

In the case of the in-house gliadin ELISA, the average optical densities (OD) of all duplicate wells were calculated and the average blank reading subtracted from each to obtain the average, blank-corrected OD for each sample.

In the case of all commercial ELISA kits, the mean OD of duplicate wells were calculated and the mean blank reading was subtracted from the mean OD of the standards, controls and patient samples. The corrected OD of the standards were plotted against the corresponding levels (log axis) using a curve-fitting system for Windows (CurveExpert 1.34, Microsoft). The mean blank OD was used as the zero standard. The antibody levels of the patient samples were determined, using the OD values, from this calibration curve by the program. It was ensured that the positive and negative assay controls fell within the stated ranges (which are established by the manufacturer and vary slightly between lot numbers). Patient samples were also recorded as positive, negative or borderline according to the manufacturers' cut-off values (table 2.3). Additionally, the standard deviation (SD) of the duplicates was calculated and used to calculate the %CV for each standard, control and sample using the formula,

$$\%CV = (SD/Mean\ reading)\ x100$$

Table 2.3 Cut-off values of commercial ELISAs, as supplied by kit manufacturers.

ELISA	ANTIBODY LEVEL (U/ml)		
	POSITIVE	BORDERLINE	NEGATIVE
IgG anti-gliadin	>9	7-9	<7
IgA anti-gliadin	>6	5-6	<5
IgA anti-tissue transglutaminase	>10	-	<10
IgG anti-GAD	>1.05	1-1.05	<1

2.2.7 Calculation of intra- and inter-assay variability

Inter-assay variability for each commercial ELISA was calculated as the average variability of the top standard supplied with each kit. For each ELISA type, the

unadjusted, average OD of the top standard was recorded from each plate that had been assayed for each ELISA type and averaged to obtain an overall mean OD of the top standard. The average SD of the individual ODs was also calculated to obtain an overall SD. The %CV was then calculated using the formula in section 2.2.6. In this way, for each ELISA type, a %CV value corresponding to the inter-assay variability was obtained.

Intra-assay variability was calculated for each individual plate. The unadjusted, average ODs of the positive control or standard 1 and its repeat sample in the last two wells of the plate were recorded. The mean and SD of these two readings were then calculated and the %CV corresponding to the intra-assay variability calculated according to the formula in section 2.2.6. Finally, for each ELISA type, an average intra-assay variability was calculated by averaging the individual plate variabilities of that ELISA type.

2.2.8 Statistical analysis

Significant differences in antibody levels between patient groups were determined using non-parametric statistical tests. Non-parametric tests were used as the sample distributions are likely to be skewed and not normally distributed. In this situation, parametric tests are invalid and non-parametric tests are the more powerful.

Initially, for all ELISA types, Kruskal-Wallis one-way analysis of variance by ranks (with tied ranks) was performed to determine whether median antibody levels differed between patient groups. If the null hypothesis was rejected a non-parametric multiple comparison test (with tied ranks) (Dunn test) was performed to determine between which of the groups significant differences occurred. Significant results were recorded as below:

P	Rating	Description
$0.01 < P < 0.05$	*	Significant
$0.001 < P < 0.01$	**	Very significant
$P < 0.001$	***	Highly significant

Antibody levels within each group were summarised according to their ranked order in box and whiskers plots. Any changes in distribution of the data between groups are evident from these plots and data series are usefully defined by several parameters all on one plot. Briefly, horizontal lines represent the median, upper (75%) and lower (25%) quartiles and the maximum and minimum values. The quartiles are 'boxed', enclosing 50% of the data range, and finally single vertical lines extend 'whiskers' to the limits. Therefore, the range of the data is also clearly represented on the plot.

Any correlations between antibody levels were determined using the Spearman's rank-order correlation, a non-parametric alternative to Pearson's correlation coefficient.

Finally, any significant differences between the results of the in-house and commercial anti-gliadin ELISAs were determined using the Mann-Whitney U test. This test was selected as it is a non-parametric substitute for the Student's t-test and is suitable for comparing two samples.

2.3 Immunohistochemistry

2.3.1 Principles of the technique

IHC can be used to identify specific proteins (both membrane-bound and cytosolic) within tissue sections. The basic principle of the method (figure 2.3a) exploits the ability of cellular components to act as antigens and interact specifically with antibodies directed against them. Briefly, tissue sections are placed onto glass or plastic microscope slides. A 'blocking' serum is applied to minimise non-specific binding by the primary antibody and secondary detection antibody during later stages. Excess 'blocking' serum is washed off and a primary antibody, directed against the protein of interest, is applied to the section. Unbound antibodies are washed off and a secondary detection antibody, directed against the F_c portion of the primary antibody, is applied to the tissue section. Visualisation of antigen location is achieved by conjugating the secondary detection antibody to a variety of labels. Alternatively, but less frequently, the primary antibody may be labelled and visualised directly. Commonly used labels include fluorescent chromophores, such as fluoroscein, or enzymes that form insoluble coloured precipitates upon addition of a suitable substrate. Frequently, ALP and HRP are used with either 5-Bromo-4-chloro-3- indolylphosphate /nitro blue tetrazolium

Figure 2.3 Immunohistochemical methods for detection of tissue/cellular antigens

ENZYME / **SUBSTRATE**

INSOLUBLE COLOURED PRECIPITATE

Enzyme label eg. HRP

Enzyme-conjugated secondary detection antibody, raised in a different species to primary, directed against Fc portion of primary antibody eg. Goat anti-human IgG

Primary antibody directed against antigen of interest eg. Human serum antibody

Tissue section containing antigen of interest

ENZYME / **SUBSTRATE**

INSOLUBLE COLOURED PRECIPITATE

Avidin/biotinylated enzyme complex

Biotinylated secondary detection antibody, raised in a different species to primary, directed against Fc portion of primary antibody eg. Horse anti-mouse antibody

Primary antibody directed against antigen of interest eg. Mouse monoclonal antibody

Tissue section containing antigen of interest

a). Indirect immunohistochemistry technique for antigen detection

b). Avidin-Biotin immunohistochemistry technique with enhanced signal for detection of low levels of antigen

Key: ☐ Avidin ○ Biotin ✳ Enzyme

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(purple precipitate) or 3,3'-Diaminobenzidine (DAB) (brown precipitate) substrates respectively. The resultant staining is permanent and can be readily seen using a light microscope.

In situations where the amount of antigen in the tissue is low, certain modifications of the technique allow an amplified end signal thus facilitating detection. One such modification is the avidin-biotin complex (ABC) system (Vector laboratories, UK) (figure 2.3b), which exploits the high binding affinity these two proteins have for one another. The secondary antibody is biotinylated and therefore adds biotin at primary antibody binding sites. Unbound biotinylated antibody is then washed off the section and enzyme-labelled avidin/biotin complex is added. The complex consists of biotinylated enzyme molecules and avidin, which form a large 3-D structure due to the fact that each avidin molecule is capable of binding four biotin molecules (www.vectorlabs.com). Staining is enhanced due to the fact that many enzyme molecules end up attached to each secondary antibody.

2.3.2 General immunohistochemistry methods

2.3.2.1 CNS tissue

Human cerebellar tissue was obtained from normal control or motor neurone disease patients at post-mortem, with informed consent from next of kin, from Professor P. Ince in the Pathology Department of The Royal Hallamshire Hospital, Sheffield. Post-mortem CNS tissue was snap-frozen in liquid nitrogen at the hospital and stored at -70^0C, in an air-tight container.

Rat cerebellar tissue was removed from an adult rat, following cervical dislocation, mounted onto a cork-board using Cryo-M-Bed tissue embedding compound (Bright Instrument Company Ltd., UK) and immediately snap-frozen in isopentane cooled on liquid nitrogen. The tissue was wrapped in foil and stored at -70^0C, in an air-tight container.

As required, cerebellar tissue was cut into 10μm sections in a cryostat (Leica, Italy) at -20^0C, and collected on polysine-coated microscope slides (BDH, UK). If necessary, slides were stored at -70^0C, in an air-tight container, until use.

2.3.2.2 Sample preparation

Serum samples were collected and stored as in section 2.1.1.1. If necessary, samples were defrosted prior to use. All samples were allowed to reach room temperature and vortexed before use. CSF samples were collected and stored as in section 2.1.1.5. Again, samples were allowed to reach room temperature and vortexed before use.

2.3.3 Avidin-Biotin amplified immunohistochemistry

After warming to room temperature, sections were fixed by immersion in acetone at 4^0C for 10min, removed and air-dried. Sections were then blocked for 60mins at RT with normal horse (if a monoclonal primary antibody was used) or goat serum (if serum was used as primary antibody) (Mouse or rabbit Vectastain Elite ™ Kits respectively, Vector Laboratories, UK) (100µl/section), diluted 3:200 in PBS, in a humid chamber to prevent the tissue drying out. Excess blocking serum was discarded by gently tapping the slides on tissue. Sections were then incubated with primary antibody, diluted in PBS (pH 7.4, see section 2.2.3.1 for preparation), for 60mins at RT (100µl/section). Sections incubated with PBS as a substitute for primary or secondary antibodies were included as negative controls. Excess antibody was removed by gently tapping the slides on tissue and the sections then washed twice in PBS for 5mins. Control slides were washed separately to avoid cross contamination. Primary antibody binding was detected by subsequent incubation of sections for 60mins at RT with either biotinylated goat anti-human IgG antibody, or biotinylated horse anti-mouse IgG antibody diluted in PBS,(100µl/section), in a humid chamber. Excess antibody was removed by gently tapping the slides on tissue and the sections then twice washed in PBS for 5mins. Bound antibody was visualised using an avidin-biotin complex containing horseradish peroxidase ('ABC' reagent, Vectastain Elite™ Kit, Vector Laboratories, UK). ABC reagent was made up 30mins prior to use to allow formation of the avidin-biotin complex by combining equal amounts of reagents A and B with PBS so that each reagent reached a final dilution of 1 in 100. Sections were incubated with ABC reagent (100µl/section) for 60mins at RT in a humid chamber. Excess complex was removed by gently tapping the slides on tissue and the sections then twice washed in PBS for 5mins. Finally, sections were immersed in a solution of 0.2mg/ml DAB (Sigma, UK) in PBS, containing 0.015% hydrogen peroxide (Sigma, UK), for 5mins. After briefly rinsing in tap-water to quench the enzyme reaction, sections were counterstained with Mayer's haematoxylin (Sigma, UK) for 2mins and then rinsed in tap-water for a further 5mins. The sections were moved to a fume-cupboard and dehydrated by immersion in

an ethanol gradient (placed for 2mins in each of 70%, 80%, 95% and 100% ethanol solutions) followed by immersion in xylene for 2mins. After being air-dried, the slides were mounted in DPX (BDH, UK).

2.3.4 Indirect immunohistochemistry

Tissue sections were warmed to RT and then washed in PBS-Tween (PBST) (0.05% Tween20 (Sigma, UK) in PBS (see section 2.2.3.1 for preparation) for 10mins at RT with gentle shaking using an orbital shaker.

Sections were then incubated for 60mins at RT in a humid chamber, to stop sections drying out, with either commercial antibodies or patient serum (100μl/section) diluted in PBST. Sections incubated with PBST as a substitute for primary or secondary antibodies were included as negative controls. After the incubation period, excess antibody was removed by gently tapping the slides on tissue paper and sections were washed three times in PBST with gentle shaking, each wash lasting 5mins. Following this wash, sections were incubated for 30mins at RT with appropriate HRP-conjugated secondary antibody, diluted in PBST. Excess secondary antibody was removed by gently tapping the slides on tissue paper and sections were again washed as above. Finally, sections were developed for up to 20mins in a solution of 0.4mg/ml DAB (Sigma, UK) in PBST with 0.0135% hydrogen peroxide (Sigma, UK). After a quick rinse in tap-water to quench the enzyme reaction, sections were moved into a fume-cupboard and dehydrated in methanol (15secs) followed by methanol/xylene (1:1) (30secs) and xylene (2mins). After being air-dried, the slides were mounted in DPX (BDH, UK).

2.3.5 Visualisation and image capture

After staining, slides were viewed using an Olympus BX60F-3 light microscope and images captured using a colour video camera (JVC 3-CCD) attached to the microscope.

2.4 Gel Electrophoresis And Western Blotting

2.4.1 Principles of the techniques

Electrophoresis is a widely used analytical technique for the separation of charged molecules from complex mixtures. SDS-PAGE is most commonly used for separation

and qualitative analysis of protein mixtures. The technique is also very useful for determination of protein RMM as proteins are separated by size. Proteins can be separated in this way as, once dissolved in high concentrations of SDS, their electrophoretic mobility in polyacrylamide gels is directly related to their RMM. SDS (CH_3-$(CH_2)_{10}$-$CH_2OSO_3^-Na^+$) is a powerful anionic detergent. Prior to being loaded onto the gel, protein samples are heated for several minutes with a sample buffer containing SDS and a reducing agent (frequently β-mercaptoethanol). The tertiary structure of the protein unfolds due to destruction of disulphide bridges by the reducing agent. The negatively charged SDS then strongly binds to the unfolded protein thus swamping its original native charge. All proteins bind the same amount of SDS per gram, therefore, the amount of negative charge is directly proportional to the size of the original protein. Samples are then loaded into the gel, which is supported between two glass plates. Up to 20 protein samples may be loaded into individual wells of a stacking gel, which is set on top of the main separating gel. The gels are placed into an electrophoresis tank and tank buffer is added. Upon application of a voltage across the gel, the negatively charged SDS-protein complexes start to move down the gel, towards the positive electrode. The stacking gel has the purpose of concentrating the samples into a sharp band before they enter the separating gel. This is achieved through exploitation of the differences in electrophoretic mobility of ions in the various buffers and gels. Briefly, glycinate ions in the tank buffer move more slowly than the chloride ions in the sample buffer and stacking gel. The SDS-protein complexes have an intermediate mobility. All ionic species must migrate at the same speed to avoid breaking the electrical circuit. To achieve this, the ionic species alter their concentrations and, as only a small amount of SDS-protein complexes are present, they concentrate in a very tight band between the glycinate and chloride boundaries. Once the separating gel is reached, the glycinate ions speed up in the higher pH environment and the interface between the glycinate and chloride ions leaves the SDS-protein complexes to migrate at their own speed. The complexes continue towards the anode at the same speed as all have the same charge per unit length. However, frictional resistance within the gel opposes this movement. Larger molecules feel more resistance and so move more slowly than smaller molecules thus separating the complexes.

Additionally, separation is achieved through the molecular sieving properties of the gel. The polyacrylamide gel has a mesh-like, porous structure through which the proteins pass. This structure is formed by the cross-linking of acrylamide monomers in the

presence of a cross-linking agent (N, N-methylene-bis-acrylamide) and the relative proportion of these two reagents is the major factor in governing pore size. Conventionally, polyacrylamide gels are defined as a percentage, which reflects the amount of acrylamide monomer and crosslinker in relation to the total gel volume. As the percentage of the gel decreases, the pore size increases. In practice, the smaller the protein, the more easily it passes through the pores of the gel whereas larger proteins are eventually prevented from moving further, or from moving at all. The ability to vary the pore size of the gel allows the most appropriate percentage gel to be selected according to the size, or likely size, of the protein of interest to be separated. The polymerisation reaction is initiated by ammonium persulphate (APS) and catalysed by N, N, N', N' – tetramethylethylenediamine (TEMED), both of which are added to the gel mixture. Finally, the protein sample buffer also contains a dye (usually bromophenol blue) which is a small molecule that moves through the gel unhindered and indicates the electrophoresis front. The separation is sensitive to any alterations in pH or ionic concentration within buffers or gels although the major problem is avoiding overheating of the system. During the separation, most of the power generated is dissipated as heat. This problem may be overcome by using a power supply capable of providing a constant power although this isn't completely effective and this must be a consideration when selecting the most appropriate voltage for the separation. Proteins on gels may be detected and quantified in a variety of ways. Often, stains such as Coomassie brilliant blue or Ponceau-Red are used to detect protein bands on the gel. Although both are highly sensitive, greater sensitivity may be achieved using the silver stain, which is capable of detecting as little as 1ng of protein (Wilson & Walker, 1995).

Alternatively, the proteins may be further examined using the technique of western blotting. Proteins are firstly transferred ('blotted') from the gel onto a piece of nitrocellulose membrane. A sandwich of gel and nitrocellulose membrane is formed, compressed in a cassette and immersed in transfer buffer between two parallel electrodes. A current is passed at right angles to the gel, causing the separated proteins to migrate out of the gel and onto the membrane. Certain stains, including Ponceau-Red, can be used to visualise proteins once they have been transferred onto nitrocellulose, which is useful in monitoring the success and reproducibility of the transfer technique. The stain may then be washed off and the blot used in further experiments. Such experiments include probing the blot with an antibody to detect a specific protein in a manner similar to both IHC and ELISA techniques. The blot is

incubated in a protein solution to 'block' all remaining hydrophobic binding sites and avoid non-specific binding of either primary or secondary antibodies. A primary antibody, directed against the protein of interest, is applied and incubated with the blot for a period. Following this period, unbound antibody is washed off and a secondary detection antibody is applied, directed against the F_c portion of the primary antibody. As with IHC and ELISA techniques, most frequently this secondary antibody is enzyme-labelled with peroxidase or phophatase enzymes and addition of appropriate substrate results in a colour change by which binding may be visualised. Increased sensitivity can be achieved through use of a chemiluminescent substrate with HRP enzyme resulting in light emission that can be measured by exposing the blot to photographic film. Alternative detection sytems include radio-labelled or fluorescently-labelled secondary antibodies.

Finally, the RMM of a protein on a gel or membrane can be determined by comparing its mobility with those contained in commercial molecular weight markers, a mixture of standard proteins with known RMMs that are run on the same gel. Alternatively, certain commercial markers can be electrophoresed, blotted onto nitrocellulose membrane and then visualised after transfer.

2.4.2 Protein extraction

Rats were sacrificed by cervical dislocation and required tissues immediately removed. All steps were carried out on ice to minimise protease action within the tissue. All reagents were stored at 4^0C until used. The tissue was twice rinsed briefly in PBS (see section 2.2.3.1 for preparation) and weighed. Working extraction buffer was then prepared by adding 1 Complete™ protease inhibitor cocktail tablet (provides inhibition of serine, cysteine and metalloproteases as well as calpains) (Roche, UK) to 50ml extraction buffer (5mM Tris-Base, 0.02M Tris-HCl, 1mM EDTA). Working extraction buffer was added to the tissue in a ratio of 10μl per milligram, which was then homogenised using a hand homogeniser and the resultant homogenate transferred into 1.5ml tubes. Triton X-100 (Sigma, UK) was added to the homogenate to a final concentration of 1% v/v and mixed well using a vortex mixer. The tissue homogenate was then incubated for 1hour, at 4^0C, during which time it was vortexed every 10mins for approximately 30secs. After incubation, the homogenate was spun at 11,000xg for 10mins using a microcentrifuge (13000 rpm in a MSE MicroCentaur) and the supernatant and pellet separated. The pellet was re-suspended in 0.5ml d.H_2O, both

supernatant and pellet extracts were aliquoted into fresh 0.5ml tubes and finally a Bicinchoninic acid protein assay performed (section 2.4.3.2) to determine the amount of protein present in the extracts.

2.4.3 Determination of protein concentration within extracts

The amount of protein extracted was determined using the Bicinchoninic acid (BCA) protein assay (BCA assay). This is a modification of the Lowry method for protein detection enabling quantification of protein levels in a sample as low as 10µg. The assay makes use of the reaction between protein and an alkaline solution of copper sulphate, which yields a purple-coloured complex. This complex is chelated by BCA, making it very stable with a maximal absorption at 562nm (Holme & Peck, 1993).

Firstly, solutions of BSA were serially diluted in d.H_2O over the range 0.1mg/ml to 20mg/ml to create a calibration curve. 20µl aliquots of BSA standards were then pipetted in duplicate into a 96-well microtitre plate (Falcon, UK). 20µl aliquots of tissue supernatant or pellet extracts and 20µl aliquots of appropriate blanks (d.H_2O or working extraction buffer) were pipetted in triplicate into the plate. Working BCA reagent was then made up by mixing 20ml Bicinchoninic acid solution (Sigma, UK) and 0.4ml of a 4% (w/v) copper (II) sulphate solution (Sigma, UK). 200µl working BCA reagent was added to each well and the plate incubated for 30mins at RT. The plate was read at 550nm on a Wallac 1420 plate reader (Version 2.00, PerkinElmer Life Sciences, USA). A protein calibration curve was created by plotting the corrected mean OD of the duplicate BSA standards against the corresponding concentrations using a curve-fitting system for Windows (CurveExpert 1.34, Microsoft, USA). The protein concentration of the tissue extracts was calculated from this curve. In addition, it was ensured that the %CV of the duplicate absorbances for the standards did not exceed 10%. %CV was calculated as described in section 2.2.5.

2.4.4 SDS-PAGE

All reagents from Sigma, UK unless specified.

2.4.4.1 Sample preparation

Sample buffer was prepared according to the recipe below, aliquoted and stored at -80^{0}C.

- Reducing sample buffer (2x):

0.125M Tris-HCl

0.14M SDS

20% v/v Glycerol

2% v/v β-mercaptoethanol

0.03mM Bromophenol blue

Samples were diluted in d.H_2O to a final concentration of 7.3mg/ml. 2x sample buffer was added to tissue extract supernatants and re-suspended pellets in the ratio 1:1 v/v. This dilution provided a final amount of 36μg protein loaded per lane in each 10μl aliquot. After mixing well using a vortex mixer, the extracts were aliquoted into 500μl aliquots and heated to 85^0C for 2.5mins in a beaker of water. After cooling, samples were stored at -80^0C.

2.4.4.2 Electrophoresis

This method is based on the Laemmli technique (1970). The apparatus used was the Hoefer 250SE Mighty Small system (Amersham Pharmacia Biotech, UK).

- Resolving gel buffer:
  1.5M Tris-HCl (pH 8.8)
- Stacking gel buffer:
  0.5M Tris-HCl (pH 6.8)
- 30% Acrylamide/bisacrylamide
- 10% SDS in d.H_2O
- TEMED
- 10% APS in d.H_2O
- Running buffer (10x):
  0.25M Tris-Base
  1.92M Glycine
  0.035M SDS
- Wide range coloured molecular weight markers, 6.5-205 kDa
- Wide range Sigmamarker™ molecular weight markers, 6.5-205 kDa

It was ensured that all equipment was clean and dry before use. Glass plates were wiped with distilled water followed by 100% ethanol to remove all traces of dirt and

grease which may interfere with gel polymerisation. Two sets of plates were assembled in the casting stand using 0.75mm spacers between the two plates. Next, a 10% resolving gel was cast as below,

2.5ml Resolving gel buffer

3.3ml Acrylamide/bisacrylamide

4.1ml d.H_2O

100µl SDS (10%)

5µl TEMED

50µl freshly prepared APS (10%)

The resolving gel buffer, water, acrylamide/bisacrylamide and SDS were combined and mixed gently to avoid introduction of air bubbles into the solution. The TEMED and APS were then added and the solution stirred briefly. Without delay, the mixture was then pipetted into the gel cast, using a plastic transfer pipette, and a thin layer of 100% ethanol pipetted over the gel to exclude air, which would inhibit polymerisation. The gel was left to set (minimum of 45mins) at RT. Once the resolving gel had set, the ethanol layer was poured off and the top of the gel rinsed several times with distilled water to remove any residual ethanol. The stacking gel (5%) was then prepared as below,

2.5ml Stacking gel buffer

1.7ml Acrylamide/bisacrylamide

5.7ml d.H_2O

100µl SDS (10%)

10µl TEMED

50µl APS (10%)

As before, the stacking gel buffer, acrylamide/bisacrylamide, water and SDS were combined first and the solution mixed gently, avoiding introduction of air into the mixture. The TEMED and APS were then added and the mixture stirred briefly. The stacking gel mixture was then quickly pipetted on top of the resolving gel using a plastic transfer pipette until it was level with the top of the plates. A 10 tooth comb was inserted between the plates and the gel left to set (minimum of 45mins) at RT. Meanwhile, the tank was assembled and filled with 1x running buffer. At this point, if necessary, samples were defrosted and mixed well using a vortex mixer. Once the stacking gel had set, the gels were secured into the tank. Pre-stained molecular weight

markers were defrosted, mixed well and loaded directly onto the gel without further preparation. The combs were gently removed and required samples (10μl /lane) and molecular weight standards (10μl/lane) loaded slowly into their allocated wells to allow even settling at the bottom of each well. The gels were then run at 150V for approximately 60mins at RT. Once the bromophenol blue dye front had reached the bottom of the gel, the power was switched off and the gels removed from the tank.

2.4.5 Western Blotting

Western blot transfer was carried out using a Hoefer semi-dry blotter (Amersham Pharmacia Biotech, UK). All reagents from Sigma, UK unless specified.

- Towbin Transfer buffer:
  0.19M Glycine
  0.025M Tris-Base
  20% (v/v) methanol
- Filter paper (Amersham Pharmacia Biotech, UK)
- Hybond™-c extra; Supported nitrocellulose membrane (Amersham Pharmacia Biotech, UK)

On completion of electrophoresis, gels were soaked in transfer buffer at RT for approximately 10mins. Sufficient filter paper (6 pieces per gel) and nitrocellulose membrane (1 piece per gel) were cut to the same size as the gel and also soaked in transfer buffer for approximately 10mins at RT. Blotting 'sandwiches' were assembled in the blotting apparatus according to figure 2.4. The gels were transferred for 60mins at RT at 100V and then the nitrocellulose membrane was removed from the apparatus and the gels and filter paper discarded. The blots were then placed in ready-to-use Ponceau-S Red stain (0.1% Ponceau S (w/v) in 5% acetic acid (v/v)) (Sigma UK) for approximately 5mins to enable visualisation of the proteins. This allowed some idea of how successful the transfer had been and acted as a check to see that lanes had been equally loaded and that there was sufficient consistency between the same samples run during different experiments. At this point, RMM marker bands were highlighted on the blot using a pencil. The Ponceau-S Red was then washed off the blot by rinsing several times in d.H_2O in preparation for immuno-probing.

2.4.6 Detection of proteins

- PBST (see section 2.3.4 for preparation)

- Blocking solution:

  5% ECL blocking agent (Amersham Pharmacia Biotech, UK) in PBST

Blots were placed in plastic, disposable trays on an orbital shaker and blocked for 60mins at RT in 25ml blocking solution, which was followed by two 5min washes in PBST. The blots were then placed in clean trays on an orbital shaker and incubated with appropriately diluted (in PBST) primary antibody overnight at 4^0C. The following day, blots were removed from the primary antibody solution and washed 1x15min then 3x5min in PBST.

Figure 2.4 Assembly of 'sandwich' for protein transfer
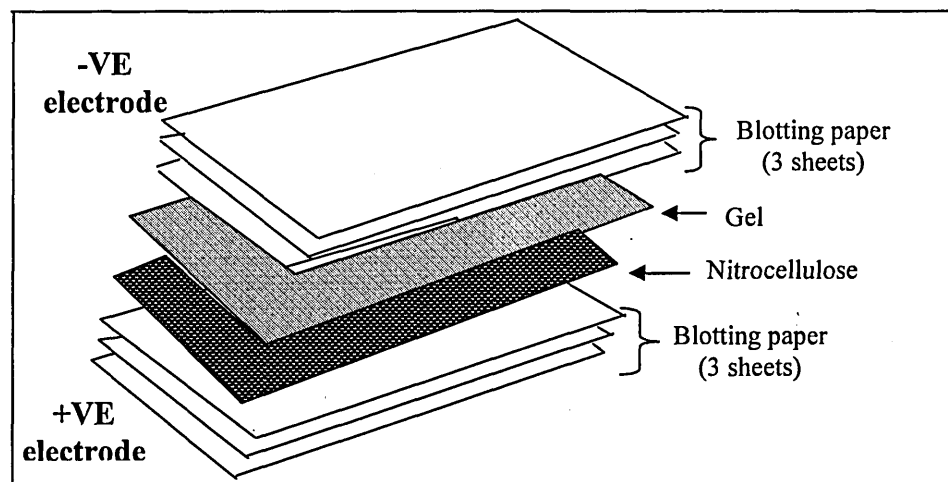
Appropriately diluted (in PBST) HRP- conjugated secondary antibody solution was added to the trays and the blots incubated for a further 60mins at RT with gentle shaking. After the incubation, blots were washed 1x15min then 3x5min in PBST. Excess PBST was removed and blots were placed protein side up onto a piece of cling-film. The required volume of ECL-Plus™ detection reagent (Amersham Pharmacia Biotech, UK) was prepared, by adding 50μl of reagent B to every 2ml of reagent A, and then added to each blot so that the entire surface of the membrane was covered before incubating for 5mins at RT. Excess reagent was removed by holding the blot gently in

forceps and holding the edge against a tissue. The blots were then placed protein side down onto a fresh piece of cling-film, which was gently wrapped around the blot to form an envelope and any air bubbles were gently smoothed out. Light emission resulting from the substrate was visualised using a UVP imager (UVP, UK) and captured using Labworks software (UVP, UK).

2.4.7 Determination of protein molecular mass

The relative mobility of each of the proteins in the RMM standard mixture was calculated as the distance moved down the gel as a percentage of the total distance moved by the dye front, using the equation below,

$$\% \text{ Relative mobility} = \frac{\text{Distance moved by protein standard of interest (mm)}}{\text{Total distance moved by dye front (mm)}} \times 100$$

A calibration curve was formed by plotting relative mobility against Log RMM for each of the protein standards in the mixture using a curve-fitting programme for Windows (CurveExpert 1.34, Microsoft, USA). The relative mobilities of unknown proteins within samples were calculated in the same way and their Log RMM determined from the calibration curve. From these values the RMM of the unknown proteins were calculated.

CHAPTER THREE

CIRCULATING GLUTEN SENSITIVITY-ASSOCIATED ANTIBODY LEVELS IN PATIENTS WITH GLUTEN ATAXIA

3.1   Introduction

3.1.1 Anti-gliadin antibodies and anti-tissue transglutaminase antibodies as serological markers of gluten sensitivity

Both anti-gliadin and anti-TG2 antibodies are produced as a result of the intestinal immune response against gluten. Their presence in the serum of gluten sensitive patients and the relatively recent availability of reliable, rapid methods of measurement (by ELISA) has resulted in these tests becoming a useful adjunct to duodenal biopsy and HLA testing for the diagnosis of CD. Additionally, these non-invasive tests facilitate the monitoring of patient response to a GFD and, together with other tests such as HLA testing, screening large populations for gluten sensitivity.

Previously, measurements of ARA, AEA and anti-gliadin antibodies were made as part of the diagnostic process of CD. However, ARA have a lower specificity for the disease (Troncone & Ferguson, 1991), and are now rarely included in current routine diagnostic tests. AEA, first described in 1983, are detected by immunofluorescence from their specific binding pattern using either monkey oesophagus or human umbilical cord as substrate (Chorzelski *et al.*, 1983, Ladinser *et al.*, 1994). Following the discovery that TG2 was the primary autoantigen recognised by AEA (Dieterich *et al.*, 1997), many diagnostic assays for their detection in serum were developed. Anti-gliadin and anti-TG2 antibody assays are today widely used serological tests for the diagnosis of CD, the combined sensitivity reaching 93% in patients where there is clinical suspicion of CD (Dahele *et al.*, 2001).

3.1.1.1 Anti-gliadin antibody assays

Initial reports of anti-gliadin antibodies in CD first appeared in the late 1950s and early 1960s (Rossi & Tjota, 1995). Isotype-specific anti-gliadin antibody tests are most usually detected by ELISA, the first method being described in 1977, although other detection methods such as immunofluorescence and RIA have also been used (Maki, 1995, Rossi & Tjota, 1995).

Production of anti-gliadin antibodies is strictly dependent on dietary exposure to gluten (Sollid, 2000). The antibodies are mainly of the IgA (IgA$_1$ and IgA$_2$) or IgG (IgG$_1$ and IgG$_4$) classes (Maki, 1995; Volta *et al.*, 1990) and are readily detected in the serum of

patients with CD. Generally, IgA anti-gliadin antibodies tend to have a greater specificity for CD and correlation with mucosal damage whereas IgG anti-gliadin antibodies have a greater sensitivity, although predictive values tend to vary and both isotypes should be tested for (Milford-Ward et al., 2001; Troncone & Ferguson, 1991) (table 3.1). It is believed that this variation is mostly due to patient selection within studies, rather than inherent properties of the assays themselves (Maki, 1995). Specificity appears more satisfactory in children (Savilahti et al., 1983) than in adults (Maki, 1995; Uibo et al., 1993) and positivity for anti-gliadin antibodies appears to increase with age in normal individuals (Uibo et al., 1993). Additionally, IgG anti-gliadin antibodies have been reported in a small proportion of patients with other GI disorders such as Crohn's disease, ulcerative colitis and cow's milk intolerance (Milford-Ward et al., 2001) as well as in otherwise healthy individuals (Troncone & Ferguson, 1991). Even so, the IgG anti-gliadin antibodies assay remains a valuable tool not least as it enables detection of IgA deficient patients with CD, a condition where subjects have a 10-20 fold increase of CD (Cataldo et al., 1998; Korponay-Szabo et al., 2003b).

As may be expected, anti-gliadin antibody levels tend to decrease once a GFD is initiated (Savilahti et al., 1983; Troncone & Ferguson, 1991) and the mucosa is allowed to recover, making them useful indicators of dietary compliance. Patients on a strict GFD have serum levels of IgA anti-gliadin antibodies similar to healthy controls but IgG anti-gliadin antibodies tend to persist at slightly higher levels for longer periods of time (Savilahti et al., 1983; Troncone & Ferguson, 1991). Re-introduction of gluten into the diet rapidly causes the reappearance of anti-gliadin antibodies but, for unknown reasons, they disappear again in a significant proportion of patients during a prolonged period (>3years) of a gluten-containing diet (Burgin-Wolff & Hadziselimovic, 1997).

3.1.1.2 Anti-tissue transglutaminase antibody assays

Production of anti-endomysial and anti-TG2 antibodies is also gluten-dependent and levels are correlated with the degree of enteropathy (Maki, 1995; Sollid et al., 1997; Tursi et al., 2003). Their levels fluctuate with gluten ingestion and normally disappear within one year on a GFD (Maki, 1995; Sollid et al., 1997). Reintroduction of gluten causes reappearance of anti-endomysial antibodies for the duration of a gluten-containing diet (Burgin-Wolff & Hadziselimovic, 1997).

Table 3.1 Sensitivity and specificity of serum anti-gliadin antibodies in untreated coeliac disease

Study number	PATIENT GROUP	IgA class		IgG class	
		Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
1	Children	97	92	-	-
2	Adults	46	98	62	97
3	Adults	90	85	76	88
4	Adults	65	100	95	98
5	Adults	67	94	78	94
6	Children	100	100	100	100
7	Adults	100	100	57	87
8	Adults	31	87	46	89
9	Children	96	100	91	99
10	Children	90	86	94	67

The above table is a summary of ten different studies investigating the sensitivity and specificity of anti-gliadin antibodies in patients with untreated CD.

Taken from Maki, 1995

Following the discovery that the primary target of AEA is actually TG2 (Dieterich *et al.*, 1997), recently developed anti-TG2 antibody ELISAs have become a more widespread tool in the diagnosis of CD. Immunofluorescent detection of AEA using monkey oesophagus or human umbilical cord was the assay of choice with a reported specificity of 96-100% and sensitivity of 90-98% (Amin *et al.*, 1999; Baldas *et al.*, 2000; Leon *et al.*, 2001; Milford-Ward *et al.*, 2001). However, good correlation between both assays has been shown (Amin *et al.*, 1999; Dieterich *et al.*, 1998; Leon *et al.*, 2001; Sulkanen *et al.*, 1998) and new TG2 ELISAs have comparable specificity (98-99%) and sensitivity (93-97%) (Baldas *et al.*, 2000; Biagi *et al.*, 1999b; Dieterich *et al.*, 1998; Milford-Ward *et al.*, 2001; Sblattero *et al.*, 2000). IgA anti-TG2 antibodies are normally measured for the purpose of diagnosis and monitoring of CD. Although IgG assays are available they have been reported to be less effective for this purpose but are useful for the detection of IgA-deficient patients with CD (Cataldo *et al.*, 2000;

Korponay-Szabo *et al.,* 2003b; Sblattero *et al.,* 2000). Originally, anti-TG2 ELISAs utilised TG2 from guinea-pig liver, which has an approximate 80% homology with human TG2 and, recently, assays using a human recombinant form of the enzyme have been made available. There is some suggestion that human recombinant TG2 assays are slightly more specific, possibly because they are able to detect antibodies directed against specific human TG2 epitopes (Amin *et al.,* 1999; Tonutti *et al.,* 2003). However, a more important factor influencing specificity appears to be the purity of the TG2 and purified guinea-pig TG2 ELISAs have been reported to have similar specificities to human recombinant TG2 ELISAs (Sblattero *et al.,* 2000).

3.1.2 Aims of this study

The presence and significance of anti-gliadin and anti-TG2 antibodies in the serum of patients with CD is well defined. In addition, both of these antibodies have been reported in patients with DH (Kumar *et al.,* 2001; Reunala & Hallstrom, 1990). Although the presence of serum anti-gliadin antibodies (IgG or IgA) is a diagnostic prerequisite of gluten ataxia, the full profile of these antibodies in conjunction with the presence of serum anti-TG2 antibodies had not been previously investigated. Finally, the presence of anti-gliadin antibodies has been reported in the CSF of a patient with neurological dysfunction and gluten sensitivity (Chinnery *et al.,* 1997).

Therefore, the aims of this study were:

o To investigate and characterise the presence of serum anti-gliadin (IgG and IgA) and anti-TG2 (IgA) antibodies in patients with gluten ataxia, using ELISA.

o To compare this serological profile with that of patients with CD only, other causes of ataxia (both genetic and sporadic (non-gluten) idiopathic ataxia) and healthy controls.

o To investigate any correlation between the levels of circulating anti-gliadin (IgG and IgA) and levels of IgA anti-TG2 antibodies.

o To investigate the presence of anti-gliadin antibodies in the CSF of patients with gluten ataxia.

3.2 Methods

3.2.1 Serum ELISAs

Initially, measurements of serum IgG and IgA anti-gliadin antibodies were made using an in-house gliadin ELISA method and then repeated using commercially available ELISA kits for comparative purposes. Measurements of serum IgA anti-TG2 were made using commercially available ELISA kits. For each kit type, serum antibody levels and inter and intra-assay variation were calculated according to the methods described in sections 2.2.6 and 2.2.7 respectively. Statistical analysis was carried out as described in section 2.2.8. Finally, for each patient, the corresponding levels of circulating antibodies were correlated using the Spearman rank order correlation as below:

o   IgG anti-gliadin antibodies and IgA anti-gliadin antibodies

o   IgG anti-gliadin antibodies and IgA anti-TG2 antibodies

o   IgA anti-gliadin antibodies and IgA anti-TG2 antibodies

3.2.1.1 In-house anti-gliadin ELISA

This method was based on an in-house ELISA for detection of anti-gliadin antibodies, which had previously been used successfully in the Immunology Department of The Northern General Hospital, Sheffield. Preliminary experiments were undertaken to optimise the method, which included investigation of antigen concentration for coating the plates, serum dilution and dilution of secondary antibody (data not shown). Following optimisation, a total of 9 gluten ataxia (untreated), 2 CD without neurological complications (untreated), 7 ataxia control and 5 healthy control serum samples were assayed for both IgG and IgA anti-gliadin antibodies, using the methods detailed in sections 2.2.2 and 2.2.3.1. Due to a lack of appropriate calibrants and controls, the inter- and intra variabilities were not calculated and statistical analysis was not carried out on the results. Variation between the average ODs from the in-house and commercial kit methods for each individual sample were assessed by calculating the %CV (as detailed in section 2.2.6) of the readings obtained by each method. If this value was 20% or less, the two methods were considered to have given comparable results. Any difference between the average OD of all samples assayed using each method were determined using the Mann-Whitney U test.

3.2.1.2 Commercial anti-gliadin IgG ELISA

A total of 33 gluten ataxia (untreated), 33 CD without neurological complications (untreated), 28 ataxia control (a mixture of familial and sporadic (non-gluten) idiopathic ataxias) and 43 healthy control serum samples were assayed according to the methods detailed in sections 2.2.2 and 2.2.3.2.

In addition, 4 gluten ataxia (untreated) and 1 healthy control CSF samples were assayed for IgG anti-gliadin antibodies according to the same methods.

3.2.1.3 Commercial anti-gliadin IgA ELISA

A total of 32 gluten ataxia (untreated), 32 CD without neurological complications (untreated), 25 ataxia control (a mixture of familial and sporadic (non-gluten) idiopathic ataxias) and 23 healthy control samples were assayed according to the methods detailed in sections 2.2.2 and 2.2.3.3.

3.2.1.4 Commercial anti-tissue transglutaminase IgA ELISA

A total of 30 gluten ataxia (untreated), 33 CD without neurological complications (untreated), 25 ataxia control (a mixture of familial and sporadic (non-gluten) idiopathic ataxias) and 42 healthy control samples were assayed according to the methods detailed in sections 2.2.2 and 2.2.4.

3.3 Results

3.3.1 Inter- and Intra-assay variability

The average inter- and intra-assay variability for each commercial ELISA kit type is shown in table 3.2.

3.3.2 In-house anti-gliadin antibody ELISA

Data from both the IgG and IgA anti-gliadin ELISAs are summarised in figures 3.1 and 3.2 respectively using box and whisker plots. Table 3.3 shows how this data compares to that obtained using commercial kits to assay the same samples. In both IgG and IgA assays, the median values of each group followed a similar pattern in both the in-house and kit ELISAs (figures 3.1 & 3.2 vs 3.4 & 3.6 respectively). The median IgG anti-gliadin antibody levels of both the gluten ataxia and CD only groups (0.73 and 1.03 OD units respectively) were higher than either the ataxia or healthy control groups, which had similar median levels (0.37 and 0.39 OD units respectively) (figure 3.1). The patients with CD only, also had a higher median IgA anti-gliadin value than any other group (0.89 OD units) whereas patients with gluten ataxia had a median level similar to that of the ataxia controls (0.05 and 0.06 OD units respectively). The median level of the healthy control group (0.20 OD units) was intermediate between these two values and the CD only group (figure 3.2).

Considerable variation was found between the actual OD values obtained with the in-house ELISA and those obtained using a commercial kit in both IgG and IgA assays (table 3.3). Although similar ODs were obtained for several samples (highlighted in grey), in some cases the difference was two-fold or more. In general, greater discrepancy was observed between IgA measurements than IgG. Variations between results followed an unpredictable pattern although both IgG and IgA OD values of all normal control samples and the majority of ataxia control samples were consistently higher when determined using the in-house ELISA method. However, the overall average ODs of all samples assayed using both the in-house ELISA and commercial ELISA kit methods were not significantly different (IgG, p = 0.61; IgA, p = 0.59).

Table 3.2 The inter- and intra-assay variability for each type of commercial ELISA kit

ELISA	AVERAGE (%)	
	Inter-assay variability	Intra-assay variability
IgG anti-gliadin	17 (n=7)	7 (n=7)
IgA anti-gliadin	24 (n=6)	10 (n=6)
IgA anti-TG2	17 (n=7)	13 (n=7)

Figure 3.1 Summary of serum IgG anti-gliadin antibody levels, measured by in-house ELISA
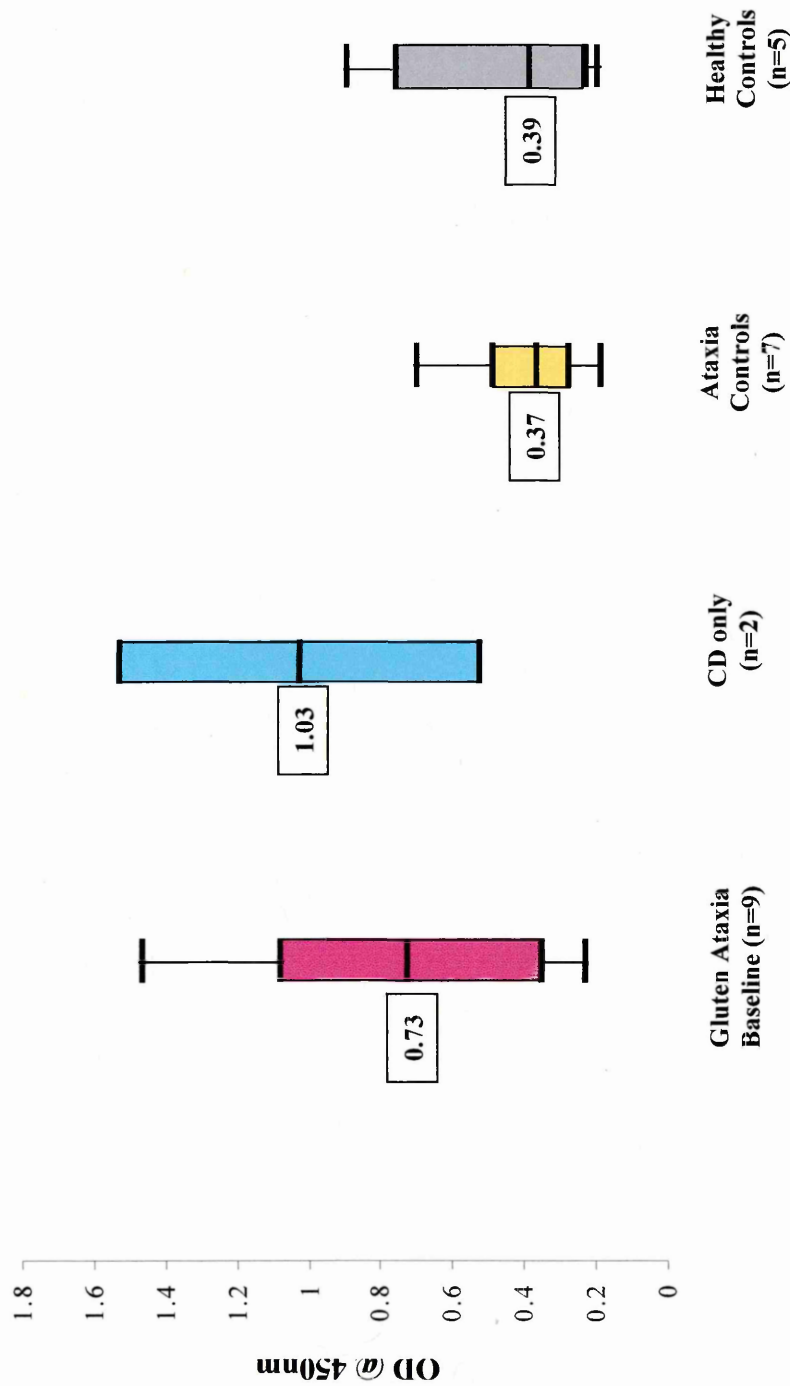
Data expressed as OD values @ 450nm in a box and whiskers plot (section 2.2.8). Median OD values of each group are shown next to the corresponding plot.

Figure 3.2 Summary of serum IgA anti-gliadin antibody levels, measured by in-house ELISA
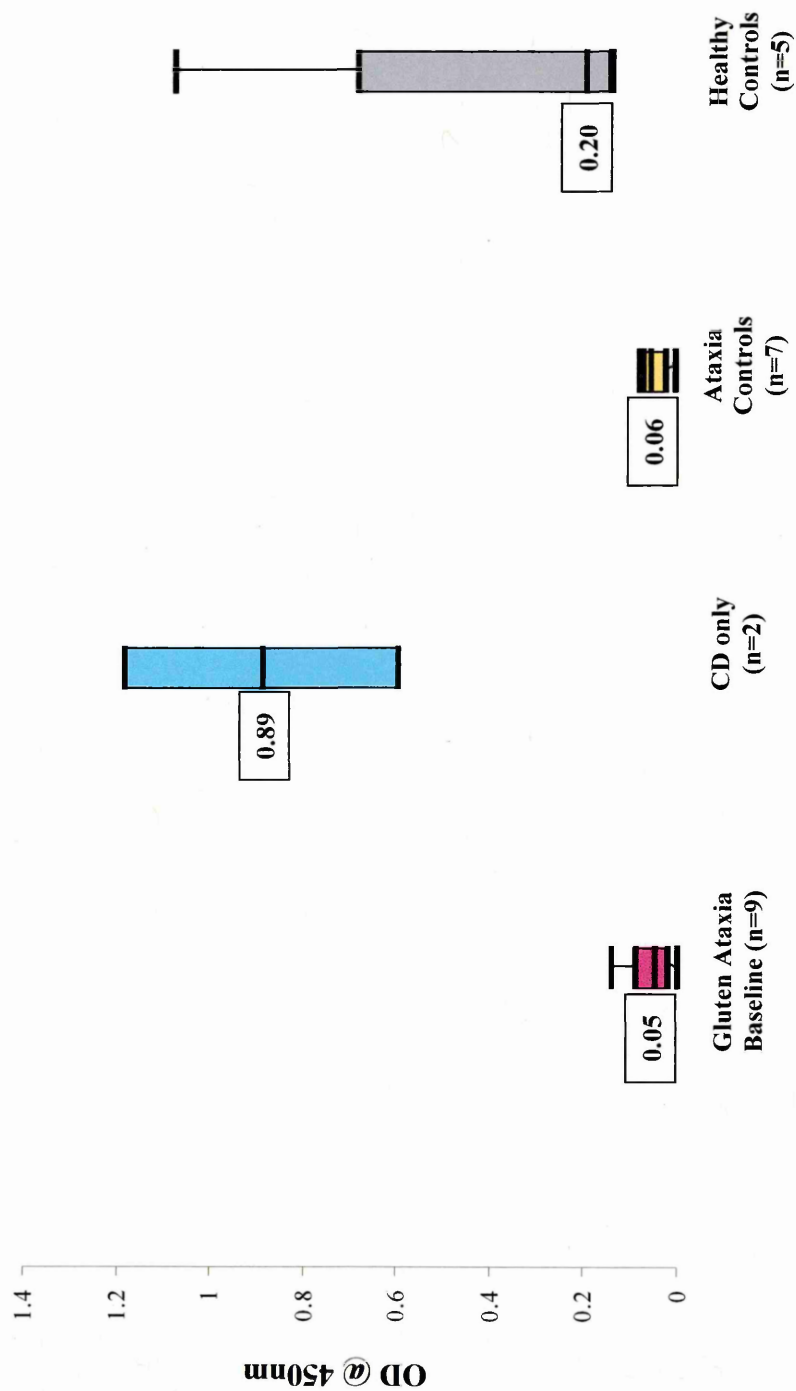
Data expressed as OD values @ 450nm in a box and whiskers plot (section 2.2.8). Median OD values of each group are shown next to the corresponding plot.

Table 3.3 Comparison of IgG and IgA anti-gliadin antibody levels obtained using in-house and commercial kit ELISAs

GROUP	Mean OD @ 450nm			
	IgG (In-house)	IgG (Commercial kit)	IgA (In-house)	IgA (Commercial kit)
Gluten Ataxia (untreated)	0.86	0.92	0.02	0.12
	0.76	0.85	0	0.02
	0.23	0.82	0.02	0.09
	0.33	0.43	0.14	0.81
	1.47	2.71	0.09	0.07
	0.73	0.47	0.06	0.04
	0.38	0.33	0.04	0
	1.30	0.46	0.09	0.09
	0.42	0.26	0.04	0.01
CD only (untreated)	1.53	1.71	1.18	2.43
	0.53	0.40	0.60	1.67
Ataxia controls	0.70	0.08	0.08	0.08
	0.37	0.09	0.07	0.1
	0.42	0.66	0.02	0.13
	0.28	0.12	0	0
	0.49	-	0.05	-
Healthy controls	0.19	0.22	0.06	0
	0.34	0.23	0.07	0.22
	0.26	0.07	0.14	0
	0.90	0.05	1.08	0
	0.62	0.17	0.20	0.04
	0.20	0.16	0.14	0.03
	0.39	0.11	0.29	0.05

Data expressed as average OD @ 450nm.

Samples with similar results (%CV <20) from each method are shaded grey.

3.3.3 Commercial IgG anti-gliadin ELISA

According to the cut-off values supplied by the kit manufacturer, 76% of gluten ataxia samples were either positive or borderline positive for IgG anti-gliadin antibodies as compared to 82% of CD only samples, 2% healthy control samples and 0% of ataxia control samples. This is represented in figure 3.3, which illustrates the distribution of sample levels of IgG anti-gliadin antibodies between groups. Samples from both control groups had similar ranges whereas samples from patients with gluten ataxia had the largest spread and those from patients with CD only had the smallest. The lowest level in each of the control groups was much lower than that of either the gluten ataxia or CD only groups (0.1U/ml compared to 0.8U/ml and 2.1U/ml respectively). The highest levels were greater in the gluten ataxia and CD only groups (31.9U/ml and 67.47U/ml respectively) than either the ataxia (6.41U/ml) or normal (10.9U/ml) control groups. Also, a greater proportion of samples from the gluten ataxia and CD only groups had levels close to the positive/negative cut-off value than either of the control groups.

The median value and inter-quartile range of each group is shown in figure 3.4. Kruskal-Wallis analysis indicated a highly significant difference between groups ($p < 0.001$). Median antibody levels of gluten ataxia patients and patients with CD only were found to be significantly higher than both the normal and ataxia control patients ($p < 0.001$). Antibody levels of patients with CD only were not significantly higher than those of gluten ataxia patients ($p > 0.05$).

3.3.4 Commercial IgA anti-gliadin ELISA

Using the cut-off values provided by the kit manufacturer, 25% of gluten ataxia samples were either positive or borderline positive as compared to 47% of CD only samples, 4% healthy controls and 0% of ataxia control samples. The distribution of all sample levels is represented in figure 3.5. More than half of the control sample levels fell below measurable limits, a greater proportion than in the other groups. Additionally, samples from both control groups were spread over a smaller range than either the gluten ataxia or CD only samples, which had similar ranges. The highest levels of the gluten ataxia and CD only groups were much higher (16.71U/ml and 100U/ml respectively) than the highest level of either the ataxia (2.07U/ml) or normal (5.97U/ml) controls.

Figure 3.3 Serum IgG anti-gliadin antibody levels of patients with gluten ataxia, coeliac disease only and ataxia and healthy controls

Data expressed in arbitrary units, defined by the kit manufacturers. The lower and upper horizontal lines represent the minimum and maximum values measurable by the assay respectively. Points lying on these lines fall either above or below these limits and their exact value was not determined. The red horizontal line represents the positive/negative threshold, as defined by the kit manufacturers.

Figure 3.4 Summary of serum IgG anti-gliadin antibody levels of patients with gluten ataxia, coeliac disease only and ataxia and healthy controls
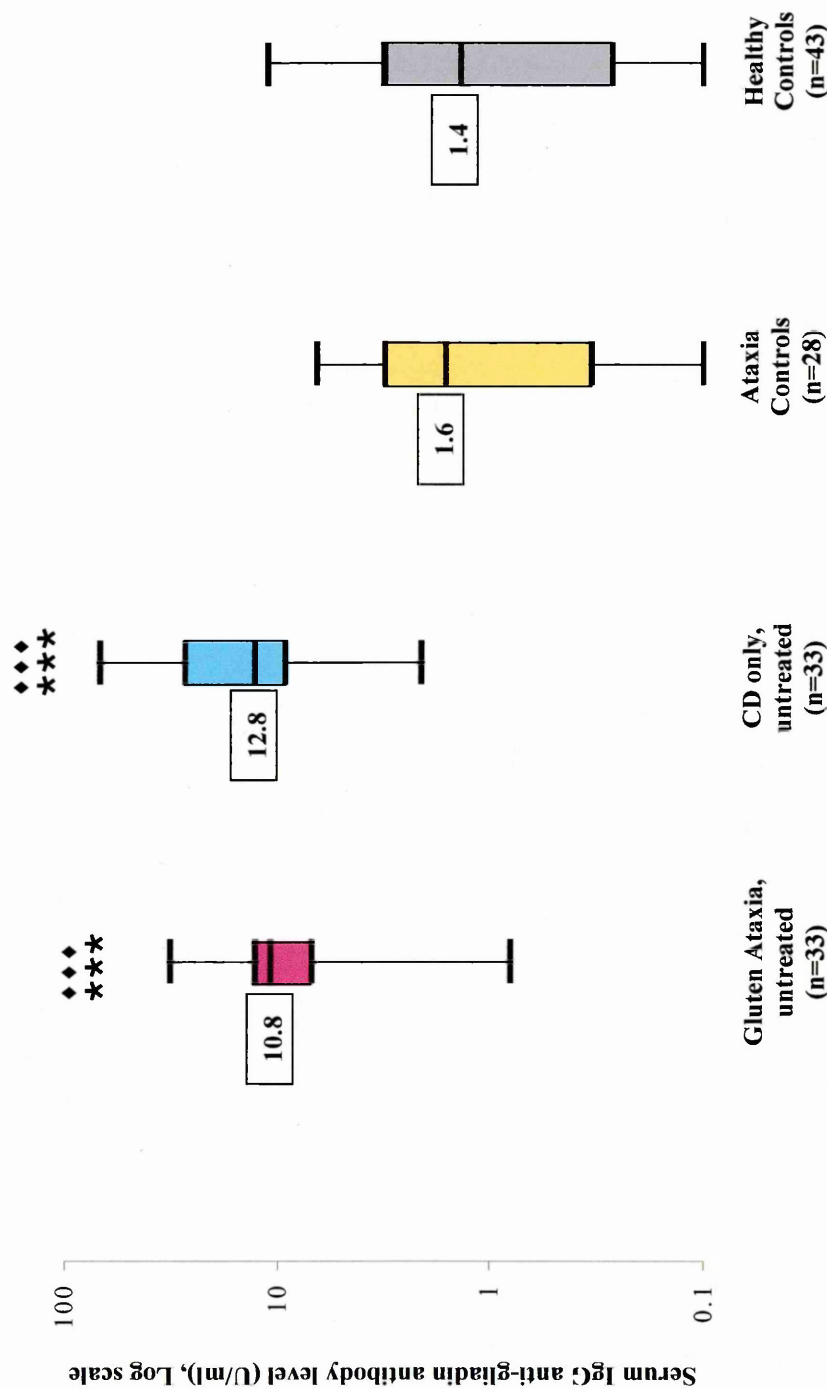
Data expressed in arbitrary units (defined by the kit manufacturers) as box and whiskers plot (section 2.2.7). ◆◆◆ = p<0.001 compared to ataxia control group. ✶✶✶ = p<0.001 compared to normal control group according to non-parametric multiple comparison. Median levels of gluten ataxia and CD groups nor control groups were not significantly different from each other. Median level of each group is shown next to the corresponding plot.

Figure 3.5 Serum IgA anti-gliadin antibody levels of patients with gluten ataxia, coeliac disease only and ataxia and healthy controls

Data expressed in arbitrary units, defined by the kit manufacturers. The lower and upper horizontal lines represent the minimum and maximum values measurable by the assay respectively. Points lying on these lines fall either above or below these limits and their exact value was not determined. The red horizontal line represents the positive/negative threshold, as defined by the kit manufacturers.

Figure 3.6 Summary of serum IgA anti-gliadin antibody levels of patients with gluten ataxia, coeliac disease only and ataxia and healthy controls
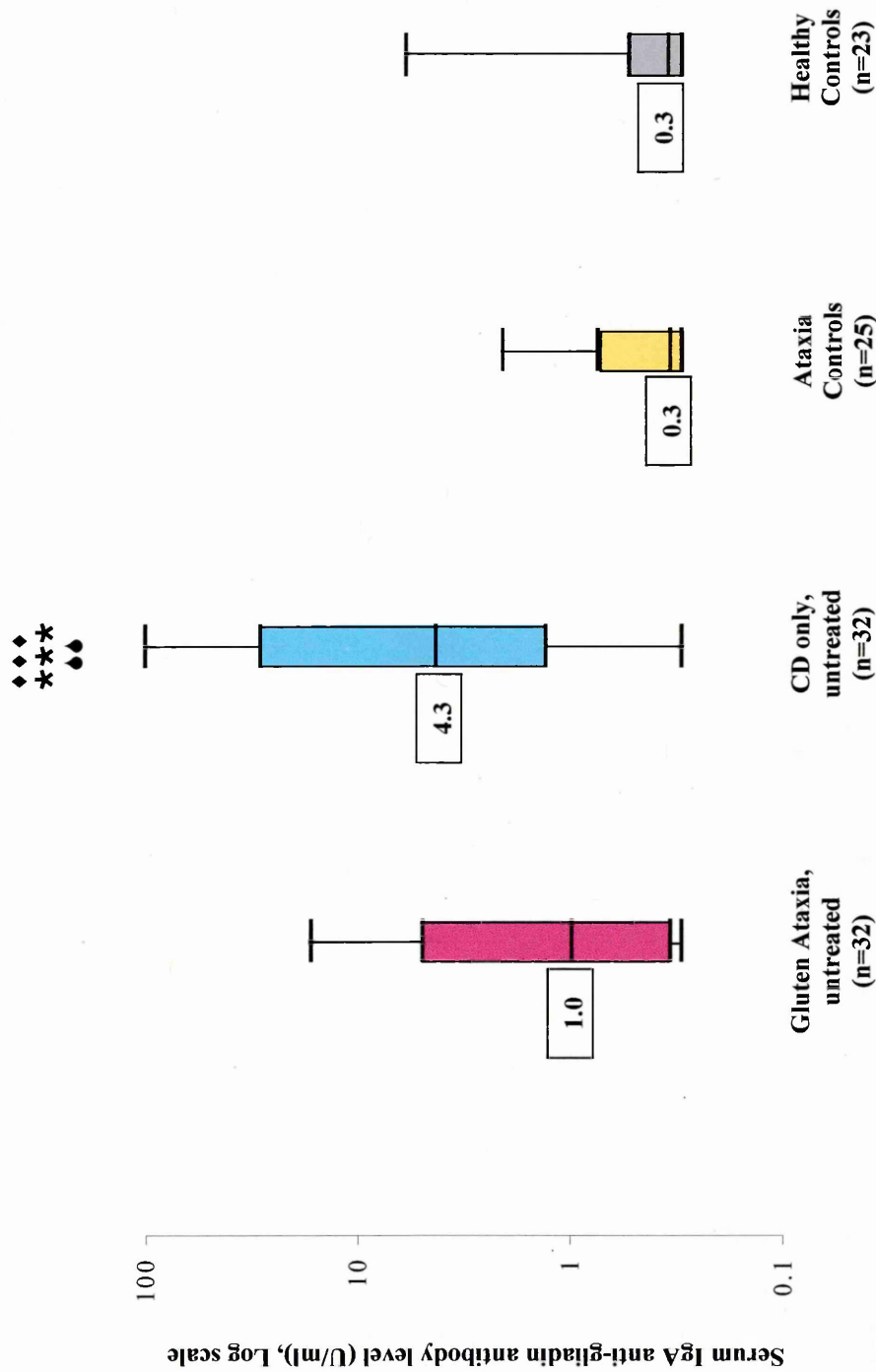
Data expressed in arbitrary units (defined by the kit manufacturers) as box and whiskers plot (section 2.2.7). ◆ ◆ ◆ = p<0.001 compared to ataxia control group. ✱✱✱ = p<0.001 compared to normal control group. ◆●=p<0.05 compared to gluten ataxia group according to non-parametric multiple comparison. Median levels of gluten ataxia and control groups were not significantly different from each other. Median level of each group is shown next to the corresponding plot.

The median antibody concentration of patients with CD only was significantly higher than in both the normal (P<0.001) and ataxia control groups (P<0.001) as well as the gluten ataxia group (P<0.05). Patients with gluten ataxia did not have significantly higher levels than either of the control groups (P>0.05) (figure 3.6).

3.3.5 Commercial IgA anti-tissue transglutaminase ELISA

The distribution of levels is shown in figure 3.7. 40% of gluten ataxia patients, 94% of CD only patients, 24% of ataxia control patients and 19% of healthy controls were positive. Patients with gluten ataxia had the largest range of antibody levels whereas the ataxia control samples had the smallest. Patients with CD only had a similar range to the normal controls, which was between the gluten ataxia and ataxia control group ranges. The lowest sample level was similar in each of the gluten ataxia (2.32U/ml), ataxia control (2.76U/ml) and normal control (1.6U/ml) groups but was slightly higher in the CD only group (6.63U/ml). The highest level from the ataxia and normal control groups 21.1U/ml and 36.3U/ml respectively) were lower than that of both the gluten ataxia and CD only groups (1227U/ml and 23000U/ml respectively).

Overall, the median IgA anti-TG2 antibody level of patients with CD was significantly increased compared to all other patient groups, (P<0.001), including the gluten ataxia group (figure 3.8). Patients with gluten ataxia had a higher median level than normal control samples (P<0.001).

3.3.6 Individual serum antibody profiles of patients with gluten ataxia

The individual serum levels of IgG and IgA anti-gliadin antibodies and IgA anti-TG2 antibodies of each patient with gluten ataxia are summarised in table 3.4.

3.3.7 Correlation of serum antibody levels

A significant positive correlation was observed between the presence of IgG anti-gliadin antibodies and the presence of IgA anti-gliadin antibodies in both patients with gluten ataxia (p<0.05) and patients with CD without neurological complications (p<0.01) (figures 3.9 and 3.10 respectively). This association was not seen in either the ataxia control group or the healthy control group (data not shown). The presence of either IgG

Figure 3.7 Serum IgA anti-tissue transglutaminase antibody levels of patients with gluten ataxia, coeliac disease only and ataxia and healthy controls
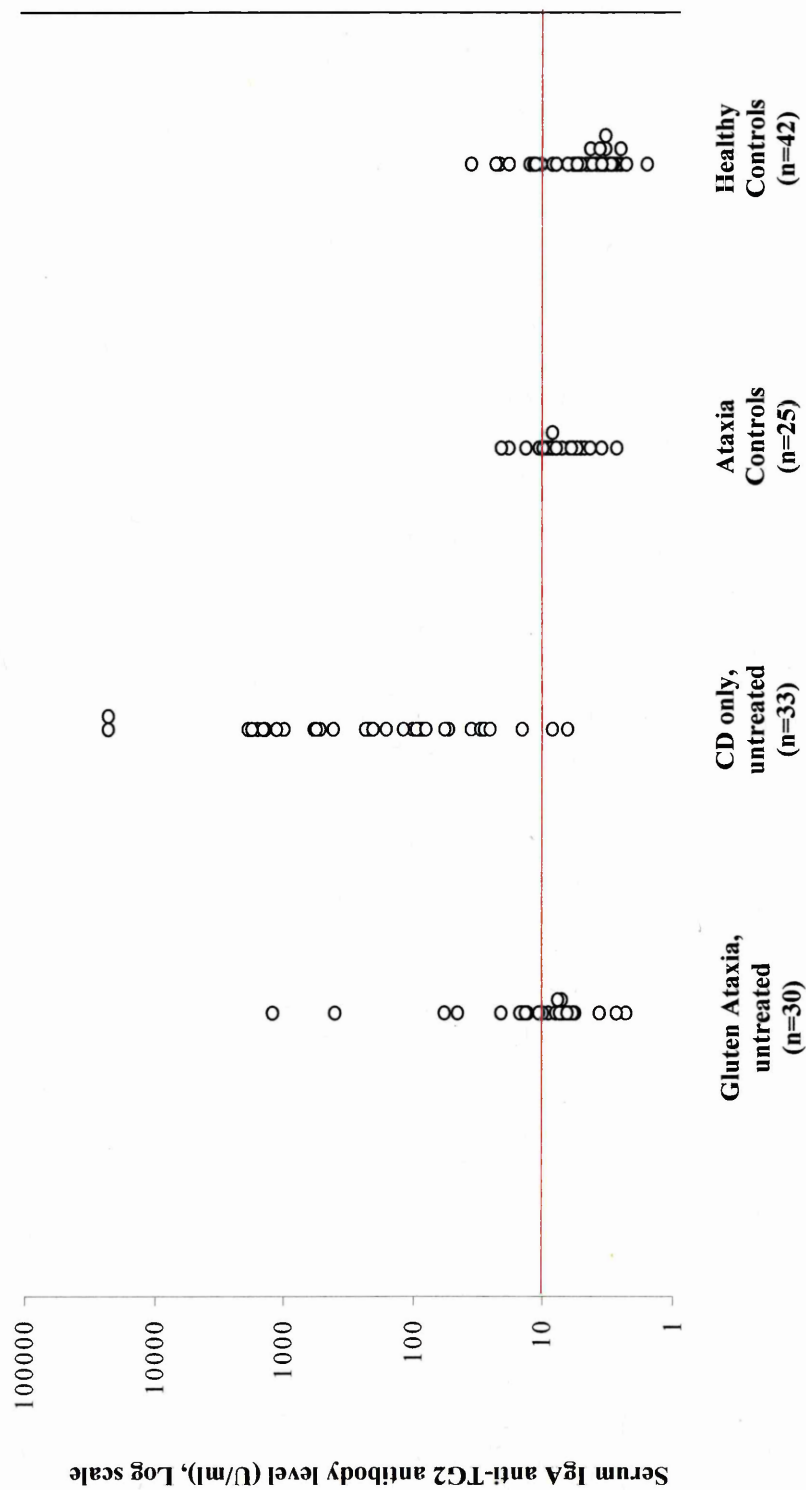
Data expressed in arbitrary units, defined by the kit manufacturers. The horizontal line represents the positive/negative threshold, as defined by the kit manufacturers.

Figure 3.8 Summary of patient serum IgA anti-tissue transglutaminase antibody levels of patients with gluten ataxia, coeliac disease only and ataxia and healthy controls
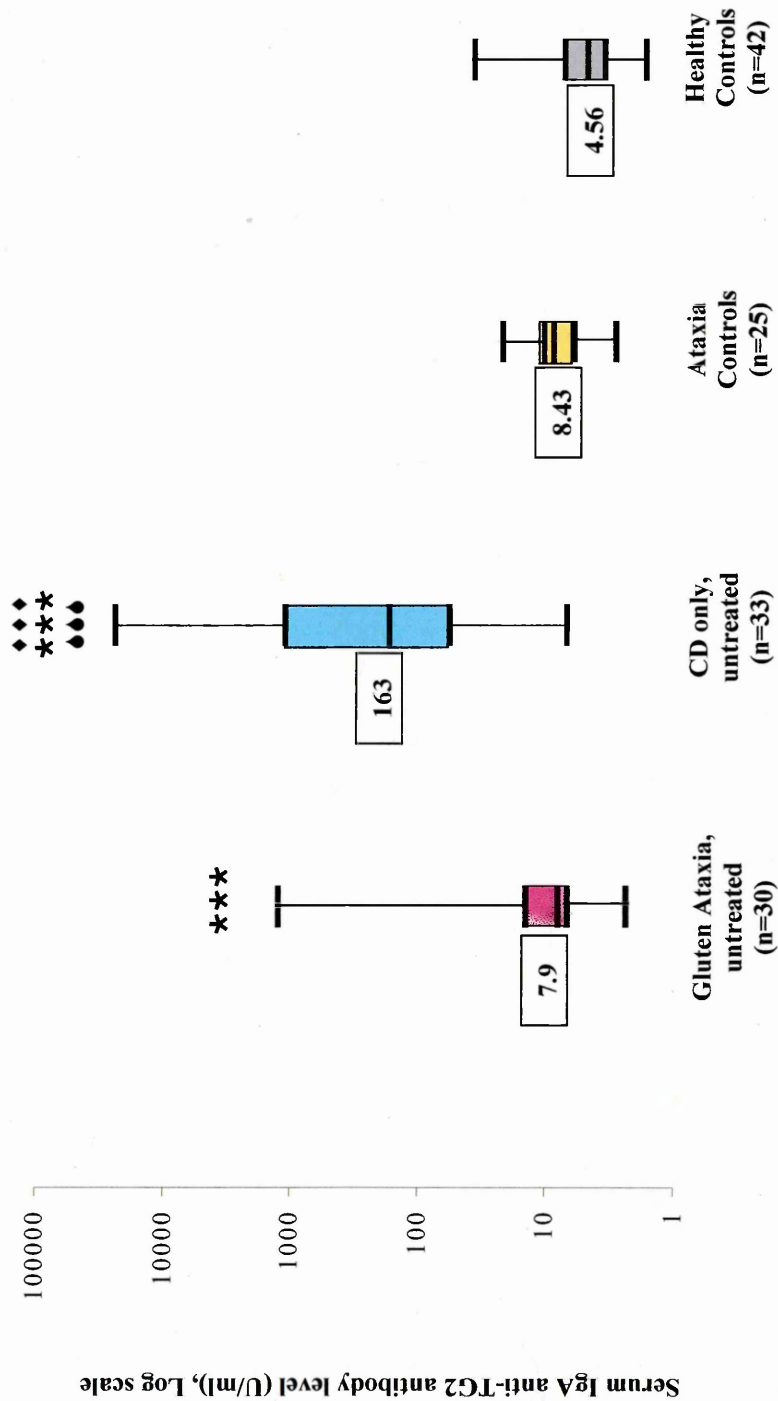
Data expressed in arbitrary units (defined by the kit manufacturers) as box and whiskers plot (section 2.2.7). ◆ ◆ ◆ = p<0.001 compared to ataxia control group, ✶ ✶ ✶ = p<0.001 compared to normal control group and ◆◆◆ = p<0.001 compared to gluten ataxia group according to non-parametric multiple comparison. Median levels of gluten ataxia and ataxia control groups were not significantly different from each other.

Median level of each group is shown next to the corresponding plot.

Table 3.4 Summary of the individual serum levels of IgG and IgA anti-gliadin antibodies and IgA anti-TG2 antibodies of each patient with gluten ataxia

Patient number	IgG AGA level (U/ml)	IgA AGA level (U/ml)	IgA TG2 level (U/ml)
1	22.7	1.1	3.7
2	2.2	ND	7.1
3	20.4	0.8	2.3
4	3.1	8.4	Not assayed
5	24.6	4.7	13.3
6	7.4	6.7	Not assayed
7	12.9	11.8	1227
8	17.1	3.8	9.2
9	12.4	ND	10.9
10	12.2	0.4	14.8
11	9.6	13.6	10.7
12	11.5	1.0	5.7
13	7.1	7.5	7
14	12.1	ND	7.7
15	12.2	5.5	408
16	3.9	ND	7.1
17	6.6	ND	7.7
18	8.5	ND	6.3
19	4.4	ND	45.6
20	12.3	ND	9.3
21	7.5	0.4	13.9
22	0.8	ND	10.1
23	31.9	1.6	21.3
24	15.4	16.7	57.9
25	5.6	ND	7.5
26	11.9	0.2	7.4
27	9.9	1.0	8.0
28	11.2	1.0	15.3
29	10.8	5.0	2.7
30	21.1	4.7	5.9
31	1.3	ND	6.6
32	9.1	1.4	6.6
33	9.3	Not assayed	Not assayed

Data expressed in arbitrary units (defined by the kit manufacturers). Patient number corresponds to that in table 2.1. ND – non-detectable (level too low to be detected). Samples highlighted in red are positive or borderline positive according to the kit cut-off values.

Figure 3.9 Correlation between circulating IgG and IgA anti-gliadin antibodies in patients with gluten ataxia
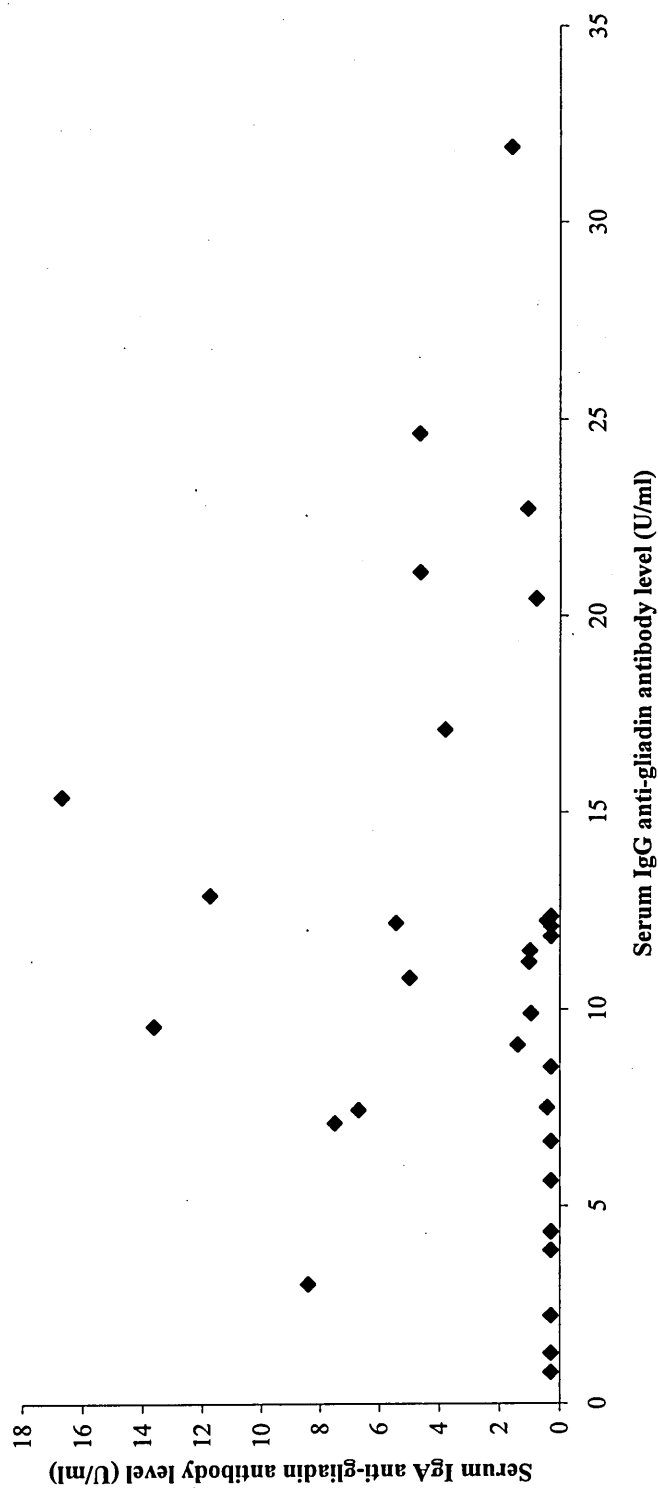
Data expressed in arbitrary units, as defined by the kit manufacturers. Correlation was assessed by Spearmans rank sum test.

A positive correlation was observed between circulating IgG and IgA anti- gliadin antibodies in patients with gluten ataxia (p<0.05).

Figure 3.10 Correlation between circulating IgG and IgA anti-gliadin antibodies in patients with coeliac disease only
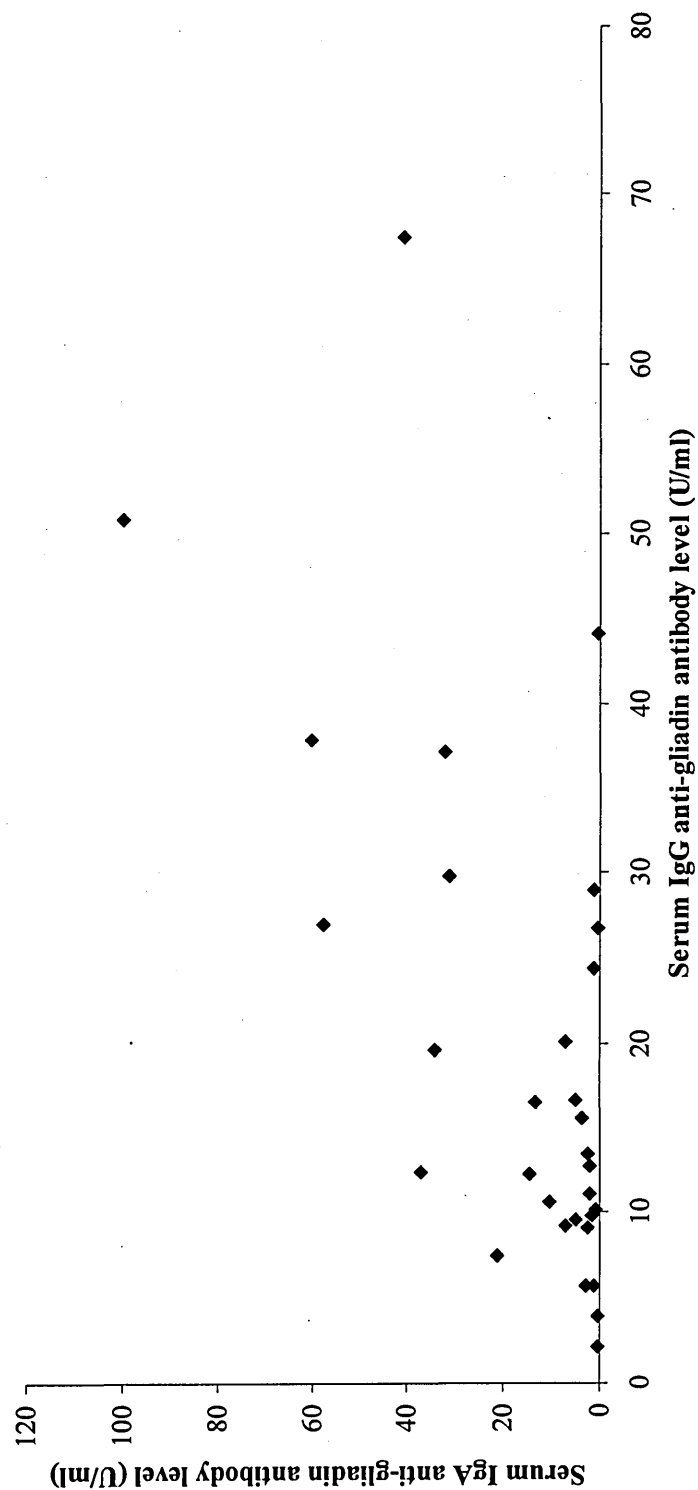
Data expressed in arbitrary units, as defined by the kit manufacturers. Correlation was assessed by Spearmans rank sum test.

A positive correlation was observed between circulating IgG and IgA anti- gliadin antibodies in patients with CD only (p<0.01).

or IgA anti-gliadin antibodies was not significantly associated with the presence of IgA anti-TG2 antibodies in patients with gluten ataxia (figure 3.11) or any control patients (data not shown). However, the presence of IgA anti-gliadin antibodies in patients with CD without neurological complications was significantly associated with the presence of IgA anti-TG2 antibodies ($p < 0.01$) (figure 3.12).

3.3.7 Presence of IgG anti-gliadin antibodies in the CSF

None of the CSF samples tested were positive according to the kit threshold value (figure 3.13), all had higher levels than the normal CSF sample, which fell below the measurable limits of the assay (data not shown). There was no correlation between serum level and CSF level, although all serum levels were higher than the corresponding CSF level.

Figure 3.11 Correlation between circulating IgA anti-gliadin and IgA anti-tissue transglutaminase antibodies in patients with gluten ataxia
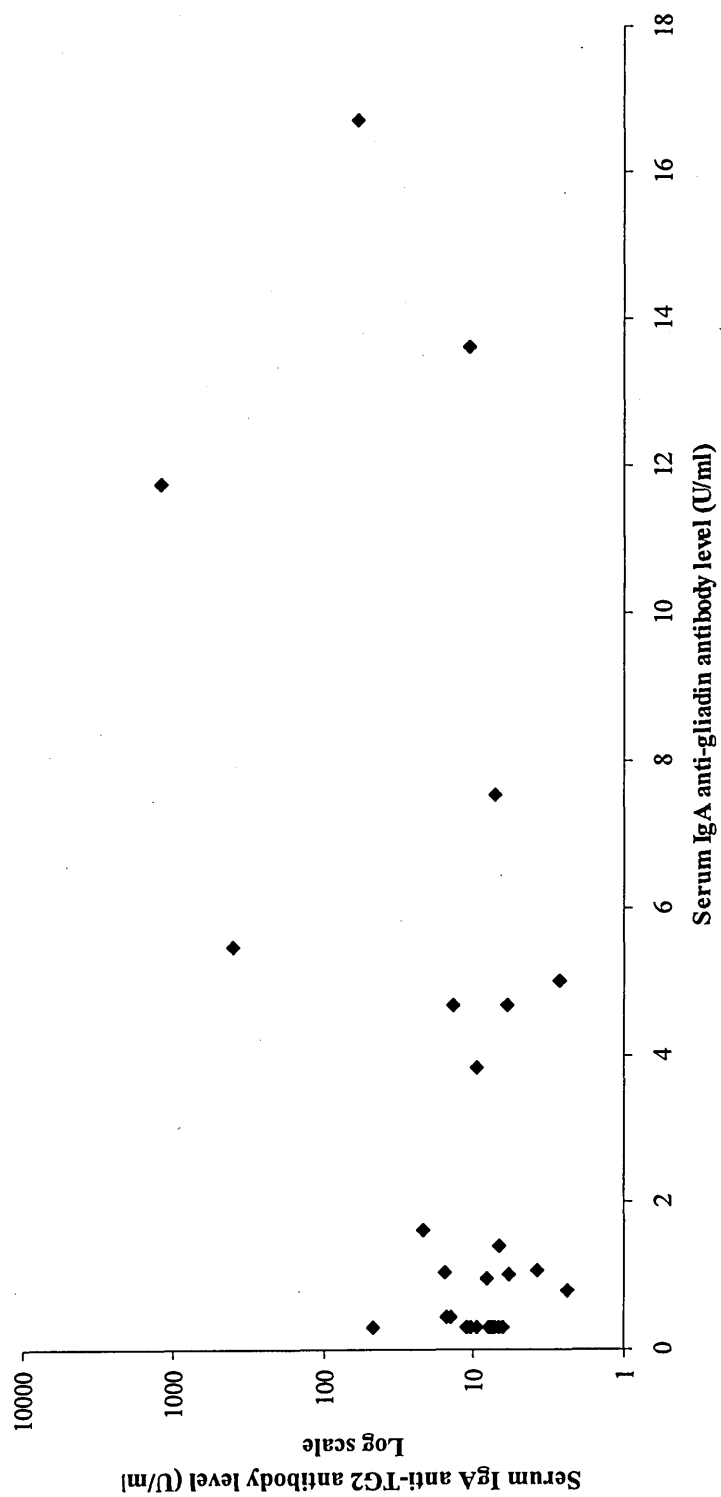
Data expressed in arbitrary units, as defined by the kit manufacturers. Correlation was assessed by Spearmans rank sum test.

A positive correlation was not observed between circulating IgA anti-gliadin and IgA anti-TG2 antibodies in patients with gluten ataxia (p>0.05).

Figure 3.12 Correlation between circulating IgA anti-gliadin and IgA anti-tissue transglutaminase antibodies in patients with coeliac disease only
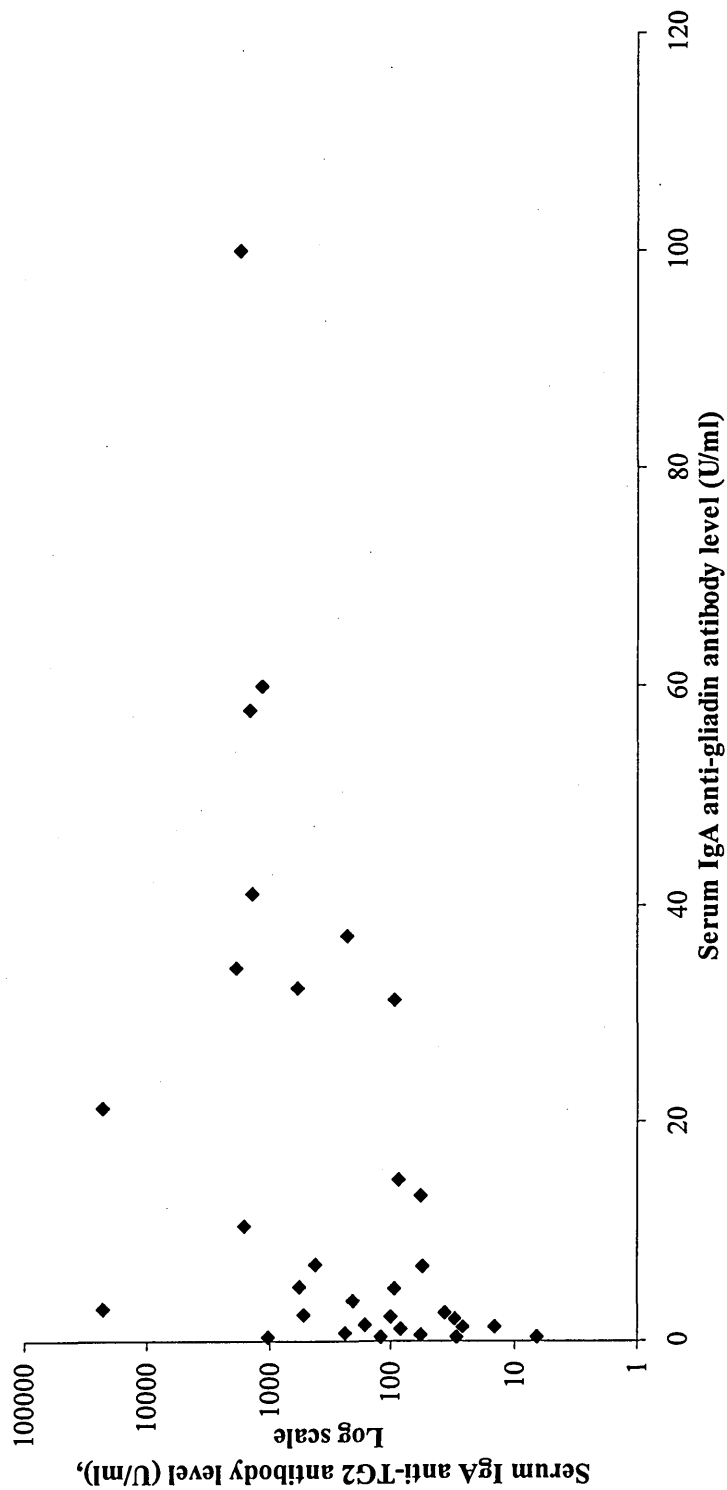
Data expressed in arbitrary units, as defined by the kit manufacturers. Correlation was assessed by Spearmans rank sum test.

A positive correlation was observed between circulating IgA anti-gliadin and IgA anti-TG2 antibodies in patients with CD only (p<0.01).

Figure 3.13 Comparison of serum and CSF IgG anti-gliadin antibody levels of patients with gluten ataxia
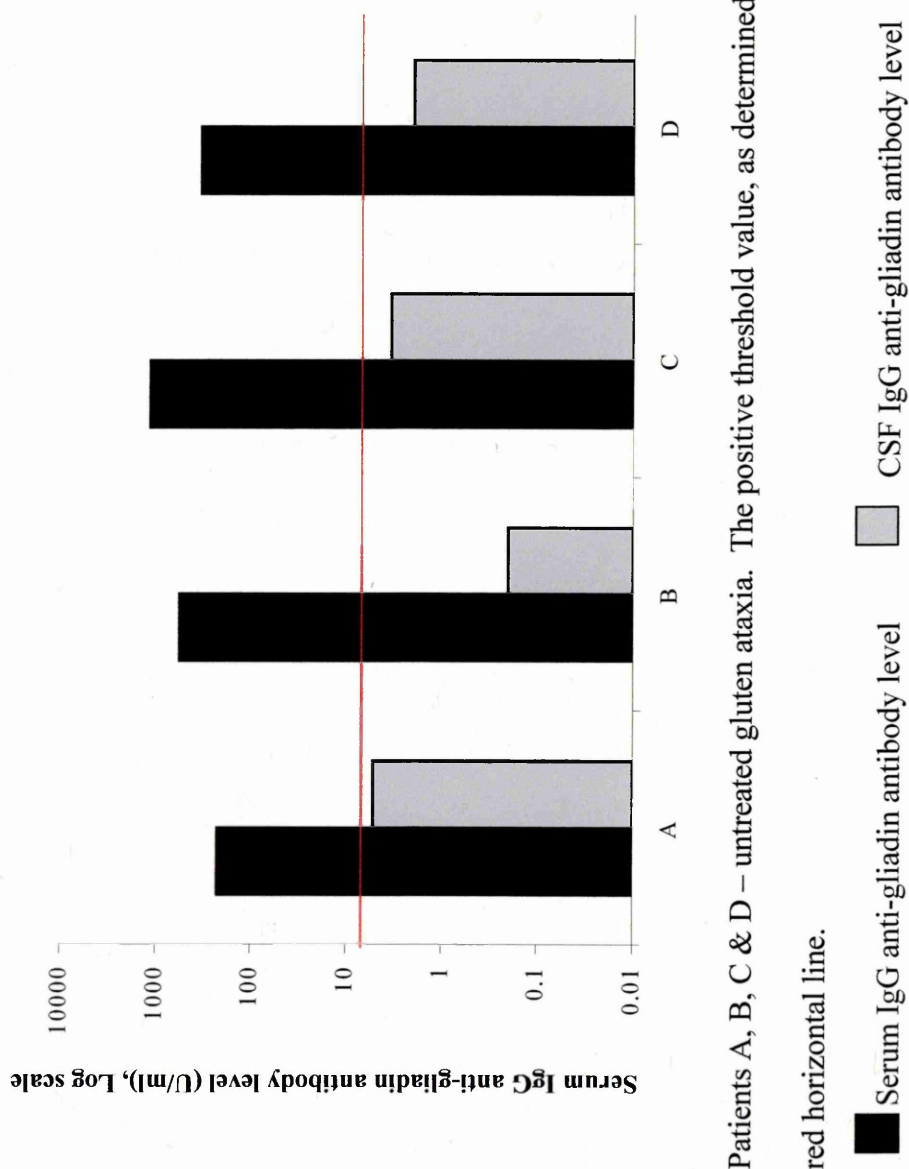
Patients A, B, C & D – untreated gluten ataxia. The positive threshold value, as determined by the kit manufacturers, is represented by the red horizontal line.

■ Serum IgG anti-gliadin antibody level   ▨ CSF IgG anti-gliadin antibody level

3.4 Discussion

It is now well-established that CD is associated with the presence of various circulating, gluten-driven antibodies, which are produced during the intestinal immune response against gluten. The majority of patients with CD have significantly increased levels of circulating antibodies directed against the primary aetiological agent, gluten (anti-gliadin antibodies) and also autoantibodies directed against the intestinal enzyme TG2. Measurement of these antibodies plays a major role in the diagnosis and monitoring of the condition. In addition, increased serum levels of these antibodies have been reported in patients with DH (Kumar *et al.,* 2001; Reunala & Hallstrom, 1990) but, to date, the presence of these antibodies in patients with gluten ataxia has not been comprehensively established.

In-house vs commercial anti-gliadin antibody ELISA

A comparison of in-house and commercial kit anti-gliadin ELISA methods found that, overall, commercial ELISA kits gave more reliable, reproducible results and the assay took less time, allowing more rapid screening of samples. Reproducibility of the in-house anti-gliadin ELISA may have been affected by factors such as non-uniform coating of the ELISA plate or slight variations in the concentration of the coating solution due to difficulties encountered related to the solubility of crude gliadin. Although, the average results obtained from each of the methods did not differ significantly (p>0.05), individual in-house ELISA results were often discordant to those obtained with the kit, by quite large margins on occasion. Discrepancies were unpredictable although generally the in-house method reported higher levels, particularly of control samples. These differences may be due to factors such as the use of higher concentrations of secondary antibody or the longer incubation times used in the in-house method. The median OD of healthy control samples, determined by the IgA anti-gliadin in-house assay, however, was considerably higher than expected. This is most likely due to the observed problems of assay reliability rather than a true reflection of sample levels as the levels of all healthy control samples were consistently lower when re-assayed using a commercial ELISA kit.

Commercial ELISA kit characteristics

The average ELISA kit intra-assay variabilities of each assay type were acceptable, the greatest being 13% in the case of the IgA anti-TG2 kit. However, the average inter-assay variabilities were greater, ranging from 17-24%. These greater variations were

found to be mainly due to the effects of one or two outlying values in each assay type, variations ranging from 13-15% with their exclusion. These outlying values may be attributable to slight differences in ambient conditions, such as temperature and light, occurring naturally between assays or kit batches. However, in each assay, all controls were within the specified ranges and assay results were considered valid.

Antibody levels of patients with coeliac disease only

As expected, the patients with CD only, examined in this thesis, had significantly elevated levels of circulating anti-gliadin antibodies (both IgG and IgA) as well as IgA anti-TG2 antibodies compared to both ataxia control and healthy control patients ($p < 0.001$). The most sensitive assay in these patients was the IgA anti-TG2 ELISA with 94% testing positive, a comparable value to the range of 81-98% quoted in similar studies (Dahele *et al.*, 2001; Dieterich *et al.*, 1998; Kumar *et al.*, 2001; Tursi *et al.*, 2003). In comparison, 82% and 47% of patients with CD only were positive for IgG or IgA anti-gliadin antibodies respectively, giving an overall total of 84% positive for IgG and/or IgA anti-gliadin antibodies. The finding that 82% of patients with CD were positive for circulating IgG anti-gliadin antibodies is comparable to the figure of 87% reported in a recent study, although the number of patients in the current study positive for IgA anti-gliadin antibodies appears to be relatively low compared to the equivalent figure of 61% in the same study (Dahele *et al.*, 2001). However, previous findings of the sensitivity of both IgG and IgA anti-gliadin ELISAs in untreated CD show wide variation (as illustrated in table 3.1). These differences may, in part, be due to differences in the positive/negative cut-offs of different assays. Alternatively, differences in gliadin peptides used to coat the plates or variation in patient selection have both been suggested as possible causes of such variation (Maki, 1995; Rossi & Tjota, 1995). The lower number of CD patients positive for IgA anti-gliadin antibodies is consistent with the reported greater specificity of these antibodies and the greater sensitivity of IgG anti-gliadin antibodies for CD (Milford-Ward *et al.*, 2001; Troncone & Ferguson, 1991). Finally, the two patients in this group who were negative for both IgA anti-TG2 antibodies and anti-gliadin antibodies may be part of the small group of patients with CD who are seronegative for all circulating antibodies associated with the disease (Rostami *et al.*, 1998).

Antibody levels of control patients

In the current study, 2/43 (5%) healthy control samples were positive for IgG anti-

gliadin antibodies and 1/23 (4%) healthy control samples was borderline positive for IgA anti-gliadin antibodies. No ataxia control patients were positive for anti-gliadin antibodies, an observation not consistent with the 8-15% of familial ataxia patients found to be positive for anti-gliadin antibodies in a recent study (Abele et al., 2003). However a prevalence of anti-gliadin antibodies in familial ataxia patients, similar to healthy control values, has been confirmed by other studies (Hadjivassiliou et al., 2003a) and it is likely that the numbers of patients in the current study were too small to confirm these observations. Although the significance of circulating anti-gliadin antibodies in otherwise healthy individuals remains uncertain, both circulating IgG and IgA anti-gliadin antibodies have been reported in a small proportion of otherwise healthy individuals with no apparent association with underlying CD (Troncone & Ferguson, 1991; Uibo et al., 1993). IgG anti-gliadin antibodies may also be found in patients with other GI disorders (Milford-Ward et al., 2001). Alternatively, these may represent cases of potential gluten sensitivity where the disorder is not yet fully apparent. One striking observation in the current study was the unexpectedly high numbers of both ataxia control and healthy control samples positive for IgA anti-TG2 antibodies (24% and 19% respectively). These values compare with reported values of between 1% and 7% in other similar studies (Dahele et al., 2001; Dieterich et al., 1998; Kumar et al., 2001). It is unclear why the current values are so high although the differences may partially be explained by assay differences. Half of the positive samples (4/8) in the current study had levels between 10-13 U/ml, just above the kit positive cut-off of 10 U/ml or above and it is possible that these samples would have been recorded as negative by a different assay. The other 4 positive samples however had individual levels well above the cut-off value. A prevalence of IgA anti-TG2 antibodies has been reported in up to 7% of patients with other GI diseases (including irritable bowel syndrome and Crohn's disease), a finding which is thought to reflect the wide distribution of TG2 throughout the GI tract (Dahele et al., 2001). IgA, IgG or IgM anti-TG2 antibodies, detected by ELISA, have also been reported in rheumatoid arthritis patients and systemic lupus erythematosus (SLE) patients as well as healthy controls (11%, 22% and 3% respectively) (Feighery et al., 2003). Therefore, it is possible that some of the anti-TG2 antibody positive control patients had other co-existing conditions causing their positive results although it is unlikely that this alone would explain the high prevalence observed. The lack of corresponding positivity for anti-gliadin antibodies implies that undiagnosed gluten sensitivity is a possible but unlikely explanation for the increased levels of anti-TG2 antibodies although one of the positive

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samples was also positive for IgG anti-gliadin antibodies, suggesting that this individual may actually have undiagnosed gluten sensitivity.

Antibody levels of gluten ataxia patients

Patients with gluten ataxia were found to have significantly elevated levels of circulating IgG anti-gliadin antibodies, ($p<0.05$), and IgA anti-TG2 antibodies, ($p<0.001$), compared to both ataxia controls and healthy controls. While not significantly different, individual levels of these antibodies in patients with gluten ataxia were generally not as high as those seen in patients with CD only. The IgG anti-gliadin antibody ELISA was the most sensitive assay in patients with gluten ataxia, a total of 73% of patients were positive which is a similar proportion to the number of patients with CD only. However, complete positivity was expected as, previously, all of these patients had tested positive for IgG anti-gliadin antibodies during the diagnostic process. However, these assays were carried out in a different laboratory where a different method was initially used and occasionally, although all not on a GFD, samples taken from patients at differing time-points were used. The antibody levels of some patients with gluten ataxia have been found to fluctuate slightly whilst on a gluten containing diet (unpublished observations). A combination of different methods and samples is the most likely reason for the discordance seen between results. Patients with gluten ataxia did not have significantly elevated levels of circulating IgA anti-gliadin antibodies compared to both ataxia and healthy controls. These levels were significantly lower ($p<0.05$) than levels of circulating IgA anti-gliadin antibodies in patients with CD only. Only 25% of patients with gluten ataxia were positive for circulating IgA anti-gliadin antibodies, giving an overall total of 79% of patients with gluten ataxia who were positive for either IgG and/or IgA anti-gliadin antibodies. This value is very similar to the corresponding value of 84% of patients with CD only. Conversely, and of note, was the large difference observed between the numbers of patients positive for circulating IgA anti-TG2 antibodies in the gluten ataxia and CD only groups. Only 40% of gluten ataxia patients had increased levels of these antibodies compared to 94% of patients with CD only. This value is also much lower than that found by Kumar and co-workers where all 33 examined DH patients were positive for circulating IgA anti-TG2 antibodies, an even greater number than the 91% of patients with CD only in the same study who were found to be positive (Kumar *et al.,* 2001). The lower proportion of IgA anti-gliadin and anti-TG2 antibody positive patients with gluten ataxia compared to those with CD only may be explained by the

relative intestinal involvement. Several recent studies have reported the much lower sensitivity of anti-gliadin and anti-TG2 assays in detecting patients with mild intestinal damage (Rostami *et al.,* 2003; Tursi *et al.,* 2001, 2003). Although numbers were small, Tursi and co-workers found that no patients with a Marsh I intestinal lesion were positive for anti-gliadin antibodies and only 8% were positive for anti-TG2 antibodies (Tursi *et al.,* 2001, 2003). The majority of patients with gluten ataxia have either Marsh 0 or Marsh I lesions, however no correlation was observed between a positive biopsy suggestive of CD associated changes (i.e. degree of mucosal damage) and antibody levels in patients with gluten ataxia. In general, patients with gluten ataxia have less mucosal damage than patients with CD only. Therefore, it seems possible that fewer are likely to be classified positive for these antibodies using positive/negative thresholds designed to detect patients with more severe mucosal damage. This has implications for the detection of patients with gluten ataxia, using such cut-off values, where anti-gliadin antibody screening is central to diagnosis. The ELISA kits are designed to detect patients with CD whose antibody titres may be up to ten times greater then those seen in normal individuals (Milford-Ward *et al*, 2001) and the cut-off values are determined accordingly. It cannot be assumed that this is also true of gluten sensitive patients where bowel involvement is not the primary manifestation, as demonstrated by the findings of the current study. Closer inspection of individual antibody levels shows that a larger number of IgG and IgA anti-gliadin antibody negative gluten ataxia samples have values that are much closer to the cut-off value than do either of the control group samples which tend to lie well below the threshold. Therefore, a number of patients with gluten ataxia, who have elevated disease associated antibodies compared to healthy controls, will be missed by the high cut-off threshold and the resulting negative status misleading (i.e. there is a reduced sensitivity of these assays for the diagnosis of gluten ataxia). It is suggested that closer examination of relative levels is necessary in the detection of patients with gluten ataxia rather than a sole reliance on positive/negative status assigned by kits designed for patients with CD. Further such considerations arise when comparing results from different laboratories and countries. The number of healthy individuals positive for anti-gliadin antibodies varies between populations and is used in the calculation of kit thresholds, which therefore vary somewhat between geographical regions. Normally, this slight variation has no effect on the assay result as patient levels are so much higher than healthy controls. However, in the case of patients with gluten ataxia, where levels are generally lower, this effect will be more pronounced leading to more gluten ataxia patients being classified as negative in some regions and

laboratories than others. This is likely to contribute to the varied reports of the prevalence of IgG anti-gliadin antibodies in patients with sporadic ataxia, which have ranged from 11.5% in Germany to 41% in the UK (Burk *et al.*, 2001a; Hadjivassiliou *et al.*, 2003a). There is, therefore, a possible risk of significant under-diagnosis of gluten ataxia in these populations.

Correlation of antibody levels

Levels of IgG and IgA anti-gliadin antibodies were positively correlated in both patients with CD only ($p<0.01$) and patients with gluten ataxia ($p<0.05$). The weaker correlation in patients with gluten ataxia may be due to the lesser intestinal involvement resulting in lower levels of IgA anti-gliadin antibodies, which have been reported to be more closely correlated with intestinal damage (Troncone & Ferguson, 1991). In addition, in patients with CD only, but not patients with gluten ataxia, IgA anti-gliadin levels were positively correlated with IgA anti-TG2 levels ($p<0.01$). This too, may be related to intestinal involvement as levels of anti-TG2 antibodies are reportedly correlated with the severity of mucosal damage (Tursi *et al.*, 2003).

In conclusion, the main findings of this study were that commercial anti-gliadin antibody ELISA kits have better reliability and reproducibility, as well as greater ease of use than the in-house assay. Consistent with previous reports (Hadjivassiliou *et al.*, 2003a), circulating IgG anti-gliadin antibodies are the most sensitive marker of gluten sensitivity in patients with gluten ataxia. However, using the high cut-off values of commercial ELISA kits, designed for the detection of patients with CD, may miss gluten sensitivity in patients with gluten ataxia who frequently have lower, although elevated, levels of circulating gluten sensitivity associated antibodies.

CHAPTER FOUR

INVESTIGATION OF THE CEREBELLAR

REACTIVITY OF CIRCULATING

ANTIBODIES IN PATIENTS WITH GLUTEN

ATAXIA BY IMMUNOHISTOCHEMISTRY

4.1 Introduction

Neuropathological findings in patients with gluten ataxia have shown the cerebellum to be the predominantly affected area. Within the cerebellum, Purkinje cells appear to be particularly susceptible to damage with the result of complete loss of these cells (figure 4.1) (Hadjivassiliou *et al.*, 1998). Preliminary evidence suggests that this damage is immune-mediated (section 1.12.3.2). As described in the previous chapter, patients with gluten ataxia all possess circulating anti-gliadin antibodies and a large proportion also possess circulating anti-TG2 antibodies.

These features are reminiscent of conditions such as paraneoplastic neurological syndromes, which arise as the indirect result of a malignancy. It is believed that an immune response, directed against tumour antigens which are normally only found in neurones, is mounted and the resultant cross-reactive immune response with neural tissue is responsible for the observed damage and clinical signs (Darnell & Posner, 2003; Sutton & Winer, 2002). Often, the resulting neurological dysfunction results before the discovery of any underlying tumour (Darnell & Posner, 2003; Sutton & Winer, 2002). Several of these syndromes have antigenic targets within the cerebellum where the immune response gives rise to similar symptoms to those seen in gluten ataxia and total Purkinje cell loss is often observed. This is known as paraneoplastic cerebellar degeneration (PCD). Patients possess antibodies against different cerebellar antigens in their serum and CSF, depending on the site of the tumour. Patients presenting with PCD most commonly possess antibodies against cdr 62 and 34 proteins (also termed Yo) (resulting from ovarian and breast carcinomas) but also against voltage-gated calcium channels (resulting from small cell lung cancer) and glutamate receptors (resulting from Hodgkins's disease) (Mason *et al.*, 1997; Peterson *et al.*, 1992; Sillevis Smitt *et al.*, 2000). There is some evidence to suggest that these antibodies may be considered pathogenic in the development of PCD (Fukunaga *et al.*, 1983; Lang *et al.*, 1983; Okano *et al.*, 1999; Sillevis Smitt *et al.*, 2000).

The knowledge of pathogenic antibodies involved in conditions sharing certain similarities with gluten ataxia, such as PCD, suggests it is conceivable that the circulating gluten sensitivity-associated antibodies present in patients with gluten ataxia may have a similar pathogenic role. Interestingly, TG2 is expressed in the cerebellum (Perry *et al.*, 1995) as well as in numerous different neural tissues including peripheral nerve, spinal cord and the forebrain (Hand *et al.*, 1993). Significant TG2 reactivity

Figure 4.1 Haematoxylin and eosin stain of a section of the cerebellar cortex of a
patient with gluten ataxia
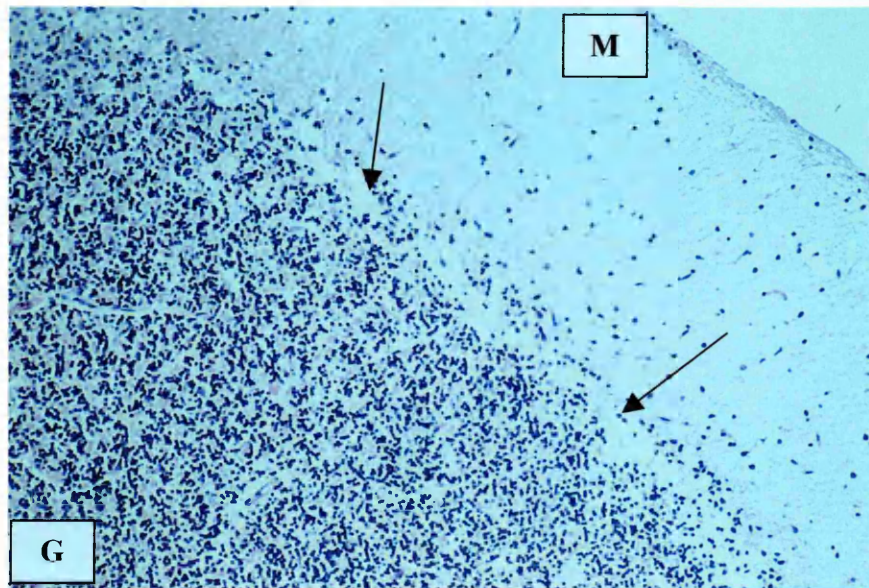
A paraffin-embedded section of post-mortem cerebellar tissue from a patient with

gluten ataxia. The loss of Purkinje cells is particularly evident (Purkinje cell layer

indicated by arrows).

M – molecular layer   G – granular layer

Photograph courtesy of Dr. M. Hadjivassiliou.

has been reported in areas such as the cerebellum, white matter, hippocampus and the frontal and temporal neocortex (Gilad & Varon, 1985; Johnson *et al.,* 1997). Recent research has shown that TG2 may play a role in neurodegenerative diseases such as Alzheimer's and Huntington's disease via a variety of different means (Lesort *et al.,* 2000). In addition, increased levels of TG2 have been reported in both of these diseases (Johnson *et al.,* 1997; Lesort *et al.,* 1999).

4.1.1 Aims of this study

With knowledge of other disorders involving immune-mediated cerebellar damage, where pathogenic antibodies play a role in the disease process, and the knowledge that patients with gluten ataxia possess circulating disease-associated antibodies, the overall aim of this study was to investigate the cerebellar reactivity of these antibodies, using IHC. Particular consideration was paid to the involvement of Purkinje cells, which appear the most susceptible to damage. This aim was achieved by completion of several separate objectives, detailed below:

o Overall assessment of cerebellar reactivity of the serum from patients with gluten ataxia by a titration study of patient serum samples. This had two objectives. Firstly, to assess and compare the pattern of cerebellar staining by serum antibodies from different patient groups and, secondly, to investigate the serological titres of anti-cerebellar antibodies in each of these groups.

o To determine whether a correlation exists between the levels of circulating anti-gliadin or anti-TG2 antibodies and the intensity or persistence of serum reactivity to cerebellar tissue.

o Comparison of the cerebellar staining pattern by serum antibodies from patients with gluten ataxia with those of other cerebellar proteins of interest (TG2 and Yo) to assess any similarities or differences.

o Investigation of the cross-reactive potential of anti-gliadin antibodies (present in all patients with gluten ataxia) with cerebellar tissue using a commercially available polyclonal antibody and pre-adsorption of anti-gliadin antibodies from gluten ataxia serum samples to determine whether these antibodies are responsible for the cerebellar staining pattern.

o Preliminary investigation of the cerebellar reactivity of CSF samples from patients with gluten ataxia and healthy control patients.

4.2 Methods

4.2.1 Titration study of patient serum samples

4.2.1.1 Human tissue

Staining was investigated using human cerebellar sections, using the indirect IHC method outlined in sections 2.3.2 and 2.3.4. The method was initially optimised in order to achieve good delineation of specific versus non-specific staining (data not shown). A total of 5 gluten ataxia (untreated), 5 CD without neurological complications (untreated), 5 ataxia controls (including both familial and sporadic cases) and 5 healthy control serum samples were used as the primary antibody at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200. In each staining run, serum samples from each group were used in order to minimise inter-experiment variation. Sections incubated with a Purkinje cell specific antibody (monoclonal anti-calbindin $D_{28}K$, table 4.1), diluted in PBST, were included as a positive control. Calbindin is a member of the family of EF-hand calcium binding proteins and is believed to function as a calcium buffer (Schwaller *et al.*, 2002). It is useful as a Purkinje cell marker as it is present in Purkinje cells at all stages of development and not present in any other cerebellar cells (Schwaller *et al.*, 2002). It is also very soluble and present throughout Purkinje cells facilitating visualisation of the cell shape and structure (Baimbridge *et al.*, 1992). Sections incubated with PBST as a substitute for primary antibody were included as negative controls. Details of secondary antibodies used are given in table 4.1.

Semiquantitative evaluation of the staining intensity was performed independently by three blinded observers. Weak or strong positive staining was recorded if Purkinje cell staining was above background levels and negative staining was recorded if Purkinje cell staining did not exceed background levels. The number of unanimous agreements between observers with regards to positivity/negativity of staining and strength of staining was calculated as a percentage of the total number of observations and quoted as a measure of inter-observer variability. In situations where classification was not unanimous, the majority classification was recorded. In the instance of staining on a particular section being classified differently by each observer (eg. weak, strong, negative), the staining was re-evaluated. Finally, for each patient, the corresponding levels of circulating anti-gliadin (IgG & IgA) and anti-TG2 (IgA) antibodies were correlated with the last dilution where positive staining was observed and also the last dilution where strong positive staining was observed to determine whether circulating

antibodies were correlated with either of these measurements. Correlation was determined using the Spearman rank-order correlation.

4.2.1.2 Rat tissue

The research group of Dr. E. Tongiorgi, at the University of Trieste in Italy, investigated the staining of rat cerebellar tissue by serum samples from the Sheffield study as part of an ongoing collaboration. This was done to confirm the results obtained from the parallel study on human tissue (section 4.2.1.1). Following cervical dislocation, brains from adult Sprague-Dawley rats were removed and fresh-frozen on dry-ice, sectioned in a cryostat (Leica, Italy) and 10-μm saggital sections collected on gelatin-coated slides (Sigma, UK). Staining was carried out using the indirect IHC method outlined in section 2.3.4. A total of 10 gluten ataxia (untreated), 19 CD without neurological complications (untreated), 6 ataxia control and 12 healthy control serum samples were used as the primary antibody at dilutions of 1:100, 1:300 and 1:600. In addition, 3 of the serum samples from patients with gluten ataxia were tested at dilutions of 1:1200 and 1:1500. Details of secondary antibodies used are given in table 4.1.

Semiquantitative evaluation of the staining intensity was performed independently by two blinded observers. Weak or strong positive staining was recorded if Purkinje cell staining was above background levels and negative staining was recorded if Purkinje cell staining did not exceed background levels.

4.2.2 Investigation of the cerebellar staining patterns of known cerebellar proteins (tissue transglutaminase and Yo)

Details of all primary and secondary antibodies used in this study are given in tables 4.2 and 4.3. In all experiments, sections incubated with either a Purkinje cell specific antibody (as described in section 4.2.1.1) or PBST as a substitute for primary antibody were included as positive and negative controls. Again, methods were optimised initially in order to achieve the best results for each antibody used (data not shown). The staining pattern of commercially available anti-TG2 antibody against rat cerebellar tissue was investigated using the indirect IHC method, outlined in sections 2.3.2 and 2.3.4.

Table 4.1 Details of the specificity, supplier and dilution of primary and secondary antibodies used in the serum titration study

Antibody Specificity	Species (supplier)	Dilution	Corresponding Secondary antibody	Secondary antibody dilution
Monoclonal anti-Calbindin D-28K	Mouse IgG1 anti-bovine kidney calbindin (Sigma, UK)	1:200	HRP Conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:500
Patient serum (Human tissue)	-	1:100 to 1:3200	HRP Conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:500
Patient serum (Rat tissue)	-	1:100 to 1:1500	HRP Conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:200

Table 4.2 Details of reagents used in the indirect IHC study of antibodies directed against known cerebellar proteins

Antibody Specificity	Species (supplier)	Dilution	Corresponding Secondary antibody	Secondary antibody dilution
Monoclonal anti-TG2	Mouse IgG anti-guinea pig TG2 (Labvision, USA)	1:50, 1:100, 1:500 (rat tissue)	HRP Conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:500
Yo positive serum 2	Obtained from the Neurology Department, The Royal Hallamshire Hospital, Sheffield, UK	1:400, 1:800 (rat tissue)	HRP Conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:500

Table 4.3 Details of reagents used in the avidin-biotin peroxidase IHC study of antibodies directed against known cerebellar proteins

Antibody Specificity	Species (supplier)	Dilution	Corresponding blocking agent	Corresponding secondary antibody
Yo positive serum 1	Kindly donated by Dr N Gregson, GKT London, UK	1:150000 (rat & human tissue)	Goat serum 3:200 (Vector Laboratories, UK)	Biotinylated goat anti-human IgG 1:200 (Vector Laboratories, UK)

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The staining patterns of two, anti-Yo antibody positive serum samples, from patients with PCD, against rat cerebellar tissue were investigated. Additionally, the reactivity of PCD serum sample 1 against human cerebellar tissue was also investigated using the avidin-biotin amplified IHC method described in sections 2.3.2 and 2.3.3. PCD serum sample 2 was investigated using the indirect IHC method described in sections 2.3.2 and 2.3.4.

4.2.3 Investigation of the cerebellar staining pattern of commercial anti-gliadin antibody with/without pre-adsorption with crude gliadin

Rabbit polyclonal horseradish peroxidase-conjugated anti-gliadin antibody (Sigma, UK) was used at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:64000, 1:128000 and 1:256 000 on human cerebellar tissue and at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200 on rat cerebellar tissue. The antibody was diluted in PBST or a saturated crude gliadin (Sigma, UK) solution (~20mg/ml) made up in PBST. The crude gliadin solution was prepared by crushing crude gliadin into a fine powder using a pestle and mortar before adding the required weight to the correct amount of PBST. The resulting solution was stirred on a magnetic stirrer whilst being heated gently for approximately 15mins. It was ensured that the temperature of the solution did not rise above 60^0C to avoid denaturation of the gliadin. Once diluted with either PBST or crude gliadin solution, the commercial anti-gliadin antibody was left for 1hour at RT to allow adsorption of anti-gliadin antibodies in the sample. Following adsorption, indirect IHC staining was performed, using the methods described in sections 2.3.2 and 2.3.4 but with omission of the secondary antibody incubation step as the anti-gliadin antibody was peroxidase-conjugated. Sections incubated with either a Purkinje cell specific antibody diluted in PBST (as described in section 4.2.1.1) or PBST as a substitute for primary antibody were included as positive and negative controls. Sections incubated with crude gliadin solution only were included as an additional negative control.

The effectiveness of adsorption of anti-gliadin antibodies was confirmed using commercial IgG and IgA anti-gliadin antibody ELISAs, carried out according to the methods described in sections 2.2.2, 2.2.3.2 and 2.2.3.3. Anti-gliadin antibody was diluted 1:800 in PBST or crude gliadin solution (~20mg/ml, prepared as detailed in

section 4.2.3) and incubated for 60mins at RT to allow adsorption of antibodies. Samples were then assayed and the results compared.

4.2.4 Investigation of the cerebellar staining pattern of patient serum with/without pre-adsorption with crude gliadin

A total of 4 gluten ataxia (untreated), 3 CD without neurological complications (untreated) and 2 healthy control serum samples were used at dilutions of 1:200 and 1:800 on human cerebellar tissue. An additional 2 gluten ataxia (untreated), 2 CD without neurological complications (untreated), 2 ataxia controls and 2 healthy control serum samples were used at a dilution of 1:400 on rat cerebellar tissue. Serum samples were diluted to the appropriate dilution in PBST or a saturated crude gliadin (Sigma, UK) solution (~20mg/ml) made up in PBST. The crude gliadin solution was prepared as in section 4.2.3. Once diluted with either PBST or crude gliadin solution, serum samples were left for 1hour at RT to allow adsorption of anti-gliadin antibodies in the sample. Following adsorption, indirect IHC staining was performed, using the methods described in sections 2.3.2 and 2.3.4. HRP-conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech), diluted 1:500 in PBST was used as the secondary antibody. Sections incubated with either a Purkinje cell specific antibody diluted in PBST (as described in section 4.2.1.1) or PBST as a substitute for primary antibody were included as positive and negative controls. Sections incubated with crude gliadin solution only were included as an additional negative control.

The effectiveness of adsorption of serum anti-gliadin antibodies was confirmed using commercial IgG and IgA anti-gliadin antibody ELISAs, using 2 serum samples from each group, carried out according to the methods described in sections 2.2.2, 2.2.3.2 and 2.2.3.3. Samples were diluted in PBST or crude gliadin solution (~20mg/ml, prepared as detailed in section 4.2.3) and incubated for 60mins at RT. Following incubation, samples were assayed and the results compared.

4.2.5 Investigation of the cerebellar staining pattern of patient CSF

A preliminary investigation of the staining pattern of CSF samples from 2 patients with gluten ataxia and 1 healthy control patient, on human cerebellar tissue, was performed using the indirect IHC methods described in sections 2.3.2 and 2.3.4. CSF samples were diluted in PBST and used at dilutions of 1:50, 1:100, 1:200 and 1:400. HRP-

conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech), diluted 1:500 in PBST was used as the secondary antibody. Sections incubated with either a Purkinje cell specific antibody diluted in PBST (as described in section 4.2.1.1) or PBST as a substitute for primary antibody were included as positive and negative controls.

4.3 Results

4.3.1 Titration study of serum samples on human cerebellar tissue

4.3.1.1 Inter-observer variability

Complete agreement between all 3 independent observers with respect to whether staining was negative or positive was overall 59%. Complete agreement with regards to whether positive staining was weak or strong was overall 78%.

4.3.1.2 Study controls

Consistently strong staining of Purkinje cells was seen using anti-calbindin D28k (figure 4.2a) as a positive control. Sections incubated with PBST, in the absence of primary antibody, showed consistently negative Purkinje cell staining (figure 4.2b).

4.3.1.3 Serum reactivity

Typical staining patterns, at all dilutions, of sera from patients with untreated gluten ataxia are shown in figures 4.3 and 4.4. In 4 of 5 sera (80%) from patients with untreated gluten ataxia, positive staining of Purkinje cells was seen at all dilutions up to 1:800. At dilutions of 1:800 positive Purkinje cell staining was seen in only 40% of sera from patients with CD only and 20% of sera from each of the ataxia and healthy control groups. In 4 of the 5 gluten ataxia sera, staining was classified as strong at some dilution. Strong staining was seen in 1 of the 4 sera up to a dilution of 1:100, in 2 of the 4 sera up to a dilution of 1:400 (figure 4.4) and up to a dilution of 1:800 in the last of the 4 sera (figure 4.3). The remaining serum sample showed positive staining at all dilutions up to 1:400 but at no dilution was the staining classified as strong. Positive staining did not persist in any sera from patients with untreated gluten ataxia above a dilution of 1:800.

Typical staining patterns, at all dilutions, of sera from patients with untreated CD without neurological complications are shown in figures 4.5 and 4.6. Sera from all 5 patients in this group showed positive staining at a dilution of 1:100. Staining was classified as strong in 4 of 5 sera at this dilution. In 3 of 5 sera, positive staining was not observed above 1:100 (figure 4.6). In one patient strong staining persisted to a dilution of 1:400 (figure 4.5) and in another weak staining persisted to a dilution of 1:800.

Figure 4.2 Immunohistochemical staining of positive and negative control sections from the titration study of serum samples on human cerebellar tissue
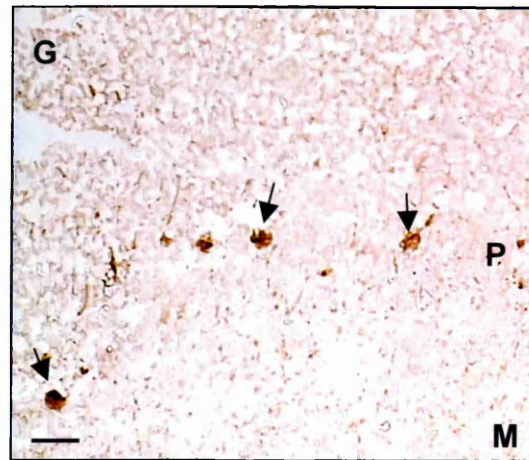
a. Positive control
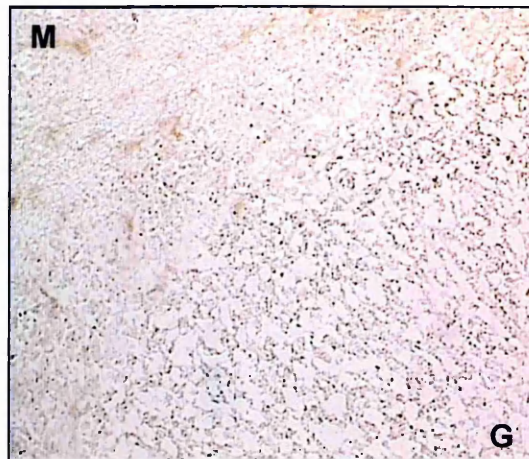
b. Negative control

The Purkinje-cell specific anti-calbindin $D_{28}K$ antibody was used as a positive control (a.) at a dilution of 1:200. PBST, as a substitute for primary antibody, was used as a negative control (b.). G - granular layer; P - Purkinje cell layer; M - molecular layer. Purkinje cells are indicated by arrows. Scale bar = 100μm

Figures 4.3 and 4.4 show the typical staining pattern, on human cerebellar tissue, by sera from patients with gluten ataxia. Serum was used at

increasing dilutions from 1:100 to 1:3200. Purkinje cell staining (indicated by arrows) was defined as weak or strong positive if above background

staining level and negative if not increased above background staining level.

G - granular layer; P - Purkinje cell layer; M - molecular layer.

Scale bar = 100μm.

Figure 4.3 Reactivity, at increasing dilutions, of sera from gluten ataxia patient no.1 on control human cerebellum
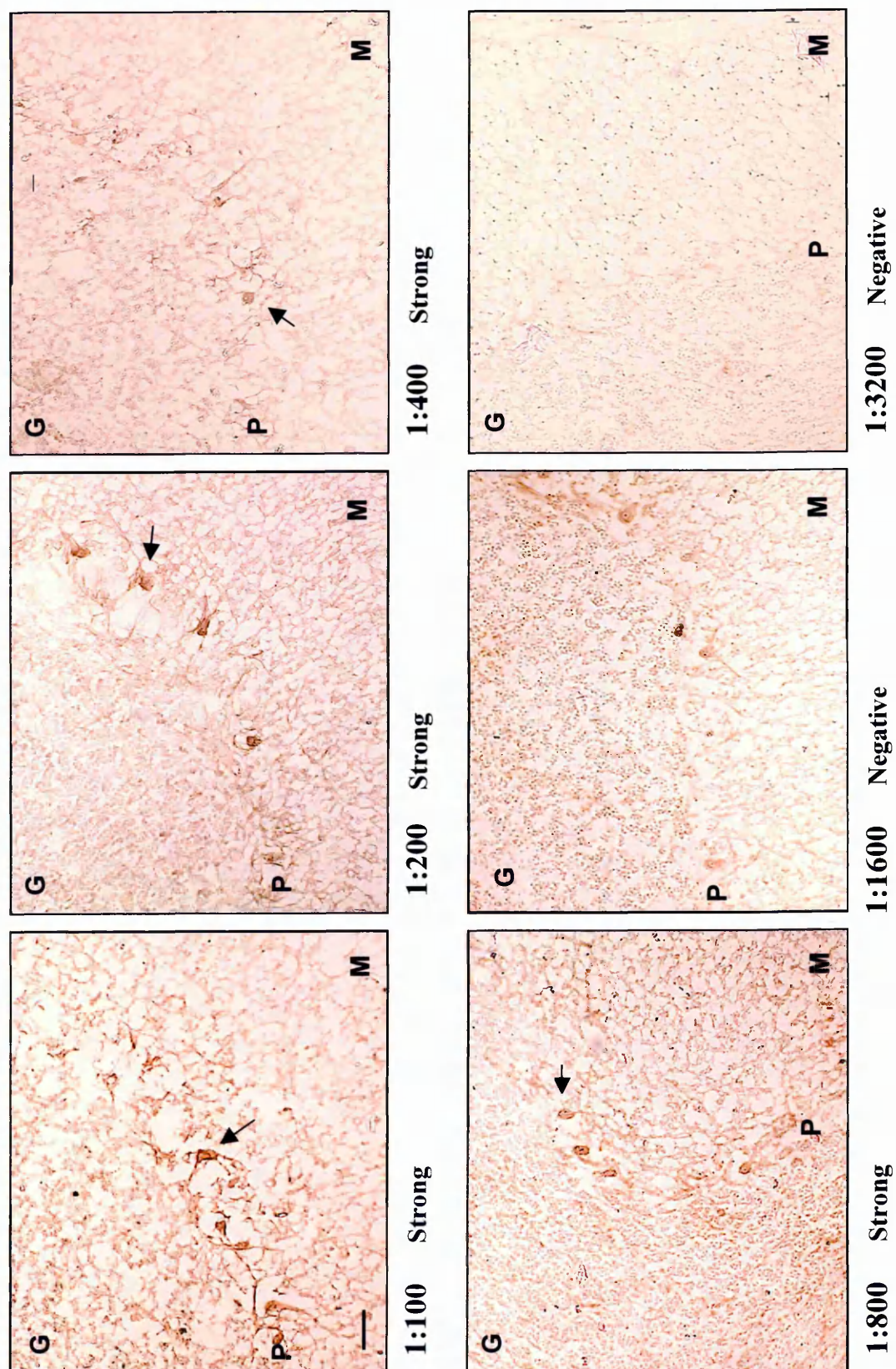
Figure 4.4 Reactivity, at increasing dilutions, of sera from gluten ataxia patient no. 17 on control human cerebellum

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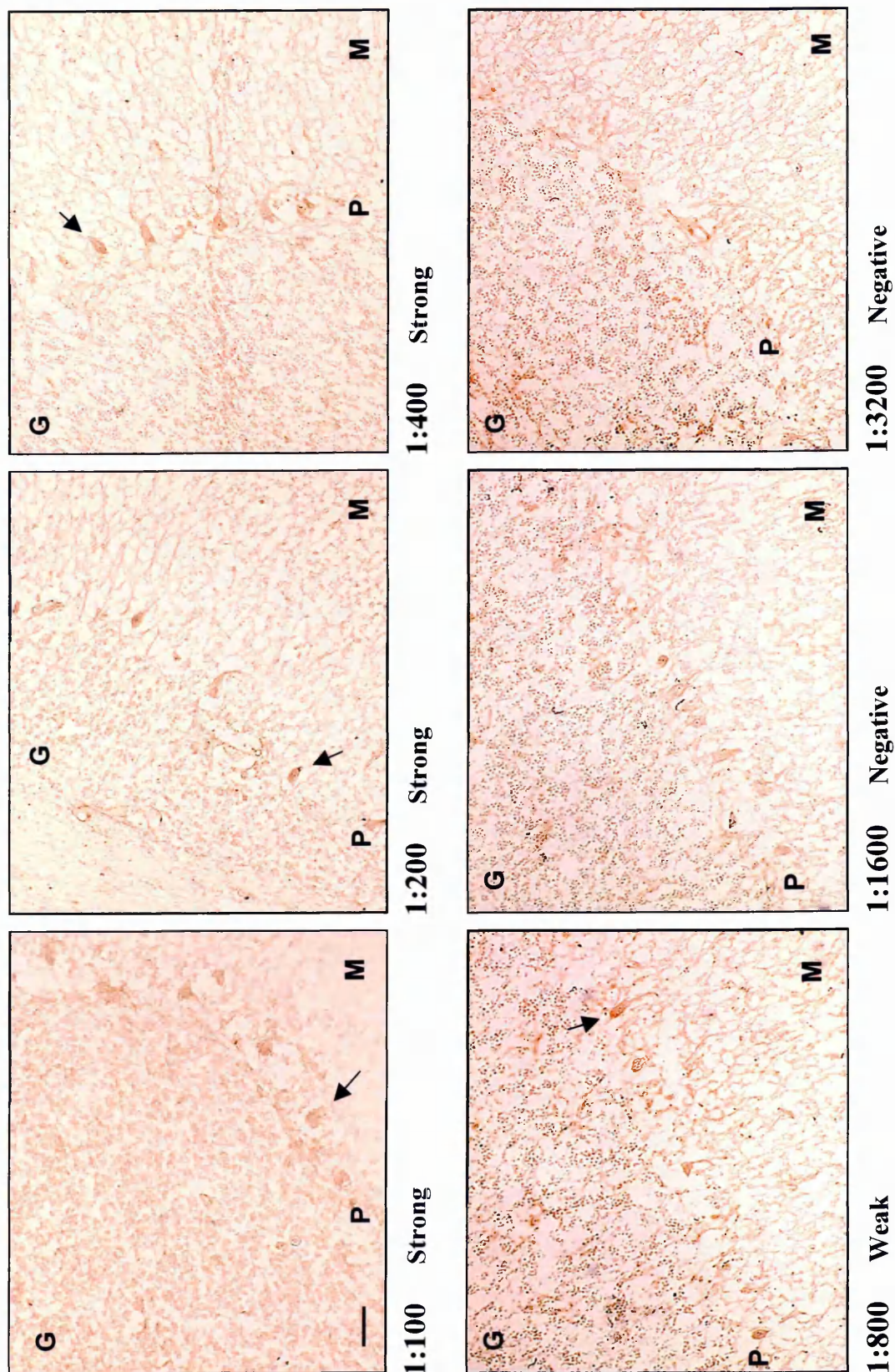
Figures 4.5 and 4.6 show the typical staining pattern, on human cerebellar tissue, by sera from patients with coeliac disease only. Serum was used at increasing dilutions from 1:100 to 1:3200. Purkinje cell staining (indicated by arrows) was defined as weak or strong positive if above background staining level and negative if not increased above background staining level.

G - granular layer; P - Purkinje cell layer; M - molecular layer.

Scale bar = 100μm.

Figure 4.5 Reactivity, at increasing dilutions, of sera from a patient with coeliac disease only on control human cerebellum
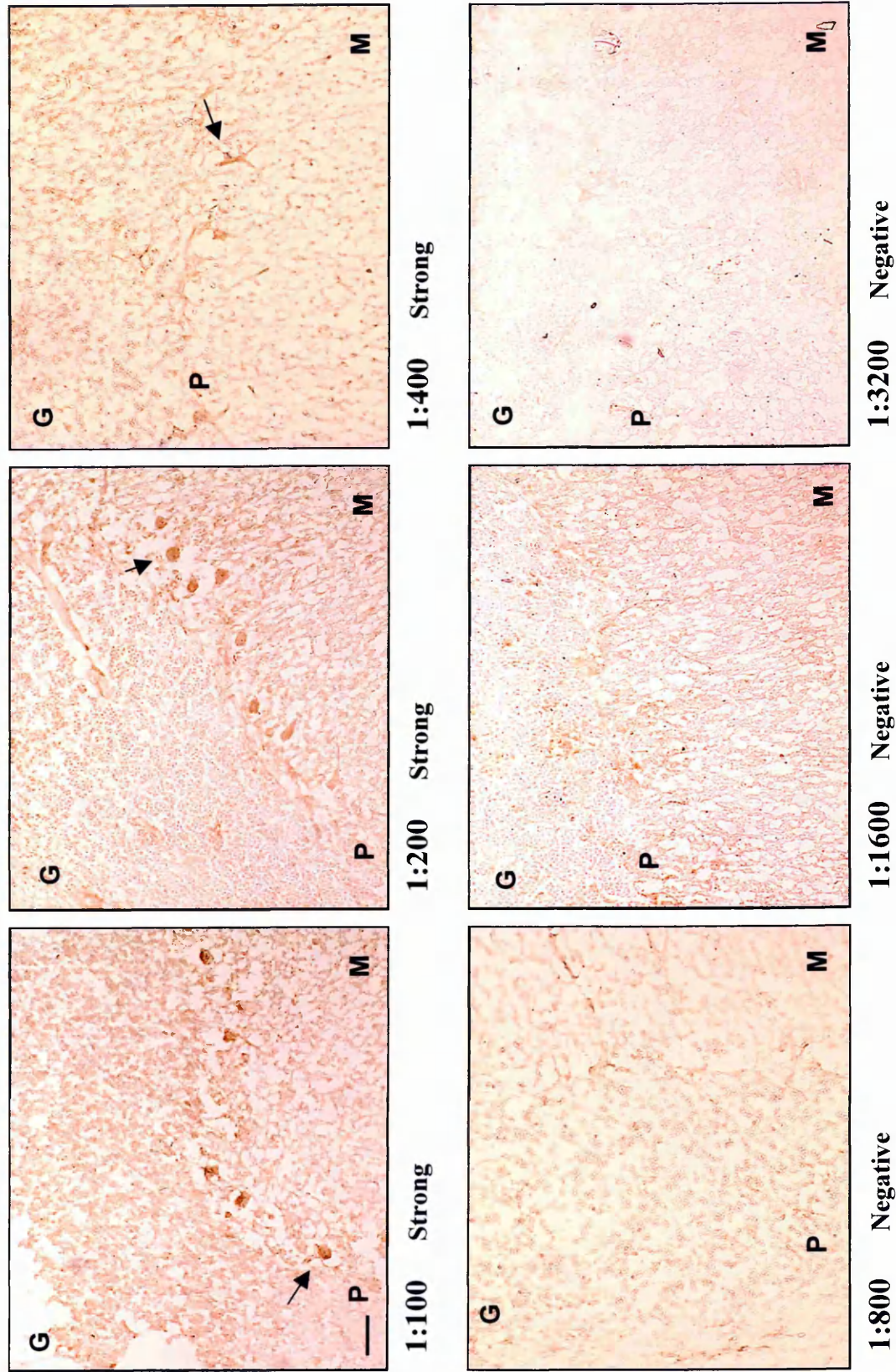
Figure 4.6 Reactivity, at increasing dilutions, of sera from a patient with coeliac disease only on control human cerebellum

1:100   Weak   1:200   Negative   1:400   Negative

1:800   Negative   1:1600   Negative   1:3200   Negative

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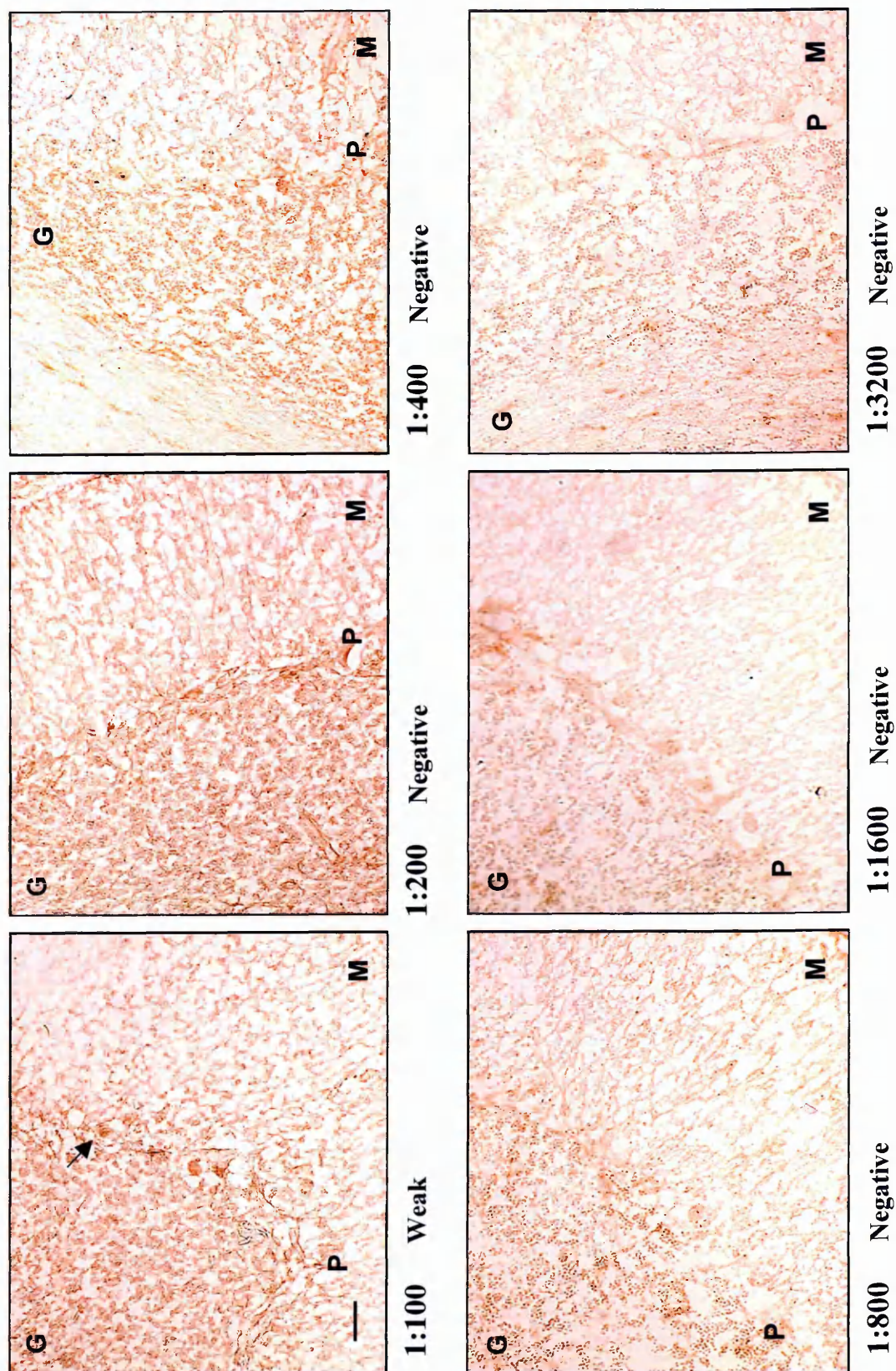
Typical staining patterns, at all dilutions, of sera from ataxia control patients are shown in figures 4.7 and 4.8. Positive Purkinje cell staining was seen in all 5 sera from ataxia control patients at a dilution of 1:100 but was only classified as strong in two of these samples (figure 4.8). Staining persisted in 2 patients to a dilution of 1:400 (figure 4.8), remaining strong until 1:200 in one, and to a dilution of 1:200 in another.

Typical staining patterns, at all dilutions, of sera from healthy control patients are shown in figures 4.9 and 4.10. Sera from 4 of 5 healthy control patients showed positive Purkinje cell staining at a dilution of 1:100 (figures 4.9 & 4.10). In two sera, this staining persisted to a dilution of 1:200 (figures 4.9 & 4.10) and persisted to 1:400 and 1:800 in the remaining 2 samples. At no dilution was any staining classified as strong. The remaining serum sample showed no positive Purkinje cell staining at any dilution.

The reactivity and intensity of staining of all serum samples tested is summarised in tables 4.4 and 4.5.

4.3.1.4 Correlation between human cerebellar staining and circulating levels of gluten sensitivity associated antibodies

No correlation was observed (p>0.05), in any patient group, between persistence of Purkinje cell staining and the titres of either circulating anti-gliadin antibodies (IgG or IgA) or IgA anti-TG2 antibodies (data not shown).

No correlation was observed (p>0.05), in any of the groups, between the strength of Purkinje cell staining and the titres of either circulating anti-gliadin antibodies (IgG or IgA) or IgA anti-TG2 antibodies (data not shown).

4.3.2 Titration study of serum samples on rat cerebellar tissue

Sera from 9 of 10 patients with untreated gluten ataxia showed strong staining of Purkinje cells at 1:100, 1:300 and 1:600 dilutions. None of the subset of sera from 3 patients with untreated gluten ataxia showed any recognisable staining at a dilution of 1:1200. Sera from 4 of 12 healthy control subjects showed weak staining at a dilution of 1:100 with no further staining detected at higher dilutions. No staining was seen using serum from 6 control patients with ataxia. The sera from 5 of 19 patients with CD without neurological complications showed strong staining of Purkinje cells at dilutions of 1:100 and 1:300 but this became weak staining at 1:600.

Figures 4.7 and 4.8 show the typical staining pattern, on human cerebellar tissue, by sera from ataxia control patients (sporadic non-gluten idiopathic ataxia and familial ataxia respectively). Serum was used at increasing dilutions from 1:100 to 1:3200. Purkinje cell staining (indicated by arrows) was defined as weak or strong positive if above background staining level and negative if not increased above background staining level.

G - granular layer; P - Purkinje cell layer; M - molecular layer.

Scale bar = 100µm.

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Figure 4.7 Reactivity, at increasing dilutions, of sera from a patient with sporadic idiopathic ataxia (non-gluten) on control human cerebellum

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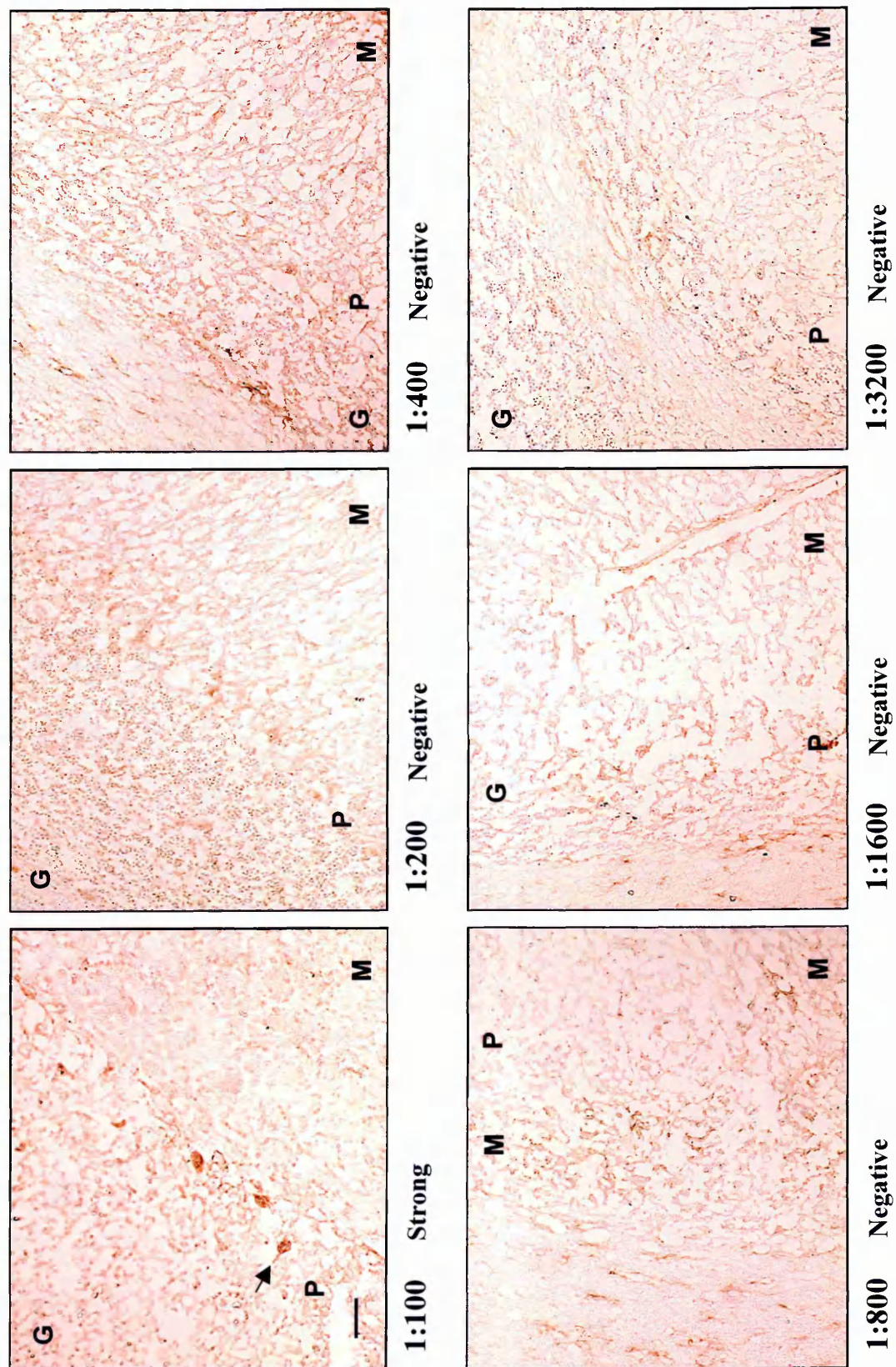
Figure 4.8 Reactivity, at increasing dilutions, of sera from a patient with familial ataxia on control human cerebellum
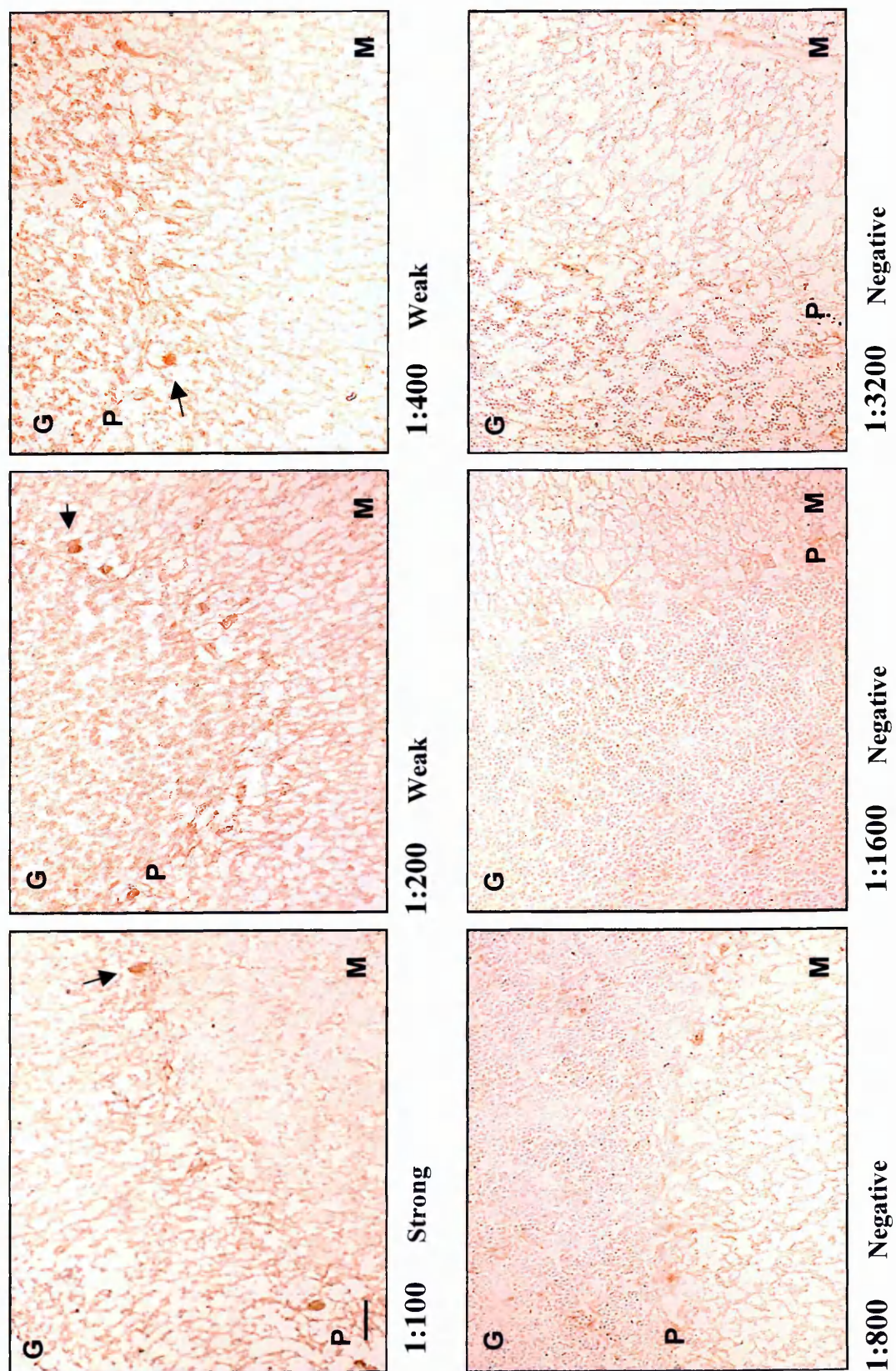
Figures 4.9 and 4.10 show the typical staining pattern, on human cerebellar tissue, by sera from healthy controls. Serum was used at increasing

dilutions from 1:100 to 1:3200. Purkinje cell staining (indicated by arrows) was defined as weak or strong positive if above background staining level

and negative if not increased above background staining level.

G - granular layer; P - Purkinje cell layer; M - molecular layer.

Scale bar = 100μm.

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Figure 4.9 Reactivity, at increasing dilutions, of sera from a healthy control on control human cerebellum

1:100 Weak

1:200 Weak

1:400 Negative

1:800 Negative

1:1600 Negative

1:3200 Negative
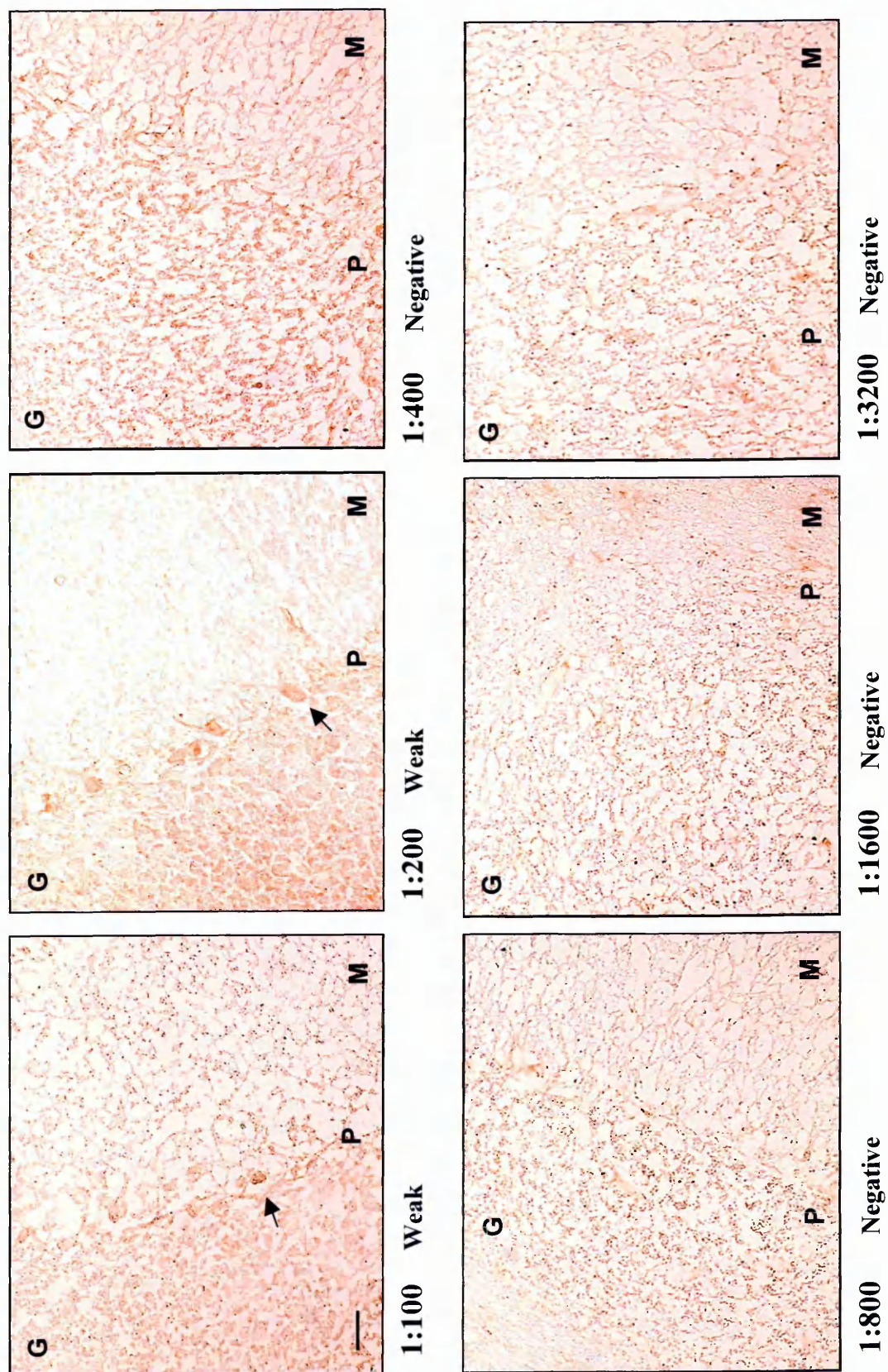
Figure 4.10 Reactivity, at increasing dilutions, of sera from a healthy control on control human cerebellum
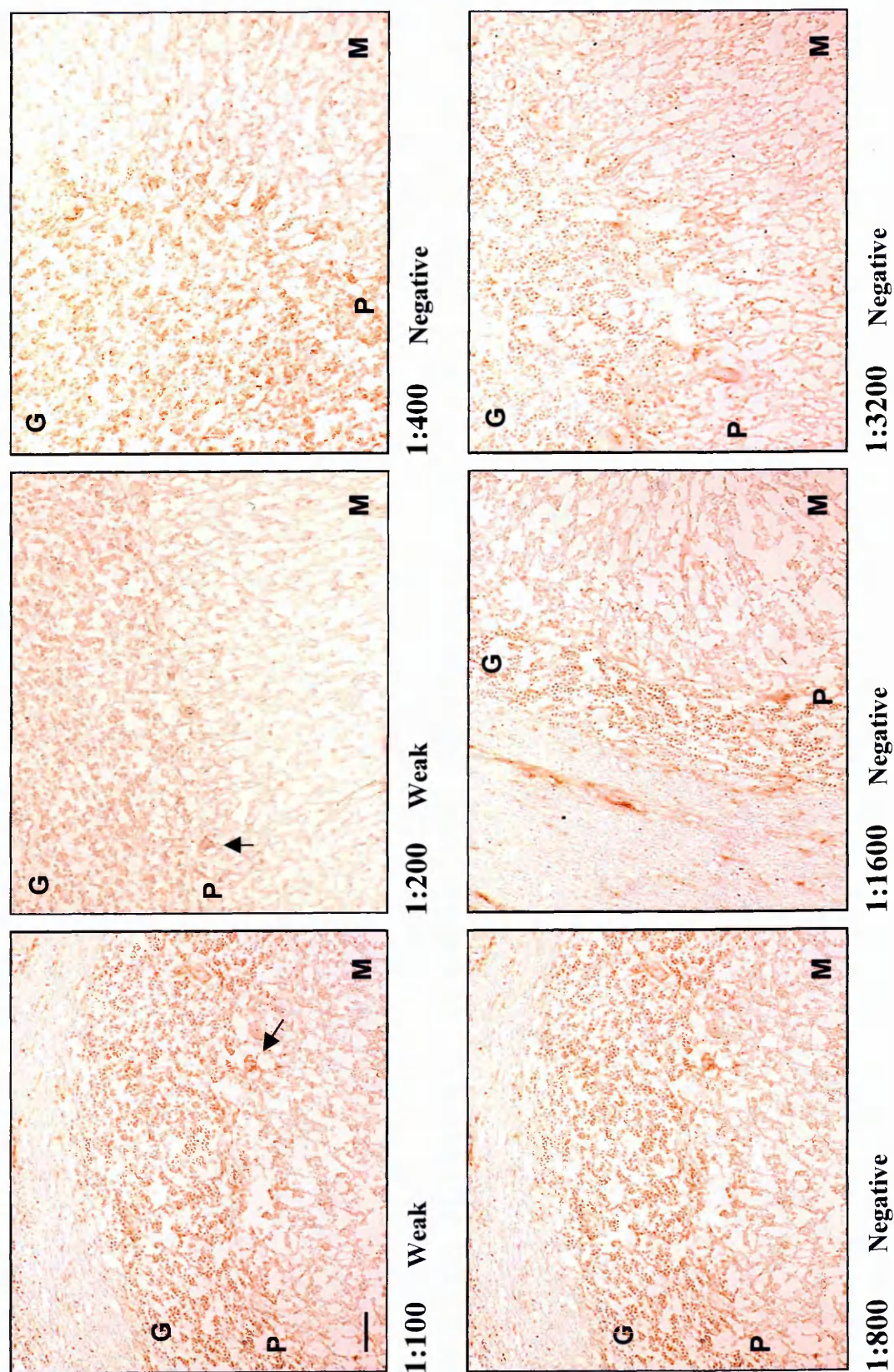
Table 4.4 Summary of individual staining patterns at all dilutions, on human cerebellar tissue, by sera used in the serum titration study

Serum dilution →	1:100	1:200	1:400	1:800	1:1600	1:3200
Patient group (Patient no).↓						
GA (17)	S	S	S	W	N	N
GA (34)	S	W	W	W	N	N
GA (1)	S	S	S	S	N	N
GA (15)	S	S	S	W	N	N
GA (8)	W	W	W	N	N	N
CD	W	N	N	N	N	N
CD	S	S	S	N	N	N
CD	S	N	N	N	N	N
CD	S	W	W	W	N	N
CD	S	N	N	N	N	N
AC	W	W	N	N	N	N
AC	S	N	N	N	N	N
AC	S	W	W	N	N	N
AC	W	N	N	N	N	N
AC	S	S	W	N	N	N
HC	W	W	N	N	N	N
HC	N	N	N	N	N	N
HC	W	W	W	W	N	N
HC	W	W	W	N	N	N
HC	W	W	N	N	N	N

KEY to groups:

GA – Gluten ataxia, untreated

CD – Coeliac disease without neurological complications

AC – Ataxia controls

HC –Healthy controls

KEY to staining:

S - Strong, positive Purkinje cell staining (shaded)

W - Weak, positive Purkinje cell staining

N - Negative Purkinje cell staining

Table 4.5 Summary of positive and strong positive staining patterns, on human cerebellar tissue, by sera used in the serum titration study

A. Summary of positive Purkinje cell staining

Group sera (n=5)	Dilutions and percentage of positive Purkinje cell staining					
	1:100	1:200	1:400	1:800	1:1600	1:3200
Gluten ataxia, untreated	100%	100%	100%	80%	0%	0%
Coeliac disease, no neurology	100%	40%	40%	20%	0%	0%
Ataxia controls	100%	60%	40%	0%	0%	0%
Healthy controls	80%	80%	40%	20%	0%	0%

B. Summary of strong positive Purkinje cell staining

Group sera (n=5)	Dilutions and percentage of strong positive Purkinje cell staining					
	1:100	1:200	1:400	1:800	1:1600	1:3200
Gluten ataxia, untreated	60%	60%	60%	20%	0%	0%
Coeliac disease, no neurology	80%	20%	20%	0%	0%	0%
Ataxia controls	60%	20%	0%	0%	0%	0%
Healthy control subjects	0%	0%	0%	0%	0%	0%

n = number of serum samples assessed

Weak staining was seen in 9 patients at all dilutions and no staining was seen in the remainder. These results are summarised in table 4.6. (Data provided by Dr. E. Tongiorgi).

Table 4.6 Summary of positive and strong positive staining patterns, on rat cerebellar tissue, by sera used in the serum titration study

A. Summary of positive Purkinje cell staining

Group sera (n)	Dilutions and percentage of positive Purkinje cell staining		
	1:100	1:300	1:600
Gluten ataxia, untreated (10)	90%	90%	90%
Coeliac disease, no neurology (19)	68%	68%	68%
Ataxia controls (6)	0%	0%	0%
Healthy controls (12)	33%	0%	0%

B. Summary of strong positive Purkinje cell staining

Group sera (n)	Dilutions and percentage of positive Purkinje cell staining		
	1:100	1:300	1:600
Gluten ataxia, untreated (10)	90%	90%	90%
Coeliac disease, no neurology (19)	26%	26%	0%
Ataxia controls (6)	0%	0%	0%
Healthy controls (12)	0%	0%	0%

n = number of serum samples assessed

4.3.3 Staining patterns of anti-tissue transglutaminase and anti-Yo antibodies on rat and human cerebellar tissue

The first serum sample from one of two patients with PCD, positive for anti-Yo antibodies, was used on both rat and human cerebellar tissue and the staining pattern was found not to differ. The staining pattern of the sample on human cerebellum is shown in figure 4.11a. Even at a dilution of 1:150,000, strong, specific, grainy cytoplasmic staining of Purkinje cells is evident. No other area appears stained. The second sample, only used on rat cerebellar tissue, differed slightly in its staining pattern. At both dilutions of 1:400 and 1:800 used, approximately 50% of Purkinje cells were uniformly, cytoplasmically stained in a pattern similar to that of the previous anti-Yo positive serum sample tested. However, the remainder of Purkinje cell staining was primarily membranous with varying degrees of cytoplasmic staining (figure 4.11b).

The staining pattern of commercial, monoclonal anti-TG2 antibody was investigated on both rat and human cerebellum. Staining patterns were similar on both tissues but positive staining was only seen at a dilution of 1:50. Figure 4.11c shows the staining on human cerebellum at an antibody concentration of 1:50. In contrast to the other antibodies investigated, anti-TG2 antibodies appeared not to specifically stain Purkinje cells but instead a weak, diffuse staining pattern was present throughout the tissue.

4.3.4 Reactivity of commercial anti-gliadin antibody with cerebellar tissue with/without pre-adsorption with crude gliadin

The staining pattern of commercial anti-gliadin antibody, at all dilutions used, differed slightly between rat and human cerebellar tissue. The main difference was that, on rat tissue the antibody positively stained the majority of Purkinje cells with uniform intensity throughout the cytoplasm and membrane of the cell. Although most Purkinje cells within human tissue were also stained with a similar pattern, a few exhibited stronger membranous or peri-nuclear staining.

Commercial polyclonal anti-gliadin antibody was found to stain rat cerebellar tissue at all dilutions up to, and including, 1:1600. Staining was not observed using the antibody at a dilution of 1:3200. Staining was present throughout the tissue but particularly within the granular layer and Purkinje cells. The antibody positively stained the

Figure 4.11 Staining patterns of anti-Yo and anti-tissue transglutaminase antibodies on control cerebellum
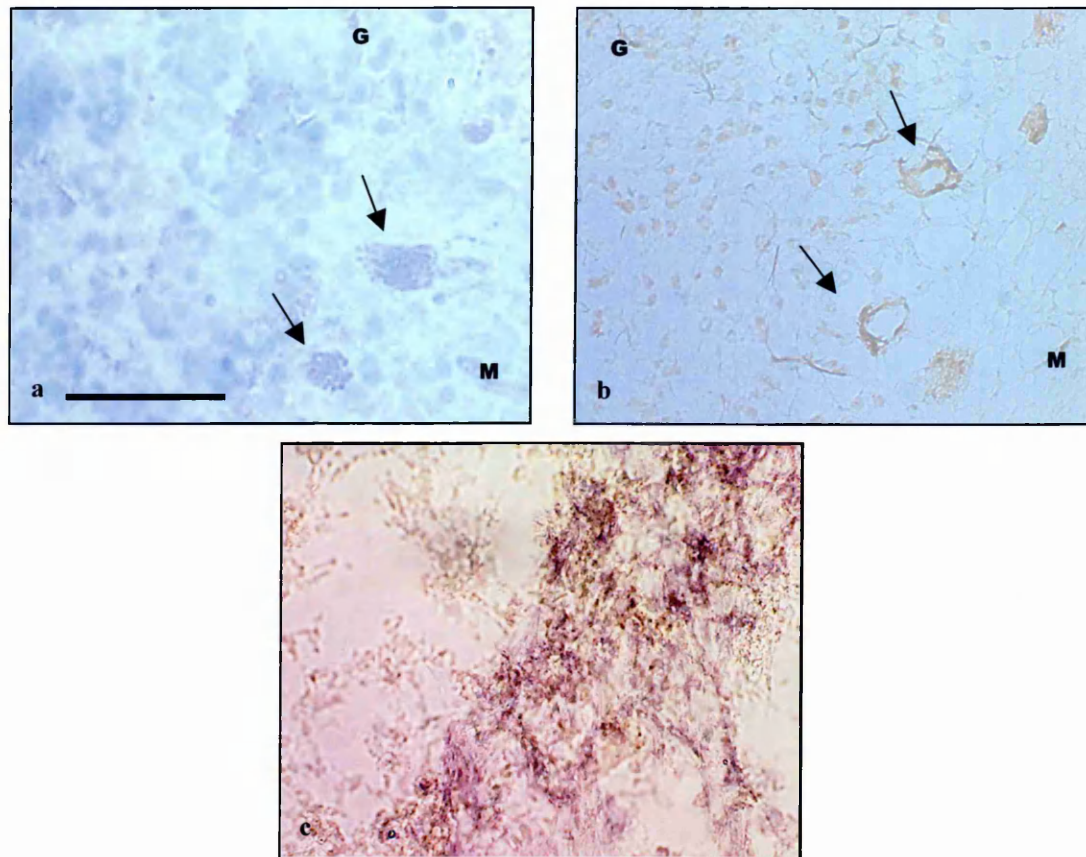
Typical staining patterns of serum from an anti-Yo antibody positive patient with PCD, diluted to 1:150000, (**a**, human control cerebellum); serum from a second anti-Yo antibody positive patient with PCD, diluted to 1:800, (**b**, rat control cerebellum) and commercial anti-TG2 antibody, diluted to 1:50, (**c**, human control cerebellum). Purkinje cells are indicated by arrows. Scale bar = 100μm.

M – molecular layer **G** – granular layer.

majority of Purkinje cells with uniform intensity throughout the cytoplasm and membrane of the cell (figure 4.12). This staining was most intense at lower dilutions and lessened in intensity as the antibody was used at higher dilutions. The antibody was found to stain rat cerebellar tissue at an optimal dilution of 1:800 (figure 4.12f). Staining with commercial anti-gliadin antibody at a dilution of 1:400 and 1:800 was abolished following adsorption of the antibody for 1hr with crude gliadin (figures 4.12d & 4.12h respectively). At dilutions of up to 1:400, staining was progressively decreased, but not completely eliminated, following adsorption of the antibody with crude gliadin.

Commercial anti-gliadin antibody was found to stain human cerebellar tissue in a similar manner to rat cerebellum. Positive staining of the tissue was observed in all dilutions tested up to, and including, 1:3200. No staining was seen when the antibody was used at a dilution of 1:6400 or above. Although some staining of molecular and granular layers by the antibody was seen, this was less intense than that produced by an equivalent dilution of the antibody on rat tissue and Purkinje cells were more strongly stained in comparison to the rest of the tissue.

The Purkinje cell staining was mostly uniformly cytoplasmic and membranous (figure 4.13), similar to that seen on rat tissue. As with rat tissue, the antibody was found to stain human cerebellar tissue at an optimal dilution of 1:800 (figure 4.13c). In contrast to rat tissue, staining of commercial anti-gliadin antibody at a dilution of 1:400 was not abolished following adsorption of the antibody for 1hr with crude gliadin (figure 4.13d). At a dilution of 1:800, staining was virtually eliminated following pre-adsorption of the antibody with crude gliadin (figure 4.13e) but was completely abolished following pre-adsorption at a dilution of 1:1600 (figure 4.13j).

Figure 4.12 Reactivity of commercial anti- gliadin antibody with/without pre-adsorption with crude gliadin on control rat cerebellum
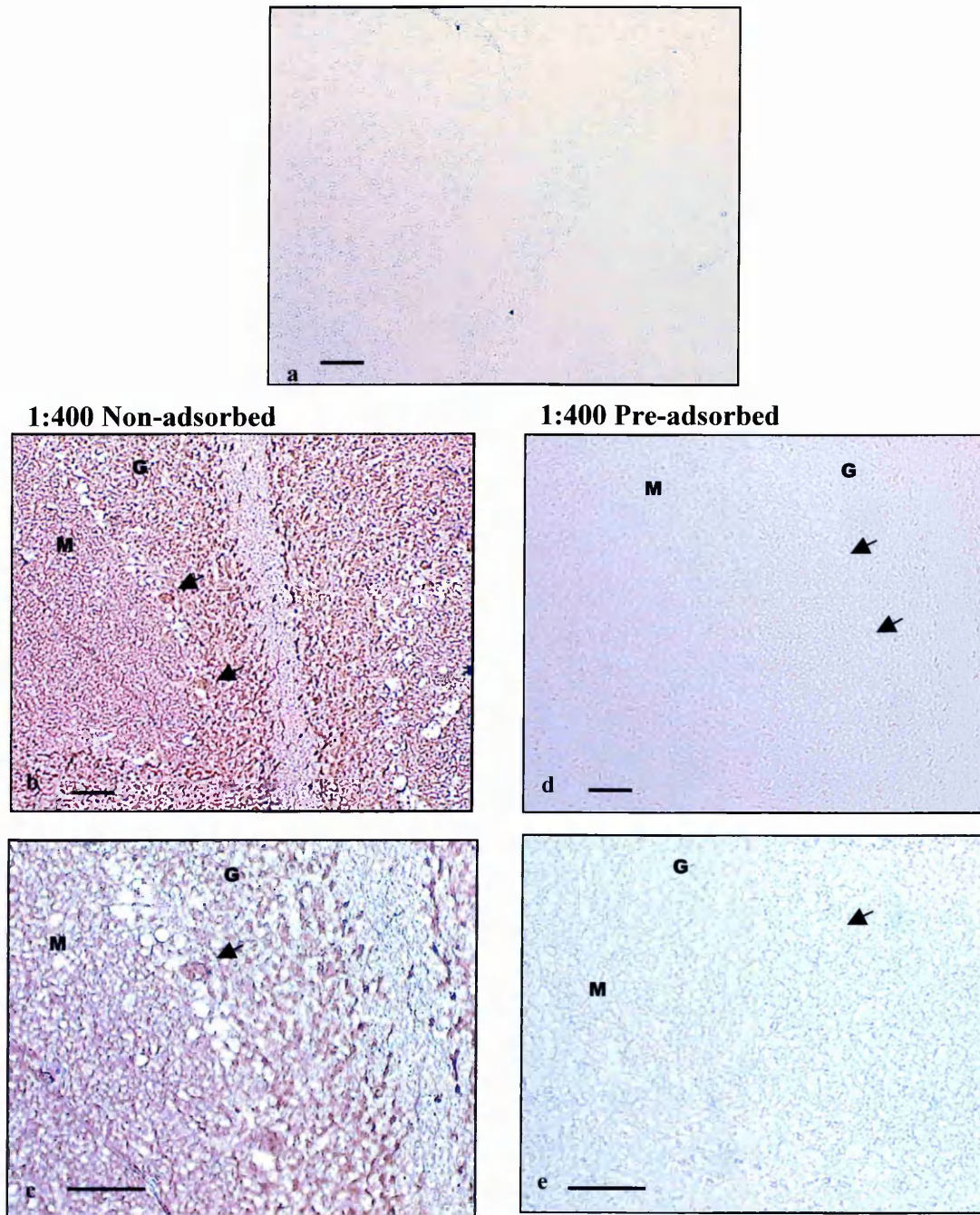
Immunohistochemistry using rat cerebellum to investigate the cross-reactivity of commercial anti-gliadin antibodies with cerebellar proteins. A typical negative control section (absence of primary antibody) is shown (**a**). Widespread staining using the commercial anti-gliadin antibody was observed, including positive Purkinje cell staining, at a dilution of 1:400 (**b** & **c**). Cerebellar staining was completely abolished following pre-adsorption of antibodies using crude gliadin (**d** & **e**). Purkinje cells are indicated by arrows. **G** – Granular layer **M** – Molecular layer. Scale bar = 100μm.

Figure 4.12 cont'd Reactivity of commercial anti- gliadin antibody with/without pre-adsorption with crude gliadin on control rat cerebellum

1:800 Non-adsorbed   **1:800 Pre-adsorbed**
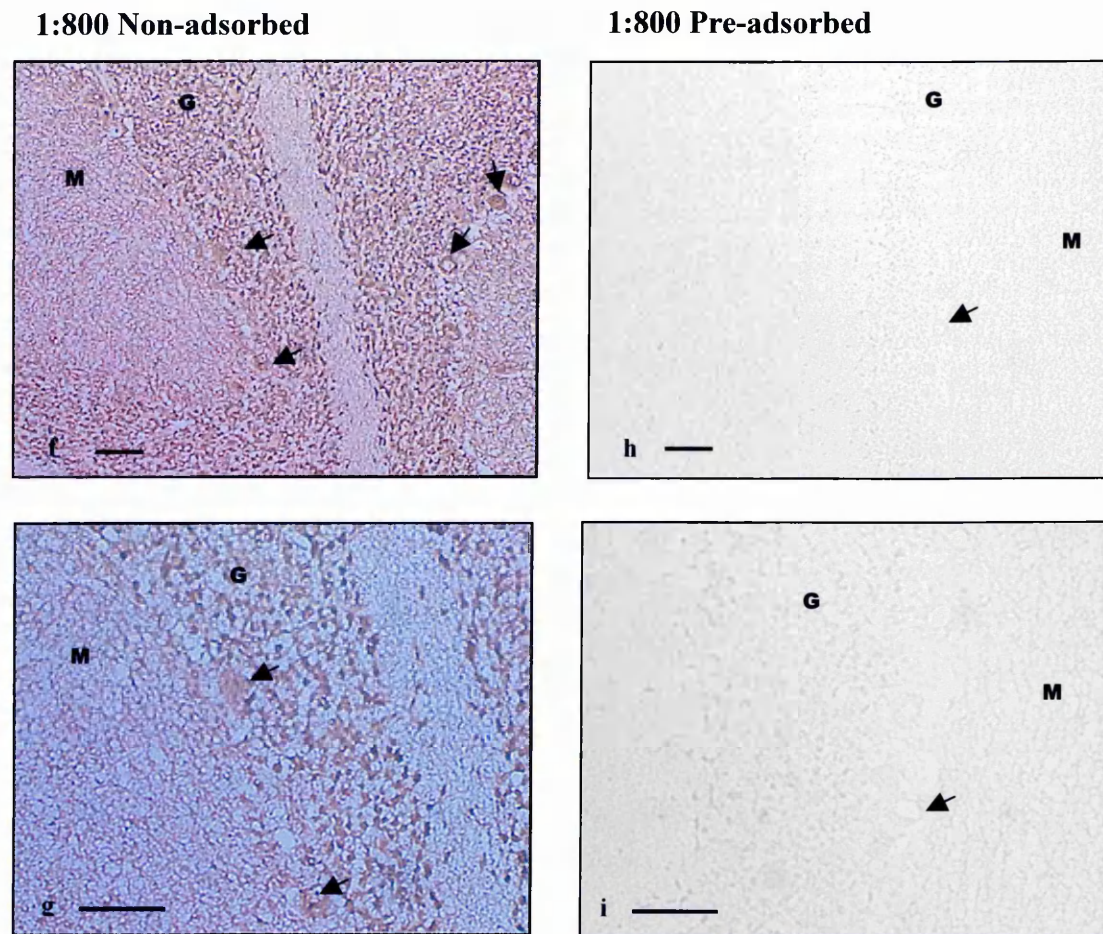
Immunohistochemistry using rat cerebellum to investigate the cross-reactivity of commercial anti-gliadin antibodies with cerebellar proteins. Widespread staining using the commercial anti-gliadin antibody was also observed, including positive Purkinje cell staining, at a dilution of 1:800 (**f** & **g**). Cerebellar staining was completely abolished following pre-adsorption of antibodies using crude gliadin (**h** & **i**). Purkinje cells are indicated by arrows. **G** – Granular layer   **M** – Molecular layer. Scale bar = 100μm.

Figure 4.13 Reactivity of commercial anti- gliadin antibody with/without pre-adsorption with crude gliadin on control human cerebellum

1:400 Non-adsorbed   **1:400 Pre-adsorbed**

1:800 Non-adsorbed   **1:800 Pre-adsorbed**
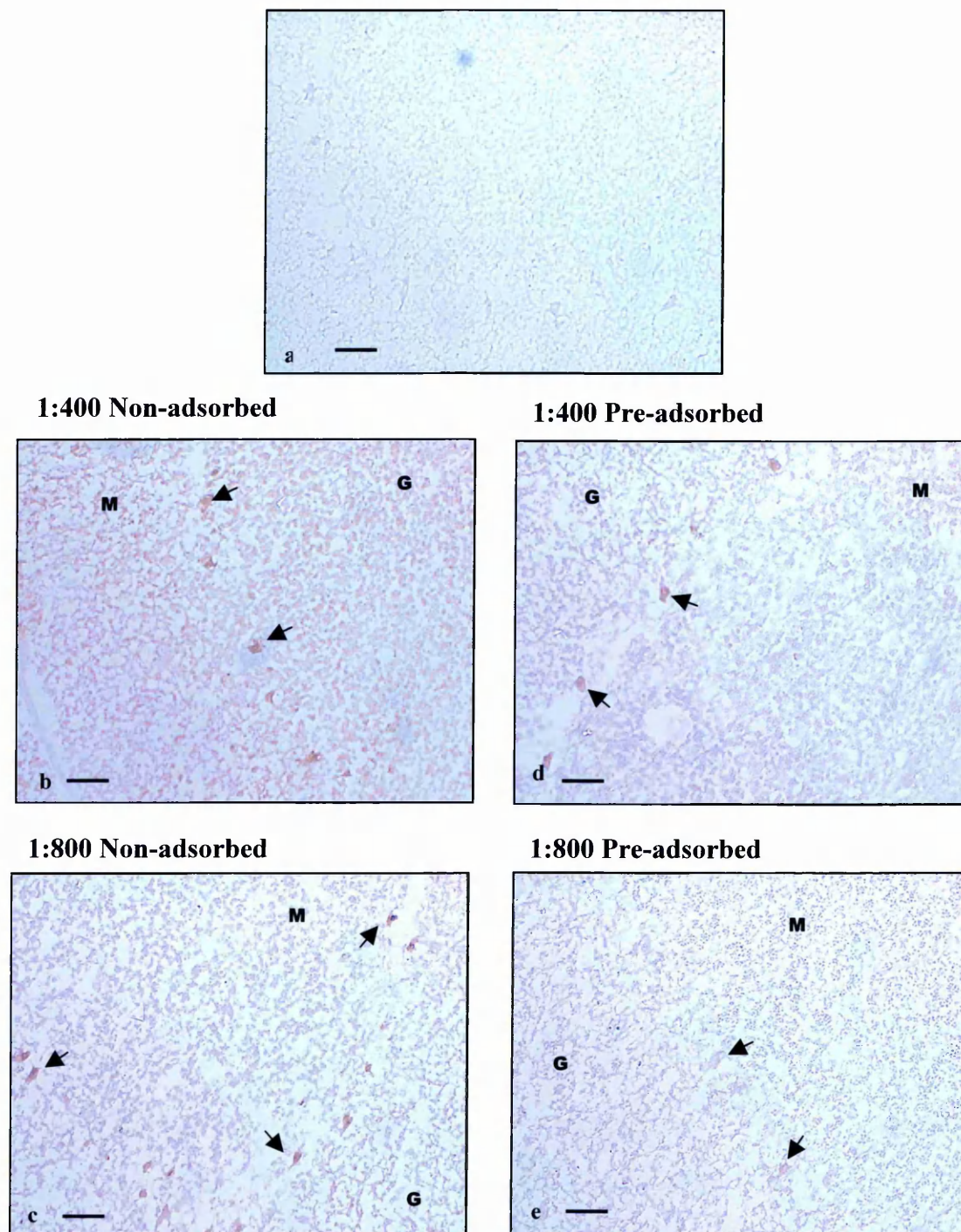
Immunocytochemistry using control human cerebellum to investigate the cross-reactivity of commercial anti-gliadin antibodies with cerebellar proteins. A typical negative control section (absence of primary antibody) is shown (**a**). Widespread staining of the commercial anti-gliadin antibody was observed, including positive Purkinje cell staining, at dilutions of 1:400 (**b**) and 1:800 (**c & f**). Cerebellar staining was reduced, but not abolished, following pre-adsorption of anti-gliadin antibodies with crude gliadin at a dilution of 1:400 (**d**). At a dilution of 1:800, staining was virtually abolished following pre-adsorption of antibodies (**e & i**). Purkinje cells are indicated by arrows. **G** – Granular layer   **M** – Molecular layer. Scale bar = 100μm.

Figure 4.13 cont'd   Reactivity of commercial anti-gliadin antibody with/without pre-adsorption with crude gliadin on human control cerebellum
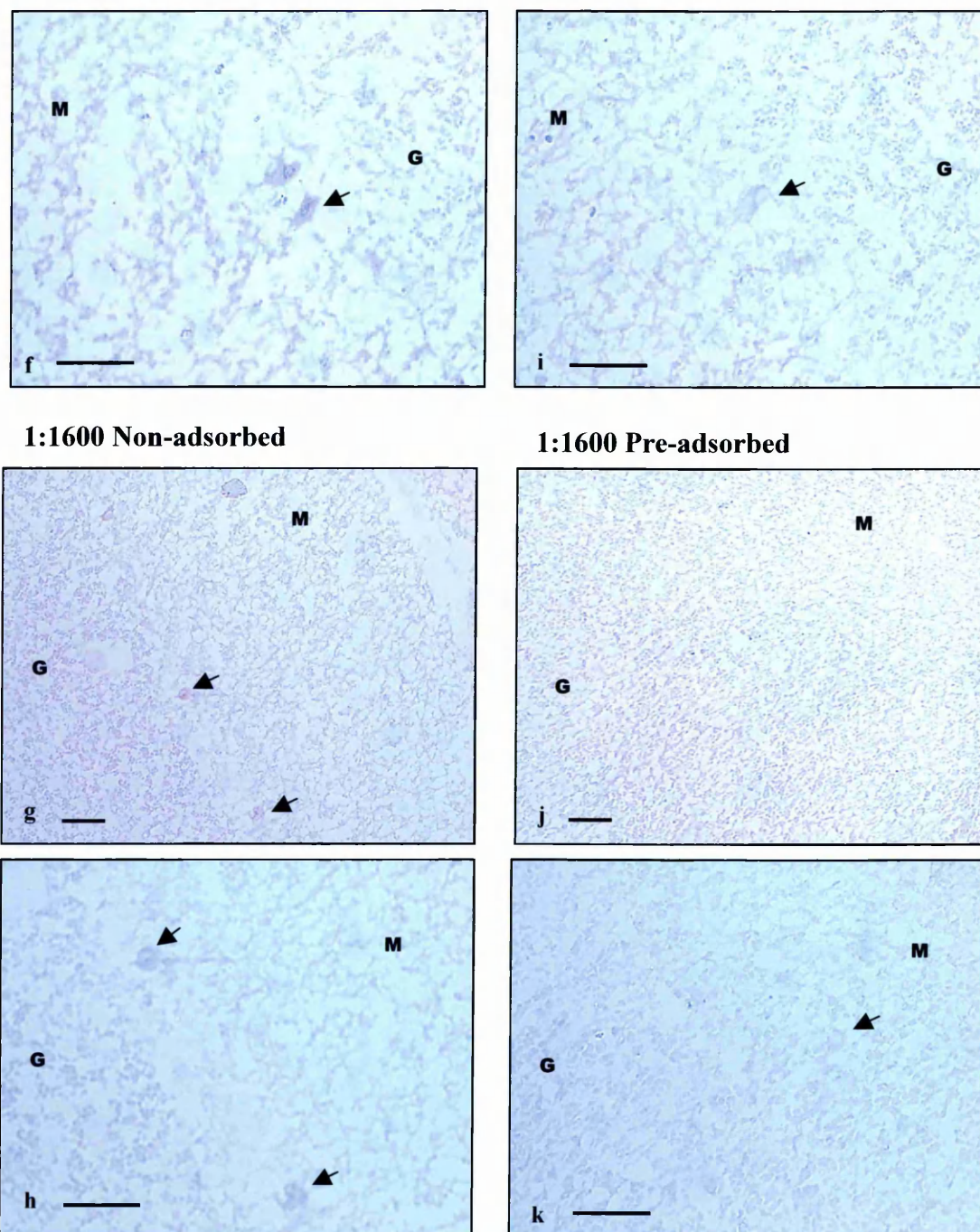
1:1600 Non-adsorbed   **1:1600 Pre-adsorbed**

Widespread staining of the commercial anti-gliadin antibody was also observed, including positive Purkinje cell staining, at a dilution of 1:1600 (**g** & **h**). This staining was abolished, following pre-adsorption of anti-gliadin antibodies with crude gliadin (**j** & **k**). Purkinje cells are indicated by arrows. **G** – Granular layer   **M** – Molecular layer. Scale bar = 100μm.

4.3.5 Reactivity of patient sera with rat cerebellar tissue with/without pre-adsorption with crude gliadin

The staining of both normal control sera tested was not affected by pre-adsorption with crude gliadin (figure 4.14h & 4.14i). Pre-adsorption of anti-gliadin antibodies also did not affect the staining of the two sera samples tested from patients with sporadic idiopathic ataxia (figure 4.14f & 4.14g). Of two serum samples from patients with CD without neurological complications, Purkinje cell staining was eliminated in one, following pre-adsorption with crude gliadin. However, staining was unchanged in the other following pre-adsorption of anti-gliadin antibodies (figure 4.14d & 4.14e). Finally, staining of both gluten ataxia sera was slightly decreased in intensity, but not completely abolished, following pre-adsorption with crude gliadin (figure 4.14b & 4.14c). In this series of patients, as previously observed, Purkinje cell staining was characterised by staining both membranous and cytoplasmic structures. However, it was noted that both sera from patients with sporadic idiopathic ataxia and serum from one patient with CD without neurological complications showed a different pattern of Purkinje cell staining. In these cases, in approximately half of the Purkinje cells stained, the staining was mainly membranous with no cytoplasmic staining (figure 4.14d & 4.14f).

4.3.6 Reactivity of patient sera with human cerebellar tissue with/without pre-adsorption with crude gliadin

Sera were found to stain human cerebellar tissue in a similar manner to rat cerebellum. The crude gliadin solution, used to pre-adsorb serum samples, when used in place of the primary antibody was similar to the negative control (PBST as primary antibody) (figure 4.15b). In these experiments, a dilution of 1:200, as opposed to 1:800, was optimal to investigate the effect of pre-adsorption of anti-gliadin antibodies between groups on staining of human cerebellum. Similar to rat cerebellar staining, the staining of both normal control sera, at both 1:200 and 1:800 dilution, was not affected by pre-adsorption with crude gliadin. Staining of 3 serum samples from patients with CD without neurological complications was virtually eliminated in 2/3 and completely eliminated in the other at a dilution of 1:200 following pre-adsorption of anti-gliadin antibodies (figure 4.15e & 4.15f). At a dilution of 1:800, serum staining of all 3 CD serum samples was completely abolished following pre-adsorption of antibodies with crude gliadin. Finally, staining of 4/4 serum samples from patients with gluten ataxia

Figure 4.14 Reactivity of sera from patients with gluten ataxia and coeliac disease only with/without pre-adsorption with crude gliadin on control rat cerebellum
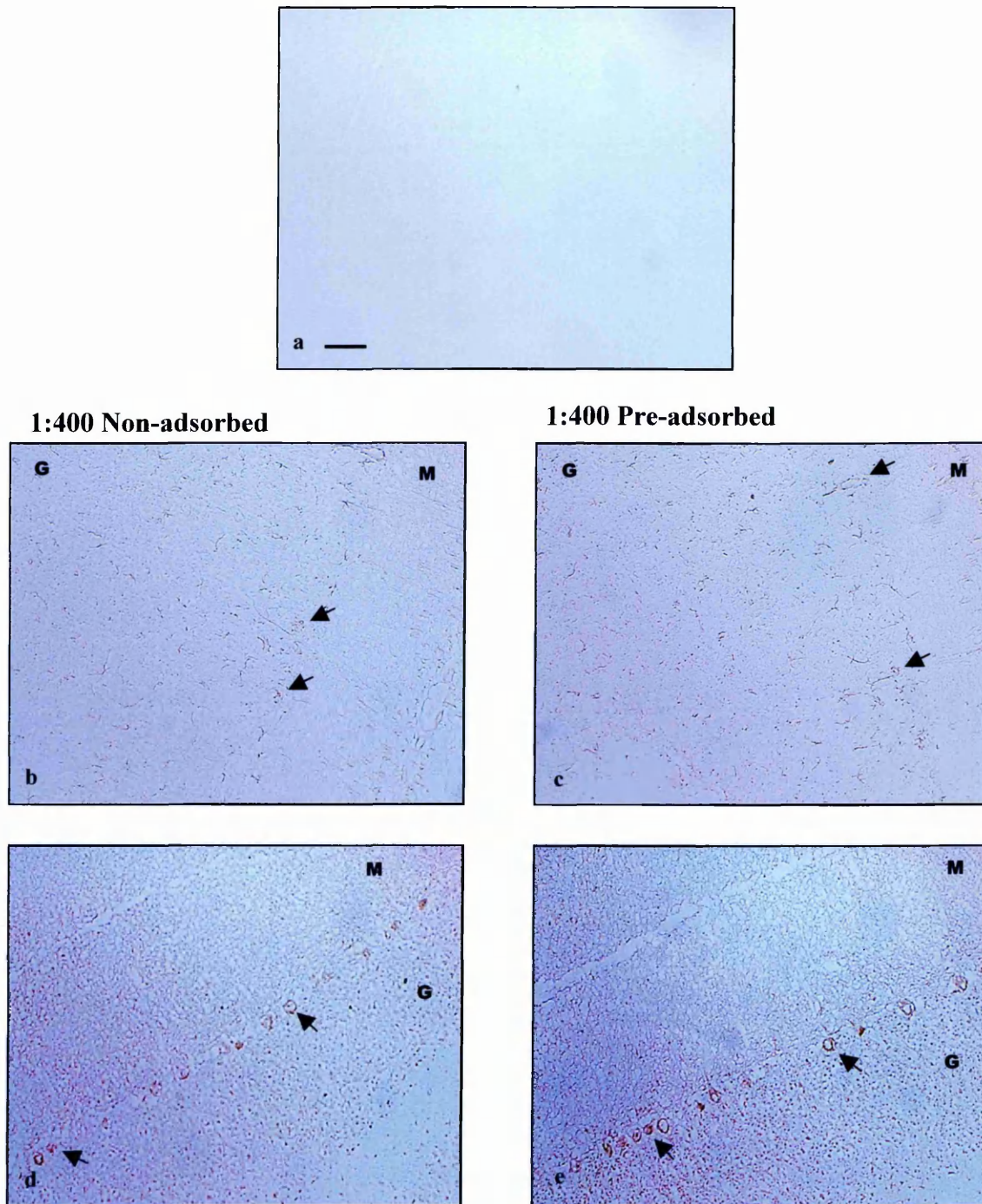
A typical negative control section (absence of primary antibody) is shown (**a**). Sera from both gluten ataxia patients and patients with CD only, diluted 1:400, positively stained Purkinje cells (**b** & **d** respectively). Pre-adsorption of both gluten ataxia patient sera and CD only sera, with crude gliadin, did not alter staining (**c** & **e** respectively). Scale bar = 100 μm. Purkinje cells are indicated by arrows. **G** – Granular layer **M** – Molecular layer.

Figure 4.14 cont'd Reactivity of sera from ataxia controls and healthy controls with/without pre-adsorption with crude gliadin on control rat cerebellum

1:400 Non-adsorbed   **1:400 Pre-adsorbed**
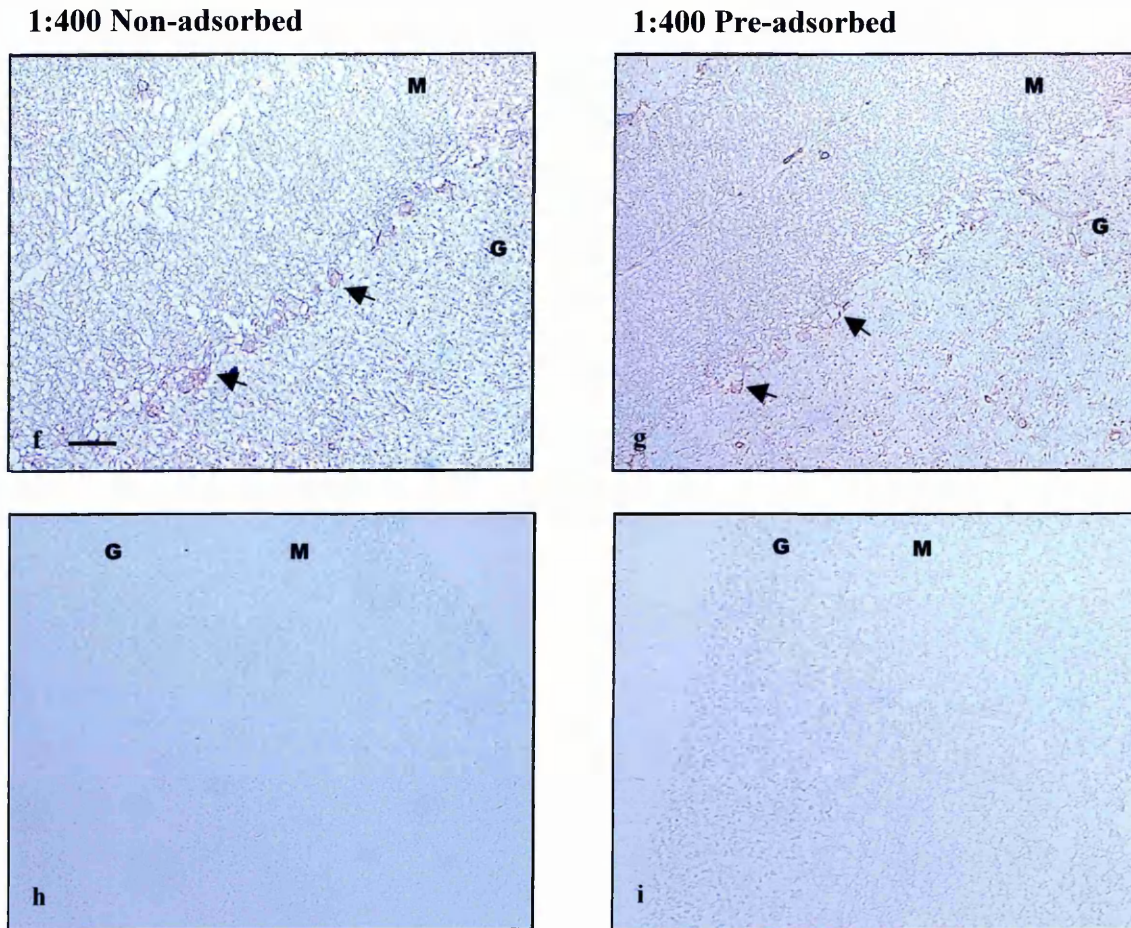
Sera from patients with idiopathic sporadic ataxia, diluted 1:400, positively stained Purkinje cells (**f**). Pre-adsorption, with crude gliadin, did not eliminate this staining (**g**). Similarly, pre-adsorption with crude gliadin (**i**) did not alter staining observed using healthy control serum, diluted 1:400 (**h**). Scale bar = 100μm.

Purkinje cells are indicated by arrows. **G** – Granular layer   **M** – Molecular layer.

Figure 4.15 Reactivity of sera from patients with gluten ataxia and coeliac disease only with/without pre-adsorption with crude gliadin on control rat cerebellum

1:200 Non-adsorbed   **1:200 Pre-adsorbed**
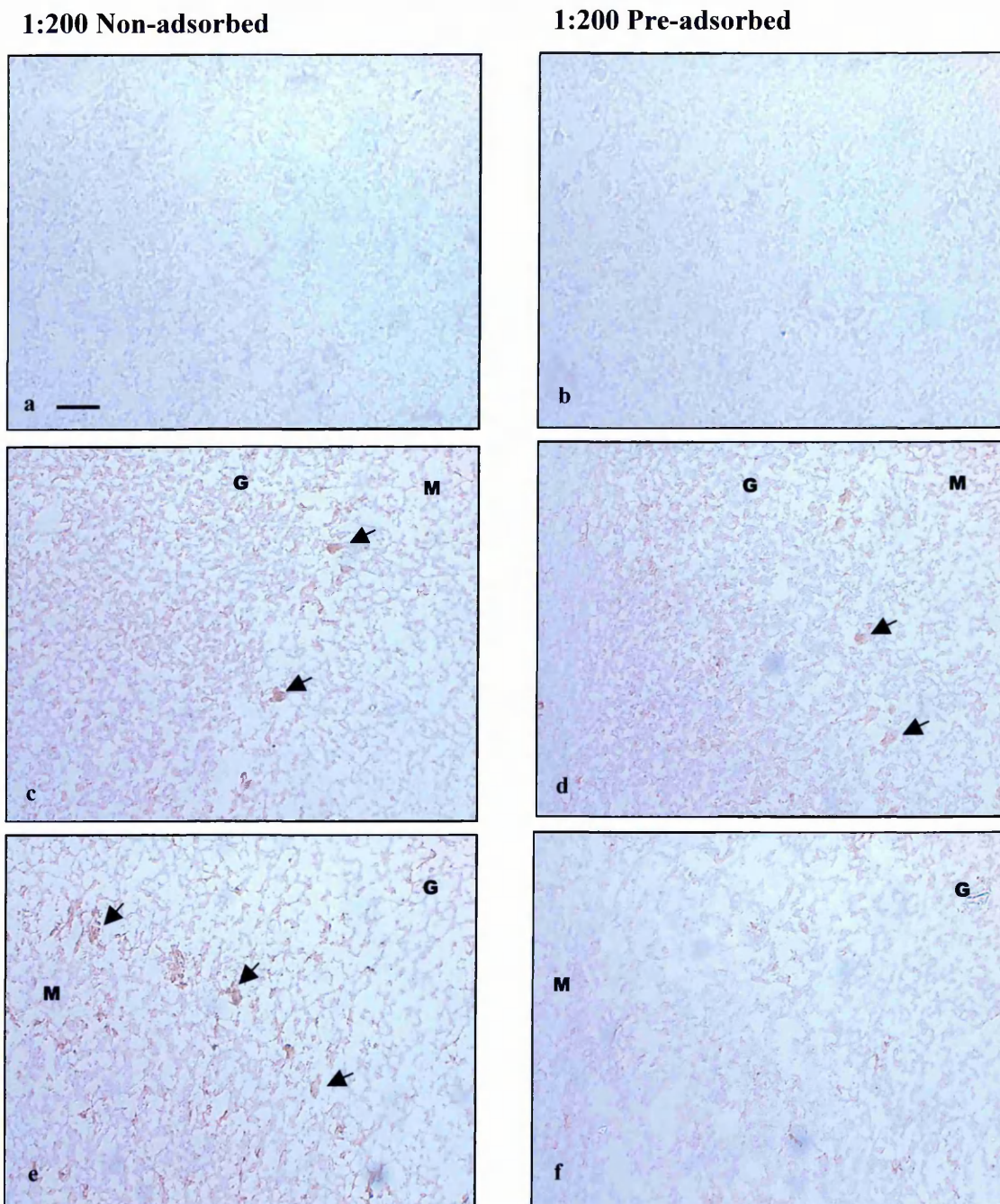
Typical negative control sections (absence of primary antibody and crude gliadin in place of primary antibody) are shown (**a** & **b** respectively). Sera from both gluten ataxia patients and patients with CD without neurological complications, diluted 1:200, positively stained Purkinje cells (**c** & **e** respectively). Pre-adsorption, with crude gliadin, of sera from patients with CD without neurological complications resulted in elimination of staining (**f**). However, pre-adsorption, with crude gliadin, of sera from patients with gluten ataxia resulted in a decrease in intensity but not elimination of Purkinje cell staining (**d**). Scale bar = 100μm. Purkinje cells are indicated by arrows. **G** – Granular layer   **M** – Molecular layer.

was decreased in intensity, but not eliminated following pre-adsorption of anti-gliadin antibodies at dilutions of both 1:200 and 1:800 (figure 4.15c & 4.15d).

4.3.7 Confirmation of adsorption of anti-gliadin antibodies using crude gliadin, determined by ELISA

ELISA confirmed the successful adsorption of anti-gliadin antibodies with crude gliadin using a crude gliadin solution of a similar concentration to that used in the experiments described above. Following adsorption of anti-gliadin antibodies with crude gliadin, the commercial anti-gliadin antibody and all sera tested were negative for IgG anti-gliadin antibodies on ELISA (figure 4.16). All, except one serum sample from a patient with CD without neurological complications, were also negative for IgA anti-gliadin antibodies on ELISA (figure 4.17).

4.3.8 Reactivity of CSF with human cerebellar tissue

No CSF sample showed reactivity with human cerebellar tissue at any of the dilutions used (data not shown).

Figure 4.16 Confirmation by ELISA of adsorption of IgG anti-gliadin antibodies, following incubation with crude gliadin, in commercial anti-gliadin antibody and sera from patients with gluten ataxia, coeliac disease only, ataxia controls and healthy controls

Data expressed in arbitrary units (defined by Hycor Biomedical Ltd). The horizontal line represents the positive/negative threshold, as defined by the kit manufacturers. ■ Sample diluted in PBST (1:100 dilution, unless stated). □ Sample diluted in crude gliadin solution (1:100 dilution, unless stated). GA – gluten ataxia, CD – CD without neurological complications, SA – sporadic ataxia, FA – familial ataxia, HC – healthy control, AGA – commercial anti-gliadin antibody.

Figure 4.17 Confirmation by ELISA of adsorption of IgA anti-gliadin antibodies, following incubation with crude gliadin, in commercial anti-gliadin antibody and sera from patients with gluten ataxia, coeliac disease only, ataxia controls and healthy controls

Data expressed in arbitrary units (defined by Hycor Biomedical Ltd). The horizontal line represents the positive/negative threshold, as defined by the kit manufacturers. ■ Sample diluted in PBST (1:100 dilution, unless stated). ☐ Sample diluted in crude gliadin solution (1:100 dilution, unless stated). GA – gluten ataxia, CD – CD without neurological complications, SA – sporadic ataxia, FA – familial ataxia, HC – healthy control, AGA – commercial anti-gliadin antibody.

4.4 Discussion

The results of these studies show that patients with gluten ataxia possess circulating IgG anti-cerebellar antibodies. IgG anti-gliadin antibodies are the most sensitive marker for gluten sensitivity in patients with gluten ataxia (see previous chapter) and in conditions, such as PCD, only IgG anti-cerebellar antibodies are considered clinically relevant (Bradwell *et al.*, 1997). In both rat and human tissue, serum from patients with gluten ataxia reacted most strongly with Purkinje cells, a result consistent with the neuropathological feature of Purkinje cell loss and the notion that these cells are most susceptible to damage in these patients (Hadjivassiliou *et al.*, 1998).

Although Purkinje cell staining was also seen using sera from other patient groups (CD without neurological complications, familial ataxia, sporadic ataxia (non-gluten)) and healthy control subjects it was only seen at high antibody concentrations, suggesting that it is relatively non-specific. Purkinje cell staining by control sera at dilutions of up to 1:100 has been reported previously and is considered non-specific (Jaeckle *et al.*, 1985).

At all increasing dilutions staining persisted in the majority of patients with gluten ataxia but only in a minority of patients from other groups. By a dilution of 1:800, positive Purkinje cell staining was evident in 4 of 5 patients with gluten ataxia compared to 1 of 5 patients with CD only, 1 of 5 healthy control subjects and no ataxia control patients.

Marked differences were also seen between groups in the strength of Purkinje cell staining. The majority of sera, from all patient groups, stained strongly with Purkinje cells at the lowest dilution but only in patients with gluten ataxia did strong staining persist in the majority of sera (3/5) at higher dilutions. At these dilutions strong staining was only seen in 1/5 patients with CD only and 1/5 ataxia control patients. Overall, strong and persistent staining of Purkinje cells by sera was only seen in the majority of patients with gluten ataxia and the lack of a similar staining pattern in ataxia control patients implies that this is not a general feature of Purkinje cell loss. These staining patterns were confirmed by experiments carried out by the group of Dr. Tongiorgi, using rat cerebellum. Both of these results from studies on rat and human tissue are in apparent disagreement with a recent report by Wiendl and co-workers who found no binding to either cell-surface or intracellular Purkinje cell antigens by gluten ataxia sera,

quantified by flow cytometry (Wiendl *et al.,* 2003). However, importantly, the neuronal cell lines used in this study may not have included Purkinje cells, the target of antibodies in gluten ataxia. In addition, the study did not include use of sera containing antibodies against Purkinje cell epitopes, such as anti-Yo antibodies, as a positive control, without which it is not possible to conclude the absence of Purkinje cell binding.

Experiments using anti-Yo positive sera, in the current study, showed granular staining of the Purkinje cell cytoplasm, consistent with the established staining pattern of this antibody (Bradwell *et al.,* 1997; Okano *et al.,* 1999). Sera from all patient groups stained Purkinje cells in a uniform diffuse manner. Most frequently, the cell bodies were most strongly stained although weaker dendritic staining was seen on occasion. Staining of the cell bodies appeared predominantly cytoplasmic although lacking the granular appearance of anti-Yo cytoplasmic staining. These experiments did not provide evidence of any distinguishing staining features, which could be reliably used in the differential diagnosis of gluten ataxia.

It is possible that this staining may be due to cross-reactivity of circulating gluten sensitivity associated antibodies (anti-gliadin or anti-TG2 antibodies) with shared epitopes on Purkinje cells. Several factors are suggestive of a limited role for circulating anti-TG2 antibodies in this respect. Firstly, the diffuse, non-specific staining pattern observed using a commercial anti-TG2 antibody does not correlate with the observed staining pattern of Purkinje cells by sera. In addition, although measured circulating anti-TG2 antibodies are of the IgA type, staining is not correlated with the presence or level of these antibodies in patients. For example, staining was observed in all patients with gluten ataxia yet antibodies are only positive in 40% of patients. Conversely, nearly all patients with CD only possess circulating anti-TG2 antibodies (94%), often at high levels, yet the same degree of Purkinje cell staining as that of patients with gluten ataxia was not generally seen. In contrast, the IHC results demonstrate the ability of commercial anti-gliadin antibody to cross-react with Purkinje cells, suggesting the existence of common epitopes. Cross-reactivity of anti-gliadin antibodies with the calcium-binding protein, calreticulin, and enterocytes has previously been demonstrated and a recent genome screen using gliadin cDNA as a probe showed partial homology with several genes (Krupickova *et al.,* 1999; Kumar *et al.,* 2000). Anti-gliadin antibodies are a common feature of almost all patients with gluten

sensitivity. This cross-reactivity may account for the Purkinje cell staining by sera from patients with CD only without neurological complications observed in the current study. One important consideration of this hypothesis is that, if Purkinje cell staining by sera is considered to be completely, or at least partially, due to the cross-reactivity of circulating anti-gliadin antibodies then the degree of staining may be expected to depend on the levels of these antibodies in the serum. It is therefore of note that levels of circulating IgG anti-gliadin antibodies were not correlated with either the persistence or strength of staining of sera from any patient group. Although, this correlation was assessed using a visual, semi-quantitative evaluation of staining and, it may be the case that a more subtle correlation would have been apparent with use of a more sensitive and precise measurement of Purkinje cell staining such as quantitive image analysis involving measurements of staining density.

In addition, the results of the adsorption experiments suggest that, in patients with gluten ataxia at least, anti-gliadin antibodies are not solely responsible for the observed staining of Purkinje cells. Adsorption with crude gliadin was sufficient to eliminate anti-gliadin antibodies, as shown by the ELISA results, yet Purkinje cell staining by sera from patients with gluten ataxia was not completely abolished. These findings suggest that patients with gluten ataxia exclusively possess additional antibodies against Purkinje cells, which are distinct from anti-gliadin antibodies.

In summary, the main findings of this study are that, sera from patients with gluten ataxia stain cerebellar Purkinje cells more strongly and persistently than sera from patients in other groups and therefore have higher titres of circulating IgG anti-Purkinje cell antibodies. Gluten sensitivity associated, circulating anti-TG2 antibodies are unlikely to contribute significantly to this staining but gluten-driven circulating anti-gliadin antibodies have been shown to cross-react with Purkinje cells, suggesting that gliadin and Purkinje cells share common epitopes. However, circulating levels of both antibodies show no correlation with the strength or persistence of Purkinje cell staining. Finally, adsorption experiments have suggested that patients with gluten ataxia possess circulating anti-Purkinje cell antibodies, which are distinct from anti-gliadin antibodies and are not found in patients from other groups. These antibodies may explain the observed cerebellar staining characteristics of patients with gluten ataxia.

CHAPTER FIVE

INVESTIGATION OF THE CEREBELLAR

SPECIFICITY OF CIRCULATING

ANTIBODIES IN PATIENTS WITH GLUTEN

ATAXIA BY WESTERN BLOTTING

5.1 Introduction

Patients with gluten ataxia possess circulating anti-gliadin antibodies, which have been shown to cross-react with cerebellar Purkinje cells, and, to a lesser extent, antibodies against TG2, a ubiquitous protein also found in the cerebellum. In addition, patients with gluten ataxia possess specific circulating anti-Purkinje cell antibodies, which are distinct from anti-gliadin antibodies and which do not appear to be present in patients with CD only or in individuals with non-gluten ataxia (see previous chapter for details). However, the Purkinje cell antigen to which these antibodies are directed is unknown. Analysis of antibody binding by western blotting in such situations, in order to gain additional information on the target protein, is commonly employed. In addition, western blotting is also widely used to confirm antibody binding to known proteins detected by IHC. For example, the specificity of PCD associated antibodies is confirmed by western blot analysis against cerebellar extracts or recombinant proteins (Bradwell *et al.*, 1997).

5.1.1 Aims of this study

The overall aim of this study was to continue the study of the humoral immune response in the cerebellum of patients with gluten ataxia by investigating the specificity of circulating antibodies from patients with gluten ataxia within the cerebellum, using SDS-PAGE and western blotting. This was achieved by several means:

- Confirmation of the cross-reactivity of anti-gliadin antibodies with cerebellar proteins and the presence of TG2 in the cerebellum by western blotting
- Screening of sera from patients with gluten ataxia for specificity to cerebellar proteins on a western blot and determination of the RMM of immunoreactive proteins.
- Comparison of this antigenic specificity of gluten ataxia sera with that of patients with CD without neurological involvement, patients with sporadic idiopathic ataxia or familial ataxia and normal healthy controls.
- Assessment of the contribution of circulating anti-gliadin or anti-TG2 antibodies to staining and determination of any reactivity to these proteins specific to patients with gluten ataxia.

5.2 Methods

5.2.1 Optimisation of protein extraction

Adult, female Wistar rats were used as the source of cerebellar tissue for SDS-PAGE. The objective of the method was to extract sufficient amounts of protein for adequate detection on a western blot. A total of four different extraction protocols were tried, including a commercial protein extraction kit (Bio-Rad, UK). Protein extractions were performed an average of 3 times for each protocol. Once the optimal method had been selected (section 2.4.2), this was used for all subsequent protein extractions.

The amount of protein in each extract (mg/ml) was calculated using the BCA protein assay, described in section 2.4.3, to enable the same amount of protein to be loaded into each lane of the polyacrylamide gel and allow comparison of results between experiments.

5.2.2 Optimisation of SDS-PAGE and western blotting

The separation of cerebellar proteins on polyacrylamide gels and their transfer onto nitrocellulose membrane was first optimised in order to facilitate subsequent detection of proteins of interest. This process included consideration of the factors detailed below:

5.2.2.1 Equipment

Optimisation of the SDS-PAGE method was carried out using a mini-protean III vertical electrophoresis system (Bio-Rad, UK). Subsequently, and throughout all the studies included in this thesis, a Hoefer mighty small SE250 vertical mini-gel system was used (Amersham Pharmacia Biotech, UK). Initially, transfer of proteins was carried out using the blotting module of the mini-protean III system (Bio-Rad, UK). Subsequently, and throughout all the studies included in this thesis, a Hoefer semi-dry blotting system was used (Amersham Pharmacia Biotech, UK), the major advantage being the use of 80% less transfer buffer in its operation with comparable results to the Bio-Rad system.

5.2.2.2 Percentage of polyacrylamide gels

A 5% stacking gel was used in all experiments. Resolving gels of 8%, 10%, 12% and 12.5% were used to compare separation of cerebellar proteins. Protein separation was assessed by staining the gel in Coomassie blue stain to visualise the proteins. Briefly, gels were placed in Coomassie blue stain (45.5% v/v methanol, 0.9% v/v acetic acid, 1.5mM Coomassie blue (Sigma, UK) in d.H_2O) overnight at RT with gentle shaking. Gels were then removed into destain (45.5% v/v methanol, 0.9% v/v acetic acid in d.H_2O) at RT with gentle shaking. The destain was changed as many times as necessary until all background staining of the gel had disappeared and the protein staining was clearly visible.

5.2.2.3 Amount of protein loaded onto gel

As the cerebellar antigen of interest was unknown, and may be present at low levels within the tissue, the amount of protein loaded in each lane influenced its subsequent detection. The objective was to find the amount of protein that was optimal for detection but that also avoided the detrimental effects on separation that can occur with too high quantities of protein. Quantities of 5μg, 10μg, 20μg, 32.5μg and 36μg of protein per lane were loaded to see which was optimal.

5.2.2.4 Time of protein transfer

Transfer times of 45mins, 60mins and 75mins, all at 100V, were used to assess optimal transfer conditions.

5.2.3 Optimisation of protein detection

In order to produce optimal detection of proteins of interest whilst minimising background and non-specific staining of nitrocellulose blots, the technique was first optimised as detailed below.

5.2.3.1 Blocking of nitrocellulose membrane

Nitrocellulose membranes were blocked in solutions of 15% milk powder in TBS (pH 8), 5% milk powder in PBS, 5% milk powder in PBST or a 5% solution of ECL blocking agent (Amersham Pharmacia Biotech, UK) in PBST. Blocking times of 60mins at RT or overnight at 4^0C were used in order to determine the optimal incubation time.

5.2.3.2 Primary antibody dilutions and incubation periods

Monoclonal anti-calbindin D28K (Sigma, UK), anti-TG2 (Labvisions, USA) and anti-GAD antibodies (Affiniti Research, UK) were incubated with nitrocellulose membranes at dilutions ranging from 1:200 to 1:10 000. Serum samples were incubated with membranes at dilutions ranging from 1:100 to 1:1000. All primary antibodies were diluted in either PBS, 'Blotto' (5% milk powder in TBST (0.05% Tween20 in TBS, pH 8)) or PBST.

Primary antibodies were incubated with membranes for periods of either 60 or 120mins at RT or overnight at 4^0C.

5.2.3.3 Secondary antibody dilutions and incubation periods

Horseradish peroxidase-conjugated anti-human IgG or anti-mouse IgG (as appropriate) secondary antibodies from Sigma, UK, Vector Laboratories, UK or Jackson Immunoresearch, USA were used. Dilutions ranged from 1:100 to 1:50 000.
All secondary antibodies were also diluted in either PBS, 'Blotto' (5% milk powder in TBST (0.05% Tween20 in TBS, pH 8)) or PBST.

An incubation period of 60mins at RT was used in all experiments.

5.2.3.4 Washing of membranes

Membranes were either washed in TBST or PBST with washing times varied from 2 x 5mins to 3 x 30mins.

5.2.3.5 Substrates and detection of antibody binding

Initially, chromogenic substrates including 0.2mg/ml solution of DAB (Sigma, UK) or opti-4CN (opti-4CN western blotting staining kit, Bio-Rad, UK) were used with development times of between 5-20mins.

A more sensitive chemiluminescent detection system was also used. Two different chemiluminescent substrates were compared, the femtolucent ECL detection kit (Chemicon, USA) and the ECL-plus™ detection kit (Amersham Pharmacia Biotech, UK). Kits were used according to the manufacturer's instruction. Initially, light emission resulting from addition of substrate was recorded using autoradiography film and developed manually using Kodak™ developer and fixer (Sigma, UK). The film

was placed in Kodak™ developer (Sigma, UK) for approximately 15secs, rinsed briefly in d.H$_2$O, placed in Kodak™ fixer (Sigma, UK) for approximately 30secs and then finally rinsed in d.H$_2$O before drying. Kodak™ (Sigma, UK) and hyperfilm™ ECL (Amersham Pharmacia Biotech, UK) films were compared. Blots were exposed to the film for periods of 15secs to 3mins. However, light emission was subsequently recorded using a UVP imager (UVP, UK) and Labworks, software (UVP, UK).

5.2.4 Detection of known cerebellar proteins (calbindin and tissue transglutaminase)

Detection of the cerebellar proteins, calbindin and TG2, was performed by probing an extract of rat cerebellum, separated on a 10% polyacrylamide gel, with appropriate commercial monoclonal antibodies. The same rat cerebellar extract (#9, section 5.3.1) was used in all experiments in order to minimise inter-experimental variation.

36μg/lane of rat cerebellar extract was loaded onto a 10% polyacrylamide gel, prepared as detailed in section 2.4.4. The first lane of each gel was loaded with 10μl coloured pre-stained molecular weight marker (Sigma, UK). SDS-PAGE and Western blotting was then performed using the optimised method, as detailed in sections 2.4.4 and 2.4.5 respectively. Proteins of interest were detected according to the optimised method detailed in section 2.4.6. Details of the primary and secondary antibodies used are given in table 5.1. Finally, the RMM of the protein bands were determined according to the method detailed in section 2.4.7. Blots were probed with anti-calbindin D28K antibody in 8 different experiments and with anti-TG2 antibody in 4 different experiments. In each case, the average RMM of resultant protein bands was calculated.

5.2.5 Detection of gliadin proteins and investigation of the cross-reactive potential of commercial anti-gliadin antibody with cerebellar proteins

A 5mg/ml gliadin solution was prepared by mixing crushed crude gliadin (Sigma, UK) with the appropriate volume of PBST. The resulting solution was heated gently overnight in a water-bath at 30^{0}C and then mixed in a ratio of 1:1 v/v with 2x sample buffer (section 2.4.4). 10μl/lane (25μg) of the solution was loaded onto a 10% polyacrylamide gel, prepared as detailed in section 2.4.4. The first lane of each gel was loaded with 10μl coloured pre-stained molecular weight marker (Sigma, UK). SDS-

Table 5.1 Details of reagents used in the detection of calbindin and tissue transglutaminase within rat cerebellar extract on a western blot

Antibody Specificity	Species (supplier)	Dilution	Corresponding secondary antibody	Secondary antibody dilution
Monoclonal anti-TG2	Mouse IgG anti-guinea pig TG2 (Labvision, USA)	1:1000	HRP conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:10 000
Monoclonal anti-Calbindin D-28K	Mouse IgG1 anti-bovine kidney calbindin (Sigma, UK)	1:1000		

PAGE and western blotting was then performed using the optimised method, as detailed in sections 2.4.4 and 2.4.5 respectively. Blots were blocked for 25mins in a 5% solution of 'quick-block'/PBST (Chemicon, USA) at RT with gentle shaking. Following 3x5min washes in PBST, blots were incubated with a peroxidase-conjugated, polyclonal rabbit anti-gliadin antibody (Sigma, UK), diluted to 1:10 000 in PBST overnight at 4^0C with gentle shaking. Blots were then washed 4x10mins in PBST and incubated in ECL-detection reagent (Chemicon, USA) for 3mins at RT with gentle shaking. The UVP imaging system detailed in section 2.4.6 was not yet available and so blots were visualised using autoradiography film. Binding was visualised by exposure of the blot to hyperfilm™ ECL (Amersham Pharmacia Biotech, UK) autoradiography film for 60secs and manual development using Kodak™ developer and fixer (Sigma, UK), as described in section 5.2.3.5. The RMM of the protein bands were determined according to the method detailed in section 2.4.7. Binding of anti-gliadin antibodies to gliadin proteins was performed in two separate experiments and the mean RMM of resultant protein bands was calculated.

In order to investigate cross-reactivity of anti-gliadin antibodies with cerebellar proteins on a western blot, 36µg/lane of rat cerebellar extract (#9, section 5.3.1) was loaded onto a 10% polyacrylamide gel, prepared as detailed in section 2.4.4. The same rat cerebellar extract was used in all experiments in order to minimise inter-experimental variation.

The first lane of each gel was loaded with 10μl coloured pre-stained molecular weight marker (Sigma, UK). SDS-PAGE and western blotting was then performed using the optimised method, as detailed in sections 2.4.4 and 2.4.5 respectively. A peroxidase-conjugated, polyclonal rabbit anti-gliadin antibody (Sigma, UK), diluted to 1:1000 in PBST, was used to probe the blot and proteins of interest were detected according to the optimised method detailed in section 2.4.6 with the omission of the secondary antibody step. The RMM of the protein bands were determined according to the method detailed in section 2.4.7. Investigation of the cross-reactivity of anti-gliadin antibodies with cerebellar proteins was performed on three separate occasions and the mean RMM of resultant protein bands was calculated.

5.2.6 Investigation of the specificity of patient serum antibody binding to cerebellar proteins

Sera from different patient groups were screened for reactivity against rat cerebellar proteins separated on a 10% polyacrylamide gel. The same rat cerebellar extract (#9, section 5.3.1) was used in all experiments in order to minimise inter-experimental variation.

Samples from patients with untreated gluten ataxia, patients with CD without neurological complications, patients with familial ataxia, patients with idiopathic sporadic ataxia (non-gluten) and healthy controls were compared (table 5.2). 10μl/lane (36μg) of rat cerebellar extract was loaded onto a 10% polyacrylamide gel, prepared as detailed in section 2.4.4. The first lane of each gel was loaded with 10μl coloured pre-stained RMM marker (Sigma, UK). SDS-PAGE and western blotting was then performed according to the optimised method detailed in sections 2.4.4 and 2.4.5 respectively. As described in section 2.4.5, Ponceau-S red stain (Sigma, UK) was used to temporarily stain the protein in the blot to assess the transfer success and allow the protein bands of the RMM marker to be indicated with pencil before destaining. At this stage, blots were cut into single lane strips. Strip orientation was enabled by the removal of the bottom left-hand corner of the strip. Reactivity of serum samples was assessed according to the optimised method detailed in section 2.4.6, using serum as primary antibody. Each strip was probed with a different primary antibody. One strip from each blot was probed with a monoclonal anti-GAD65/67 antibody to allow an estimate of inter-experimental variation. Further details of the samples screened and

secondary antibodies used are given in table 5.2. Staining was visualised using the UVP imaging system detailed in section 2.4.6. The RMM of the protein bands were determined according to the method detailed in section 2.4.7. The RMM of GAD was calculated for each blot and used to determine the inter-experimental variation (%CV). The average RMM and the standard deviation of the values were calculated and used to calculate %CV as below,

$$\%CV = (SD/Mean\ reading)\ x100$$

In order to determine which bands may be due to binding of IgG anti-gliadin or anti-TG2 antibodies within positive serum samples, the mean RMM (as reported in sections 5.3.4 and 5.3.5) of the respective gliadin or TG2 protein band was used. Any bands at the calculated RMM ± inter-experimental variability were considered likely to be produced by binding of these specific antibodies. The percentage of the total number of patients whose serum was positive for anti-gliadin antibodies on ELISA and that also produced a band suggestive of binding of anti-gliadin antibodies, was calculated. This was repeated for patients whose serum was positive for anti-TG2 antibodies.

Table 5.2 Summary of sera and detection antibodies used in screening studies of sera reactivity against rat cerebellar proteins on a western blot

Patient Group	n	Serum dilution	Corresponding secondary antibody	Secondary antibody dilution
Gluten Ataxia (untreated)	14	1:1000	HRP conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:10 000
CD without neurological complications (untreated)	10			
Familial ataxia	7			
Sporadic idiopathic ataxia (non-gluten)	2			
Healthy controls	11			
Stiff-person syndrome	1			

n= number of patients in each group

5.3 Results

5.3.1 Optimisation of protein extraction

Table 5.3 shows the protein yields obtained for each extraction carried out using each of the four extraction methods. Method 4 was selected as optimal and used in all protein extractions thereafter.

Table 5.3 A comparison of the results obtained from four different protein extraction methods

Extraction #	Method used	Tissue used	Weight of tissue used (g)	Protein yield (mg/ml)	
				Pellet fraction	Supernatant fraction
1	1	Rat cerebellum	2.9	0.6	-
2	1	Rat cerebellum	0.6	<0.1	-
3	1	Human cerebellum	0.016	<0.1	-
4	2	Rat cerebellum	3.62	6.5	-
5	2	Rat cerebellum	1.7	2.3	-
6	2	Rat cerebellum	2.92	2.0	4.0
7	3	Rat cerebellum	0.15	-	4.7
8	4	Rat cerebellum	0.365	5.5	7.0
9	4	Rat cerebellum	0.616	5.49	7.33

- = Indicates fraction not tested

A typical BSA standard curve (from extraction #9), used in the BCA assay to calculate protein yield following the extraction procedure, is shown in Figure 5.1. Data is expressed as Mean ± standard error of the mean (SEM).

5.3.2 Optimisation of SDS-PAGE and western blotting

A 10% resolving gel was found to give the best overall separation over a wide range of RMMs, important as the cerebellar antigen of interest was unknown, and was used in all subsequent experiments. It was found that, generally, the greater the amount of protein loaded in each lane, the better the protein detection.

Figure 5.1 A typical BSA standard curve used to determine the protein concentration
of tissue extracts
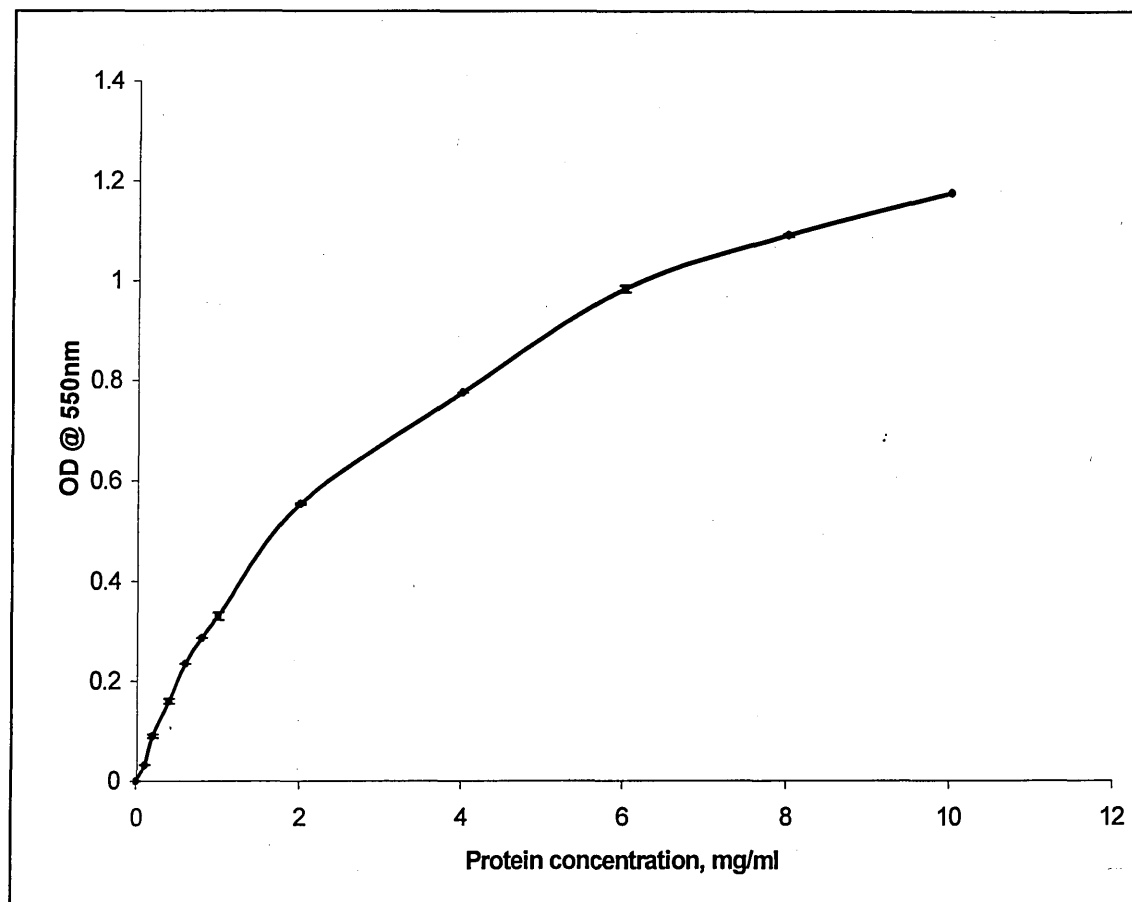
BSA solutions ranging in concentration from 0.1-20mg/ml were prepared and used in

the BCA protein assay as standards (in duplicate). Protein concentration is plotted

against the corresponding blank-corrected, average OD to produce a standard curve.

The unknown protein concentration of tissue extracts is determined using this curve by

using the blank-corrected, average OD reading of the sample to determine the

corresponding protein concentration. Data is expressed as MEAN ± SEM.

Initially, protein amount was limited due to poor protein yields from extractions from tissue. In addition, higher amounts of protein were problematic to load because the resultant increase in volume necessary to load the specified amount of protein caused sample wells to overflow. As the protein extraction method was improved, higher amounts of protein contained within lesser volumes were able to be loaded. The maximum, 36µg per well was found to produce the best results and was used in all subsequent experiments. Typical separation of rat cerebellar proteins (extraction #9), 36µg/lane, on a 10% polyacrylamide gel, visualised by Coomassie blue staining, is shown in figure 5.2.

A transfer time of 60mins at 100V was optimal for transfer of proteins from the polyacrylamide gel onto nitrocellulose membrane.

5.3.3 Optimisation of protein detection

Once transfer of proteins onto nitrocellulose membrane had been completed, blocking solutions of 5% milk powder in PBST and 5% ECL blocking agent in PBST were found to be equally as effective at minimising non-specific background staining of proteins. However, a 5% solution of ECL blocking agent in PBST was used in all experiments included in this thesis due to the standardised formulation of a commercially available blocking-agent, minimising variation between experiments. Incubation of membranes in blocking solution overnight at 4^0C or 60mins at RT were equally as effective. An incubation period of 60mins at RT was selected due to its convenience.

Adequate washing of the membrane between incubation steps was critical to the success of detection of antibody binding with minimal non-specific background binding. PBST was the most efficient washing solution and washes of 1x15mins followed by 3x5mins between each step resulted in the best outcome.

Dilution of primary and secondary antibodies (from Jackson Immunoresearch) in PBST with incubation periods of overnight at 4^0C and 60mins at RT, respectively, gave the best results. Optimal dilutions of primary and secondary antibodies were found to vary according to the detection and imaging systems used. Generally, lower dilutions were required for chromogenic detection than for the more sensitive chemiluminescent detection system. Optimal dilutions also varied according to whether the

Figure 5.2 Typical separation of rat cerebellar proteins on a 10% polyacrylamide gel, visualised by Coomassie blue stain
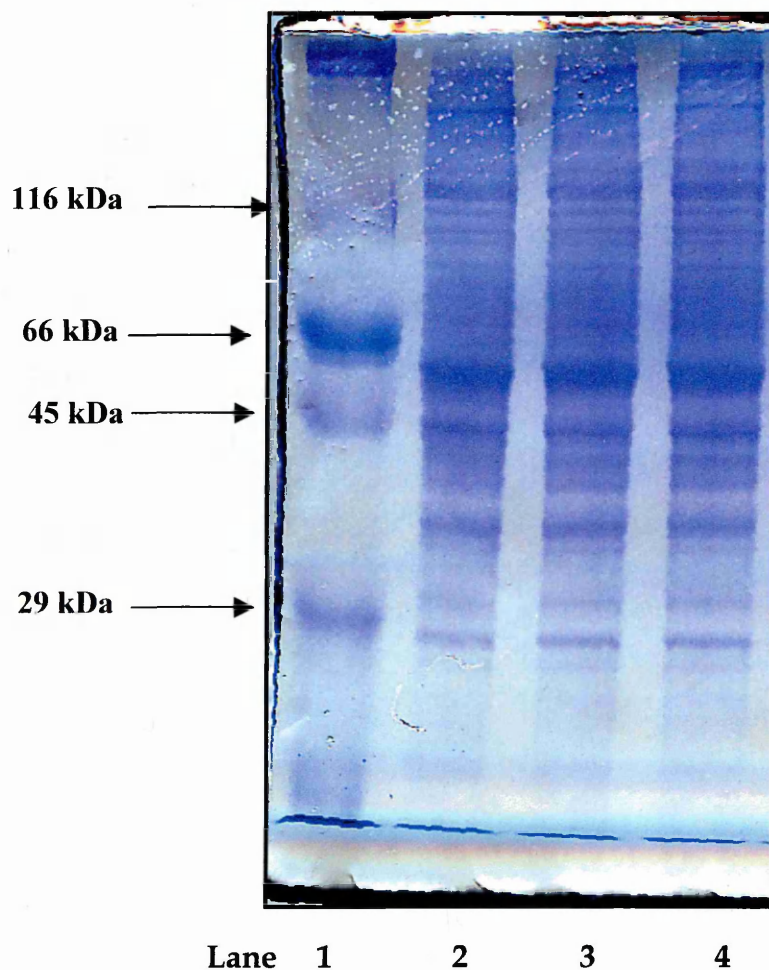
A 10% polyacrylamide gel was loaded as follows,

Lane 1   10μl coloured pre-stained RMM marker (Sigma, UK)

Lanes 2-4   10μl/lane (36μg protein) rat cerebellar protein extract (supernatant, extraction #9)

Proteins were separated by SDS-PAGE for 60mins at 150V. Following separation, proteins were visualised by Coomassie blue stain.

chemiluminescent signal was detected using manual development of autoradiography film or using the UVP imager (table 5.4).

Table 5.4 Optimal dilutions of primary and secondary antibodies for the detection of proteins using both chromogenic and chemiluminescent detection systems

ANTIBODY	DILUTION			
	CHROMOGENIC		CHEMILUMINESCENT	
	DAB	Opti-4CN	Manual Development	UVP imager
Anti-calbindin $D_{28}K$	ND	1:500	1:5000	1:1000
Anti-TG2	ND	ND	1:5000	1:1000
Anti-GAD	ND	ND	-	1:10 000
Anti-gliadin	-	-	1:10 000	1:1000
Patient sera	ND	ND	1:5000	1:1000
Secondary antibody (peroxidase-conjugated anti-mouse or anti-human IgG)	-	1:5000	1:50 000(mAb) 1:10 000(serum)	1:10 000

ND – Not detectable   mAb – monoclonal antibodies

Binding of monoclonal calbindin D28k was detected following exposure to opti-4CN chromogenic substrate for 5mins. Overall, chemiluminescent detection of monoclonal antibody binding produced superior results with the added advantage of the permanency of autoradiography film or direct computer-stored images. Binding of serum antibodies could not be detected using chromogenic detection systems, only by chemiluminescent detection. Chemiluminescent detection was best achieved using the ECL-plus™ reagent. Manual imaging of the signal was most successful when hyperfilm™ECL autoradiography film was used with a 15secs (serum) or 30secs (monoclonal antibodies) exposure time to the membrane. However, once available, the UVP imager was found to have greater ease of use and efficiency whilst producing comparable results.

Optimisation of the chromogenic detection of monoclonal calbindin $D_{28}K$ antibody is shown in figure 5.3 and the resultant optimal primary and secondary antibody dilutions for this system are summarised in table 5.4.

Figure 5.3 Determination of the optimal dilution of monoclonal calbindin D28k for detection of antibody binding to rat cerebellar extract using a chromogenic detection system
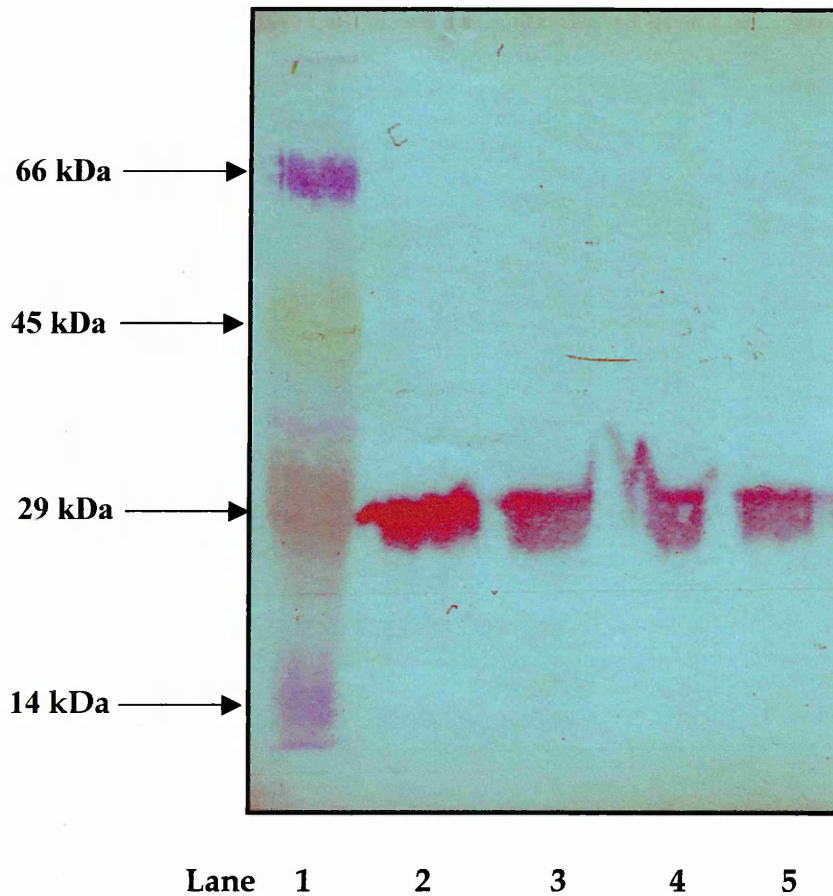
A 10% polyacrylamide gel was loaded as follows,

Lane 1   10μl coloured pre-stained molecular weight marker (Sigma, UK)

Lanes 2-5   10μl/lane (35μg protein) rat cerebellar protein extract (extraction #8)

Following separation and transfer, optimal detection of binding of monoclonal anti-calbindin D28k was established using the following antibody dilutions,

Lane	2	3	4	5
Primary antibody dilution	1:200	1:500	1:1000	1:2000

Horseradish-peroxidase conjugated anti-mouse IgG secondary antibody was used at a dilution of 1:5000. Visualisation was attained by exposure to Opti-4CN substrate for 5mins. The optimal anti-calbindin D28k and secondary antibody dilutions for this system were 1:500 and 1:5000, respectively.

The optimisation of chemiluminescent detection of monoclonal calbindin $D_{28}K$ antibody and serum antibody binding using the manual chemiluminescent imaging method is shown in figures 5.4 and 5.5 respectively. The resultant optimal dilutions of primary and secondary antibodies for the detection of these proteins are summarised in table 5.4.

5.3.4 Detection of known cerebellar proteins (calbindin and tissue transglutaminase)

Typical staining of the cerebellar proteins, calbindin and TG2, within a rat cerebellar extract on a western blot, using monoclonal antibodies, is shown in figure 5.6. Immunodetection of calbindin using a monoclonal anti-calbindin D28k antibody resulted in a single band of an average RMM of 26.4kDa (range 23-30kDa). Detection of TG2 using a monoclonal anti-TG2 antibody resulted in two visible bands, the upper having an average RMM of 69.6kDa (range 61- 79kDa) and the lower having an average RMM of 48.2kDa (range 44-57kDa).

5.3.5 Detection of gliadin proteins and investigation of the cross-reactive potential of commercial anti-gliadin antibody with cerebellar proteins

Probing of separated gliadin proteins on a western blot using a polyclonal anti-gliadin antibody resulted in the visualisation of two bands. The upper band was found to have an average RMM of 48.5kDa (weights 48 and 49kDa) and the lower band was found to have an average RMM of 39.5kDa (weights 41 and 38kDa) (figure 5.7a).

Probing of separated cerebellar proteins on a western blot using a polyclonal anti-gliadin antibody demonstrated the presence of several bands. Two bands appeared consistently with average RMMs of 43.2kDa (range 41-45kDa) and 32.8kDa (range 30-37kDa) (figure 5.7b).

Figure 5.4 Determination of the optimal secondary antibody dilution for detection of monoclonal calbindin D28k antibody binding to rat cerebellar extract using a chemiluminescent detection system
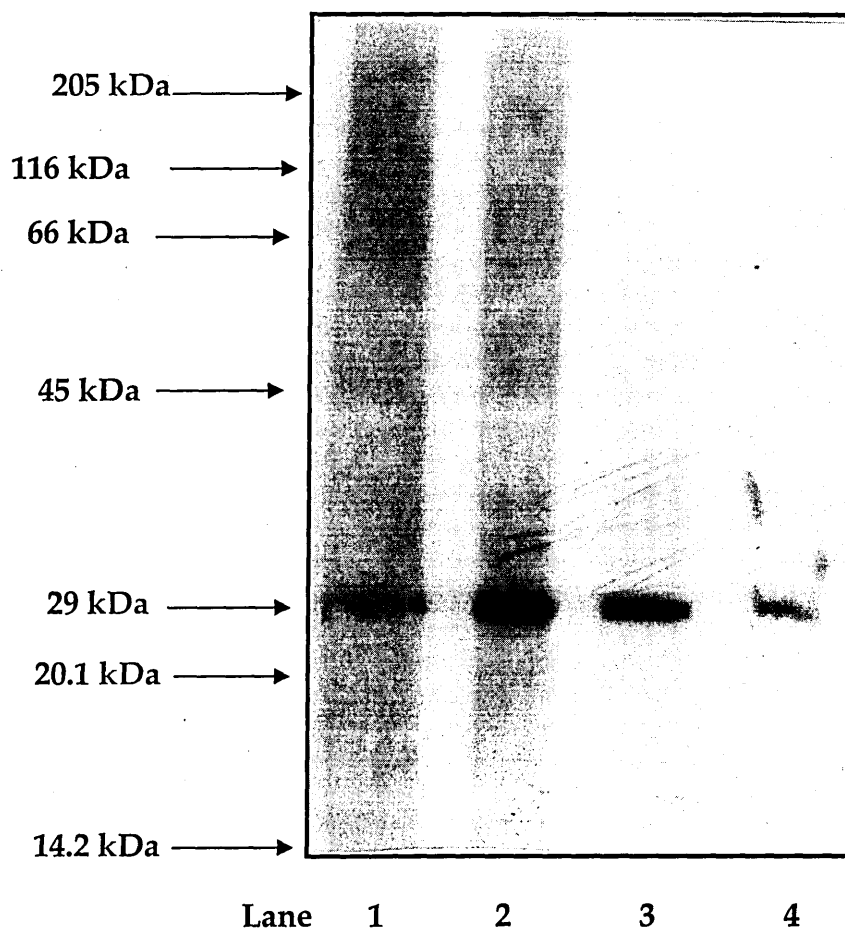
A 10% polyacrylamide gel was loaded as follows,

Lanes 1-4   10μl/lane (35μg protein) rat cerebellar protein extract (extraction #8)

Following separation and transfer, optimal detection of binding of monoclonal anti-calbindin D28k at a dilution of 1:5000 was established using horseradish-peroxidase conjugated anti-mouse IgG secondary antibody at the following dilutions,

Lane	1	2	3	4
Secondary antibody dilution	1:5000	1:10 000	1:50 000	1:100 000

Visualisation was attained by exposure to ECL substrate (Chemicon, USA) followed by exposure to autoradiography film for 30secs and manual development of film. The optimal anti-calbindin D28k and secondary antibody dilutions for this system were 1:5000 and 1:50 000, respectively.

A 10% polyacrylamide gel was loaded as follows,

All lanes   10µl/lane (35µg protein) rat cerebellar protein extract (extraction #8)

Following separation and transfer, optimal detection of binding of serum antibodies was established using serum and horseradish-peroxidase conjugated anti-human IgG secondary antibody at the following dilutions,

LANE	Primary antibody (serum) dilution	Secondary antibody dilution
1	1:100	1:5000
2	1:100	1:10 000
3	1:100	1:50 000
4	1:500	1:5000
5	1:500	1:10 000
6	1:500	1:50 000
7	1:1000	1:5000
8	1:1000	1:10 000
9	1:1000	1:50 000

Visualisation was attained by exposure to ECL substrate (Chemicon, USA) followed by exposure to autoradiography film for (a) 15secs, (b) 30secs or (c) 60secs and manual development of film.

Figure 5.5 Determination of the optimal conditions for detection of serum antibody binding to rat cerebellar extract using a chemiluminescent detection system
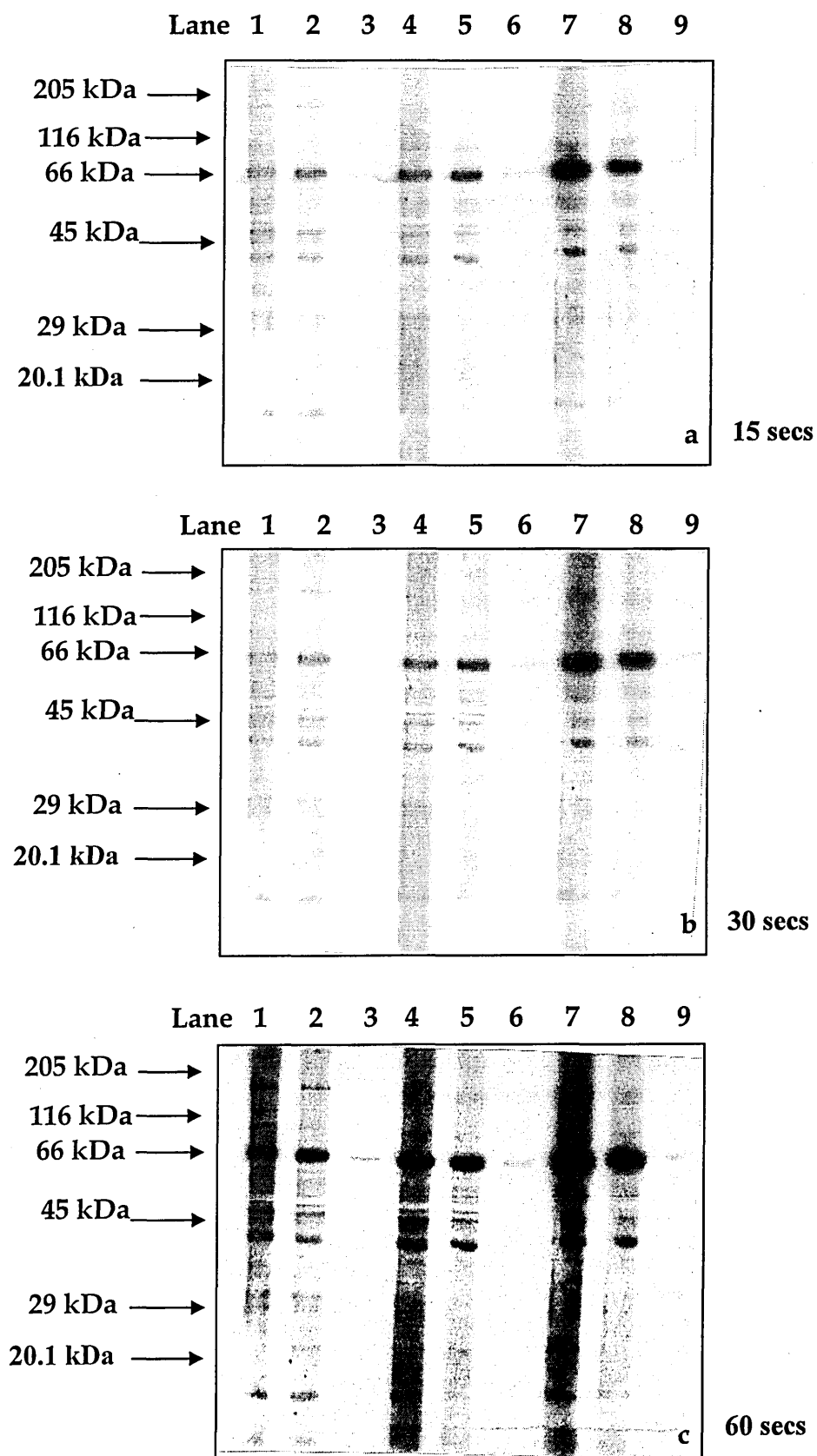
Figure 5.6 Typical binding to known cerebellar proteins (calbindin and TG2) using commercial monoclonal antibodies detected by use of a chemiluminescent detection system
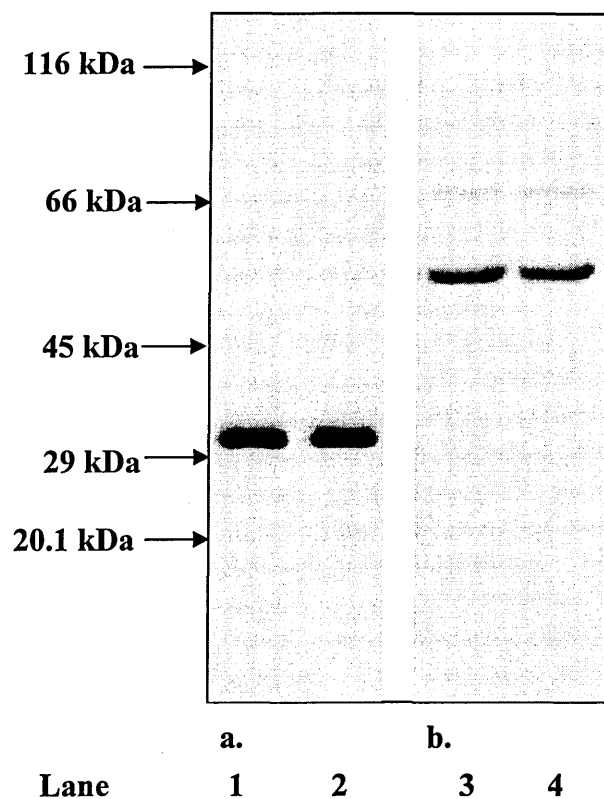
A 10% polyacrylamide gel was loaded as follows,

Lanes 1-4   10μl/lane (36μg protein) rat cerebellar protein extract (extraction #9)

Following separation and transfer, the blot was probed with (**a**). monoclonal anti-calbindin D28k antibody, 1:1000 (**b**). monoclonal anti-TG2 antibody, 1:1000. Binding was visualised using horseradish-peroxidase conjugated anti-mouse IgG secondary antibody at a dilution of 1:10 000. The blot was then incubated with ECL-plus™ reagent and imaged using a UVP imager.

Figure 5.7 Binding of a polyclonal anti-gliadin antibody to gliadin proteins and rat
cerebellar proteins detected using a chemiluminescent detection system
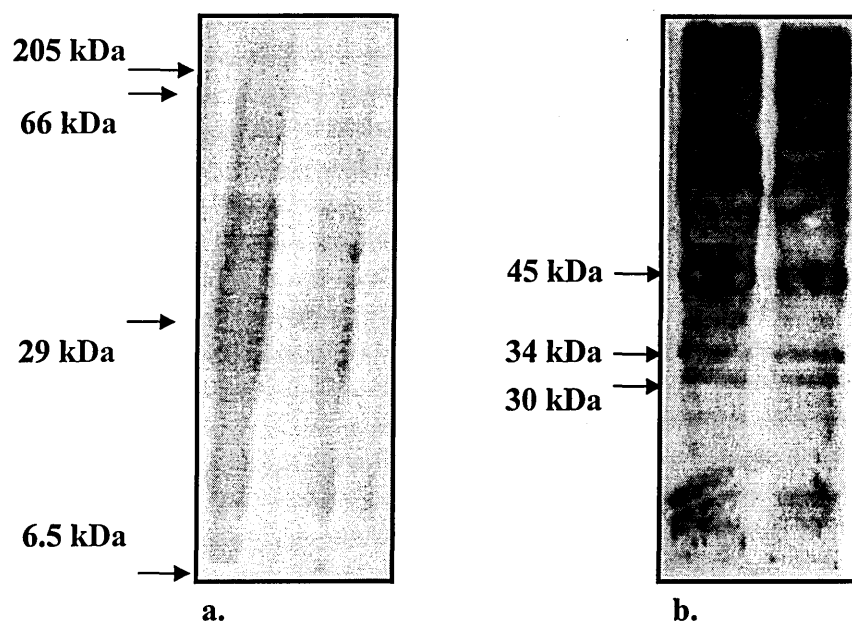
A 10% polyacrylamide gel was loaded with either **(a)**. 10μl/lane (25μg protein) crude

gliadin solution or **(b)**. 10μl/lane (36μg protein) rat cerebellar protein extract (extraction

#9).

Following separation and transfer, the blot was probed with horseradish-peroxidase

conjugated anti-gliadin antibody at a dilution of either **(a)**. 1:10 000 or **(b)**. 1:1000. The

blot was then incubated with either **(a)**. ECL substrate (Chemicon, USA) followed by

exposure to autoradiography film for 60secs and imaged by manual development of film

or **(b)**. ECL-plus™ reagent and imaged using a UVP imager.

5.3.6 Specificity of patient serum antibody binding to cerebellar proteins

Inter-experimental variation of the positive control (anti-GAD65/67 antibody) was 8%. The reactivity and RMM of protein bands for all sera tested are shown in figure 5.8(a-g). These results are summarised in table 5.5. Bands possibly due to binding of serum anti-gliadin or anti-TG2 antibodies, among antibody positive patients, are highlighted in table 5.5. Bands at 69.6 ± 8% kDa (64-75kDa) or 48.2 ± 8% kDa (44-52kDa) were considered to be possibly due to binding of anti-TG2 antibodies and bands at 43.2 ± 8% kDa (40-47kDa) or 32.8 ± 8% kDa (30-35kDa) were considered to be possibly due to binding of anti-gliadin antibodies. Using these criteria, overall, 65% of sera positive for anti-TG2 antibodies on ELISA also exhibited a band on a blot possibly due to binding of anti-TG2 antibodies. The corresponding value for anti-gliadin antibodies was 58%. However, 62% and 55% of sera negative for anti-gliadin and anti-TG2 antibodies respectively also exhibited bands at these RMMs.

Bands at wide ranges of RMMs were seen in sera from all groups. No band appeared specific to patients with gluten ataxia (table 5.5). However, bands within certain RMM ranges were observed more frequently in certain groups. For example, sera from patients with gluten ataxia and from patients with CD without neurological complications showed high RMM bands (100-101kDa) more frequently than other groups (21% and 20% respectively compared to no reactivity in other groups apart from 9% of healthy control sera). Bands with a RMM of 77 or 78kDa were observed in 2/14 (14%) sera from patients with gluten ataxia and 1 of 2 sera from patients with sporadic ataxia (non-gluten) but not with any other sera. RMM bands between 80-83kDa were observed in 3/14 (21%) sera from patients with gluten ataxia and in 2/7 (29%) sera from patients with familial ataxia but only 1/10 (10%) sera from patients with CD without neurological complications and 1/11 (9%) sera from healthy controls. Interestingly, RMM bands between 24-28kDa were seen in 6/14 (43%) sera from patients with gluten ataxia and 1 of 2 sera from patients with sporadic ataxia (non-gluten). These bands were also seen with sera from other groups but not at a frequency of more than 18%. Similarly, RMM bands between 56-59kDa were seen in 7/14 (50%) sera from patients with gluten ataxia and 1 of 2 sera from patients with sporadic ataxia (non-gluten) but at a frequency of not more than 36% in sera from other groups. In addition, bands between 56-59kDa were more likely to be strong in the gluten ataxia group (57%) than in other groups.

Figure 5.8 Binding of antibodies within patient sera samples to rat cerebellar proteins, separated by SDS-PAGE and western blotting, detected using chemiluminescence

A 10% polyacrylamide gel was loaded with 10μl/lane (36μg protein) rat cerebellar protein extract (extraction #9). Following separation, the blot was cut into strips (1 lane/strip) and each strip was probed with patient sera at a dilution of 1:1000. A total of, 14 sera from patients with gluten ataxia, 10 sera from patients with CD without neurological complications, 7 sera from patients with familial ataxia, 2 sera from patients with sporadic ataxia (non-gluten), 1 serum from a patient with stiff-person syndrome and 11 sera from healthy control patients were tested. One strip from each blot was probed with anti-GAD65/67 antibody (1:10 000 dilution) as a positive control. Binding was visualised by incubation with horseradish-peroxidase conjugated-anti human IgG (1:10 000 dilution) and development in ECL-plus™ substrate. Finally, blots were imaged using a UVP imager.

GA –gluten ataxia (patient number corresponding to table 2.1 is shown in brackets), FA – familial ataxia, SA – sporadic ataxia (non-gluten), CD – CD without neurological complications, SPS – stiff-person syndrome, HC – healthy control. RMMs of visible bands were calculated as detailed in section 2.4.7. Weights of intense bands are in bold. The serological antibody status of anti-gliadin (AGA) and anti-tissue transglutaminase (TG2) antibodies for each patient is provided for reference. Previous experiments indicated expected bands for AGA at approximately 33 and 43kDa and for anti-TG2 antibodies at approximately 48 and 70kDa. The inter-experimental variation of the positive control (anti-GAD65/67 antibody) was 8%. Therefore, bands at 69.6 ± 8% kDa (64-75kDa) or 48.2 ± 8% kDa (44-52kDa) were considered to be possibly due to binding of anti-TG2 antibodies and bands at 43.2 ± 8% kDa (40-47kDa) or 32.8 ± 8% kDa (30-35kDa) were considered to be possibly due to binding of anti-gliadin antibodies.

NA – not applicable

Figure 5.8a Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
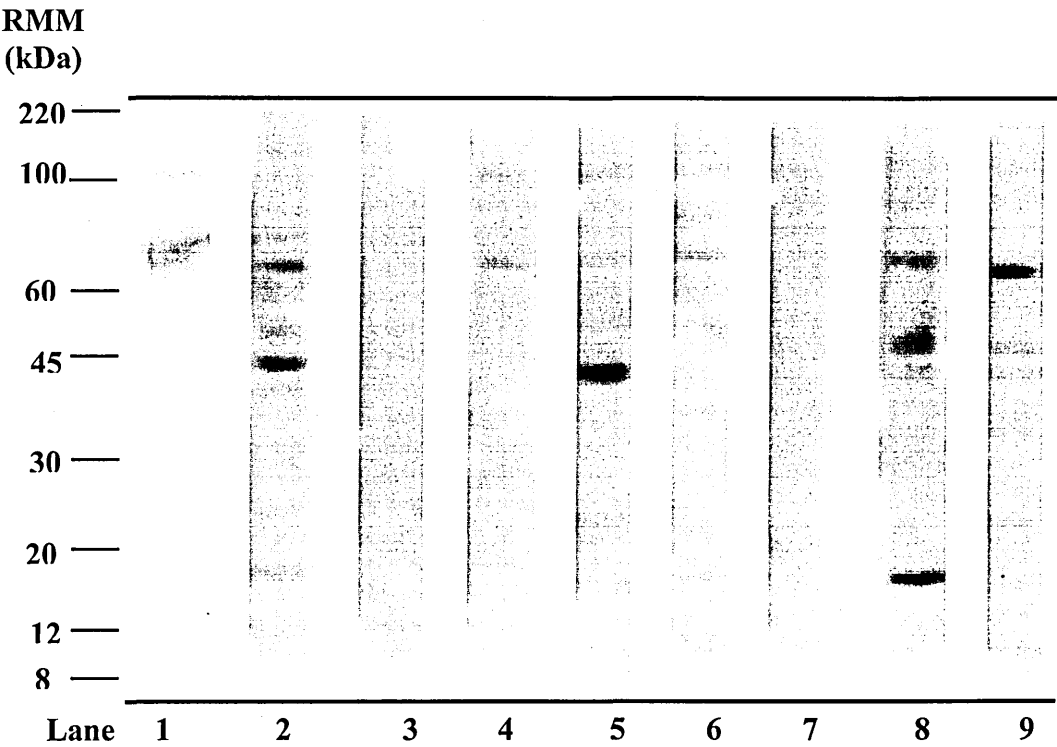
LANE	1	2	3	4	5	6	7	8	9
GROUP (patient number)	GAD + control	GA (15)	GA (30)	GA (25)	GA (27)	CD	CD	SPS	HC
AGA	NA	+	+	-	+	+	+	NA	-
TG2	NA	+	-	.-	-	+	+	NA	-
MAJOR VISIBLE BANDS (kDa)	68	65 44	-	65	43	83 70	-	65 48 17	63 48

Figure 5.8b Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
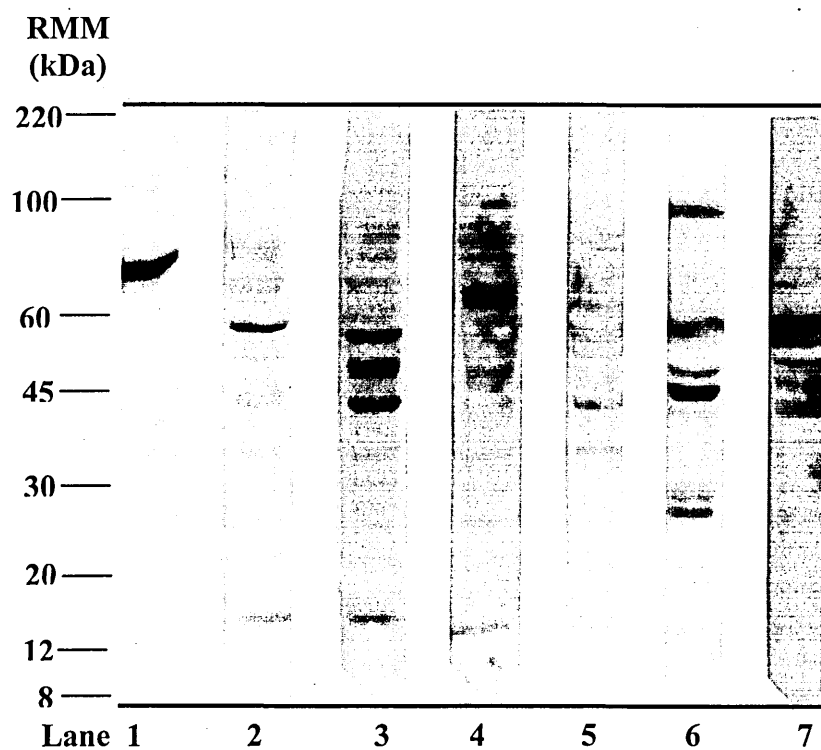
LANE	1	2	3	4	5	6	7
GROUP (patient number)	GAD + control	CD	CD	FA	FA	GA (24)	HC
AGA	NA	-	+	-	-	+	-
TG2	NA	+	+	-	-	+	-
MAJOR VISIBLE BANDS (kDa)	71	55	54 48 41	62	41	93 58 48 32 28 26	58 49 41
		15	15	14			

Figure 5.8c Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
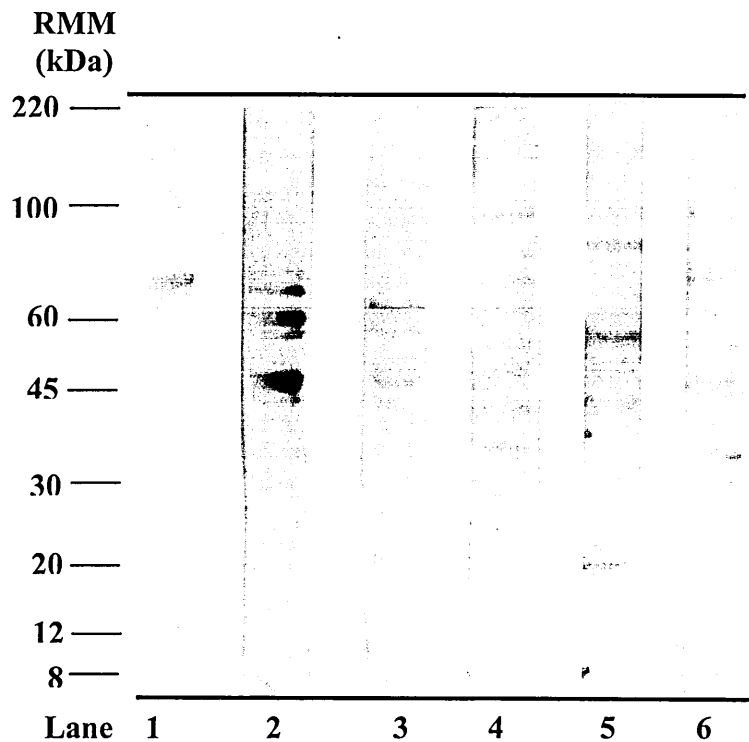
LANE	1	2	3	4	5	6
GROUP (patient number)	GAD + control	CD	FA	FA	GA (28)	HC
AGA	NA	+	-	-	+	-
TG2	NA	+	+	-	+	-
MAJOR VISIBLE BANDS (kDa)	70	67 59 56 46	-	-	56	-

Figure 5.8d Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
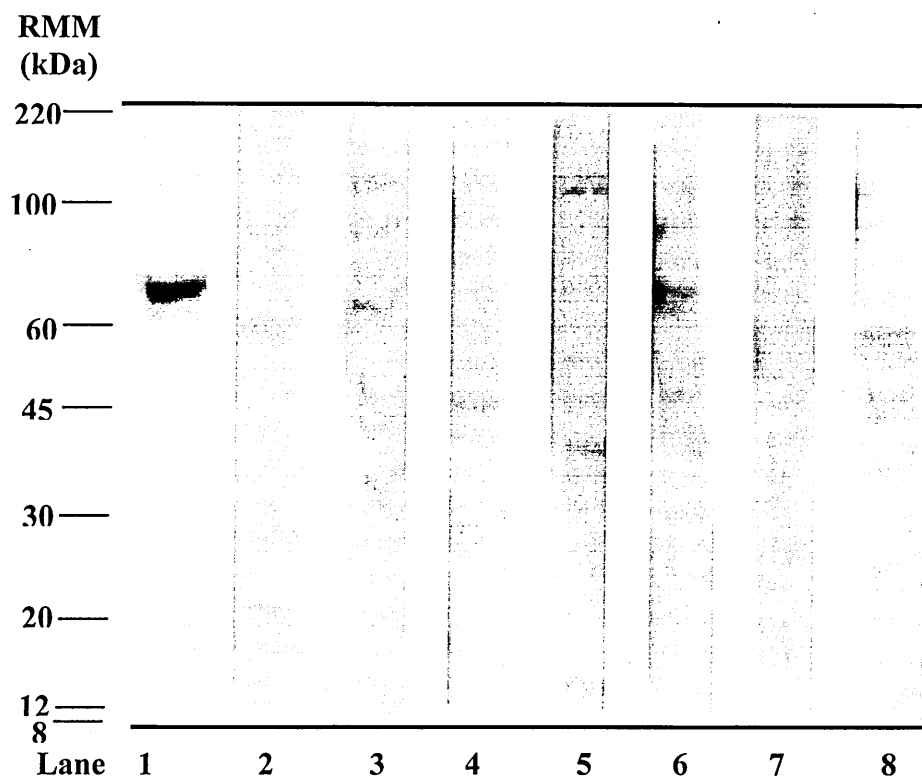
LANE	1	2	3	4	5	6	7	8
GROUP (patient number)	GAD + control	HC	HC	CD	CD	GA (23)	GA (29)	SA
AGA	NA	-	-	-	+	+	+	-
TG2	NA	-	-	+	+	+	-	+
MAJOR VISIBLE BANDS (kDa)	69	-	-	-	39	67	-	-

Figure 5.8e Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
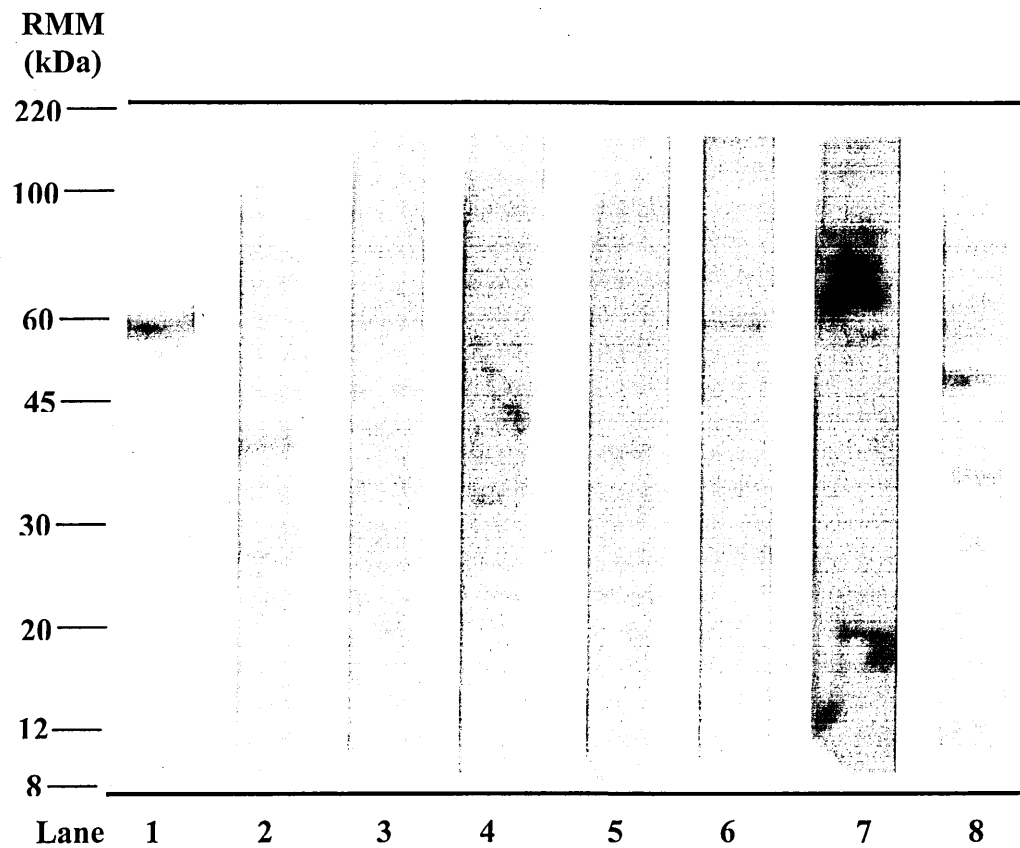
LANE	1	2	3	4	5	6	7	8
GROUP (patient number)	GAD + control	HC	HC	CD	CD	GA (13)	GA (21)	GA (11)
AGA	NA	-	-	+	+	+	+	+
TG2	NA	+	+	+	+	-	+	+
VISIBLE BANDS (kDa)	58	-	-	-	-	58	-	50

Figure 5.8f Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
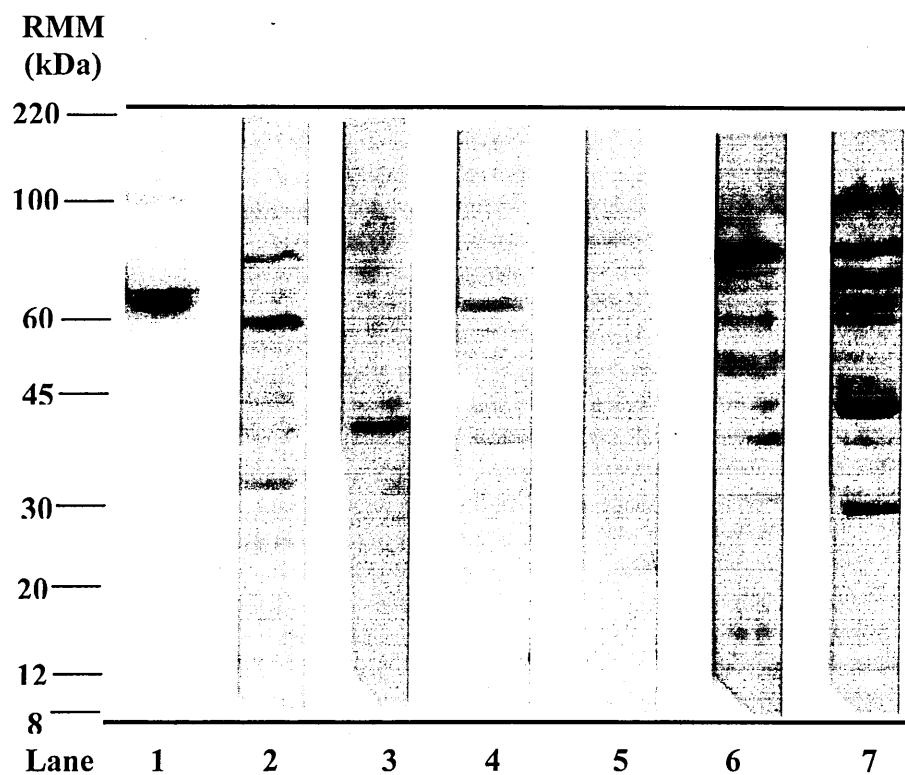
LANE	1	2	3	4	5	6	7
GROUP (patient number)	GAD + control	HC	HC	FA	FA	GA (16)	GA (22)
AGA	NA	-	-	-	-	-	-
TG2	NA	-	-	-	-	-	+
VISIBLE BANDS (kDa)	62	76 58 43 38 30	39	62	-	78 51	80 70 65 59 43 28

Figure 5.8g Binding of antibodies within patient serum samples to rat cerebellar proteins, detected using chemiluminescence
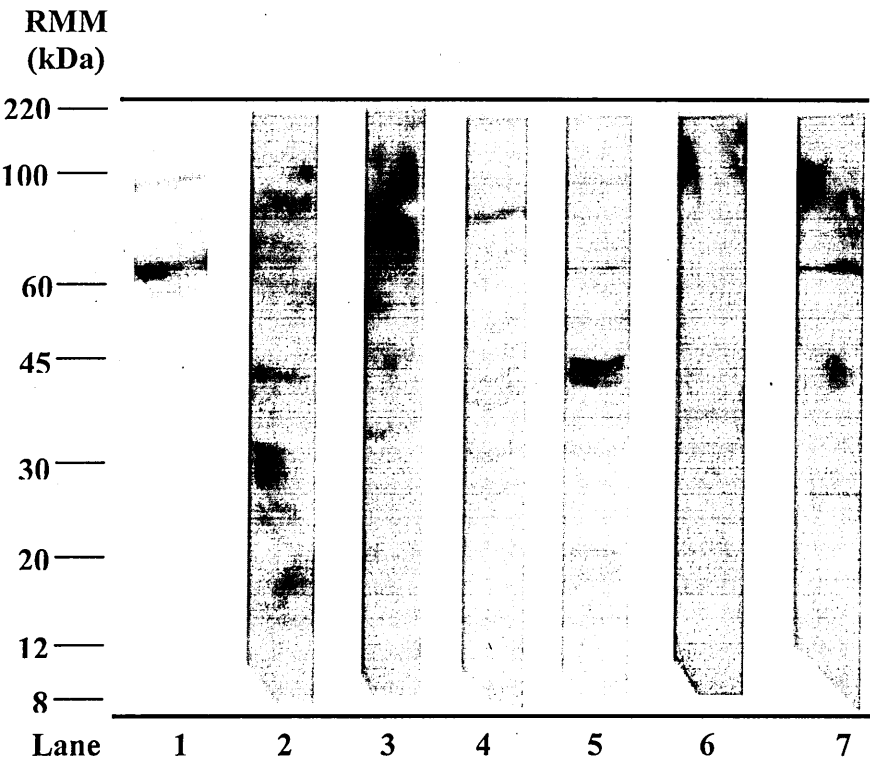
LANE	1	2	3	4	5	6	7
GROUP (patient number)	GAD + control	HC	HC	SA	FA	CD	GA (32)
AGA	NA	-	-	-	-	+	+
TG2	NA	+	-	+	-	+	-
VISIBLE BANDS (kDa)	61	-	-	77	63 43	-	63

Table 5.5 Summary of reactivity of sera from patients with gluten ataxia, coeliac disease only, familial ataxia, sporadic ataxia (non-gluten), stiff-person syndrome and healthy controls with rat cerebellar proteins separated on a western blot, detected using chemiluminescence

Molecular weight (kDa) → / PATIENT (no.)	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-99	100-109
GA (15)	-	-	-	-	44	-	65	-	-	-	-
GA (30)	-	-	-	-	-	-	-	-	-	-	-
GA (25)	-	-	-	-	43	-	65	-	-	-	-
GA (27)	-	-	-	-	-	-	-	-	-	-	-
GA (24)	-	-	26 28	32	48	58	-	-	-	93	-
GA (28)	-	-	-	-	-	56	-	-	-	-	-
GA (23)	-	-	-	-	-	-	67	-	-	-	-
GA (29)	-	-	-	-	-	-	-	-	-	-	-
GA (13)	-	-	-	-	-	58	-	-	-	-	-
GA (21)	-	-	-	-	-	-	-	-	-	-	-
GA (11)	-	-	-	-	-	50	-	-	-	-	-
GA (16)	-	-	-	-	-	51	-	78	-	-	-
GA (22)	-	-	28	-	43	59	65	70	80	-	-
GA (32)	-	-	-	-	-	-	63	-	-	-	-
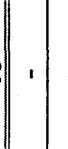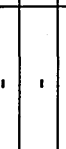
GA – gluten ataxia (patient number corresponding to table 2.1 shown in brackets). Major bands are shown in bold. Bands likely to be due to anti-TG2 ▦ (64-75kDa and 44-52kDa) or anti-gliadin ▦ (40-47kDa and 30-35kDa) antibodies within antibody positive serum are highlighted (molecular weight within 8% of known protein weight). Where RMM overlap occurs, cells are shaded with both relevant patterns.

Table 5.5 cont'd Summary of reactivity of sera from patients with gluten ataxia, coeliac disease only, familial ataxia, sporadic ataxia (non-gluten), stiff-person syndrome and healthy controls with rat cerebellar proteins separated on a western blot, detected using chemiluminescence

Molecular weight (kDa) → PATIENT	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-99	100-109
SA	-	-	-	-	-	-	-	-	-	-	-
SA	-	-	-	-	-	-	-	77	-	-	-
SPS	-	17	-	-	48	-	65	-	-	-	-
FA	-	14	-	-	-	-	62	-	-	-	-
FA	-	-	-	-	41	-	-	-	-	-	-
FA	-	-	-	-	-	-	-	-	-	-	-
FA	-	-	-	-	-	-	-	-	-	-	-
FA	-	-	-	-	-	-	62	-	-	-	-
FA	-	-	-	-	-	-	-	-	-	-	-
FA	-	-	-	-	43	-	63	-	-	-	-
CD	-	-	-	-	-	-	-	70	83	-	-
CD	-	-	-	-	-	-	-	-	-	-	-
CD	-	15	-	-	-	55	-	-	-	-	-

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SA – sporadic ataxia (non-gluten), SPS- stiff-person syndrome, FA – familial ataxia, CD- CD without neurological complications. Intense bands are shown in bold. Bands likely to be due to binding of anti-TG2 ▨ (64-75kDa and 44-52kDa) or anti-gliadin ▨ (40-47kDa and 30-35kDa) antibodies within antibody positive serum are highlighted (molecular weight within 8% of known protein weight). Where RMM overlap occurs, cells are shaded with both relevant patterns.

Table 5.5 cont'd Summary of reactivity of sera from patients with gluten ataxia, coeliac disease only, familial ataxia, sporadic ataxia (non-gluten), stiff-person syndrome and healthy controls with rat cerebellar proteins separated on a western blot, detected using chemiluminescence

Molecular weight (kDa) → / PATIENT	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-99	100-109
CD	-	15	-	-	41 [48]	54	-	-	-	-	-
CD	-	-	-	-	46	56 59	67	-	-	-	-
CD	-	-	-	-	-	-	-	-	-	-	-
CD	-	-	-	-	-	-	-	-	-	-	-
CD	-	-	-	-	-	-	-	-	-	-	-
CD	-	-	-	-	-	-	-	-	-	-	-
CD	-	-	-	-	-	-	-	-	-	-	-
HC	-	-	-	-	48	-	63	-	-	-	-
HC	-	-	-	-	41 49	58	-	-	-	-	-
HC	-	-	-	-	-	-	-	-	-	-	-
HC	-	-	-	-	-	-	-	-	-	-	-
HC	-	-	-	-	-	-	-	-	-	-	-
HC	-	-	-	-	-	-	-	-	-	-	-

CD- CD without neurological complications, HC – healthy control. Intense bands are shown in bold. Bands likely to be due to binding of anti-TG2 ▦ (64-75kDa and 44-52kDa) or anti-gliadin ▦ (40-47kDa and 30-35kDa) antibodies within antibody positive serum are highlighted (molecular weight within 8% of known protein weight). Where RMM overlap occurs, cells are shaded with both relevant patterns.

Table 5.5 cont'd Summary of reactivity of sera from patients with gluten ataxia, coeliac disease only, familial ataxia, sporadic ataxia (non-gluten), stiff person syndrome and healthy controls with rat cerebellar proteins separated on a western blot, detected using chemiluminescence

Molecular weight (kDa) → PATIENT	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90-99	100-109
HC			20 28	34	41 41 47	58					
HC		12 16 18	23	**30 38**	**43**	**58**		76		92	
HC				39							
HC					42				82		
HC				33							

HC – healthy control. Intense bands are shown in bold. Bands likely to be due to binding anti-TG2 ▦ (64-75kDa and 44-52kDa) or anti-gliadin ▦ (40-47kDa and 30-35kDa) antibodies within antibody positive serum are highlighted (molecular weight within 8% of known protein weight). Where RMM overlap occurs, cells are shaded with both relevant patterns.

5.4 Discussion

The antigen to which the circulating anti-Purkinje cell antibodies associated with gluten ataxia are directed remains unknown. Screening of sera using cerebellar proteins on a Western blot failed to identify a specific target protein associated with gluten ataxia. In sera from all groups multiple immunoreactive bands were observed. The failure to identify the autoantigen associated with gluten ataxia using western blotting may be due to sub-optimal extraction of the protein or because it is present in too low amounts in the whole cerebellar extract used to be adequately detected. Alternatively, denaturation of the protein prior to electrophoresis may prohibit recognition by the autoantibodies. However, bands with a RMM of 56-59kDa were observed more frequently when sera from patients with gluten ataxia was used (50% samples) than sera from other groups (22% ataxia control, 20% CD only, 36% healthy control samples). In the same way, 43% sera from patients with gluten ataxia exhibited a band with a RMM of 24-28kDa compared to 22% ataxia control, 10% CD only and 18% healthy control samples. It is possible that the proteins represented by these bands are important in the pathogenesis of the condition and possible candidate proteins within the cerebellum remain to be identified. Bands at a RMM of 77-78kDa were only seen in patients with gluten ataxia or sporadic ataxia (non-gluten) (19% of total samples). Bands at a RMM of 80-83kDa were seen in a total of 24% of samples from patients with gluten ataxia or familial ataxia but only 9% and 10% of healthy control and CD only samples respectively. Bands with higher RMMs of 100-101kDa were seen in a total of 21% of samples from gluten sensitive individuals (gluten ataxia and CD only) but in no samples from other groups apart from one healthy control sample.

Subsequent western blot analysis to assess and/or confirm the possible contribution of binding of anti-gliadin and anti-TG2 antibodies to cerebellar staining by sera was limited by the high numbers of antibody negative sera also exhibiting bands in the expected RMM range. This analysis is also of limited use due to the inability to positively identify the immunoreactive protein without sequencing. However, 58% and 65% of all anti-gliadin and anti-TG2 antibody positive samples, respectively, showed corresponding bands on a western blot, which may possibly may be attributable to the binding of these antibodies. Comparison of bands before and after pre-adsorption of antibodies with the appropriate protein will help to clarify the relative involvement of these antibodies to staining.

Finally, the cross-reactivity of commercial anti-gliadin antibody with cerebellar proteins was confirmed by western blotting. Commercial anti-gliadin antibody bound to two cerebellar proteins with RMMs of approximately 33 and 43kDa, which remain unidentified. It is likely, although not certain, that these are the Purkinje cell proteins stained in the IHC experiments. Interestingly calreticulin, the protein with which anti-gliadin antibodies have been shown to cross-react with, is reported to be present in cerebellar Purkinje cells (Perrin *et al.,* 1991). Autoantibodies to calreticulin have also been reported in a proportion of patients with other autoimmune diseases, such as SLE (Sanchez *et al.,* 2000). However, the protein has an apparent RMM on western blot of ~60kDa, which is not consistent with the RMMs of the bands detected by commercial anti-gliadin antibody but may account for some of the bands close to this RMM produced by sera from anti-gliadin antibody positive patients (Sanchez *et al.,* 2000; Tuckova *et al.,* 1997). Reactivity to proteins of RMMs of ~33 and 43kDa was not apparent in all anti-gliadin antibody positive sera from patients with gluten ataxia or CD only, which may suggest that commercial and serum anti-gliadin antibodies have varying specificities.

In summary, the main findings of this study were that sera, from all groups, were found to react with numerous cerebellar proteins, detected by western blotting. None were specific to patients with gluten ataxia. Bands with a RMM of between 24-28kDa and 56-59kDa were observed more frequently when sera from patients with gluten ataxia was used than sera from other groups. The proteins represented by these bands may be associated with gluten ataxia. 58% and 65% of anti-gliadin and anti-TG2 antibody positive samples respectively showed corresponding bands on a western blot that possibly may be attributable to the binding of these antibodies although analysis of this type has limitations. Finally, the cross-reactivity of commercial anti-gliadin antibodies with cerebellar proteins was confirmed. The antibody bound to proteins with RMMs of ~ 43kDa and 33kDa.

CHAPTER SIX

INVESTIGATION OF A POSSIBLE RELATIONSHIP BETWEEN GLUTEN ATAXIA AND CIRCULATING ANTI-GLUTAMIC ACID DECARBOXYLASE ANTIBODIES

6.1 Introduction

6.1.1 Glutamic acid decarboxylase

GAD is an intracellular enzyme involved in the synthesis of the inhibitory neurotransmitter GABA (figure 6.1).

Figure 6.1 Synthesis of γ-aminobutyric acid from glutamic acid
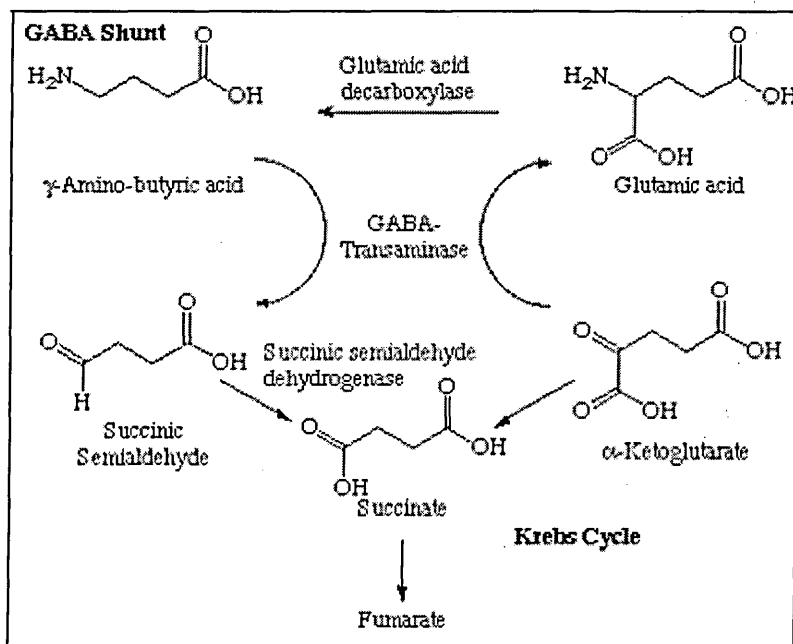
GAD catalyses the removal of the carboxyl group adjacent to the α-amino group from glutamic acid to produce GABA and carbon dioxide. GABA is broken down by GABA-transaminase to form succinic semialdehyde.

Taken from www.neurosci.pharm.utoledo.edu/MBC3320/GABA.htm

Like GABA, GAD is ubiquitously present in a variety of cells and tissues as well as plants and various unicellular organisms. In particular, GABA and GAD are found in both the peripheral and central (especially hippocampal, striatal and cerebellar neurones) nervous systems and the pancreas (islet cells). GABA is the neurotransmitter used by cerebellar Purkinje cells and is also present in the enteric nervous system.

GAD exists as two isoforms, GAD_{65} with a RMM of 65kDa which is encoded by the GAD2 gene on chromosome 10 and GAD_{67} with a RMM of 67kDa encoded by the

GAD1 gene on chromosome 2. However, there is evidence for a common ancestral gene and the two isoforms have an overall 65% homology at the amino acid level (Lernmark, 1996). The GAD isoforms are extremely well conserved between species, for example porcine GAD_{65} and GAD_{67} are 96% and 95% identical to human and rat isoforms respectively (Suzuki et al, 1995). Evidence suggests that constitutive levels of GABA are provided by GAD_{67} and GAD_{65} provides reserve levels according to localised demand (Ji et al., 1999). Both isoforms are usually found together in tissues although some tissue-specific differences in the relative amounts of each isoform are apparent (table 6.1).

Cellular distribution also differs between isoforms. For example within neurones, GAD_{67} is distributed throughout the neurone whilst GAD_{65} is primarily found in nerve terminals (Soghomonian & Martin, 1998). GABA is released from nerve terminals via synaptic vesicles. GAD may be associated with these vesicles, where it plays a role in the accumulation of GABA into the vesicle, or present in a soluble cytosolic pool. The relative ratio of GAD_{65} to GAD_{67} associated with vesicles varies between cells, depending on the overall expression of the two isoforms. Overall, the amount of vesicle-bound GAD_{65} is higher than the amount of vesicle-bound GAD_{67}. It has been shown that only GAD_{65} contains the correct information for vesicular binding, which is associated with amino acids 24-31 within the N-terminal although the protein(s) to which it binds are still unknown (Lernmark, 1996; Soghomonian & Martin, 1998).

GAD_{67} tends to be at greater concentrations within the soluble pool although it may also be vesicle-bound by a heterodimeric interaction with GAD_{65} (Soghomonian & Martin, 1998).

GAD exists either as an active holoenzyme containing bound co-factor (pyridoxal 5'-phosphate) or as an inactive apoenzyme lacking the co-factor. GAD_{67}, responsible for constitutive GABA levels, tends to be found as the holo-GAD form whilst GAD_{65}, responsible for reserve GABA levels, tends to be found as apo-GAD. However, little is known about the regulation of levels of apo- and holo-GAD and its importance in maintaining GABA levels (Lernmark, 1996).

Table 6.1 Human glutamic acid decarboxylase isoforms

	GAD_{65}	GAD_{67}
Gene	GAD2	GAD1
Chromosome	10	2
mRNA (kb)	5.6	3.7
Protein (RMM x 10^3)	65	67
Brain	++	+
Testis	+	±
Islets	+ +	±
Human beta cells	++	-
Human non beta cells	±	+

Taken from Lernmark, 1996

6.1.2 Glutamic acid decarboxylase and disease

GAD_{65} is a shared autoantigen in IDDM, SPS and polyendocrine autoimmune syndrome. The responses, involving both humoral and cellular mechanisms, are mainly directed against GAD_{65}, but to a lesser extent, also GAD_{67}. This may be due to the higher tissue concentration of GAD_{65}, but more likely is the fact that immunological tolerance to GAD_{67} is established due to the early expression of the GAD_{67} gene, before the GAD_{65} gene in development (Lernmark, 1996). The trigger for autoimmunity to GAD is not fully known. It has been speculated that the sequence similarity of GAD to various viruses, particularly the Coxsackie B4 virus (Kaufman *et al*, 1992) which allows an immune response to be mounted against GAD following infection due to molecular mimicry. However, this notion is not consistent (Cainelli *et al*, 2000; Lernmark, 1996) and it seems likely that a combination of genetic and environmental factors are necessary for overt autoimmunity to GAD and the development of diseases such as IDDM, SPS and autoimmune polyendocrine syndrome. In addition, the nature of the immune response to GAD seems to define, to some degree, which clinical manifestation will develop.

All of these diseases are associated with the expression of certain HLA class II molecules: primarily HLA DQ2, HLA DQ8 and HLA DR3 and DR4 (Abraham *et al*, 2001), thus sharing the same genetic susceptibility as gluten sensitive individuals. Spontaneous T-cell reactivity to GAD has been shown in mice expressing these genes and, interestingly, expression of DQ6 or DR2 appeared to confer resistance or neutrality (Abraham *et al*, 2001). However, the presence of susceptible HLA genes alone does not induce IDDM in mice (Abraham *et al*, 2001), supporting the theory that, as in the case of gluten sensitivity, multiple genetic and environmental factors promote development of these diseases. Finally, 1-2% of healthy individuals have circulating anti-GAD_{65} antibodies (Hao *et al*, 1999) yet only 10-20% of these will go on to develop IDDM (Lernmark, 1996) suggesting that differences in the type of immune response may also influence disease development.

6.1.2.1 Insulin dependent diabetes mellitus

IDDM is an autoimmune disease associated with an immune response against various pancreatic antigens and consequent destruction of insulin-producing beta-cells necessitating regular administration of synthetic insulin. GAD_{65} has been shown to be a major autoantigen in the disease and approximately 60-80% of newly diagnosed sufferers have circulating GAD_{65} antibodies (Rharbaoui *et al*, 1998; Schloot *et al*, 1999). Anti-GAD_{67} antibodies, when present, are exclusively associated with the presence of anti-GAD_{65} antibodies and competitively bind to epitopes on GAD_{65} (Lernmark, 1996). Anti-GAD antibodies tend to be present for long periods of time before clinical onset of the disease and anti-GAD antibody assays have proved useful in identifying at-risk patients.

IDDM associated anti-GAD_{65} antibodies recognise several conformational epitopes located within the middle and C-terminus portions of the GAD_{65} protein (Lernmark, 1996; Powers *et al*, 1999; Rharbaoui *et al*, 1998; Schloot *et al*, 1999). The immune response to GAD in IDDM is of a Th1 type, mediated primarily by CD4$^+$ T-cells (Dalakas & Floeter, 1999; Lernmark, 1996), although a mixed Th1/Th2 response has been reported in mice (Abraham *et al*, 2001). Petersen and co-workers (1999) suggested that progression to clinical onset of IDDM is associated with a decrease in Th2 response to GAD and that dominance of a Th2 response is associated with non-development of IDDM, possibly explaining why many anti-GAD_{65} positive people do

not develop IDDM. Functionally, anti-GAD$_{65}$ antibodies associated with IDDM do not inhibit the enzymatic activity of GAD$_{65}$ (Powers *et al*, 1999).

6.1.2.2 Stiff-person syndrome

SPS is a rare neurological disorder characterised by progressive muscle rigidity and stiffness together with episodic spasms primarily involving axial and limb muscles. The disease is thought to have an autoimmune pathogenesis and also has an association with expression of certain HLA class II molecules. GAD, is a major autoantigen and approximately 60% of patients have circulating anti-GAD$_{65}$ antibodies. Interestingly, anti-GAD$_{65}$ antibody titres tend to be 10-100 fold higher than in IDDM patients (Lernmark, 1996; Schloot *et al*, 1999). This may be due to the relatively late-onset of SPS, which allows titres to gradually increase prior to clinical manifestation. Alternatively, in contrast to IDDM, SPS is associated with a Th2 type immune response (Dalakas & Floeter, 1999; Lernmark, 1996; Schloot *et al*, 1999), which may also explain the higher titres. Additionally, several differences exist between IDDM-associated and SPS associated anti-GAD$_{65}$ antibodies themselves. SPS associated antibodies also target conformational epitopes located in the C-terminus and middle sections but additionally target a linear epitope located in the N-terminus of the protein and are also able to inhibit the enzymatic activity of GAD$_{65}$ (Lernmark, 1996; Powers *et al*, 1999; Schloot *et al*, 1999). Approximately 30% of SPS patients also have IDDM (Schloot *et al*, 1999). This may be due to the fact that IDDM requires a dominant Th1 response for clinical onset or due to the difference in target epitopes. Powers and co-workers (1999) observed that SPS patients who subsequently developed IDDM were more likely to express HLA DQ2-DR3 markers leading to the hypothesis that subtle differences in HLA class II molecules may differentiate between disease manifestation.

6.1.2.3 Autoimmune polyendocrine syndrome

Autoimmune polyendocrine syndrome patients also tend to have higher titres of anti-GAD$_{65}$ antibodies than are found in IDDM patients (Klemetti *et al*, 2000). Autoimmune polyendocrine syndrome is also associated with a Th2 dominant immune response and the antibodies also inhibit GAD enzymatic activity. In common with IDDM-associated anti-GAD$_{65}$ antibodies, epitopes within the middle and C-terminus (different to that recognised in IDDM) regions are targeted (Powers *et al.*, 1999). Klemetti and co-workers (2000) found that 12% of patients also had IDDM but that anti-GAD antibodies were present in 41% of patients without clinical IDDM.

Interestingly, the authors also found a dissociation of cellular and humoral immune responses. Increased antibody titres and T-cell proliferative responses to GAD rarely occurred within the same patient. This provides further evidence for differences in the immune response, which may influence manifestation of the different diseases.

6.1.2.4 Ataxia

The presence of anti-GAD antibodies in association with sporadic idiopathic ataxia was first reported in 1988 (Solimena *et al.*, 1988). This has since been confirmed by a number of case-reports and a larger study of 14 patients with anti-GAD antibodies and ataxia (Honnorat *et al.*, 2001). It has been suggested that the mechanism of ataxia associated with anti-GAD antibodies is also immune-mediated. There is convincing *in vitro* evidence to suggest that anti-GAD antibodies, present in the CSF of such patients with ataxia, interfere with inhibitory GABAergic synaptic transmission in the cerebellum by acting presynaptically to decrease GABA synthesis and release (Dinkel *et al.*, 1998; Ishida *et al*, 1999; Mitoma *et al.*, 2000, 2003; Takenoshita *et al.*, 2001).

6.1.3 Aims of this study

Conditions associated with anti-GAD antibodies share the same genetic susceptibility as patients with gluten sensitivity. Humoral reactivity to GAD has also been reported in patients with sporadic ataxia. Furthermore, it was noted that 4/5 SPS patients attending the neurology clinic in Sheffield had circulating anti-gliadin antibodies and expressed the HLA DQ2 allele. This prompted the investigation of whether, conversely, patients with gluten ataxia, with circulating anti-gliadin antibodies, may also have raised levels of circulating anti-GAD antibodies.

Therefore, the overall aim of this study was to investigate the potential relationship between gluten sensitivity, ataxia and anti-GAD antibodies. This was achieved by several means:
- To investigate the presence of serum anti-GAD (IgG) antibodies in patients with gluten ataxia, using ELISA.
- To compare these levels with patients with CD only, patients with other causes of ataxia (both genetic and sporadic (non-gluten) idiopathic ataxia), SPS and healthy controls.

- To investigate any correlation between the levels of circulating anti-GAD (IgG) antibodies and anti-gliadin (IgG and IgA) or IgA anti-TG2 antibodies.
- Comparison of the cerebellar staining pattern by serum from patients with gluten ataxia with that of GAD, using IHC, to assess any similarities or differences.
- Assessment of the contribution of circulating anti-GAD (IgG) antibodies to serum staining with cerebellar proteins on a western blot.

6.2 Methods

6.2.1 Investigation of circulating IgG anti-glutamic acid decarboxylase antibodies using ELISA

A total of 29 gluten ataxia (untreated), 32 CD without neurological complications (untreated), 25 sporadic ataxia (non-gluten), 16 familial ataxia, 41 healthy control and 3 SPS serum samples were assayed according to the methods detailed in sections 2.2.2 and 2.2.5. Antibody levels and inter- and intra-assay variabilities were calculated according to the methods described in sections 2.2.6 and 2.2.7 respectively. In addition, a further 39 gluten ataxia (untreated), 32 CD without neurological complications (untreated), 65 sporadic ataxia (non-gluten) and 22 familial ataxia serum samples were assayed using the same commercial assay by the Immunology Department of The Northern General Hospital, Sheffield. Results were pooled and statistical analysis performed according to the method described in section 2.2.8. Finally, for each patient (not including those assayed at The Northern General Hospital), the corresponding levels of circulating antibodies were correlated using the Spearman rank order correlation as below:

o   IgG anti-gliadin antibodies and IgG anti-GAD antibodies

o   IgA anti-gliadin antibodies and IgG anti-GAD antibodies

o   IgA anti-TG2 antibodies and IgG anti-GAD antibodies

6.2.2 Investigation of the cross-reactivity of anti-gliadin and anti-tissue transglutaminase antibodies with glutamic acid decarboxylase using ELISA

Commercial polyclonal peroxidase-conjugated rabbit anti-gliadin antibody (Sigma, UK) or monoclonal mouse anti-guinea pig TG2 antibody (Labvision, USA) were assayed at dilutions of 1:100 and 1:500 using a commercial anti-GAD IgG ELISA according to the methods detailed in sections 2.2.2 and 2.2.5.

These antibodies, as well as 4 gluten ataxia (untreated), 1 CD without neurological complications (untreated), 1 ataxia control and 2 healthy control serum samples were also assayed at a dilution of 1:100 both before and after adsorption of anti-gliadin antibodies by crude gliadin. Samples were diluted 1:100 in PBST or crude gliadin solution (~20mg/ml, prepared as detailed in section 4.2.3) and incubated for 60mins at

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RT to allow adsorption of antibodies. Samples were then assayed according to the methods detailed in sections 2.2.2 and 2.2.5.

6.2.3 Investigation of the cerebellar staining pattern of commercial glutamic acid decarboxylase antibody

The staining pattern of a commercially available monoclonal, mouse anti-GAD antibody (Affiniti Research, UK) against rat cerebellar tissue were investigated using the indirect IHC method, outlined in sections 2.3.1 and 2.3.3. The antibody was used at a dilution of 1:500 or 1:1000 in PBST and binding was detected using a HRP-conjugated, goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech)) at a dilution of 1:500 in PBST.

In all experiments, sections incubated with either a Purkinje-cell specific antibody (section 4.2.1) or PBST as a substitute for primary antibody were included as positive and negative controls.

6.2.4 Detection of glutamic acid decarboxylase within rat cerebellar extract by western blotting

Detection of GAD was performed by probing an extract of rat cerebellum, separated on a 10% polyacrylamide gel, with a commercial monoclonal antibody. The same rat cerebellar extract (#9, section 5.3.1) was used in all experiments in order to minimise inter-experimental variation.

36µg/lane of rat cerebellar extract was loaded onto a 10% polyacrylamide gel, prepared as detailed in section 2.4.4. The first lane of each gel was loaded with 10µl coloured pre-stained molecular weight marker (Sigma, UK). SDS-PAGE and western blotting was then performed using the optimised method, as detailed in sections 2.4.4 and 2.4.5 respectively. GAD was detected according to the optimised method detailed in section 2.4.6. Details of the primary and secondary antibodies used are given in table 6.2. The RMM of the protein band was determined according to the method detailed in section 2.4.7. Blots were probed with monoclonal anti-GAD antibody in 10 different experiments. The average RMM of resultant protein bands was calculated.

Table 6.2 Details of reagents used in the detection of glutamic acid decarboxylase within a cerebellar extract on a western blot

Antibody Specificity	Species (supplier)	Dilution	Corresponding secondary antibody	Secondary antibody dilution
Monoclonal anti-GAD65/67	Mouse IgG (Affiniti Research, UK)	1:10 000	Horseradish Peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech))	1:10 000

6.2.5 Investigation of the specificity of patient serum antibody binding to glutamic acid decarboxylase by western blotting

The results of the sera screening experiment described in section 5.3.6 were re-assessed to determine which bands may be due to binding of IgG anti-GAD antibodies within positive serum samples. The average RMM of the positive control GAD protein band from this study was used. Any bands at the calculated RMM ± inter-experimental variability were considered likely to be produced by binding of anti-GAD antibodies. The percentage of the total number of patients whose serum was positive for IgG anti-GAD antibodies on ELISA and that also produced a band suggestive of binding of anti-GAD antibodies, was calculated.

The persistence of commercial and serological anti-GAD antibody reactivity was assessed and compared using commercial anti-GAD antibody or serum from a patient with untreated gluten ataxia, previously found to have GAD reactivity on western blot in the sera screening experiment. 10μl/lane (36μg) of rat cerebellar extract was loaded onto a 10% polyacrylamide gel, prepared as detailed in section 2.4.4. The first lane of each gel was loaded with 10μl coloured pre-stained molecular weight marker (Sigma, UK). SDS-PAGE and western blotting was then performed according to the optimised method detailed in sections 2.4.4 and 2.4.5 respectively. The blot was probed with either commercial monoclonal anti-GAD65/67 antibody (Affiniti Research, UK) at dilutions of 1:10 000, 1:100 000 or 1:500 000 in PBST or patient serum at dilutions of

dilutions of 1:10 000, 1:100 000 or 1:500 000 in PBST or patient serum at dilutions of 1:5000, 1:50 000 or 1:100 000 in PBST according to the optimised method detailed in section 2.4.6. Peroxidase-conjugated, goat anti-human or anti-mouse IgG (Jackson Immunoresearch Laboratories, USA (UK distributor, Stratech)) diluted 1:10 000 in PBST was used as the appropriate secondary antibody. Staining was visualised using the UVP imaging system detailed in section 2.4.6.

6.3 Results

6.3.1 Inter- and Intra-assay variability

The average inter- and intra-assay variability of the commercial anti-GAD IgG ELISA kit was 5.2 and 4.7% respectively (n=4).

6.3.2 Presence of circulating IgG anti-glutamic acid decarboxylase antibodies in patients with gluten ataxia

According to the cut-off values supplied by the kit manufacturer, 56% of gluten ataxia samples and 59% of CD only samples were positive for IgG anti-GAD antibodies as compared to 29% sporadic (non-gluten) ataxia, 13% familial ataxia and 12% of healthy control samples. This is represented in figure 6.2, which illustrates the distribution of IgG anti-GAD antibody levels between groups. The median value and inter-quartile range of each group is shown in figure 6.3. Kruskal-Wallis analysis indicated a highly significant difference between groups ($P<0.001$). The median antibody levels of gluten ataxia patients and patients with CD without neurological complications, which were not significantly different ($p>0.05$), were significantly higher than those of patients with sporadic (non-gluten) ataxia, familial ataxia and healthy controls (all $p<0.001$). Although more patients with sporadic (non-gluten) ataxia than patients with familial ataxia were positive for circulating IgG anti-GAD antibodies, the median levels of these two groups were not significantly different ($p>0.05$). The median level of each of these groups also did not differ significantly from the median level of healthy control patients ($p>0.05$).

6.3.3 Correlation of circulating levels of gluten sensitivity associated antibodies and IgG anti-glutamic acid decarboxylase antibodies

Levels of circulating IgG anti-GAD antibodies in patients with untreated gluten ataxia were not significantly correlated with levels of either IgG anti-gliadin antibodies or IgA anti-TG2 antibodies ($p>0.05$) (data not shown). However, levels of circulating IgG anti-GAD antibodies were weakly correlated with levels of IgA anti-gliadin antibodies in these patients ($p<0.05$) (figure 6.4).

Figure 6.2 Serum IgG anti-glutamic acid decarboxylase antibody levels, measured by ELISA
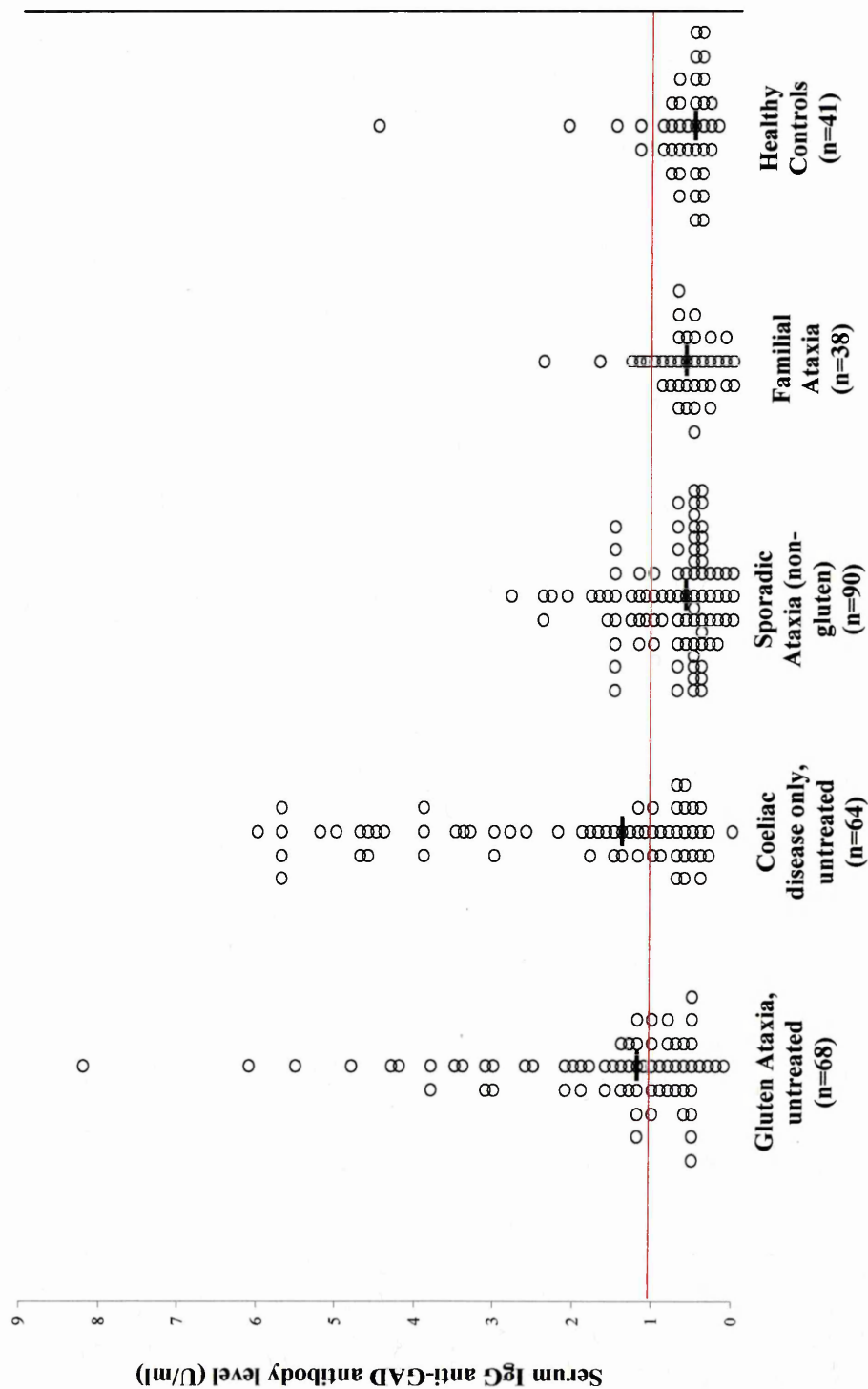
Data expressed in arbitrary units, defined by the kit manufacturers. The horizontal line represents the positive/negative threshold, as defined by the kit manufacturers.

Figure 6.3 Summary of serum IgG anti-glutamic acid decarboxylase antibody levels, measured by ELISA
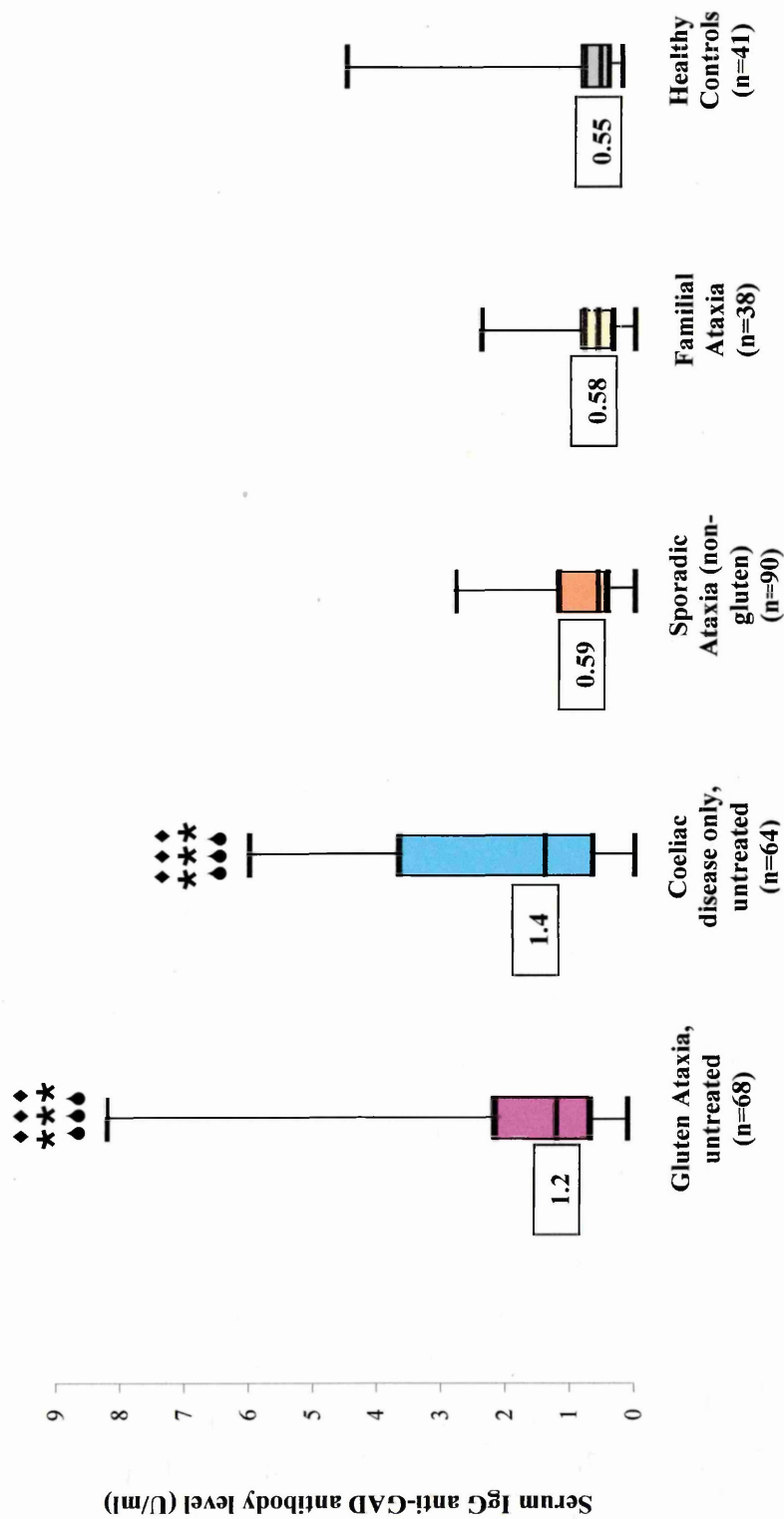
Data expressed in arbitrary units (defined by the kit manufacturers) as box and whiskers plot (section 2.2.7). ◆ ◆ ◆ = p<0.001 compared to sporadic ataxia control group, ✱ ✱ ✱ = p<0.001 compared to familial control group and ● ● ● = p<0.001 compared to healthy control group according to non-parametric multiple comparison. Gluten ataxia and CD only groups did not differ significantly. Median levels of control groups were not significantly different from each other. Median level of each group is shown next to the corresponding plot.

Figure 6.4 Correlation between circulating IgG anti-GAD and IgA anti-gliadin antibodies in patients with gluten ataxia

Data expressed in arbitrary units, as defined by the kit manufacturers. Correlation was assessed by Spearmans rank sum test.

A positive correlation was observed between circulating IgG anti-GAD and IgA anti-gliadin antibodies in patients with gluten ataxia (p<0.05).

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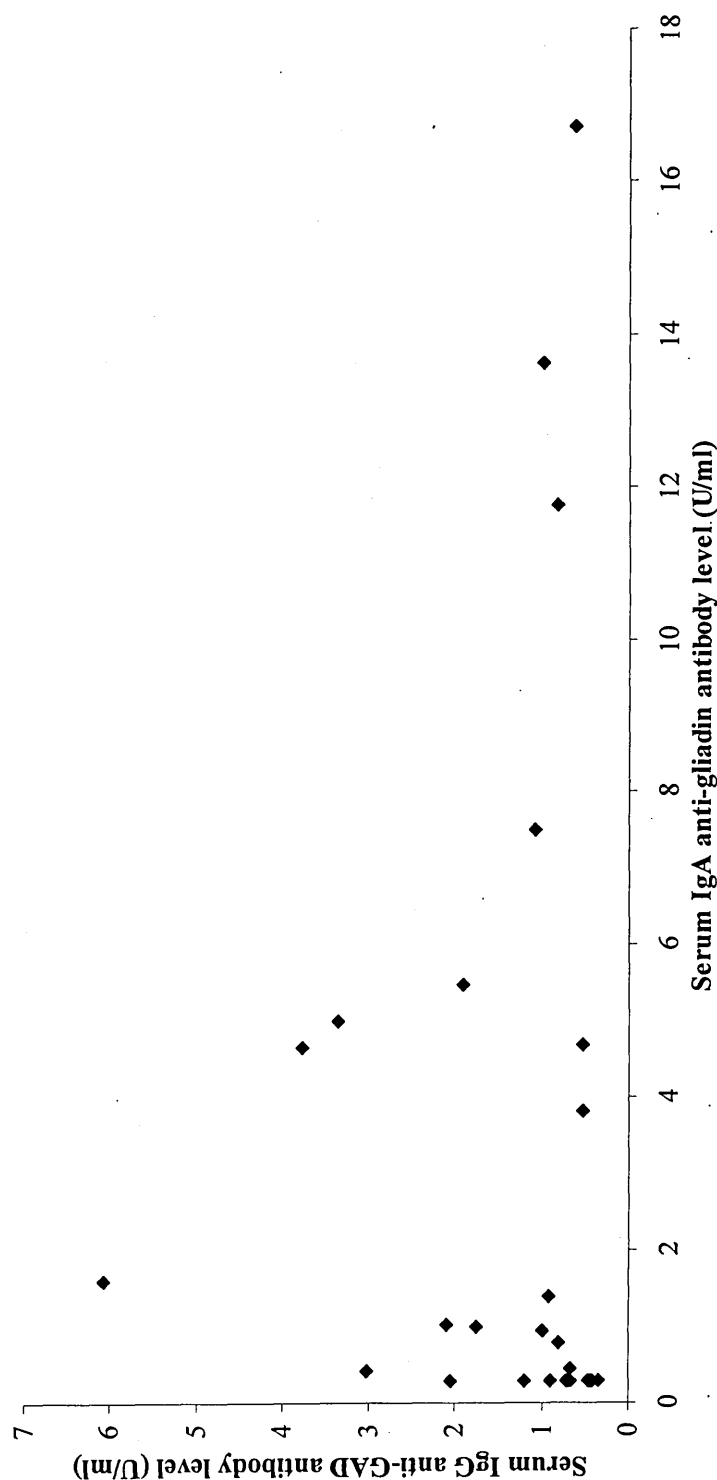
6.3.4 Individual anti-glutamic acid decarboxylase antibody profiles of patients with gluten ataxia

The individual serological levels of IgG anti-GAD antibodies of each patient with gluten ataxia are summarised in table 6.3.

6.3.5 Cross-reactivity of anti-gliadin and anti-tissue transglutaminase antibodies with glutamic acid decarboxylase and the effects of pre-adsorption of sera with crude gliadin on IgG anti-glutamic acid decarboxylase antibody levels

Commercial anti-gliadin and anti-TG2 antibodies showed no cross-reactivity with GAD on the IgG anti-GAD ELISA. Pre-adsorption of patient serum anti-gliadin antibodies using crude gliadin did not affect the IgG anti-GAD levels of any sample tested, determined by IgG anti-GAD ELISA (figure 6.5).

6.3.6 Staining pattern of commercial anti-GAD antibody on rat cerebellar tissue

Commercial anti-GAD antibody, used at dilutions of 1:500 and 1:1000, weakly stained rat cerebellum. Purkinje cells were particularly stained with both cytoplasmic and, stronger, membranous staining evident (figure 6.6).

6.3.7 Detection of GAD protein within rat cerebellar extract

Detection of GAD using a monoclonal anti-GAD65/67 antibody produced a doublet (although usually appearing as a single thick band) at an average RMM of 65.3kDa (range 60-71kDa) (figure 6.7).

Table 6.3 Summary of the individual levels of IgG anti- glutamic acid decarboxylase antibodies in patients with gluten ataxia

Patient number	IgG anti-GAD level (U/ml)	Patient number	IgG anti-GAD level (U/ml)
1	Not assayed	18	0.52
2	2.05	19	0.9
3	0.8	20	0.72
4	Not assayed	21	3.01
5	0.54	22	1.2
6	Not assayed	23	6.09
7	0.83	24	0.63
8	0.52	25	0.68
9	0.34	26	0.41
10	0.67	27	1.00
11	0.99	28	2.1
12	1.76	29	3.36
13	1.09	30	3.78
14	0.66	31	0.67
15	1.9	32	0.91
16	0.45	33	Not assayed
17	0.43	34	Not assayed

Data expressed in arbitrary units (defined by the kit manufacturers). Patient number corresponds to that in table 2.1. Samples defined as positive according to the kit manufacturers are shown in red.

Figure 6.5 Cross-reactivity of commercial anti-gliadin and anti-tissue transglutaminase antibodies with glutamic acid decarboxylase and the effect of adsorption of anti-gliadin antibodies, using crude gliadin, on the anti-glutamic acid decarboxylase levels of these antibodies and serum samples from patients with gluten ataxia, coeliac disease only, ataxia controls and healthy controls, as determined by ELISA
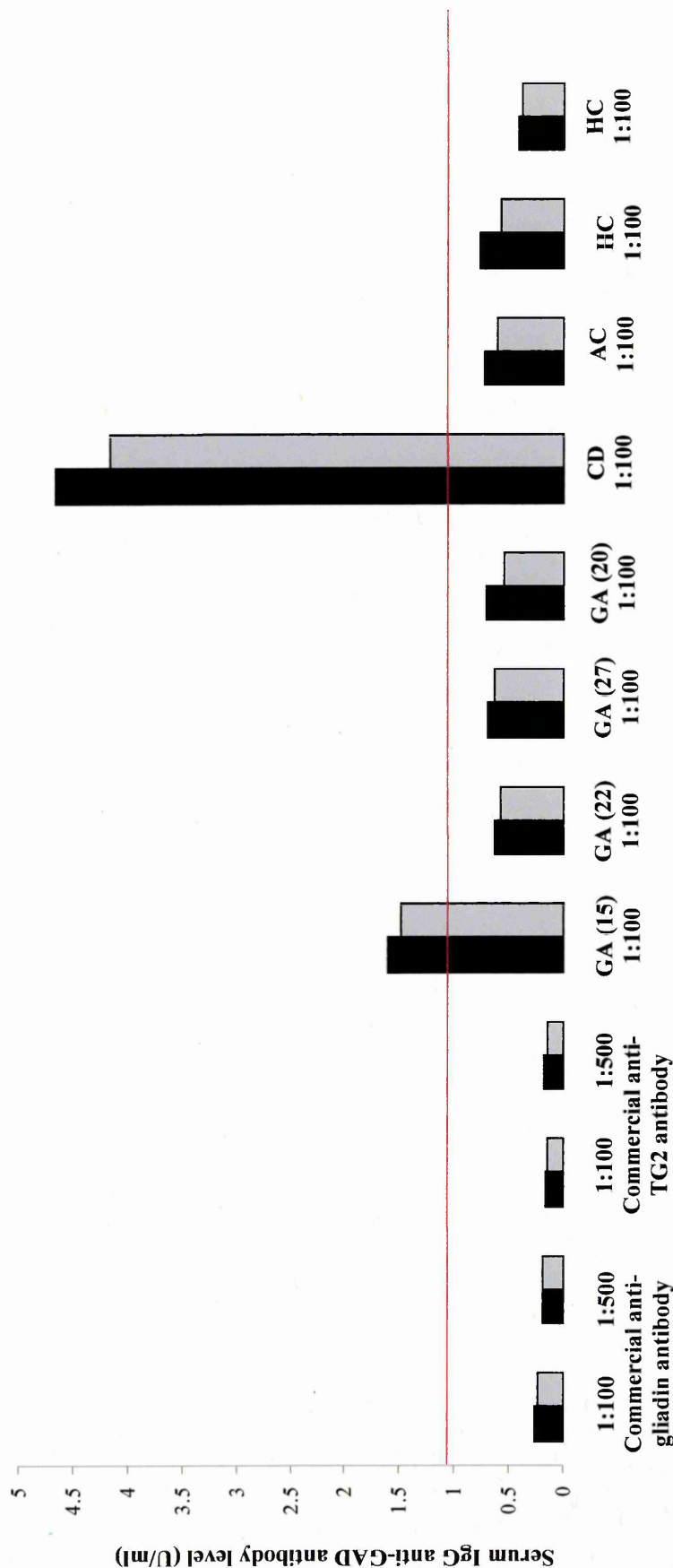
Data expressed in arbitrary units (defined by Biomerica). The horizontal line represents the positive/negative threshold, as defined by the kit manufacturers. ■ Sample diluted in PBST (1:100 dilution, unless stated). ▨ Sample diluted in crude gliadin solution (1:100 dilution, unless stated). GA – gluten ataxia (number in brackets corresponds to patient number in table 2.1), CD – CD only, AC – ataxia control, HC – healthy control.

Figure 6.6 Staining pattern of commercial anti-glutamic acid decarboxylase antibody on control rat cerebellum, as determined by IHC
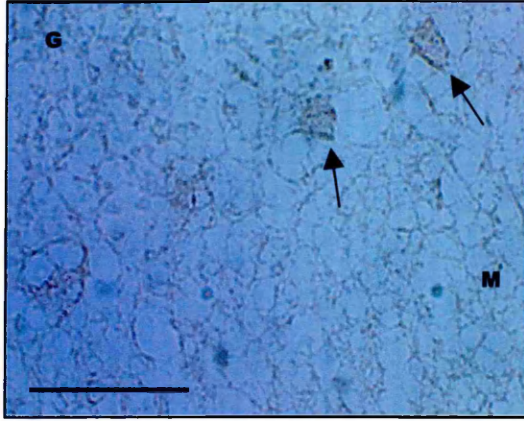
Typical staining pattern of commercial anti-GAD antibody, diluted to 1:500, on rat control cerebellum. Purkinje cells are indicated by arrows. **M** – molecular layer **G** – granular layer. Scale bar = 100 μm.

Figure 6.7 Binding to glutamic acid decarboxylase within rat cerebellar extract of a commercial monoclonal antibody, detected by chemiluminescence
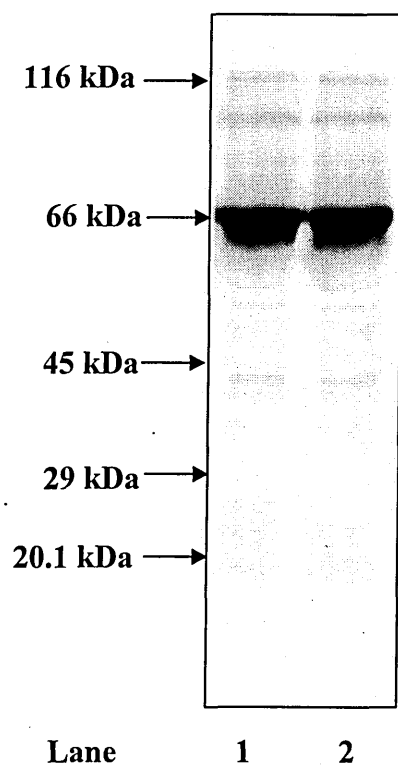
A 10% polyacrylamide gel was loaded as follows,

Lanes 1-2   10μl/lane (36μg protein) rat cerebellar protein extract (extraction #9)

Following separation and transfer, the blot was probed with monoclonal anti-GAD$_{65/67}$ antibody, 1:10 000. Binding was visualised using horseradish-peroxidase conjugated anti-mouse IgG secondary antibody at a dilution of 1:10 000. The blot was then incubated with ECL-plus™ reagent and imaged using a UVP imager.

6.3.8 Specificity of patient serum antibody binding to GAD

The inter-experimental variability of the sera screening experiment was 8%. Therefore, for antibody positive serum, bands at 65.3 ± 8% kDa (60-71kDa) were considered possibly due to binding of anti-GAD antibodies. These bands are highlighted in table 6.4. Using these criteria, overall, 60% of sera positive for anti-GAD antibodies on ELISA also exhibited a band on a blot likely to be due to binding of IgG anti-GAD antibodies.

Reactivity of both commercial anti-GAD antibody and the sera from a patient with untreated gluten ataxia against GAD protein within rat cerebellar extract on a western blot decreased in intensity but persisted up to the highest dilutions tested (1:500 000 and 1:100 000 respectively) (figure 6.8).

Table 6.4 Summary of reactivity of sera from patients with gluten ataxia, coeliac disease only, familial ataxia, sporadic ataxia (non-gluten), stiff-person syndrome and healthy controls likely to be against glutamic acid decarboxylase within rat cerebellar extract on a western blot, detected by chemiluminescence

RMM (kDa) → PATIENT (no.)	60-69	70-79
GA (15)	65	-
GA (30)	-	-
GA (25)	65	-
GA (27)	-	-
GA (24)	-	-
GA (28)	-	-
GA (23)	67	-
GA (29)	-	-
GA (13)	-	-
GA (21)	-	-
GA (11)	-	-
GA (16)	-	78
GA (22)	65	70
GA (32)	63	-

RMM (kDa) → PATIENT	60-69	70-79
SA	-	-
SA	-	77
SPS	65	-
FA	62	-
FA	-	-
FA	-	-
FA	-	-
FA	62	-
FA	-	-
FA	63	-
CD	-	70
CD	-	-
CD	-	-

GA – gluten ataxia (patient number corresponding to table 2.1 shown in brackets), SA – sporadic ataxia (non-gluten), SPS – stiff-person syndrome, FA – familial ataxia, CD – CD only without neurological complications. Bands likely to be due to binding of anti-GAD (RMM within 8% of known protein RMM) (60-71kDa) antibodies within antibody positive serum are shaded ▨ . Intense bands are shown in bold.

Table 6.4 cont'd Summary of reactivity of sera from patients with gluten ataxia, coeliac disease only, familial ataxia, sporadic ataxia (non-gluten), stiff-person syndrome and healthy controls likely to be against glutamic acid decarboxylase within rat cerebellar extract on a western blot, detected by chemiluminescence

RMM (kDa) → PATIENT	60-69	70-79
CD	-	-
CD	67	-
CD	-	-
CD	-	-
CD	-	-
CD	-	-
CD	-	-
HC	63	-
HC	-	-
HC	-	-
HC	-	-
HC	-	-
HC	-	-
HC	-	-
HC	-	**76**
HC	-	-
HC	-	-
HC	-	-

CD – CD only without neurological complications, HC – healthy controls. Bands likely to be due to binding of anti-GAD (RMM within 8% of known protein RMM) (60-71kDa) antibodies within antibody positive serum are shaded ▨. Intense bands are shown in bold.

Figure 6.8 Persistence of the reactivity of commercial and serological anti-glutamic

acid decarboxylase antibodies to glutamic acid decarboxylase protein within

rat cerebellar extract, detected by chemiluminescence

| Lane | 1 | 2 | 3 | 4 | 5 | 6 |
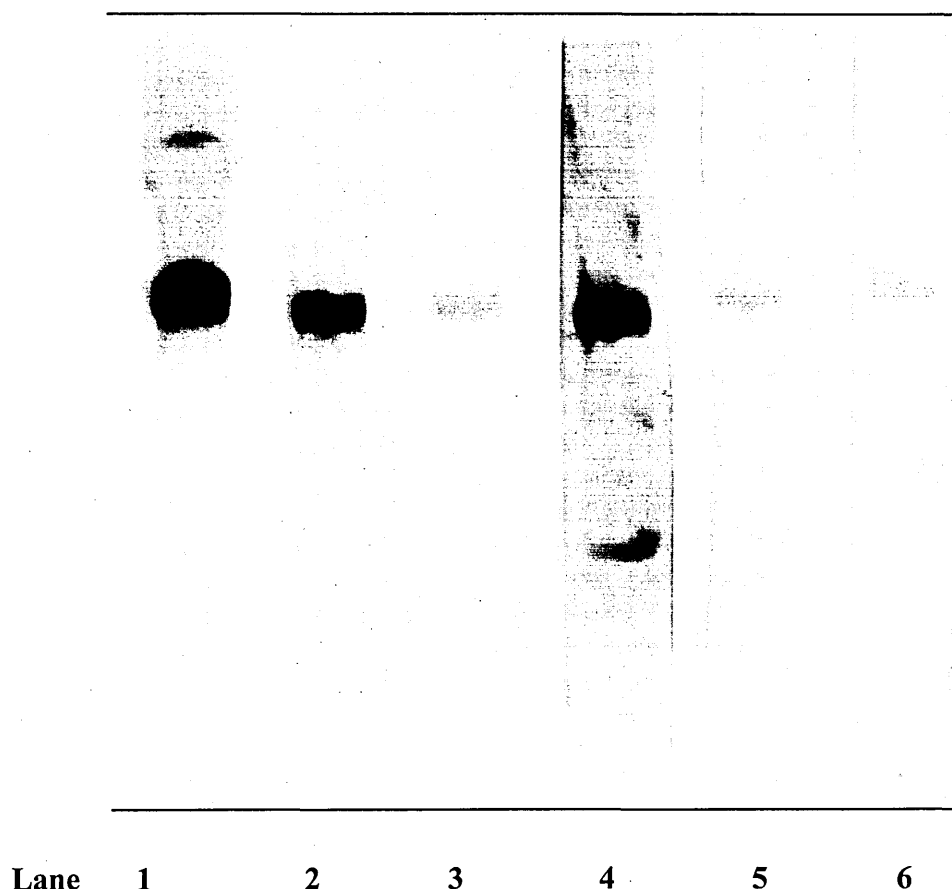
A 10% polyacrylamide gel was loaded as follows,

Lanes 1-6   10μl/lane (36μg protein) rat cerebellar protein extract (supernatant,

extraction #9)

Following separation and transfer, the blot was probed with monoclonal anti-GAD$_{65/67}$

antibody at dilutions of 1:10 000 (lane 1), 1:100 000 (lane 2) or 1:500 000 (lane 3) or

serum from a patient with untreated gluten ataxia (number 15 corresponding to table

2.1) at dilutions of 1:5000 (lane 4), 1:50 000 (lane 5) or 1:100 000 (lane 6). Binding

was visualised using horseradish-peroxidase conjugated anti-mouse IgG secondary

antibody at a dilution of 1:10 000. The blot was then incubated with ECL-plus™

reagent and imaged using a UVP imager.

6.4 Discussion

Investigation of the relationship between gluten sensitivity, ataxia and anti-GAD antibodies has provided an additional target area for further research related to the humoral immune response in gluten ataxia. The results of these demonstrate that patients with gluten ataxia and CD only have increased levels of circulating IgG anti-GAD antibodies compared to other groups (p<0.001), which are not explained by an increased prevalence of co-existing diseases associated with these antibodies such as IDDM compared to control groups. These findings are in contrast to a recent study by Uibo and co-workers who found no relationship between immune reactivity to GAD and gliadin (Uibo *et al.,* 2001). These differences may be due to the different study populations examined. Uibo and co-workers examined a randomly selected adult population whilst the patients in the current study may be considered at an increased likelihood for GAD reactivity due to the presence of a co-existing autoimmune condition with a shared genetic susceptibility to other GAD-associated autoimmune disorders. Therefore, such an association may be more difficult to detect in a general population and may only become evident if a greater number than the 200 individuals investigated by Uibo is considered.

The prevalence of anti-GAD antibodies in patients with gluten ataxia and CD only was similar (56% and 59% respectively) suggesting that the presence of these antibodies may be associated with gluten sensitivity in general. ELISA studies have shown that the observed reactivity of serum on the anti-GAD antibody ELISA is not due to cross-reactivity of circulating anti-gliadin or anti-TG2 antibodies with GAD. Levels of circulating IgG anti-GAD antibodies in patients with gluten ataxia were not correlated with levels of circulating IgG anti-gliadin or IgA anti-TG2 antibodies but appear to be weakly correlated with IgA anti-gliadin antibodies (p<0.05). In addition, although it is not possible to positively identify the immunoreactive protein without sequencing of the band, further analysis of western blots showed that 60% of sera from patients with gluten ataxia, positive for anti-GAD antibodies on ELISA, also exhibited a band possibly due to the binding of IgG anti-GAD antibodies.

GAD is present in the enteric nervous system and it seems possible that exposure of the enzyme to immune cells during the immune response against gluten may result in the production of anti-GAD antibodies. This may explain the observed correlation of these antibodies with circulating IgA anti-gliadin antibodies. Patients with more severe

mucosal damage (for example patients with CD only) may then be expected to have higher levels of antibodies, in a similar manner to the way in which levels of anti-gliadin and anti-TG2 antibodies are reportedly associated with the degree of mucosal damage (Rostami et al., 2003; Tursi et al., 2001, 2003). However, this is not the case. It has been suggested that anti-GAD antibodies may arise via cross-linking by TG2 and presentation to the appropriate immune cells, a suggestion supported by the fact that IgG anti-TG2 antibodies have been reported in up to 42% of patients with IDDM (Kim et al., 2002). This notion is also supported by the high prevalence of IgA anti-TG2 antibodies in patients with CD and gluten ataxia although no correlation between circulating levels of the two antibodies was observed in the current study.

Alternatively, immunoreactivity to GAD may be the result of epitope spreading of gluten sensitivity associated antibodies or a reflection of a general increased propensity to autoimmunity as a result of immune hyper-responsivity in these patients. Patients with CD only have been shown to be at an increased risk of developing autoimmune diseases, such as IDDM (Sategna Guidetti et al., 2001; Ventura et al., 1999), which shares a genetic susceptibility with gluten sensitivity of the presence of HLA DQ2 or DQ8 (Abraham et al, 2001). Using anti-GAD antibodies as a marker of current or pre-clinical diabetes, the incidence of anti-GAD antibodies in gluten ataxia patients is much higher than would be expected due to the previously reported association between these conditions. This suggests an interesting possible link between these disorders, which may be more extensive than previously appreciated and requiring further investigation.

Anti-GAD antibodies have previously been reported in association with cases of cerebellar ataxia, most frequently in the presence of other autoimmune diseases (anti-GAD associated ataxia) (Abele et al., 1999; Honnorat et al., 2001; Saiz et al., 1997). The presence of anti-GAD antibodies has been suggested as an indicator that these individuals represent a subgroup of patients with sporadic idiopathic ataxia with an autoimmune origin and an immune-mediated pathogenesis (Honnorat et al., 2001). Although the presence of anti-GAD antibodies in these patients may be attributed to the association with co-existing IDDM or polyendocrine autoimmunity, similarly elevated anti-GAD antibodies have been reported in patients with anti-GAD associated ataxia without these disorders (Honnorat et al., 2001). In addition, the anti-GAD levels of these patients have been found to be significantly higher than the corresponding levels in patients with IDDM only and similar to the levels seen in patients with SPS

(Honnorat *et al.*, 2001; Saiz *et al.*, 1997). In the current study, samples from patients with IDDM were not available to screen for comparative purposes although levels of anti-GAD antibodies in gluten ataxia patients were similar to those of two sera from anti-GAD antibody positive patients with SPS.

Several similarities between ataxia associated with anti-GAD antibodies and gluten ataxia are apparent although the significance of these observations is unclear. Both conditions are associated with a progressive ataxia of gait and the presence of oligoclonal bands (10/14 patients with anti-GAD associated ataxia and 12/28 patients with gluten ataxia) although the majority of patients with anti-GAD associated ataxia have been reported to be female whereas this is not the case in patients with gluten ataxia (Honnorat *et al.*, 2001; Saiz *et al.*, 1997). In one study, two patients with anti-GAD associated ataxia also had IgA anti-gliadin antibodies although this was reported as a probable coincidental finding by the authors (Honnorat *et al.*, 2001). The presence of IgG anti-gliadin antibodies, a more sensitive marker of gluten ataxia, was not reported. Although not a significant difference compared to controls, the finding that 29% of patients with sporadic idiopathic ataxia, in the absence of any other indicators of gluten sensitivity, have increased circulating levels of anti-GAD antibodies is also interesting in this regard. It is possible that these patients represent a subgroup with anti-GAD associated ataxia although it is not known whether these patients have co-existing autoimmune diseases, which may increase the likelihood of anti-GAD antibodies. In addition, all were found to express either DQ2 or DQ1, susceptibility genes associated with autoimmune conditions and found in at least 98% of patients with gluten ataxia. However, antibody levels of positive patients were generally not as high as those of patients with CD only or gluten ataxia and the median level of this group was not statistically different to those of other control groups. The significance of this group of patients with regard to the possible relationship between anti-GAD associated ataxia and gluten ataxia remains unclear. It is unlikely that these entities are identical as these patients are not gluten sensitive although it seems possible that these disorders represent a more general group of patients with ataxia associated with anti-GAD antibodies related to HLA DQ2/DQ8 susceptible autoimmune conditions in general.

The finding that patients with familial ataxia did not have significantly increased levels of anti-GAD antibodies compared to controls is consistent with previous reports of their absence in patients with ataxia and no evidence of autoimmune disorders (Saiz *et al.*,

1997) and suggests that these antibodies too, are specific and not merely an epiphenomenom of Purkinje cell loss.

Finally, the observed prevalence of anti-GAD antibodies in healthy control patients (12%) is higher than previously reported in other studies (Honnorat *et al.,* 2001; Tuomilehto *et al.,* 1994). This difference may be due to factors such as the use of different detection methods (ELISA vs RIA) or a population variation in the incidence of clinical and pre-clinical IDDM.

In summary, the main findings of this study were that patients with gluten sensitivity (gluten ataxia and CD only) have significantly increased levels of circulating IgG anti-GAD antibodies. These antibodies, detected by ELISA, are directed against GAD and not a result of cross-reactivity of circulating anti-TG2 or anti-gliadin antibodies in gluten sensitive individuals. Although of limited use, western blot analysis showed that 60% of sera from patients with gluten ataxia, positive for anti-GAD antibodies on ELISA, also exhibited a band on a blot possibly due to the binding of IgG anti-GAD antibodies. Levels of circulating IgG anti-GAD antibodies in patients with gluten ataxia were not correlated with levels of circulating IgG anti-gliadin or IgA anti-TG2 antibodies but appear to be weakly correlated with IgA anti-gliadin antibodies. A smaller proportion of patients with sporadic idiopathic ataxia also have increased levels of IgG anti-GAD antibodies compared to healthy controls and patients with familial ataxia, although this difference was not significant. These patients may represent a subgroup of patients with ataxia associated with anti-GAD antibodies similar to that which has been previously described by other authors.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 Discussion of results

Gluten ataxia is part of the spectrum of gluten sensitivity, ataxia being the primary presentation followed by the subsequent detection of previously undiagnosed gluten sensitivity. The cerebellum is the primary target organ in a manner analogous to the skin in DH or the small intestine in CD. The strong association with the HLA haplotypes found in CD, presence of associated circulating antibodies and oligoclonal bands, neuropathological findings and the clinical response to a GFD are all convincing evidence for the inclusion of gluten ataxia within the spectrum of gluten sensitivity and also the hypothesised immune-mediated pathogenesis of the condition. It is suggested that this immune response results in damage to the cerebellum and the associated clinical symptoms. The studies contained within this thesis have aimed to elucidate the involvement of the humoral immune response in the pathogenesis of gluten ataxia.

The results included in this thesis demonstrate that, in common with patients with other manifestations of gluten sensitivity (CD and DH), patients with gluten ataxia have elevated levels of circulating anti-gliadin and anti-TG2 antibodies. In addition, the majority of patients with gluten ataxia have elevated levels of circulating antibodies directed against GAD. IHC studies have shown that patients with gluten ataxia also possess high titres of antibodies capable of reacting with cerebellar Purkinje cell proteins, providing evidence for the existence of a humoral immune response, although the target antigen remains unidentified. Interestingly, the ability of anti-gliadin antibodies to cross-react with cerebellar Purkinje cell proteins has been confirmed by both IHC and western blotting studies, suggesting the existence of common epitopes. However, adsorption experiments indicate that patients with gluten ataxia also possess additional anti-Purkinje cell antibodies, which are distinct from anti-gliadin antibodies. It is hypothesised that these, as yet unidentified antibodies, may be responsible for the Purkinje cell damage observed in gluten ataxia.

It is logical to first consider whether these anti-Purkinje cell antibodies are related to the anti-gliadin, anti-TG2 or anti-GAD antibodies, which have already been identified in these patients. In order for an antibody to play a causal role in disease pathogenesis it must be present in all patients. In addition, the infrequency of histologically evident mucosal damage in patients with gluten ataxia suggests that any neuropathic antibodies are not dependent on the presence of severe mucosal damage for their production.

By these criteria, gluten-associated anti-TG2 antibodies are unlikely to be a requirement for neuronal damage as they are found in less than half of patients with gluten ataxia. The dissimilarity of cerebellar staining patterns of sera from patients with gluten ataxia and monoclonal anti-TG2 antibody is also consistent with the idea that cerebellar antibody reactivity is, at least primarily, mediated by antibodies other than those directed against TG2. In contrast, all patients with gluten ataxia possess anti-gliadin antibodies, which are capable of cross-reacting with cerebellar Purkinje cells. Also, in contrast to anti-TG2 antibodies, production of anti-gliadin antibodies, directed against the universal exogenous aetiological factor of gluten sensitivity, is not dependent on the presence of histological mucosal damage. It seems possible that circulating anti-gliadin antibodies may somehow be involved in the humoral pathogenesis of gluten ataxia.

However, one major consideration of this hypothesis must be that the majority of patients with CD only and DH also have high circulating levels of both of these antibodies yet don't develop neurological complications. Some, as yet unknown, differentiating factor must determine whether a neurological immune response is initiated or established and elucidation of this factor will provide further knowledge of the pathogenesis of gluten ataxia.

Although patients with gluten ataxia share the same genetic HLA susceptibility as other gluten sensitive individuals, the expression of additional, as yet unidentified, genes may result in a tendency for a predominantly neurological manifestation as opposed to other target organs. Alternatively, it is clear that patients with gluten ataxia generally suffer less intestinal mucosal damage than do patients with CD. It is likely that both groups consume similar levels of gluten and, often, the diagnosis of gluten ataxia at a late age means that these patients have been consuming gluten for a longer period yet the intestine appears less susceptible to immunological damage. It seems possible therefore that, here too, genetics or some other subtle difference in the intestinal immune response to gluten may be responsible for this difference. For example, IDDM, SPS and autoimmune polyendocrine disorder are all associated with the presence of anti-GAD antibodies and it has been suggested that slight differences in the immune response to GAD determines the clinical manifestation (Lernmark, 1996). One such difference is the dominance of a Th1 versus a Th2 immune response. Progression to clinical onset of IDDM has been reported to be associated with a decrease in the Th2 response to GAD (Petersen *et al.,* 1999) whilst SPS is associated with a dominance of the Th2 response,

along with additional differences in the properties of anti-GAD antibodies which also distinguish the two disorders (Schloot *et al.,* 1999). Analagous to this situation, onset of different manifestations of gluten sensitivity may be related to the relative balance between a Th1 and Th2 response to gluten. A Th1 response is responsible for most of the intestinal damage associated with CD and it may be that, in patients with gluten ataxia, this response is somehow shifted towards a Th2 response due to differences in the immune recognition of gluten, thereby avoiding much of the Th1-mediated intestinal damage but still resulting in disease associated antibody production. Interestingly, intestinal T-cells from patients with DH have been shown to produce more IL-4, a cytokine involved in antibody production, than T-cells from patients with CD only (Hall *et al.,* 2000). It may be the case that a similar process occurs in patients with gluten ataxia. The selection of Th1/Th2 immunity may be affected by many factors including alterations in the sequence of the T-cell epitope, antigen dose and route and relative affinity to the T-cell receptor (Murray, 1998). Differences in the intestinal immune response to gluten may also explain the presence of anti-Purkinje cell antibodies in patients with gluten ataxia. The gluten T-cell epitopes that have been identified to date in patients with CD may not be as immunodominant in patients with gluten ataxia. Although anti-gliadin antibodies from both patients with CD and gluten ataxia cross-react with Purkinje cell epitopes, additional gluten epitopes may be more predominant in patients with gluten ataxia. Again, this may be in an analagous manner to anti-GAD antibody associated conditions where manifestation of a particular disorder is partly dependent on the target epitope of GAD recognised by antibodies (Lernmark, 1996; Powers *et al.,* 1999). Anti-gliadin antibodies in patients with gluten ataxia directed against different gluten epitopes may have a greater avidity or cross-reactive potential for Purkinje cell epitopes. In addition, with time these epitopes may be more likely to undergo epitope spreading than epitopes recognised by anti-gliadin antibodies in patients with CD without neurological complications. Epitope spreading refers to the process by which antibody recognition of new epitopes occurs, on the same or different proteins to the primary disease-inducing epitope. This process has been implicated in the pathogenesis of other autoimmune diseases, including IDDM and multiple sclerosis (MS), and has also been suggested as the mechanism responsible for the increased risk of autoimmune diseases associated with CD (Martucci & Corazza, 2002). It is conceivable then, that some process of epitope spreading may occur in patients with gluten ataxia, but not CD only, resulting in the observed anti-Purkinje cell antibodies that are not adsorbed by crude gliadin in these patients. Alternatively, antigenic

neoepitopes may be produced by the cross-linking of gliadin and TG2 in the intestine, which, in some cases, may produce antibodies distinct from anti-gliadin antibodies that cross-react with Purkinje cell epitopes resulting in an immune response targeting these cells.

Although initial evidence has suggested that anti-TG2 antibodies are unlikely to play a direct role in neurological damage, differences in the affinity of circulating antibodies to different transglutaminases may also play a role in the formation of anti-Purkinje cell antibodies. For example, patients with DH have recently been shown to have a serological profile suggesting a higher affinity for epidermal transglutaminase (TG3) than TG2. In addition, these patients possess an antibody population specific for TG3, which is not found in patients with CD only (Sardy et al., 2002). It is possible therefore, that a similar situation exists in patients with gluten ataxia, who may have a serological profile suggestive of a higher affinity towards a neural transglutaminase, or a subgroup of antibodies directed against this enzyme, not seen in patients with other manifestations of gluten sensitivity. This hypothesis is intriguing in light of the recent discovery of a novel transglutaminase (TG6), which is primarily expressed in the brain including Purkinje cells (Grenard et al., 2001).

The full significance of the finding that both patients with CD and gluten ataxia have elevated levels of circulating anti-GAD antibodies is still not known. The fact that anti-GAD antibodies are present in just over half of patients with gluten ataxia suggests that these antibodies are unlikely to play a causative role in the pathogenesis of gluten ataxia. However, these antibodies have been shown to be pathogenic mediating suppression of GABA release from cerebellar neurones (Dinkel et al., 1998; Ishida et al., 1999; Mitoma et al., 2000, 2003; Takenoshita et al., 2001). It has been hypothesised that a cerebellar GAD dysfunction could lead to pathological changes and neuronal cell death (Honnorat et al., 1995). It seems possible therefore, that anti-GAD antibodies, when present, may have some additional or modulatory effect on the disease process.

Alternatively, the anti-Purkinje cell antibodies present in patients with gluten ataxia may be unrelated to any other previously described circulating disease associated antibody. Determination of the target antigen will shed more light on the nature of these antibodies. IHC studies suggest that the antigen may be cytoplasmic. Although

antibodies directed against cytoplasmic antigens are often thought to be non-pathogenic, this does not preclude the possibility that these antibodies are neurotoxic. Purkinje cells have previously been shown to be capable of taking up large molecules from the CSF, including immunoglobulins, which may provide a mechanism for pathogenic antibodies directed against cytoplasmic components (Borges *et al.,* 1985). In addition, antibodies have been shown to penetrate living cells, including neurones (Alarcon-Segovia *et al.,* 1996; Wenthold *et al.,* 1984). In this way, antibodies could theoretically be internalised by Purkinje cells and cause pathological effects in this manner.

In order for a neuronal immune response to occur, immune cells must gain access to the cerebellum. Even under normal circumstances, it has been shown that activated T- and B-cells as well as small amounts of IgG can enter the CNS across an intact blood-brain barrier (BBB) (Hickey, 2001). IgG antibodies are able to pass the BBB by passive diffusion and enter the CSF at a fraction of approximately 1/500 of the serum concentration (Wurster, 2003). This diffusion may therefore be quite significant in situations where the serum antibody concentration is high. This mechanism is likely to explain the observations in the case study by Chinnery and co-workers, which suggested that anti-gliadin antibodies may be able to cross the BBB (Chinnery *et al.,* 1997). In other neurological inflammatory diseases, such as MS, the BBB is initially impaired thus allowing access of immune cells to the brain (Markovic-Plese & McFarland, 2001). Impairment of the BBB by factors such as inflammation caused by infection or some abnormality in proteins regulating permeability may occur in patients with gluten ataxia but not in gluten sensitive patients without neurological complications. A novel modulator of intestinal permeability, zonulin, a human analogue to the zonula occludins toxin (ZOT) found in *Vibrio cholerae,* has been shown to be upregulated in CD (Fasano *et al.,* 2000). Related zonula occludens proteins are also present in the BBB (Pachter *et al.,* 2003) and it is possible that these may be affected in a similar manner in gluten ataxia. However, dysfunction of these proteins still doesn't yet fully explain why only some gluten sensitive patients are affected and not others.

In other conditions such as PCD and SPS, the presence of antibodies in the CSF has been established as well as confirmation of intrathecal synthesis in some cases (Dalakas *et al.,* 2001; Darnell & Posner, 2003). The preliminary studies contained within this thesis failed to detect the presence of anti-gliadin antibodies in the CSF of gluten ataxia patients or any reactivity of CSF with Purkinje cells by IHC. This suggests that

intrathecal anti-gliadin antibody production is unlikely in gluten ataxia patients, which is consistent with a report by Burk and co-workers of no anti-gliadin antibodies or any other abnormal values in the CSF of twelve patients with gluten ataxia (Burk *et al.,* 2001). However, further studies are needed to clarify this situation with regard to both anti-gliadin and other antibodies in the CSF of these patients.

Finally, a primary role for the humoral response in the pathogenesis of gluten ataxia cannot be assumed. The involvement of a, possibly primary, T-cell response in PCD has been described (Albert *et al.,* 1998; Tanaka *et al.,* 1999) in addition to the established antibodies directed against onconeural antigens. There is also strong evidence for the involvement of a T-cell response in the pathogenesis of gluten ataxia. Marked lymphocytic infiltration of the cerebellum, consisting mainly of T-cells (CD4 and CD8) has been observed in patients with gluten ataxia at post-mortem (Hadjivassiliou *et al.,* 1998) and there is upregulation of IP-10, a T-cell chemoattractant, in the CSF of patients with gluten ataxia (Hadjivassiliou *et al.,* 2003b). The relative contributions of both the humoral and the cellular immune responses to the pathogenesis of gluten ataxia have yet to be described. Access of T-cells to the cerebellum and initiation of a cell-mediated immune response may be the differentiating factor for development of gluten ataxia and it remains to be seen whether antibodies play a causative role in the cerebellar damage or are merely produced secondary to a primary T-cell mediated response.

7.2 Future work

The work contained within this thesis has raised many further questions related to the immune pathogenesis of gluten ataxia, which need to be answered and provide opportunities for further work.

These questions include:

o *What is the antigen recognised by the circulating anti-Purkinje cell antibodies identified in patients with gluten ataxia?*
   This question is currently being addressed by ongoing work. To date, protein sequencing of two immunoreactive bands from a 1-D SDS-PAGE gel has been completed and ongoing work is concentrated on the preparation of gel digests of target proteins for identification using MALDI-TOF and ion spray analysis, although this method may be complicated by the complex nature of the proteins

within the band (i.e. the presence of several proteins at each position). This problem may be overcome by the use of 2-D electrophoresis, where proteins undergo a second separation based on their isoelectric point, thus allowing sequencing of single proteins. Further purification and concentration of the target protein prior to electrophoresis may also aid its identification. Initial attempts to purify and isolate the antigenic protein from serum using immunoprecipitation have, to date, been unsuccessful although positive control proteins (calbindin and GAD) have been precipitated onto a gel successfully using this method. Isolation of antibody from the serum using protein-A affinity chromatography may also be an alternative approach.

o *What is the in vitro effect of these antibodies on Purkinje cell function?*
Purkinje cells have previously been isolated from rat embryos and cultured following published techniques (Furuya *et al.,* 1998) with some success although the method remains to be optimised. Once established, Purkinje cell cultures may be used in a variety of in vitro techniques designed to answer this question including ion-channel assays and electrophysiology.

o *What is the role of the T-cell mediated neurological response in gluten ataxia?*
It is important to investigate the potential role of the T-cell immune response in gluten ataxia, as well as the humoral response. Isolation of T-cells from the intestine of patients with gluten ataxia and comparison with patients with CD only may provide information as to whether the T-cell response may be different in different manifestations of gluten sensitivity. Comparative experiments may include assays to determine the effects on T-cells of stimulation with various disease-related peptides such as various transglutaminases, GAD and gluten and analysis of the cytokine profiles of these cells through cellular assays or flow cytometry.

o *What are the distinguishing features between development of gluten ataxia and other manifestations of gluten sensitivity?*
This is a broad question encompassing many different areas, which will provide valuable insights into the pathogenesis and identification of gluten sensitive patients who may develop gluten ataxia. Studies may include: analysis of genotypes in patients with gluten ataxia and comparison with other gluten sensitive individuals;

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investigation of any impairment or abnormality of the BBB in gluten ataxia that may account for entry of immune cells to the CNS; further analysis of CSF samples from patients with gluten ataxia for the presence of specific antibodies or T-cells and evidence of any intrathecal antibody synthesis; further analysis of the roles of specific circulating antibodies in gluten ataxia, including identification of target B-cell epitopes and comparison with patients with CD only as well as investigation of the presence of antibodies directed against specific transglutaminases that may be specific to patients with gluten ataxia.

CHAPTER EIGHT

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PUBLICATIONS RELEVANT TO THIS THESIS

ABSTRACTS AND MEETINGS

Williamson C.A., Simpson J.E., Grunewald R.A., Davies-Jones G.A.B., Woodroofe M.N., Hadjivassiliou M. "The immune response in ataxia associated with coeliac disease". Poster presentation: SET for Britain meeting, The Houses of Parliament, London, UK. April 2000.

Williamson C.A., Simpson J.E., Grunewald R.A., Davies-Jones G.A.B., Woodroofe M.N., Hadjivassiliou M. "Gluten ataxia: Immunological mechanisms of neural damage". Poster presentation: Ninth International Symposium on Celiac Disease, Hunt Valley, Maryland, USA. August 2000. Abstract published in *Journal of Pediatric Gastroenterology and Nutrition.* **31(Suppl. 3)**: S22.

Williamson C.A., Simpson J.E., Grunewald R.A., Davies-Jones G.A.B., Woodroofe M.N., Hadjivassiliou M. "Gluten ataxia: Immunological mechanisms of neural damage". Poster presentation: Annual Congress of the British Society for Immunology, Harrogate, UK. December 2000. Abstract published in *Immunology.* **101(Suppl. 1)**: 53.

Williamson C.A., Grunewald R.A., Tongiorgi E., Woodroofe M.N., Hadjivassiliou M. "The humoral response in the pathogenesis of gluten ataxia". Oral presentation: International Society of Neuroimmunology Sixth International Congress, Edinburgh, UK. September 2001. Abstract published in *Journal of Neuroimmunology.* **118**: 149.

Williamson C.A., Grunewald R.A., Woodroofe M.N., Hadjivassiliou M. "An investigation of anti-cerebellar antibodies in gluten ataxia". Poster presentation: V European Meeting on Glial cell function in health and disease (Euroglia 2002), Rome, Italy. May 2002. Abstract published in *Glia.* **Suppl.1 May 2002.**

Williamson C.A., Grunewald R.A., Woodroofe M.N., Hadjivassiliou M. "An investigation of anti-cerebellar antibodies in gluten ataxia". Poster presentation: Tenth International Symposium on Celiac Disease, Paris, France. June 2002.

PAPERS

Hadjivassiliou M., Boscolo S., Davies-Jones G.A.B., Grunewald R.A., Not T., Sanders D.S., Simpson J.E., Tongiorgi E., Williamson C.A., Woodroofe N.M. (2002). The humoral response in the pathogenesis of gluten ataxia. *Neurology.* **58**: 1221-1226.

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The humoral response in the pathogenesis of gluten ataxia

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Abstract—*Objective:* To characterize humoral response to cerebellum in patients with gluten ataxia. *Background:* Gluten ataxia is a common neurologic manifestation of gluten sensitivity. *Methods:* The authors assessed the reactivity of sera from patients with gluten ataxia (13), newly diagnosed patients with celiac disease without neurologic dysfunction (24), patients with other causes of cerebellar degeneration (11), and healthy control subjects (17) using indirect immunocytochemistry on human cerebellar and rat CNS tissue. Cross-reactivity of a commercial IgG antigliadin antibody with human cerebellar tissue also was studied. *Results:* Sera from 12 of 13 patients with gluten ataxia stained Purkinje cells strongly. Less intense staining was seen in some but not all sera from patients with newly diagnosed celiac disease without neurologic dysfunction. At high dilutions (1:800) staining was seen only with sera from patients with gluten ataxia but not in control subjects. Sera from patients with gluten ataxia also stained some brainstem and cortical neurons in rat CNS tissue. Commercial anti-gliadin antibody stained human Purkinje cells in a similar manner. Adsorption of the antigliadin antibodies using crude gliadin abolished the staining in patients with celiac disease without neurologic dysfunction, but not in those with gluten ataxia. *Conclusions:* Patients with gluten ataxia have antibodies against Purkinje cells. Antigliadin antibodies cross-react with epitopes on Purkinje cells.

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Gluten sensitivity is an immune-mediated disease triggered by the ingestion of gluten in genetically susceptible individuals.[1] Genetic susceptibility in gluten sensitivity is apparent by the fact that up to 90% of patients with celiac disease (CD) express the HLA class II molecules DQ2. The remaining 10% express HLA DQ8.

Gastrointestinal symptoms because of involvement f the small bowel (CD or gluten sensitive enteropathy) re a common presenting feature. However clinical ⟨anifestations can be diverse, for example, the pruˑtic vesicular rash of dermatitis herpetiformis.[2] ˑeurologic manifestations were first reported in 16 ⟨atients with established CD who had gait ataxia, ith involvement of other areas of the central and ⟨eripheral nervous systems.[3] We have suggested ⟨hat gluten sensitivity can be presented solely with ⟨eurologic dysfunction,[4] with ataxia (gluten ataxia) ⟨eing the most frequent presentation.[5] Up to 90% of ⟨atients with dermatitis herpetiformis and 33% of ⟨atients presenting with neurologic dysfunction associated with gluten sensitivity also have CD.[4] The ⟨emaining patients have no histologic evidence of ⟨mall bowel involvement but have serologic markers ⟨erum anti-gliadin antibodies) and genetic suscepti-⟨ility (HLA DQ2) consistent with gluten sensitivity.

Based on a large epidemiologic study (>200 patients), gluten ataxia was found to account for 40% of cases with idiopathic sporadic cerebellar degeneration.[6] This figure was lower in two smaller studies.[7,8] Another study[9] found similarly high prevalence, but in both sporadic and familial ataxias. The number of patients (26 sporadic and 24 familial) was too small to derive any meaningful conclusions about the prevalence of gluten ataxia among familial ataxias. A larger study (117 sporadic and 55 familial)[6] found the prevalence of gluten sensitivity among familial ataxias to be the same as healthy control subjects. Possible reasons for these differences include that the prevalence of antigliadin antibodies in the population varies depending on the antigliadin assays used (5%[8] to 13%[6]) and that there is a possible geographic variability in the prevalence of gluten ataxia as there is in the prevalence of CD.

Neuropathologic findings found in patients with gluten ataxia when autopsied showed perivascular cuffing with inflammatory cells, predominantly affecting the cerebellum, and resulting in loss of Purkinje cells implying that the neurologic insult may be immune mediated. It is yet unknown whether such immune-mediated damage is primarily cellular or antibody driven. This article presents a study of the

Table *Summary of immunocytochemistry results using human and rat cerebellum*

| Tissue substrate | Group sera (n) | Dilutions and percentage of strong positive staining | | | | | |
		1:200	1:400	1:800	1:100	1:300	1:600
Human cerebellum	Gluten ataxia (5)	100%	100%	60%			
	Celiac disease no neurology (5)	40%	40%	0%			
	Other ataxias (5)	20%	0%	0%			
	Healthy control subjects (5)	20%	0%	0%			
Rat cerebellum	Gluten ataxia (10)				90%	90%	90%
	Celiac disease no neurology (19)				26%	26%	0%
	Other ataxias (6)				0%	0%	0%
	Normal control subjects (12)				0%	0%	0%

humoral response that may be implicated in such damage.

Methods. *Patient and control sera.* This was a dual center study carried out in Sheffield, UK, and Trieste, Italy. Sera from gluten ataxia and ataxia control patients were collected in Sheffield and used by both groups. Sera from healthy control subjects and patients with CD without neurologic dysfunction were collected at each center after informed consent was obtained. The South Sheffield Research Ethics Committee approved the study protocol. Sera were collected from 13 patients upon diagnosis with gluten ataxia (defined by the presence of IgG antigliadin antibodies and the absence of any other cause of ataxia including paraneoplastic cerebellar degeneration) attending the gluten sensitivity/neurology clinic at the Department of Clinical Neurology, The Royal Hallamshire ospital, Sheffield. Sera from 24 patients with newly diag-. osed CD but no evidence of neurologic dysfunction and 11 atients with other causes of cerebellar degeneration (2 riedreich ataxia, 5 autosomal dominant ataxia, 3 sporadic taxia, 1 ataxia postcerebellitis) were used as disease con- ol patients. Finally, samples from 17 healthy individuals ere used as healthy control subjects. All samples were tored at −70 °C immediately after collection.

CNS tissue. Immunocytochemistry using human cere- ellum was performed in Sheffield, UK. Immunocytochem- stry using rat cerebellum was performed in Trieste, Italy. uman cerebellar tissue from a patient with motor neuron ·sease was obtained at autopsy after consent from rela- ·ves. The tissue was snap-frozen in liquid nitrogen and tored in an airtight container at −70 °C. It was then ectioned in a cryostat (Bright Instruments, UK), and 10- m-thick sections were collected on Polysine-coated slides BDH, UK).

Rat CNS tissue was obtained from adult Sprague- awley rats. Procedures involving animals and their hus- andry were performed in accordance with European ommunity directives. Rat brains were fresh-frozen on dry ce, sectioned in a cryostat (Leica), and 10-μm sagittal ections were collected on gelatin-coated slides (Sigma, ∴). The slides were dried overnight at room temperature d stored at −80 °C until required.

Immunocytochemistry on rat and human CNS tissue. n human CNS tissue, sera from 5 patients from each oup were used at dilutions of 1:100, 1:200, 1:400, 1:800,

1:1,600, and 1:3,200. Anti-calbindin D28k antibody (Sig- ma) diluted 1:200 with phosphate buffered saline/0.05% Tween 20 (PBST) was used as a positive control for Pur- kinje cell staining; negative controls included sections in- cubated without either serum or antihuman IgG secondary antibody.

On rat CNS tissue, sera from 11 patients with gluten ataxia, 19 patients with CD but no neurologic illness, 6 patients with other causes of ataxia, and 12 healthy con- trol subjects were analyzed. All sera were used at dilutions of 1:100, 1:300, and 1:600. In addition, a subset of 3 sera from patients with gluten ataxia also was tested at 1:1,200 and 1:1,500.

CNS sections were warmed to room temperature and then washed in PBST for 10 minutes at room temperature. Sections were incubated for 1 hour at room temperature with either patient or healthy control sera. After incuba- tion with sera, sections were washed in PBST and incubated for 30 minutes at room temperature with a horseradish peroxidase-conjugated goat anti-human IgG antibody (Jack- son Immunoresearch Laboratories) diluted 1:200 (for rat tis- sue) or 1:500 (for human tissue) with PBST. After a second wash in PBST, sections were developed for 20 minutes at room temperature using a peroxidase substrate solution of 0.4 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.0135% hydrogen peroxide (Sigma). Sections were de- hydrated in methanol, cleared in methanol/xylene (1:1) fol- lowed by xylene, and then mounted.

Semiquantitative evaluation of the signal intensity of the rat and human CNS sections was performed indepen- dently by two blinded observers. Weak or strong positive staining was recorded if Purkinje cell staining went above background levels, and negative staining was recorded if Purkinje cell staining did not exceed background levels. Patient and control samples were run simultaneously with a concordance rate of 75% between the observers.

Based on the optimal serum dilution for rat CNS tissue, 1:600, reactivity with other rat brain structures using sera from 10 patients with gluten ataxia (and also 6 patients with CD but no neurologic dysfunction and 12 healthy control subjects) was investigated using the above method.

Immunocytochemistry on human CNS tissue using anti- gliadin antibody and patient sera before and after adsorp- tion with crude gliadin. Sera (four patients with gluten ataxia, three patients with CD without neurologic dysfunc- tion, and two healthy control subjects) were diluted to
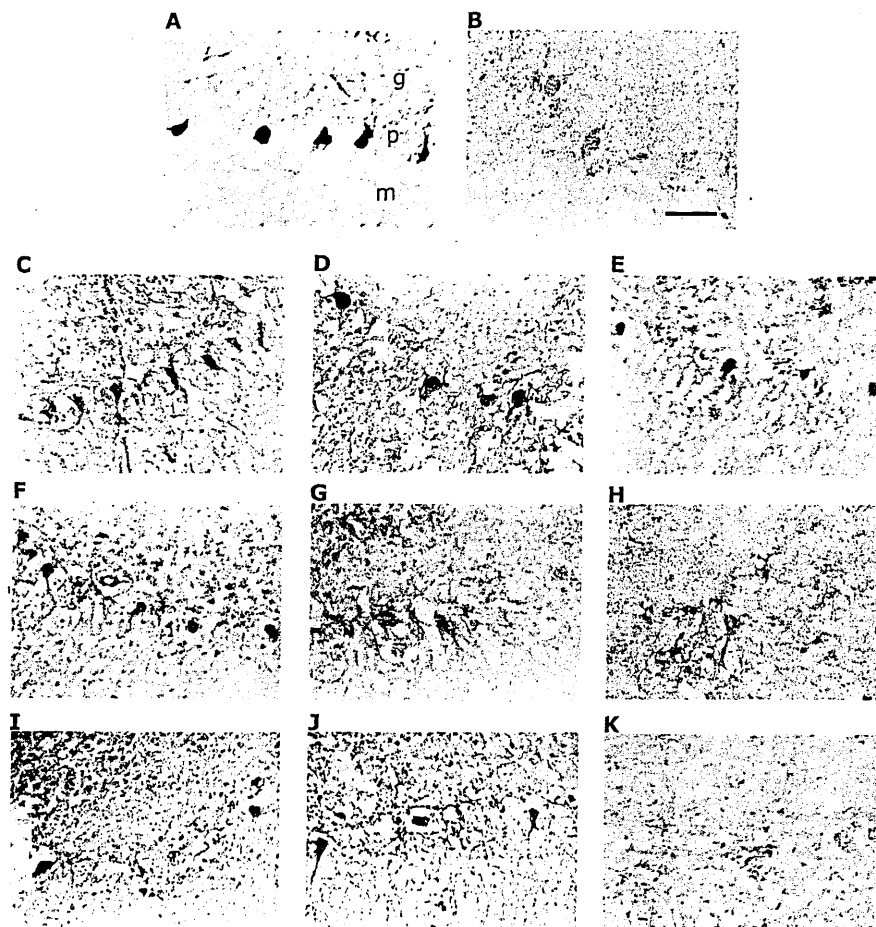
Figure 1. Immunohistochemical staining of human cerebellar Purkinje cells using anti-calbindin $D_{28}K$ antibody (A, positive control), anti-human IgG only (B, negative control), and sera at different dilutions from a patient with gluten ataxia (C-E), a healthy blood donor (F-H), and a patient with celiac disease without neurologic dysfunction (I-K). Dilutions used were 1:200 (C,F,I), 1:400 (D,G,J) and 1:800 (E,H,K). Calibration bar = 65 μm. g = granular layer; p = Purkinje cell layer; m = molecular layer.

:200 and 1:800 in PBST or in a saturated crude gliadin (Sigma) solution (~20 mg/mL) made up in PBST. Commercial rabbit polyclonal peroxidase-conjugated predominantly IgG anti-gliadin antibody (Sigma) was diluted from 1:100 to 1:256,000 in either PBST or a saturated crude gliadin (Sigma) solution (~20 mg/mL) in PBST. Once diluted, sera and commercial anti-gliadin antibody were incubated for 1 hour at room temperature to allow adsorption of anti-gliadin antibodies by the crude gliadin. The effectiveness blocking of antigliadin antibodies was tested by an anti-gliadin ELISA assay on the sera after adsorption. Immunocytochemistry was performed as above with adsorbed and nonadsorbed samples.

Results. *Characteristics of patients with gluten ataxia.* The mean age of the 13 patients with gluten ataxia was 61 years (range 46–79). The mean duration of the ataxia was [?] years (range 3–25). Ten patients had the HLA DQ2, one had DQ8, and two had DQ1. All patients were by definition positive for IgG antigliadin antibodies; four also had IgA antigliadin antibodies. Screening for antiendomysium (AEA) immunoreactivity and reaction with purified human tissue transglutaminase[10] (tTG) revealed only one patient with gluten ataxia that was strongly positive for AEA and tTG. Duodenal biopsies revealed CD in five patients, results were normal in six patients, and the procedure was not performed in two patients.

Immunocytochemistry on human CNS tissue. Consistently strong staining of Purkinje cells was seen using anti-calbindin $D_{28}K$ (figure 1A) as a positive control. Sec-

tions incubated either without primary or without secondary antibody showed consistently absent Purkinje cell staining (see figure 1B).

In the 5 patients with gluten ataxia, positive staining of Purkinje cells was seen at all dilutions up to 1:800 (see figure 1, C, D, and E) with strong staining up to 1:400. The staining persisted at higher dilutions in three patients. At dilutions of 1:800, sera from the control groups showed no staining (see figure 1, H and K). However, at dilutions of 1:200, staining was seen in 2 of 5 patients with CD but no neurologic illness (see figure 1I), in 1 of 5 patients with ataxia of a different cause, and in 1 of 5 healthy control subjects (see figure 1F).

Immunocytochemistry on rat CNS tissue. Sera from 12 of 13 patients with gluten ataxia showed strong staining of Purkinje cells at 1:100, 1:300, and 1:600 dilutions (figure 2, A through C), with marked staining particularly of cell somas and dendritic processes even at dilutions of 1:600. None of the subset of sera from three patients with gluten ataxia tested at 1:1,200 dilution showed any recognizable staining. In contrast, sera from 4 of 12 healthy control subjects showed only weak staining at 1:100 dilution (see figure 2D), with no further staining detected at higher dilutions (see figure 2, E and F). No staining was seen using serum from six control patients with ataxia. The sera from 5 of 19 patients with CD without neurologic dysfunction showed strong staining of Purkinje cells at 1:100 and 1:300 dilutions (see figure 2, G and H) but only weak staining at a dilution of 1:600 (see figure 2I). Weak staining was seen in nine patients and no staining was
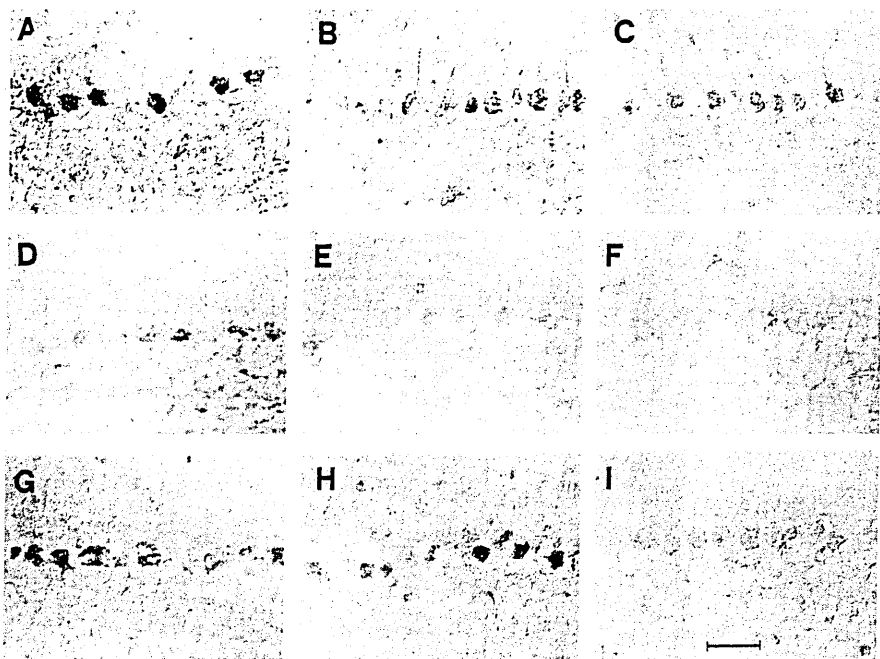
Figure 2. Immunohistochemical staining of rat cerebellar Purkinje cells using sera at different dilutions from a patient with gluten ataxia (A-C), a healthy blood donor (D-F), and a patient with celiac disease without neurologic dysfunction (G-I). Dilutions used were 1:100 (A,D,G), 1:300 (B,E,H) or 1:600 (C,F,I). Calibration bar = 65 μm.

seen in six. Sera from 10 patients with gluten ataxia showed weak or absent staining of the striatum, corpus callosum, hippocampus, brain blood vessels, choroid plexus, and ependyma. The more consistently stained cells in the cortex were large stellate-like cells (not pyramidal neurons) in cortical layers IV and V (figure 3A) and small polymorphic cells in the layer VI (see figure 3B), especially in the frontal cortex. Large neurons of the deep cerebellar nuclei (see figure 3C) and neurons of the reticular formation and pontine gray in the brainstem (see figure 3D) also stained. Sera from two of six patients with CD but no neurologic illness showed staining of cortical layer VI but no staining of any other structures. The sera from healthy control subjects did not stain cells in any brain area except for one case with weak staining in cortical layer VI.

Reactivity of sera and anti-gliadin antibody with human cerebellar tissue after adsorption with crude gliadin. Commercial rabbit anti-gliadin antibody stained human cerebellar Purkinje cells, at an optimal dilution of 1:800, with a pattern identical to that seen using sera from patients with gluten ataxia.

Staining with commercial anti-gliadin antibody at a dilution of 1:1,600 (figure 4A) was abolished after adsorption of the antibody for 1 hour at room temperature with crude gliadin (see figure 4B). Comparative adsorption experiments were performed using sera (dilution 1:200) from patients with gluten ataxia (see figure 4; C and D) and patients with CD but no neurologic dysfunction (see figure 4, E and F). The staining persisted but was weaker even after adsorption with gliadin when using sera from patients with gluten ataxia. The staining was eliminated when using sera from patients with CD and no neurologic dysfunction. None of the postadsorption sera tested positive for the presence of antigliadin antibodies.

A summary of all immunocytochemistry results can be seen in the table.

Discussion. These results suggest that patients with gluten ataxia have circulating antibodies directed against cerebellar Purkinje cells, with some reactivity seen with deep cerebellar nuclei brainstem and cortical neurons. Some weak staining with both rat and human cerebellum was seen using sera from other patient groups. At low dilutions some sera from normal control subjects also showed weak staining. Such staining of Purkinje cells at low dilutions is thought to be nonspecific and has been described before.[11] Strong and persistent staining at higher dilutions was only seen when using sera from patients with gluten ataxia. The absence of staining using sera from the group of patients with other causes of ataxia suggests that this is not just an epiphenomenon of Purkinje cell loss. However, the presence of similar although weaker staining with sera from only some patients with CD without neurologic dysfunction implies that these antibodies are not necessarily neurotoxic. The demonstration of reactivity of commercial IgG antigliadin antibody with human Purkinje cells may be the reason why sera from patients with CD but no neurologic dysfunction stain Purkinje cells. However, patients with gluten ataxia possess additional antibodies against Purkinje cells as demonstrated by the adsorption experiments. These antibodies are different to antigliadin antibodies and appear to be present exclusively in patients with gluten ataxia. Antigliadin antibodies are present in almost all patients with celiac disease with gastrointestinal symptoms. Yet only 6% to 10% of patients with established CD develop neurologic dysfunction. One hypothesis may be that antigliadin antibodies only become neurotoxic if they gain access to the CNS. Antigliadin antibodies have been found in the CSF in patients with gluten sensitivity and neurologic dysfunction.[12] The mechanism by which and reason why antigliadin antibodies may gain access to the CNS remains obscure. The presence of
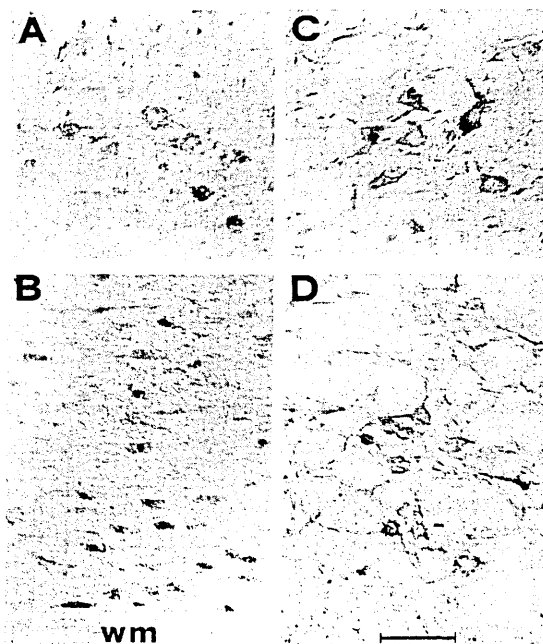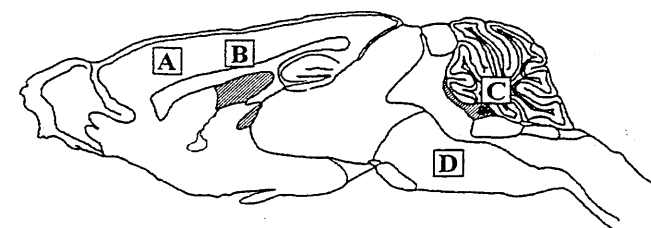
Figure 3. Immunohistochemical staining obtained using serum from a newly diagnosed untreated patient with gluten ataxia in different brain areas (dilution 1:600). Purkinje cell staining is shown in figure 2A. The approximate position of the high magnification photographs is indicated by the letters marked on the camera lucida drawing of a sagittal section of rat brain (top panel). In the cortex, only a subset of cells was stained, comprising principally horizontal or polymorphic cells in layers V (A) and VI (B). The deep cerebellar nuclei stained strongly at the level of neuronal cell somas and processes (C), as did the pontine gray nuclei (D). Calibration bar = 65 μm. wm = white matter.
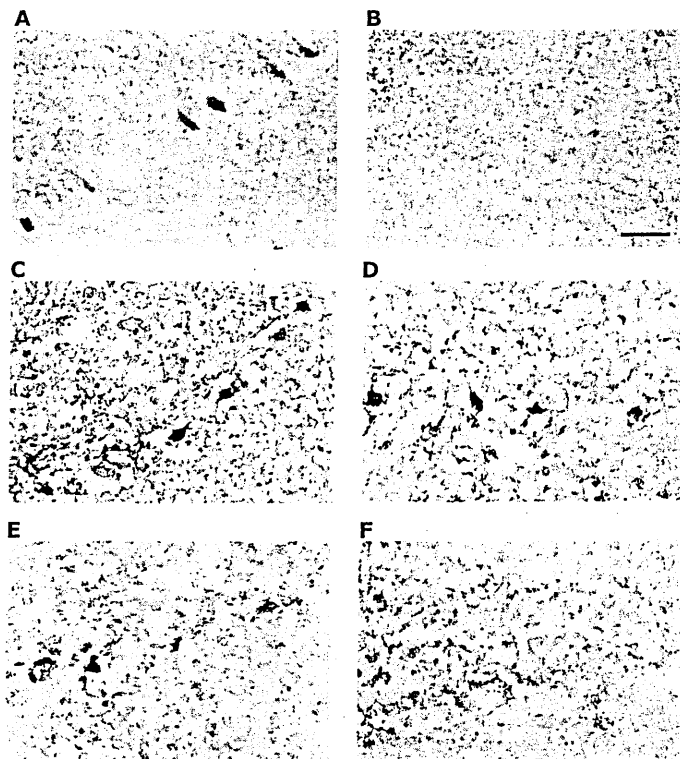

Figure 4. Immunohistochemical staining of human Purkinje cells using a commercial rabbit anti-gliadin antibody at a dilution of 1:1600 (A), serum from a patient with gluten ataxia at dilution 1:200 (C), and serum from a patient with celiac disease without neurologic dysfunction at dilution 1:200 (E), both before (A,C,E) and after adsorption (B,D,F) of antigliadin antibodies by incubation with crude gliadin. Calibration bar = 65 μm.

lymphocytic infiltration of the perivascular space and the neuropil by CD4 and CD8 T cells[13] in cases of gluten ataxia suggests cell-mediated responses may also play a part. Cell-mediated inflammation may compromise the blood–brain barrier allowing for the entry of antigliadin antibodies into the CNS.

It remains unclear why some patients with gluten sensitivity present solely with neurologic dysfunction whereas others have gastrointestinal symptoms or a pruritic rash. Genetic susceptibility may have a small role. Although 70% of patients with gluten ataxia have the HLA DQ2 (found in 90% of patients with CD) and 10% have the HLA DQ8 (usually found in the remaining 10% of patients with CD), 20% have the HLA DQ1. HLA DQ1 has not yet been reported in association with celiac disease but may be an important difference in genetic susceptibility to gluten ataxia compared with CD.

The staining of Purkinje cells using sera from patients with gluten ataxia agrees with the neuropathologic findings from postmortem examination of patients with gluten ataxia.[3,5] The most consistent finding is the loss of Purkinje cells. Although the cerebellum and in particular the Purkinje cells appear to be most susceptible to damage in patients with gluten ataxia, other areas of the brain are not spared. Our results demonstrate that sera from patients with gluten ataxia react with both brainstem and cortical neurones in rat CNS tissue. Such involvement has been reported both clinically and neuropathologically: patients with gluten ataxia may exhibit myoclonus (possibly cortical in origin[14]), dementia,[15] and brainstem signs.[16]

The cross-reactivity of anti-gliadin antibodies with cerebellar Purkinje cells suggests that gliadin proteins and cerebellar Purkinje cells share common epitopes. Such common epitopes also have been demonstrated to exist between gliadin proteins and enterocytes.[17] A recent study investigating human genome search in CD using gliadin cDNA as probe demonstrated that several genes have partial gliadin homology.[18] The nature of the antigens recognized by sera from patients with gluten ataxia is still unknown. A specific large scale screening approach of

uman brain expression libraries is envisaged to chieve the identification of antigens recognized by e gluten ataxia antibodies. Characterization of ese antibodies by immunoblotting may provide a eful marker for the diagnosis of gluten ataxia in a anner analogous to the use of specific antibodies as arkers of paraneoplastic cerebellar degeneration.

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Gluten ataxia in perspective: epidemiology, genetic susceptibility and clinical characteristics

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Summary

We previously have described a group of patients with gluten sensitivity presenting with ataxia (gluten ataxia) and suggested that this disease entity may account for a large number of patients with sporadic idiopathic ataxia. We have therefore investigated the prevalence of gluten sensitivity amongst a large cohort of patients with sporadic and familial ataxia and looked at possible genetic predisposition to gluten sensitivity amongst these groups. Two hundred and twenty-four patients with various causes of ataxia from North Trent (59 familial and/or positive testing for spinocerebellar ataxias 1, 2, 3, 6 and 7, nd Friedreich's ataxia, 132 sporadic idiopathic and 3 clinically probable cerebellar variant of multiple stem atrophy MSA-C) and 44 patients with spordic idiopathic ataxia from The Institute of eurology, London, were screened for the presence f antigliadin antibodies. A total of 1200 volunteers ere screened as normal controls. The prevalence of tigliadin antibodies in the familial group was eight ut of 59 (14%), 54 out of 132 (41%) in the sporic idiopathic group, five out of 33 (15%) in the SA-C group and 149 out of 1200 (12%) in the normal controls. The prevalence in the sporadic idiopathic group from London was 14 out of 44 (32%). The difference in prevalence between the idiopathic sporadic groups and the other groups was highly significant (P < 0.0001 and P < 0.003, respectively). The clinical characteristics of 68 patients with gluten ataxia were as follows: the mean age at onset of the ataxia was 48 years (range 14–81 years) with a mean duration of the ataxia of 9.7 years (range 1–40 years). Ocular signs were observed in 84% and dysarthria in 66%. Upper limb ataxia was evident in 75%, lower limb ataxia in 90% and gait ataxia in 100% of patients. Gastrointestinal symptoms were present in only 13%. MRI revealed atrophy of the cerebellum in 79% and white matter hyperintensities in 19%. Forty-five percent of patients had neurophysiological evidence of a sensorimotor axonal neuropathy. Gluten-sensitive enteropathy was found in 24%. HLA DQ2 was present in 72% of patients. Gluten ataxia is therefore the single most common cause of sporadic idiopathic ataxia. Antigliadin antibody testing is essential at first presentation of patients with sporadic ataxia.

troduction

e term 'gluten sensitivity' refers to a state of heightened unological responsiveness to ingested gluten in genetic-susceptible individuals (Marsh, 1995). Gastrointestinal ptoms caused by involvement of the small bowel (coeliac ase, also known as gluten-sensitive enteropathy) have in past been considered the most common presenting re. Prevalence studies have shown that coeliac disease affects up to 1% of the normal population (Fasano, 2001). It has been estimated that for every one patient with coeliac disease who presents with gastrointestinal complaints, there are seven patients with coeliac disease who have no gastrointestinal symptoms (Fasano, 2001). There is evidence to suggest that the small bowel is no longer the sole protagonist in gluten sensitivity. An itchy vesicular rash due

to skin involvement (dermatitis herpetiformis) has been recognized as a separate manifestation since 1966 (Marks *et al.*, 1966). Although neurological complications have been reported in association with established coeliac disease (Cooke and Smith, 1966), we have shown that gluten sensitivity can present solely with neurological dysfunction (Hadjivassiliou *et al.*, 1996). Ataxia (gluten ataxia) is the most common neurological manifestation of gluten sensitivity (Hadjivassiliou *et al.*, 1998). Organ-specific manifestations can occur in isolation or in combination with one another. Only a proportion of patients presenting with neurological dysfunction associated with gluten sensitivity will also have an enteropathy (Hadjivassiliou *et al.*, 1999). The remaining patients have no histological evidence of small bowel involvement but have serological markers (serum antigliadin antibodies) in keeping with gluten sensitivity, a situation analogous to dematitis herpetiformis. Genetic susceptibility in the form of the HLA typing may play an important role in this disease. HLA DQ2 is found in up to 90% of patients with coeliac disease.

Referral bias in units with an interest in genetic ataxias has produced an impression of relative rarity of idiopathic sporadic cases. One aspect of this study was to look at the prevalence of familial and sporadic ataxias in an unselected population from North Trent served by the Department of Neurology, The Royal Hallamshire, Sheffield. We have suggested that gluten ataxia may account for a large number of patients with idiopathic sporadic ataxia. We present the prevalence of gluten sensitivity amongst a large cohort of patients with sporadic and familial ataxia and investigate possible genetic predisposition to gluten sensitivity amongst these groups. In addition, we present the clinical and radiological characteristics of the largest ever reported cohort of 68 patients with gluten ataxia.

Methods
Patient selection
Over the last 8 years, we have reviewed prospectively and followed-up at 6 monthly intervals all patients with the diagnosis of cerebellar degeneration seen in a specially formed ataxia clinic based at the Department of Neurology at the Royal Hallamshire Hospital, Sheffield. The patients were identified from the disease register of the Department of Neurology, referrals from other neurology consultant colleagues based in North Trent and from the database of the regional Department of Molecular Genetics. Patients were divided into three groups. The first group consisted of patients with a family history of ataxia (autosomal recessive or dominant) and/or positive genetic testing for one of spinocerebellar ataxias (SCAs) 1, 2, 3, 6 and 7, and Friedreich's ataxia. The second group consisted of patients with sporadic idiopathic cerebellar degeneration without clinical evidence of the cerebellar variant of multiple system atrophy (MSA-C). The third group consisted of patients with

clinically probable MSA-C as defined by the consensus statement on the diagnosis of multiple system atrophy (Gilman *et al.*, 1999). Patients with a history of alcohol abuse, prolonged use of the anticonvulsant medication phenytoin, laboratory evidence of multiple sclerosis, vitamin E deficiency, paraneoplastic cerebellar degeneration and viral cerebellitis were excluded. In an attempt to eliminate possible referral bias associated with our department's involvement in research into the neurological manifestations of gluten sensitivity, we also screened a group of patients with sporadic idiopathic ataxia identified from the database of the ataxia clinic of the Institute of Neurology, Queen Square, London. All of these patients had been labelled as suffering from idiopathic late onset cerebellar ataxia, a term introduced by the late Professor Harding but now synonymous with sporadic idiopathic ataxia. Normal volunteers from the Trent region attending their primary care physician for unrelated problems (e.g. obtaining repeat prescription, accompanying relatives to their GP, common cold, etc.) were screened for the presence of IgG and/or IgA antigliadin antibodies to estimate the prevalence of such antibodies in the local population. The study was approved by the South Sheffield Ethics Committee. All patients were tested for antigliadin antibodies, and the first 169 consecutive patients (apart from the normal volunteers group) also had their HLA type determined. All patients underwent full neurological examination. Patients with gluten ataxia were referred for duodenal biopsy.

Antigliadin antibody estimation and HLA typing
IgG and IgA antigliadin antibody estimation was done using a commercially available enzyme-linked immunoassay kit (ELISA) kit (Cogent Diagnostics Ltd, Edinburgh, UK) according to the manufacturers' instructions. Briefly, 96-well microtitre plates pre-coated with purified gliadin were used. Test serum, positive and negative controls (all diluted to 1:100) or standards were added and incubated at room temperature for 30 min. Antigliadin antibodies were detected following incubation at room temperature for 15 min with mouse anti-human IgG and IgA horseradish peroxidase conjugate and subsequent development with tetra-methyl benzidine (TMB) substrate at room temperature for 15 min. Sample optical density units were converted to Cogent arbitary units (U/ml) using the pre-calibrated standards. Concentrations of controls and standards had to fall within pre-defined limits for acceptance of assay validity.

HLA typing was performed at the regional blood transfusion unit.

Duodenal biopsies
Duodenal biopsies were taken from the distal duodenum using biopsy forceps, through a conventional forward viewing endoscope (Key-Med, Southend, UK). Four biopsies were taken from the third part of the duodenum. The presence of
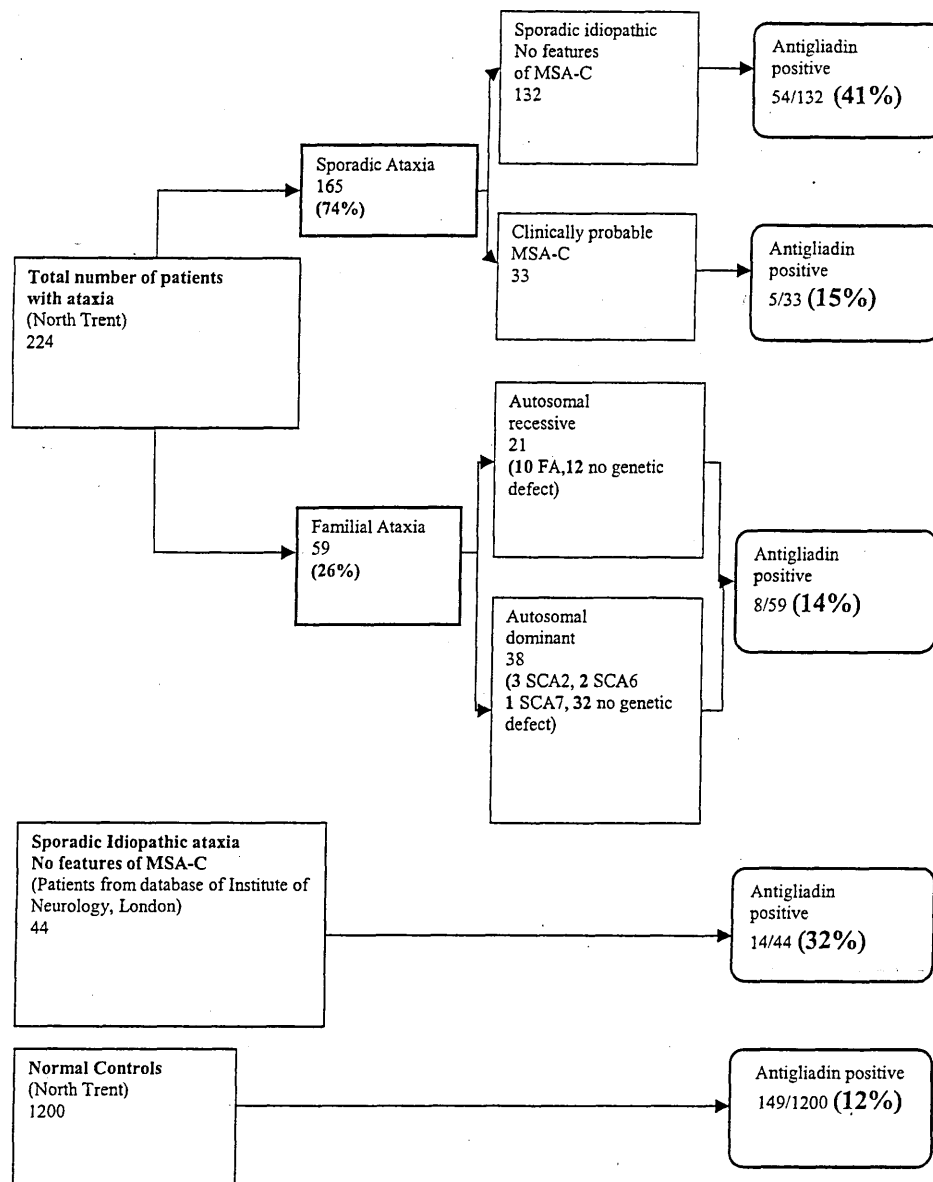
Fig. 1 The prevalence of antigliadin antibodies in ataxia and control groups. FA = Friedreich's ataxia; SCA = spinocerebellar ataxia, MSA-C = cerebellar variant of multiple system atrophy.

gluten-sensitive enteropathy was established by histological examination looking for evidence of crypt hyperplasia, villous atrophy and increase in intraepithelial lymphocytes.

Statistical analysis

The χ^2 test was used for comparing the prevalence of antigliadin antibodies in each of the groups with that of the normal controls group.

Results

Figure 1 summarizes the prevalence of IgG and/or IgA antigliadin antibodies in the various groups. A total of 268 patients with ataxia were screened, 224 from the Trent region and 44 patients with sporadic idiopathic ataxia without evidence of MSA-C taken from the ataxia database of the Institute of Neurology, London. Of the 224 patients from the Trent region, 59 had a family history (36 autosomal dominant, 23 autosomal recessive) and/or were positive on genetic testing for SCA 2 (three patients), SCA 6 (two patients), SCA 7 (one patient) and Friedreich's ataxia (10 patients). A total of 132 patients had sporadic idiopathic ataxia without any evidence of MSA-C. Thirty-three patients had clinically probable MSA-C. All 165 patients from the latter two groups tested negative for SCA 1, 2, 3, 6 and 7, and Friedreich's ataxia. Forty-four patients with sporadic idio-pathic ataxia without evidence of MSA-C taken from the

database of the Institute of Neurology, London were screened for gluten sensitivity but only 31 attended for clinical examination. A total of 1200 normal volunteers were screened as controls.

The prevalence of significant titres of circulating IgG and/ or IgA antigliadin antibodies amongst healthy control subjects from the Trent region was 149 out of 1200 (12%). This was not significantly different from the prevalence in patients with familial ataxia, where the antigliadin antibodies were present in eight out of 59 (14%). Of these eight patients, three had Friedreich's ataxia, one had SCA2 and one had SCA7. The remaining three had no genetic diagnosis. The prevalence in familial ataxia compares with 54 out of 132 (41%) in the group with sporadic idiopathic ataxia without features of MSA-C group from North Trent ($P < 0.0001$), five out of 33 (15%) in the group with clinically probable MSA-C

Table 1 *Characteristics of 68 patients with gluten ataxia*

Male to female ratio	35 : 33
Mean age at onset of ataxia	48 years
(range)	(14–78 years)
Mean duration of ataxia	9.7 years
(range)	(1–40 years)
Occular signs	84%
Dysarthria	66%
Upper limb ataxia	75%
Lower limb ataxia	90%
Gait ataxia	100%
Gastrointestinal symptoms	13%
Cerebellar atrophy on MRI	79%
White matter hyperintensities on MRI	19%
Sensorimotor axonal neuropathy on neurophysiology	45%
Gluten-sensitive enteropathy on biopsy	24%
HLA DQ2	72%

(not significant) and 14 out of 44 (32%) in the group with sporadic idiopathic ataxia from The Institute of Neurology, London ($P < 0.003$). Three of the eight antigliadin antibody-positive patients in the familial group had IgA antigliadin antibodies, but all were also IgG antigliadin positive. One of the five antigliadin antibody-positive patients in the MSA-C group was positive for IgA antigliadin antibody only. Only six out of 68 patients with gluten ataxia had IgA without IgG antigliadin antibodies.

HLA DQ2 was found in 27 out of 70 (39%) patients tested with sporadic idiopathic ataxia and in 13 of 31 patients tested (40%) with familial ataxia (not all patients from each group had HLA typing performed). The prevalence of HLA DQ2 amongst normal controls from the Trent region was 35% (data from the regional Blood Transfusion Unit); thus the prevalence of DQ2 in patients with sporadic idiopathic ataxia (without gluten sensitivity) and in those with familial ataxia was not significantly different from the control population. HLA DQ2 was present in 49 out of 68 (72%) patients with gluten ataxia (54 patients from North Trent plus 14 from The Institute of Neurology, London). Six percent had the HLA DQ8 and the remaining 22% had HLA DQ1.

Table 1 summarizes the clinical, radiological and neurophysiological characteristics of 68 patients with gluten ataxia (54 patients from North Trent plus 14 from The Institute of Neurology, London). There were 35 men and 33 women. The mean age at the onset of the ataxia was 48 years (range 14–81 years). The mean duration of the ataxia was 9.7 years (range 1–40 years). Ocular signs (spontaneous and gaze-evoked nystagmus and/or abnormal saccades) were observed in 84% of patients. Dysarthria was present in 66%. Upper limb ataxia was present in 75%, lower limb ataxia in 90% and gait ataxia in 100% of patients. Gastrointestinal symptoms (diarrhoea, abdominal bloating, weight loss) were present in only 13% of
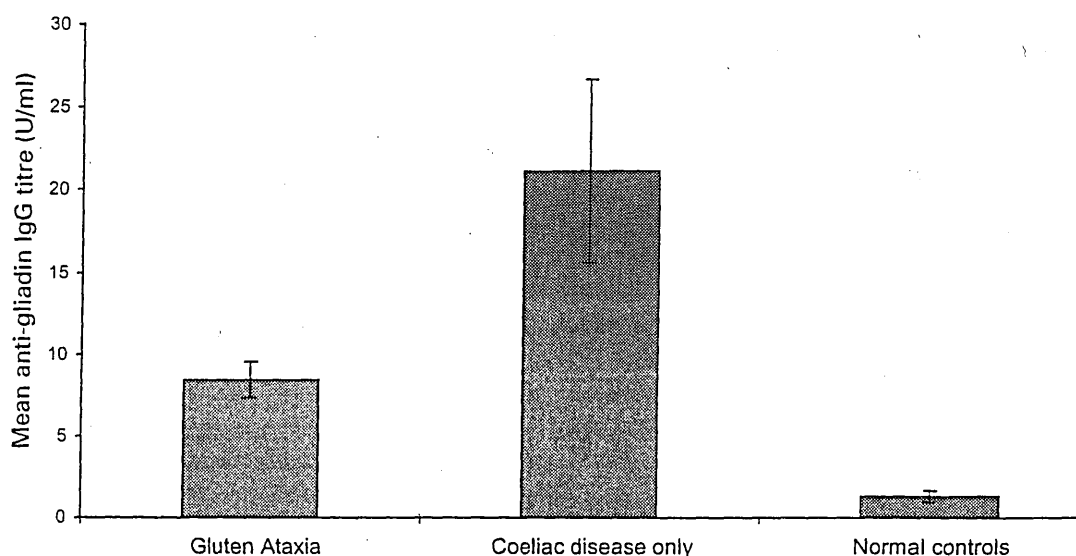

Fig. 2 Quantitative IgG antigliadin antibody titre in 30 patients with gluten ataxia, 13 patients with coeliac disease without neurological illness and 12 healthy control subjects.

patients. There was no correlation between the presence of gastrointestinal symptoms and evidence of an enteropathy on biopsy. MRI revealed atrophy of the cerebellum in 79% and white matter hyperintensities in 19% of patients. Forty-five percent of patients had neurophysiological evidence of a sensorimotor axonal neuropathy. Evidence of coeliac disease was found in 12 out of 51 (24%) patients with gluten ataxia who underwent gastroscopy and duodenal biopsy. IgG antigliadin antibody titre was higher in a cohort of 13 patients with coeliac disease and no neurological deficit when compared with a cohort of 30 patients with gluten ataxia (Fig. 2).

Discussion

In our experience of 59 patients with familial ataxia and/or positive genetic testing for SCA 1, 2, 3, 6 and 7, and Friedreich's ataxia, genetic diagnosis was only achieved in 16 (27%), leaving 73% without a genetic diagnosis. Sporadic idiopathic ataxias still account for the vast majority of patients with ataxia (74%). Of these, 20% had clinically probable MSA-C. Thus, in the majority of patients, the aetiology of ataxia still remains obscure. Our finding that gluten ataxia accounts for up to 41% of cases of sporadic idiopathic ataxia makes it the single most common cause of ataxia in this cohort of patients. This figure was slightly lower (32%) in patients with sporadic idiopathic ataxia from The Institute of Neurology in London, but the difference was not significant. The prevalence of gluten sensitivity amongst familial ataxias as well as clinically probable MSA-C from the Trent region was no different from that found in the normal population, suggesting no aetiological link between these types of ataxia and gluten sensitivity. There was, however, a small but not significant trend for the prevalence of gluten sensitivity in the MSA-C group to be higher than in the other control groups. This may reflect the fact that definite diagnosis of MSA-C on clinical grounds is impossible. Thus it is conceivable that a small number of patients with clinically probable MSA-C may prove to have gluten ataxia. This can only be determined by their response to gluten-free diet or by the pattern of progression of the disease.

Following our first publication on gluten ataxia (Hadjivassiliou *et al.*, 1998), other groups have published prevalence figures. The small number of patients screened and the lack of information on the prevalence of gluten sensitivity amongst the control population limits the value of some reports.

In a study from Italy (Pellecchia *et al.*, 1999), patients with sporadic ataxia were found to be more likely to have coeliac disease (three out of 24) than a group of patients with familial ataxia (zero out of 23). The authors did not quote the prevalence of antigliadin antibodies in their normal population and did not report any patients with circulating antigliadin antibodies who did not have coeliac disease. All their patients with ataxia and coeliac disease had circulating IgG antigliadin antibodies but not necessarily IgA antigliadin

or antiendomysium antibodies. Yet the last two antibodies are reported to be highly specific for gluten-sensitive enteropathy (Fasano, 2001). This observation suggests that IgA antigliadin and antiendomysium antibodies may lack sensitivity and specificity when used in a neurological population. Their observations support our contention that IgG antigliadin antibody is a better marker of the whole spectrum of gluten sensitivity irrespective of the organ involved. Possible reasons why these authors did not find patients with ataxia and circulating IgG antigliadin antibodies who did not have an enteropathy (representing 76% of our patients with gluten ataxia) include the fact that their antigliadin assay is set so as to have high specificity for coeliac disease perhaps at the expense of low sensitivity. This is what we have demonstrated by showing that the IgG antigliadin antibody titre in patients with gluten ataxia is lower than that in patients with coeliac disease without neurological illness. By increasing the antigliadin assay specificity for coeliac disease (i.e. increasing the threshold antibody titre defined as 'positive'), the sensitivity for the diagnosis of gluten ataxia will be reduced. Given that only 24% of our patients with gluten ataxia had gluten-sensitive enteropathy, potentially 76% of these patients may remain undiagnosed if such a high threshold value for positivity is adopted.

In a study from Germany (Bürk *et al.*, 2001), the prevalence of gluten ataxia amongst sporadic idiopathic ataxias was found to be 12 out of 104 (11.5%). Like us, the authors found a number of patients with positive IgG antigliadin antibodies and no enteropathy, but the appropriate HLA for coeliac disease. The prevalence of IgG antigliadin antibodies amongst normal controls was said to be 5%. This is lower than the 12% we have found in this study but again may relate to the assay. The authors did not attempt to separate those patients with clinically probable MSA-C from the idiopathic ataxia group. A more recent study from Germany (Abele *et al.*, 2002) found the prevalence of gluten sensitivity in the sporadic ataxia group to be 13% compared with 6% in patients with genetic ataxias and 5% in the normal population. The prevalence of antigliadin antibody positivity in patients with MSA-C was 9%. The authors, however, included not just patients with clinically probable MSA-C but also patients with possible MSA-C, thus potentially reducing further the accuracy of the clinical diagnosis of MSA-C.

A study from the USA (Bushara *et al.*, 2001) found a high prevalence of antigliadin antibody positivity in both sporadic (27%) and familial ataxias (37%). The numbers screened were small (26 sporadic and 24 familial). The prevalence of IgG antigliadin antibody alone, however, was higher in the sporadic ataxia group (15%) than the familial group (8%). Those authors offer no figure for the prevalence of antigliadin antibodies in the normal population for comparison.

A study from Finland (Luostarinen *et al.*, 2001) found the prevalence of coeliac disease in sporadic ataxias (44 patients) to be 16.7%, with the prevalence of coeliac disease in the normal population being ~1.6%.

A report from Spain (Combarros *et al.*, 2000) describes 32 patients with idiopathic ataxia who were screened but not found to have antigliadin antibodies. From the limited clinical data provided, 16 of these patients had features suggestive of MSA-C, leaving only 16 patients with sporadic idiopathic ataxia. The authors offer no figure for the prevalence of antigliadin antibodies in the normal population.

Finally, a much smaller study from Ireland (Lim *et al.*, 2001) reported coeliac disease in three of seven patients presenting with sporadic ataxia.

Despite possible methodological differences (e.g. antigliadin assay) and geographical variations that may contribute to differences in prevalence, all but one of these studies confirm our original findings of the existence of gluten ataxia as a disease entity. No combination of clinical features is sufficiently specific to enable a clinical diagnosis of gluten ataxia to be made with confidence, except perhaps in patients with established coeliac disease. The clinical features described in Table 1 are also seen in patients with other forms of sporadic and inherited ataxia, and this emphasizes the importance of maintaining a low threshold for suspicion of gluten sensitivity in patients presenting with ataxia.

IgG antigliadin antibodies by definition remain the best diagnostic marker for gluten ataxia. Although it is generally accepted that IgG antigliadin antibodies have a very high sensitivity for gluten-sensitive enteropathy, they are said by most gastroenterologists to lack specificity. In the context of a range of mucosal abnormalities seen in this disease, ranging from normal to irreversible hypoplastic atrophic lesions (Marsh, 1995), and the concept of potential coeliac disease (Maki *et al.*, 1991), meaning a histologically normal mucosa but altered T-cell subpopulations, IgG antigliadin antibodies may be the only available immunological marker for the whole spectrum of gluten sensitivity, of which gluten-sensitive enteropathy is only a part. Further support for our contention comes from our HLA studies. Within the group of patients with gluten ataxia (defined by the presence of IgG antigliadin antibodies), we have found an HLA association similar to that seen in patients with coeliac disease: 72% of patients have the HLA DQ2 (35% in the general population), 6% have the HLA DQ8 and the remainder have HLA DQ1.

We have looked at the prevalence of HLA DQ2 in both familial and sporadic idiopathic ataxia in an attempt to clarify whether the high prevalence of gluten ataxia in sporadic idiopathic ataxias may be related to a high prevalence of this HLA type within this group. Our results show no significant differences of the prevalence of this HLA type amongst normal controls, familial or sporadic idiopathic ataxias. The high prevalence of gluten ataxia in the group of patients with sporadic idiopathic ataxias cannot simply be explained on the grounds of the prevalence of HLA DQ2 within this group. It is likely, however, that the prevalence of HLA DQ2 in the general population may have some influence on the prevalence of gluten-related diseases. It would be interesting to study the prevalence of gluten ataxia in Japan where the prevalence of HLA DQ2 is only 1%.

The introduction of more coeliac disease-specific serological markers such as endomysium and, more recently, tissue transglutaminase antibodies may have helped in diagnosing gluten-sensitive enteropathy, but their sensitivity as markers of other manifestations of gluten sensitivity (where the bowel is not affected) is, by definition, low. This reflects our findings with patients with gluten ataxia. Endomysium and tissue transglutaminase antibodies are positive in the majority but not necessarily all of the gluten ataxia patients with an enteropathy. Patients with an enteropathy represent only a small proportion of patients with gluten ataxia (24%).

Intestinal mucosal damage in gluten-sensitive enteropathy is both humoral and T cell mediated. Such inflammation is not, however, confined to the gut, as activated HLA-restricted gliadin-specific T cells and antigliadin antibodies are found systemically (Sollid and Thorsby, 1993; Jensen *et al.*, 1995). Antigliadin antibodies are also found in the CSF (Chinnery *et al.*, 1997). Post-mortem findings from two of our patients with gluten ataxia has shown perivascular cuffing with both CD4 and CD8 cells. This inflammation was seen primarily in the white matter of the cerebellum. There was also marked but patchy Purkinje cell loss. We have also found antibodies against Purkinje cells in patients with gluten ataxia (Hadjivassiliou *et al.*, 2002b). Our research suggests that antigliadin antibodies cross-react with epitopes on Purkinje cells from human and rat cerebellum. Characterization of the anti-Purkinje cell antibodies by immunoblotting may provide a useful marker for the diagnosis of gluten ataxia in a manner analogous to the use of antiendomysium antibodies as a marker for coeliac disease, or the anti-Yo and other antibodies in paraneoplastic cerebellar degeneration. This will eliminate the potential problem of overdiagnosing gluten ataxia by 12% given that antigliadin antibodies are found in 12% of the normal population. What is unclear at present (due to lack of definition) is whether the prevalence of gluten sensitivity (with or without an enteropathy) in the general population could be as high as 12%.

Evidence is emerging that a gluten-free diet may be beneficial in the treatment of gluten ataxia in terms of both the symptoms of ataxia and the neurophysiological assessment of the peripheral neuropathy (Hadjivassiliou *et al.*, 2002a). The timing of the diagnosis and treatment of these patients appears to be crucial because of the loss of Purkinje cells which is irreversible. Thus, antigliadin antibodies should be an essential part of the investigation of patients with sporadic idiopathic ataxia at first presentation, and a gluten-free diet should be advised even in the absence of an enteropathy.

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