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THE IMMUNOGENETICS OF COMMON VARIABLE IMMUNODEFICIENCY

Dissertation submitted for the degree of Doctor of Medicine

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For Laura

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THE IMMUNOGENETICS OF COMMON VARIABLE IMMUNODEFICIENCY

Summary of a dissertation submitted for the degree of Doctor of Medicine, University of Adelaide, by Charles Grenfell Mullighan.

The aetiology of common variable immunodeficiency (CVID) is unknown. This thesis examines the role of polymorphisms in immunoregulatory genes in conferring susceptibility to this complex, heterogeneous disease.

It is shown that several HLA haplotypes are positively and negatively associated with CVID. No single allele or amino acid is associated with CVID more significantly than any other, suggesting that the primary HLA association is with an allele common to these haplotypes, such as in the HLA class III region. Patient subgrouping according to clinical and immunophenotypic criteria demonstrates that HLA alleles are associated with disease severity: HLA-DQB1 alleles are associated with immunophenotypic abnormalities, and tumour necrosis factor alleles with granulomatous disease.

Subsequently, the development of a unified method of efficiently genotyping large numbers of DNA polymorphisms simultaneously is described. Genes encoding adhesion molecules, cytokines, complement components and chemokine, hormone and immunoglobulin receptors are examined in CVID. Mutant alleles of the complement component mannose-binding lectin are associated with early age of onset of disease and autoimmunity in CVID, reinforcing the ostensible role of this molecule in innate immunity. Polymorphisms in the interleukin-10 gene, a key regulator of immune responses and antagonist of TNF production, interact with TNF polymorphisms in conferring susceptibility to granulomatous CVID. Polymorphisms in the vitamin D receptor gene and interleukin-6 genes are associated with multiple immunophenotypic abnormalities, providing further evidence that these abnormalities are genetically determined. The exceptional utility of this multiple candidate gene approach in dissecting the complex pathogenesis of CVID suggests

that this technique will be also be a powerful tool in the elucidation of the genetic basis of a broad range infectious and inflammatory conditions.

In summary, the immunogenetic predisposition to CVID is explored, revealing important new associations that increase understanding of this complex disease.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Charles Grenfell Mullighan
October 1 1997

CONTRIBUTION OF THIS THESIS TO MEDICAL SCIENCE AND PRACTICE

This study examined the genetic basis of common variable immunodeficiency (CVID). This complex, heterogenous disease is the commonest symptomatic primary immune deficiency, and a cause of substantial morbidity and premature mortality. Clinical, genetic, and immunophenotypic data on the largest recorded cohort of CVID patients was analysed. In addition, a unified method of simultaneously genotyping large numbers of DNA polymorphisms was developed. This permitted the efficient analysis of genes encoding adhesion molecules, cytokines, complement components and chemokine, hormone and immunoglobulin receptors, as well as classical HLA genes.

It was shown that polymorphisms in immunoregulatory genes influence the clinical manifestations of CVID. First, tumour necrosis factor and interleukin-10 alleles reciprocally interacted in conferring susceptibility to granulomatous CVID. Both genes encode mutually antagonistic immunoregulatory molecules of key importance. Secondly, mutant alleles of the complement component mannose-binding lectin were associated with early age of onset of disease and autoimmunity, reinforcing the ostensible role of this molecule in innate immunity. Finally, polymorphisms in the vitamin D receptor gene, HLA-DQB1 and interleukin-6 genes were associated with multiple immunophenotypic abnormalities, providing further evidence that these abnormalities are genetically determined.

In summary, the contributions of this study to medical knowledge and practice include an increased understanding of the immunopathogenesis of the CVID, and of the genetic regulation of the immune response. In addition, novel genotyping technology was developed that will be a powerful tool in the elucidation of the genetic basis of a broad range of infectious and inflammatory conditions.

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ABBREVIATIONS

ANCA	anti-neutrophil cytoplasmic antibody
AF	aetiologic fraction
APC	antigen presenting cell
ARMS	amplification refractory mutation system
bp	base pairs
CCR5	CC chemokine receptor 5
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity test
cDNA	complementary DNA
CI	confidence interval
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen
ConA	concanavalin A
CVID	common variable immunodeficiency
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP)
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FcγR	Fc gamma receptor
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
gpBIIIa	glycoprotein B IIIa
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HH	hereditary haemochromatosis
HIgM	hyper IgM syndrome
HITS	heparin-induced thrombocytopenia syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
hsp70	heat shock protein 70
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IDDM	insulin-dependent diabetes mellitus
IFNγ	interferon gamma
Ig	immunoglobulin
IgAD	IgA deficiency
IgH C	immunoglobulin heavy chain constant genes
IHW	International Histocompatibility Workshop
IL (-2, -4, -6, etc)	interleukin-2, -4, -6 etc
IU	international units
JRA	juvenile rheumatoid arthritis
kb	kilobase
kD	kilo Dalton
LTα	lymphotoxin alpha
LTβ	lymphotoxin beta

MASP	mannose-binding protein associated serine protease
Mbp	megabase pairs
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MI	myocardial infarction
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mRNA	messenger RNA
MS	multiple sclerosis
NDS	Nuffield Department of Surgery
NK	natural killer
NLB	nuclear lysis buffer
ns	not significant
OR	odds ratio
p	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PF	preventive fraction
PHA	phytohaemagglutinin
PWM	pokeweed mitogen
RA	rheumatoid arthritis
RBC	red blood cell
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RR	relative risk
SBP	systolic blood pressure
SCID	severe combine immunodeficiency
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SSOP	sequence-specific oligonucleotide probe
SSP	sequence-specific primers
TAP	transporters associated with antigen processing
TBE	Tris/boric acid/EDTA
TCR	T cell receptor
TDMH	10x base buffer (T), dNTP (D), $MgCl_2$ (M), H_20 (H)
TGFβ	transforming growth factor beta
Th (1,2)	T helper (1,2)
TNF	tumour necrosis factor
TNFRI, II, III	tumour necrosis factor receptor I, II, III
TRIS	2-amino-2-hydroxymethylpropane-1,2-diol
U	units
VDR	vitamin D receptor
VNTR	variable number tandem repeat
v/v	volume/volume
XLA	X-linked agammaglobulinmaemia
WHO	World Health Organisation
w/v	weight/volume

PUBLICATIONS ARISING FROM THIS THESIS

Mullighan CG, Welsh KI, Bird AG. HLA associations of common variable immunodeficiency. Immunology 1995;86:56.

Mullighan CG, Bunce M, Welsh KI. High resolution HLA-DQB1 typing by PCR-SSP. Tissue Antigens 1997; in press.

Mullighan CG, Fanning GC, Chapel HM, Welsh KI. Tumour necrosis factor and lymphotoxin alpha polymorphisms associated with common variable immunodeficiency: role in the pathogenesis of granulomatous disease. J Immunol 1997; in press.

Mullighan CG, Marshall SE, Fanning GC, Briggs DC, Welsh KI. Rapid haplotyping of mutations in the Duffy gene by PCR-SSP. Tissue Antigens 1997; in press.

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Manuscripts in preparation

Mullighan CG, Marshall SE, Bunce M, Fanning GC, Lympany P, Welsh KI. A unified method of genotyping multiple DNA polymorphisms simultaneously applied to common variable immunodeficiency.

Mullighan CG, Chapel HM, Welsh KI. A comprehensive reevaluation of the role of major histocompatibility complex polymorphisms in common variable immunodeficiency.

Fanning GC, Mullighan CG, Marshall SE, Koss K, Black CM, Welsh KI. TNF, LTα and related cytokine polymorphisms are associated with increased creatinine kinase activity and proximal muscle weakness in Systemic Sclerosis.

1. Introduction

1.1. COMMON VARIABLE IMMUNODEFICIENCY

Common variable immunodeficiency (CVID) is the commonest symptomatic primary immunodeficiency (Cunningham Rundles, 1989; Sneller et al, 1993; 1995). The clinical and immunological features of CVID are highly variable, suggesting that CVID may represent a heterogenous group of conditions, all of which share a fundamental defect in B lymphocyte differentiation, hypogammaglobulinaemia, inability to produce specific antibodies to previously encountered antigens, and susceptibility to recurrent infection. A number of other clinical features, such as autoimmunity, lymphoproliferative disease and malignancy are variably present. The aetiology of CVID is unknown. A genetic predisposition is suggested by familial clustering of CVID and the related condition IgA deficiency (IgAD), however the fundamental immunological defects in CVID and the molecular mechanisms responsible remain elusive.

1.1.1. Epidemiology

CVID prevalence rates in Caucasoid populations range from 1/50 000 to 1/200 000 (Fasth, 1982; McCluskey et al, 1989), affecting males and females approximately equally. Recent United Kingdom data report 17.4 cases per million males, and 13.3 per million females (Toolan et al, 1996). As the commonest clinical feature of CVID - sinopulmonary infection - is common in non-immunodeficient individuals, referral to tertiary immunological clinics is often delayed, or does not occur. Consequently these figures probably substantially underestimate prevalence. CVID may manifest at any age, but presentation in early childhood or in the second or third decades is typical (Hermans et al, 1976; Asherson, 1980; Cunningham Rundles, 1989).

1.1.2. Clinical features

1.1.2.1. Infection

Recurrent infection of the sinopulmonary tract with encapsulated bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* is an almost

universal manifestation of CVID. Infections may be severe, and if treatment is delayed, may result in irreversible lung damage. A small proportion of patients present with infections with organisms such as *Pneumocystis carinii*, mycobacteria, *Mycoplasma spp.* or fungi (Hermans et al, 1976; Asherson, 1980; Cunningham Rundles, 1989; Sneller et al, 1993), suggesting impaired cellular immunity as well as antibody deficiency. Most viral infections are handled normally in CVID (Webster, 1994), but there is an abnormally high occurrence of varicella zoster virus (VZV) (Asherson, 1980; Hermaszewski et al, 1993) and herpes simplex virus (HSV) reactivation (Straus et al, 1984). Severe human cytomegalovirus (HCMV) infection has also been reported (Freeman et al, 1977; Wong et al, 1995). An unusual manifestation of enterovirus infection, echoviral meningoencephalitis or dermatomyositis, has been observed in CVID, but is more commonly associated with X-linked agammaglobulinaemia (XLA) (McKinney et al, 1987; Rudge et al, 1996).

1.1.2.2. Autoimmunity

Approximately 20% of patients develop autoimmune disease, implying a fundamental defect in immunoregulation (Asherson, 1980; Conley et al, 1986; Cunningham Rundles, 1989; Hermaszewski et al, 1993; Lee et al, 1993). The commonest diseases are pernicious anaemia (Twomey et al, 1969; Borriello et al, 1985), autoimmune blood dyscrasias (Webster et al, 1981b; Leickly et al, 1987; Wong et al, 1995), alopecia, vitiligo (Spickett et al, 1991), and an aseptic polyarthropathy resembling rheumatoid arthritis (Conley et al, 1986; Espinoza et al, 1995). Less common autoimmune manifestations include autoimmune hepatitis and Sjögren's syndrome. Treatment is often difficult, as standard immunosuppressive therapy may further impair cellular and humoural immunity.

1.1.2.3. Gastrointestinal disease

Infectious and non-infectious enteropathies resulting in diarrhoea and malabsorption are common. *Giardia lamblia, Salmonella, Shigella and Campylobacter spp.* are the commonest pathogens (Webster, 1976; Ward et al, 1984; Hermaszewski et al, 1993; Washington et al, 1996). All typically cause

more severe and chronic diarrhoea and malabsorption than in immunocompetent hosts, and may require repeated and prolonged anti-microbial therapy.

Non-infectious malabsorption is well-recognised in CVID, and may cause hypoalbuminaemia, vitamin deficiency and worsening of hypogammaglobulinaemia. This enteropathy may histologically resemble coeliac disease (Ament et al, 1973; Ament, 1985). It is difficult to treat, and usually unresponsive to immunosuppressive agents, withdrawal of gluten or high dose immunoglobulin. True coeliac disease is uncommon (Webster et al, 1981a). Inflammatory bowel disease, particularly resembling microscopic colitis, also occurs more commonly in CVID than in the general population (Abramowsky et al, 1988; Teahon et al, 1994).

Lymphoproliferative disease commonly involves the gastrointestinal tract in CVID and may present similarly to the enteropathies described above.

1.1.2.4. Lymphoproliferative disease and malignancy

1.1.2.4.1. Lymphoma

Lymphoproliferative disease occurs in several forms in CVID. The overall risk of malignant lymphoma is at least 30 times that of the general population (Kinlen et al, 1985; Cunningham Rundles et al, 1991b; Sander et al, 1992; Espanol et al, 1993; Hermaszewski et al, 1993; Sneller et al, 1993). The majority of such lymphomas occur in females, resulting in a 400-fold increased risk of lymphoma in female patients in the fifth and sixth decades of life (Buckley, 1992). Non-Hodgkin's lymphoma (NHL) is the usual histologic type, although Hodgkin's disease and leukaemias are also more frequent in CVID. The tumours are commonly extranodal, particularly involving the gut. They are usually B cell-derived, but may be of T cell origin, and are often high grade and poorly responsive to treatment. Monitoring of CVID patients with polyclonal lymphoproliferative disease suggests that some lymphomas may be preceded by 'benign' lymphoproliferative disease or sarcoid-like granulomata for many years (Montalban et al, 1989; Chiaramonte et al, 1994).

1.1.2.4.2. Polyclonal lymphoproliferative disease

Nonmalignant lymphoproliferative disease is commoner in CVID than lymphoma, and may take one of three main forms (Sander et al, 1992). *Reactive lymphoid hyperplasia*, the most common lesion, is characterised by polyclonal lymphocyte proliferation with preservation of lymphoid architecture. *Atypical reactive lymphoid hyperplasia* is also polyclonal but lymphoid architecture is destroyed by the lymphoid infiltrate. This may be superficially mistaken for malignant lymphoma, but immunohistochemistry demonstrating admixture of T and B cells, or lymphocyte receptor gene rearrangement studies distinguishes the two conditions. When involving the gut, this process is termed nodular lymphoid hyperplasia (NLH), and consists of multiple discrete B lymphocyte aggregates confined to the lamina propria and submucosa (Ajdukiewicz et al, 1972; Caballero et al, 1984; Bastlein et al, 1988; Sander et al, 1992). NLH may be asymptomatic, or cause malabsorption (Bennett et al, 1987), haemorrhage (Freiman et al, 1985) or progress to lymphoma (Bastlein et al, 1988; Montalban et al, 1989; Chiaramonte et al, 1994). Non-caseating sarcoid-like *granulomata* are often observed in extra-nodal sites in CVID (Sander et al, 1992), especially the skin (Pierson et al, 1993; Levine et al, 1994), lung (James, 1992), brain (Misbah et al, 1992) and abdominal viscera (Mike et al, 1991; Pierson et al, 1993). These granulomata may be asymptomatic and ephemeral, or cause severe respiratory embarrassment or fulminant hepatitis. Diagnostic confusion and inappropriate treatment may also occur. The pathogenesis of granulomata and non-malignant lymphoproliferative disease is discussed in detail in chapter 4.

1.1.2.4.3. Non-lymphoid malignancy

Excess mortality from malignancy in CVID is also attributable to a 50-fold increased risk of gastric adenocarcinoma (Hermans et al, 1972; Kinlen et al, 1985; den Hartog et al, 1992; Hermaszewski et al, 1993). This is probably due to achlorhydria, as non-immunodeficient patients with pernicious anaemia and achlorhydria also have a increased risk of gastric cancer (Twomey et al, 1970; Webster, 1976; Eriksson et al, 1981). Immunodeficiency *per se* is not likely to be an important pathogenic factor, as individuals immunosuppressed long-term for other reasons do not exhibit a comparable increase in risk (Hanto et al, 1981).

1.1.3. Immunological assessment and diagnostic evaluation

Early diagnosis and thorough evaluation of end-organ complications such as bronchiectasis, coupled with vigorous treatment is essential in order to avoid unnecessary morbidity and mortality (Chapel, 1994). Parenteral IgG replacement is effective in reducing the frequency and severity of infections, and can also prevent, delay or even partly reverse bronchiectasis (Roifman et al, 1987; Eibl et al, 1989; Buckley et al, 1991; Sneller et al, 1993).

The hallmark of CVID is reduced serum IgG concentration in the absence of other secondary causes of immunodeficiency. Several other tests may be used corroborate the diagnosis, and exclude other conditions. These include enumeration of total leukocyte and lymphocyte counts, and lymphocyte subpopulations (usually by flow cytometry), measurement of specific antibodies, and assessment of the humoral response to immunisation (1995). Evaluation of T cell function is optional, and may be assessed *in vitro* by stimulation with mitogens (e.g. phytohaemagglutinin, concanavalin A or pokeweed mitogen), recall antigens (tetanus toxoid, candida) or antibodies to T cell membrane proteins (CD2, CD3, CD28, TcR). Proliferative responses may be assessed by incorporation of ^{3}H-thymidine or expression of activation markers (e.g. CD69); and cytokine production measured by intracellular flow cytometry or ELISA (North et al, 1996). Diagnostic and pathogenic implications of these assays are discussed below.

1.1.4. Laboratory abnormalities in CVID

1.1.4.1. Antibody levels and B cell function

Whilst the hallmark of CVID is a reduced serum concentration of IgG, the degree of reduction is variable, ranging from undetectable to mildly reduced IgG levels. Serum IgA is usually reduced or absent, and IgM is reduced in approximately 50% of cases. Hypogammaglobulinaemia is usually permanent, although levels may fluctuate, and rare cases of spontaneous normalisation of serum IgG levels, accompanied by amelioration of clinical symptoms, have been reported (Seggev, 1991; Seligmann et al, 1991; Johnson et al, 1997).

Peripheral blood B lymphocyte numbers are normal in the majority of patients, but are reduced or absent in 10-15% (Cunningham Rundles, 1989; Spickett et al, 1992). B cell function *in vivo* is clearly abnormal. In addition to low total IgG, most patients fail to mount appropriate specific antibody responses to infection or diagnostic immunisation, particularly to polysaccharide antigens. The ability of CVID B cells to secrete immunoglobulin in response to *in vitro* stimulation (such as with PWM) is variable, and has formed the basis for a classification of CVID patients (discussed below) (De Gast et al, 1980; Saiki et al, 1982; Bryant et al, 1990).

1.1.4.2. T cell phenotype and function

Approximately 30% of patients have reversal of the peripheral blood T lymphocyte CD4+:CD8+ ratio (Phan-Dinh-Tuy et al, 1981; Reinherz et al, 1981; Aiuti et al, 1983; Tedder et al, 1985; Wright et al, 1990; Jaffe et al, 1992; Spickett et al, 1992), attributable to CD4+ T lymphopenia (Lebranchu et al, 1991; Spickett et al, 1992), and/or CD8+ lymphocytosis (Pandolfi et al, 1983; Wright et al, 1990). CD4+CD45RA+ ('naive') T cell lymphopenia (Lebranchu et al, 1990; Lebranchu et al, 1991; Richards et al, 1992), and B lymphopenia (Cunningham Rundles, 1989; Spickett et al, 1992) are also common findings. Patients with CD8+ lymphocytosis typically have increased lymphocyte HLA-DR expression and CD8+CD57+ lymphocytosis (Wright et al, 1990; Baumert et al, 1992), suggesting substantial T cell activation. Raise serum β2-microglobulin and neopterin levels in patients with these immunophenotypic abnormalities also suggests an abnormal immune response (North et al, 1991a; Aukrust et al, 1992). Although T cell numbers and proliferative response to mitogens are often (but not always) normal (North et al, 1991b), more subtle T cell responses, such as cytokine production and proliferative responses to recall antigens, are commonly abnormal (Sneller et al, 1990a; Sneller et al, 1990b; Fischer et al, 1993a; Fischer et al, 1994a; Fischer et al, 1994c; Stagg et al, 1994;).

It is unclear whether these immunophenotypic disturbances are merely markers of a disturbed immune response, or if these quantitative abnormalities directly contribute to the pathogenesis of hypogammaglobulinaemia. The CD8+CD57+

expansion is particularly intriguing. This subset is also expanded following acute lymphotropic viral infections, such as with human immunodeficiency virus (Gupta, 1986), Epstein-Barr virus (Crawford et al, 1981) or cytomegalovirus (Wang et al, 1993). Indeed immunophenotypic abnormalities such as this led to speculation that CVID may result from a lymphotropic virus infection (Spickett et al, 1988). Furthermore, CD8+CD57+ cells suppress both B lymphocyte proliferation and immunoglobulin secretion (Clement et al, 1984), and removal of these cells from allogeneic CVID lymphocyte cultures results in restoration of *in vitro* immunoglobulin secretion in some CVID patients (Jaffe et al, 1993b). Similarly, the presence of CD4+CD45RA+ lymphopenia strongly correlates with B lymphopenia and profoundly abnormal immunoglobulin secretion *in vitro* (Farrant et al, 1994), yet it is not known if depletion of this subset is directly pathogenically relevant. The role of cellular abnormalities in the pathogenesis of CVID is discussed in detail below. Correlation between immunophenotypic abnormalities and clinical features is explored below and in chapters 3 and 4.

1.1.4.3. Correlation between lymphocyte phenotype and clinical features

Extensive clinical heterogeneity suggests that CVID may consist of several discrete diseases. Several classification systems for CVID, based on various combinations of clinical, immunophenotypic and functional lymphocyte abnormalities have been proposed. For example, associations of clinical features such as splenomegaly and lymphadenopathy with immunophenotypic and functional abnormalities such as CD4+ and CD4+CD45RA+ lymphopenia, CD8+CD57+ lymphocytosis and impaired T cell proliferation *in vitro* have been reported (Wright et al, 1990; Aukrust et al, 1995; Aukrust et al, 1996). Classification according to the ability of CVID B cells to secrete IgG, IgA and IgM when stimulated *in vitro* has also been described (De Gast et al, 1980; Saiki et al, 1982; Bryant et al, 1990). Correlation between impaired antibody secretion *in vitro* and immunophenotypic abnormalities such as CD4+ lymphopenia have been described.

A classification system comprehensively encompassing all clinical and laboratory features has recently been described (Spickett et al, 1997) (table 1.1). This proposes

that CVID comprises (at least) 4 distinct groups of patients. It is suggested that patients with no circulating B cells may not be true CVID, but rather XLA or autosomal 'XLA-like' immunodeficiency; 'group A' patients have marked immunophenotypic abnormalities and clinical features such as granulomata and splenomegaly, in addition to impaired *in vitro* immunoglobulin secretion; 'group B' patients have minor immunophenotypic abnormalities and secrete IgM but not IgG *in vitro*; and 'group C' patient have 'mild' disease, normal antibody secretion *in vitro*, no lymphoproliferative disease and a preponderance of autoimmune disease. It has also been suggested that each of these groups may have a discrete genetic diathesis, such as a preponderance of the HLA A*01-B*0801-DRB1*0301 haplotype in group C, to account for the high rate of autoimmune disease. However, this is only a working classification, and the clinical/immunophenotypic/genetic correlations described require confirmation in large groups of well characterised patients.

Table 1.1: Proposed classification of CVID

This classification has attempted to unify pre-existing classification systems based on clinical and laboratory criteria. Adapted from Spickett et al, 1997. nk = not known.
*The use of the term hyper-IgM to describe a subgroup of CVID patients is based on the observation that expression of CD40L is subnormal in many patients. However this is controversial, as serum IgM levels are not elevated in CVID, and CD40L mutations have not yet been demonstrated in CVID.

	CVID subgroup			
	CVID without B cells	**Group A**	**Group B**	**Group C**
B cells	None	Low	Normal	Normal
IgG production *in vitro*	No	No	No	Yes
IgM production *in vitro*	No	No	Yes	Yes
Lymphopenia	B cell	B cell, CD4+ T cell	Minor T cell	Rare/absent
β2-microglobulin	Normal	Raised	Slightly raised	Normal
Neopterin	nk	Raised	nk	nk
PHA-induced proliferation	Normal	Reduced	nk	Normal
Antigen-specific T-cell proliferation	Normal	Reduced	Reduced	Reduced
Other features	Exclude XLA (genetics to detect *btk* mutations)	Granulomata, splenomegaly	Exclude X-HIgM (genetic/functional CD40L studies)	Autoimmune disease ?MHC-association
(Proposed) clinical name	**XLA** (X-linked agammaglobulinaemia)	**GAD** (Granulomatous antibody deficiency)	X-linked or autosomal **HIgM** (immunodeficiency with hyper-IgM*)	**VAD** (Variable antibody deficiency)

1.1.4.4. Differential diagnosis

CVID is a diagnosis of exclusion, and care must be taken to distinguish CVID from other primary antibody deficiencies (such as X-linked

agammaglobulinaemia (XLA) and immunodeficiency with hyper-IgM) in addition to causes of secondary immunodeficiency, such as drug therapy, covert lymphoid malignancy and protein-losing states. Like 10-15% of CVID patients, XLA is characterised by grossly reduced circulating B cell numbers. Now that the genetic defect in XLA has been localised to the *btk* gene (Vetrie et al, 1993), the conditions can usually be distinguished by mutation analysis.

1.1.5. Pathogenesis of CVID

The majority of CVID patients have normal numbers of circulating B cells, suggesting that hypogammaglobulinaemia is caused by a failure of CVID B cells to mature into immunoglobulin secreting plasma cells. This failed B cell maturation could result from an intrinsic B cell defect, or from a lack of appropriate T cell help, due to either T or accessory cell abnormalities.

1.1.5.1. A B lymphocyte defect?

Early studies of CVID B lymphocyte function demonstrated defective antibody production by co-cultures of B and T cell from CVID patients and controls (Choi et al, 1972; Waldmann et al, 1974b; Siegal et al, 1976; De La Concha et al, 1977; Siegal et al, 1978). Whilst it was claimed that intrinsic B cell defects were responsible for hypogammaglobulinaemia, the abnormalities observed may have resulted from allogeneic effects. Subsequent studies demonstrated that B cells from some patients fail to proliferate or secrete immunoglobulin to T-dependent or T-independent stimuli, whereas B cells from other patients proliferate but fail to secrete normal amounts of antibody (Pereira et al, 1982; Saiki et al, 1982; Bryant et al, 1990). These reports resulted in classification systems for CVID based on *in vitro* immunoglobulin secretion (De Gast et al, 1980; Saiki et al, 1982; Bryant et al, 1990), and generated a widely held view that the failure of B cell differentiation in CVID was due to a variably expressed B cell defect. However these experiments assayed B cell function after non-physiological stimulation (such as with PWM) and did not determine the role, if any, of the known quantitative and qualitative T cell abnormalities present in many patients (Spickett et al, 1990; Jaffe et al, 1993a).

The identification of costimulatory pathways (such as CD40-CD40L), and soluble factors (such as IL-4, IL-10 and TGFβ) as crucial determinants of B cell activation and immunoglobulin synthesis enabled more accurate *in vitro* evaluation of B cell function in CVID. Several investigators have shown that stimulation of B cells with anti-CD40 and IL-10 restores B cell proliferation and antibody secretion, particularly with the addition of IL-4 (Nonoyama et al, 1993; Nonoyama et al, 1994; Punnonen et al, 1997). While this improvement has not been observed in all patients studied, Eisenstein *et al* have demonstrated further correction of antibody secretion upon IL-10 and allogeneic T cell stimulation after initial 7 day stimulation with anti-CD40 and IL-10. This led the authors to propose that hypogammaglobulinaemia results from reversible B cell anergy (Eisenstein et al, 1994). This data also suggests that the primary abnormality in CVID may lie at the T cell level. The subsequent demonstration of abnormal T cell cytokine secretion and CD40L expression (discussed below) in many CVID corroborates this hypothesis. However it remains unclear whether some CVID patients do in fact have some form of intrinsic B cell abnormality, albeit one that may be overcome with prolonged stimulation *in vitro*. Further work is required to resolve this issue.

1.1.5.2. A T lymphocyte defect?

Considerable evidence suggests that T lymphocyte defects observed in a significant proportion of patients are responsible for the immunodeficiency seen in all patients. The range of infectious, lymphoproliferative and autoimmune complications seen in CVID also suggests that T cell function is highly abnormal in many patients. Furthermore, the critical role of T lymphocytes in the generation of humoral immune responses suggests that T cell defects may represent the primary abnormality in CVID. Humoral immune responses require the induction of B cell activation, proliferation and differentiation, which in turn requires costimulation and help from activated T lymphocytes. T cell activation itself requires costimulatory interaction with antigen-presenting cells (eg. dendritic cells, macrophages and activated B cells). Once T cells are activated, B cells are stimulated by soluble factors (such as the cytokines IL-2, -4, -5, -6, -10, -13 and TGFβ) and direct cell-cell interaction (eg. via the CD40-CD40L axis).

Abnormalities of T cell activation are highly likely to impair antibody responses, and have been extensively investigated in CVID. Such a T cell abnormality could occur at several stages: abnormal activation of T cells by accessory cells; the inability of T cells to provide costimulatory signals (e.g. the expression of CD40L) or help in the form of cytokines. Each has been examined.

Early reports described abnormal T cell help and increased "suppressor" function in CVID (Waldmann et al, 1974a). More recent studies have documented impaired T cell proliferation to mitogens and diminished responsiveness to recall antigens *in vitro* (Fischer et al, 1993a; Fischer et al, 1994a; Stagg et al, 1994; Funauchi et al, 1995). The exact proportion of CVID patients exhibiting such a proliferative abnormality varies between studies, but several groups have reported figures in the vicinity of 50-60% (Fischer et al, 1993b; Fischer et al, 1994a; Fischer et al, 1994b; Stagg et al, 1994; Funauchi et al, 1995). CVID T cells from the majority of these patients also produce abnormally low quantities of cytokines when stimulated *in vitro,* particularly IFNγ, IL-2, -4 and -5 (Fiedler et al, 1987; Pastorelli et al, 1989; North et al, 1990; Sneller et al, 1990b; Fischer et al, 1993b). Similar results using purified CD4+ T cells suggest that it is indeed T cell, rather accessory cell abnormalities that are responsible for these abnormalities.

Unsurprisingly, *in vitro* T cell abnormalities vary between patients. Patients with reversal of the CD4+:CD8+ ratio and CD8+ lymphocytosis exhibit distinct *in vitro* as well as clinical abnormalities. In particular, CD4+ T cells from these patients produce normal amounts of IL-2, and CD8+ cells display increased cytotoxic activity and *increased* IFNγ production. Purified CD8+ T cells from these patients have been reported to suppress allogeneic B cell immunoglobulin production more potently than control CD8+ T cells (Jaffe et al, 1993a; Jaffe et al, 1993b). Removal of these CD8+ cells restored autologous B cell immunoglobulin secretion in some, but not all patients with reversal of the CD4+:CD8+ ratio and CD8+CD57+ lymphocytosis. This is the first clear evidence of active suppression of immunoglobulin production by CVID T lymphocytes, but requires corroboration.

In an attempt to elucidate the basis of these T cell defects, T and B lymphocyte antigen receptor signal transduction in CVID has been investigated. Initial reports of impaired signal transduction were anecdotal and conflicting, with some authors reporting defective B cell receptor transduction (Kondo et al, 1993), and others abnormalities in T cell, but not B cell receptor signal transduction (Fischer et al, 1994b). Fischer *et al.* have examined proliferative responses and antigen receptor signalling in 9 patients, with a range of immunophenotypic abnormalities (Fischer et al, 1993a; Fischer et al, 1994a). T cell proliferative and cytokine responses to PMA and ionomycin were normal, but responses to recall antigens were grossly impaired. Recall antigen responses are dependant on intact TcR function, whereas PMA and ionomycin bypass the TcR. Defective TcR signal transduction was confirmed in pulse-chase experiments (Fischer et al, 1996). Furthermore, antigen presenting cell function was normal in these patients. These results implicate abnormal TcR-mediated T cell activation in some CVID patients, and have been confirmed by the demonstration of abnormal tyrosine kinase phosphorylation, an essential step in TcR signal transduction, in two subsequent studies (Majolini et al, 1997; Schwartz et al, 1997). The precise location of the defect is unknown. However expression of the key tyrosine kinases $p56^{lck}$ and ZAP-70 in patients with abnormal TcR signalling is normal (Majolini et al, 1997), suggesting a location at or near the TcR itself. Finally, it is important to note that whilst this work elegantly localises a T cell abnormality to the TcR in some patients, abnormal T cell transduction is not a universal finding (Fischer et al, 1994a; Majolini et al, 1997), and other mechanisms must be responsible in the remaining patients.

A further possibility is that delivery of costimulatory signals, a necessary requirement for activation of unprimed T cells, is deficient. Signalling via CD28, a key T cell costimulatory molecule, appears to be intact, and direct CD28 stimulation§ cannot correct the defect in T cell activation in CVID (Fischer et al, 1994c). Similarly, immunoglobulin class switching and affinity maturation in B cells also requires the delivery of two signals. The CD40-CD40 ligand (CD40L) axis is probably the most important B cell costimulatory pathways (the first signal being interaction of membrane immunoglobulin with antigen). It has been

demonstrated that B cell expression of CD40 is normal (Eibl et al, 1995), and that stimulation of CVID B cells with anti-CD40 and IL-4 results in normal class switching and antibody secretion (Nonoyama et al, 1993). However, it has been shown that CD40L expression by activated T cells is reduced in a subset of patients (Farrington et al, 1994; Brugnoni et al, 1996). As CD40-CD40L interaction is critical for immunoglobulin class switching (Banchereau et al, 1994; Kawabe et al, 1994), this abnormality provides a potential explanation for the variability of IgG and IgM secretion by CVID B cells *in vitro* (Bryant et al, 1990). The basis for abnormal CD40L expression by CVID T cells is unknown. It is possible that this abnormality is due to abnormal TcR signalling, however further work is required to correlate the two abnormalities.

1.1.5.3. An accessory cell defect?

Studies specifically investigating accessory cell function in CVID are relatively scarce. Abnormal dendritic cell function (Farrant et al, 1985; Rozynska et al, 1989), macrophage antigen presentation (Eibl et al, 1982b; Mannhalter et al, 1986), and T cell priming (Eibl et al, 1982a) have been described in small groups of patients and anecdotal reports, but found to be intact in other studies (Ko et al, 1983; Rozynska et al, 1989). A more recent study has examined antigen presentation by both peripheral blood monocytes and Epstein-Barr virus-transformed B cells in 7 CVID patients, demonstrating that both monocytes and B cells presented (tetanus toxoid) antigen normally to CD4+ antigen-specific T cell lines from healthy controls (Thon et al, 1997). Whilst it is possible that accessory cell defects are present in a minority of patients, these findings together with the profound T cell abnormalities observed in the absence of accessory cells (or in the presence of functionally normal accessory cells) suggest that accessory cell defects are not important in the pathophysiology of CVID in the majority of patients.

It remains possible that accessory cell dysfunction may contribute to complications of CVID, such as lymphoproliferative disease (Aukrust et al, 1994). This is discussed in chapter 4.

1.1.5.4. *Conclusions*

Although the fundamental defect(s) in CVID remain elusive, there is compelling evidence that defective T lymphocyte function plays a role in the pathogenesis of hypogammaglobulinaemia in the majority of patients. Abnormal T cell proliferation, cytokine secretion and TcR signal transduction - events of critical importance in the generation of humoral immune responses - have been observed by multiple investigators in at least 50-60% of patients. However the contribution of intrinsic B cell defects remains unresolved. Although B cells from the majority of CVID patients secrete immunoglobulin of all isotypes in response to *in vitro* stimulation, levels of secretion are often below those of, or require greater stimulation than, normal controls. If B cell function *is* defective in some subgroups of CVID, further work is required to determine whether this is due to abnormalities in specific B cell activation pathways, or perhaps due to abnormalities in cells important for normal B cell ontogeny, such as bone marrow stromal cells. Similarly T cell abnormalities could result from abnormal maturation of T cell precursors in the thymus. Thus it is possible that one or more defects in cells other than lymphocytes could result in long-lasting T and B cell abnormalities.

Furthermore, the aetiology of the many functional cellular abnormalities described above remains unknown, although several explanations have been suggested. The immunophenotypic abnormalities seen in CVID are highly similar to those seen in several lymphotropic virus infections, particularly herpesvirus infections (Carney et al, 1981; Gratama et al, 1987; Tompkinson et al, 1987), which may cause hypogammaglobulinaemia (Pahwa et al, 1983). Despite promising early reports (Spickett et al, 1988; Döcke et al, 1991), exhaustive studies of large numbers of patients has not shown any evidence of an increased rate of herpesvirus infection in CVID (Mullighan et al, 1996). Similarly, an initial report of a high rate of hepatitis C infection, a hepatotropic and lymphotropic flavivirus, in Italian CVID patients has not been confirmed in UK patients (O'Leary et al, 1996; Quinti et al, 1996; Webster et al, 1996). Although the delayed onset of CVID renders this "viral hypothesis" attractive, supporting evidence is lacking.

An additional confounding factor of previous functional studies in CVID is that most patients examined were receiving parenteral immunoglobulin replacement at the time of study. Such treatment exerts profound immunomodulatory effects, including reversal of the CD4+:CD8+ ratio, suppression of lymphocyte proliferation, and increased plasma neopterin levels, suggesting *in vivo* monocyte activation (Aukrust et al, 1997). This should be considered when inferring conclusions from the functional data presented above.

1.1.6. Evidence for a genetic predisposition to CVID/IgAD

Substantial evidence does exist however, for a genetic diathesis in a proportion of CVID patients, and it is the role of genetic polymorphisms of immunoregulatory molecules that forms the basis of this dissertation. Due to the clustering of both CVID and IgA deficiency (IgAD) in families, the similar clinical features and presence of arrested B cell differentiation in both conditions, it has been suggested that the two conditions share a related genetic pathogenesis. Furthermore, far more work has invetigated the genetic basis of IgAD than CVID, due to the higher prevalence of IgAD. For these reasons, previous studies of the genetic basis of both IgAD and CVID are reviewed in this and the following chapter.

1.1.6.1. Racial variation in prevalence of immunodeficiency

The frequency of IgAD varies markedly in different population groups. IgAD is found in one in every 500 to 700 European Caucasoids, but the frequency in Japanese and American Afro-Caribbeans is much lower (Lawton et al, 1972; Kanoh et al, 1986). This data suggests that genetic factors are important, although the action of an environmental agent cannot be excluded. Less data is available for CVID, due to the much lower frequency of this condition than IgAD, but the clinical and laboratory features shared by the two conditions, in addition to the familial co-occurrence of IgAD and CVID suggest that the two conditions have a common genetic predisposition.

1.1.6.2. Familial clustering of antibody deficiency and autoimmunity

Rates of immunodeficiency and autoimmunity in relatives of CVID patients are substantially higher than in the general population (Friedman et al, 1977;

Cunningham Rundles, 1989; Vorechovsky et al, 1991). Selective IgA deficiency and CVID may coexist in kindreds with more than one antibody deficient member (Vorechovsky et al, 1995b), suggesting that a proportion of CVID and IgAD cases share a genetically determined pathogenesis (Volanakis et al, 1993). 11 to 25% of IgAD or CVID probands have been reported to have at least one other hypogammaglobulinaemic relative, a figure that varies with geographical location and intensity of screening of relatives. It has been estimated that the relative risk of CVID or IgAD for siblings of IgA-deficient individuals is 50 times that of the general population (Vorechovsky et al, 1995b).

1.1.6.3. Mode of inheritance

Several patterns of inheritance in families multiplex for CVID and/or IgAD have been described, particularly autosomal dominant and autosomal recessive patterns (Vorechovsky et al, 1995b). The low frequency of affected individuals within kindreds suggests either incomplete or delayed penetrance of a single disease-causing gene, a substantial environmental component, or the involvement of multiple genes. The polygenic hypothesis is supported by the extensive clinical heterogeneity observed in CVID. It is also possible that *familial* CVID and IgAD represent two closely related conditions, but that *sporadic* CVID (the majority of cases) is a fundamentally different disorder, with or without a genetic diathesis. In the absence of large case-control gene association studies, or family studies, it is impossible to say which, if any, of these theories is accurate.

In marked contrast to the plethora of data concerning cellular abnormalities, relatively few studies have investigated the genetics of CVID. Most data has been obtained from relatively small candidate gene association studies of major histocompatibility complex (MHC) polymorphism. The role of MHC polymorphism in CVID is discussed in detail in the chapters 3 and 4. Before introducing the structure, function and genetics of MHC, and its relevance to CVID, the small number of non-MHC genetic studies will be summarised. Previous HLA studies in CVID are discussed in detail in chapter 3.

Most genetic associations have first been reported for IgAD, and subsequently extended to CVID. Consequently some candidate genes that have only been associated with IgAD, but are of possible relevance to CVID, are discussed.

1.1.6.4. *Chromosome 9: the PAX genes*

The PAX (paired-box-containing) genes encode a family of tightly developmentally regulated transcription factors (Vorechovsky et al, 1995a). In particular, *PAX5* encodes a B-cell specific activator protein that binds to IgH regulatory sequences, and has been implicated in class switching. *PAX5* knockout mice are profoundly hypogammaglobulinaemic. However, no mutations in, or linkage to the *PAX5* gene have been detected in 45 families multiplex for CVID and/or IgAD (Vorechovsky et al, 1995a).

1.1.6.5. *Chromosome 14: immunoglobulin gene deletions*

The human immunoglobulin heavy chain constant regions (IgH C) are encoded by 9 genes encompassing 300 kb. Interest in these genes was stimulated by the finding of IgH deletions in healthy individuals with low Ig levels (Mignone et al, 1984; Lefranc et al, 1990). Whilst IgH deletions have since been found to be responsible for some cases of XLA-like agammaglobulinaemia (Yel et al, 1996), similar results have not been found for CVID or IgAD. Several IgH C haplotypes have been defined by the restriction enzyme *Bam*HI (Bech-Hansen et al, 1983), and the "H2" haplotype has been found to be weakly associated with IgAD (Olsson et al, 1992), but not CVID (Olsson et al, 1991). Similarly, no mutations or deletions have been found in the *VpreB* genes of 5 CVID patients (VpreB is thought to be specifically expressed by, and regulate the development of, B lymphocytes) (Kato et al, 1994).

1.1.6.6. *Chromosome 18*

Structural aberrations of chromosome 18 have been associated with hypogammaglobulinaemia. Hemizygous long or short arm deletions or ring chromosomes have been associated with IgAD and/or IgG2 or IgG4 subclass deficiency (Stewart et al, 1968; Feingold et al, 1969; Ruvalcaba et al, 1969; Lewkonia et al, 1980; Truedsson et al, 1995). It is not known if the extent of deletion

correlates with the severity of immunodeficiency. Apart from anecdotal reports (Slyper et al, 1997), chromosome 18 abnormalities are not associated with IgG1 deficiency, an almost universal feature of CVID, suggesting that chromosome 18 defects are not important in the majority of patients.

1.1.6.7. Chromosome 6: the MHC

Most studies investigating CVID genetics have examined polymorphisms of the MHC. Before examining previous findings in detail (chapter 3) it is useful to review the salient features of HLA organisation, function and genetics.

1.2. THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX

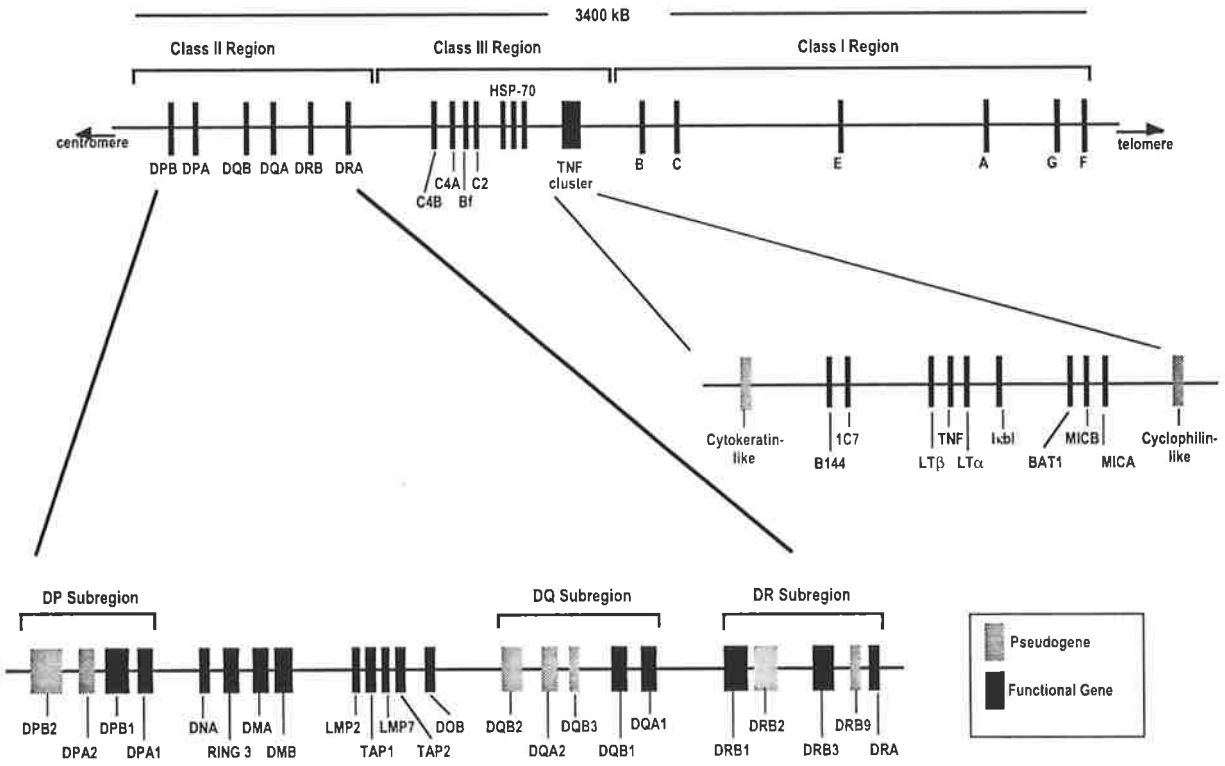
The human MHC, also known as HLA (human leukocyte antigens) occupies 4 Mbp on the short arm of chromosome 6 (6p21.3), and is one of the most gene dense and polymorphic regions of the human genome (Trowsdale, 1996). The MHC contains over 100 genes, many of which encode proteins with key roles in the immune response, particularly antigen presentation. Polymorphisms in HLA genes, or the products they encode, have been associated with susceptibility to vast array of autoimmune and infectious diseases (Hall et al, 1996; Thorsby, 1997).

1.2.1. Organisation

The MHC region is divided into three regions, classes I, II and III. Class I genes lie at the telomeric end of the MHC, and encode the classical transplantation antigens HLA-A, -B, and -C. The centromeric class II region, originally identified as an immune response locus, encodes, amongst other proteins, HLA-DP, -DQ, and -DR. The class II region also contains several antigen-processing genes: *TAP-1*, *TAP-2*, *LMP-2* and *LMP-7* (involved in class I peptide presentation) and *DMA* and *DMB* (involved in class II presentation). Like HLA-A, -B and -C, these molecules are members of the immunoglobulin superfamily. The class III region lies between class I and II, and encodes a heterogeneous group of proteins, many of which also have important immunological roles, including complement components (C2, C4, factor B), cytokines (tumour necrosis factor, lymphotoxin α and β) and chaperones (hsp 70). The class III region is the most gene dense of the

MHC, and also encodes a wide variety of non-immune proteins (eg. 21-hydroxylase), proteins with homology to other immunological or MHC molecules (MICA, MICB, 1C7) and a large number of poorly characterised genes. It is likely that HLA class III contains genes for many more key immunoregulatory molecules. The class III region, and 2 of its products, TNF and LTα, are discussed in detail in chapter 4. A schematic diagram of MHC organisation is given in figure 1.1.

Figure 1.1: Organisation of the human major histocompatibility complex



1.2.2. Roles of HLA encoded molecules

Some of the functionally most important (and most polymorphic) HLA-encoded molecules are the classical class I and II proteins. Class I molecules present mainly cell-derived, or "endogenous", peptide fragments of 9-11 amino acids to HLA class I-restricted CD8+ T lymphocytes. This enables CD8+ T cells to recognise and kill cells harbouring foreign or inappropriately expressed peptides, such as virally-infected or tumour cells. HLA class I molecules are expressed by most nucleated cells. Some "immunologically privileged" sites, such as the

central nervous system, testes and placenta, express little or no class I, preventing inappropriate cytotoxic immune responses in these sites (such as rejection of the fetus). Although expressed at lower levels than HLA-A and -B, HLA-C appears to have important additional roles in regulating NK cell function.
Each of the three classical class I loci (HLA-A, -B and -C) encodes a single α chain, which with the non-HLA encoded, non-polymorphic β2-microglobulin, forms a class I molecule. The α chain has three domains. The α1 and α2 domains are the most distal from the cell surface, form the peptide binding site, and are the site of most HLA class I genetic polymorphism. The α3 domain interacts with β2-microglobulin, stabilising the class I complex in the cell surface. The HLA class I region also encodes several non-classical functional class I-like molecules, such as *HLA-E, -F and -G*, and several pseudogenes, such as *HLA-H, -J and -K.*

HLA class II molecules present mostly extracellular (or "exogenous") peptide fragments of highly variable length (10-34 amino acids) to CD4+ T lymphocytes, which, when stimulated, induce inflammatory and humoral immune responses. Expression of class II molecules is more restricted than class I, and is largely limited to professional antigen presenting cells (APCs) such as dendritic cells, macrophages and B lymphocytes.

The main cell-surface expressed class II molecules are HLA-DP, -DQ and -DR. Each is an heterodimer of an α and a β chain, the genes for which are arranged as matched pairs (*DRA* and *DRB*, *DQA* and *DQB* etc). Each α chain preferentially pairs with its corresponding β chain, although cross-pairing may occur. The DR, DP and DQ regions also contain pseudogenes, although the number of DRB genes and pseudogenes depends on the haplotypes present. A final pair of class II genes, *DMA* and *DMB*, are important in loading of peptide onto classical class II molecules, but are not thought to present significant amounts of peptide. The class II region also encodes molecules involved in class I antigen presentation, including the *LMP* and *TAP* genes.

By facilitating the induction of HLA-restriction and self-tolerance, class I and class II molecules are also vital for normal T lymphocyte maturation in the thymus. The function of class III encoded molecules is discussed in chapter 4.

1.2.3. HLA class I and II gene polymorphism

The HLA class I and II genes are amongst the most polymorphic in the human genome. Polymorphism in the HLA region has been generated by a variety of mechanisms, including substitution, recombination and conversion, and maintained, it is thought, by outbreeding and the selection of polymorphic variants that promote a beneficial immune response to infection, or do not confer susceptibility to non-infectious disease (Kaufman, 1996). Over 600 class I and II HLA alleles have been formally recognised (Bodmer et al, 1996).

1.2.4. Nomenclature

Throughout this thesis alleles are named in accordance with the currently accepted method of nomenclature (Bodmer et al, 1996; Krausa et al, 1996). Alleles are denoted by their gene locus and a four digit number. The first two digits denote the (usually serologically defined) specificity, and the last two digits the allele number within the specificity. Thus HLA-A*0205 denotes allele 5 of HLA*02. Synonymous variants are denoted by a fifth digit (eg. HLA-DQB1*06011 and *06012). Two extra digits are used to denote variants of identical amino acid sequence, but different levels of cell surface expression, with a final letter to denote low expression (L) or no expression (N). For example, the identification of an A*2402 variant with reduced expression has resulted in renaming of the normal allele as A*2402101, and the allele with low expression as A*2402102L. The first two digits only are used where HLA typing has not permitted differentiation of all alleles (eg. DRB1*15 rather than DRB1*1501). Antigenic nomenclature is used when results from early, low resolution studies are cited (e.g. DR3 rather than DRB1*0301).

Another feature of the HLA genes, in addition to extensive polymorphism, is the high degree of linkage disequilibrium between polymorphic loci; i.e. the occurrence of combinations of alleles on the same chromosome more frequently

than that expected given the individual allele frequencies. These combinations of alleles in linkage disequilibrium often take the form of conserved "ancestral" haplotypes, or supratypes, that contain additional distinctive features such as duplications and deletions. This hinders accurate interpretation of the biological significance of disease associations. This concept is discussed below, and in chapters 3 and 4.

1.2.5. Mechanisms of HLA associations with disease

A vast array of diseases have been associated with HLA polymorphisms (Tirawi et al, 1985) (table 1.2). It is beyond the scope of this introduction to comprehensively discuss all known mechanisms of HLA disease association. The key points relevant to the present study have been outlined. More detailed reviews may be found in Lechler, 1994 and Hall et al, 1996.

Positive and negative associations have been described for many diseases (including CVID), and consequently positively associated HLA alleles are often termed 'susceptibility factors', and negatively associated alleles 'protective'. However when both positive and negative associations exist in one disease (such as CVID) it is not clear whether both truly influence disease susceptibility or progression, or if (for example) an apparent negative association is merely due to the strength of a genuine positive association; that is, a 'reciprocal' association. This concept is discussed in the context of CVID in chapter 3.

For most HLA-associated diseases, the presence of a positively-associated HLA allele alone is not sufficient to induce disease. This is suggested by disease concordance rates for HLA-identical siblings of less than 100% (often much lower), and the large number of unaffected individuals carrying disease-associated HLA alleles (Thorsby, 1997). It is likely that, even in strongly HLA-associated diseases such as ankylosing spondylitis (Schlosstein et al, 1973), additional genetic and or environmental factors are required to induce disease.

The majority of HLA-associated diseases are autoimmune, either T lymphocyte-mediated (eg. IDDM), or autoantibody-mediated (eg. pemphigoid skin diseases).

However many other diseases have been associated with HLA polymorphisms, including infections (eg. malaria) (Hill et al, 1991), apparently non-immunological diseases such as hereditary haemochromatosis (Feder et al, 1996), and conditions of unknown aetiology, the classic example being narcolepsy, whose HLA-DRB1*15 association is one of the strongest described (Juji et al, 1984). With few exceptions, the mechanisms of disease association are poorly understood, and consequently many explanations have been proposed (table 1.3) (Lechler, 1994; Hall et al, 1996). The role of HLA in conferring disease susceptibility or affording protection is likely to vary according to the nature of disease, and mechanisms are not necessarily mutually exclusive.

Table 1.2: Examples of HLA-associated diseases

Adapted from Thorsby, 1997. Relative risk denotes how much more frequently the disease occurs in individuals carrying versus those not carrying the HLA allele. *HLA-HFE* 282Tyr may represent a truly disease-causing mutation (see text).

		Frequency (%)		
Disease	**Association**	**Patients**	**Controls**	**Relative risk**
Ankylosing spondylitis	B*27	>95	9	>150
Reiter's disease	B*27	>80	9	>40
Acute anterior uveitis	B*27	68	9	>20
Subacute thyroiditis	B*35	70	14	14
Psoriasis vulgaris	Cw6	87	33	7
Narcolepsy	DQB1*0602	>95	33	>38
Grave's disease	DRB1*0301	65	27	4
Myasthenia gravis	DRB1*0301	50	27	2
Addison's disease	DRB1*0301	69	27	5
Rheumatoid arthritis	DRB1*0401/4/5/8	81	33	9
Juvenile rheumatoid arthritis	DRB1*08	38	7	8
Coeliac disease	DQA1*0501 & DQB1*0201	99	28	>250
Multiple sclerosis	DRB1*1501-DQB1*0602	86	33	12
Type 1 diabetes	DRB*0301, *04, DQB1*0302 (Asp57)	81	23	14
Hereditary haemochromatosis	HLA-HFE 282Tyr	>90	10	>100

Table 1.3: Mechanisms of HLA associations with disease

Theories marked with an asterisk are discussed in the text.

Mechanism
Presentation of pathogenic peptide*
Peptide contribution by HLA molecules*
Inability to present protective peptide*
Chemical modification of peptide
Chemical modification of HLA molecules, altering peptide binding*
Regulation of T cell development*
Binding and internalisation of microorganisms
Superantigen binding
Regulation of antibody formation
Molecular mimicry*
Linkage disequilibrium*
Altered non-immunological function of HLA molecule*

1.2.5.1. *Peptide presentation*

Many theories relate HLA-mediated disease association to the key physiological function of HLA molecules, namely peptide presentation (reviewed in Lechler, 1994, and Hall et al, 1996). Much of the polymorphism in HLA molecules resides in the peptide binding groove, and facilitates the binding and presentation of a wide range of peptides to T cells. Differences in HLA alleles between individuals may influence the range of peptides presented, and the efficacy of an immune response. For example, an effective T cell response may protect against infectious disease, but if too vigorous may promote excessive inflammation or autoantibody production, and predispose to autoimmune disease. HLA molecules may also induce disease by inappropriate presentation of pathogenic peptides, or by presenting peptides in unusual or inappropriate sites, as has been suggested for ankylosing spondylitis.

It has become apparent that the majority of self peptides presented by HLA molecules are derived from HLA molecules themselves (Rammensee, 1995). Thus, HLA molecules could influence the immune response not only by T cell restriction, but also as a source of peptides. Presentation of an HLA-derived peptide in competition with a disease-promoting, stimulatory peptide, could explain the proposed protective effect of HLA alleles. Alternatively, HLA-derived peptides could, via similarity to microbial peptides, promote an excessive immune response and induce disease. This has been suggested for ankylosing spondylitis, rheumatoid arthritis and primary biliary cirrhosis.

1.2.5.2. *Thymic T cell repertoire selection*

Normal T lymphocyte maturation in the thymus is critically dependant on interaction with HLA class I and II molecules. If immature thymocytes fail to recognise HLA-peptide complexes, or bind with too great an avidity, T cells fail to receive signals necessary for survival, apoptose and die (negative selection) (Ashton-Rickardt et al, 1994). Interactions of intermediate affinity allow egress of mature, CD4+ or CD8+, MHC class I or II restricted T lymphocytes. This process is thought to be influenced by the nature of peptide presented by HLA in the thymus, as well as by the specificity of the TcR, and the HLA molecules

themselves. Selection of potentially autoreactive or pathogen-specific T cells will thus be strongly influenced by HLA haplotype. TcR-HLA interactions in the thymus also depend on the level and site of HLA expression, which is a function of HLA gene dosage, and of polymorphisms regulating HLA gene expression. Consequently, both gene dosage of HLA alleles and HLA promoter polymorphism may confer susceptibility to disease. This has been observed for rheumatoid arthritis (DRB1*04 homozygosity is associated with more severe disease) (van Zeben et al, 1991; Winchester, 1994) and juvenile rheumatoid arthritis (associated with several haplotypes carrying DQA1 promoter polymorphisms) (Scholz et al, 1994), although it is not clear if this is a result of central or peripheral effects.

1.2.5.3. "Altered self": modification of HLA molecules and peptide

In general, T lymphocytes are tolerant (do not respond) to self peptide expressed at appropriate levels and anatomical sites by self-HLA molecules. However if either HLA molecules or peptide are modified, tolerance may be broken. Thus, tissue-specific oxidation of cysteine at amino acid 67 of HLA-B*27 has been proposed as a mechanism for the association of ankylosing spondylitis with this allele (Benjamin et al, 1990). Likewise, the abnormally glycosylated IgG characteristic of rheumatoid factor-positive rheumatoid arthritis (Parekh et al, 1985), may, via inappropriate presentation of IgG-derived peptide, provide a potential mechanism for the HLA-mediated predisposition to RA (Hall et al, 1996).

1.2.5.4. Non-immunological function of HLA molecules

Most of the theories described above, whilst conceptually attractive, are lacking in directly corroborative evidence. However, in the case of hereditary haemochromatosis (HH), a recently described polymorphism in a non-classical HLA gene may represent a genuine disease-causing allele. HH has long been known to be associated with HLA-A*03 (Simon et al, 1976). Recently a class I-like gene, *HLA-HFE*, has been cloned, and 2 polymorphisms identified, at codons 63 (His→Asp) and 282 (Cys→Tyr) (Feder et al, 1996). 282Tyr is in linkage disequilibrium with HLA-A*03 (Mullighan et al, 1997a). Homozygosity for

282Tyr is present in over 90% of HH patients (Feder et al, 1996). HLA-HFE is specifically expressed in close proximity to sites of iron absorption in intestinal epithelia (Parkkila et al, 1997), suggesting a critical role in iron metabolism, and a direct causative role of the HLA-HFE mutations. This example of a preliminary HLA association (A*03) subsequently shown to be secondary to linkage disequilibrium with an apparently causative allele (*HLA-HFE* 282Tyr) demonstrates the importance of considering linkage disequilibrium when interpreting the significance of HLA associations.

1.2.5.5. Linkage disequilibrium

The concept of linkage disequilibrium, the occurrence of *cis* combinations of alleles more frequently than would be expected from individual allele frequencies, has been introduced. Many disease-associated HLA alleles are indeed known to be in linkage disequilibrium with other HLA alleles. For example, SLE is associated with all alleles on the common Caucasoid haplotype A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 (commonly referred to as the "A1-B8-DR3" haplotype). However it is not known which, if any, of these alleles represents the true disease associated allele. Indeed, the primary association with this haplotype may be with the HLA class III complement component allele *C4AQ0* (Arnett et al, 1992). This illustrates that the HLA association of primary importance may be with hitherto unidentified genes or alleles, and that these associations may explain other observed HLA associations by linkage disequilibrium.

1.2.6. Relevance of HLA to CVID

HLA molecules substantially influence both cellular and humoral immune responses at a number of levels. HLA molecules are also critical for normal T cell maturation. As T cell number and function may be profoundly abnormal in many CVID patients, it is feasible that HLA polymorphism may contribute to these T cell abnormalities at thymic or post-thymic levels.

In addition, the HLA region is known to be important in the humoral immune response, both by influencing the interaction of T helper cells with antigen presenting cells, or by encoding soluble factors critical for normal

immunoglobulin production (such as TNF and LTα, see chapter 4). It is thus possible that HLA polymorphism might significantly influence the efficacy of antibody responses.

Finally, many autoimmune diseases are known to be HLA associated. Both organ-specific and non-organ specific autoimmunity is a feature in many CVID patients, suggesting that autoimmune disease in CVID may be independently HLA associated. Indeed, it has previously been suggested that both autoimmune disease and antibody deficiency (IgAD) share a common, HLA class III mediated susceptibility (French et al, 1990).

1.3. AIMS OF THIS WORK

This thesis has two aims: the first is to clarify the role of HLA polymorphisms in CVID. As is discussed in chapter 3, previous HLA studies in CVID have yielded weak and conflicting results, possibly due to disease heterogeneity. Consequently it has been a priority to collect as much clinical and laboratory data as possible in order to examine associations with disease subgroups. This work is described in chapters 3 and 4.

The second aim of this work is to examine the role of other immunoregulatory polymorphisms in CVID. Very few previous studies have examined polymorphisms in non-HLA molecules involved in the humoral immune response, such as cytokines and adhesion molecules. Many of these polymorphisms are known to significantly influence the production or function of these molecules (such as the levels of cytokine transcription, or the interaction of adhesion molecules with their ligands). Chapters 5 and 6 describe a unified method of genotyping multiple immunogenetic polymorphisms, and the application of this technique to CVID.

2. Materials and Methods

2.1. PATIENT SELECTION

A total of 179 Caucasoid CVID patients were recruited from English and Welsh specialist clinical immunology centres. Demographic, diagnostic and clinical characteristics of the patient group are presented in detail in chapters 3 and 4.

2.2. CONTROL SELECTION

2.2.1. HLA controls

605 Caucasoid cadaveric kidney donors were used as controls for the HLA and non-HLA genetic association studies described in chapters 3-5. These controls represent consecutive cadaveric organ donors from around the United Kingdom genotyped at the Oxford Tissue Typing laboratory for transplantation purposes. This population has been extensively characterised and utilised for a variety of HLA studies, and has the expected HLA allelic frequencies for UK Caucasoids (Bunce et al, 1995; Bunce et al, 1996).

2.2.2. Healthy controls for lymphocyte phenotyping

50 non-smoking Caucasian volunteer controls were recruited for examination of HLA associations with peripheral blood lymphocyte numbers. This cohort is discussed in chapter 3.

2.3. HLA GENOTYPING

HLA class I, II and III genotyping protocols are detailed in chapters 3 and 4.

2.4. MOLECULAR BIOLOGY PROTOCOLS

2.4.1. Basic reagents.

RED CELL LYSIS BUFFER (RCLB). Composition: 0.144M ammonium chloride (NH_4Cl) and 1mM sodium bicarbonate ($NaHCO_3$). Preparation: 15.4g of NH_4Cl and 1.68g of $NaHCO_3$ were dissolved in two litres of sterile distilled water.

NUCLEAR LYSIS BUFFER (NLB): Composition: 10mM Tris-HCl pH8.2, 0.4M Sodium chloride (NaCl) and 2mM di-sodium EDTA pH 8.0 (Na_2EDTA). Preparation: 23.37g of NaCl was dissolved in 900ml of distilled water. 10ml of 1M Tris-HCl pH8.2 and 10ml of Na_2EDTA pH8.0 added and made up to 1l with distilled water.

NLB+SDS BUFFER. Preparation: 300ml of NLB was combined with 20ml of 10% w/v SDS.

SODIUM DODECYL SULPHATE (SDS). Preparation: 100g of sodium dodecyl sulphate was dissolved in 1l of distilled water in a fume hood, and stored at room temperature.

STANDARD PK BUFFER. Composition: 1% w/v sodium dodecyl sulphate and 2mM Na_2EDTA pH 8.0. Preparation: 10ml of 200mM Na_2EDTA and 100ml of 10% w/v sodium dodecyl sulphate were added to 890ml of distilled water and stored at room temperature.

10MG/ML PROTEINASE K FOR DNA EXTRACTIONS. Preparation: 100mg of Proteinase K (Gibco) was dissolved in 10ml of standard PK buffer (2.1.5). The proteinase K solution was stored in appropriate aliquots at -70°C until required.

95% ETHANOL. Preparation: 950ml of absolute ethanol was mixed with 50ml distilled water.

75% ETHANOL. Preparation: 700ml of absolute ethanol was mixed with 300ml distilled water.

6M NaCl. Preparation: 350.64g of sodium chloride was dissolved with warming in 800mls of distilled water and then topped up to 1l with distilled water.

10X CONCENTRATED BASE BUFFER. Composition: 670mM Tris-base pH8.9, 166mM ammonium sulphate, and 1% Tween 20. Preparation: 40.568g Tris base was dissolved in 400ml dH_2O and adjusted to pH 8.9 with concentrated hydrochloric acid. 10.96g of ammonium sulphate was dissolved in the Tris solution. The solution was filtered through a 0.22 micron filter into an

autoclaved bottle. 5ml of Tween 20 was added and the solution was topped up to 500ml with dH_20. The buffer was stored at -70°C.

TDMH BUFFER. This is made from 10x base buffer stock, and makes 65ml. Composition: 16.9 ml 10x Base buffer, 0.4ml each dCTP, dGTP, dATP and dTTP from 100mM stock (Promega), 13.5ml 25mM Magnesium chloride, 33.4ml distilled water. Preparation: ingredients were mixed in a sterile vessel and stored in appropriate aliquots at -70°C.

2.4.2. DNA methods.

2.4.2.1. Preparation of a cell pellet from fresh whole blood prior to DNA extraction.

10ml of anticoagulated blood was centrifuged at 500g for 7 minutes. The resulting buffy coat was aspirated into a 15ml polypropylene tube (Anachem) to which 10ml of RCLB was added. Erythrocytes were lysed for at least 5 minutes and the sample was then centrifuged for 5 minutes at 500g. The supernatant was poured off, and the remaining pink cell pellet resuspended in 10ml RCLB and centrifuged again for 5 minutes at 500g. The supernatant was again poured off leaving a white cell pellet, which was then either stored at -70°C or used for DNA extraction, as described below.

2.4.2.2. Preparation of a cell pellet from frozen whole venous blood prior to DNA extraction.

Samples were thawed at 37°C and mixed with five times the volume of ice cold swelling buffer A in a 50ml Falcon tube. After a five minutes incubation on ice the sample was centrifuged at 500g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 50ml of swelling buffer B, incubated on ice for 5 minutes before being centrifuged at 500g for 10 minutes. The previous step was repeated. DNA was then extracted as described in section 2.2.6. Whole frozen blood is not a clean source of DNA and a second chloroform extraction was often necessary to ensure all protein was removed.

2.4.2.3. DNA extraction from a buffy coat pellet.

3.2ml of NLB + SDS buffer was added to the pellet and 120µl of 10mg/ml proteinase K in standard PK buffer was added. The sample was incubated for at least 6 hours at 42°C in a rotisserie oven (Hybaid). The sample was then shaken after the addition of 1ml of 6m NaCl followed by 3ml of chloroform until the sample became milky. The sample was centrifuged for 25 minutes at 1200g, resulting in three visible phases: a lower organic phase containing chloroform and dissolved lipid, a DNA rich upper phase, with an intervening interface contained precipitated proteins. The upper aqueous phase (3ml) was aspirated into 20ml Universal tube, avoiding the transfer of protein from the interface. 10ml of 95% ethanol was added and the sample was gently rocked until a fluffy DNA precipitate was formed. The sample was then centrifuged at 500g for 3 minutes and washed in 70% ethanol and centrifuged at 500g for another 3 minutes. The precipitate was transferred to a 0.5ml Eppendorf tube and the excess 70% ethanol was drained onto a tissue. The DNA sample was dried in a centrifugal evaporator (Savant) for 5 minutes before being resuspended in 200-400µl of sterile distilled water. The DNA was fully resuspended before use.

2.4.2.4. Modified DNA extraction method.

This modified DNA method is cheaper, quicker and gives better quality DNA than the method described in section 2.4.2.3. The method has been used for all DNA preparations in the Oxford Tissue Typing laboratory since August 1996.

Cell pellets were evenly resuspended in 3.2ml of NLB + SDS buffer and then mixed vigorously with 1ml of 6M NaCl and 3ml of chloroform until the samples became milky. The sample was centrifuged for 25 minutes at 1200g, resulting in three visible phases: a lower organic phase containing chloroform and dissolved lipid, a DNA rich upper phase, with an intervening interface containing precipitated proteins. The upper aqueous phase (3ml) was aspirated into a 20ml Universal tube, avoiding the transfer of protein from the interface. 10ml of 95% ethanol was added and the sample was gently rocked until a fluffy DNA precipitate was formed. The sample was then centrifuged at 500g for 3 minutes and washed in 70% ethanol and centrifuged at 500g for another 3 minutes. The

precipitate was transferred to a 0.5ml Eppendorf tube and the excess 70% ethanol was drained onto a tissue. The DNA sample was dried in a centrifugal evaporator (Savant) for 5 minutes before being resuspended in 200-400µl of sterile distilled water. The DNA was fully resuspended before use.

2.4.2.5. Calculation of DNA concentration.

A 100 fold dilution of DNA was made by diluting 10µl of DNA in 990µl of distilled water and optical density readings at 260 nm (l max of DNA) and 280nm (l max of protein) were taken using quartz cuvettes. An OD_{260} reading of 1 is equivalent to 50µg/ml of DNA thus the following formula was used:

Equation 2.1: Calculation of DNA concentration

$$\text{DNA conc. } (\mu g/\mu l) = \frac{OD_{260} \times 50 \times 100}{1000}$$

A measure of purity (with respect to protein) was made by comparing OD_{260} to OD_{280}: a ratio of 1.8 - 2.0 indicated pure DNA.

2.4.3. Synthesis of oligonucleotide primers for PCR-SSP.

Primers were manufactured at crude 0.2µM scale by R & D Systems, Abingdon, Oxon, Life Technologies, Paisley, UK; MWG technologies, Milton Keynes, UK; or in house using a Beckman 2000M oligonucleotide synthesiser. All allele-specific primers used in the PCR-SSP assays were designed to have a *Tm* of between 58-62°C using equation 2.2. All primers were tested empirically to establish efficiency, and modified as required. Specific aspects of primer design and optimisation are discussed in chapters 5 and 6.

Equation 2.2: Calculation of primer annealing temperature

$$\text{Tm(C)} = [(A+T)\times 2] + [(G+C)\times 4]$$

Molecular weight of primers were calculated using equation 2.3, where *n*A = number of adenine bases in the DNA sequence, for example, and N is the total number of bases (Powell, 1995). (63 x N-1) accounts for the weight of the phosphate groups.

Equation 2.3: Calculation of primer molecular weight

$$\text{Molecular weight} = (249 \times n\text{A}) + (240 \times n\text{T}) + (265 \times n\text{G}) + (225 \times n\text{C}) + (63 \times N - 1) + 2$$

Oligonucleotide concentration was estimated by diluting a 1µl sample of oligonucleotide in 499µl of distilled water and a reading was taken at OD_{260} using a Ultrospec III spectroscope (Pharmacia). The amount of oligonucleotide present was calculated using equation 2.4, where 1 OD_{260} unit = 33µg oligonucleotide.

Equation 2.4: Calculation of oligonucleotide mass

$$\text{Mass of oligonucleotide } (\mu g) = 33 \times OD_{260}$$

2.4.3.1. Establishing working concentration of primers.

Primers were stored at -20°C at a concentration of 2000µg/ml before use in primer mixes. Final concentrations of between 0.077 and 11µg/µl were used in individual PCR reactions, determined individually by titration.

Allele-specific primers were tested in the following manner: First, the 796 bp DRB1 control primers (numbers 63 and 64, used as controls for the majority of reactions and listed in the appendices) were empirically titrated in water to which 62.5mg/L cresol red was added. A dilution of controls giving good amplification over a wide range of DNA samples was used. The final concentration of control primers was typically 0.077µg/µl. Control primer stock solution (without allele-specific primers) was typically made up in one litre volumes and frozen at -30°C in 50ml volumes. Allele-specific primers were subsequently titrated in the control primer stock as described below.

Initially all primers were tested at 0.77µg/µl per PCR reaction. The primer mix was tested with several expected positive and negative DNA samples to screen for false positive and false negative reactions. If false negative reactions were obtained the primer concentrations were tested using increasing concentrations of both primers (typical range 0.77-11µg/µl). If false positive amplifications were observed the primers were tested in decreasing concentration (typical range 0.77-0.0077µg/µl) until a strong true positive was identified and no false positives were visible. On occasion these titrations were not effective, and therefore

primers were tested by asymmetric titration. Asymmetric titration involves keeping the concentration of the sense primer constant whilst varying the concentration of the antisense primer, and vice versa. Asymmetric titrations were especially useful for reducing the presence of false positive bands of incorrect size relative to the expected amplicon. If false positive amplification could not be abolished by asymmetric titration, the length or composition of primers was modified (such as by incorporation of mismatches).

2.4.4. Preparation and storage of primer mixes for PCR.

Bulk (1-50ml) primer mixes were stored at -30°C in 1ml aliquots until required. 62.5mg/L Cresol red stock were added to each primer mix to aid dispensing and visualisation of small amounts of primer mixes. Primer mixes were dispensed in 5µl amounts into 200µl strip tubes (Advanced Biotechnology) or into 96 or 192 well PCR plates (Costar) with a repeating electronic multichannel pipette. The tubes or plates were pre-dispensed with 10µl of mineral oil (Sigma) to prevent evaporation of stored mixes. Once the primer mixes were dispensed the plates or tubes were sealed in polythene bags and stored at -70°C until required.

2.4.5. PCR mixtures.

All PCR-SSP adhered to the "TDMH method" using base buffer as described by Bunce et al (Bunce, 1996). Primers were stored in ready tested 5µl volumes, whereupon for each 13µl PCR reaction a 3µl volume of Taq/DNA/water mixture was mixed with 5µl of TDMH before being added to the 5µl of primers. This method facilitated the use of semi-automatic dispensing and allowed greater manipulation of primer concentrations without wasteful use of dNTPs.

2.4.6. Setting up PCR reactions using the TDMH method (using base buffer).

Trays or strips of tubes containing 5µl primer mixes were thawed at room temperature prior to use. 50µl TDMH, 28µl distilled H_2O, 0.375µl 5unit/µl Taq polymerase (Advanced Biotechnology) and 1.5µl target DNA at a concentration between 0.1-0.5µg/ml were mixed for every eight PCR reactions required.

Each PCR reaction contains ingredients in the proportions PM:TDMH:OE = 5:5:3 where PM is the primer mix, TDMH is the PCR buffer and OE is the other ingredients in the PCR reaction. The final concentration of the ingredients per 13µl PCR reaction are: 67mM Tris Base pH8.8, 16.6mM ammonium sulphate, 2mM magnesium chloride, 0.01% (v/v) Tween 20, 200µM of each dNTP, approximately 1-4µM of each allele-specific primer, 0.1µM of DRB1 control primers (primers 63 & 64) or 2µM of APC control primers (primers 210 & 211), between 0.1 and 0.01µg DNA and 0.1875 units Taq Polymerase (Advanced Biotechnology, London).

Each reaction was set up under 10µl PCR mineral oil (Sigma). 8µl of the TDMH/DNA/Taq mixture was added to each 5µl primer mix using an electronic dispensing pipette. After adding the PCR mixture the PCR plates were sealed with a self-adhesive plastic sealer (Costar) and tubes were sealed with the PCR caps (Advanced Biotechnology). The plates/tubes were briefly centrifuged to ensure that all the PCR mixture was submerged under the PCR mineral oil. The PCR plates/tubes were dipped in paraffin oil and blotted onto tissues before insertion into the PCR machine blocks. This fine coating of oil ensured a better contact between the block and the vessel and hence ensured more reliable PCR. The concentration of the DNA was seldom measured as most PCR-SSP assays worked over a wide range of DNA samples, even for reactions generating very large amplicons. However, if a DNA sample was insufficiently concentrated to amplify consistently in all PCR reactions, more DNA was added to the PCR mixture while adding less water, thus keeping the essential 5:5:3 ratio. If reaction failure was considered to be due to poor quality DNA, extra Taq polymerase was added.

2.4.6.1. PCR amplification of heparin-contaminated DNA using Heparinase II.

Immediately prior to use a 10 unit vial of heparinase II was added to 100µl of dH_20 to make a 0.1 unit/µl solution (this stock solution could not be re-frozen). For eight PCR reactions, 1µl of DNA was incubated for 45 minutes at 37°C with 20µl (2 units) of heparinase II. The DNA could then be used for PCR using the

TDMH method described in section 2.6.2 with modified volumes thus: 50µL TDMH, 8µl distilled H_2O, 0.375µl 5unit/µl Taq polymerase (Advanced Biotechnology), and 21.5µl Heparinase II-treated DNA.

2.4.7. PCR machines and thermal cycling parameters.

The following PCR machines were used for PCR-SSP: PTC-200 (96 or 192 well machine; MJ Research Ltd), PTC-100 96v (96 well machine; MJ Research Ltd), and GeneAmp 9600 (96 well machine Perkin Elmer). Identical thermal cycling parameters were used for HLA class I and II genotyping (chapter 3), TNF and LTα genotyping (chapter 4), and non-HLA genotyping (chapter 5), and are listed in Table 2.1.

Table 2.1: PCR-SSP thermal cycling parameters

Number of cycles	Temperature (°C)	Duration (seconds)
1	96	60
5	96 70 72	20 45 45
21	96 65 72	25 50 45
4	96 55 72	25 60 120
1	20	30

2.4.8. Electrophoresis reagents.

LOADING BUFFER. Composition: 300ml Glycerol, 250ml 2xTBE (See 2.8.4 recipe), 450ml dH_20, 0.25g Bromophenol blue (Sigma) *or* 0.25g Xylene CyanoleFF blue (Sigma) *or* 0.25g Orange G disodium (Sigma). Preparation: the aqueous ingredients were first combined and the powdered dye was dissolved into the solution. Loading buffer was stored at room temperature. 6µl of loading buffer was added to a typical 13µl PCR reaction.

2x TBE BUFFER. Composition: 0.18M Tris-borate and 0.004M EDTA. Preparation: 216g of Tris-base and 110g of boric acid were dissolved in 9l of distilled water. 8ml of 0.5M EDTA pH 8.3 was added and the solution was topped up to 10l with distilled water.

0.5x TBE BUFFER. Composition: 0.045M Tris-borate and 0.001M EDTA. Preparation: 750ml of distilled water was combined with 250ml of 2xTBE

1% AGAROSE GEL. Composition: 10g agarose (Gibco), 1l 0.5xTBE and 10µl 10mg/ml ethidium bromide (Sigma). Preparation: 10g of agarose was added to 400ml 0.5xTBE in a one litre Schott bottle. The agarose was dissolved by microwaving (650 watt) for 4-5 minutes, interrupting the microwaving occasionally to release pressure and swirl undissolved agarose powder into suspension. Once fully dissolved, the agarose solution was then topped up to one litre and 10ml of 10mg/ml ethidium bromide solution (Sigma) added and mixed. Agarose was stored at 50°C until required.

2.4.9. Electrophoresis and photography.

2.4.9.1. Addition of gel loading buffers to PCR reactions.

Plate sealers were removed from the 96 or 192 well PCR plates and 6µl of gel loading buffer was added to each vessel. The loading buffer was added by using a 1ml plastic syringe attached to a Hamilton repeating dispenser with a 6 lane stream splitter attached to enable multi-well dispensing.

2.4.9.2. Electrophoresis conditions.

Horizontal gel electrophoresis equipment (Flowgen and Anachem) was used for all gel electrophoresis. 0.5 to 1 cm thick 1% agarose gels were prepared utilising gel combs of the same spacing as multichannel pipettes to facilitate gel loading. The electrophoresis running buffer was 0.5 x TBE. 18µl of each PCR reaction was loaded into a submerged horizontal gel using 8 or 12 lane multichannel pipettes. The samples were electrophoresed for 30 minutes at 200 v/cm, or 25 minutes at 250 v/cm, producing similar separation of PCR products.

2.4.9.3. Gel photography.

Upon completion of electrophoresis, the PCR products were visualised by placing the gel on a 320nm wavelength transilluminator. The gel was photographed using a Polaroid MP4 Camera system (Polaroid). Polaroid black & white 667 film (Fahrenheit Supplies) was used in conjunction with a Wratten 22 filter (Sigma). Photographs were taken with 0.5 second exposures at f5.6.

2.5. PERIPHERAL BLOOD IMMUNOPHENOTYPING

Lymphocyte subpopulations on all samples collected during the course of this study, and the majority of samples collected previously, were enumerated by standard dual colour flow cytometry using a Becton Dickinson FACScan (Chapel et al, 1990). Blood samples were analysed within 6 hours of collection. Total leukocyte and lymphocyte counts used for calculation of absolute numbers of lymphocyte subsets were measured by the Haematology laboratories of the Churchill and John Radcliffe Hospitals using a Coulter Counter.

2.5.1. General reagents

10% FACS LYSE SOLUTION. Preparation: 50 ml FACS Lyse (Becton Dickinson) was mixed with 450 ml distilled water.

PHOSPHATE BUFFERED SALINE (PBS). Preparation: 10 PBS tablets (Oxoid) were dissolved in 1l of sterile distilled water.

1% PARAFORMALDEHYDE. Preparation: 5 ml formaldehyde was mixed with 500 ml PBS, incubated at 56°C for at least 1 hour, and allowed to cool.

2.5.2. Monoclonal antibodies

Fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labelled monoclonal anti-CD45, -CD14, -CD3, -CD4 -CD8, -CD19, -CD20, -CD45RA, -CD45RO, and -CD57 (Becton-Dickinson and Dako, UK) were used at standard dilution, as per manufacturer's instructions, to stain EDTA-anticoagulated peripheral blood.

2.5.3. Staining procedure

5 µl of labelled monoclonal antibody was added to labelled 12mm Falcon tubes. Blood samples were gently mixed, and 50µl of blood added to each tube. Tubes were then incubated at room temperature for 10 minutes in the dark. 1 ml of 10% FACS Lyse solution was added to each tube, and the tube then gently vortexed. Tubes were again incubated for 10 minutes in the dark, then centrifuged for at 500g for 5 minutes at room temperature. Supernatant was discarded into a bucket of neat bleach. 1 ml of sheath fluid (Becton Dickinson) was added to each tube followed by gentle vortexing and centifugation at 500g for

5 minutes at room temperature. Supernatant was discarded into a waste bucket. Cells were resuspended in 1% paraformaldehyde and vortexed. Stained samples were then stored in the dark at 4°C and analysed using the Lysis II program (Becton-Dickinson) within 24 hours.

2.6. DATA MANAGEMENT AND STATISTICAL ANALYSIS

2.6.1. General data management

All clinical, immunophenotypic and genotypic data was stored in Filemaker Pro v3.0 (Claris Corporation), and manipulated in either Filemaker Pro or Microsoft Excel 5.0 (Microsoft Corporation). Statistical analysis and graphing was performed using KnowledgeSEEKER and Statview 4.5 (Abacus Concepts). Amino acid sequence comparisons described in chapter 3 were performed using the Windows-based Disease Discriminator program (Martin Barnardo et al, unpublished data).

2.6.2. Phenotype, gene and allele frequencies

The following calculations are used extensively in chapter 3, 4 and 5, and are described here to avoid repetition.

Phenotype frequencies were obtained by counting the number of individuals in a population positive for an allele. Allele frequencies were obtained by directly counting the number of chromosomes bearing an allele, and gene frequencies were obtained using the Bernstein estimator (Farewell et al, 1984) (equation 2.5)

Equation 2.5

p=gene frequency. f=allele frequency

$$p = 1 - \sqrt{1 - f}$$

Alleles at each polymorphism were said to be in Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($p>0.05$) from those expected when analysed by chi-square.

2.6.3. Analysis of disease associations

Significance of associations between alleles (or haplotypes) and disease (or disease subgroups) was assessed in several ways. Associations between two categorical variables (e.g. the presence or absence of allele A in patients v. controls) were assessed using Chi-square (with Yates' continuity correction, where appropriate) and Fisher's exact tests using KnowledgeSEEKER or StatView, after tabulation of 2xn data in contingency tables. The basic layout of a 2x2 contingency table for the comparison of two categorical variables with 2 categories each is given in table 2.2. The formulae used for calculation of the chi-square and Fisher's exact test statistics may be found in Altman, 1991a. Contingency table data was also analysed by the calculation of:

- **RELATIVE RISK (RR)**: the product of 'true positives' and 'true negatives' divided by the product of false positive and negatives (i.e. (axd)/(bxc) from the contingency table (table 2.2). Where low numbers prevented meaningful calculation of this statistic, the modification of Woolf and Haldane was used (equation 2.6) (Wolff, 1955; Haldane, 1956). Where significant, the 95% confidence interval for the RR was also calculated (described in Altman, 1991a, p. 269)

- **ODDS RATIO (OR)**: the proportion of patients that are positive divided by the proportion of controls that are positive (Svejgaard et al, 1974) (equation 2.7). Whilst this estimates how much more frequent a characteristic is in one group compared to another, the relative risk calculation in equation 2.6 is more commonly used, and confusingly is often referred to as the odds ratio. The nomenclature described here will be used throughout this thesis.

Estimates of the strength of associations between characteristics such as alleles and disease were also expressed by the aetiologic fraction (AF) for positive associations, and the preventive fraction (PF) for negative associations (Svejgaard et al, 1974; Svejgaard et al, 1983). The former is widely used in epidemiology, and is also known the δ value. Broadly speaking, the AF value indicates how much of the disease is "due to" the disease-associated factor. Calculation of AF is only

applicable when RR is greater than one. When the RR is less than one (negative associations) the preventive fraction calculation is used.

Table 2.2: The 2x2 contingency table

	Number of individuals	
	Character (eg. allele) present	Character absent
Patients	a	b
Controls	c	d

Equation 2.6: Calculation of relative risk (RR)

This includes the modification of Woolf, 1955 and Haldane, 1956.

$$RR = \frac{(a+0.5)\times(d+0.5)}{(b+0.5)\times(c+0.5)}$$

Equation 2.7: Calculation of Odds Ratio (OR)

$$OR = \frac{a}{a+b} \Big/ \frac{c}{c+d}$$

Equation 2.8: Calculation of aetiologic fraction (AF)

a and b are values taken from the 2x2 contingency table. hp =a/(a+b), the frequency of character in patients.

$$EF = \left(\frac{RR-1}{RR}\right)\left(\frac{a}{a+b}\right) = \left(\frac{RR-1}{RR}\right)h_p$$

Equation 2.9: Calculation of preventive fraction (PF)

$$PF = \frac{(1-RR)h_p}{RR(1-h_p)+h_p}$$

2.6.4. Linkage disequilibrium

Where disease associations were detected with multiple alleles known to be in linkage disequilibrium (e.g. extended HLA haplotypes), data was further stratified and analysed according to the method of Svejgaard and Ryder (Svejgaard et al, 1994) in order to detect the strongest association. This method is discussed in detail in chapter 3.

Several of the non-HLA genes studied contain multiple polymorphic sites, and associations between alleles were unknown prior to this study. Thus, linkage disequilibrium was initially assessed by chi-square analysis of 2x2 contingency tables. Probable haplotypes thus identified were directly counted, and the resulting haplotype frequencies used to calculate Δ, a measure of the difference between observed and expected haplotype frequencies. Standardised Δ values, Δs, were calculated using Equation 2.10. The theory relating to these calculations of linkage disequilibrium is discussed at length in Hedrick, 1987; Lewontin, 1988

Equation 2.10

P_{ab} = observed frequency of haplotype *ab*, and P_a and P_b = frequencies of alleles *a* and *b*.

$$\Delta s = \frac{\Delta}{\Delta \max} = \frac{P_{ab} - (P_a \times P_b)}{P_a(1 - P_b)}$$

Linkage disequilibrium data was also evaluated using the correlation coefficient *r*, calculated using Equation 2.11.

Equation 2.11

χ^2 = chi-square value, n = number of observations

$$r = \sqrt{\frac{\chi^2}{n}}$$

2.6.5. Correction of probability values

p values were corrected for multiple comparisons according to Equation 2.12, where pc is the corrected p value, p the uncorrected one, and n the number of comparisons (Svejgaard et al, 1994). This approximates to multiplying the uncorrected p value by the number of comparisons made. As discussed in chapter 6, when large numbers of comparisons were made, frequencies were initially examined in the first half of the patient cohort (as reported in Mullighan et al, 1995), and significant associations were then tested in the subsequently recruited cohort. If reproducible, associations were said to be significant.

Equation 2.12

$$pc = 1-(1-p)^n$$

2.6.6. Associations between continuous variables

Correlation between continuous variables (such as lymphocyte subset counts, chapter 3) was measured by calculating Pearson's correlation coefficient (Altman, 1991b). This is usually expressed as r, but is termed r_p in this thesis to avoid confusion with the correlation coefficient for categorical variables detailed in Equation 2.11. Correlation between multiple continuous variables are shown in a correlation matrix (chapter 3). Deviation of r_p values from the null hypothesis was assessed by Fisher's r to Z test. Additionally the $100r_p^2$value was calculated for each significant correlation. This represents the percentage of data variability that is "explained" by the association between the two variables.

2.6.7. Associations between nominal and continuous variables

The unpaired t-test was used to compare the means of continuous variables in two groups of patients (eg. the mean CD4+:CD8+ ratio in patients HLA-DQB1*0603 positive or negative, or mean CD8+ counts in patients with normal or reversed CD4+:CD8+ ratios; see chapter 3).

3. HLA class I and II polymorphisms in CVID

3.1. INTRODUCTION

The structure, function and genetics of the human major histocompatibility complex were reviewed in chapter 1, and the theoretical relevance of HLA polymorphism to CVID discussed. In summary, HLA class I and II molecules are critical elements in the ontogeny of T lymphocytes, and in the genesis of normal immune responses. Furthermore, the HLA region also encodes several important immunoregulatory molecules, such as complement components and cytokines. It is possible that polymorphisms in HLA genes might, by contributing to the profound quantitative and qualitative lymphocyte abnormalities characteristic of CVID, be responsible for the known heritable susceptibility to CVID. It is also possible that HLA polymorphism might confer susceptibility to other characteristic features of CVID, such as autoimmunity and lymphoproliferative disease, which have been shown to be HLA-associated in non-immunodeficient individuals. This chapter describes a detailed analysis of the associations of CVID with HLA class I and II polymorphisms. The following chapter examines associations with polymorphisms of the HLA class III cytokine genes TNF and LTα. Before these are presented, previous studies of HLA polymorphism in CVID will be reviewed.

3.1.1. Previous HLA studies in CVID and IgA deficiency

As was discussed in chapter 1, most genetic studies in primary antibody deficiencies have examined associations with HLA polymorphisms. Relatively few studies have specifically investigated CVID alone. In view of the familial clustering of IgA and CVID, the similar patterns of infection in both conditions, and a common B lymphocyte abnormality (arrested terminal plasma cell differentiation), it has been suggested that IgAD and CVID share common aetiologic factors, or in fact may represent polar ends of clinical spectrum induced by a single genetic defect. Thus most studies have examined HLA associations with IgAD and CVID together, or have inferred associations with CVID on the basis of those detected with IgAD. For these reasons, both IgAD and CVID HLA

associations will be reviewed. Most studies, like the present one, are unmatched case-control association studies. Due to the rarity of the condition and the difficulty accessing kindreds with multiple immunodeficient members, most previously reported family studies are anecdotal, or have investigated small numbers of kindreds. One small HLA linkage study has been reported.

HLA associations with primary immunodeficiencies were first described in 1974 (Hors et al, 1974). At that time HLA typing techniques were serologically-based and relatively low resolution. The HLA-C locus was poorly characterised, and the class II loci undiscovered. Associations with HLA-A1 and -B8 were amongst the first identified (Hors et al, 1974; Ambrus et al, 1977; Smith et al, 1978), and were to be confirmed in many subsequent studies of both IgAD and CVID (table 3.1). However, these positive associations with A1 and B8 were not universal, with some authors subsequently finding no association with these antigens (Buckley et al, 1977). Furthermore, early studies detected positive associations with other antigens subsequently shown to be negatively associated, such as HLA-A2 (Evers et al, 1977). These conflicting associations probably resulted at least partly from the admixture of multiple immunodeficiencies: the ability to distinguish immunodeficiencies at the time of these studies was limited, and the patient groups examined included conditions now shown to be caused by discrete non-HLA mutations, such as severe combined immunodeficiency (SCID) and X-linked agammaglobulinaemia (XLA) (Hors et al, 1974; Buckley et al, 1977; Evers et al, 1977).

The majority of subsequent HLA association studies have examined IgA deficiency, principally because of the high frequency of this condition, and the ease of procurement of samples from blood donor banks. Approximately 60% of IgAD individuals are asymptomatic, and previous studies have examined either these presumably healthy donors, or symptomatic individuals recruited from hospital clinics. Positive associations with A1 and B8 in IgAD were confirmed and extended. Positive associations were also detected with A19, A28, A29, B14, B17 and B40, and negative associations with A3 and B7 were described. Association studies are listed in table 3.1, and summarised in table 3.3.

After the identification of the HLA-D region, it became apparent that HLA class II antigens in linkage disequilibrium with those class I antigens previously shown to be associated with IgAD were also associated with CVID/IgAD. For example, HLA-DR3 was found to be associated with IgAD in several studies, and is in linkage disequilibrium with HLA-A1 and -B8 (Ostergaard et al, 1979; Hammarström et al, 1983; Hammarström et al, 1984). Accordingly, subsequent studies reported associations of IgAD with combinations of antigens in linkage disequilibrium, or haplotypes, such as A1-B8-DR3. Other positively associated haplotypes included A1/A28-B14, B14-DR1, DR1-DQ5, DR7-DQ2[1]. The HLA class II haplotype DR2(15)-DQ6 has been shown to be negatively associated with both IgAD and CVID in a number of studies (Ambrus et al, 1977; Ostergaard et al, 1979; Cobain et al, 1983; Heikkila et al, 1984; Cuccia Belvedere et al, 1989; Schaffer et al, 1989; Olerup et al, 1990; Olerup et al, 1992; Clerici et al, 1993).

The positive association with the A1-B8-DR3 haplotype has been frequently confirmed in both IgAD and CVID. In order to determine which allele on this haplotype is responsible for the association, several investigators have stratified and re-examined HLA data with conflicting results. Indeed, primary associations with A1 (Strothman et al, 1986), B8 (Oen et al, 1982) and DR3 (Hammarström et al, 1983) have been described. It has thus been suggested that the primary associations is with alleles shared by A1-B8-DR3 and other associated haplotypes, such as A28-B14. This hypothesis has been supported by family studies describing associations between both CVID and IgAD and several "extended" HLA haplotypes (particularly the A1-B8-DR3 and A29-B44-DR7 haplotypes, table 3.2) that incorporate a variety of deletions and mutations, particularly class III complement and cytokine genes (Cobain et al, 1985; Wilton et al, 1985; Bucin et al, 1991; Ashman et al, 1992; Volanakis et al, 1992). Despite intensive efforts to determine which alleles on these haplotypes are primarily associated with IgAD and CVID, this issue has not been resolved, although two hypotheses have been proposed.

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[1] Haplotypes are denoted by antigens separated by hyphens (eg. A1-B14). Alternative antigens are separated by a forward slash (eg. A1/A28; thus A1/A28-B14 represents both the A1-B14 and A28-B14 haplotypes).

3.1.1.1. *The HLA class III hypothesis*

The first hypothesis resulted from studies of HLA class III gene polymorphism (mainly in IgAD). The A1-B8-DR3 and A29-B44 extended haplotypes both contain deletions, duplications or defects in the HLA class III C4A, C2 and 21-hydroxylase (*CYP21A*) loci (Wilton et al, 1985; Schaffer et al, 1989; Keyeux et al, 1990; Bucin et al, 1991; Howe et al, 1991; Volanakis et al, 1992). It was thus suggested that as these haplotypes share HLA class III polymorphisms, the susceptibility gene(s) for IgAD and CVID are located in or very near the class III region (French et al, 1990). It was suggested that these HLA class III associations could explain, by linkage disequilibrium, the vast majority of previously reported HLA class I and II associations (Wilton et al, 1985). This hypothesis was supported by the cloning of an increasing number of HLA class III genes with sequence homology to other immunoregulatory molecules (Aguado et al, 1996). Thus while alleles of known class III genes, may not be directly responsible for immunodeficiency, other linked class III alleles on these extended haplotypes may represent the true causative elements.

3.1.1.2. *The HLA-DQB1 codon 57 hypothesis*

An alternative hypothesis has been proposed by Olerup et al. These authors have suggested that homozygosity for non-aspartate amino acids at codon 57 of HLA-DQB1 represents the primary HLA association with both IgAD and CVID (Olerup et al, 1990; Olerup et al, 1991; Olerup et al, 1992). HLA-DQB1 codon 57 is highly polymorphic, is critical for peptide binding (Kwok et al, 1995; Kwok et al, 1996), and has been implicated in other diseases such as insulin-dependent diabetes mellitus (Todd et al, 1987). In a large study of HLA class II polymorphism in IgAD, positive associations with the DR17-DQ2, DR7-DQ2 and DR1-DQ5 haplotypes were detected, in addition to a negative association with the DR2(15)-DQ6 haplotype (Olerup et al, 1990). Comparison of amino acid sequence at the polymorphic amino-terminal domain of DQB1 showed that the three positively associated "susceptibility" haplotypes possess a neutral alanine (A) or valine (V) at codon 57, whereas the "protective" (negatively associated) DR15-DQ6 haplotype has a negatively charged aspartate (D) at this position. This finding was confirmed in a second group of IgAD patients, and identical, albeit weaker

associations found in CVID (Olerup et al, 1992). Furthermore, a sib-pair analysis in the same study found linkage between IgAD and the HLA class II region. C4A and CYP21A polymorphism were also examined in this latter study. Whilst associations similar to previous studies were detected, these were found to be statistically secondary to the positive association with DQB1*0201 (which is found on the A1-B8-DR3 haplotype).

Unfortunately, primacy of the DQB1 codon 57 association has not been confirmed by subsequent family studies (Volanakis et al, 1992; Vorechovsky et al, 1995b). This hypothesis has also been criticised on the grounds that whilst both insulin-dependent diabetes mellitus (IDDM) and IgAD have been associated with Ala57, not all Ala57-bearing haplotypes are associated with IDDM and IgAD (French et al, 1991). Furthermore, a recent study of the Sardinian population has demonstrated that the DR3 haplotype is associated with IDDM and coeliac disease (as in other Caucasoid populations) but not IgAD (Burrows et al, 1997). In contrast, the IgAD/CVID-associated haplotypes all share class III alleles (French et al, 1990). However this criticism does not explain the observation, that the class III associations, whilst strong, are statistically secondary to those with DQB1*0201.

Even though it is not possible to conclude which (if either) hypothesis is correct, it would appear that a susceptibility gene to IgAD, and possibly CVID, resides in, or in close proximity to, the HLA class II or III regions. What is not clear, is whether this gene is involved in antigen-specific immune regulation (such as DQB1*0201, as suggested by Olerup et al), or whether the genetics are more complex, and involve a non antigen-specific immunoregulatory molecule with or without a contribution from classical HLA.

3.1.2. Limitations of previous HLA association studies in CVID

Previous HLA studies in CVID have four serious limitations: the small number of patients examined; the lack of definition of patient subgroups by clinical and laboratory criteria; low resolution typing of limited numbers of HLA loci; and, in the case of amino acid polymorphism studies, the lack of examination of any other polymorphic HLA codons apart from DQB1 codon 57.

Compared to IgAD, there has been a paucity of studies specifically investigating HLA associations with CVID. Apart from an isolated case report of homozygous C2 deficiency associated with pan-hypogammaglobulinaemia (Seligmann et al, 1979), the first studies specifically examining HLA associations with CVID did not appear until 1989 (Schaffer et al, 1989). When family studies and pooled studies are excluded, only 145 CVID patients in 3 studies have been examined (Schaffer et al, 1989; Howe et al, 1991; Olerup et al, 1992), compared to over 1000 IgAD individuals in over 20 reports. This is perhaps surprising, as despite the higher overall prevalence of IgAD (1:200-1:700 Caucasoids), most cases of IgAD are asymptomatic, and CVID is the commonest *symptomatic* primary immunodeficiency.

CVID is characterised by extensive clinical and laboratory heterogeneity. In addition to hypogammaglobulinaemia, other clinical features (eg. autoimmunity and lymphoproliferative disease) and laboratory abnormalities (particularly peripheral blood immunophenotypic abnormalities) are variably present. Thus it is possible that CVID represents several discrete diseases with different genetic diatheses, or that genetic factors determine the likelihood of developing such features. However no previous HLA studies in CVID have examined associations with CVID subgroups defined by clinical or laboratory criteria.

Also, interpretation of previous studies is hindered by the restricted number of HLA loci examined. Studies such as those of Olerup et al described above, whilst ascribing susceptibility to the HLA class II region, have not examined HLA class I polymorphism. Without doing so it is difficult to conclude what associations, if any, exist with "extended" haplotypes, as proposed for IgAD. Similarly, the C4A and TNFα *EcoR*I associations reported by Howe et al, whilst interesting in isolation, do little to determine which regions of HLA are primarily associated with CVID (Howe et al, 1991) in the absence of additional data.

Finally, the HLA typing techniques used in previous studies (serology and PCR-RFLP) are unable to distinguish all HLA allelic variants that differ in amino acid sequence. This is essential if the role of DQB1 codon 57 is to be resolved, as some

of the Asp57-bearing DQB1*06 subtypes commonly found in Caucasoids cannot be distinguished from non-Asp57 variants without recourse to higher-resolution genotyping techniques.

3.1.3. Aims of this chapter

The aim of this chapter was to address the limitations of previous HLA association studies in CVID by examining a large number of CVID patients; by using high-resolution genotyping techniques of both class I and II HLA loci; and by examining associations with subgroups of patients stratified on the basis of clinical and laboratory features. In addition, the controversy over the role of DQB1 codon 57 in CVID has been addressed by examining associations with all polymorphic HLA-A, -B, -Cw, -DRB1 and -DQB1 codons.

Table 3.1: Previous HLA association studies in CVID and IgA deficiency

Studies are listed in chronological order, with earliest studies first. HLA typing by "serology" refers to complement-dependent lymphocyte microcytotoxicity. Associations are positive unless marked ('-'). Alleles found to be associated individually are listed on separate lines or separated by commas. Alleles in associated haplotypes are separated by hyphens. Subjects labelled as IgAD patients often presented with problems not related to recurrent infection. Associations marked with an asterisk are not significant after correction for multiple comparisons.
ns = not significant, nd = none detected, na = not applicable, RR = relative risk, χ^2 = Chi-square statistic. CYP21A = 21-hydroxylase gene. PID = primary immunodeficiency

Reference	Disease (number)	Loci (technique)	Association	Significance
Hors et al, 1974	CVID (8), IgAD (2) Other PID (25)	A, B (serology)	**A1**	p<0.001
Ambrus et al, 1977	IgAD symptomatic patients(26)	A, B (serology)	**A1** **B8** **A1-B8**	χ^2=4.5; p<0.05 χ^2=5.3; p<0.05 χ^2=6.1; p <0.05
Evers et al, 1977	AT (4) IgAD (3) XLA (3),SCID (1) IgG subclass deficiency (2)	A, B (serology)	**A2** **A9 (-)** **B8 (-)**	p<0.02 <0.02 <0.04
Buckley et al, 1977	IgAD (15), CVID (14) other PID (37)	A, B (serology)	nd	
Seignalet et al, 1978	IgAD patients (47) CVID (3)	A, B (serology)	**A19** **B5** **Bw17**	p<0.05* p<0.05* p<0.05*
Smith et al, 1978	IgAD and IDDM (8)	A, B (serology)	**B8**	$p<5x10^{-5}$
Ostergaard et al, 1979	IgAD patients (22)	A, B, DR (serology)	**A1-B8**	p<0.05
Seligmann et al, 1979	CVID (case report)	C2 (serum levels)	**Homozygous C2 deficiency**	na
Oen et al, 1982	IgAD donors (62)	A, B (serology)	**A1** **B8** **B17**	χ^2=4.5; p <0.05* χ^2=12.3; $p=5x10^{-4}$ χ^2=9.3; p<0.005*
Jersild et al, 1983	IgAD (32)	A, B (not stated)	**B8**	p=0.014
Hammarström et al, 1983	IgAD donors (21)	A, B, Cw, DR (serology)	**A1*** **B8*** **DR3** **A28***	χ^2=3.2; p<0.05 χ^2=7.1; p <0.01 χ^2=11.1; $p<5x10^{-4}$ χ^2=2.7; p=0.05
Cobain et al, 1983	IgAD donors (44) & patients (37)	A, B (serology)	**A28** **B14** **A1-B14** **A28-B14** **not A1-B8** **A3-B7 (-)**	$p=7x10^{-4}$ $p<6x10^{-6}$ $p=7x10^{-4}$ p=0.002 p=0.09 not stated
Heikkila et al, 1984	IgAD donors (37)	A, B, Cw (serology)	**A1** **B8** **A1-B8** **A28-B8** **B7 (-)**	p<0.02* p<0.001 p<0.001 p<0.02* p<0.02*
Hammarström et al, 1984	IgAD donors (36) & patients (35)	A, B, Cw, DR (serology)	**B8 in donors** **DR3 in donors** **B40 in patients**	p<0.001 p<0.001 p<0.05
Strothman et al, 1986	IgAD donors (36)	A, B (all 36), DR (17) (serology)	**A1** **B14** **A2 (-)** **B7 (-)**	χ^2=10.2 p<0.005 χ^2=14.4 p<0.0005 p<0.01 p<0.005

Table 3.1 continued

Reference	Disease (number)	Loci (technique)	Association	Significance
Klemola et al, 1988	IgAD patients (62)	A, B, Cw, DR (Serology)	**A1, B8, B13, Cw6, DR3** **DR7** **DR2 (-)**	all p<0.0005 p<0.01* p<0.0005
Strothman et al, 1989	IgAD donors (46)	A, B, Cw, DR (Serology)	**DR3** with IgAD **DR7** with anti-IgA Ab **DR1** (-, anti IgA Ab)	p<0.05; RR 2.07 p<0.05; RR 2.94 p<0.05; RR 2.42
Schaffer et al, 1989	IgAD (16) CVID (19)	A, B, C, DR; (Serology) C4A, C4B, Bf, CYP21A (RFLP)	**C2, C4A, CYP21A** in association with **A1-B8-DR3**	CVID p<0.0001 IgAD p<0.01
Cuccia Belvedere et al, 1989	IgAD patients (44)	A, B, Cw, DR; (Serology) C4A, C4B, Bf (immunofixation)	**B14** **DR1** **DQw1** **C4A2** **C4B2** **B14-DR1** **A33-B14-DR1** **A28-B14-DR1** **BfS-C4A2-C4B2**	p=8x10^{-7}; RR 7.4 p=2x10^{-5}; RR 3.8 p=0.005; RR 2.0 p=0.005; RR 2.9 p=0.03; RR 2.2 p=3x10^{-5}; RR 16.4 p=7x10^{-4}; RR 11.5 p=0.002; RR 46.8 p=0.002; RR 8.9
Olerup et al, 1990	IgAD (96)	DR, DQ (PCR-RFLP)	**DR17-DQ2** (IgAD) **DR17-DQ2** (anti-IgA Ab) **DR1-DQ5** **DQB1 non-Asp57** **DR2-DQ6 (-)**	p<10^{-4}; RR 4.9 p<0.005 p<0.005; RR 2.2 p<10^{-7}; RR 5.9 p<0.001
Bucin et al, 1991	IgAD (110)	A, B, DR; (Serology) C4A, Bf (immunofixation)	**B8** **DR3** **C4AQ0** homozygosity **A1-B8-DR3** **B7 (-)** **DR2 (-)**	χ^2=13.53 p=0.004 χ^2=15.01 p=0.001 χ^2=9.35 p=0.01 χ^2=13.46; p=10^{-3} χ^2=19.47; p=10^{-4} χ^2=18.78; p=10^{-5}
Cunningham Rundles et al, 1991a	IgAD (36)	A, B, Cw, DR (Serology) C4A, C4B (immunofixation)	**C4AQ0, C4BQ0** (60%) **B38** (25%)	na: no controls studied
Howe et al, 1991	CVID (40)	DR, DQ, C4, TNF *EcoRI*, (RFLP)	**C4AQ0** **TNF *EcoRI*** in DR3+	χ^2=4.93; p<0.05 χ^2=5.1; p<0.025
Olerup et al, 1992	IgAD (69) CVID (86)	DRB1, DQA1, DQB1, DPA, DPB, C2, C4A, 21OH (PCR-RFLP)	**DR1-DQ5, DR7-DQ2** **DPw1** **21OH deletions** (secondary to DQ) **C4AQ0** (secondary to DQ) **DR15-DQ6** (-)	as in Olerup et al, 1990 IgAD p<0.005* CVID p<0.05* IgAD p<10^{-7}; RR4.4 CVID p<0.01; RR2.1 IgAD p<10^{-10}; RR4.4 CVID p<0.01; RR2.1 as in Olerup et al, 1990
Clerici et al, 1993	IgAD patients (45)	A, B, CW, DR, DQ (Serology)	**DR1; DR1-DQw1** **DR3-DQw2** **A1-B8-Cw7-DR3-DQw2** **B35-Cw4-DR1-DQw1** **B14-DR1-DQw1** **DR2 (-); DR4 (-)**	p<0.0001; p<0.002 p<0.002 p<0.00003 p<0.002 p<0.04 p<0.01; p<0.03
Machulla et al, 1994	IgAD patients (11), donors (21)	A, B (32, Serology) DR (24, Serology, PCR-SSO, PCR-SSP)	**A19, A29** **B8; DR3** **A2 (-)** **B35 (-)**	p<0.05 p<0.01; p<0.05 p<0.05 p<0.05
Mullighan et al, 1995	CVID (49)	A, B, Cw, DR, DQ (PCR-SSP)	**DRB1*15-DQB1*0602**	p<0.02
Fiore et al, 1995	IgAD patients (25)	DRB1, DQA1, DQB1 (PCR-SSOP)	**DRB1*0102** **DRB1*1302/3** **DRB1*0102-DQB1*0501** **DRB1*0404-DQB1*0302** **DRB1*0301-DQB1*0201** **DRB1*0101 (-)**	p=0.022; RR 4.02 p=0.035; RR2.63 p=0.015; RR 4.64 p=0.022 p=0.021; RR2.98

Table 3.2: Family studies of HLA in CVID and IgA deficiency

†The "A1-B8-DR3" and "A29-B44-DR7" extended haplotypes described in these reports are [A*01-Cw*0701-B*0801-TNFa5-HSP7.5-C2a-Bf0.4-G11.15-C4AQ0-C4BSf-DRB1*0301-DQB1*0201] and[A*29-B*44-TNFa9-HSP9-C2b-Bf0.6-G4.5-C4AL-C4BS-DRB1*0701-DQB1*0201] respectively

Reference	Disease; (no. kindreds; relatives; patients)	Loci studied (technique)	Associations
Van Thiel et al, 1977	IgAD (1; 40; 3)	A, B (serology)	**A2-B8-DR3**
Evers et al, 1977	Mixture (see table 3.1) (11; 24;11)	A, B (serology)	nd
Cleland et al, 1978	IgAD (2; 31; 16)	A, B (serology)	nd
Perez Jimenez et al, 1981	IgAD (2;11;3)	A, B (serology)	**B8**
Fuchs et al, 1984	CVID, IgAD (1;9;5 CVID, 1 IgAD)	A, B, Cw, DR (serology)	nd
Wilton et al, 1985 and **Cobain et al, 1985**	IgAD (13; not stated; 17)	A, B, DR (serology) C4A, C4B, Bf (immunofixation)	Non-functional **C4A** and **C4B** alleles (in association with the A1-B8-DR3†,Bw65(14)-C4A2-C4B1/2-BfS and Bw15-C4A6-C4B1-BfS haplotypes)
Lakhanpal et al, 1988	IgAD (1; 20; 6)	A, B, DR (serology)	Maternal (but not paternal) **A1-B8**
Cuccia Belvedere et al, 1989	IgAD (35; 83; 42)	A, B, C, DR (serology) C4A, C4B, Bf (immunofixation)	No associations
Keyeux et al, 1990	IgAD (1; 2; 2)	C4AQ0, CYP21A	**C4A-21OH deletions**
Cunningham Rundles et al, 1991a	IgAD (10; 16; 10)	A, B, Cw, DR (serology) C4A, C4B (immunofixation)	No clear associations
Bucin et al, 1991	IgAD (6; 39; 9)	A, B, DR, (serology) C4, Bf (Quantitation)	**A1-B8-DR3** extended haplotype†
Ashman et al, 1992	IgAD and CVID (1; 13; 2 IgAD, 3 CVID)	A, B, DR, DQ (PCR-SSOP) TNF, hsp-70 C2, Bf, G11, C4A, C4B (RFLP)	**A1-B8-DR3** extended haplotype†
Volanakis et al, 1992	IgAD & CVID (21; 79; 12 IgAD, 19 CVID)	A, B (serology) DR, DQB1 (PCR-SSOP) C4B, C4A, G11, C2, hsp-70 TNF (RFLP)	**A1-B8-DR3** and **A29-B44-DR7** extended haplotypes† No DQB1 codon 57 association
Olerup et al, 1992	IgAD sib-pair analysis	DRB1, DQA1, DQB1, DPA, DPB, C2, C4A, CYP21A (PCR-RFLP)	**Linkage to class II**
Vorechovsky et al, 1995b	34 kindreds, 12 with IgAD & CVID, 27 with autosomal dominant inheritance Linkage study in 16 kindreds	9 6p microsatellites DQB1 codon 57 (PCR)	**No linkage to MHC** (including DQB1 codon 57) in kindreds with autosomal dominant inheritance
Powis et al, 1994	CVID and IgAD (21; 79; 18 CVID, 11 IgAD)	TAP1, TAP2 (PCR-SSP)	No associations
de Asis et al, 1996	CVID (1 kindred, 3 CVIDs studied only)	A, B, Cw (Not stated)	No associations

Table 3.3: Summary of HLA associations with CVID and IgA deficiency

Associations are listed as originally described; for the majority of studies this is as serologically derived antigenic equivalents. Where a study has identified an association with a broad specificity only (eg. A9) rather than with one of the "splits" (eg. A23 or A24), both are listed, with the broad specificity followed by the split in brackets (A9(23,24)). Allelic nomenclature is only listed where used by the authors of the original report.
†The "A1-B8-DR3" and "A29-B44-DR7" extended haplotypes are listed in full in the previous table legend.

Antigenic/allelic associations	Positive / negative	Associated with CVID or IgAD
A1	+	CVID & IgAD
A2	+	CVID & IgAD
A3	-	CVID & IgAD
A19	+	IgAD
A9(23,24)	-	CVID & IgAD
A28(68,69)	+	IgAD
A29	+	IgAD
B5	+	IgAD
B7	-	IgAD
B8	+ and -	CVID (+) & IgAD (+ & -)
B13	+	IgAD
B14(64,65)	+	IgAD
B17(57,58)	+	IgAD
B38	+	IgAD
B40	+	IgAD
Cw6	+	IgAD
C2a	+	CVID & IgAD
C4AQ0	+	CVID & IgAD
CYP21A	+	CVID & IgAD
C4B2	+	IgAD
BfS-C4A2-C4B2	+	IgAD
TNF EcoR/ 5.5 kb	+	CVID
DR1	+	IgAD
DRB1*0101	+	IgAD
DRB1*0102	-	IgAD
DR2(15,16)	-	CVID & IgAD
DR3(17,18)	+	CVID & IgAD
DR4	-	IgAD
DRB1*0402, 0404	+	IgAD
DR7	+	IgAD
DQw1(5,6)	+	IgAD
DQ2	+	IgAD
DQ5	+	IgAD
DQ6	-	IgAD
DQB1 codon 57 Asp	-	CVID & IgAD

Haplotypic association	Positive / negative	Associated with CVID or IgAD
A1-B8-DR3	+	CVID & IgAD
A1-B14	+	IgAD
A28-B14-DR1	+	IgAD
B35-Cw4-DR1-DQw1	+	IgAD
DR1-DQ5 (DRB1*0102-DQB1*0501)	+	CVID & IgAD
DR3(17)-DQ2	+	CVID & IgAD
DRB1*0404-DQB1*0302	+	IgAD
DR2(15)-DQ6	-	CVID & IgAD
DR7-DQ2	+	CVID & IgAD
DPw1	+	CVID & IgAD
Extended haplotypes†		
A1-B8-DR3	+	CVID & IgAD
A29-B44-DR7	+	CVID & IgAD

3.2. RESULTS

3.2.1. Patient and control characteristics

3.2.1.1. Patient recruitment

179 unrelated Caucasoid patients were recruited from tertiary referral immunodeficiency clinics from England and Wales from October 1995 to May 1997. All fulfilled World Health Organisation (WHO) diagnostic criteria for CVID (WHO, 1995). All were receiving parenteral replacement immunoglobulin therapy at the time of study.

3.2.1.2. Demographic characteristics

Of the 179 patients studied, 73 were female, and 106 male. Basic demographic data (age at time of study, age at diagnosis, and serum IgG at diagnosis), is summarised in table 3.4.

Table 3.4: Basic CVID patient demographic data

	Mean (range; SD)
Age at study	43.7 (5-74; 16.6)
Age at diagnosis	33.4 (3-72; 17.8)
IgG at diagnosis	2.1 (0-5.2; 1.6)

3.2.1.3. Clinical features

Clinical data were available for 112 patients, and was collected independently of immunophenotyping and genotyping studies. Sufficient clinical, radiological and histologic data was available for 90 patients to enable classification into subgroups on the basis of the presence or absence of granulomata and splenomegaly. These patients were examined at weekly to six-monthly intervals over 2 to 22 years (mean 9.7 years). All patients received at least 2 chest X-rays during this period. CT chest examination was also performed in 48 patients. Splenic size was assessed by upper abdominal ultrasonography or computerised tomography in all 90 patients. All clinically suspicious lesions were biopsied to confirm the presence of granulomata, and to exclude malignancy and infection. No patients refused biopsy.

In the entire cohort, 8 patients had intravenous immunoglobulin-associated hepatitis C (HCV) infection prior to the time of study (Healey et al, 1996). Two

HCV-positive patients were included in the clinical analyses. In these, only clinical, radiological, histologic and immunophenotypic data obtained prior to HCV infection was included. The time of infection was clearly documented, and confirmed by retrospective analysis of stored sera for HCV RNA by RT-PCR (Healey et al, 1996). Similarly, for those patients receiving corticosteroid therapy, only clinical and laboratory data obtained prior to initiation of steroid treatment were used.

3.2.2. Controls for HLA associations studies

605 UK Caucasoid cadaveric kidney donors were used as controls for HLA association studies. These controls represent consecutive donors from around the United Kingdom genotyped at the Oxford Tissue Typing laboratory for transplantation purposes. This population has been extensively characterised and utilised for a variety of HLA studies, and has the expected HLA allelic frequencies for UK Caucasoids (Bunce et al, 1995; Bunce et al, 1996).

3.2.3. HLA class I and II associations - all patients

3.2.3.1. HLA class I and II typing

Genotyping of 8 HLA class I (HLA-A, -B, -Cw) and class II (HLA-DRB1, -DRB3, -DRB4, -DRB5 and -DQB1) loci was performed using the polymerase chain reaction and sequence specific primers (PCR-SSP) as previously described (Bunce et al, 1995; Bunce et al, 1996).

3.2.3.2. Initial analysis

Basic HLA association data with individual alleles, or groups of alleles, are presented in table 3.5. The previously described associations with alleles of the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 and A*02-Cw*0702-B*07-DRB1*1501-DQB1*0602 haplotypes were confirmed. Several other associations were detected, namely positive associations with B*14 and Cw*0802, and a negative association with DQB1*0603.

3.2.3.3. Haplotype analysis

These associations were further analysed by assessing frequencies of these haplotypes in the patient and control groups, either as haplotypic "fragments"

(pairs of adjacent alleles in linkage disequilibrium), or as full "extended" haplotypes. This data is shown in table 3.6. Frequency data for haplotypes previously associated with CVID or IgAD, but not found to be associated in this study (eg. A29-B44) is not shown.

Associations with the A1-B8-DR3 and B7-DR15-DQ6 haplotypes were confirmed. Furthermore, an increased rate of homozygosity for the A1-B8-DR3 haplotype was detected in the patient group. Positive associations with the B*14 containing haplotypes, such as Cw*0802-B*14 and B*14-DR*0701, were also detected.

3.2.3.4. Determination of the strongest associations

This data confirms previous reports of association of CVID with several extended haplotypes, and on initial inspection suggests that some alleles, or combinations of alleles, are more strongly associated than others. For example A1*-Cw*0701 ($p<0.0001$) appears to be more strongly associated than DRB1*0301-DQB1*0201 ($p=0.04$). However in order to determine which, if any alleles on these haplotypes are most strongly associated with CVID, further data stratification and analysis was required.

A method of determining the strongest association of alleles in linkage disequilibrium has been described by Svejgaard and Ryder (Svejgaard et al, 1994), as follows. X and Y are two alleles in linkage disequilibrium already known to be individually disease-associated. To determine which of X or Y is more strongly associated with disease, data is stratified in order to ask several additional questions: eg. is X is associated with disease in Y-negative as well as Y-positive patients? If, for example, X is associated in both Y-positive and Y-negative patients, it can be concluded that X contributes to disease susceptibility independently of Y. Further analyses investigate whether the X and Y associations differ significantly, or if a combined association with disease exists. Data stratification and analyses are explained in detail in table 3.7.

This approach has been successfully applied to multiple sclerosis (MS) (Svejgaard et al, 1994). MS has been shown in previous studies to be associated with both

HLA-B7 and HLA-Dw2, antigens that are in linkage disequilibrium (Jersild et al, 1973). Tests analogous to those outlined above show that Dw2 is increased in B7-negatives in addition to B7-positives. Furthermore a significant difference (in favour of Dw2) in the Dw2 and B7 associations exist, and a combined B7-Dw2 association does not exist. This is strong evidence that the strongest association is with Dw2, and the B7 association with MS is observed due to linkage disequilibrium.

The situation with CVID is more complex, in that 3 haplotypes are positively or negatively disease-associated, and more than two alleles on each haplotype are associated. Consequently, for each of the A1-B8-DR3, B7-DR15-DQ2 and B14-related haplotypes, associations were compared between all possible pairs of alleles. For example, the strength of the A*01 association was compared with each of the Cw*0701, B*0801, DRB1*0301 and DQB1*0201 associations. Each of the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201, A*02-Cw*0702-B*07-DRB1*1501-DQB1*0602 and Cw*0802-B*14-DRB1*0701-DQB1*0201 haplotypes has been analysed using this approach. Data is summarised in tables 8.1, 8.2 and 8.3 in appendix 8.2.

This approach confirmed the strong positive association of CVID with the A1-B8-DR3 haplotype, but no single allele on this haplotype was more significantly associated with CVID than any other. It is possible that the extremely strong linkage disequilibrium between alleles on this haplotype masks a primary association with one allele. A vastly larger number of patients and controls would be required to detect differences in the strength of A1-B8-DR3 allelic associations. For example, a 50-fold greater number of patients (9000) and controls (30 000) would be necessary to detect a weak difference in the Cw*0701 and B*0801 associations.

Several observations from this data stratification suggest the association with the A1-B8-DR3 haplotype involves multiple alleles, ie. requires the presence of the haplotype per se, rather than only one allele. First, as stated above, tests for independence of associations with A1-B8-DR3 alleles were negative (i.e.

comparisons [3], [5] and [8] in table 8.1, produce equally increased RR values). Secondly, tests for dependence showed lack of association with one allele in the absence of any other allele on the same haplotype (tests [4] and [6]). Finally, associations between A1-B8-DR3 alleles were stronger in patients than in controls (RR values in comparison [9] are greater than [10]; the reverse was true for χ^2 and χ^2 p values, but this reflects the 3-fold greater number of controls examined). The implication of this data is that another allele on the A1-B8-DR3 haplotype not examined in this study is primarily associated with disease, and the observed HLA-A, -B, -Cw, -DRB1 and -DQB1 associations are due to linkage disequilibrium.

Upon analysis of B*14 containing haplotypes, it was demonstrated that B*14 was more strongly associated with CVID than the linked alleles A28, DR7 and DQ2 (although the p values for these latter 2 analyses do not reach significance). B14 was also associated with CVID in the absence of A1, but this was not reflected as a significant difference between the 2 associations, as A1 was also associated with CVID as part of the A1-B8-DR3 haplotype. It was not possible to determine if B*14 or Cw*0802 is more strongly associated with CVID.

The centromeric alleles on the A2-B7-DR15 haplotype (ie. B*07, Cw*0702, DRB1*1501 and DQB1*0602) were more strongly negatively associated with CVID than A*02. However from the present analysis, none of the remaining alleles were more significantly associated with CVID than any others.

3.2.3.5. Summary

Thus in this study, significant positive associations with the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 and Cw*0802-B*14-DRB1*0701 haplotypes were detected, in addition to a negative association with the A*02-Cw*0702-B*07-DRB1*1501-DQB1*0602. Associations were detected both with individual alleles on these haplotypes, and with full haplotypes, or portions of haplotypes. Stratification of A1-B8-DR3 association data suggested that no single allele was associated with disease, rather a combined association was present. Similarly, no difference in the B*14 and Cw*0802 associations was detected. These findings suggest that a linked allele, possibly shared by the A*01-Cw*0701-B*0801-

suggest that a linked allele, possibly shared by the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 and Cw*0802-B*14-DRB1*0701-DQB1*0201 haplotypes, is responsible for the observed HLA associations with CVID. This analysis does not exclude a primary association with an amino acid, or group of amino acids, at a polymorphic HLA codon, and this hypothesis is examined in the next section of this chapter.

Table 3.5: HLA associations with CVID (individual alleles)

Most associations are accounted for by alleles lying on the A1-B8-DR3-DQ2, C7-B7-DR15-DR6, or B14-containing haplotypes. no. = number. RR = relative risk. AF = aetiological fraction. PF = preventive fraction (derivation in chapter 2).

	Patients (n=179)		Controls (n=605)						
Allele	**no.**	**%**	**no.**	**%**	**χ^2**	**χ^2 p**	**Fisher's p**	**RR (95% CI)**	**AF/PF**
A*01	80	44.7	217	35.9	4.57	0.03	0.04	1.45 (1.03-2.03)	0.14
A*02	73	40.8	296	48.9	3.68	0.06	0.06	0.72 (0.51-1.01)	0.14
Cw*0701	75	41.9	183	30.2	8.49	0.001	0.01	1.66 (1.18-2.35)	0.17
Cw*0702	23	12.8	168	27.8	16.69	<0.0001	<0.0001	0.39 (0.24-0.63)	0.17
Cw*0802	25	14.0	45	7.4	7.24	0.01	0.01	2.03 (1.21-3.42)	0.07
B*07	24	13.4	162	26.8	13.64	0.0001	0.0001	0.43 (0.27-0.69)	0.15
B*0801	64	35.8	150	24.8	8.36	0.001	0.01	1.69 (1.18-2.42)	0.15
B*14	25	14.0	45	7.4	7.24	0.007	0.01	2.03 (1.21-3.42)	0.07
DRB1*15	23	12.8	168	27.8	16.69	<0.0001	<0.0001	0.39 (0.24-0.63)	0.17
DRB1*0301	61	34.1	151	25.0	4.55	0.03	0.04	1.56 (1.09-2.23)	0.12
DQB1*0201	96	53.6	162	26.8	2.88	0.01	0.01	3.15 (2.23-4.45)	0.37
DQB1*06	47	26.3	246	40.7	12.25	0.00	0.00	0.52 (0.36-0.76)	0.19
DQB1*0602	20	11.2	159	26.3	17.89	<0.0001	<0.0001	0.36 (0.22-0.59)	0.17
DQB1*0603	10	5.6	73	12.1	6.13	0.01	0.01	0.45 (0.23-0.89)	0.06
Frequency data for alleles found to be associated with CVID(± IgAD) in previous studies, but not the current study									
A*03	42	23.5	159	26.3	0.57	ns	ns		
A*23	4	2.2	11	1.8	0.13	ns	ns		
A*24	29	16.2	87	14.4	0.36	ns	ns		
B*44	55	30.7	187	30.9	0.93	ns	ns		
DRB1*0101-2/4	35	19.6	116	19.2	0.01	ns	ns		
DRB1*0701	57	31.8	161	26.6	1.88	ns	ns		
DQB1*0501	50	27.9	176	29.1	0.09	ns	ns		
Frequency data for alleles found to be associated with IgAD but not CVID in previous studies, but not the current study									
A*28	10	5.6	40	6.6	0.62	ns	ns		
A*29	14	7.8	44	7.3	0.06	ns	ns		
A*33	5	2.8	8	1.3	1.83	ns	ns		
Cw*0602	30	16.8	122	20.2	1.03	ns	ns		
Cw*1602	0	0.0	2	0.3	0.59	ns	ns		
B*13	6	3.4	22	3.6	0.32	ns	ns		
B*4001	17	9.5	74	12.2	1.03	ns	ns		
B*17	14	7.8	62	10.2	1.00	ns	ns		
DRB1*0103	2	1.1	11	1.8	0.42	ns	ns		
DRB1*04	53	29.6	211	34.9	1.72	ns	ns		

Table 3.6: Associations of haplotypes and haplotype fragments with CVID

Frequencies are given for haplotype fragments (pairs of adjacent alleles) followed by entire "extended" haplotype frequencies. RR = relative risk. OR = odds ratio. AF/PF = aetiologic/preventive fraction (see chapter 2 for derivation).

	Patients		Controls							
Haplotype	+	(%)	+	(%)	χ^2	χ^2 p	Fisher p	RR	OR	AF/PF
A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201										
A*01-CW*0701	61	34.1	117	19.3	17.10	<0.0001	<0.0001	2.16 (1.49-3.12)	1.76	0.18
homozygote	8	4.5	8	1.3	6.94	0.008	0.01	3.48 (1.29-9.42)	2.25	0.03
Cw*0701-B*0801	62	34.6	143	23.6	7.82	0.005	0.007	1.71 (1.20-2.46)	1.50	0.14
homozygote	14	7.8	13	2.1	13.33	0.0003	0.0008	3.85 (1.77-8.34)	2.38	0.06
B*0801-DR*0301	52	29.1	117	19.3	7.70	0.006	0.007	1.71 (1.20-2.46)	1.49	0.12
homozygote	10	5.6	5	0.8	16.68	<0.0001	0.0003	6.76 (2.28-20.1)	3.03	0.05
DR*0301-DQB1*0201	60	33.5	156	25.8	4.14	0.04	0.05	1.45 (1.01-2.08)	1.33	0.1
homozygote	11	6.1	11	1.8	9.40	0.002	0.007	3.53 (1.5-8.28)	2.27	0.04
A*01-Cw*0701-B*0801-DR*0301-DQB1*0201	43	24.0	86	14.2	9.67	0.002	0.003	1.91 (1.27-2.89)	1.61	0.11
homozygote	4	2.2	2	0.3	6.59	0.01	0.03	6.19 (1.12-34.1)	2.96	0.02
(A*02/A*03)-Cw*0702-B*0701-DRB1*1501-DQB1*0602										
A*02-Cw*0702	8	4.5	73	12.1	8.61	0.003	0.003	0.36 (0.17-0.76)	0.41	0.07
A*03-Cw*0702	10	5.6	88	14.5	10.14	0.002	0.001	0.36 (0.18-0.71)	0.41	0.09
Cw*0702-B*0701	18	10.1	154	25.5	19.12	<0.0001	<0.0001	0.33 (0.20-0.56)	0.40	0.17
B*0701-DRB1*1501	10	5.6	92	15.2	1.295	0.0008	0.0006	0.34 (0.18-0.68)	0.40	0.1
DRB1*1501-DQB1*0602	21	11.7	158	26.1	16.22	<0.0001	<0.0001	0.38 (0.23-0.63)	0.45	0.16
A*02-Cw*0702-B*0701-DRB1*1501-DQB1*0602	3	1.7	36	6.0	5.34	0.02	0.02	0.31 (0.09-1.02)	0.33	0.04
A*03-Cw*0702-B*0701-DRB1*1501-DQB1*0602	4	2.2	49	8.1	7.54	0.006	0.004	0.29 (0.10-0.81)	0.32	0.05
Cw*0702-B*0701-DRB1*1501-DQB1*0602	8	4.5	87	14.4	12.74	0.0004	0.0001	0.29 (0.14-062)	0.34	0.1
B*14-related haplotypes										
A*01-B*14	9	5.0	12	2.0	4.91	0.03	0.04	2.65 (1.1-6.38)	1.92	0.03
Cw*0802-B*14	24	13.4	44	7.3	6.56	0.01	0.01	1.99 (1.17-3.37)	1.63	0.07
B*14-DR*01	5	2.8	8	1.3	1.83	ns	ns	-	-	-
B*14-DRB1*0701	13	7.3	20	3.3	5.36	0.02	0.03	2.32 (1.13-4.75)	1.78	0.04
DRB1*0701-DQB1*0201	50	27.9	120	19.8	5.33	0.02	0.02	1.57(1.07-2.30)	1.40	0.1
DRB1*01-DQB1*05	34	19.0	114	18.8	0.002	ns	ns	-	-	-

Table 3.7: Example of data stratification to compare the strength of associations of alleles in linkage disequilibrium

Both HLA-A*01 and -Cw*0701 are associated with CVID, individually and as a haplotype. The analysis below stratifies patients and controls on the basis of the presence or absence of A*01 and Cw*0701 in order to determine which of these alleles is more strongly associated with disease. *Basic data* shows the number of patients and controls positive for one allele only, for both, and for neither. This data forms the basis for entries in a 2x2 contingency table. 10 comparisons are performed, in 4 categories. *Comparisons* [1] and [2] confirm that both A*01 and Cw*0701 are individually associated with disease. Secondly, tests for independence show that A1 is only associated with CVID in patients who are also Cw*0701 positive ([3] and [4]), and vice versa ([5] and [6]). Comparison [7] shows that there is no significant difference in the strength of associations. Rather, a combined association exists ([8]). Finally, comparisons [9] and [10] confirm the strong linkage disequilibrium between A*01 and Cw*0701 in both patients and controls.

Basic Data

A*01	Cw*0701	CVID	controls
+	+	61	117
+	-	19	100
-	+	14	66
-	-	85	322
	total	179	605

Comparisons

Comparison	Entries of 2x2 table				RR	χ^2	p	Interpretation
Individual associations								
[1] A*01 v non A*01	80	99	217	388	1.45	4.20	0.04	A*01 associated with CVID? - YES
[2] Cw*0701 v non Cw*0701	75	104	183	422	1.66	7.97	0.005	Cw*0701 associated with CVID? - NO
Is A*01 associated independently of Cw*0701?								
[3] + + v - +	61	14	117	66	2.40	6.74	0.009	A*01 associated in Cw*0701 positives? - YES
[4] + - v - -	19	85	100	322	0.73	1.73	ns	A*01 associated in Cw*0701 negatives? - NO
Is Cw*0701 associated independently of A*01?								
[5] + + v + -	61	19	117	100	2.70	11.23	0.001	Cw*0701 associated in A*01 positives? - YES
[6] - + v - -	14	85	66	322	0.82	0.70	ns	Cw*0701 is associated in A*01 negatives? - NO
Difference in associations, or combined association?								
[7] + - v - +	19	14	100	66	0.89	0.23	ns	Significant difference in A*01 & Cw*0701 associations? - NO
[8] + + v - -	61	85	117	322	1.97	11.14	0.001	Significant combined A*01-Cw*0701 association? - YES
Association between A*01 and Cw*0701 in patients?								
[9]	61	19	14	85	18.60	67.58	$<10^{-16}$	A*01 & Cw*0701 in linkage disequilibrium in patients? - YES
Association between A*01 and Cw*0701 in controls?								
[10]	117	100	66	322	5.67	88.10	$<10^{-21}$	A*01 & Cw*0701 in linkage disequilibrium in controls? - YES

3.2.4. Amino acid associations

As mentioned above, it has been suggested that the primary HLA association with CVID is with non-aspartate amino acids at HLA-DQB1 codon 57 (Olerup et al, 1990; Olerup et al, 1991; Olerup et al, 1992). It has been proposed that this association explains all other observed HLA class I, II and III associations by linkage disequilibrium, including associations with alleles on the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201, A*02-Cw*0702-B*07-DRB1*1501-DQB1*0602 and Cw*0802-B*14-DRB1*0701 haplotypes detected above.

However the validity of these studies is limited by the methodology employed. The PCR-RFLP HLA typing used was not capable of differentiating all DQB1 alleles that occur in Caucasoids and that differ in amino acid sequence. Furthermore, the authors only examined amino acid polymorphism at one HLA codon (DQB1 codon 57). It is possible that this association may be no more significant than with other polymorphic HLA class I and II codons. For these reasons, high resolution HLA-DQB1 genotyping using PCR-SSP was developed, capable of distinguishing all recognised DQB1 alleles that differ in amino acid sequence. Amino acid polymorphism was then analysed at every polymorphic HLA-A, -B, -Cw, -DRB1 and -DQB1 codon.

3.2.5. HLA-DQB1 high resolution genotyping

Oligonucleotide primers complementary to intron 1 and exons 2-4 of DQB1 were designed according to published HLA-DQB1 nucleotide sequences (Marsh et al, 1995; Mersch et al, 1996; Saito et al, 1996; Trachtenberg et al, 1996; Vilches et al, 1996; Williams et al, 1996; Bodmer et al, 1997; Olerup et al, 1997; Thye et al, 1997) and the unpublished DQB1*0613 sequence registered with Genbank (accession number U77344). Most primer pairs were designed utilising the high degree of sequence variability in exon 2 of HLA-DQB1. Primers complementary to exon 3 were used to distinguish DQB1*0202-3 from *0201 (reaction 2). A combination of exon 3 and exon 4 primers was used to generate a DQB1*0201-specific amplicon (reaction 1). Primers sequences are listed in table 3.8. Primer annealing positions relative to the DQB1 nucleotide sequence are given in appendix 8.1.

Thirty-two reactions were used. The primers, primer concentration, and specificity of each reaction are listed in table 3.9. Buffer composition, control primer concentration, reaction conditions, PCR cycling parameters and amplicon detection were as previously described in chapter 2.

Reactions 2-32 incorporate a DRB1-specific control primer pair, producing a 796 base pair amplicon, to verify successful amplification (Bunce et al, 1995). However, reaction 1 (DQB1*0201) generates an allele-specific amplicon of approximately 700 base pairs, too close in size to the 796 bp control amplicon used for reactions 2-32 to be resolved by 1% agarose gel electrophoresis. Consequently, primers specific for the adenomatous polyposis coli gene, generating a 256 bp amplicon, were used for this reaction (Sadler et al, 1994).

As preliminary validation, and in order to identify as many different DQB1 alleles as possible, 344 Caucasoid cadaveric kidney donors previously DQB1 genotyped by medium resolution PCR-SSP (Bunce et al, 1993; Bunce et al, 1995) were successfully HLA-DQB1 subtyped. Primer mixes were further validated using cell lines listed in table 3.10. The genotypes of these cell lines were in agreement with those reported by the 11th Histocompatibililty Workshop (Dong et al, 1992), with the exception of HO301, which was found to be DQB1*0609, rather that *0605. Reference cell lines were not available for DQB1*0203, *0304-7, *0504, *0605-8 and *0610-13. DNA samples for DQB1*0306, *0612 and *0613 were typed successfully. Examples of high resolution HLA-DQB1 typing are given in figure 3.1.

This method will detect all currently recognised (as at July 1997) HLA-DQB1 alleles: DQB1*0201-3, *0301-7, *0401-2, *0501-4, and *0601-13. Synonymous variants are not distinguished (DQB1*05031/*05032, *06011/*06012 and *06051/*06052). In addition, this method permits differentiation of all heterozygous DQB1 combinations, with the exception of combinations involving several alleles that have not been detected in Caucasoid populations. Heterozygous combinations that are not distinguished by this method are listed in table 3.11.

Table 3.8: Oligonucleotide primers used in high-resolution HLA-DQB1 subtyping

All primers anneal to exon 2 of DQB1 unless otherwise marked. †The italicised nucleotides anneal to the last 7 nucleotides of intron 1.

Primer no.	Sense / antisense	DQB1 annealing position (codon numbers)	Primer sequence
77	S	15-21	5'gCTACTTCACCAACgggACC
79	S	21-26	5'ACggAgCgCgTgCgggg
82	S	24-30	5'gTgCgTCTTgTgAgCAgAAg
88	S	20-26	5'gACggAgCgCgTgCgTCT
181	S	19-26	5'gACggAgCgCgTgCgTTA
347	S	6-13	5'TTTCgTgCTCCAgTTTAAggC
348	S	43-48	5'gACgTgggggTgTACCgC
349	S	21-27	5'ggAgCgCgTgCgTCTTgTA
353	S	24-30	5'gTgCgTCTTgTgACCAgATA
624	S	23-30	5'CgTgCgTCTTgTAACCAgAC
627	S	7-14	5'*CgTgTA*CCAgTTTAAgggCC
628	S	7-14	5'*CgTgTA*CCAgTTTAAgggCA
629	S	22-27	5'gAgCgCgTgCgTCTTgTg
851	S	5-9†	5'*CCCgCAg*AggATTTCgTgTT
852	S	5-9†	5'*CCCgCAg*AggATTTCgTgTA
969	S	24-30	5'TgCggggTgTgACCAgAC
970	S	24-30	5'gTgCggggTgTgACCAgAT
971	S	17-23	5'CACCAACgggACCgAgCT
972	S	17-23	5'CACCAACgggACCgAgCg
973	S	129-135 (exon 3)	5'TCCggTggTTTCggAATgg
988	S	129-135 (exon 3)	5'gTCCggTggTTTCggAATgA
78	AS	77-84	5'TggTAgTTgTgTCTgCATACg
89	AS	57-63	5'CTgTTCCAgTACTCggCgT
111	AS	84-89	5'CCgCggAACgCCACCTC
112	AS	84-89	5'CgTgCggAgCTCCAACTg
350	AS	74-80	5'TgCACACCgTgTCCAACTC
351	AS	74-80	5'TgCACACCCTgTCCACCg
619	AS	48-54	5'CCTgCggCgTCACCgCC
620	AS	70-75	5'CAACTCCgCCCgggTCCT
621	AS	70-75	5'AACTCCgCCCgggTCCC
622	AS	57-64	5'gCTgTTCCAgTACTCggCAT
630	AS	48-54	5'CCTgCggCgTCACCgCg
831	AS	57-64	5'TggCTgTTCCAgTACTCggCgg
853	AS	57-63	5'TgTTCCAgTACTCggCgCT
967	AS	57-63	5'gCTgTTCCAgTACTCggCAA
968	AS	47-53	5'gCggCgTCACCgCCC gA
974	AS	172-177 (exon 3)	5'gTgCTCCACgTggCAggT
987	AS	49-54	5'CCCCAgCggCgTCACCA
990	AS	191-198 (exon 4)	5'TgCTCTgggCAgATTCAgAT

Table 3.9: Primer mixes used for high-resolution HLA-DQB1 subtyping

*This reaction uses a smaller control amplicon (256 bp) than all other reactions (796 bp).

Lane number	Primer mix number	Sense primer number	Conc (mM)	Antisense primer	Conc (mM)	Specificity (DQB1 alleles)	Product size (base pairs)
1*	404	988	3.4	990	3.4	*0201	700
2	405	973	0.68	974	3.4	*0202,*0203	144
3	406	82	3.4	89	3.4	*0203	120
4	317	181	6.8	89	6.8	*03,*0601	128
5	148	181	3.4	112	3.4	*0301,*0304	207
6	407	347	3.4	831	3.4	*0304	207
7	362	353	3.4	831	3.4	*0302,*0307	101
8	150	88	6.8	89	6.8	*0203,*03032,*0306	129
9	142	77	3.4	350	3.4	*0305	195
10	369	88	3.4	78	3.4	*0306	190
11	408	852	3.4	987	3.4	*0307	156
12	409	971	3.4	78	3.4	*0401	202
13	410	972	3.4	78	3.4	*0402	202
14	411	79	3.4	967	3.4	*0501	130
15	412	969	3.4	853	3.4	*0502	118
16	413	969	3.4	968	3.4	*0503	87
17	414	970	3.4	853	3.4	*0504	119
18	333	347	3.4	111	3.4	*0601	248
19	367	851	3.4	630	3.4	*0602,*0610,*0613	153
20	334	629	3.4	630	3.4	*0602,*0610,*0611,*0613	96
21	338	348	1.7	621	1.7	*0602-3,*0608,*0610-11,*0613	96
22	342	628	3.4	350	3.4	*0602-4,*0607-13,*0302-3/5-7	218
23	335	349	3.4	350	3.4	*0603-5,*0607-9,*0612	176
24	340	349	3.4	622	3.4	*0603,*0607	127
25	366	852	1.7	622	1.7	*0603,*0607,*0611, (weak*03)	183
26	364	349	3.4	621	3.4	*0603,*0608,*0612	155
27	339	349	3.4	619	3.4	*0604-6,*0609,*0612	97
28	337	624	3.4	620	3.4	*0604,*0607	155
29	341	627	1.7	350	1.7	*0605	218
30	336	349	3.4	351	3.4	*0606	176
31	365	851	3.4	853	3.4	*0610	180
32	415	851	3.4	967	0.68	*0613	184

Table 3.10: Reference cell lines used for validation of high-resolution HLA-DQB1 subtyping

DQB1 allele	Cell line
*0201	WT49
*0202	BOWEN, BUP (BURKHARDT)
*0301	ATH, BM15, BM16, BR1P, JHA, RML
*0302	BOLETH, BSM, FS
*03032	DBB
*0401	KT3
*0402	BTB'A', RSH, SPL, SPACH
*0501	BVR, RAINTER, RENNEY
*0502	AZH
*05031	RAJIN, WT52
*06011	BGE
*0602	PGF
*0603	CB6B, WDV
*0604	DAUDI
*0609	HO301

Table 3.11: DQB1 allelic combinations indistinguishable by high resolution DBQ1 subtyping

Indistinguishable combinations of alleles are listed by rows. eg. a DQB1*0203/*0202 heterozygote is indistinguishable from an *0203 homozygote.

Indistinguishable DQB1 allele combinations	
*0203 / *0202	*0203 / *0203
*0303 / *0306	*0306 / *0306
*0602 / *0610	*0610 / *0610
*0602 / *0613	*0613 / *0613
*0603 / *0608	*0603 / *0603
*0610 / *0613	*0610 / *0610

Figure 3.1: Examples of high-resolution HLA-DQB1 genotyping by PCR-SSP

Sample 1

Positive lanes: 4, 18, 22, 23, 27, 28
DQB1 genotype: *0601, *0604

Direction of electrophoresis

Sample 2

Positive lanes: 2, 22, 23, 26, 27.
DQB1 genotype: *0202, *0612

LEGEND:
PCR products have been electrophoresed through 1% agarose and photographed after UV illumination.
48 reactions have been used for each sample.
Each lane has a control amplicon with or without a allele-specific amplicon.
All lanes use a 796 bp control amplicon, with the exception of lane 1.
Lane 1 uses a 250 bp control amplicon, due to the large size of the DQB1*0201 specific product
See text for composition and specificity of each primer mix.

3.2.5.1. *Analysis of amino acid associations*

Each polymorphic residue in HLA-A, -B, -Cw, -DRB1 and -DQB1 residue was analysed for specific amino acid associations with CVID. This analysis was performed using the PC-based Disease Discriminator program (Martin Barnardo et al, unpublished data), in several stages, as follows. First, frequencies of individual amino acids at each polymorphic codon were measured in patients and controls. Secondly, frequencies of groups or "sets" of amino acids were compared, to determine (for example) if acidic, neutral or basic amino acids predispose to disease (or afford protection). These steps were performed by the program. Finally, the amino acid sequences of each allele at each HLA locus was examined, in order to determine if any observed associations truly reflect a primary association with an amino acid (or groups of amino acids), or rather an association with an allele containing that amino acid. If the latter is the case, several amino acids common to an allele would be associated with comparable significance. This final stage was performed manually by examination of peptide sequence alignments for each locus.

Significant associations with individual amino acids or groups of amino acids are summarised in tables 3.12 and 3.13. The principle finding from this analysis was that whilst many amino acids (or groups of amino acids) at both HLA class I and II loci are significantly associated with CVID, the majority of these associations correspond to the previously detected associations with the A1-B8-DR3, A2-B7-DR15 or B14-DR7 haplotypes. Several amino acid associations were detected that do not correspond to previously allelic associations, (such as in HLA-Cw codon 9 and DRB1 codons 85 and 161) but these were not highly significant.

A significant difference in the distribution of amino acids at codon 57 (A, S, D or V) between patients and controls was detected, primarily due to an increased frequency of alanine (A) and decreased frequency of aspartate (D) in patients ($p=0.0003$). However a primary association with non-Asp at codon 57 (as previously described) was not detected. Examining the group associations at DQB1, a positive association with non-Asp amino acids at codon 57 was detected (ie A and V, $p=0.007$), however this result is of comparable or weaker significance

that a large number of other DQB1 (and other HLA) amino acid associations that all correspond to the positive and negative haplotypic associations described above.

An increased frequency of DQB1 codon 57 non-Asp homozygosity in patients was observed (table 3.14). Again, like many other amino acid associations, this finding corresponds to the DQB1*0201 and *0602 associations described above, and is not of primary importance.

Data from these analyses is listed comprehensively in appendix 8.3.

Table 3.12: Summary of HLA class I amino acid associations with CVID

Results for each HLA locus are listed as individual amino acid associations, and associations with amino acid groups. Associations are listed in descending order of significance. Column one lists the Chi-square p value. The codon and associated amino acids are listed in the next column, with positively associated amino acids followed by "+", and negatively associated amino acids by "-". The final column lists those alleles previously associated with CVID that carry the associated amino acid at that codon. Other common alleles also positive for an amino acid but not previously associated with CVID are listed in brackets. Only common alleles or groups of alleles are listed. The amino acid code is given in appendix 8.3 eg. HLA-A codon 166D is positively associated with CVID, and 166E negatively (p=0.009). This is corresponds to the positive association of CVID with A*01, which has 166D, and negative association with A*02, which has 166E. An example of an association with a group of amino acids is HLA-A codon 77; D or S are significantly less frequent in CVID than in controls. This corresponds to the A*02 negative association (A*02 is 77D). A comprehensive listing of all significant amino acid associations is given in appendix 8.3.

Individual amino acid associations

p value	codon (aa)	Corresponds to association with:
HLA-A		
0.009	166 (D+, E-)	A*01 (D), A*02 (E)
0.009	167 (G+, W-)	A*01 (G), A*02 (W)
0.01	67 (M+, V-),	A*01 (M), A*02 (V)
0.01	150 (V+, A-)	A*01 (V), A*02 (A)
0.01	158 (V+, A-)	A*01 (V), A*02 (A)
0.01	163 (R+, T-)	A*01 (R), A*02 (T)
0.01	90 (D+, A-)	A*01 (D), A*02 (A)
0.02	105 (P+, S-)	A*01 (P), A*02 (S)
0.02	76 (A+, V-)	A*01 (A), A*02 (V)
0.02	77 (N+, D-)	A*01 (N), A*02 (D)
0.04	142 (I+, T-)	A*01 (I), A*02 (T)
0.04	145 (R+, H-)	A*01 (R), A*02 (H)
0.04	156 (R+, L-)	A*01 (R), A*02 (L)
HLA-C		
No significant individual allelic associations		
HLA-B		
0.0001	67 (F+ Y-)	*0801 (F), *0702 (Y)
0.0001	163 (T+ E-)	*0801 (T), *14 (T), *0702 (E)
0.0002	9 (D+ Y-)	*0801 (D), *0702 (Y)
0.0009	156 (D+ R-)	*0801 (D), *0702 (R)
0.001	178 (T+ K-)	*0801 (T), *14 (T), *0702 (K)
0.002	71 (T+ A-)	*0801 (T), *14 (T), *0702 (A)
0.005	114 (N+ D-)	*0801 (N), *14 (N), *0702 (D)
0.006	11 (A+ S-)	*0801 (A) *0702 (S)
0.007	70 (N+ Q-)	*0801 (N), *14 (N), *0702 (Q)
0.03	74 (D+ Y-)	*0801, *14 & *0702 (all D)
0.04	12 (M+ V-)	*0801 (M), *14 (V), *0702 (V)

Associations with groups of amino acids

p value	codon (set of aa)	Corresponds to association with:
HLA-A		
0.003	77 set (D S -)	A*02 (D) (*25 & *32) (S)
0.004	152 set (V E -)	A*02 (V or E); (*25, *26) (E); *(24, *29) (V)
0.007	156 set (W L -)	all except *01 are W, L or Q
0.009	152 set (W V E -)	A*02 (V or E); (*25, *26) (E); (*24, *29) (V); (*30) (W)
0.02	152 set (W V -)	A*02 (V); (*24, *29) (V); (*30) (W)
0.03	156 set (W R +)	A*01 (R)
0.04	156 set (L Q -)	all except *01 are W, L or Q
0.04	97 set (M R -)	all except *01, *30 (I)
HLA-C		
0.02	9 set (F S)	not explained
HLA-B		
<0.0001	163 set (L E -)	*0702 (E) (also *15, *35 L)
<0.0001	156 set (W R -)	*0702, *15 (R)
0.0001	114 set (D H -)	*0702 (D)
0.0001	9 set (Y H -)	*0702 (Y)
0.0004	67 set (Y M S -)	*0702 (Y)
0.0005	67 set (Y S -)	*0702 (Y)
0.0005	67 set (F C +)	*0702 (Y)
0.001	67 set (Y M -)	*0702 (Y)
0.001	70 set (S Q -)	*0702 (Q)
0.001	67 set (Y M C S -)	*0702 (Y)
0.001	67 set (Y C S -)	*0702 (Y)
0.005	97 set (R V N +)	not explained
0.006	70 set (K Q -)	*0702 (Q) (also *27 K)
0.007	70 set (Q K S -)	*0702 (Q) (also *27 K)

Table 3.13: Summary of HLA class II amino acid associations with CVID

Note that most associations, either with individual amino acids or with groups of amino acids, correspond to the previously described associations with alleles on the DR3-DQ2, DR15-DQ6 haplotypes (ie. positive associations with DRB1*0301 and DQB1*0602, and negative associations with DRB1*15 and DQB1*0602). DQB1 codon 57 associations were detected (italicised and bolded), but a non-Asp (i.e. A or V) association was not of primary significance, in contrast to previous reports. All significant amino acid associations are listed in appendix 8.3.

Individual amino acid associations			**Associations with groups of amino acids**		
p value	**codon (aa)**	**Corresponds to associations with:**	**p value**	**codon (set of aa)**	**Corresponds to associations with:**
HLA-DRB1			***HLA-DRB1***		
<0.0001	73 (G+ A-)	*0301 (G) *15 (A)	<0.0001	37 set (Y S -)	*15 (S); (*04, *08, *13) (Y)
<0.0001	133 (R+ L-)	*0301 (R) *15 (L)	<0.0001	37 set (Y L S -)	as above; *12 (L)
<0.0001	142 (V+ M-)	*0301 (V) *15 (M)	<0.0001	74 set (A E L -)	*15 (A); (*13) (A); *08 & *14 (E/L)
0.0002	96 (H+ Q-)	*0301 (H) *15 (Q)	<0.0001	11 set (P V L -)	*15 (P); *04, *10 (V); *01 (L)
0.0004	37 (N+ S-)	*0301 (N) *15 (S)	<0.0001	13 set (H F R -)	*15 (R); *04 (H); *01 (F)
0.0004	71 (K+ A-)	*0301 (K) *15 (A)	<0.0001	96 set (Q Y E -)	*15 (Q); *04 (Y); *01 (E)
0.0007	11 (S+ G+ P-)	*0301 (S), *0701 (G), *15 (P)	<0.0001	13 set (H F R G -)	*15 (R); *04 (H); *01 (F); *08 (G)
0.001	74 (Q+ R+ A-)	*0301 (R), *0701 (Q), *15 (P)	<0.0001	74 set (A E -)	*15 (A); (*13) (A), (*08 & *14) (E/L)
0.002	77 (N+ T-)	*0301 (N), *15 (T)	<0.0001	74 set (A L -)	*15 (A); (*13) (A), (*08 & *14) (E/L)
0.002	13 (S+ Y+ R-)	*0301 (S), *0701 (Y), *15 (R)	<0.0001	13 set (R H -)	*15 (R); *04 (H);
0.005	26 (Y+ F-)	*0301 (Y), *15 (F)	<0.0001	11 set (P V -)	*15 (P); *04, *10 (V); *01 (L)
0.009	32 (H+ Y-)	*0301 (H), *15 (Y)	<0.0001	96 set (Y Q -)	*15 (Q); *04 (Y);
0.02	60 (S+ Y-)	*0701 (S), *15 (Y)	<0.0001	13 set (H R G -)	*15 (R); *04 (H); *08 (G)
0.03	85 (A+)	not explained (*11 and *12 are A+)	0.0002	13 set (G R -)	*15 (R); *08 (G)
0.03	161 (P+ L-)	not explained (*12 is P+)	0.0005	96 set (E Q -)	*15 (Q); *01 (E)
0.04	57 (V+ D-)	*0701 (*12) (D)			
0.04	149 (H+ Q-)	*0301 (H)			
0.04	233 (R+ T-)	*0301 (*11, *12) (R)			
HLA-DQB1			***HLA-DQB1***		
<0.0001	87 (L+ F-)	*02 (L), *0602, *0603 (F)	<0.0001	71 set (T A -)	*06, (*03, *04) (T); *05(A)
<0.0001	52 (L+ P-)	*02 (L), *06 (P)	<0.0001	30 set (Y H -),	all except *02 (S in *02)
<0.0001	55 (L+ R-)	*02 (L), *06 (*05) (R)	<0.0001	37 set (Y D -)	all except *02 (I in *02)
0.0001	74 (A+ E-)	*02 (A), *06 *03) (E)	<0.0001	55 set (P R -)	all except *02 (L in *02)
0.0001	77 (R+ T-)	*02 (*05) (R), *06 (*03-*04) (T)	<0.0001	71 set (T A D -)	all except *02 (K in *02)
0.0002	75 (V+ L-)	*02 (*04-*05) (V); *06 (*03) (L)	<0.0001	74 set (S E -)	all except *02 & *0606 (K in *02)
0.0002	30 (S+ Y-)	*02 (S); *06 (*03-*04) (Y)	0.0003	9 set (F L -)	*0601 (L); *0602, *04 (L)
0.0002	67 (I+ V-)	*02 (*04) (I); *06 (V)	0.0004	70 set (E G -)	*0601-2/8, (*04, *05)
0.0003	220 (H+ R-)	*02 (*03-*04) (H); *06 (*05) (R)	0.0006	71 set (T D -)	all except *02, *05
0.0003	221 (H+ Q-)	*02 (*03-*04) (H); *06 (*05) (Q)	***0.005***	***57 set (D V -)***	****0601-3, *0301/3, *04 (D); *0501, *0604/9 (V)***
0.0003	***57 (A+ D-)***	****02 (*0302/4-5) (A); *06 (*0301, *04) (D)***			
0.0003	71 (K+ T-)	*02 (K); *06 (*03) (T)	***0.007***	***57 set (A V +)***	****02, *0302/4-5/7 (A) *0501, *0604/9 (V)***
0.0003	38 (V+ A-)	*02 (*05) (V); *06 (*03-04) (A)			
0.0005	203 (I+ V-)	*02 (*03, *04, *0601) (I); *06 (*05) (V)			
0.0006	53 (L+ Q-)	*02 (*03, *04) (L); *06 (*05) (Q)			
0.0006	84 (Q+ E-)	*02 (*03, *04) (Q); *06 (*05) (E)			
0.0006	85 (L+ V-)	*02 (*03, *04) (L); *06 (*05) (V)			
0.0006	89 (T+ G-)	*02 (*03, *04) (T); *06 (*05) (G)			
0.0006	90 (T+ I-)	*02 (*03, *04) (T); *06 (*05) (I)			
0.0008	125 (A+ G-)	*02 (*03, *04) (A); *06 (G)			

Table 3.14: DQB1 codon 57 genotypes in CVID

Genotypes are listed as frequencies of Asp/non-Asp heterozygote and homozygotes, and compared with frequencies reported previously. Genotype frequencies are similar in the two patient cohorts, but the Asp/Asp frequency is higher in patients an the present report, and the non-Asp/non-Asp frequency lower.

	Genotype - this study n (%)			Genotype (Olerup et al, 1992) % only given		
	Asp/Asp	Asp / non-Asp	non-Asp / non-Asp	Asp/Asp	Asp / non-Asp	non-Asp / non-Asp
CVID n=179	22 (12.3)	82 (45.8)	75 (41.9)*	10%	53%	37%‡
Controls n=605	128 (21.1)	298 (49.3)	179 (29.6)*	32%	49%	19%‡
		*χ^2=9.01, p=0.003; RR=1.71 (1.22-2.42; AF 0.17)			‡p<0.001, RR=2.5	

3.2.6. HLA associations with patient subgroups defined by clinical criteria

The extensive clinical and laboratory heterogeneity characteristic of CVID has been emphasised in chapter 1. It is possible that genetic factors contribute to the development of features such as autoimmunity, granulomata and immunophenotypic abnormalities, thus it was of interest to examine HLA associations with patient subgroups stratified on the basis of these features.

3.2.6.1. Clinical stratification

Of 112 CVID patients with accessible clinical data, 37 had active or quiescent autoimmune disease[2] (table 3.15). Six patients had more than one type of autoimmune disease.

20 of 90 patients (22.2%) had histologically confirmed non-caseating granulomata in at least one of the following organs: lung (10 patients), liver (7), spleen (6), skin (2), eye (2), brain (1) and bone marrow (1) (table 3.16). A total of 41 biopsies confirming granulomata were performed: five biopsies in 1 patient, 3 biopsies in 4 patients, 2 biopsies in 9 patients and a single biopsy in 6 patients. Granulomata were confirmed by biopsy in 2 or more organs in 12 patients. A further 14 biopsies in these patients did not detect additional granulomata. In 15 patients with suggestive lesions in whom granulomata were not detected by biopsy, definitive diagnoses were reached in 12: fungal or *Staphylococcal* infection (6 patients), nodular lymphoid hyperplasia (3), lymphoma (2) or inflammatory bowel disease (1). Of the remaining 3 patients, 2 had non-granulomatous lymphocytic pulmonary infiltration, and 1 non-granulomatous lymphadenopathy. 14 patients had bone marrow biopsies performed for diagnostic purposes, with no granulomata detected.

There was a significant association between the presence of granulomata and splenomegaly. 14 of 20 patients with granulomata also had splenomegaly, compared to 13 of 70 patients without granulomata (Fisher's exact test $p<0.0001$).

[2] This data does not include inflammatory bowel disease or coeliac disease. Coeliac disease has been excluded in view of the difficulty in histologically distinguishing true coeliac disease from other conditions with subtotal villous atrophy seen in CVID, and the inability to use conventional serological tests to assist diagnosis.

The accurate diagnosis of granulomata in CVID is difficult, and it is possible that some clinically silent lesions were not detected, thus introducing bias into the clinical and genetic analyses described in this study. To minimise this potential problem, rigorous stratification criteria were used. Four of the commonest sites of granulomata in CVID were repeatedly examined clinically (skin) and radiologically (liver, spleen and lung). All suspicious lesion were biopsied, on multiple occasions where possible: 14 patients had more than one biopsy confirming granulomata, and biopsy-confirmed granulomata were detected in at least 2 organs in 12 patients. Patients not meeting these stringent criteria were excluded from the clinical analyses. Whilst it remains possible that some patients were wrongly classified due to a false negative biopsy result, the criteria employed were as stringent as possible in order to avoid bias.

3.2.6.2. HLA associations with autoimmunity

As in the entire patient group, alleles of the B7-DR15-DQ6 haplotype were less frequent in patients with autoimmune disease than in controls (table 3.17). A positive association with the A1-B8-DR3 haplotype was not detected. Furthermore, apart from A*02, no significant differences in HLA allele frequencies between autoimmune and non-autoimmune CVID subgroups were detected.

Autoimmunity in CVID was not associated with any other clinical or immunophenotypic parameters examined. Granulomata were present in autoimmune and non-autoimmune CVID in approximately equal proportions: 6 of 29 (20.7%) autoimmune patients had granulomata, compared with 14 of 61 non-autoimmune patients (23.0%).

3.2.6.3. HLA associations with granulomata and splenomegaly

Alleles of the A1-B8-DR3 haplotype were more frequent in CVID patients with granulomata, and B7-DR15-DQ6 alleles less frequent, but only the B*0801 association reached statistical significance (table 3.18). No unique HLA class I and II associations were evident with this subgroup, nor were there any significant differences in allele frequency between granulomatous and non-granulomatous

CVID patients. Likewise, no unique associations were detected with patients with or without splenomegaly.

Table 3.15: Autoimmune disease in 112 CVID patients

ITP = idiopathic thrombocytopaenic purpura; AIHA = autoimmune haemolytic anaemia, IDDM = insulin-dependent diabetes mellitus. CAH = chronic active hepatitis (HCV negative). The patient with aspermia was included as "autoimmune" and the basis of the presence of anti-sperm antibodies.

Type of autoimmune disease	number of patients (%)
ITP	12 (10.00)
Pernicious anaemia	9 (8.04)
Vitiligo	7 (6.25)
Anti-IgA antibodies	4 (3.57)
Inflammatory arthropathy	4 (3.57)
AIHA	3 (2.68)
IDDM	2 (1.79)
Aspermia	1 (0.89)
CAH	1 (0.89)
Alopecia	1 (0.89)

Table 3.16: Sites of granulomata in 20 of 90 CVID patients

Site of granulomata	Number of patients (%)
Lung	10 (11.1)
Liver	7 (7.78)
Spleen	6 (6.67)
Skin	2 (2.22)
Eye	2 (2.22)
Brain	1 (1.11)
Bone marrow	1 (1.11)

Table 3.17: HLA associations with "autoimmune" CVID

HLA allele frequencies have been compared between CVID patients with and without autoimmune disease, and between CVID patients with autoimmunity and HLA (cadaveric donor) controls. No unique HLA associations with autoimmune CVID were detected. A positive A1-B8-DR3 association with this patient subgroup was not detected. ns = not significant.

	Controls (n=605)		Autoimmune CVID (n=37)		Autoimmune CVID v controls		Non-autoimmune CVID (n=75)		Autoimmune v. non-autoimmune CVID	
Allele	no.	%	no.	%	χ^2	p	no.	%	χ^2	p
A*01	217	35.9	14	37.8	0.06	ns	36	48.0	1.04	ns
Cw*0701	183	30.2	13	35.1	0.39	ns	31	41.3	0.40	ns
B*0801	150	24.8	9	24.3	0.00	ns	25	33.3	0.95	ns
DRB1*0301	157	26.0	10	27.0	0.02	ns	22	29.3	0.07	ns
DQB1*0201	256	42.3	19	51.4	1.16	ns	36	48.0	0.11	ns
A*02	296	48.9	20	54.1	0.37	ns	21	28.0	7.25	0.007
Cw*0702	168	27.8	4	10.8	5.11	0.02	9	12.0	0.03	ns
B*07	162	26.8	5	13.5	3.19	0.07	8	10.7	0.20	ns
DRB1*15	168	27.8	4	10.8	5.11	0.02	9	12.0	0.03	ns
DQB1*0602	159	26.3	4	10.8	4.40	0.04	10	13.3	0.14	ns
B*14	45	7.4	8	21.6	9.26	0.002	8	10.7	2.43	ns

Table 3.18: HLA associations with "granulomatous" CVID

Trends toward positive associations between A1-B8-DR3 alleles, and negative associations with B7-DR15-DQ6 alleles were seen with granulomatous CVID, but this only reaches significance for HLA-B*0801.

	Controls (n=605)		Granulomatous CVID (n=20)		Granulomatous CVID v controls		Non-granulomatous CVID (n=70)		Granulomatous v. non-granulomatous CVID	
Allele	**no.**	**%**	**no.**	**%**	χ^2	**p**	**no.**	**%**	χ^2	**p**
A*01	217	35.9	9	46.7	0.74	ns	33	47.1	0.00	ns
Cw*0701	183	30.2	9	46.7	1.86	ns	30	42.6	0.08	ns
B*0801	150	24.8	9	46.7	3.70	0.05	22	30.9	1.37	ns
DRB1*0301	157	26.0	5	26.7	0.00	ns	22	30.9	0.10	ns
DQB1*0201	256	42.3	11	53.3	0.73	ns	34	48.5	0.11	ns
A*02	296	48.9	9	46.7	0.30	ns	27	38.2	0.37	ns
Cw*0702	168	27.8	5	26.7	0.01	ns	8	11.8	2.20	ns
B*07	162	26.8	3	13.3	1.36	ns	9	13.2	0.00	ns
DRB1*15	168	27.8	3	13.3	1.53	ns	9	13.2	0.00	ns
DQB1*0602	159	26.3	3	13.3	1.28	ns	10	14.7	0.00	ns
B*14	45	7.4	1	6.7	0.01	ns	8	11.8	0.33	ns

3.2.7. Stratification on the basis of peripheral blood immunophenotype

As discussed in chapter one, immunophenotypic abnormalities are characteristic of CVID, have been associated with clinical features such as splenomegaly (Wright et al, 1990), and have been used to identify subgroups of CVID patients (Wright et al, 1990; Spickett et al, 1997). It is thus of interest to examine HLA associations with subgroups of patients defined by blood immunophenotype.

In order for such a study to be valid, it is also necessary to examine associations between HLA alleles and blood immunophenotype in non-immunodeficient controls. Variables such as the peripheral blood CD4+:CD8+ ratio have been shown to be heritable, and HLA polymorphism is a possible controlling factor.

3.2.7.1. Patient immunophenotypic data

Immunophenotyping was performed by dual colour flow cytometry as described in chapter 2. Basic lymphocyte subsets were measured in 157 patients including total lymphocytes; CD3+, CD3+CD4+ and CD3+CD8+ T cells; and B cells (CD19+ or CD20+). These measurements were repeated in 81 patients, 2 months to 7 years apart. CD45RA and RO positive subsets of CD4+ and CD8+ lymphocytes and CD8+CD57+ lymphocyte counts were measured in 71 patients, and repeated in 32 patients. Where multiple measurements were recorded, mean values were used for statistical analysis. Nine patients had received hepatitis C contaminated immunoglobulin prior to the time of study. Only immunophenotypic data obtained prior to infection was analysed.

Basic immunophenotypic data for T and B lymphocyte subsets is given in table 3.19. 45 of 157 patients (28.7%) had reversal of the peripheral blood CD4+:CD8+ ratio (<0.9, table 3.20). CD4+ lymphopenia was present in 17 of these 45 individuals (37.8%), and CD8+ lymphocytosis in 28 (62.3%). 63 of 157 patients (40.1%) were B lymphopenic. Circulating B cells were undetectable in 13 patients. Of these 13, 7 were female (mean age at diagnosis 20.9 years; range 6-32, standard deviation 11.7), and 6 male (mean age at diagnosis 39.8; 5-61; range 5-61; SD 25.9).

3.2.7.2. *Correlation between immunophenotypic parameters in all patients*

Strong correlations were detected between many of the lymphocyte subset parameters enumerated, and are comprehensively listed in appendix 8.4. Positive correlations were detected between total T cells, CD4+ and CD8+ T cells, CD45RA and RO isoforms of CD4 and CD8 cells, and B cells. As expected, the CD4+:CD8+ ratio was positively correlated with CD4+ counts, and negatively with CD8+ counts. CD8+CD57+ lymphocyte number was negatively correlated with CD4+ T cell, CD4+CD45RA+ T cell and B cell number. These findings were confirmed when associations between CD8+CD57+ cell number and specific lymphocyte subset abnormalities are examined (see below).

3.2.7.3. *Associations between specific immunophenotypic abnormalities*

Associations between specific immunophenotypic abnormalities were also detected, either by comparison of means, or contingency table analysis. For example, the mean CD4+ count was lower (unpaired t test $t=2.78$, $p=0.006$), and the mean CD8+ count higher ($t=-6.716$, $p<0.0001$), in patients with reversal of the CD4+:CD8+ ratio. Furthermore, reversal of the CD4+:CD8+ ratio was specifically associated with CD4+ lymphopenia ($\chi^2=5.02$, $p=0.02$), and CD8+ lymphocytosis ($\chi^2=45.5$, $p<0.0001$).

Similar t test and contingency table analysis demonstrated that CD4+ lymphopenia was associated with both CD4+CD45RA+ lymphopenia (t test and χ^2 $p=0.005$), and B lymphopenia (t test $p=0.002$, χ^2 $p=0.0001$). As will be discussed below, it is important to note that CD4+ lymphopenia was not associated with total absence of circulating B cells (n=13). Furthermore, absolute absence of B cells was not associated with specific clinical features (autoimmunity or granulomata) or variability in other immunophenotypic parameters, with the exception of the mean percentage of CD8+ lymphocytosis (t 2.78, $p=0.007$; χ^2 3.98, $p=0.04$).

Analyses of specific associations with CD8+CD57+ lymphocytosis are summarised in table 3.21 and corroborate the correlations detected in all patients. In

particular, CD8+CD57+ lymphocytosis was specifically associated with CD4+ lymphopenia and B lymphopenia.

Table 3.19: Summary of CVID immunophenotypic data

Counts are listed as number of cells $x10^9$/litre. na = normal range not available. no = number of patients studied. Normal values represent 5-95 centile ranges for healthy adult Caucasians. These reference ranges are those in routine use in the Oxford Radcliffe Hospital Clinical Immunology Laboratory, and are derived from Hannet et al, 1992, and Hulstaert et al, 1994.

Parameter (normal range)	Mean	SD	no	Minimum	Maximum	Number outside normal range
CD3+	1.31	0.83	157	0.275	5.64	19 (<0.6)
CD3+CD4+ (0.4-1.0)	0.66	0.42	157	0.12	2.735	40 (<0.4)
CD3+CD8+ (0.2-0.7)	0.61	0.56	157	0.043	4.92	39 (>0.7)
CD4+:CD8+ (>0.9)	1.49	1.03	157	0.19	7.781	45 (<0.9)
B cells (0.1-0.5)	0.19	0.19	157	0	0.89	63 (<0.1); 13 (<0.01)
CD4+CD45+RA (>30% total CD4+)	0.25	0.28	71	0.005	1.5	36 (<30% total CD4+)
CD4+CD45RO+	0.38	0.28	71	0.01	2.08	na
CD8+CD45RA+	0.37	0.30	71	0.01	1.4	na
CD8+CD45RO+	0.23	0.20	71	0.005	1.1	na
CD8+CD57+	0.22	0.25	71	0.01	1.43	na
Proportion of CD8+ also CD57+ (<40% total CD8+)	0.38	0.19	71	0.04	0.89	35 (>40% total CD8+)

Table 3.20: Contribution of aberrant CD4+ and CD8+ counts to reversal of the CD4+:CD8+ ratio

Abnormally high and low counts are represented by up and down arrows respectively. To simplify the table, all CD4+ counts of >1.0 have been grouped as normal.

	↓CD8 ↓CD4	%	↓CD8 N CD4	%	N CD8 ↓ CD4	%	N CD8 N CD4	%	↑CD8 ↓CD4	%	↑CD8 N CD4	%
normal (n=112)	10	8.9	5	4.5	13	11.6	72	64.3	0	0	12	10.7
reversed (n=45)	1	2.2	0	0.0	13	28.9	3	6.7	3	6.7	25	55.6
Total (n=157)	11	7.0	5	3.2	26	16.6	75	47.8	3	1.9	37	23.6

Table 3.21: Associations between CD8+CD57+ lymphocytosis and lymphocyte subset abnormalities

Unpaired t-tests on the left of the table demonstrate the significant variance of the mean number of CD8+ cells also CD57+ in patients grouped by normality or reversal of several basic immunophenotypic parameters. The contingency table analyses on the right show that CD8+CD57+ lymphocytosis is specifically associated with CD4+ and B lymphopenia.

Summary of variance in mean %CD8s CD57+ counts with lymphocyte subset abnormalities (unpaired t test)			Summary of contingency table analyses between CD8+CD57+ lymphocytosis and other lymphocyte subset abnormalities (chi-square test)		
Grouping parameter	t value	p value	Grouping parameter	χ^2	p value
CD3+	1.32	0.19	CD3+	3.2	0.07
CD4+	2.02	0.04	CD4+	3.84	0.04
CD8+	-1.81	0.06	CD8+	0.09	0.77
CD4+:CD8+	-2.96	0.004	CD4+:CD8+	1.13	0.29
B cells	3.84	0.0003	B cells	4.98	0.03
CD4+CD45RA+	-1.90	0.05	CD4+CD45RA+	3.83	0.05

3.2.7.4. Control immunophenotypic data

50 volunteer healthy Caucasoid adults were recruited from hospital personnel. None were smokers, or were taking regular medication, and none had engaged in strenuous physical exercise in the 24 hours prior to peripheral blood sampling. The mean age of the healthy controls was 32.7 (range 23-61). 32 were male, and 18 female. Total lymphocyte, T cell, CD4+ and CD8+ counts were measured.

These results are summarised in table 3.22. Four individuals (8%) had a CD4+:CD8+ ratio of less than 1.0. Reversal of the ratio in all 4 was due to a CD4+ lymphopenia, rather than CD8+ lymphocytosis. Lymphocyte counts were remeasured in 3 individuals, at least 2 weeks after initial measurement, confirming inversion of the ratio. All 4 individuals with reversal of the CD4+:CD8+ ratio were HIV antibody negative. Lymphocyte subsets were measured twice in 13 individuals. Mean duration to second measurement was 13 days (range 9 days to 4 months). The CD4+:CD8+ ratio remained constant between repeated measurements (the mean variation was ±5.7%) even though considerable variability was detected in CD4+ and CD8+ numbers (mean variation ±25.5% and ±24.6% respectively). The CD4+:CD8+ ratio of 3 female patients sampled on two occasions at least two weeks apart was similarly stable (mean variation ±4.6%, range ±2.9-6.9%).

3.2.7.5. *HLA associations with lymphocyte phenotype*

3.2.7.5.1. Patients

Significant differences in HLA-DQB1 associations were detected between patients stratified on the basis of normality or reversal of the CD4+:CD8+ ratio (table 3.23). HLA-DQB1*06 was less frequent in patients with reversal of the CD4+:CD8+ ratio than in those with normal ratios (χ^2 p=0.001, RR 0.19). This was attributable to reduced frequencies of both DQB1*0602 (χ^2 p=0.02, RR 0.21) and DQB1*0603 (χ^2 p=0.04, RR 0.11) in patients with reversal of the CD4+:CD8+ ratio. A positive association between DQB1*0301 and reversal of the CD4+:CD8+ ratio was detected. Furthermore, the mean CD4+:CD8+ ratio was significantly different in patients grouped by DQB1*06 positivity (ie. all DQB1*06 alleles), or DQB1*0603 positivity, but not DQB1*0602 positivity. Results have also been depicted in box plots in figure 3.2.

No other associations between HLA class I and II polymorphisms and immunophenotypic parameters were detected. Notably, no associations, or trends toward associations, were detected with patients with absent circulating B cells.

3.2.7.5.2. Controls

No HLA alleles were associated with the CD4+:CD8+ ratio in controls. A weak association between HLA-A*03 and a higher ratio was found, however this was not significant after correction for multiple comparisons (average CD4+:CD8+ ratio in those negative for HLA-A*03 was 0.73 (n=34), and in those positive for A*03 0.95 (n=16), p uncorrected = 0.01). Likewise, there were no significant associations between HLA polymorphisms and variability in absolute CD3+, CD4+ or CD8+ T cell counts.

Table 3.22: Summary of lymphocyte counts and the CD4+:CD8+ ratio in 50 healthy adults

Counts are given as number of cells x 10^9 per litre.

	Total lymphocytes	CD3+ (%)	CD4+ (%)	CD8+ (%)	CD4+:CD8+ ratio
Average	1.87	1.34 (71.5)	0.8 (42.4)	0.5 (27.2)	1.63
Minimum	0.83	0.56 (53)	0.22 (27.0)	0.27 (18)	0.73
Maximum	2.87	2.38 (87)	1.64 (62)	0.89 (40)	2.82
SD	0.48	0.39 (7.7)	0.27 (7.4)	0.15 (5.6)	0.48

Table 3.23: Relationships between DQB1*06 alleles and the CD4+:CD8+ ratio in CVID

Associations between HLA-DQB1 alleles and the CD4+:CD8+ ratio have been analysed by comparing the mean ratio in patients positive or negative for a DQB1 allele or group of alleles using the unpaired t test (left side of table). A negative value for mean difference indicates that the CD4+:CD8+ ratio is lower in those patients positive for the DQB1 allele in question. Specific DQB1 associations with reversal of the ratio have been analysed by contingency table analysis (right side of table). A relative risk (RR) of less than one indicates that the allele(s) in question are negatively associated with reversal of the CD4+:CD8+ ratio.

Summary of variance in mean CD4+:CD8+ values with HLA-DQB1 alleles (unpaired t test)

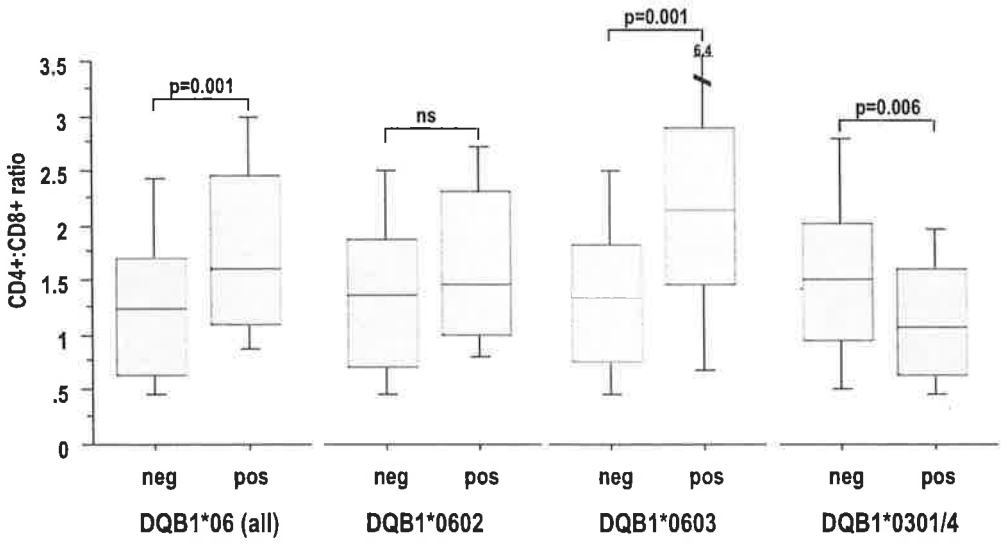
Allele(s)	Mean difference	t	p value
DQB1*06 (all subtypes)	-0.594	-3.30	0.001
DQB1*0602	-0.165	-0.70	ns
DQB1*0603	-1.217	-3.37	0.001
DQB1*0301	0.473	2.77	0.006

Summary of contingency table analyses between the CD4+:CD8+ ratio and HLA-DQB1 alleles (Chi-squared and Fisher's exact tests)

Allele(s)	Normal ratio patients with allele (n=112)	Reversed ratio patients with allele (n=45)	χ^2	χ^2 p	Fisher's exact p	RR
DQB1*06	38 (33.9%)	4 (8.9%)	9.04	0.001	0.0007	0.19
DQB1*0602	20 (17.9%)	2 (4.4%)	4.06	0.02	0.02	0.21
DQB1*0603	10 (8.9%)	0 (0%)	4.29	0.04	0.03	0.11
DQB1*0301	31 (27.7%)	21 (46.6%)	4.4	0.02	0.02	2.29

Figure 3.2: Box plots of variation in the CD4+:CD8+ ratio in patient groups defined by DQB1 genotype

DQB1*06 was associated with a higher, and *0301 with a lower CD4+:CD8+ ratio.



3.3. DISCUSSION

This chapter has described the largest and most detailed HLA class I and II case control association study in CVID thus far reported. In addition, many of the limitations of previous studies have been addressed. Specifically, high resolution techniques have been utilised to genotype a large number of HLA class I and II loci in 179 patients. By examining polymorphisms in multiple class I and II loci spanning a large region of the human major histocompatibility complex, it has been possible to examine associations with haplotypes, as well as with individual alleles. In order to resolve the controversial hypothesis regarding the role HLA-DQB1 codon 57 in conferring susceptibility to CVID, amino acid associations at every polymorphic HLA-A, -B, -Cw, -DRB1 and -DQB1 codon have been investigated. As a number of clinical and laboratory features are variably present in CVID, and may be genetically influenced, HLA associations have also been examined with subgroups of patients stratified by clinical criteria (eg. autoimmunity and granulomata) and immunophenotypic abnormalities (eg. the CD4+:CD8+ ratio).

Three main HLA associations were detected with CVID: positive associations with the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 and B*14-DRB1*0701-DQB1*0201 haplotypes, and a negative association with the A*02-B*07-DRB1*15-DQB1*0602 haplotype. Several further observations may be made regarding these associations. First, the relative risk and aetiological/preventive fraction data suggest that these associations are relatively weak: relative risks rarely are seldom more than 3 or less than 0.4 for positive and negative associations respectively. This is in contrast to diseases such as IDDM and ankylosing spondylitis, with relative risks of approximately 14 and 150 respectively (chapter 1, table 1.2). Even strongly HLA-linked disease such as these are known to be associated with at least one non-HLA gene. An exception is the high rate of homozygosity for the A1-B8-DR3 haplotype in CVID (RR>6) although the actual numbers involved are small. Four patients compared with 2 controls are homozygous for the entire "extended" haplotype ($p=0.01$, RR=6.19); and 10 patients and 5 controls are homozygous for the B*0801-DRB1*0301 part of the haplotype that spans the HLA class III region ($p<0.0001$, RR=6.76).

It is not possible from this study to estimate the contribution of HLA polymorphisms to genetic susceptibility to CVID. However the previously reported figure of a relative risk of 50 for siblings of antibody deficient individuals (Vorechovsky et al, 1995b) would suggest that other non-HLA factors are also important. Furthermore, the associations are with haplotypes commonly found in non-immunodeficient Caucasoids. Indeed, in one study of multiplex immunodeficient kindreds with CVID and/or IgAD inherited in an autosomal dominant fashion, no linkage to the MHC was detected (Vorechovsky et al, 1995b). Thus whilst these haplotypes may confer susceptibility (or, in the case of the B7-DR15-DQ6 haplotype, afford protection), it would seem logical that other factors, genetic and/or environmental, are required to induce immunodeficiency.

These haplotypic associations confirm previous HLA association studies in both IgAD and CVID (Ambrus et al, 1977; Oen et al, 1982; Hammarström et al, 1983; Hammarström et al, 1984; Schaffer et al, 1989; Olerup et al, 1990; Bucin et al, 1991; Olerup et al, 1992; Clerici et al, 1993). However, many of the HLA associations previously described in IgAD have not been detected in this study. There are two possible explanations for this observation. First, it is possible that different HLA associations exist for the two conditions, although the associations detected here are among those most frequently confirmed in IgAD, suggesting that a common HLA mediated susceptibility may exist. Alternatively, the associations not confirmed in this study (table 3.3) may be artefactual, due to the small numbers of patients examined and/or low-resolution typing techniques used in previous studies. Indeed, many of these associations have only been described in one report. By using medium to high resolution DNA based methods, problems inherent in serological HLA typing (such as cross-reactivity and missed alleles), have been avoided. It remains possible that unique HLA associations predispose to specific clinical and laboratory abnormalities characteristic of CVID. This issue is discussed below.

A more complex issue to address is which, if any, of the alleles on the associated haplotypes represents the primary, or disease-causing allele. This is difficult to

answer when a relatively small number of loci (HLA-A, -Cw, -B, -DRB1, and -DQB1) spanning a large area of highly polymorphic genome are studied. However by stratifying HLA data it is often possible to demonstrate which region of a haplotype is most strongly associated, thus enabling more localised future studies. Initial analysis of individual allelic associations suggested that the strongest associations were with class I and II loci flanking the HLA class III region such as HLA-B and HLA-DRB1, rather than with HLA-A. Detailed analysis (Svejgaard et al, 1994) was performed for all combinations of alleles on each of the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201, A*02-Cw*0702-B*07-DRB1*1501-DQB1*0602 and Cw*0802-B*14-DRB1*0701-DQB1*0201 haplotypes. With the exception of weaker associations with HLA-A alleles, none of the remaining alleles were more strongly associated than any others on these haplotypes. The stratification analyses strongly suggest that HLA associations in CVID require the presence of the haplotypes (or parts of the haplotypes) rather than just one allele.

One possible explanation for the multiple haplotypic associations observed is the "HLA class III" hypothesis proposed by Wilton and French et al (Wilton et al, 1985; French et al, 1990). These authors have examined polymorphisms in HLA class III genes such as C4A and CYP21A, and found that IgAD is associated with rare alleles and deletions in these genes. These authors suggested that these alleles (or linked alleles in close proximity) were primarily associated with IgAD, and explained all observed HLA class I and II associations by linkage disequilibrium. Indeed, the A1-B8-DR3 and B14-DR7 haplotypes positively associated with CVID, but not the negatively associated B7-DR15-DQ6 haplotype, share polymorphisms in and near the class III region (Briggs, 1993). The appeal of this hypothesis is enhanced by the knowledge that these haplotypes share substantial deletions in the exceptionally gene-dense class III region. Thus a deleted region might contain a gene encoding a molecule essential for normal humoral immune responses. Many HLA class III genes encode proteins with substantial homology to members of the immunoglobulin superfamily. It is possible that one or more of these class III products is of importance in T cell activation and/or immunoglobulin secretion. For example, several of the

molecules associated with the T cell receptor complex are members of the immunoglobulin superfamily. If one or more of these proteins is encoded by HLA class III genes, and is deleted in CVID patients carrying one of the disease-associated haplotypes, it could explain the abnormal TcR signal transduction and defective T cell activation characteristic of a subgroup of CVID (Fischer et al, 1996). Thus it will be of interest to examine HLA polymorphisms in those patients with defective TcR function. The finding of increased A1-B8-DR3 homozygosity in CVID would further support this hypothesis, by suggesting that homozygosity for such a deletion confers susceptibility. However such recessivity is not a universal finding in familial IgAD/CVID (Vorechovsky et al, 1995b).

This speculation does not, however, formally refute the hypothesis of Olerup, who has suggested that homozygosity for non-aspartic acid at DQB1 codon 57 represents the primary HLA association with CVID, and that all other (class I, II and III) observed HLA associations are secondary to linkage disequilibrium (Olerup et al, 1990; Olerup et al, 1991; Olerup et al, 1992). This is a fundamentally different concept from that espoused above, as it suggests that the disease associated HLA gene (DQB1) encodes a product directly involved in antigen-specific immune responses (ie. presentation of peptide) rather than exerting a non-antigen specific effect (such as regulation of immunoglobulin gene transcription or TcR signalling). Although the association described by Olerup in both IgAD and CVID cannot be disputed, the interpretation of primary biological significance is flawed by the lack of examination of amino acid variability in other polymorphic HLA codons in addition to DQB1 codon 57. This discrepancy has been addressed in the present study by examining every polymorphic codon in HLA-A, -B, -Cw, -DRB1 and -DQB1. Similar associations with DQB1 codon 57 were detected (and with codon 57 non-Asp homozygosity) but these were of comparable strength, or weaker, than a variety of associations with polymorphic HLA-A, -B, -DRB1 and (additional) -DQB1 residues. With few exceptions, all of these associations correspond to, and are explained by, the three haplotypic associations described above. If an amino acid or group of amino acids at one HLA codon were truly primarily associated with CVID, an association of much

greater significance would be expected. This has not been detected, and this hypothesis is unlikely to be correct. Furthermore, unlike the present study, the techniques used by Olerup were not capable of detecting all HLA-DQB1 subtypes found in Caucasoids, and thus prior results should be interpreted with caution.

HLA associations were also examined with patient groups stratified according to clinical and immunophenotypic criteria. This is an important analysis, as CVID is heterogeneous, and classification systems have been proposed on the basis of clinical and laboratory abnormalities. For example, a group of patients has been described characterised by a high prevalence of autoimmune disease but a lack of granulomatous disease or immunophenotypic abnormalities (Spickett et al, 1988). Given the known association of A1-B8-DR3 with several autoimmune diseases, it has been suggested that this haplotype may be preferentially associated with this subgroup (Spickett et al, 1988). However, no significant differences in HLA allele frequencies were observed between autoimmune and non-autoimmune patients. This is perhaps unsurprising given the wide variety of autoimmune diseases present. Moreover, similar proportions of autoimmune and non-autoimmune patients had granulomatous disease (20.7% and 23.0% respectively) suggesting that autoimmune disease is not a reliable marker of a CVID disease subgroup. In contrast, granulomatous disease is strongly associated with several clinical and immunophenotypic abnormalities, and would appear to be a more valid marker of a CVID disease subgroup (examined in detail in the following chapter).

It has also been suggested that patients with absent circulating B cells (13 in the present study) do not share immunophenotypic and clinical features seen in other CVID patients, and may represent undiagnosed cases of X-linked agammaglobulinaemia (or, in female patients, an XLA-like disorder resulting from and autosomal mutation) (Spickett et al, 1988). Accordingly, in this study B cell absence was not associated with clinical complications such as autoimmunity and granulomata, or with immunophenotypic abnormalities such as CD4+ lymphopenia. This is in marked contrast to the strong association between CD4+ lymphopenia and reduced, but not absent, B cell number. Furthermore, patients

with absent B cells do not share the HLA associations with the remainder of patients with CVID. This suggests that investigation of these patients for *btk* mutations, in order to diagnose XLA, will be of interest. However the finding of an association between B cell absence and CD8+CD57+ lymphocytosis suggests that those patients with absent B cells and high CD8+CD57+ number do genuinely represent CVID, and that not all hypogammaglobulinaemic patients with absent B cells will have XLA[3].

Significantly different HLA associations were detected in patients defined by normality or reversal of the CD4+:CD8+ ratio, namely a reduced frequency of DQB1*0602 and *0603 and an increased frequency of DQB1*0301 in patients with a ratio of less than 0.9. There are at least two possible explanations for this observation. The first is that HLA alleles such as DQB1*06 serve as markers of disease progression. For example, it is possible that DQB1*06 positive patients are more likely to develop immunophenotypic abnormalities over time. This is not likely as reversal of the CD4+:CD8+ ratio is usually present at diagnosis, and remains stable over time. It is also important to note that reversal of the ratio, when present, is usually observed prior to the initiation of intravenous immunoglobulin therapy, which is known to increase CD8+ cell number, and reduce the CD4+:CD8+ ratio (Aukrust et al, 1997). It is more likely that subgroups of CVID, here defined by the CD4+:CD8+ ratio, have different genetic associations. Reversal of the peripheral blood CD4+:CD8+ ratio has previously been associated with other immunophenotypic and clinical abnormalities such as CD8+CD57+ lymphocytosis and splenomegaly (Wright et al, 1990). Furthermore, this association is not likely to be artefactual as no association were detected between HLA polymorphisms and the CD4+:CD8+ ratio in 50 healthy adults. This highlights the need for further large studies of HLA polymorphisms in CVID, using high-resolution genotyping techniques, and accompanied by comprehensive clinical and laboratory data.

In summary, several HLA haplotypes were found to be positively and negatively associated with CVID. It was not possible to determine which regions of these

[3] At the time of writing, all patients with reduced or absent B cells included in this study were being analysed for the presence of *btk* mutations.

haplotypes were most strongly associated, and no one polymorphic HLA codon represented the primary association with CVID. The observation that the positively associated haplotypes share extensive deletions and polymorphisms in and around the HLA class III region, suggests that a disease susceptibility gene may lie in this region. No significant differences in HLA class I and II associations were found with CVID subgroups defined by clinical features. Significant differences in HLA-DQB1 allelic associations were demonstrated between patients with normal or reversed peripheral blood CD4+:CD8+ ratios. These alleles may serve as markers for patient subgroups with distinct genetic predisposition.

Finally, these results indicate several potential avenues of investigation. The finding of strong class I and II associations around the HLA class III region, and the class III polymorphisms known to be shared by the disease-associated haplotypes suggest that further exploration of HLA class III polymorphism in CVID will be of considerable interest. Two such genes, tumour necrosis factor and lymphotoxin alpha, are the subject of the next chapter. Polymorphisms in a variety of non-HLA immunoregulatory genes are the subject of chapters 5 and 6.

4. TNF and LTα polymorphisms in CVID

4.1. INTRODUCTION

4.1.1. Overview

In the previous chapter, CVID was shown to be associated with several common Caucasoid HLA class I and II haplotypes. The principle associations were positive associations with the A*01-B*0801-Cw*0701-DRB1*0301-DQB1*0201 and B*14-DRB1*07 haplotypes, and a strong negative association with the HLA-Cw*0702-B*0701-DRB1*1501-DQB1*0602 haplotype. These associations were strongest with loci flanking the HLA class III region: HLA-B and HLA-DRB1. This suggests that CVID may be primarily associated with class III loci, and the observed associations with HLA class I and II alleles are explained by linkage disequilibrium with class III alleles. The class III region is of considerable interest, as it encodes several key immunoregulatory molecules. This chapter examines the role of polymorphisms of tumour necrosis factor and lymphotoxin alpha, two class III-encoded molecules with important roles in humoral and granulomatous inflammatory responses, both of which are perturbed in CVID.

4.1.2. HLA class III

The HLA class III region lies telomeric to HLA class II and centromeric to class I (see figure 1.1, chapter 1), and spans 1100 kb of DNA (Aguado et al, 1996). The class III region contains genes encoding molecules with key immunological roles, including the complement components C2, C4 and factor B, and the cytokines tumour necrosis factor (TNF, previously known as TNFα), lymphotoxin alpha (LTα, formerly known as TNFβ) and lymphotoxin β. Many other genes of known and unknown function lie in the class III region, on average one gene every 15 kb. TNF and LTα are attractive candidate genes for investigation in CVID for several reasons: both cytokines have important roles in determining the nature of immune responses; several TNF and LTα alleles are in linkage disequilibrium with HLA class I and II alleles previously associated with CVID; and several abnormalities of TNF production in CVID have been previously identified.

4.1.3. TNF and LTα

TNF and LTα are related cytokines synthesized by a wide range of cell types, particularly activated macrophages and T cells. TNF was originally identified by virtue of its anti-tumour activity, but was soon found to be a major mediator of cellular and inflammatory responses (Gruss et al, 1995; Aguado et al, 1996). TNF is cytotoxic to a wide range of cell types, has profound effects on cellular metabolism, and induces the acute phase response, fever and cachexia. TNF also exerts several important immunological actions, such as enhancing T cell proliferation, upregulating adhesion molecule expression, recruiting leukocytes, modulating T cell receptor expression, enhancing NK cell function, and influencing B cell responses.

LTα is synthesised almost exclusively by T lymphocytes (Paul et al, 1988; Ruddle, 1992). LTα exerts similar actions *in vitro* to TNF, although often with less potency. LTα can also act via the TNF receptors, suggesting a degree of redundancy in the actions of these two cytokines. However LTα knockout mice display a distinct phenotype, suggesting that this cytokine does have unique and important actions.

Both TNF and LTα are members of a superfamily whose members share structural and functional similarities (other members include CD27L, CD30L, CD40L, OX40L, FasL and LTβ) (Gruss et al, 1995). Several members of this superfamily, including TNF and LTα, exist in biologically active form as trimers, and act by causing receptor multimerisation. TNF is a type II membrane protein that may be cleaved to produce a biologically active soluble form. In contrast, LTα is secreted as a homotrimer, but may be expressed at the cell surface by associating with another member of the TNF ligand superfamily, LTβ. LTβ is also a type II membrane protein, and is encoded by a gene 2 kb centromeric to TNF.
Both homotrimeric TNF and LTα exert their actions via 2 specific cell surface receptors, the p60 TNF receptor I (TNFRI) and p80 TNFRII. The heteromeric cell surface form of LTα is structurally different, and may act via the recently identified receptor for LTβ, TNFR related protein (TNFR-RP or TNFRIII, also a

member of the TNF receptor superfamily). This latter interaction may explain the unique immunoregulatory actions of LTα (Crowe et al, 1994).

4.1.4. The role of TNF and LTα in the immune response

Human and murine studies suggest that TNF and LTα are essential for normal humoral immune responses (Douni et al, 1995; Pasparakis et al, 1996). TNF or p55 TNF-R knockout mice are unable to form organised splenic follicular dendritic cell (FDC) networks or germinal centres upon immunisation (Le Hir et al, 1996; Pasparakis et al, 1996). Furthermore, TNF or TNF-R knockout mice fail to form structured primary B lymphocyte follicles prior to immunisation (Douni et al, 1995). These effects of TNF are probably mediated by the TNFRI, as these abnormalities are not apparent in TNFRII knockout mice (Matsumoto et al, 1996). Studies in knockout mice have shown that LTα is also essential for normal development of germinal centres and peripheral lymphoid organs (De-Togni et al, 1994; Banks et al, 1995; Matsumoto et al, 1996). Although serum IgG levels are relatively normal in these knockout mice, specific antibody responses are impaired and IgA levels are reduced.

Both TNF and LTα are important growth factors and costimulatory signals for human B cell growth, differentiation and activation, and facilitate class-switching and polyclonal immunoglobulin synthesis (Kehrl et al, 1987; Aversa et al, 1993; Macchia et al, 1993; Boussiotis et al, 1994). The known crucial role of other members of the TNF ligand superfamily, such as CD40L (DiSanto et al, 1993; Korthäuer et al, 1993), in class switching and maturation of antibody responses would support a similar role for TNF (and LTα).

TNF has been implicated in a diverse range of pathologic states, including septic shock, autoimmune conditions such as rheumatoid arthritis, infectious conditions such as cerebral malaria (McGuire et al, 1994), malignancies and graft-versus-host disease. TNF is critical for the pathogenesis of infectious and non-infectious granulomata, including those seen in *Mycobacterial* and *Corynebacterial* infection, schistosomiasis, and sarcoidosis (Joseph et al, 1993; Boros, 1994; Lukacs et al, 1994; Myatt et al, 1994; Senaldi et al, 1996). This latter

point is of particular interest, as granulomata are a feature of a discrete subgroup of CVID patients.

4.1.5. TNF and LTα genetics

The genes encoding TNF and LTα lie in the HLA class III region on the short arm of chromosome 6 (Nedwin et al, 1985; Carroll et al, 1987; Dunham et al, 1987). The genes occupy a 7 kb stretch of DNA, separated by approximately 1.1 kb, LTα lying telomerically. Both genes incorporate four exons and three introns. The genes are homologous, most strikingly in the fourth exon. Both are polymorphic. The TNF cluster contains six microsatellites (Jongeneel et al, 1991; Udalova et al, 1993; Greenberg et al, 1997), 10 biallelic single nucleotide polymorphisms (Partanen et al, 1988; Messer et al, 1991; Ferencik et al, 1992; Wilson et al, 1992; D'Alfonso et al, 1994; Hamann et al, 1995), and a nucleotide insertion polymorphism (Brinkman et al, 1995) (figure 4.1). Many of the single nucleotide polymorphisms lie in areas of the genes critical for regulation of transcriptional activity (Goldfield et al, 1991), and thus may affect levels of expression of these cytokines.

4.1.5.1. Functional effects of the polymorphisms, and previous studies of TNF and LTα polymorphisms in disease

Abnormalities of TNF production have been identified in multiple inflammatory conditions, such as rheumatoid arthritis (di Giovine et al, 1988). Furthermore, transgenic rodents overexpressing TNF are more susceptible to RA (Keffer et al, 1991), and blockade of TNF action may also ameliorate the arthropathy of RA (Elliott et al, 1993). Functional studies and rodent models have also implicated TNF in the pathogenesis of other inflammatory conditions such as SLE (Jacob et al, 1988) and IDDM (Mandrup-Poulsen et al, 1987). Coupled with the known HLA class I and II associations with these diseases, these observations have led to speculation that TNF and LTα polymorphisms may have an important role in disease pathogenesis and explain the previously identified class I and II HLA associations.

Many studies have identified associations between TNF and LTα alleles and susceptibility to, or severity of a variety of infectious and inflammatory diseases

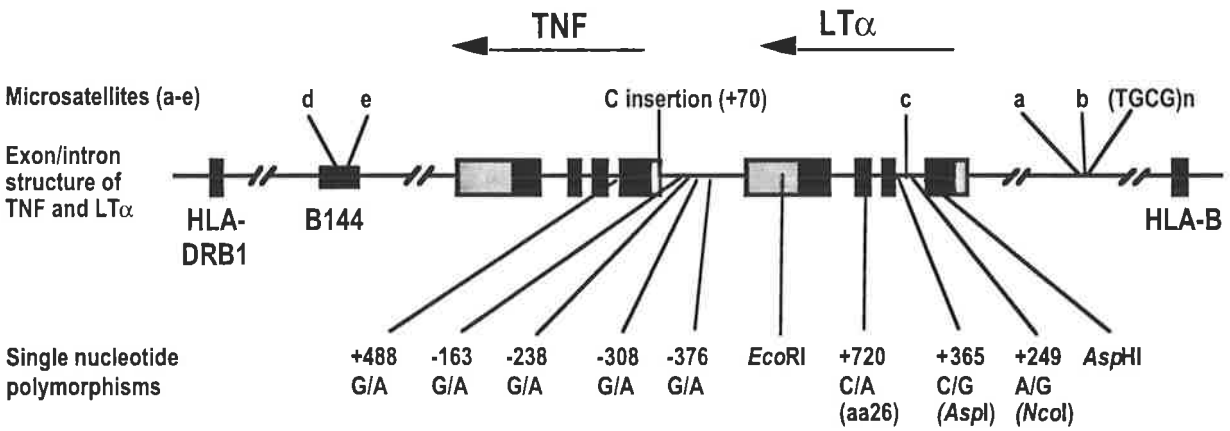
(Wilson et al, 1995b), including rheumatoid arthritis (Danis et al, 1995; Wilson et al, 1995a; Hajeer et al, 1996; Brinkman et al, 1997), systemic lupus erythematosus (Hajeer et al, 1996), asthma (Moffatt et al, 1997), meningococcal disease (Westendorp et al, 1997), malaria (McGuire et al, 1994) and inflammatory bowel disease (Bouma et al, 1996b). This has led to suggestions that TNF and LTα represent true genetic risk factors for disease. However, whilst inter-individual variability of both TNF and LTα production is known to be genetically determined, definitive conclusions are hampered by the strong linkage disequilibrium between TNF/LTα alleles and HLA class I and II alleles (Wilson et al, 1996). For example, peripheral blood mononuclear cells of HLA-DR2 positive individuals produce relatively lower levels of TNF *in vitro* than A1-B8-DR3 or -DR4 positive individuals (Bendtzen et al, 1988; Jacob et al, 1990; Wilson et al, 1992; Abraham et al, 1993; Pociot et al, 1993; Wilson et al, 1993; Bouma et al, 1996a). LTα polymorphisms have also been associated with variability in LTα production *in vitro*(Messer et al, 1991; Pociot et al, 1991; Whichelow et al, 1996). However it is not known if this variability is due to polymorphisms in the cytokine genes themselves, or to allelic variation in other linked MHC genes. Few studies have examined both HLA and TNF/LTα polymorphisms in order to determine which alleles are primarily associated with disease. One exception was the identification of homozygosity for TNF -308A as a strong risk factor for cerebral malaria (McGuire et al, 1994). This association was shown to be independent of HLA class I and II allelic associations, and it was suggested that this allele directly contributed to high TNF production and disease pathogenesis. However a subsequent report has suggested that the presence of this allele does not correlate with severity of sepsis, and that observed associations with TNF -308A are secondary to associations with the linked LTα *Nco*I polymorphism (Stuber et al, 1995).

This highlights the difficulty of accurately determining the functional effect of these polymorphisms. There are few studies formally examining the functional effect of TNF polymorphisms, and none for LTα. Moreover, the available data for TNF is conflicting. In experiments using reporter gene constructs and nuclear factor binding assays, Wilson *et al* detected a 7-fold increase in TNF transcription

in the presence of the uncommon TNF -308A (*TNF2*) allele (Wilson et al, 1997). However subsequent studies have failed to demonstrate any transcriptional effect of this polymorphism (Stuber et al, 1995; Brinkman et al, 1996). In addition, family studies suggest that the TNF -308 and -238 polymorphisms alone are unable to account for the entire genetically determined variability in TNF production (Westendorp et al, 1997). This suggests that other polymorphisms in the TNF gene, or other genes, are important determinants of TNF expression, and may be pathogenically relevant to a variety of infectious and inflammatory conditions.

Figure 4.1: Location of polymorphisms in the TNF and LTα genes

The homologous TNF and LTα genes lie on the short arm of chromosome 6 separated by 1 kb. HLA-DRB1 and TNF are separated by approximately 850 kb, and LTα and HLA-B by 250 kb. The positions of the 10 single nucleotide polymorphisms, the nucleotide insertion and 6 microsatellite polymorphisms in the TNF and LTα genes are depicted (the TNF cluster d and e microsatellites lie in intron 3 of the B144 gene). Exons are represented by shaded boxes, and translated regions of the gene by black boxes. Arrows represent direction of transcription. Polymorphisms genotyped in this study are shown in figure 4.2.



4.1.6. TNF and LTα in CVID

4.1.6.1. Patient subgroups - granulomatous CVID

A number of authors have attempted to delineate subgroups of CVID patients by clinical or laboratory criteria (Bryant et al, 1990). Due to technical difficulty, these classifications are not widely used in clinical practice. However several reports have described a clinically distinct subgroup of patients where recurrent infections are accompanied by multisystem non-caseating sarcoid-like granulomata, splenomegaly and lymphadenopathy (Wright et al, 1990; Aukrust et al, 1996; Fasano et al, 1996). The presence of granulomata often results in diagnostic confusion with sarcoidosis (James, 1992). Many CVID patients exhibit peripheral blood immunophenotypic abnormalities, such as CD4+ T lymphopenia (Spickett et al, 1990; Lebranchu et al, 1991; Farrant et al, 1994), inversion of the CD4+:CD8+ ratio (Reinherz et al, 1981; Spickett et al, 1990; Wright et al, 1990; Farrant et al, 1994), and CD8+CD57+ lymphocytosis (Wright et al, 1990; Baumert et al, 1992). Several of these abnormalities, particularly CD4+ lymphopenia and inversion of the CD4+:CD8+ ratio, have been associated with clinical features such as splenomegaly (Wright et al, 1990; Aukrust et al, 1995). Similar immunophenotypic associations with granulomata have been suggested (Wright et al, 1990), however the relatively small number of reported cases of CVID with granulomata prevent definite conclusions. Abnormal B and T lymphocyte responses *in vitro* have been described in this "granulomatous" subgroup of patients, but these are not universal findings. The pathogenesis of these features is unknown.

4.1.6.2. Previous studies of TNF and LTα in CVID

Recent reports have documented abnormally high activity of the TNF axis in CVID patients with CD4+ lymphopenia and splenomegaly. Serum TNF:soluble TNF receptor levels are significantly elevated in these patients, and spontaneous monocyte TNF secretion *in vitro* is abnormally high (Aukrust et al, 1995; Aukrust et al, 1996). It is not known whether this abnormal TNF production is caused by infection, is secondary to other cellular anomalies, or indicates a genetically distinct subgroup, characterised by high TNF responder phenotype. The status of TNF activity in granulomatous CVID has not been definitively

reported: several, but not all, of the patients in the studies of Aukrust *et al* had granulomata.

Genetic and functional studies of LTα in CVID are lacking. A weak association of CVID with the LTα restriction fragment length polymorphism, *EcoR*I, has been described (Howe et al, 1991). However, the low carriage rate of this marker (1% of normal Caucasoids) limits its use as a marker (Partanen et al, 1988).

4.1.7. Aims of this chapter

The existence of a discrete subgroup of CVID patients with high TNF production, coupled with the important immunoregulatory functions of TNF and LTα suggests that a detailed examination of multiple TNF and LTα polymorphisms in a large group of well characterised CVID patients would be of considerable interest. Accordingly, 6 biallelic TNF and LTα polymorphisms were genotyped in 150 Caucasoid CVID patients, 90 of whom had comprehensive clinical and immunophenotypic data available (as described in chapter 3), and 200 controls. Results were analysed in conjunction with HLA class I and II genotype data in order to determine the relative importance of any associations detected.

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4.2. RESULTS

4.2.1. Patient and control details

150 of the 179 Caucasian CVID patients described in chapter 3 were studied in this chapter[4]. Clinical and peripheral blood immunophenotypic data was collected for 90 patients, as described in chapter 3.

The 605 consecutive Caucasian cadaveric renal allograft donors described in chapter 4 were used as controls for HLA class I and II genotyping. 200 of these were used as controls for TNF and LTα genotyping.

4.2.2. Genotyping

4.2.2.1. HLA class I and II

8 locus HLA class I and II genotyping (HLA-A, B, Cw, DRB1, DRB3-5 and high resolution -DQB1) was performed in all patients and controls as described in chapter 4.

4.2.2.2. TNF and LTα

Six biallelic single nucleotide TNF and LTα polymorphisms were genotyped by PCR-SSP (Fanning et al, 1997) in all patients and 200 controls: -308, -238 and +488 (TNF); and +249, +365 and +720 (LTα). By using forward and reverse allele-specific primers, this technique is able to formally demonstrate combinations of alleles on the same chromosome (in *cis*, or "haplotypes"). Of the 8 possible haplotypes for each of TNF and LTα, only 4 have been described for TNF, and 4 for LTα (Fanning et al, 1997). These haplotypes, and the positions of the polymorphisms are illustrated in figure 4.2. Examples of TNF and LTα genotyping are given in figure 4.3.

4.2.3. Peripheral blood immunophenotyping

Enumeration of basic T and B lymphocyte subsets, in addition to CD45 isoforms and CD8+CD57+ counts was described in chapter 3.

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[4] Owing to lack of DNA, not all patients could be TNF and LTα genotyped

4.2.4. Statistics

HLA, TNF and LTα association data was analysed using Chi-squared and Fisher's exact tests. The unpaired t test and z test were used to analyse associations between clinical parameters and lymphocyte phenotype data. A p value corrected for multiple comparisons of <0.05 was considered to be significant.

Figure 4.2: ***Location of 6 biallelic TNF and LTα polymorphisms, and previously detected haplotypes***

Haplotypes represent alleles in *cis*, ie. lying on the same chromosome. Of the 4 LTα haplotypes previously described, only LTα-1, 2 and 3 were detected in the present study.

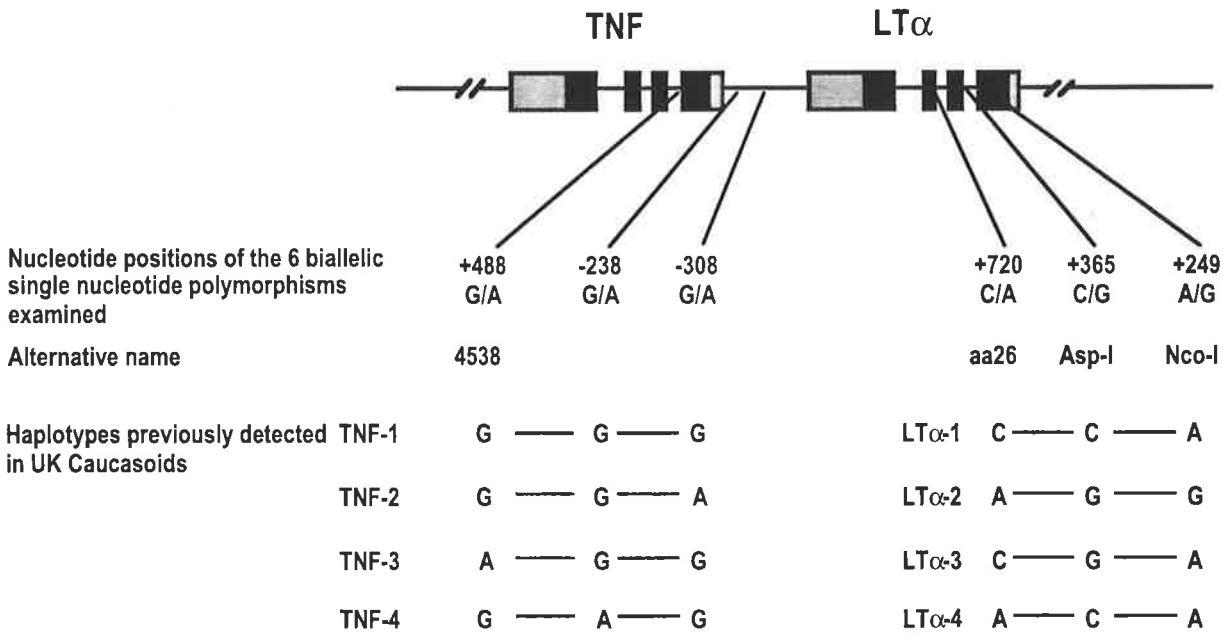
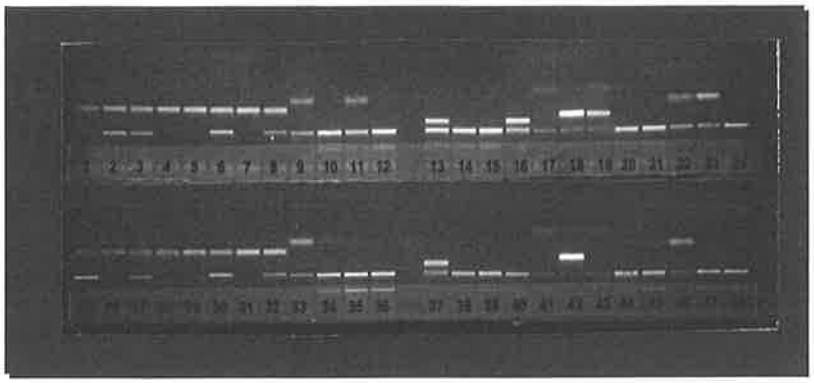


Figure 4.3: Examples of tumour necrosis factor and lymphotoxin alpha genotyping.



Direction of electrophoresis

Sample	Lanes	Positive lanes	Specificity
1	1-24	2, 6, 11	**TNF-2**
		3, 8, 9	**TNF-3**
		13, 18, 22	**LT-1**
		16, 19, 23	**LT-2**
2	25-48	1, 6, 9	**TNF-1**
		3, 8, 9	**TNF-3**
		13, 18, 22	**LT-1 (homozygous)**

LEGEND:
PCR products have been electrophoresed through 1% agarose and photographed after UV illumination.
Each row represents a sample. The first 12 lanes in each row are TNF specific, and the second 12 LT specific.
Each lane has a control amplicon with or without a haplotype-specific amplicon.
In the first 8 lanes of each row, the control amplicon is uppermost (250 bp) with haplotype-specific bands below.
In the remaining lanes, the control band is lowermost (796 bp), with the haplotype specific bands above.
Haplotypes are shown in figure 4.2
Method from Fanning et al, 1997.

4.2.5. Clinical parameters

In view of the relevance of the results of the previous chapter, important clinical characteristics of the patient cohort are summarised here. Of 90 patients with available clinical data, 20 (22.2%) had histologically confirmed non-caseating granulomata in at least one organ. There was a significant association between the presence of granulomata and splenomegaly (14 of 20 patients with granulomata also had splenomegaly, compared to 13 of 70 patients without granulomata; Fisher's exact test $p<0.0001$).

4.2.6. Immunophenotypic abnormalities

Immunophenotypic abnormalities were described at length in the previous chapter. Salient findings for the 90 patients with comprehensive clinical data are summarised in table 4.1. In brief, the peripheral blood CD4+:CD8+ ratio was reversed in 31 patients (34.4%). Of the 51 patients for whom CD45 isoforms and CD8+CD57+ lymphocytes were measured, 26 had CD4+CD45RA+ lymphopenia, and 24 (47.1%) had CD8+CD57+ lymphocytosis.

The presence of granulomata strongly correlated with CD3+ lymphopenia (t test $p=0.004$), B lymphopenia ($p=0.0009$), depletion of CD4+CD45RA+ cells ($p=0.0003$), and CD8+CD57+ lymphocytosis ($p=0.001$) (Table 4.2). Weaker correlations were detected with a lower CD4+ count and CD4+:CD8+ ratio. Similar associations between splenomegaly and immunophenotypic abnormalities were also detected (data not shown). This correlation of clinical and immunophenotypic abnormalities confirms previous reports (Wright et al, 1990; Aukrust et al, 1996).

Table 4.1: Peripheral blood immunophenotypic data

Counts are expressed as number of cells x 10^9/l. n/a = (reference range) not available. Reference range derivation is discussed in chapter 3.

Lymphocyte subset	Normal range (x10^9 cells/l)	Number	Mean (range; SD)	Number of patients outside reference range
CD3+	0.6-1.5	90	1.36 (0.28-6.49; 0.95)	14 (<0.6x10^9 cells/l)
CD3+CD4+	0.4-1.0	90	0.65 (0.18-2.74; 0.44)	24 (<0.4x10^9 cells/l)
CD3+CD8+	0.2-0.7	90	0.63 (0.04-3.92; 0.58)	25 (>0.7x10^9 cells/l)
CD4+:CD8+	>1.0	90	1.48 (0.19-7.78; 1.1)	31 (<1.0x10^9 cells/l)
B cells (CD19+/CD20+)	0.1-0.5	90	0.21 (0-0.94; 0.22)	27 (<0.1x10^9 cells/l); absent in 7 patients
CD4+CD45RA+	n/a	51	0.24 (0.01-1.5; 0.31)	n/a
CD4+CD45RA+ / CD4+ (%)	>30% total CD4+	51	30% (1-75; 23)	26 (<30% total CD4+)
CD8+ also CD57+ (%)	<40%	51	37 (4-89; 19)	24 (>40% total CD8+)

Table 4.2: Correlation between granulomata and peripheral blood immunophenotypic abnormalities

Counts are expressed as number of cells x 10^9/l. ns = not significant.

Lymphocyte subset	Granulomata n=20	No Granulomata n=70	p value (t test)
Mean CD3+	0.8	1.36	0.004
Mean CD3+CD4+	0.51	0.66	0.02
Mean CD3+CD8+	0.69	0.61	ns
Mean B cells	0.11	0.22	0.0009
Mean CD4+:CD8+	1.41	1.73	0.04
Mean CD4+ also CD45RA+ (%)	11	35	0.0001
Mean CD8+ also CD57+ (%)	52	33	0.002

4.2.7. TNF, LTα allele and haplotype frequencies in all patients

The uncommon TNF -308A allele was more frequent in the patient group than in controls (χ^2=4.91, p=0.02; RR=1.62, CI 1.06-2.49; AF 0.19) (table 4.3). This finding was corroborated by an identical positive association with the TNF haplotype bearing this allele, TNF-2 (table 4.4).

4.2.7.1. A high rate of TNF -308A homozygosity in CVID

Although these associations with phenotype frequencies are not significant after correction for multiple comparisons, the number of patients homozygous for TNF -308A and TNF-2 was significantly greater in patients than in controls (18 (12%) patients compared with 8 (4%) controls, χ^2 p=0.007, p corrected = 0.04; RR=3.27, CI 1.38-7.75). A corresponding negative association was detected with TNF -308G (χ^2=15.17 p=0.0001; RR=0.17, CI 0.06-0.46, PF=0.81).

Significant negative associations were detected with LTα +365C, and the corresponding LTα-1 haplotype (both p=0.003).

4.2.7.2. Linkage disequilibrium analysis - determination of the strongest associations

The observed associations between TNF and LTα alleles and CVID may be pathogenically important, but interpretation of these associations must take linkage disequilibrium with HLA class I and II alleles into account. TNF -308A and its haplotype, TNF-2 are known to lie on the common Caucasoid haplotype A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 (Wilson et al, 1993; Fanning et al, 1997). Similarly, LTα +365C/LTα-1 are associated with the Cw*0702-B*0702-DR*15-DQB1*0602 haplotype (Fanning et al, 1997). The strongest associations are with HLA-B: LTα-1 with HLA-B*07, and TNF-2 with HLA-B*08 (Wilson et al, 1993; Fanning et al, 1997). As described in chapter 3, CVID is associated with these extended haplotypes (summarised in Table 4.5). Moreover, these associations appear strongest with loci flanking the HLA class III region (containing the TNF and LTα genes), especially HLA-B. From this preliminary analysis it is difficult to ascertain which loci, HLA-B or TNF/LTα, are of primary importance. To address this issue, data was stratified and further analysed according to the method of Svejgaard and Ryder, as described in chapter 3

(Svejgaard et al, 1994). No significant differences between the HLA-B and TNF/LTα associations were detected Rather, associations with B*07—LTα-1 together, and B*08—TNF-2 together, were demonstrated, reflecting the previously described strong linkage disequilibrium between these alleles (Wilson et al, 1993; Fanning et al, 1997). Thus it remains possible that the class III allelic associations are of primary importance, but clarification of this issue will require examination of a much greater number of patients.

4.2.8. TNF +488A is associated with "granulomatous" CVID

Stratification of data on the basis of clinical criteria revealed a highly significant association between the presence of granulomata and the uncommon TNF +488A allele (Table 4.6). 8 of 20 (40%) patients with granulomata were TNF +488A positive, compared to 4 of 70 (5.7%) non-granulomatous patients (Fisher's exact test p=0.0005; RR=11.0, CI 2.9-42.4; AF 0.36), and 31 of 200 (15%) controls (Fisher's p=0.009, RR=3.63, CI 1.37-9.62). This allele was also significantly associated with other features associated with granulomata, including splenomegaly (8 patients (29.6%) with splenomegaly were TNF +488A positive, compared with 4 patients (6.7%) without splenomegaly, Fisher's exact test p=0.005; RR=5.76, CI 1.56-21.29; AF=0.24); CD3+ lymphopenia (p=0.009); and CD8+CD57+ lymphocytosis (p=0.002). No other significant TNF or LTα associations were detected for patient subgroups defined by other immunophenotypic or clinical criteria (including age at diagnosis, autoimmunity and other end-organ complications such as bronchiectasis).

Table 4.3: Associations of CVID with TNF and LTα alleles

Table 4.3 lists associations with alleles at individual polymorphisms, whereas table 4.4 lists associations with TNF and LTα haplotypes. Phenotype frequencies are shown (the number of patients positive for an allele or haplotype), rather than allele frequencies (the number of positive chromosomes). ns = not significant.

Gene	Position of polymorphism	Allele	Patients total=150 n (%)	Controls total=200 n (%)	χ^2 p	p corrected
TNF	+488	G	145 (73)	198 (99)	ns	ns
		A	19 (13)	31 (16)	ns	ns
	-238	G	142 (71)	199 (99.5)	ns	ns
		A	15 (7.5)	24 (12)	ns	ns
	-308	G	130 (65)	195 (98)	0.0001	0.0006
		A	74 (49)	75 (37)	0.02	0.11
LTα	+720	C	116 (58)	174 (87)	ns	ns
		A	91 (45)	112 (56)	ns	ns
	+365 (Asp-1)	G	131 (66)	174 (87)	ns	ns
		C	70 (35)	126 (63)	0.003	0.01
	+249 (NcoI)	A	116 (58)	174 (87)	ns	ns
		G	91 (46)	112 (56)	ns	ns

Table 4.4: Associations of CVID with TNF and LTα haplotypes

		Patients total=150 n (%)	Controls total=200 n (%)	p value	p corrected
TNF haplotype (+488 — -238 — -308)	TNF-1 (G—G—G)	123 (62)	180 (90)	ns	ns
	TNF-2 (G—G—A)	74 (49)	75 (37)	0.02	0.11
	TNF-3 (A—G—G)	15 (7.5)	31 (16)	ns	ns
	TNF-4 (G—A—G)	15 (7.5)	23 (12)	ns	ns
LTα haplotype (+720 — +365 — +249)	LTα-1 (C—C—A)	70 (35)	126 (63)	0.003	0.01
	LTα-2 (A—G—G)	91 (46)	115 (58)	ns	ns
	LTα-3 (C—G—A)	73 (37)	88 (44)	ns	ns

Table 4.5: Association of CVID with HLA class I and II alleles

The principle associations are shown arranged into haplotypes, and alleles listed telomeric to centromeric. Positive associations are shown in bold, and negative associations italicised. These associations have been reported previously, thus p values have not been corrected. ns = not significant.

Allele (antigenic equivalent)	Patients (all) total = 150 n (%)	Controls total = 605 n (%)	p value
A*01	68 (45%)	217 (36%)	0.03
Cw*0701	64 (43%)	183 (30%)	0.004
B*0801	58 (39%)	150 (25%)	0.001
DRB1*0301	52 (35%)	157 (26%)	0.03
DQB1*0201	81 (54%)	256 (42%)	0.01
A*02	63 (42%)	296 (49%)	ns
A*03	34 (23%)	159 (26%)	ns
Cw*0702	22 (15%)	168 (28%)	0.0007
B*07	23 (15%)	172 (27%)	0.0008
DRB1*15	21 (14%)	172 (28%)	0.001
DQB1*0602	19 (13%)	159 (26%)	0.004

Table 4.6: The TNF +488A association with granulomatous CVID

*Granulomatous v non-granulomatous CVID: Fisher's exact test p=0.0005, RR 11, CI 2.86-42.38, AF=0.36.
†Granulomatous CVID v. controls p=0.009

	Granulomatous CVID total = 20 n (%)	Non-granulomatous CVID total = 70 n (%)	Controls total = 200 n (%)
TNF +488A positive	8*† (40%)	4 (5.7%)	31 (15%)
TNF +488A negative	12 (60%)	66 (94.2%)	169 (84.5%)

4.3. DISCUSSION

4.3.1. Summary of findings

There were two major findings of this study. Firstly, TNF and LTα alleles were significantly associated with CVID, but it was not possible to determine whether or not these associations were of primary importance. Secondly, when patients were stratified by clinical criteria, the granulomatous disease subgroup was strongly associated with an uncommon TNF allele, TNF +488A.

4.3.2. The role of TNF 488A in the pathogenesis of granulomata

A detection of a genetic association of granulomatous CVID with the uncommon TNF +488A allele is a novel finding. Associations were also detected between TNF +488A and other features associated with granulomata, namely splenomegaly, CD3+ lymphopenia and CD8+CD57+ lymphocytosis. Given the high TNF activity previously documented in such patients (Aukrust et al, 1994; Aukrust et al, 1995; Aukrust et al, 1996), this association is of particular interest. As discussed above, the estimated contribution of genetic factors to variability in TNF production between individuals is 60%, but cannot be explained by the effects of the TNF -238 and -308 polymorphisms alone (Westendorp et al, 1997). Thus it is likely that other polymorphisms, in TNF or other genes, regulate transcriptional activity of the TNF gene. The TNF +488 polymorphism has only recently been described (D'Alfonso, 1996) and its functional relevance is unknown, but in view of these results, it is possible that the presence of TNF +488A, or alleles associated with it, results in higher TNF production in these patients.

TNF +488A was not in linkage disequilibrium with any HLA class I or II alleles associated with CVID. Although the number of patients and controls carrying this allele was small (50 in the present study) no trends toward disequilibrium were detected. This association may indeed be primary, although further studies of polymorphism of both TNF and the flanking HLA class III region will be necessary, in addition to functional studies.

Paradoxically, abnormal lymphocyte activation is a feature of "granulomatous" CVID patients. It is not known if granulomata, splenomegaly and lymphadenopathy in CVID result from abnormally high activity of the TNF axis, or whether profound macrophage activation in granulomata contributes to high TNF levels. It is suggested that in this subgroup of patients, aberrant immune activation results in increased production of TNF, which in turn stimulates granuloma formation. Furthermore, there are several reports of improvement of granulomata and splenomegaly with corticosteroid treatment (Fasano et al, 1996; Spickett et al, 1996), which is known to abrogate monocyte TNF secretion. This mechanism may also underlie the formation of sterile non-caseating granulomata in other clinical situations, such as sarcoidosis and inflammatory bowel disease. Aberrant lymphocyte activation associated with TNF production may also lead to another feature of CVID, autoimmunity, although no associations were detected in the present study.

The TNF +488 association with this "granulomatous" subset of CVID patients is not absolute: only 40% of granulomatous patients carries this allele. This suggests that other polymorphisms influencing the production or action of TNF may be of importance in the remaining patients (such as the TNF receptor genes, which are known to be polymorphic) (Kaufman et al, 1994).

4.3.3. A role for TNF and LTα polymorphisms in the pathogenesis of hypogammaglobulinaemia?

Several other TNF and LTα associations were detected with the CVID: positive associations with TNF -308A/TNF-2, and negative associations with LTα-+365C/LTα-1. In view of the known importance of both TNF and LTα in humoral immunity, these associations provide a potential mechanism for the pathogenesis of hypogammaglobulinaemia. Despite stratification and re-analysis of data, it was not possible to conclude whether these, or HLA class I and II alleles, were primarily associated with CVID. As discussed earlier, evidence regarding the direct effect of this allele on TNF gene expression is conflicting. Further evaluation of the functional effects of the TNF and LTα polymorphisms is required.

It is feasible that the combination of associations with TNF and LTα, both of which act through the TNF receptors, confers susceptibility to CVID. Alternatively, these polymorphisms may synergise with other immunogenetic polymorphisms in the pathogenesis of hypogammaglobulinaemia.

The mechanisms by which genetically determined variability in TNF and/or LTα production might generate hypogammaglobulinaemia are unknown. One possible mechanism is impairment of TcR signal transduction and thus T lymphocyte activation. Chronically elevated TNF levels have been shown to attenuate T cell receptor signalling and T lymphocyte IL-2 production in mice (Cope et al, 1997). These abnormalities of TcR signalling and cytokine production are well documented in CVID, thus it will be of interest to examine associations between TcR function and TNF levels and genotype.

It is also possible that the primary associations detected with the entire patient group are not with TNF/LTα or class I or II alleles, but with other linked HLA genes in close proximity to TNF. Over 50 transcriptional units have been detected in the class III region (Trowsdale et al, 1994; Aguado et al, 1996). Several of these, such as B144 and 1C7, are very close to the *TNF/LTA/LTB* cluster (see figure 1.1, chapter 1). These products of these genes are predicted to have substantial homology to members of the immunoglobulin superfamily, such as CD28, CTLA-4 and the T cell receptor. Furthermore, these genes are expressed in lymphoid cells, and are inducible by IFNγ, suggesting an immunoregulatory function (Holzinger et al, 1995; Nalabolu et al, 1996), and are polymorphic (Abraham et al, 1992; de Baey et al, 1995). Thus whilst the precise immunoregulatory roles of B144 and 1C7 are unknown, these genes are highly attractive candidates for investigation in CVID.

If genetically determined variability in TNF production is important in the generation of granulomata in CVID, it is likely that polymorphism in other HLA or non-HLA genes is important, as the TNF +488A allele was only present in 40% of granulomatous patients. One obvious candidate is IL-10, a T cell derived cytokine that negatively regulates inflammatory 'type 1' immune responses,

inhibits monocyte TNF production, and promotes humoral 'type 2' responses (Fiorentino et al, 1989; Armstrong et al, 1996). The role of polymorphism in IL-10, and other immunoregulatory genes in CVID is explored in the following chapters.

5. Mannose-binding lectin polymorphism in CVID

5.1. INTRODUCTION

Hypogammaglobulinaemia is the *sine qua non* of common variable immunodeficiency. However, despite this universal manifestation, the clinical features of CVID are highly heterogeneous. For example, there is poor correlation between serum IgG concentration and frequency and severity of infection. The hypothesis examined in this chapter is that in the absence of adequate levels of antibody, compensatory immune mechanisms assume greater importance in host defence, and influence CVID phenotype. For example, impairment of these compensatory mechanisms may further increase susceptibility to infection, or predispose to complications of CVID, such as autoimmunity.

It is possible that complement may compensate for hypogammaglobulinaemia in CVID, and in particular the mannose-binding lectin (MBL) pathway of complement activation[5]. Of the many components of the complement cascade critical for normal complement activation, MBL is an interesting candidate for investigation in CVID for two reasons. First, MBL activates both classical and alternative pathways of complement in a non-antigen specific, non-antibody dependent fashion, and has been proposed as an important innate host defence mechanism (Turner, 1996). Secondly, preliminary evidence suggests that MBL is an important immune defence in young children, prior to the attainment of fully functional adaptive immunity. Several allelic variants of the polymorphic MBL gene have been associated with low serum levels of MBL, and with an increased risk of recurrent infection, particularly in children (Sumiya et al, 1991; Garred et al, 1995; Summerfield et al, 1997). This suggests that MBL is important during physiological hypogammaglobulinaemia in the first years of life, and thus may also be important in compensating for other hypogammaglobulinaemic states,

[5] In order to avoid confusion with myelin basic protein, which shares the acronym MBP, it has been suggested that the term 'mannan- (or mannose-) binding lectin' should replace 'mannose-binding protein', and thus 'MBL' should replace 'MBP' (Turner, 1996).

such as CVID. Before a summary of previous studies of MBL and disease, and further discussion of the relevance of this pathway to CVID, the basic features of MBL function and genetics are presented.

5.1.1. MBL structure and function

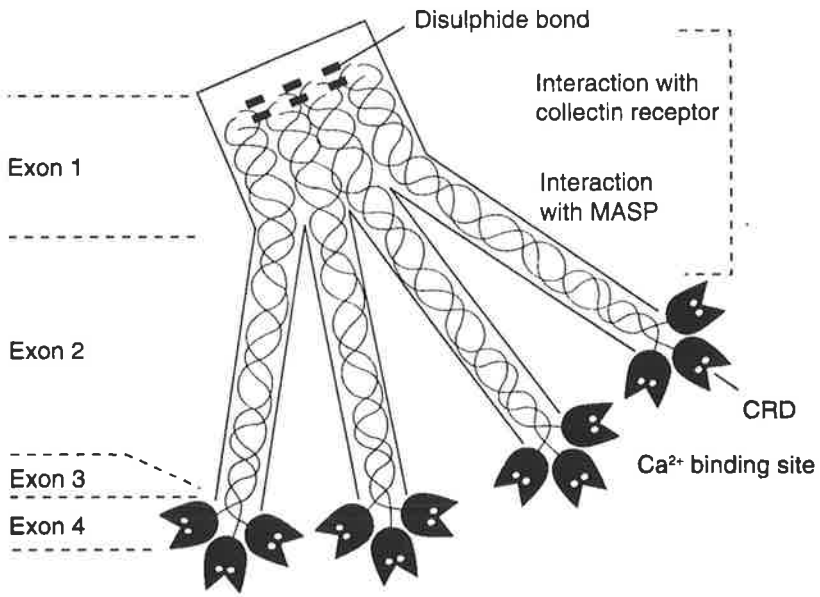
MBL is a member of the collectin (calcium-dependent lectin) family (Sastry et al, 1993). MBL has diverse IgG, IgM and C1q-like immunological functions, including opsonisation of pathogens (Kawasaki et al, 1983), activation of both classical (Ikeda et al, 1987) and alternative complement pathways (Schweinle et al, 1989), and activation of macrophages through the C1q receptor (Tenner et al, 1995). By binding sugars such as N-acetylglucosamine, fucose, glucose and mannose, but not sialic acid or galactose, MBL distinguishes oligosaccharides on bacterial and protozoal glycoproteins from those on mammalian glycoproteins. As MBL may bind to a large range of sugars, it is capable of recognising a wide range of pathogens. Although data regarding interaction with specific pathogens is limited, MBL has been shown to bind to yeasts (eg. *Candida albicans* and *Cryptococcus neoformans*) (Tabona et al, 1995), viruses (including HIV and influenza A) (Haurum et al, 1993a; Haurum et al, 1993b), protozoa (eg. *Leishmania spp.*) (Green et al, 1994b), and a variety of bacterial species (including *Salmonella*, *Streptococci*, *E. coli*, *Neisseria* and *Listeria spp.*), although binding affinity experiments suggest that MBL binding is impaired by bacterial capsules (Kuhlman et al, 1989; van Emmerik et al, 1994).

MBL is structurally similar to C1q, the first component of the classical complement pathway, but in contrast to MBL, C1q does not possess lectin domains or sugar-binding activity. MBL can activate the classical pathway either by substituting for C1q (Lu et al, 1990), or through the MBL-associated serine proteases, MASP 1 and 2 (Matsushita et al, 1992; Thiel et al, 1997). MASP 1 and 2 are highly homologous to the C1q-associated C1r and C1s serine proteases, and it has been suggested that the MBL pathway of complement activation antedates the development of specific immunity (Thiel et al, 1997).

The basic structure of MBL is a trimer of 32 kD peptides, each peptide containing a collagen like region and calcium dependent carbohydrate recognition domain (Sastry et al, 1989) (figure 5.1). Association of these basic trimers into pentamers and hexamers is required for full function (Lu et al, 1990), although these higher order polymers account for only 25% of circulating MBL (Lipscombe et al, 1995). Upon binding oligosaccharides, conformational changes render MBL functionally active.

Figure 5.1: Tetrameric mannose-binding lectin

Each of the four subunits of tetrameric human MBL consists of three identical 32 kD chains that combine to produce a disulphide linked N-terminal region, a collagenous triple helix, a neck and a C-terminal cluster of three lectin (carbohydrate recognition) domains. The collagenous regions interact with MASP-1 and -2. Figure taken from Turner, 1996



5.1.2. MBL genetics

The MBL gene has been localised to chromosome 10q11.2-q21. Six polymorphisms in the MBL gene have been identified, three in exon 1 and three in the 5′ untranslated and promoter regions of the gene (Sumiya et al, 1991; Lipscombe et al, 1992; Madsen et al, 1994; Madsen et al, 1995) (figure 5.2). All but one polymorphism (P/Q at nucleotide position +4 relative to the transcription initiation site) have been associated with variance in levels of circulating MBL, as determined by enzyme-linked immunosorbent assay. A synonymous nucleotide substitution at codon 44 has also been described in one patient (AAC→AAT, both encoding asparagine) (Mead et al, 1997).

5.1.2.1. Polymorphisms in exon 1 of the MBL gene

The polymorphisms in exon 1 result in amino acid substitutions in the collagen-like domain of the MBL peptide (table 5.1). The uncommon alleles at each of these codons (52Cys, 'D'; 54Asp, 'B'; and 57Glu, 'C') are commonly referred to as 'mutations' in view of their deleterious effect on MBL assembly and serum MBL levels. Both codon 54Asp (B) and 57Glu (C) alleles disrupt the glycine repeats of the collagen-like domain, resulting in unstable trimers that cannot form functional multimers (Lipscombe et al, 1995) (figure 5.3). The Arg→Cys codon 52 substitution does not disrupt the collagen helix, but by introducing an extra disulphide bond may accelerate MBL degradation. Immunoblotting and chemiluminescent experiments have shown that individuals homozygous for the codon 54 and 57 mutations have very little functional serum MBL (as determined by ELISA detecting the complement-fixing pentamer and hexamer forms of MBL), and the protein present predominantly consists of low-order oligomers unable to fix complement (Kurata et al, 1993; Lipscombe et al, 1995). Codon 54 or 57 mutation heterozygotes have approximately 1/8 to 1/4 normal MBL levels (Sumiya et al, 1991; Garred et al, 1992; Lipscombe et al, 1992; Madsen et al, 1994). Serum MBL levels in 52Cys heterozygotes are approximately 50% that of wild-type individuals (Madsen et al, 1994). Median serum concentrations for the different codon 54 genotypes have been reported in Caucasoids: wild type, 1630 ng/ml; 54Asp heterozygote 358 ng/ml and 54Asp homozygote ~10 ng/ml (Lipscombe et al, 1992). The effect of coding mutations on serum MBL levels is also modified by age (Terai et al, 1993; Lau et al, 1995; Thiel et al, 1995), ethnicity (Garred et al, 1992; Lipscombe et al, 1992; Lipscombe et al, 1996) and polymorphisms in the 5' region of the MBL gene (Madsen et al, 1995) (discussed below).

With the exception of 52Cys, each of these 'mutant' alleles has been detected at high frequency in at least one population (Lipscombe et al, 1992; Madsen et al, 1994). 54B has been detected at a (gene) frequency of 0.19 in Caucasoids (but is rare in Afro-Caribbeans); and 57Glu has been found to have a gene frequency of 0.29 in Gambian Afro-Caribbeans (but is rare in Caucasoids (Lipscombe et al, 1992; Madsen et al, 1994).

5.1.2.2. *Polymorphisms in the 5' region of the MBL gene*

The 5' region of the MBL gene has several features characteristic of acute phase proteins, such as heat shock and glucocorticoid response elements (Sastry et al, 1989; Taylor et al, 1989), and indeed is a modest acute phase reactant (Thiel et al, 1992; Arai et al, 1993). Thus it is not surprising that MBL levels are strongly influenced by polymorphisms in the MBL promoter region. Alleles at the -550 (H and L) and -221 (X and Y) polymorphisms are in linkage disequilibrium and have only been detected in three of four possible haplotypes: *HY*, *LY* and *LX* (Madsen et al, 1995). When in *cis* with a normal coding region ('A'), *HY* is associated with the highest MBL levels, *LY* with intermediate levels, and *LX* with the lowest levels (Madsen et al, 1995). Each of these promoter haplotypes is common in Caucasoids (frequencies of 0.38, 0.38 and 0.34 respectively). Promoter haplotype frequencies vary substantially in different populations. For example, the *LY* haplotype is more common (0.65) and *HY* less frequent (0.11) in Afro-Caribbeans (Madsen et al, 1995).

A polymorphism in the 5' untranslated region of the MBL gene, at nucleotide position +4 (alleles P and Q) has also been identified (Madsen et al, 1994). The effect of this polymorphism on circulating MBL levels is unknown.

The high frequency of both coding and promoter alleles, and variability in frequencies between different populations, has resulted in speculation that low levels of MBL may be advantageous in certain circumstances. For example, the entry of certain intracellular pathogens such as *Mycobacteria* into cells is enhanced by complement-mediated opsonisation and phagocytosis. As a direct opsonin and activator of complement, MBL may facilitate intracellular infection. Consequently low MBL levels may protect against infection with intracellular pathogens (Garred et al, 1994). MBL levels have been reported to be higher in Ethiopian lepromatous leprosy patients than in controls (Garred et al, 1994), although MBL genotyping was not performed in this study, and the difference in levels may be partly explained by an acute-phase response raising MBL levels in the leprous patients.

Table 5.1: Polymorphisms in the MBL gene

Note that only the uncommon alleles that result in disruption of assembly of the MBL multimer have been denoted "names" (D for 52Arg, B for 54Asp and C for 57 Glu). A coding region without any of these alleles has been denoted "A". na=not applicable

	Polymorphic site	Nucleotides	Amino acids	Alternative allele names (amino acid)
Promoter / 5' UTR	-550	G/C	na	H / L
	-221	C/G	na	X / Y
	+4	C/T	na	P / Q
Exon 1	codon 52	CGT/TGT	Arg/Cys	D (Cys)
	codon 54	GGC/GAC	Gly/Asp	B (Asp)
	codon 57	GGA/GAA	Gly/Glu	C (Glu)

Figure 5.2: Organisation of exon 1 and the 5' region of the MBL gene

The six polymorphic sites in the MBL gene are indicated by arrows. Promoter and transcriptional response elements are also indicated: The TATA, CCAAT and GC boxes are involved in basal transcription; the glucocorticoid response element (GRE) and heat shock element (HSE) have been implicated in inducible transcription in other genes. UT = 5' untranslated element. Diagram adapted from (Madsen et al, 1995).

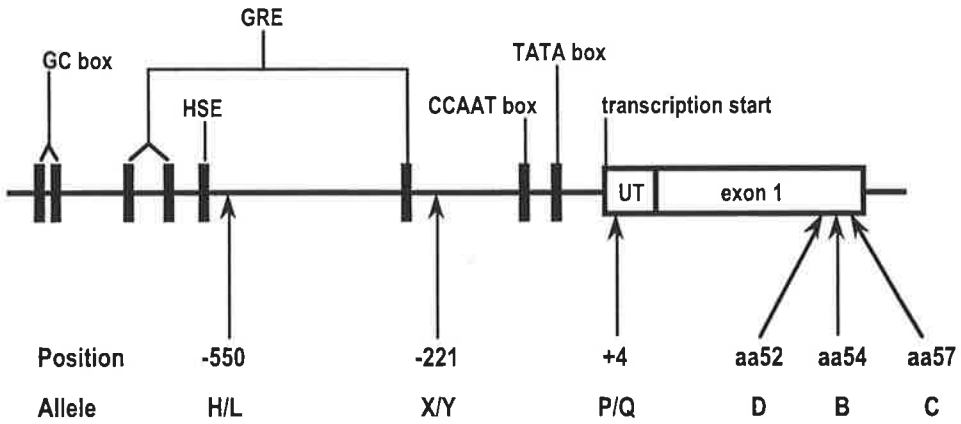
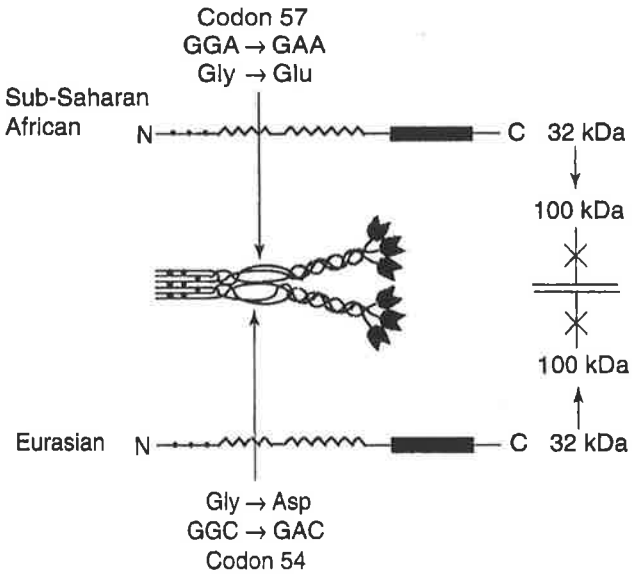


Figure 5.3: Effect of codon 54 and 57 mutations on MBL polymer assembly

Both the codon 54 and 57 mutations result in the replacement of axial glycines in the collagen-like helix with structurally disruptive dicarboxylic acids. Consequently there is little assemble of stable, complement fixing higher-order MBL polymers. Figure taken from Turner, 1996.



5.1.2.3. *MBL polymorphisms and disease*

MBL is theoretically most important in host defence at times when fully functional adaptive immunity is abnormal, or has not yet been attained. The latter is exemplified by the immunological immaturity and physiological hypogammaglobulinaemia characteristic of early childhood.

A childhood syndrome of recurrent infection, diarrhoea, and failure to thrive associated with defective pathogen opsonisation was first reported almost thirty years ago (Miller et al, 1968; Soothill et al, 1976; Candy et al, 1980). In 1989, this opsonic defect was shown to be associated with low serum MBL levels in 10 children with no other identified cause of immunodeficiency (Super et al, 1989). Low MBL levels, recurrent infection and defective serum opsonisation were subsequently demonstrated to be associated with the 54Asp MBL mutation in three children (Sumiya et al, 1991). This finding, and the subsequent identification of the codon 52 and 57 polymorphisms, generated considerable interest in the relationship between MBL mutations, serum MBL levels and recurrent infection.

A study of 229 children investigated in tertiary referral clinics for suspected immunodeficiency detected a high frequency of MBL coding mutation homozygosity (19 of 229 (8.3%) patients compared with 1 of 123 (0.8%) healthy controls, p=0.002) leading the authors to suggest that MBL coding mutation homozygosity predisposes to recurrent infection in childhood (Garred et al, 1995). A subsequent study of 626 paediatric hospital admissions, classified into infectious and non-infectious groups, found the frequency of MBL coding mutations in the infection group to be twice that of the non-infection group (146 of 345 (42.3%) compared with 64/272 (23.5%), p<0.0001) (Summerfield et al, 1997). Both heterozygotes and homozygotes were found to be at increased risk of infection. Additionally, 13 of the 17 homozygotes presented with severe infection, including septicaemia in 6 cases (Summerfield et al, 1997).

Only one small study has investigated the relationship of MBL mutations with infection in adults (Summerfield et al, 1995). In a report of 5 adults with severe and unusual infections (one of whom had IgA deficiency), 3 individuals were homozygous for MBL coding mutations, and 2 heterozygous.

MBL coding mutations have also been associated with increased susceptibility to human immunodeficiency virus (HIV) infection (Garred et al, 1997), adverse outcome from hepatitis B virus (HBV) (Thomas et al, 1996) and HIV infection (Garred et al, 1997). Furthermore, systemic lupus erythematosus (SLE), a systemic autoimmune disease previously associated with other complement deficiencies, has also been found to be associated with both MBL promoter and coding alleles (Davies et al, 1995; Lau et al, 1996; Sullivan et al, 1996; Davies et al, 1997).

These studies have resulted in a widely held view that both homozygosity and heterozygosity for MBL coding mutations predisposes to recurrent infection, in children and adults (Sumiya et al, 1996; Turner, 1996). However these studies have several important limitations and should be interpreted with caution. Approximately 30% of Caucasoids will have at least one MBL coding mutation (Turner et al, 1993; Lipscombe et al, 1996), many of whom will never experience unusually frequent or severe infections. This would suggest that the presence of

MBL coding mutations alone (or at least heterozygosity for mutations) is not sufficient to cause recurrent infection in all individuals. Furthermore, the infections ascribed to the presence of MBL mutations are heterogeneous, with no clear unifying pattern, in contrast to the majority of primary immunodeficiencies (Snowden et al, 1995).

Secondly, the frequencies of MBL mutations in the control groups used by Garred and Summerfield are much lower than those predicted from the gene frequency (Snowden et al, 1995). It has been suggested that this may result from the use of healthy blood donors as controls, who are positively screened for health and thus not representative of the general population (Snowden et al, 1995). Indeed, mutation frequencies in non-blood donor controls comparable to those found in patients with recurrent infection (Garred et al, 1995) have been detected (Snowden et al, 1995). This highlights the need for carefully chosen, representative control groups in all studies examining the role of immunogenetics in host defence.

Thirdly, the only data associating MBL mutations with recurrent infections in adults is based on 5 cases (Summerfield et al, 1995). It is not possible to extrapolate a critical role of MBL in host defence to the general adult population from such limited anecdotal evidence.

Fourthly, only two of the above studies have examined serum MBL levels (Garred et al, 1995; Summerfield et al, 1995), and neither of these studies have examined MBL levels on more than one occasion. This is important, as MBL levels vary substantially between individuals with the same genotype (Madsen et al, 1995), and in response to stress (Thiel et al, 1992; Arai et al, 1993). Furthermore, the patients described in all these studies were recruited from tertiary referral immunology clinics or inpatient paediatric units, and thus are highly selected, and not representative of the general population.

Finally, none of these studies examined the non-coding MBL polymorphisms, at least two of which (-550 and -221) significantly influence serum MBL levels

(Madsen et al, 1995). In reviews of the MBL literature, several authors have stated that variance in MBL levels is almost entirely explained by the three coding mutations, and have dismissed these 5' polymorphisms as being of trivial importance (Sumiya et al, 1996; Turner, 1996). This conclusion would seem premature, given that individuals homozygous for the *HYA* haplotype ('A' denoting the absence of coding mutations) have a six-fold higher level of MBL that *LXA* homozygous individuals (p=0.0006) (Madsen et al, 1995). Furthermore, the serum MBL level of *LXA* homozygotes is comparable to *HYA/O* heterozygotes ('O' represents a chromosome carrying a coding mutation); and levels in *LXA/O* heterozygotes are comparable to coding mutation homozygotes (Madsen et al, 1995). One implication of this data is that some of the individuals reported to have no coding mutations in the studies above would have had circulating MBL levels comparable to an individual carrying one or more copies of a coding mutation. The effect of the promoter polymorphisms should thus not be ignored.

These issues highlight the need for large prospective population-based studies of both children and adults incorporating genotyping of all MBL polymorphisms, in conjunction with repeated measurement of serum MBL levels, in order to elucidate the relationship between MBL alleles and susceptibility to infection. It is possible that the clinical significance of MBL genotype will depend on other immunogenetic factors, such as Fc gamma receptor polymorphism, C4 alleles and IgG allotype, as has previously been suggested (Turner, 1996). Interaction with these factors may contribute to the extensive variability in the nature of infections previously associated with MBL mutations. Consequently it will be necessary in future studies to control for these factors in order to determine the true biological significance of defects in the MBL pathway.

5.1.3. Hypothesis

The frequency and severity of infections in CVID is highly variable, and correlates poorly with serum antibody levels. The hypothesis tested in this chapter is that the MBL pathway is an important compensatory mechanism for

hypogammaglobulinaemia, and that abnormalities in this pathway (suggested by mutations in the MBL gene) may adversely influence disease phenotype.

This hypothesis was tested by examining all known MBL gene polymorphisms in CVID patients, and in patient subgroups. This entailed the development of a simple PCR-SSP assay capable of genotyping all six MBL polymorphisms, and assigning alleles as being in *cis* or *trans* (haplotypes). This assay was used to genotype a large group of CVID patients who were subsequently stratified on the basis of clinical and laboratory criteria.

5.2. RESULTS

5.2.1. Development of MBL genotyping by PCR-SSP

5.2.1.1. Basic primer design

Combinations of sense and antisense allele-specific primers were used to detect all possible *cis* combinations of MBL alleles at each of the six MBL polymorphisms: -550, -221, +4 and codons 52, 54 and 57. This 'double ARMS' or 'PCR-haplotyping' approach was first described in 1991 (Lo et al, 1991b), and has been extensively used for HLA genotyping (Lo et al, 1991a; Bunce et al, 1995). The principles of haplotyping by PCR-SSP are described in figure 5.4. Where a primer spanned a polymorphic site (in addition to that specifically detected by the 3' terminal nucleotide of the primer), alternative bases were incorporated into the primer sequence, corresponding to the alleles of that additional polymorphism. This was necessary for the codon 52 and codon 54 specific primers, as codons 52, 54 and 57 lie in close proximity. Primers are listed in table 5.2.

5.2.1.2. Adjustment of primer length

Primer length was initially calculated using the primer annealing temperature equation (chapter 2, equation 2). However, on initial testing, very poor amplification was obtained with several primers. Extension of primers by several bases (at the 5' end, preserving 3' allelic specificity) rectified this problem. These primers are marked in table 5.2. An example of the effect of primer extension is shown in figure 5.5.

5.2.1.3. Adjustment of primer composition

All allele-specific primers were initially designed so that all bases (except for the allele specific terminal 3' base) were complementary to the consensus MBL nucleotide sequence (Genbank accession number X15954) (Taylor et al, 1989). However reactions using primers specific for 54Asp and +4Q frequently produced false positive amplification. Both represent inappropriate extension of a T/C mismatch, a feature of PCR-SSP that has been reported previously (Kwok et al, 1990; Huang et al, 1992). This problem was overcome by the introduction of deliberate mismatches several bases from the 3' termini of these primers. Several primers for each allele were evaluated, with the mismatch position ranging from

4-8 nucleotides from the 3' end. Primers that produced strong true positives, but no false positives (even in the presence of high-quality DNA at high concentration) were selected. The position of the mismatches is indicated in table 5.2.

Buffer composition, amplification conditions and thermal cycling parameters were identical to those listed in chapter 2. Primer concentrations are listed in table 5.3.

5.2.1.4. Reaction mixes

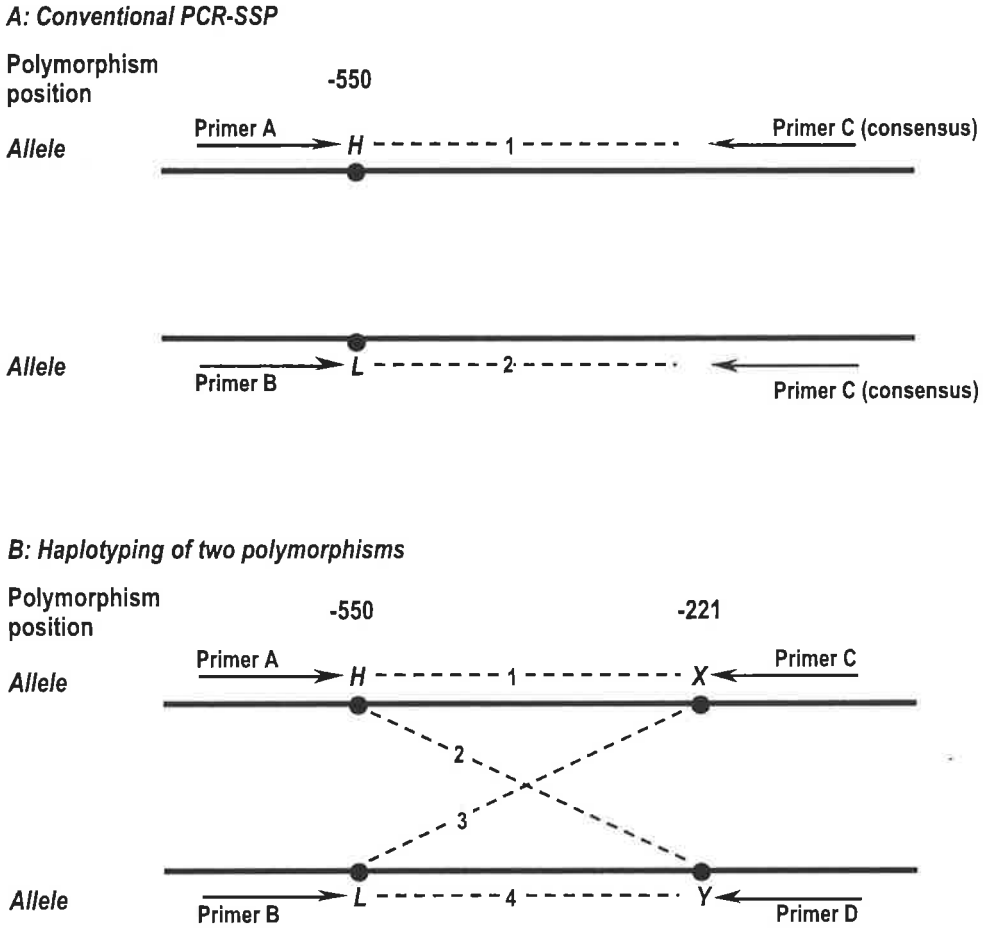
A full "set" of 48 reactions was initially used (table 5.3). This set detects all possible *cis* combinations of MBL alleles. In addition, because each allele is detected by several primer mixes (in combination with alleles at several different polymorphic sites), a degree of redundancy exists. This is useful when poor quality DNA samples are genotyped, or where new populations are studied with potentially new haplotypes. Examples of the full 48 reaction set are given in figure 5.6.

Reactions 1-36 of the full genotyping set all use DRB1-specific control primers (generating a 796 bp amplicon) to verify successful amplification. Control primers generating a 256 bp amplicon (specific for the adenomatous polyposis coli gene) were used in reactions 37-48 in view of the larger haplotype-specific product size. These primers were also used in HLA-DQB1 subtyping primer mixes (chapter 3) and are listed in table 5.2.

Genotyping of exon 1 polymorphisms was validated by confirming genotype of heterozygous and homozygous samples kindly provided by Adrian Hill, Richard Bellamy (Wellcome Centre for Human Genetics, Oxford UK) and Malcolm Turner (Institute for Child Health, St Mary's Hospital, London, UK). These samples had previously been genotyped by PCR-SSOP and PCR-heteroduplex analysis.

After genotyping in excess of 300 Caucasoid samples, the strong linkage disequilibrium between the MBL alleles became apparent. For example, the *HX* promoter haplotype was not detected; the 52Cys coding mutation was only found with the *HYP* promoter haplotype; the 54Asp mutation with *LYP*; and 57Glu with *LYQ* (confirming previous reports) (Madsen et al, 1995). Thus if these linkage disequilibria are assumed to be universally true (at least in Caucasoids), the 48 reaction set can be abridged to 12 reactions. This 12 reaction set will detect all promoter haplotypes and all X/Y-coding haplotypes. Although several combinations of haplotypes cannot be formally distinguished by this set (eg *HYPD* and *LYPB*), these haplotypes can be inferred from known linkage disequilibrium. Primer mixes used in this abridged set are marked in table 5.3. Examples are given in figure 5.7.

Figure 5.4: Haplotyping of multiple polymorphisms by PCR-SSP



Legend: Chromosomes are denoted by solid lines, polymorphisms by filled circles, alleles by italicised capitals, primers by arrows, and reaction mixes by numbered dashed lines.

Figure A illustrates the principles of conventional PCR-SSP. A pair of allele-specific primers (with specificity conferred by the terminal 3′ nucleotide of the primer) are designed so that each primer is specific for one allele at a single polymorphism (illustrated here using the MBL -550 promoter polymorphism, with primers A and B specific for alleles H and L respectively). Each of these primers is used in conjunction with a consensus (ie. non-allele specific) primer to generate two allele specific primer pairs (illustrated by dashed lines). Reaction 1 is specific for -550H, and reaction 2 for -550L

Figure B demonstrates haplotyping of two polymorphisms (here the MBL -550 and -221 promoter polymorphisms) by PCR-SSP. Each of forward primers A (-550H) and B (-550L) are combined with each of the reverse allele-specific primers C (-221X) and D (-221Y) to generate 4 reaction mixes specific for *cis* combinations of alleles (haplotypes). For example reaction 2, using forward primer A (-550H) and reverse primer D (-221Y), will only detected the *HY* haplotype. This technique has been used to haplotype all 6 MBL polymorphisms, and many of the polymorphisms in the following chapter.

Table 5.2: Primers used in MBL haplotyping

Both MBL allele-specific and control primers are listed. *Primers requiring 5′ extension by 3 nucleotides to ensure efficient amplification. ‡Deliberate mismatches introduced to abolish false positive amplification are italicised (nucleotide 'A' 5 bases from the 3′ end of primer 1150, and nucleotide 'g' 7 bases from the 3′ end of primer 1209. Where a primer spanned another polymorphic site, alternative bases (corresponding to the alleles of that polymorphism) were used, and are here enclosed in parentheses and separated by a forward slash.

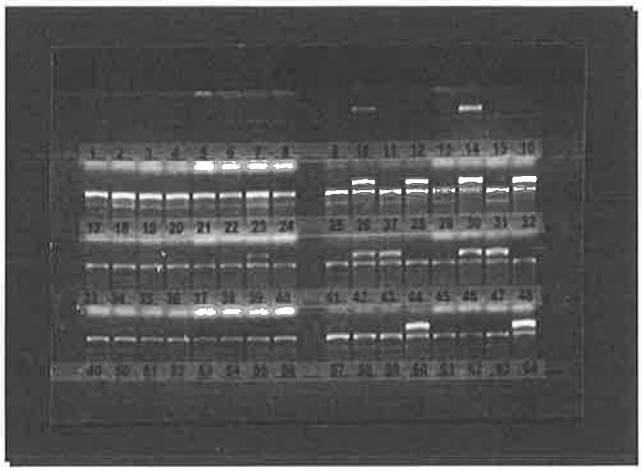
Primer number	Specificity	Sense/ antisense	Sequence (5' to 3')
63	DRB1 (control)	S	TgCCAAgTggAgCACCCAA
210	APC (control)	S	ATgATgTTgACCTTTCCAggg
611	-221 X	S	CATTTgTTCTCACTgCCACC
612	-221Y	S	CATTTgTTCTCACTgCCACg
614	+4 P	S	TAggACAgAgggCATgCTC
981*	-550 L	S	TgCTTACCCAggCAAgCCTgTC
982*	-550 H	S	TgCTTACCCAggCAAgCCTgTg
1150‡	+4 Q	S	TAggACAgAgggCA*A*gCTT
64	DRB1 (control)	AS	gCATCTTgCTCTgTgCAgAT
211	APC (control)	AS	TTCTgTAACTTTTCATCAgTTgC
587	codon 57 C	AS	CCTggTTCCCCCTTTTCTC
588	codon 57 wild type	AS	CCTggTTCCCCCTTTTCTT
589	codon 52 D	AS	CTCCCTTggTg(C/T)CATCACA
590	codon 52 wild type	AS	CTCCCTTggTg(C/T)CATCACg
592	codon 54 wild type	AS	CCCCTTTTCT(C/T)CCTTggTgC
1028*	-221 X	AS	CTggAAgACTATAAACATgCTTTCg
1029*	-221Y	AS	CTggAAgACTATAAACATgCTTTCC
1209‡	codon 54 B	AS	CCCCTTTTCT(C/T)CC*g*TggTgT
1129*	+4 P	AS	CTCAgTTAATgAACACATATTTACCg
1130*	+4 Q	AS	CTCAgTTAATgAACACATATTTACCA

Table 5.3: Primer mixes used for MBL haplotyping

PM no. = primer mix number. Primer and primer mix numbers are a unique identifying numbers used by the Oxford Transplant Centre Tissue Typing Laboratory. Volumes of primer per ml refer to prediluted primer stock at 2000 µg/ml. conc = concentration. *Haplotypes that have not been detected. ‡Reactions that are used in the abridged (12 reaction) MBL genotyping set.

		Sense primer			Antisense primer			Control primers	Specificity	Product size
Lane	PM no.	no.	volume per ml	conc (mM)	no.	volume per ml	conc (mM)			
1	648	614	10	3.4	589	10	3.4	63+64	P — 52Cys	256
2	649	614	5	1.7	590	5	1.7	63+64	P — 52wt	256
3*	677	1150	20	6.8	589	20	6.8	63+64	Q — 52Cys	256
4	678	1150	20	6.8	590	20	6.8	63+64	Q — 52wt	256
5	676	614	30	10.2	1209	30	10.2	63+64	P — 54Asp	265
6	653	614	5	1.7	592	5	1.7	63+64	P — 54wt	265
7*	679	1150	20	6.8	1209	30	10.2	63+64	Q — 54Asp	265
8	680	1150	20	6.8	592	20	6.8	63+64	Q — 54wt	265
9*	656	614	10	3.4	588	10	3.4	63+64	P — 57Glu	272
10	657	614	10	3.4	587	10	3.4	63+64	P — 57wt	272
11	681	1150	40	13.6	588	40	13.6	63+64	Q — 57Glu	272
12	682	1150	20	6.8	587	20	6.8	63+64	Q — 57wt	272
13	668	611	20	6.8	1129	20	6.8	63+64	X — P	264
14*	669	611	40	13.6	1130	20	6.8	63+64	X — Q	264
15	670	612	10	3.4	1129	10	3.4	63+64	Y — P	264
16	671	612	40	13.6	1130	40	13.6	63+64	Y — Q	264
17*	511	611	10	3.4	589	10	3.4	63+64	X — 52Cys	481
18*	673	611	20	6.8	1209	20	6.8	63+64	X — 54Asp	490
19*	513	611	10	3.4	588	10	3.4	63+64	X — 57Glu	497
20‡	514	611	10	3.4	590	10	3.4	63+64	X — 52wt	481
21‡	515	611	10	3.4	592	10	3.4	63+64	X — 54wt	490
22‡	516	611	10	3.4	587	10	3.4	63+64	X — 57wt	497
23‡	517	612	10	3.4	589	10	3.4	63+64	Y — 52Cys	481
24‡	672	612	20	6.8	1209	20	6.8	63+64	Y — 54Asp	490
25‡	519	612	10	3.4	588	10	3.4	63+64	Y — 57Glu	497
26‡	520	612	10	3.4	590	10	3.4	63+64	Y — 52wt	481
27‡	521	612	10	3.4	592	10	3.4	63+64	Y — 54wt	490
28‡	522	612	10	3.4	587	10	3.4	63+64	Y — 57wt	497
29*	640	982	20	6.8	1028	20	6.8	63+64	H — X	368
30‡	641	982	20	6.8	1029	20	6.8	63+64	H — Y	368
31‡	642	981	20	6.8	1028	20	6.8	63+64	L — X	368
32‡	643	981	20	6.8	1029	20	6.8	63+64	L — Y	368
33	664	982	20	6.8	1129	20	6.8	63+64	H — P	592
34*	665	982	20	6.8	1130	20	6.8	63+64	H — Q	592
35	666	981	20	6.8	1129	20	6.8	63+64	L — P	592
36	667	981	40	13.6	1130	40	13.6	63+64	L — Q	592
37	523	982	10	3.4	589	10	3.4	210+211	H — 52Cys	809
38*	675	982	20	6.8	1209	20	6.8	210+211	H — 54Asp	818
39*	525	982	10	3.4	588	10	3.4	210+211	H — 57Gly	825
40	526	982	10	3.4	590	5	1.7	210+211	H — 52wt	809
41	527	982	10	3.4	592	10	3.4	210+211	H — 54wt	818
42	528	982	30	10.2	587	30	10.2	210+211	H — 57wt	825
43*	529	981	10	3.4	589	10	3.4	210+211	L — 52Cys	809
44	674	981	20	6.8	1209	20	6.8	210+211	L — 54Asp	818
45	531	981	20	6.8	588	20	6.8	210+211	L — 57Glu	825
46	532	981	10	3.4	590	10	3.4	210+211	L — 52wt	809
47	533	981	10	3.4	592	10	3.4	210+211	L — 54wt	818
48	534	981	20	6.8	587	20	6.8	210+211	L — 57wt	825

Figure 5.5: Effect of MBL primer extension on amplification effiency



Direction of electrophoresis

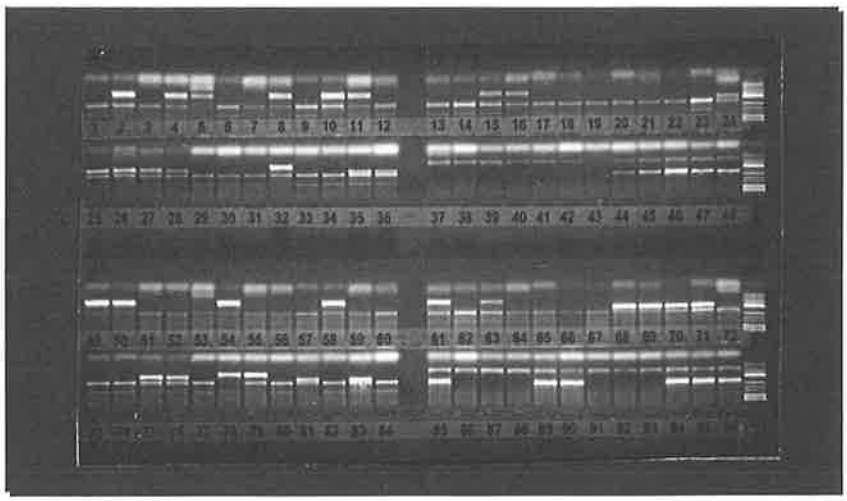
Lane	S primer	vol	AS primer	Vol	Specificity	Lane	S	Vol	AS	Vol	Specificity
1	982	20	781	20	H-X	9	982	5	1028	5	H-X
2	982	20	782	20	H-Y	10	982	5	1029	5	H-Y
3	981	20	781	20	L-X	11	981	5	1028	5	L-X
4	981	20	782	20	L-Y	12	981	5	1029	5	L-Y
5	982	40	781	40	H-X	13	982	20	1028	20	H-X
6	982	40	782	40	H-Y	14	982	20	1029	20	H-Y
7	981	40	781	40	L-X	15	981	20	1028	20	L-X
8	981	40	782	40	L-Y	16	981	20	1029	20	L-Y

Legend:

H/L-X/Y specific primer mixes are shown. Each row represents a different sample.
Lane numbering in the table refers to the first sample, but applies to the other 3 samples also (17-32, 33-48, 49-64).
The first 8 reactions of each row use the original H/L (sense) and X/Y (antisense) primers,
designed according to the primer annealing temperature equation (chapter 2).
Very poor amplification is seen with these mixes.
For the revised reactions, both sense and antisense primers were extended by 3 bases at the 5' end.
Vastly improved amplification is seen.

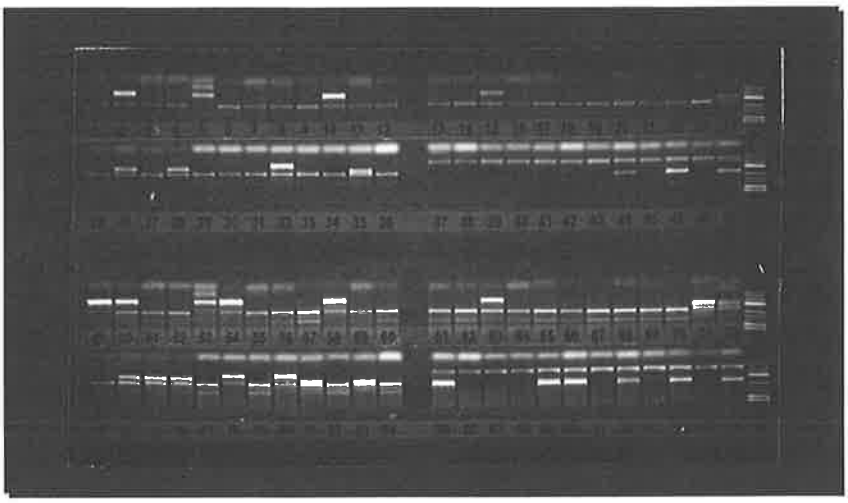
Figure 5.6: Example of comprehensive MBL haplotyping by PCR-SSP

Sample 1 (lanes 1-48) and sample 2 (lanes 49-96).



Direction of electrophoresis

Sample 3 (lanes 1-48) and sample 4 (lanes 49-96).



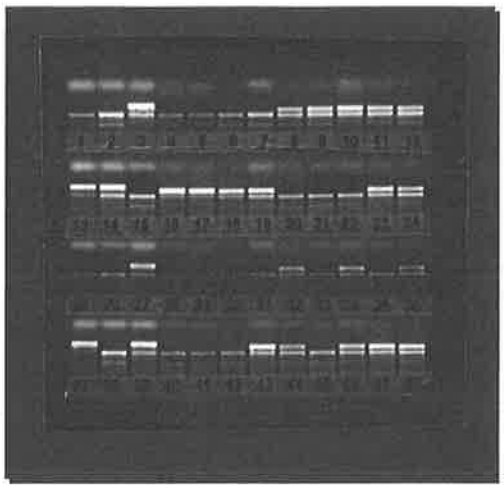
Legend:

4 samples have been genotyped, with 48 reactions per sample.
PCR products have been electrophoresed through 1% agarose impregnated with ethidium bromide prior to photography after UV illumination.

Sample	Positive lanes	MBL haplotypes
1	2, 4, 5, 8, 10, 11, 15, 16, 24-28, 32, 35-36, 44-48	**L-Y-P-wt-B-wt** **L-Y-Q-wt-wt-C**
2	49, 50, 54, 58, 61, 63, 68-71, 75-76, 78-79, 81, 83, 85, 89-90, 94-96	**H-Y-P-D-wt-wt** **L-X-P-wt-wt-wt**
3	2, 5, 10, 15, 24, 26, 28, 32, 35, 44, 46, 48	**L-Y-P-wt-B-wt** **(homozygous)**
4	49-50, 53-54, 58, 63, 71-72, 74-76, 78, 80-81, 83, 85, 89-90, 92, 94, 96	**H-Y-P-D-wt-wt** **L-Y-P-wt-B-wt**

Figure 5.7: Examples of abridged MBL genotyping using PCR-SSP

Samples 1-4.



Direction of
Electrophoresis

Legend:

The same 4 samples genotyped for figure 5.6 have been genotyped here.
The abridged 12 reaction set detects H/L-X/Y (first 3 lanes), and X/Y-exon1 haplotypes (latter 9 lanes).
Thus +4 (P/Q) genotype is not directly demonstrated, but can be inferred
from the known linkage disequilibrium between MBL alleles.

Sample	Lanes	Positive lanes	MBL haplotypes
1	1-12	3, 8-12	**L-Y, Y-wt-B-wt** **L-Y, Y-wt-wt-C**
2	13-24	13-14, 16-19, 23-24	**H-Y, Y-D-wt-wt** **L-X, X-wt-wt-wt**
3	25-36	27, 32, 34, 36	**L-Y, Y-wt-B-wt** **(homozygous)**
4	37-48	37, 39, 43, 44, 46, 48	**H-Y, Y-D-wt-wt** **L-Y, Y-wt-B-wt**

5.2.2. Control MBL allele and haplotype frequencies

100 Caucasoid cadaveric allograft donors were used as a control population for MBL genotyping (this population was described in chapter 3). Control allele, genotype and haplotype frequencies are listed in tables 5.5, 5.6 and 5.7, and are very similar to those reported previously (Madsen et al, 1995; Mead et al, 1997).

Only 7 haplotypes were detected (table 5.7), confirming and extending the findings of Madsen et al (Madsen et al, 1995). Promoter alleles were in linkage disequilibrium with each other and with exon 1 alleles. For example, 52Cys was always in *cis* with the *HYP* promoter haplotype; 54Asp with *LYP*; and 57Glu with *LYQ*. Frequencies of full haplotypes (including the +4 alleles P and Q) have not been previously reported. Thus full haplotype frequencies generated by this study have been compared with frequencies from more restricted genotyping studies on the basis of the known linkage disequilibrium between the +4 alleles and other MBL alleles (table 5.7).

5.2.3. MBL allele and haplotype frequencies in CVID

Allele and genotype frequencies for individual alleles, promoter haplotype and full haplotypes were also examined in 163 Caucasoid CVID patients (table 5.8)[6]. No significant differences between overall CVID and control frequencies were detected.

MBL allele and haplotype frequencies were also examined in subgroups of patients stratified on the basis of clinical and immunophenotypic criteria (table 5.4). 102 of the 163 patients genotyped had sufficient clinical data to permit classification as autoimmune (33 patients, 32.4%) or non-autoimmune (69 patients, 67.6%). 98 of 163 had data available concerning date of onset of symptoms, and date of diagnosis. The accurate determination of age of onset of symptoms of recurrent infection is retrospective, and therefore subject to reporter error. However it is unlikely that this potential problem has biased the data detailed below, as this applies to all individuals, irrespective of MBL genotype.

[6] Not all of the 179 patients described in chapter 3 were examined, due to lack of DNA.

MBL genotyping was performed after diagnosis of CVID, and thus did not influence the timing or rate of referral to immunodeficiency clinics.

Stratification according to infection history is inherently unreliable, due to the confounding effects of factors such as antibiotic use, adequacy of immunoglobulin replacement, and the presence of permanent end-organ damage (such as bronchiectasis) on the frequency and severity of infection. Consequently this stratification was not performed.

Three important associations between MBL genotype and clinical features were detected. Early age of onset of symptoms and age at diagnosis were both associated with the MBL coding mutations and 'low-producing' promoter haplotypes. Autoimmune disease was associated with the MBL +4Q allele, and the *LYQA* haplotype. These associations are discussed in detail below.

Table 5.4: Clinical and laboratory criteria used for patient stratification

MBL frequencies were assessed in patient subgroups defined by several clinical and immunophenotypic criteria. Significant associations are discussed in detail below.

Criterion	Significant association?
Clinical	
Sex	no
Age at onset of symptoms	yes - with coding mutations and promoter haplotypes
Age at diagnosis	yes - with coding mutations and promoter haplotypes
Autoimmunity	yes - with +4Q (*LYQA*)
Splenomegaly	no
Granulomata	no
Immunophenotypic	
CD3+	no
CD3+CD4+	no
CD3+CD8+	no
B cells (CD19+ or CD20+	no
CD4+CD45RA+	no
CD4+CD45RO+	no
CD8+CD45RA+	no
CD8+CD45RO+	no
CD8+CD57+	no

5.2.4. MBL coding mutations are associated with early age of onset of CVID

The presence of MBL coding mutations was significantly associated with earlier age of onset of symptoms in CVID. Mean age of onset of symptoms in those patients heterozygous or homozygous[7] for any of the coding mutations was 14.51 years, compared with 25.04 years in those patients with no mutations (unpaired t test t=3.17, p=0.002, table 5.9). This observation was further analysed by examination of age of onset of symptoms in groups of patients positive for each of the 3 mutations. Onset of symptoms was earlier in each of the 52Cys, 54Asp and 57Glu positive patient groups than in patients with no mutations, although these differences did not reach significance for the 52Cys and 54Asp groups individually. Whilst only one patient carried the 57Glu mutation, the age of onset in this patient was substantially lower than in CVID patients with no mutations (5 years compared with a mean of 25.04 years). Furthermore, earlier age of onset was observed both in patients heterozygous (p=0.013) and homozygous (p=0.012) for coding mutations. Mean age of onset was lower in homozygotes than heterozygotes (6.17 years compared with 16.03 years), but due to the low number of MBL coding mutation homozygotes (6 patients) this difference was not statistically significant (t=1.835, p=0.075). The low age of onset of symptoms in coding mutation homozygotes was striking: 5 of 6 patients experienced severe recurrent upper respiratory infections before the age of 10, and the sixth by age 15 (ages of onset 3, 3, 3, 5, 8 and 15 years). Consequently, compared with patients with no coding mutations, homozygosity was strongly associated with onset of symptoms prior to age 10 (Fisher's exact test p=0.005; odds ratio 17.5, 95% CI 1.86-164.5). B lymphocyte counts were available for 5 of the MBL coding mutation homozygotes, and were normal in all 5.

The presence of MBL coding mutations was also associated with earlier age of diagnosis (table 5.10), reflecting the strong correlation between age of onset and age at diagnosis (correlation coefficient 0.719, CI 0.604-0.806, Z=8.55, p<0.0001, see figure 5.10). Mean age at diagnosis in patients with any coding mutations

[7] MBL coding mutation "homozygosity" here refers to coding mutation on both chromosomes, and includes homozygosity for one MBL coding mutation (eg 54 Asp homozygotes); and compound heterozygotes (eg 52Cys and 54Asp)

(whether hetero- or homozygous) was 29.8 c.f. 38.0 years (t=2.31, p=0.02). Age at diagnosis was even lower in coding mutation homozygotes (18.7 years), but because of the relatively small number of homozygotes, this difference did not reach significance (t=1.99, p=0.051). Box plots depicting the relationship between age of onset and diagnosis with the presence or absence of mutations are given in figures 5.8 and 5.9.

5.2.5. Promoter haplotypes also influence age of onset, in both coding mutation wild type and heterozygous individuals

Age of onset data was further stratified according to promoter haplotype. Coding mutation heterozygotes also carrying the (low-producing) *LXP* haplotype (in *cis* with a normal coding region, ie. *LXPA*) had a lower mean age of onset of symptoms (6.83 years) than patients with a coding mutation and the (high-producing) *HYPA* haplotype (18.30 years, p=0.003). Analogously, in patients without coding mutations, the low producing *LXPA* haplotype was associated with a lower age of onset than in patients without this haplotype, although this difference did not attain significance (21.22 compared with 29.16 years, p=0.106). No significant differences in age of onset were detected in patient groups defined by presence or absence of the (intermediate-producing) *LYA* haplotype, in patients with or without coding mutations.

5.2.6. MBL +4Q is associated with autoimmunity in CVID

The Q allele of the +4 polymorphism of the MBL 5' untranslated region was strongly associated with autoimmunity in CVID. 14 of 33 (42.4%) autoimmune CVID patients carried this allele, compared with 10 of 69 (14.4%) non-autoimmune patients (χ^2=9.68, p=0.0019, OR 4.35, 95% CI 1.66-11.38), and 31 of 100 (31.0%) controls (p=0.161, OR 1.64, CI 0.72-3.69). This was attributable to an elevated frequency of the *LYQA* haplotype in patients with autoimmunity (13 of 33 compared with 9 of 69; p=0.0025; OR 4.33, CI 1.61-11.66). Restriction of the autoimmune group to only those patients with presumed autoantibody-mediated conditions (ie. autoimmune haemolysis, vitiligo, pernicious anaemia, ITP, anti-IgA antibodies, aspermia) did not increase or decrease the significance of these associations. No associations were observed with other MBL alleles or

haplotypes previously demonstrated to be associated with autoimmunity (i.e. 54Asp, 57Glu and *HY* promoter haplotype and SLE) (Davies et al, 1995; Sullivan et al, 1996).

Table 5.5: MBL phenotype and allele frequencies in 100 Caucasoid controls

Phenotype frequencies (the proportion of individuals carrying an allele) and allele frequencies (the proportion of chromosomes carrying an allele) are listed for each of the 6 MBL promoter, 5' UTR and coding polymorphisms. Previously reported frequencies are taken from [a](Mead et al, 1997) and [b](Madsen et al, 1995), and are similar to those detected here.

Position	Allele	Phenotype		Allele		Previously reported Caucasoid frequencies
		count n=100	frequency	count n=200	frequency	
-550	H	65	0.65	79	0.40	0.383[a]
	L	86	0.86	121	0.61	0.617[a]
-221	X	39	0.39	45	0.23	0.238[a]
	Y	94	0.94	155	0.78	0.763[a]
+4	P	95	0.95	164	0.82	
	Q	31	0.31	36	0.18	
codon 52	Cys	11	0.11	11	0.06	0.06[b]
	wt	100	1	189	0.95	
codon 54	Asp	25	0.25	27	0.14	0.16[b]
	wt	98	0.98	173	0.87	
codon 57	Glu	3	0.03	3	0.02	0.00[b]
	wt	100	1	197	0.99	

Table 5.6: MBL genotype frequencies in 100 Caucasoid controls

"Genotype" is here used to refer to heterozygous/homozygous combinations of alleles at a single polymorphism. [a]Previously reported frequencies are taken from (Mead et al, 1997).

Position		Observed		Expected			Previously reported Caucasoid frequencies[a]
		Number n=100	Frequency	Number n=100	Frequency		
-550	HH	14	0.14	16	0.16	0.80	
	HL	51	0.51	48	0.48		
	LL	35	0.35	37	0.37		
-221	XX	6	0.06	5	0.05	0.87	
	XY	33	0.33	35	0.35		
	YY	61	0.61	60	0.60		
+4	PP	69	0.69	67	0.67	0.49	
	PQ	26	0.26	30	0.30		
	QQ	5	0.05	3	0.03		
codon 52	Cys/Cys	0	0	0	0.003	0.84	0.03
	Cys/wt	11	0.11	10	0.10		0.103
	wt/wt	89	0.89	89	0.89		
codon 54	Asp/Asp	2	0.02	2	0.02	0.99	0.023
	Asp/wt	23	0.23	23	0.23		0.228
	wt/wt	75	0.75	75	0.75		
codon 57	Glu/Glu	0	0	0	0.000	0.99	0
	Glu/wt	3	0.03	3	0.03		0.026
	wt/wt	97	0.97	97	0.97		

Table 5.7: MBL haplotype frequencies in 100 Caucasoid controls

Frequencies of promoter and full MBL haplotypes are given. The haplotypes actually investigated by Madsen et al (Madsen et al, 1995) are listed in parentheses.

	Patients		Homozygotes		Chromosomes		Previously reported Caucasoid frequencies
	count n=100	frequency	count	frequency	count n=200	frequency	
Promoter haplotypes							
HY	65	0.65	15	0.15	80	0.40	0.383
LY	61	0.61	15	0.15	76	0.38	0.379
LX	38	0.38	6	0.06	44	0.22	0.238
HX	0	0	0	0	0	0	
Full haplotypes							
HYPA	58	0.58	10	0.1	69	0.345	0.333 (HYA)
LXPA	38	0.38	6	0.06	44	0.22	0.238 (LXA)
LYQA	28	0.28	4	0.04	32	0.16	0.225 (LYA)
LYPA	14	0.14	0	0	14	0.07	
LYP-52wt-54Asp-57wt	25	0.25	2	0.02	27	0.135	0.125 (LYB)
HYP-52Cys-54wt-57wt	11	0.11	0	0	11	0.055	0.05 (HYD)
LYQ-52wt-54wt-57Glu	3	0.03	0	0	3	0.015	0.029 (LYC)

Table 5.8: Comparison of MBL allele and haplotype frequencies between CVID patients and controls

No significant differences were detected in allele or haplotype frequencies or genotype frequencies. ns = not significant. RR = relative risk.

	Patients n=163				Controls n=100				χ^2	p	RR
	+	%	-	%	+	%	-	%			
codon 52Cys	20	0.12	143	0.88	11	0.11	89	0.89	0.01	ns	1.11
codon 54Asp	44	0.27	119	0.73	25	0.25	75	0.75	0.05	ns	1.10
codon 57Glu	4	0.02	159	0.98	3	0.03	97	0.97	0.44	ns	0.79
Any coding mutation	65	0.40	98	0.60	37	0.37	63	0.63	0.11	ns	1.13
coding mutation homozygosity	6	0.04	157	0.96	4	0.04	96	0.96	0.21	ns	0.89
***HYA* / coding mutation heterozygous**	30	0.18	133	0.82	16	0.16	84	0.84	0.11	ns	1.17
***LYA* / coding mutation heterozygous**	19	0.12	144	0.88	10	0.1	90	0.90	0.05	ns	1.16
***LXA* / coding mutation heterozygous**	10	0.06	153	0.94	7	0.07	93	0.93	0.29	ns	0.85
HYA / HYA	17	0.10	146	0.90	10	0.10	90	0.90	0.01	ns	1.03
HYA / LYA	16	0.10	147	0.90	14	0.14	86	0.86	1.53	ns	0.67
HYA / LXA	24	0.15	139	0.85	17	0.17	83	0.83	0.45	ns	0.84
LYA / LYA	10	0.06	153	0.94	4	0.04	96	0.96	0.22	ns	1.47
LYA / LXA	20	0.12	143	0.88	8	0.08	92	0.92	0.78	ns	1.55
LXA / LXA	10	0.06	153	0.94	6	0.06	94	0.94	0.05	ns	0.99
Breakdown of coding mutation homozygotes	Patients		Controls								
54Asp homozygous	3		2								
52Cys and 54Asp	2		2								
54Asp and 57Glu	1		0								

Table 5.9: Associations between mean age of onset of symptoms in CVID and MBL coding mutations

MBL coding mutation	count (n=98)	Mean age at onset of symptoms	SD	Unpaired t test			
				comparison	mean diff.	t	p
None	56	25.0	17.7				
Any mutation	42	14.5	12.5	no mutations	10.53	3.17	0.002
52Cys heterozygous	12	16.3	14.5	no mutations	8.75	1.70	0.093
54Asp heterozygous	23	16.4	12.0	no mutations	8.59	1.912	0.060
57Glu heterozygous	1	5 (1 patient only)	na	no mutations	na	na	na
All heterozygotes	36	16.0	12.9	no mutations	9.01	2.53	0.013
All homozygotes	6	6.2	4.8	no mutation	18.87	2.59	0.012
				heterozygotes	9.86	1.84	0.075

Table 5.10: Associations between age at diagnosis and MBL coding mutations

MBL coding mutation	count (n=98)	Mean age at diagnosis	SD	Unpaired t test			
				comparison	mean diff.	t	p
None	56	38.0	18.1				
Any mutation	42	29.8	14.5	no mutations	8.12	2.31	0.023
52Cys heterozygous	12	29.6	11.0	no mutations	8.38	1.53	0.130
54Asp heterozygous	23	32.0	16.5	no mutations	5.97	1.32	0.191
57Glu heterozygous	1	5 (1 patient only)	na	no mutations	na	na	na
All heterozygotes	36	30.6	14.8	no mutations	6.84	1.85	0.068
All homozygotes	6	18.7	12.3	no mutation	16.57	1.99	0.051
				heterozygotes	11.94	1.86	0.070

Figure 5.8: Box plot depicting variation in age at diagnosis and onset with the presence of MBL coding mutations

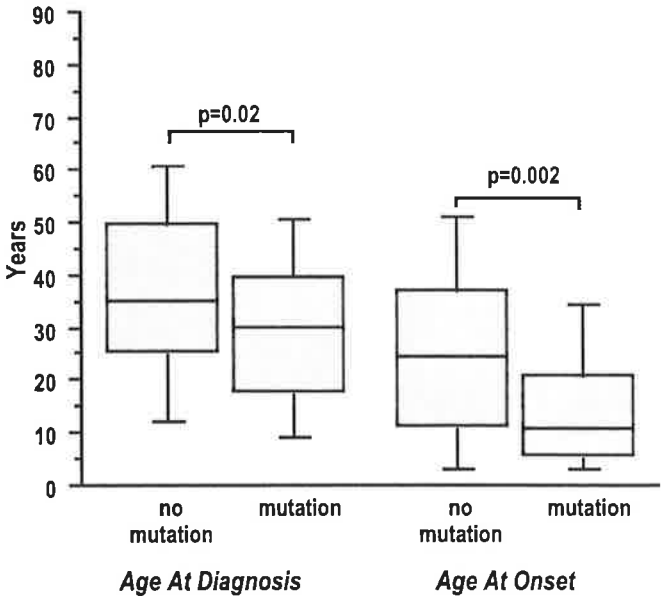


Figure 5.9: Box plot of age of diagnosis and onset in relation to MBL coding mutation homozygosity

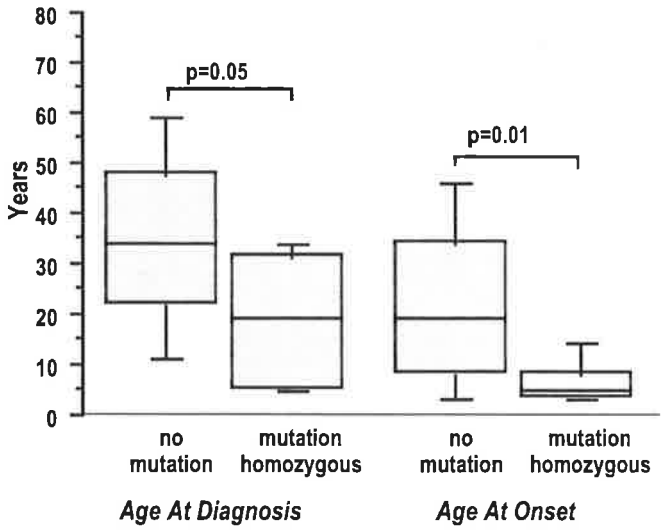
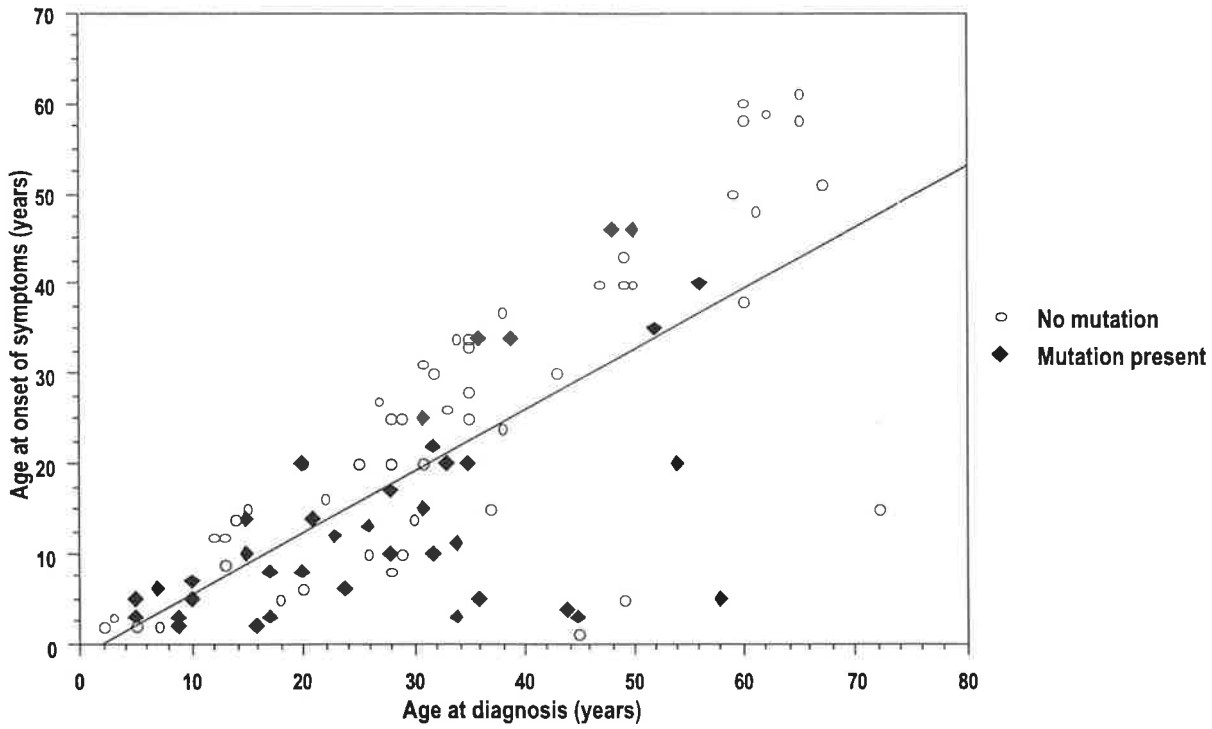


Figure 5.10: Relationship between age at onset of symptoms, age at diagnosis, and presence of MBL coding mutations in CVID

The scatter plot below visually demonstrates the strong correlation between age of onset of symptoms and age at diagnosis in CVID (r_p=0.719, 95% CI 0.604-0.806, $100r_p^2$=51.7; Z=8.55, p<0.0001), and the clustering of MBL coding mutations in patients with early age of onset and presentation



5.3. DISCUSSION

This chapter has described the development of a simple PCR-SSP method for comprehensive genotyping of all six known polymorphisms in exon 1 and the 5' region of the MBL gene. This method has several important advantages over previously reported methods. First, all polymorphisms are identified using one PCR-based method and set of amplification conditions. This is in stark contrast to other reports utilising different methods for different polymorphisms (Madsen et al, 1995), or technically difficult methods, such as PCR-heteroduplex analysis (Jack et al, 1997). Furthermore this method can assign alleles as being in *cis* or *trans*. The utility of this approach is demonstrated by two specific examples. Alternative techniques are able to detect the presence of two coding mutations, but are unable to formally demonstrate that the mutations lie on different chromosomes, which would be expected to have a more profound effect on circulating MBL levels than both mutations on one chromosome, and the other structurally normal. Secondly, the low producing MBL promoter genotypes only influence serum MBL level when in *cis* with a normal coding region (Madsen et al, 1995). Thus although the both promoter haplotypes and coding mutations have been associated with SLE in one study (Sullivan et al, 1996), the *cis/trans* orientation of promoter and coding alleles was not determined, rendering the significance of the promoter associations difficult to interpret. Haplotyping of the -550 and -221 alleles, and the -221 and coding alleles, has been previously described, but not using identical reaction conditions (Madsen et al, 1994; Sullivan et al, 1996). Indeed, Madsen et al were unable to use PCR-SSP to haplotype the -550 polymorphism (Madsen et al, 1994), probably because of the need for primer elongation, as described above. By enabling the efficient and unequivocal demonstration of MBL haplotypes, this technique will prove useful in the accurate evaluation of the relationship between haplotypes and circulating MBL levels.

The full haplotyping technique using 48 reactions detected only 7 of 64 possible MBL haplotypes in 263 Caucasoids. Consequently, for future studies of Caucasoid populations, it will be possible to use a much smaller set of 12 reactions.

However until MBL haplotypes have been thoroughly examined in non-Caucasoid populations, the full primer mix set will prove useful.

Having developed this assay, allele and haplotype frequencies were examined in CVID. No associations with MBL alleles or haplotypes were detected with the entire patient group. This suggests that MBL polymorphisms do not directly contribute to CVID pathogenesis. In contrast, highly significant associations were detected between MBL alleles and haplotypes and age of onset, age at diagnosis, and autoimmunity in CVID. Whilst it was not possible to directly examine the relationships between MBL alleles and infection severity (due to difficulty in stratification), these findings support the hypothesis proposed above that MBL is an important compensatory mechanism in the presence of hypogammaglobulinaemia. This is reinforced by the strikingly early presentation of CVID patients homozygous for coding mutations, and the earlier age of onset of patients carrying the low producing *LXPA* haplotype.

Although the concept of a genetic predisposition to CVID is widely accepted, no satisfactory explanation exists for the considerable variability in age of onset in CVID. The associations with MBL genotype described above represent the first evidence that this variability in age of onset is at least partly genetically determined.

The association of autoimmune CVID with a MBL haplotype associated with low MBL production, *LYQA*, supports the involvement of complement deficiencies in the pathogenesis of autoimmune disease. Similar associations in non-immunodeficient individuals (an increased frequency of coding mutations in SLE) have also been described (Davies et al, 1995; Lau et al, 1996; Sullivan et al, 1996; Davies et al, 1997). However, the exact mechanism underlying this increased susceptibility to autoimmune disease, in CVID or otherwise, remains elusive.

It has been suggested that MBL deficiency may impair effective immune complex clearance, resulting in immune complex mediated autoimmune diseases such as

SLE (Davies et al, 1995; Sullivan et al, 1996; Davies et al, 1997). Indeed, other early complement component deficiencies (C1q, C2, C4) are associated with lupus-like disease (Lachmann, 1991; Arnett et al, 1992; Moulds et al, 1992; Walport, 1993), and polymorphisms in several other genes involved in immune complex clearance, such as C4A, complement receptors 1 and 3, and Fc gamma receptor III, are also associated with SLE (Clark et al, 1990; Kumar et al, 1991; Cornillet et al, 1992; Witte et al, 1992). The role of MBL in immune complex clearance has not been clearly defined, but the similarity of MBL to C1q, and the known interaction of MBL with hypoglycosylated IgG (Malhotra et al, 1995) suggests that MBL may participate in clearance of immune complexes.

However, if impaired immune complex clearance is responsible for autoimmunity in CVID, it would be expected that immune complex mediated diseases such as SLE or synovitis would worsen upon the commencement of immunoglobulin replacement therapy. Immunoglobulin therapy may generate large quantities of circulating immune complexes, especially at the initiation of treatment, when endogenous antibody levels are lowest, and microbial load highest. However such a flare of autoimmune disease does not occur; indeed, conditions such as synovitis and SLE often improve with immunoglobulin administration (Cooper et al, 1975; Lee et al, 1993). Furthermore, this explanation would not explain the occurrence of non-immune complex mediated autoimmune diseases seen in CVID, such as autoimmune cytopenias.

Alternatively, complement deficiencies such as low MBL or C4 levels, in conjunction with hypogammaglobulinaemia, may impair microbial antigen clearance, resulting in chronic abnormal immune activation, and the generation of autoreactive T cells and/or autoantibodies, and thus autoimmune disease (Sleasman, 1996). Impaired clearance of an autoimmunity-triggering organism due to MBL deficiency has also been suggested (Davies et al, 1995). Furthermore, several of the conditions considered to be autoimmune in CVID may in fact represent infection rather than autoimmunity. Arthritis resembling aseptic polyarthritis due to *Ureaplasma* and *Mycoplasma* infection is well recognised in CVID (Lee et al, 1993), and it is possible that MBL deficiency, in conjunction with

hypogammaglobulinaemia, renders CVID patients particularly susceptible to infection with these organisms. In summary, it is likely that MBL deficiency predisposes to autoimmunity in CVID by more than one mechanism.

The lack of association between autoimmunity and other MBL genotypes associated with low MBL levels (such as coding mutations or the *LXPA* haplotype) is difficult to explain, but may reflect the small number of patients with autoimmunity included in this study (33 patients). Clarification of the importance of the observed *LYQA* association awaits further investigation of the relationship between MBL promoter haplotypes and serum MBL levels. In the only previous study investigating MBL promoter genotype and MBL levels, the *LYA* haplotype (ie. including both *LYQA* and *LYPA* haplotypes) was associated with low serum MBL levels (Madsen et al, 1995). Unfortunately, due to low sample numbers, a proper comparison of the effects of the P and Q alleles (ie. *LYQA* v. *LYPA*) on MBL production was not possible. Measurement of serum MBL levels in this group of CVID patients will also be of interest, and this work is in progress.

Finally, the observed influence of MBL alleles on the manifestations of CVID suggests that further investigation of the role of other immunogenetic polymorphisms in the pathogenesis and expression of CVID will be of interest. This is the subject of the following chapter.

6. Simultaneous genotyping of multiple immunogenetic polymorphisms

6.1. INTRODUCTION

The identification of large numbers of polymorphisms in genes encoding molecules critical for normal immune responses has provided the opportunity to rigorously analyse the molecular basis of genetic susceptibility to a wide range of infections and inflammatory conditions. A number of gene polymorphisms have been individually implicated in disease pathogenesis, but interpretation of the biological significance of these individual associations is often limited by the complex interaction of gene products in physiological responses. Consequently, simultaneous investigation of multiple polymorphisms is important to understand both normal physiological responses and disease pathogenesis. Examination of multiple gene polymorphisms is particularly desirable in order to examine complex disease associations, such as where gene products are known to interact functionally (e.g. cytokines), or where disease results from the synergistic action of several genes (e.g. oncogenesis). The involvement of multiple genes in disease pathogenesis is often suggested by genome-wide linkage studies (eg. inflammatory bowel disease and IDDM). Genotyping of multiple immunoregulatory candidates is also of interest in disease where previously identified genetic associations, such as with HLA alleles, cannot account for the entire susceptibility to disease.

This rationale is particularly pertinent to common variable immunodeficiency. As discussed in chapter 3, the known HLA associations do not account for the entire genetic predisposition to CVID. Thus it is highly likely that polymorphisms in at least one non-MHC gene contributes to CVID susceptibility. Furthermore, the extensive heterogeneity of the CVID phenotype suggests that multiple genetic factors may be important both in conferring susceptibility, and also in determining severity or progression of disease. The highly variable clinical and laboratory manifestations of CVID have resulted in speculation that CVID consists of multiple diseases, all of which share defective B lymphocyte differentiation and immunoglobulin secretion (Spickett et al, 1997). If this is true,

it is possible that each discrete disease subtype has a unique genetic diathesis. Alternatively, it is possible that CVID represents one, or a limited number of different diseases, and that phenotypic variability results from the action of additional genes that are not directly involved in the pathogenesis of hypogammaglobulinaemia. This hypothesis is supported by the associations described in chapters 4 and 5 of the TNF +488A allele with granulomatous CVID; and the associations between mannose binding lectin genotypes and age of onset and autoimmunity in CVID. Of course, both hypotheses may be correct: that CVID represents multiple diseases with distinct genetic diatheses, and additional genetic elements influence phenotype and predispose to complications such as autoimmunity. Whichever argument is true, this complexity carries the implication that simultaneous examination of multiple candidate genes in large numbers of well-characterised patients is essential if the genetic predisposition to CVID is to be accurately elucidated.

The cytokine network is an excellent example of the utility of genotyping multiple polymorphisms in order to dissect complex gene interactions. Individual cytokines have been shown to have profound effects on lymphocyte function, and thus the strength, nature and duration of immune responses. However the cytokine network is characterised by pleiotropy, redundancy, synergism and antagonism (Balkwill et al, 1989; Paul, 1989). A simplified classification groups cytokine into two mutually antagonistic groups according to the nature of the immune responses they promote. 'Type 1' cytokines such as IL-2 and IFNγ promote cell-mediated immunity, and 'type 2' cytokines such as IL-4, -5, -6, -10, and -13 promote humoral immune responses (Romagnani, 1995; Mosmann et al, 1996; Romagnani, 1997). Thus it is possible that the presence of a single allele in one cytokine gene is not alone sufficient to confer susceptibility to disease, but rather the simultaneous presence of multiple allelic variants of synergistic or antagonistic cytokines is required. The importance of cytokines in the generation of immune responses, the profound abnormalities of cytokine production in CVID (Fiedler et al, 1987; Pastorelli et al, 1989; North et al, 1990; Sneller et al, 1990b; Fischer et al, 1993b; Hauber et al, 1995), and previous

associations between cytokine polymorphisms and CVID (chapter 4), suggest that investigation of cytokine polymorphism is highly relevant to CVID.

This is illustrated using the association between the TNF +488A and granulomatous CVID described in chapter 4. It was demonstrated that 40% of granulomatous patients were TNF +488A positive, compared with 5.7% of non-granulomatous patients. It was hypothesised that +488A promotes high TNF production, and thus the formation of granulomata. But the observation that only 40% of granulomatous patients carried this allele suggests that other alleles, in TNF or other genes, are responsible for the generation of granulomata in the remaining patients, if indeed all such granulomata are genetically determined. The production and secretion of TNF is tightly regulated, with cytokines such as IL-4 and IL-10 suppressing, and IFNγ promoting monocyte TNF release (de Waal Malefyt et al, 1991; Ralph et al, 1992; Donnelly et al, 1995; Armstrong et al, 1996; Joyce et al, 1996). Thus it is possible that polymorphisms in these, or other cytokines may synergise with or antagonise the effect of TNF +488A. Alternatively, polymorphisms in these cytokines may be independently responsible for promoting granulomata in patients not carrying TNF +488A. A logical candidate gene is that encoding IL-10. The promoter of this gene contains at least 3 polymorphisms, at least one of which is functionally relevant (Turner et al, 1997), and IL-10 profoundly suppresses monocyte TNF production (Ralph et al, 1992; Armstrong et al, 1996). If high TNF levels are a prerequisite for granuloma formation in CVID, it is feasible that IL-10 polymorphisms associated with low IL-10 production would further promote the generation of granulomata.

Examination of multiple gene polymorphisms is also attractive in diseases where the abnormality responsible for disease could reside at any one or more stages of a complex physiological process. As discussed in chapter 1, the fundamental abnormality of CVID is arrested B lymphocyte differentiation and impaired immunoglobulin secretion. Whilst there is considerable evidence for, and stigmata of, a T cell defect in many patients (low circulating T cell numbers, impaired proliferation and cytokine secretion etc.), abnormalities in any stage of T cell activation, from T cell signal transduction to expression of adhesion

molecules and cytokines, may be responsible for hypogammaglobulinaemia. Consequently it is desirable to examine polymorphisms in as many genes involved in the genesis of normal immune responses as possible. The previously reported associations between polymorphisms in genes involved in host defence, and susceptibility to parasitic, bacterial and mycobacterial infections further support this argument (Hill, 1996). Candidates potentially relevant to CVID are illustrated in figure 6.1.

Two final arguments support the study of multiple candidate genes in CVID. First, previous studies of CVID pathophysiology have highlighted several interesting candidate genes. For example, in addition to the cytokines discussed above, defects in lymphocyte trafficking and adhesion molecule expression have been identified in CVID (Baumert et al, 1992; Zhang et al, 1996), and it is possible that these defects are genetically determined. The second argument concerns the desirability of this approach when information from family-based linkage studies is limited. This is certainly true in CVID. Although the familial occurrence of CVID and IgAD has been highlighted, multiplex kindreds are rare, and thus the attainment of sufficient statistical power for linkage studies is difficult. Only one formal MHC-linkage study in CVID/IgAD has been described (Vorechovsky et al, 1995b), and no non-MHC linkage studies have been reported. Candidate gene studies are a logical alternative, until the ongoing genome-wide linkage study in CVID and IgAD has been completed. Moreover, once loci have been identified by linkage studies, multiple candidate gene analysis is very useful to examine associations in more detail.

6.1.1. Aims and scope of this chapter

The aim of this chapter was to examine as many immunogenetic polymorphisms of theoretical relevance to CVID as possible in a large group of well-characterised CVID patients.

To expedite this analysis as efficiently as possible, major methodological developmental work was necessary. The wide variety of disparate and incompatible methods available prior to this study would have entailed

considerable repetition, expense and potential error when multiple polymorphisms were investigated. It was important to streamline genotyping methodology in order to maximise efficiency, reproducibility and simplicity. Consequently a unified PCR-SSP method capable of simultaneously genotyping large numbers of nucleotide substitutions and deletions using identical amplification conditions was developed. This technique was applied to a number of immunoregulatory genes including cytokines (IL-1α, -1β, -4, -6, -10, TGFβ); the chemokine receptor CCR5; the vitamin D receptor; Fcγ receptor IIA; adhesion molecules (L- and E-selectin, ICAM-1); and CTLA-4.

Simultaneously, assays for a number of other polymorphisms not directly relevant to CVID were developed as part of other disease studies performed by the immunogenetics group in the Nuffield Department of Surgery. These polymorphisms are of considerable interest to a wide range of inflammatory and acquired immunodeficiency conditions, including inflammatory bowel disease, solid organ transplantation, IDDM, coeliac disease and systemic sclerosis. Methodological details are listed in the appendix.

6.1.2. Selection of candidate gene polymorphisms

Genes were selected for genotyping according to four criteria: known or theoretical involvement of a gene product in the pathogenesis of CVID; the presence of previously described polymorphisms; previous associations with disease; and suitability for genotyping using PCR-SSP (table 6.1, figure 6.1). Few candidates fulfilled all criteria. All polymorphisms, with the exception of the IL-6 intron 4 substitution, had been previously described. Studies directly examining the functional consequences of many of the polymorphisms genotyped are limited or non-existent, and it is possible that many polymorphisms are of hitherto unrecognised physiological importance. Also, many of the genes studied have not been thoroughly examined for sequence variation in multiple individuals. Thus, even in those cases where a candidate polymorphism would not be expected to exert any functional effect itself (such as a synonymous substitution in the coding sequence of a gene), polymorphisms may serve as a useful indicator for the involvement of other (unidentified) linked allelic

variants of the same gene in disease pathogenesis. Thus, some polymorphisms were genotyped as markers of involvement of a gene, rather than a specific allele, in disease causation.

Several previously described polymorphisms, potentially relevant to CVID, were not genotyped due to the methodological unsuitability. These polymorphisms were mostly variable number of tandem repeat (VNTR, or microsatellite) polymorphisms, which may be readily amplified using the PCR conditions described below, but not in an allele-specific fashion. Detection thus depends on resolution of small differences in product size, which is difficult using 1% agarose gel electrophoresis. For example, many cytokines have microsatellite repeat polymorphisms in the 5′ or 3′ regions of the gene (Jacob et al, 1993), including IL-2 (Epplen et al, 1994), IL-4 (Mout et al, 1991), IL-6 (Bowcock et al, 1989) and IL-10 (Eskdale et al, 1995). Genotyping of these polymorphisms was deferred in order to establish a unified method of genotyping to maximise efficiency. Consequently all polymorphisms described below are either nucleotide substitutions or deletions, readily typed using PCR-SSP.

Essential features of each of the genes and gene products studied, the polymorphisms genotyped, and previously described disease associations are summarised below.

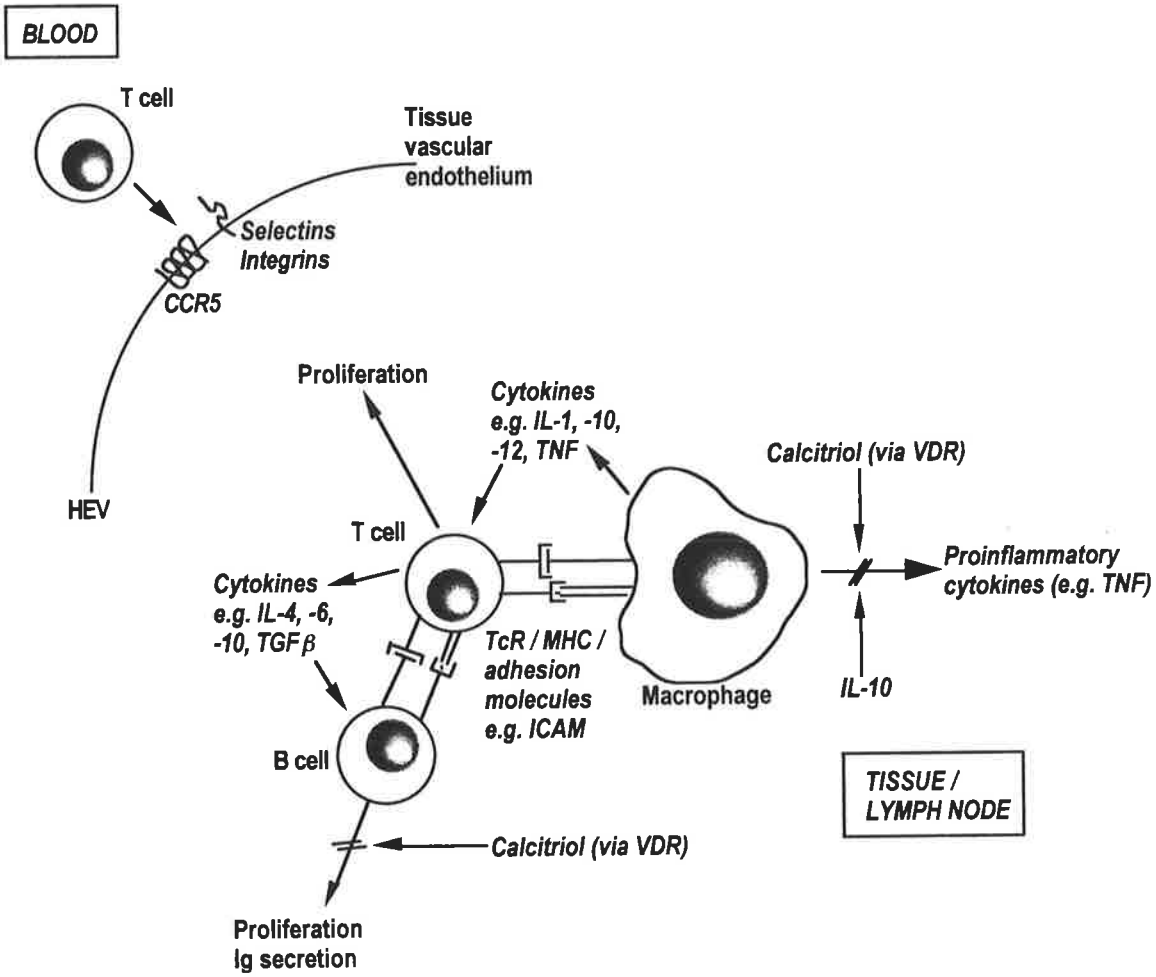
Table 6.1: Summary of features of immunogenetic polymorphisms selected for genotyping in CVID

Key features of the non-HLA immunogenetic polymorphisms selected for genotyping in CVID, and their theoretical relevance to CVID, are summarised. For full discussion, see text and table 6.2. Nucleotide variants at polymorphisms are represented as lower case letters separated by a forward slash.
JRA = juvenile rheumatoid arthritis, IDDM = insulin -dependent diabetes mellitus, IHD = ischaemic heart disease, HITS - heparin-induced thrombocytopenia syndrome, OP = osteoporosis, HP = primary hyperparathyroidism

Polymorphism genotyped	Previously described?	Known to be functionally relevant?	Previously associated with disease?	Theoretical relevance to CVID
IL-1α -889 c/t	yes	no	yes - JRA	Lymphocyte differentiation, inflammation
IL-1β +3962 c/t	yes	yes	yes - IDDM	Lymphocyte differentiation, inflammation
IL-4 -590 c/t	yes	yes	yes - atopy	Humoral immunity
IL-6 +3247 a/g	no	no	no	Humoral immunity
IL-10 -1082 a/g, -819 c/t, -592 c/a	yes	yes (-1082)	no	Humoral immunity, Th1/Th2 regulation
TGFβ -988, -800, aa 10, aa 263	yes	no	yes (aa 25) - IHD	Humoral immunity
E selectin aa 128, aa 554	yes	no	yes (aa 128) - IHD	Leukocyte trafficking
L-selectin aa 49, aa 26	yes	no	no	Leukocyte trafficking and lymphocyte recirculation
ICAM-1 aa 241, aa 469	yes	no	yes (aa 241) - IBD	Leukocyte trafficking and costimulation
CTLA-4 -308, +49	yes	no	yes - IDDM, Graves'	Regulation of T cell proliferation
FcγRIIA aa 131	yes	yes	yes - infection, HITS	Fc binding affinity
VDR exon 2 *FokI*, 3' *ApaI*, *TaqI*	yes	yes	yes - OP, HP	Regulation of T and B cell and monocyte responsiveness
CCR5 Δ32	yes	yes	HIV	?Role in lymphocyte trafficking, inflammation

Figure 6.1: Theoretical involvement of immunoregulatory candidates in the pathogenesis of CVID

The basic features of lymphocyte extravasation and proliferation are illustrated. Molecules potentially involved in the pathogenesis of CVID are shown in italics.



6.1.3. Candidate genes examined

6.1.3.1. Interleukin-1

The IL-1 family comprises IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RA). IL-1α and IL-1β are both potent agonists at the IL-1 receptor, and IL-1RA is a pure receptor antagonist. IL-1 is primarily an inflammatory cytokine (reviewed in Dinarello, 1996). IL-1 exerts profound systemic and local tissue effects similar to those of TNF. At low concentrations, IL-1 promotes local tissue inflammation. IL-1 induces macrophages and vascular endothelium to promote coagulation, and produce further proinflammatory cytokines (e.g. IL-6), chemokines, and mediators such as prostaglandins, leukotrienes and nitric oxide. Like TNF, at high concentrations IL-1 has profound effects on multiple organ systems and may induce shock. IL-1 also promotes bone resorption and inhibits cartilage formation, and thus has been the focus of investigation in erosive arthropathies such as rheumatoid arthritis. In contrast to TNF, IL-1 does not directly cause tissue injury; is not lethal at high dose; does not directly cause cell lysis, and does not upregulate MHC expression.

IL-1 has significant haematological effects. IL-1 induces the formation, and acts synergistically with, colony-stimulating factors (GM-, G-, and M-CSF), and raises circulating neutrophil and platelet levels. The role of IL-1 in antigen-specific immune responses is less clear. IL-1 amplifies T cell activation by inducing IL-2 and IL-2R expression, particularly in the presence of IL-6, but this appears to be physiologically more important for thymocytes that mature T cells. The effects of IL-1 on B lymphocyte function are poorly understood but it appears that IL-1 may synergise with IL-6 to induce B lymphocyte proliferation.

The genes for IL-1α and β, IL-1RA and IL-1 receptor type one have been cloned, sequenced, and mapped to chromosome 2q13 (March et al, 1985; Clark et al, 1986; Furutani et al, 1986; Bensi et al, 1987; Modi et al, 1988; Lafage et al, 1989; Copeland et al, 1991; Lennard et al, 1992; Nicklin et al, 1994). All are polymorphic. IL-1α contains a dinucleotide repeat polymorphism (Todd et al, 1991), and a 46 bp intron 6 VNTR (Bailly et al, 1993) in addition to a nucleotide substitution at -889 of the IL-1α promoter (McDowell et al, 1995). The 46 bp repeat sequence has been

shown to bind the transcription factor Sp1, and an inverse correlation between the number of repeats and IL-1α production has been demonstrated (Bailly et al, 1996). Four nucleotide substitutions in the IL-1β gene have been described (table 6.1) (di Giovine et al, 1992; Pociot et al, 1992; Guash et al, 1996). Of the IL-1β polymorphisms, only the exon 5 +3962 polymorphism has been examined for effect upon IL-1β production (Pociot et al, 1992). The less common +3962T allele was significantly associated with higher monocyte IL-1β secretion in a dose-dependent fashion (Pociot et al, 1992). This is somewhat surprising, as the polymorphism does not produce a change in amino acid sequence, but may be explained by the known linkage disequilibrium with other polymorphisms. The IL-1RA gene contains an 86 bp VNTR in intron 2 (Tarlow et al, 1993), and 4 nucleotide substitutions from exon 2 to exon 4 (Guash et al, 1996).

Several of the IL-1 polymorphisms have been associated with disease. The IL-1α -889 polymorphism has been associated with juvenile rheumatoid arthritis (McDowell et al, 1995; Ploski et al, 1995), and the IL-1β +3962 polymorphism with IDDM (Pociot et al, 1992; Pociot et al, 1994). IL-1RA VNTR alleles have been associated with several autoimmune and inflammatory conditions including ulcerative colitis (Mansfield et al, 1994), alopecia areata (Tarlow et al, 1994), lichen sclerosus (Clay et al, 1994), psoriasis (Cork et al, 1993), SLE (Blakemore et al, 1994), Graves' disease (Blakemore et al, 1995) and IDDM (Mandrup-Poulsen et al, 1994). The presence of multiple associations with diseases characterised by autoimmunity and inflammation suggests a role for members of the IL-1 family in the pathogenesis of CVID, particularly in the development of complications such as autoimmunity and granulomata. The IL-1 nucleotide substitutions are amenable to PCR-SSP typing, as described below. The IL-1α -889 and IL-1β exon 5 +3963 polymorphisms were genotyped in this study. The IL-1RA nucleotide substitutions have only recently been described, and genotyping by PCR-SSP is currently being developed.

6.1.3.2. Interleukin-4

IL-4 was first identified as a T-cell derived cytokine important for mitogen-activated B cell IgG1 production (de Vries et al, 1996). IL-4 has since emerged to be

a pleiotropic cytokine of key importance for the generation of type 2 immune responses, and indeed is almost exclusively synthesised by Th2 lymphocytes. IL-4 induces B lymphocyte activation and proliferation, and upregulation of MHC class II, low-affinity IgE receptor (FcεRII), IL-4R, IL-2R and CD40 expression. IL-4 induces IgG4 and IgE synthesis by B lymphocytes, for which induction of CD40 expression is thought to be critical. Thus IL-4 is an interesting candidate for investigation in disease where the humoral response is either excessive (e.g. atopy) or deficient (e.g. CVID). Indeed, high IgE levels in atopic individuals have been linked to the IL-4 gene-containing cytokine cluster in chromosome 5q31.1 (Meyers et al, 1994; Neely et al, 1994). Several nucleotide substitutions in the 5' region of the IL-4 gene have been identified (Arai et al, 1989; Borish et al, 1994; Borish et al, 1995; Rosenwasser et al, 1995; Song et al, 1996), two of which (-590 and -81) have been associated with higher IL-4 transcriptional activity (Rosenwasser et al, 1995; Song et al, 1996), and high IgE levels in some studies of atopic families, (Rosenwasser et al, 1995), but not others (Walley et al, 1996). The -590 C/T polymorphism was examined in this study.

6.1.3.3. Interleukin-6

Originally named 'B-cell differentiation factor', IL-6 was first identified as a T-cell derived cytokine that enhanced B cell antibody synthesis (Tanaka et al, 1996). IL-6 is also produced by, and has profound effects on cells of the haematopoietic, endocrine, hepatic and neural systems. The regulation of IL-6 production by cytokines and growth factors is highly complex. In the immune system IL-6 is produced by B and T lymphocytes and monocytes, and acts relatively late in the B lymphocyte differentiation pathway. Whilst it does not induce B cell proliferation, it is critical in the induction of both polysaccharide and protein-specific antibody responses. Specific antibody formation, and particularly mucosal IgA production, is grossly impaired in mice with targeted IL-6 gene deletions. Furthermore, in conjunction with IL-4, IL-6 is critical in promoting immunoglobulin class-switching to IgE. IL-6 also contributes to the acute phase response by inducing hepatocyte protein synthesis, but does not share the profound systemic effects of IL-1 and TNF; in general IL-6 is an anti-inflammatory cytokine.

The IL-6 gene has been localised to chromosome 7p21-p15 (Ferguson-Smith et al, 1988), and several RFLP-defined nucleotide substitutions and microsatellite polymorphisms have been identified (Bowcock et al, 1988; Bowcock et al, 1989; Fugger et al, 1989b; Jacob et al, 1993; Linker-Israeli et al, 1996). The functional significance of these polymorphisms is unknown. *Msp*I and *Bgl*II polymorphisms have been investigated in rheumatoid arthritis, juvenile chromic arthritis and SLE, with no significant associations detected (Fugger et al, 1990). A weak association between an *Xba*I RFLP in the 3' region of the IL-6 gene and SLE has been reported (Linker-Israeli et al, 1996).

Unfortunately the precise locations of these RFLP sites in the IL-6 gene are not known. The intronic A→G substitution at nucleotide +3247 of the IL-6 gene genotyped in this study was identified by comparison of IL-6 gene sequences obtained from the literature and from the Genbank/EMBL databases. This substitution introduces a *Msp*I site, but it is not known if this corresponds to that described by Fugger et al (Fugger et al, 1989a).

6.1.3.4. Interleukin-10

IL-10 was first identified as a potent inhibitor of T lymphocyte type 1 cytokine synthesis (Fiorentino et al, 1989). IL-10 is secreted by, and influences the function of T lymphocytes, macrophages and activated B lymphocytes (Moore et al, 1993; Briere et al, 1996). As such it is a powerful controller of the balance of humoral and inflammatory immune responses. IL-10 markedly enhances B cell proliferation and differentiation (Rousset et al, 1992; Itoh et al, 1995), an effect additive with IL-4, and induces IgM, IgG1-3 and IgA secretion, but not IgG4 or IgE. This effect is most marked in B cells also stimulated through CD40. Recombination experiments have demonstrated that IL-10 is an important switch factor for IgG and IgA synthesis, but not IgE. TGFβ (discussed below) synergistically enhances IL-10 induced IgA synthesis. Furthermore, Il-10 induces specific antibody secretion by antigen-primed memory B cells.

The IL-10 pathway is one of the few cytokine pathways investigated in detail in humoral immunodeficiencies. B cells from IgAD patients stimulated with CD40

alone produce only IgG and IgM, but high amounts of IgA if IL-10 is added (Briere et al, 1994; Islam et al, 1994b). IL-10 mediated restoration of IgG and IgA secretion by CVID B lymphocytes *in vitro* has also been demonstrated in several reports (Nonoyama et al, 1993; Zielen et al, 1993; Briere et al, 1996). B cells of some CVID B cells appear to require additional stimuli to restore immunoglobulin secretion, such as IL-2 (Nonoyama et al, 1994). It is thus possible that hypogammaglobulinaemia in CVID is associated with defects in the IL-10 pathway.

IL-10 profoundly inhibits monocyte proinflammatory cytokine secretion (e.g. TNF) (de Waal Malefyt et al, 1991; Fiorentino et al, 1991a; Armstrong et al, 1996) and T lymphocyte type 1 cytokine secretion (e.g. IFNγ) (Fiorentino et al, 1989; Fiorentino et al, 1991b; D'Andrea et al, 1993). Conversely, IL-10 secretion and humoral immune responses are inhibited by type 1 cytokines such as IFNγ and IL-12 (Donnelly et al, 1995; Romagnani et al, 1995). The down-regulatory effects of IL-10 on the development of cellular immune responses suggests that quantitative abnormalities of this cytokine may be involved in the pathogenesis of complications of CVID such as granulomata.

The IL-10 gene has been mapped to chromosome 1q31-1q32 (Kim et al, 1992; Eskdale et al, 1997a). Three single nucleotide and two dinucleotide repeat polymorphisms have been identified in the 5′ region of the IL-10 gene (Eskdale et al, 1995; Eskdale et al, 1997a; Turner et al, 1997). Several weak associations between microsatellite alleles and SLE, an autoimmune disease characterised by high IL-10 production and B cell hyperactivity, have been described (Eskdale et al, 1997b). The three single nucleotide promoter polymorphisms, at positions -1082, -819 and -592, were genotyped in this study. Data regarding the functional effects of these polymorphisms is limited, but one study has examined IL-10 production following ConA stimulation of healthy control lymphocytes, and demonstrated significantly lower IL-10 production in individuals carrying the -1082A allele (Turner et al, 1997). This effect is consistent with the position of this polymorphism in a region of the IL-10 promoter containing positive regulatory elements (Kube et al, 1995).

6.1.3.5. *Transforming growth factor beta 1 (TGFβ1)*

TGFβ is a pleiotropic cytokine with both stimulatory and inhibitory effects on a variety of cell types, and has a particularly important role in fibrogenesis and wound repair (Stavnezer, 1995; Stavnezer, 1996). TGFβ exists as three isoforms, with similar but not identical spectra of activity. TGFβ1 and 3 are more potent that TGFβ2, and each isoform has unique regulatory elements tightly regulating expression. Most studies have examined TGFβ1. B cells and CD4+ and CD8+ T cells synthesise both TGFβ1 and TGFβ receptors, particularly after stimulation. TGFβ1 inhibits B cell proliferation and may induce apoptosis. However the addition of cytokines such and IL-2 and IL-4 *in vitro* overcomes these effects. TGFβ1 also facilitates class switching to IgA, and increases synthesis of most immunoglobulin isotypes except IgE, particularly in concert with IL-10. Thus like IL-10, TGFβ1 is an interesting candidate for investigation in humoral immunodeficiency. Limited data is available concerning the role of TGFβ1 in IgAD. Whilst IgAD peripheral blood lymphocytes express normal amounts of TGFβ1 mRNA, serum levels of TGFβ are reduced in some patients (Muller et al, 1995). Unstimulated IgAD lymphocytes do not contain Igα mRNA (Islam et al, 1994a), but if stimulated with TGFβ1 and PMA, germline α transcripts are increased to control levels (Islam et al, 1994b). Thus IgAD (and theoretically CVID) may be associated with defective production of mature, active TGFβ1 (Islam et al, 1994b).

The TGFβ1 gene has been localised to chromosome 19q13.1 and sequenced (Derynck et al, 1987; Kim et al, 1989). Seven polymorphisms have been described: three substitutions at nucleotides -988, -800 and -509 in the 5′ region of the gene (numbered relative to the transcription initiation site), a C insertion at nucleotide +72 in the untranslated region of the gene, and 3 nucleotide substitutions resulting in coding changes in the signal peptide and precursor region of TGFβ1 (Cambien et al, 1996) (table 6.1). The effects of these polymorphisms on TGFβ1 expression have not been directly investigated. Allele frequencies have been investigated in patients with ischaemic heart disease, and weak associations between codon 25 Pro allele and both increased risk of myocardial infarction and

risk of hypertension detected (Cambien et al, 1996). The -800, -509, codon 10 and codon 263 polymorphisms were examined in this study.

6.1.3.6. *L- and E- selectin*

Adhesion molecules are critically involved in the movement and recirculation of lymphocytes and inflammatory cells between lymphoid organs, the circulation, and peripheral tissues (Springer, 1990; Bevilacqua, 1993). The selectins, L-selectin (CD62L), E-selectin (CD62E) and P-selectin (CD62P) are involved in the earliest stage of leukocyte migration: rolling and tethering to vascular endothelium. L-selectin is expressed constitutively by all leukocytes, with particularly high expression on naïve T cells. By binding to GlyCAM-1, L-selectin serves as a homing molecule for lymphocytes to lymph node high endothelial venules, and thus assists lymphocyte recirculation and the generation of humoral immune responses. L-selectin may also assist leukocyte migration across non-lymphoid endothelium, however the ligand for L-selectin in non-lymphoid tissue is unknown. E-selectin is exclusively expressed by cytokine activated endothelium, and recognises sialylated carbohydrate groups found on the surface of granulocytes, monocytes and lymphocytes. E-selectin is unequivocally important in neutrophil migration (E-selectin blockade prevents neutrophil accumulation) but its role in lymphocyte biology is less clear. It is thought that E-selectin may assist homing of memory T-lymphocytes. P-selectin is stored in platelet and endothelial cytoplasmic granules (Weibel-Palade bodies), recognises similar ligands to E-selectin, and mediates neutrophil and monocyte binding. L-selectin is crucial for the homing of naïve T cells to lymph nodes, and impairment of this homing may hinder the genesis of normal antibody responses. This suggests a role for the selectins in the pathogenesis in CVID, and elevated serum L-selectin levels and low lymphocyte L-selectin expression have indeed been demonstrated in CVID (Zhang et al, 1996). The role of selectins in the recruitment of leukocytes to sites of inflammation also raises the possibility of involvement of these molecules in the inflammatory complications of CVID, such as granulomata.

The three selectins map to chromosome 1q22-25 (Watson et al, 1990), have been cloned (Bevilacqua et al, 1989; Tedder et al, 1989; Johnston et al, 1990) and are polymorphic (Powers et al, 1993; Warnecke et al, 1993; Vora et al, 1994; Wenzel et al, 1994a; Wenzel et al, 1994b; Wenzel et al, 1996). The selectin genes contain at least 15 nucleotide substitutions (some of which may be identical to previously described RFLPs) (Wenzel et al, 1996), four of which result in coding changes in E- and L-selectin, and were genotyped in this study. E-selectin alleles have been associated with premature atherosclerosis (Wenzel et al, 1994a; Wenzel et al, 1996), but no data exists concerning allele frequencies in autoimmunity or immunodeficiency.

6.1.3.7. Intercellular adhesion molecule 1 (ICAM-1)

ICAM-1 is an integral membrane protein and member of the immunoglobulin superfamily expressed by B and T lymphocytes, in addition to keratinocytes, endothelial cells and fibroblasts. It is a ligand for the β_2 integrins LFA-1 (CD11aCD18) and Mac-1 (CD11bCD18), which are expressed by most leukocytes. The main functions of β_2 integrin/ICAM-1 interactions are the enhancement of T helper cell activation (Damle et al, 1992), and leukocyte adhesion and extravasation (Springer, 1990). Thus, like the selectins, both molecules are potential candidates for investigation in a very broad range of diseases characterised by an abnormal immune response.

The ICAM-1 gene lies on chromosome 19p13.3-2 (Simmons et al, 1988; Staunton et al, 1988; Voraberger et al, 1991). Three single nucleotide polymorphisms and a dinucleotide repeat polymorphism have been identified (Vora et al, 1994; Fernandez-Reyes et al, 1997). The three nucleotide substitutions in exons 2, 4 and 6 result in coding changes in the first, third and fifth immunoglobulin-like domains (table 6.1). The first Ig-like domain represents the LFA-1 binding site, and the third domain the Mac-1 binding site, thus at least two of these polymorphisms are potentially functionally important (Vora et al, 1994). Associations between Arg 241 and ANCA-negative ulcerative colitis, and ANCA-positive Crohn's disease, have been described (Yang et al, 1995). Both exon 4 and exon 6 (Ig domain 3 and 5) polymorphisms were genotyped in the present

investigation. PCR-SSP genotyping of the exon 2 codon 29 polymorphism was also examined, but preliminary investigation confirmed previous findings that the 29Met allele was absent in Caucasoids, and only found in Afro-Caribbeans. Therefore this polymorphism was not examined in the CVID patient group, as all were Caucasoids.

6.1.3.8. CTLA-4

CTLA-4 is another member of the immunoglobulin superfamily involved in T lymphocyte activation. The CTLA-4 gene (Dariavach et al, 1988; Harper et al, 1991), and the tightly linked CD28 gene, lie on chromosome 2q33, and both encode T cell molecules intimately involved in the regulation of T cell proliferation (Robey et al, 1995). CTLA-4 is a strong candidate gene for T-cell mediated diseases, as it mediates T cell apoptosis, and negatively regulates T cell activation (Lenschow et al, 1993; Green et al, 1994a; Gribben et al, 1995; Waterhouse et al, 1995). Exon three of the gene contains a polymorphic dinucleotide repeat (Polymeropoulos et al, 1991), alleles of which have been associated with Graves' disease (Yanagawa et al, 1995). A T/C substitution at position -308 of the CTLA-4 promoter (Deichmann et al, 1996), and three substitutions at positions 49, 272 and 439 of exon 1 have been described (Dariavach et al, 1988). The A→G transition at position +49 results in a Thr→Ala substitution in the CTLA-4 leader peptide. The +49G allele (codon 17 Ala) has been associated with both IDDM and Graves' disease (Nistico et al, 1996; Donner et al, 1997; Marron et al, 1997). The -308 and +49 substitutions were genotyped in this study.

6.1.3.9. Fc gamma receptor IIA (FcγRIIA)

The receptors for the Fc domain of IgG (FcγR) provide a critical link between humoral and cellular immune responses, for example by facilitating phagocytosis, endocytosis, antibody-dependent cellular cytotoxicity (ADCC), release of inflammatory mediators and antigen presentation (van de Winkel et al, 1993). 12 isoforms in three main classes (FcγRI-III) are encoded by a gene cluster on chromosome 1q21.1. Fc gamma RIIA (FcγRIIA, CD32) is the sole IgG Fc receptor capable of interacting with human IgG2, the IgG subclass specific for

bacterial capsular polysaccharides. The FcγRIIA receptor is polymorphic in its ability to bind murine IgG1, and in its isoelectric focusing pattern. This polymorphism is determined by two single nucleotide substitutions resulting in a Glu→Trp substitution at amino acid 27 in the first Ig-like domain (27Q→W) and an Arg→His change at amino acid 131 in the second Ig-like domain (131 R→H) (Clark et al, 1989; Warmerdam et al, 1990). FcγRIIA-131H has a high affinity for human IgG2, whereas 131R binds human IgG2 weakly (Warmedam et al, 1991). Several interesting disease associations have been identified with these alleles. FcγRIIA-131R homozygosity has been found to be more frequent in children with meningococcal septic shock (Bredius et al, 1994), and FcγRIIA-131H homozygosity less frequent in children with recurrent bacterial respiratory tract infections (Sanders et al, 1994), suggesting that this polymorphism is an important determinant of resistance to infection *in vivo*. In further support of the functional importance of this polymorphism, the low affinity R131 allotype has been associated with lupus nephritis in some, but not all studies (Duits et al, 1995; Botto et al, 1996; Salmon et al, 1996); and a high frequency of the high affinity FcγRIIA-131H allele has been detected in heparin-induced thrombocytopenia (HITS), an antibody-mediated condition (Brandt et al, 1995; Burgess et al, 1995). Thus, like the MBL polymorphisms described in the previous chapter, FcγRIIA polymorphism may potentially influence the severity of the CVID phenotype.

6.1.3.10. The vitamin D receptor (VDR)

The active metabolite of vitamin D, 1,25 dihydroxyvitamin D3 (1,25D3, calcitriol) has a central role in calcium homeostasis, and the role of polymorphisms in the vitamin D receptor (VDR) gene in the pathogenesis of osteoporosis has generated intense interest and controversy (Ralston, 1997). However calcitriol also exerts important immunoregulatory functions, including monocyte activation (Rigby et al, 1992), inhibition of monocyte immunoglobulin receptor expression (Boltz Nitulescu et al, 1995), and suppression of lymphocyte proliferation (Lemire et al, 1984; Iho et al, 1985; Haq, 1989; Koren et al, 1989; Yang et al, 1993b), immunoglobulin production (Lemire et al, 1984; Iho et al, 1986; Provvedini et al, 1986; Yang et al, 1993b) and cytokine synthesis (Tsoukas et al, 1984; Iho et al, 1985 ; Bhalla et al, 1986; Reichel et al, 1987). These actions are effected via the vitamin D

receptor, which is expressed by human monocytes, and B and T lymphocytes (Provvedini et al, 1983; Walter et al, 1984; Provvedini et al, 1989). The vitamin D axis might thus have a key role in the development of immunopathology in CVID.

Five polymorphisms in the vitamin D receptor gene on chromosome 12q12-14 (Baker et al, 1988) have been described. A C→T transition at the junction of intron 1 and exon 2 creates an initiation codon (ATG) three codons proximal to a downstream start site, and introduces a *Fok*I site (Saijo et al, 1991; Gross et al, 1996). Four RFLP-defined nucleotide substitutions have been described in the 3' region of the gene: *Bsm*I (alleles *B* and *b*, where *b* is recognised by the restriction enzyme), *Eco*RV (*E/e*) and *Apa*I (*A/a*) sites in intron 8, and a synonymous *Taq*I (*T/t*) site in exon 9 (Faraco et al, 1989; Morrison et al, 1992; Morrison et al, 1994). Of these, the *Bsm*I, *Apa*I and *Taq*I polymorphisms have been most intensively studied. The *Bsm*I and *Taq*I alleles are in complete linkage disequilibrium (i.e. only *Bt* and *bT* haplotypes exist). In contrast, the *Apa*I polymorphism is not in complete disequilibrium, and three haplotypes exist: *baT*, *bAT* and *BAt*. The *Fok*I, *Apa*I and *Taq*I polymorphisms were genotyped in this study.

Despite the plethora of reports examining associations between VDR genotype and disease, data regarding the functional effects of the VDR polymorphisms is very limited. Experiments using minigene constructs containing 3.2 kb segments of the 3' end of the VDR gene coupled with a luciferase reporter, have demonstrated that the *BAt* haplotype supports significantly greater luciferase activity, and presumably VDR expression, than the *baT* haplotype (Morrison et al, 1994). It has thus been inferred that homozygosity for the *Taq*I t allele is associated with higher VDR expression, and greater sensitivity to the immunological effects of 1,25D3. No data exists concerning the effect of the *Apa*I polymorphism, or the *in vivo* effect any of the polymorphisms. VDR alleles, and *Taq*I alleles in particular have been associated with a variety of diseases including osteoporosis (Morrison et al, 1994; Spector et al, 1995), tuberculosis and chronic hepatitis B infection (Bellamy et al, submitted), risk of heterosexual HIV infection (Ali et al, submitted), prostate cancer (Taylor et al, 1996), primary

hyperparathyroidism (Carling et al, 1995) and Crohn's disease (JD Simmons, unpublished data).

6.1.3.11. CC-chemokine receptor 5 (CCR5)

Few molecules have attracted such intense interest as CCR5. CCR5 is a G protein-coupled receptor for the β (or CC) chemokines MIP-1α, RANTES and MIP-1β (D'Souza et al, 1996; Baggiolini et al, 1997). CCR5 is a co-receptor for monocyte/macrophage-tropic strains of HIV-1 (Alktahib et al, 1996; Choe et al, 1996; Deng et al, 1996; Dragic et al, 1996), and a 32 bp deletion (Δccr5) in the CCR5 gene on chromosome 3p21 (Combadiere et al, 1996; Raport et al, 1996; Samson et al, 1996a) confers protection against HIV-1 infection, and disease progression (Dean et al, 1996; Liu et al, 1996; Samson et al, 1996b; Michael et al, 1997). The mutant protein resulting from the Δccr5 deletion lacks the last 3 transmembrane segments of the CCR5 protein, as well as the regions involved in G-protein coupling (Samson et al, 1996b). Whilst the ligands for this receptor are important chemoattractants for inflammatory cells and lymphocytes, the relevance of Δccr5 to non-HIV immunopathology unknown. As discussed above, trafficking of lymphocytes through lymph nodes via high-endothelial venules (HEV) is critical for the genesis of normal humoral immune responses. Chemokines and chemokine receptors appear to be important in this process, by inducing firm integrin-mediated attachment of lymphocytes to the HEV, although the specific chemokine and chemokine receptors involved remain to be determined (Kraal et al, 1997). Preliminary evidence suggests marked upregulation of CCR5 in inflammatory bowel disease (Dr Lee Brettman, unpublished data). The Δccr5 deletion is thus of potential relevance to both the pathogenesis of hypogammaglobulinaemia and the inflammatory complications of CVID, and was genotyped in the CVID cohort.

The potential involvement of each of these candidate genes in the pathogenesis of CVID is schematically represented in figure 6.1. Details of polymorphisms examined in CVID polymorphisms are listed in table 6.2.

Table 6.2: Location of single nucleotide polymorphisms genotyped by PCR-SSP.

Sequence accession numbers cited refer to those sequences used for primer design. Positions of polymorphisms are listed both in relation to the first nucleotide encoding the mature peptide, and according to the numbering of the reference sequence. Many polymorphisms were first described as RFLPs, and these are given where previously described in the literature. The location of several polymorphisms relative to the coding initiation site is not given where the reference sequence is divided into several discontinuous segments, each with its own numbering. Amino acids are denoted by capital letters, and nucleotides by lower case.

IL-1α genotyping was established by Dr Sara Marshall. TGFβ genotyping was developed by Dr Penny Lympany.

*This position was originally reported as nucleotide 188 of reference sequence X16150 (Wenzel et al, 1996), but is incorrect.

JRA = juvenile rheumatoid arthritis. IDDM = insulin-dependent diabetes mellitus, [IgE] = serum IgE concentration, MI = myocardial infarction, SBP = systolic blood pressure, IBD = inflammatory bowel disease, HITS = heparin-induced thrombocytopenia syndrome, nk = not known, na = not applicable.

†Unpublished sequence kindly provided by Dr Nigel Morrison.

Table 6.2: Location of single nucleotide polymorphisms genotyped by PCR-SSP.

Gene	Chromosomal location	Genbank accession number	Position of polymorphism				Restriction site	Functional effect?	Disease associations
			by coding start site	by reference sequence	nucleotide change	amino acid change			
IL-1α	2q13	X03833	-889	549	c/t	na	*Nco*I (c)	nk	JRA
IL-1β	2q13	X04500	+3962 (exon 5)	5887	c/t	synonymous	*Taq*I (c)	yes	IDDM
IL-4	5q31.1	M23442	-590	582	c/t	na	*Bsm*FI (c)	yes	atopy, [IgE]
IL-6	7p21-p15	Y00081	+3247 (intron 4)	4470	a/g	na	*Msp*I (g)	nk	
IL-10	1q31–32	Z30175	-1082	192	a/g	na		yes	
			-819	455	c/t	na	*Mae*III (c)	nk	
			-592	782	c/a	na	*Rsa*I (a)	nk	
TGFβ	19q13.1	J04431	-988	375	c/a	na		nk	
		J04431	-800	563xp	g/a	na		nk	
		J04431	-509	854	c/t	na		nk	
		J04431	+72	1434-1440	c insertion	na		nk	
		X02812	+28	870	t/c	10 L/P		nk	
		X02812	+73	915	g/c	25 R/P		nk	MI, SBP
		X02812	+790	1632	c/t	263 T/I		nk	
E-selectin	1q22-25	M61888	na	153	a/c	128 S/R		nk	atherosclerosis
		M61892	na	388	c/t	554 L/F		nk	atherosclerosis
L-selectin	1q22-25	M32408	na	94	c/g	49 T/S		nk	
		M32409	na	265	t/c	206 F/L		nk	
ICAM-1	19p13.3-2	X59287	na (exon 2)	224	a/t	29 K/M		nk	
		X59288	na (exon 4)	657	g/a	241 G/R		nk	IBD
		X59288	na (exon 6)	1548	g/a	469 E/K	*Bst*UI (g)	nk	
CTLA-4	2q33	M74363	-308	875	c/t	na	*Mse*I (t)	nk	
			+49 (exon 1)	1241	a/g	17 T/A	*BstE*II (a)	nk	IDDM, Graves'
FcγRIIA	1q21.1	M90724	na (exon 4)	512	g/a	131 A/H		yes	Infection, HITS
VDR	12q12-14	J03258	-3 (intron 1)	113	g/t	New start site	*Fok*I (t)	nk	OP
		unpublished†	intron 8	1078 bp before *Taq*I	a/g	na	*Bsm*I (g)	?yes	
		unpublished†	intron 8	80 bp before *Taq*I	t/g	na	*Apa*I (g)	?yes	
		J03258	+1055 (exon 9)	1171	t/c	synonymous	*Taq*I	yes	OP, HP
CCR5	3p21	U54994	553-585 (exon 1 of 1)	608-639	32bp deletion	termination	*Eco*RI	yes	HIV-1 infection

6.2. RESULTS

6.2.1. Establishment of genotyping by PCR-SSP

All primers were designed such that allelic specificity was determined by the terminal 3' nucleotide of the primer, with the exception of the CCR5 and Δccr5 primers, where the deletion-specific primer spanned the entire deletion site. Primers sequences and specificities are listed in table 6.3. Reaction mix composition is detailed in table 6.4. PCR buffer composition, thermal cycling parameters, agarose gel electrophoresis and product detection were identical to the protocols described in chapter 2. An example of simultaneous genotyping of multiple non-HLA polymorphisms is shown in figure 6.2.

6.2.2. Methodological comments

This method has demonstrated that large numbers of nucleotide substitution and deletion polymorphisms may be successfully and reliably genotyped simultaneously using PCR-SSP. It should be noted that the reaction conditions described in this chapter are identical to those employed for MBL, TNF and LTα genotyping (chapters 4 and 5). This approach has previously been used very successfully for HLA class I and II genotyping (Bunce et al, 1995). Indeed, the fact that the reaction conditions used here are identical to those widely used for HLA genotyping (Bunce et al, 1995) further simplifies disease-association studies involving examination of both HLA and non-HLA genotype.

Technically this approach has several advantages over previously described methods such as PCR-RFLP and PCR-SSOP. Only two steps are required (PCR and agarose gel electrophoresis) minimising equipment requirements and facilitating automation. The use of restriction enzymes and radioactivity is avoided. Gel interpretation is simple, requiring the scoring of the presence or absence of an amplicon, rather than, for example, the interpretation of complex restriction digests. Thus a single DNA sample may be analysed for all these polymorphisms in two hours. Cost-efficiency may be further enhanced by PCR-multiplexing, the incorporation of multiple allele-specific primer pairs in one reaction mixture. Although this somewhat reduces ease of interpretation, results obtained are at least comparable, if not superior to those obtained by PCR-RFLP.

6.2.2.1. PCR-haplotyping by PCR-SSP

The use of forward and reverse allele-specific primers enables the direct demonstration of *cis* combinations of alleles between polymorphic sites ('haplotyping', or 'double ARMS') (Dutton et al, 1991; Lo et al, 1991b). PCR-haplotyping was first established as a highly useful tool for HLA class I and II genotyping (Lo et al, 1991a) and has now been successfully used to genotype many non-classical HLA and non-HLA genes: ICAM-1, CTLA-4, TGFβ, the vitamin D receptor, Apo-E, *HLA-HFE*, and the Duffy gene, in addition to the TNF, LTα and MBL polymorphisms discussed in previous chapters. This approach has several benefits. First, it greatly simplifies the genotyping of alleles determined by two or more polymorphisms. For example, the Apo-E ε2, ε3 and ε4 alleles are determined by combinations of single nucleotide polymorphisms at codons 112 and 158 of the Apo-E gene. PCR-haplotyping enabled the design of 3 unique ε2, ε3 and ε4-specific primer mixes, avoiding the need for large numbers of reactions and interpretation of patterns of reactions in order to assign Apo-E type, as previously described (Main et al, 1991).

Secondly, PCR-haplotyping permits the discrimination of alleles of primary importance in disease pathogenesis from other alleles associated with disease due to linkage disequilibrium. This has been highly useful for the analysis of the TNF and LTα polymorphisms described in chapter 4, and has also been useful in the analysis of polymorphisms in the *HLA-HFE* gene, alleles of which may directly induce hereditary haemochromatosis (Mullighan et al, 1997a).

Haplotyping may also be used to further enhance efficiency by minimising the number of reaction mixes needed to genotype multiple polymorphisms within one gene. This was discussed for MBL in the previous chapter, and IL-10 serves as another useful example. The 3 biallelic nucleotide substitutions in the IL-10 gene generate 8 (2x2x2) possible haplotypes. 12 reactions are necessary to detect all possible *cis* combinations of alleles (primer mixes 604-615, table 6.4). However after genotyping more than 200 Caucasoid samples, it became apparent that only 3 haplotypes were present in Caucasoids, confirming previous reports (Turner et al, 1997). These three haplotypes (-1082a/-819c/-592c, a-t-a and g-c-c) were thus

distinguished by three haplotype-specific primer mixes: mixes 604 (-1082a/-592c, representing a-c-c), 608 (-1082a/-819t; a-t-a) and 611 (-1082g/-819c; g-c-c). These three 3 haplotype specific reactions are illustrated in figure 6.2. As emphasised for MBL, such assumptions regarding linkage disequilibrium should not be made when novel, poorly characterised populations are investigated.

Using the buffer composition, *Taq* polymerase and thermal cycling parameters described herein, reliable haplotype-specific amplification was obtained for polymorphisms up to 1.5 kb apart. It is possible to haplotype alleles further apart, however modification of amplification conditions or *Taq* polymerase is necessary (GC Fanning, unpublished data).

6.2.2.2. *Genotyping of deletion and insertions by PCR-SSP*

PCR-SSP was first described as a tool for the examination of single nucleotide polymorphisms (Newton et al, 1989), and has mostly been used for this purpose. Theoretically this technique should also be applicable to the detection of insertions and deletions. This has been confirmed by the successful genotyping the CCR5 32 bp deletion (Δccr5) in an allele-specific fashion. This is a significant advance over alternative existing methods utilising generic amplification of DNA fragments encompassing the deletion site, and scoring on the basis of amplicon size after prolonged electrophoresis through high concentration gels (Liu et al, 1996). The +72C nucleotide insertion in the TGFβ gene (Cambien et al, 1996) has also been successfully genotyped by PCR-SSP using conditions identical conditions to those described here (Dr Penny Lympany, manuscript in preparation). However, it would be expected that for a deletion to be successfully typed by PCR-SSP it should, like Δccr5, involve exactly the same sequence of deleted nucleotides in every individual. This is not the case for many deletions.

6.2.2.3. *Limitations of PCR-SSP*

The main limitation of the PCR-SSP technique was that imposed by the nucleotide sequence flanking the polymorphic site under investigation. The high stringency step-down thermal cycling parameters necessitated the use of primers capable of annealing at high temperature (70°C and 65°C for the first 5

and subsequent 21 PCR cycles, respectively). This was difficult to achieve if the nucleotide sequence adjacent to the polymorphism was AT rich. If so, annealing, and thus band intensity, was weak or absent. This problem was rarely insurmountable: extension of the 5′ primer end (thus preserving 3′ allelic specificity) usually overcame this problem. This was necessary for the IL-6 and CCR-5 primers, in addition to the MBL primers described in the previous chapter. However despite this modification it was not possible to achieve reliable allele-specific amplification for several polymorphisms (such as the complement receptor 1, CD35) without modification of thermal cycling parameters. It is emphasised that this is an uncommon problem.

Table 6.2: Location of single nucleotide polymorphisms genotyped by PCR-SSP.

Sequence accession numbers cited refer to those sequences used for primer design. Positions of polymorphisms are listed both in relation to the first nucleotide encoding the mature peptide, and according to the numbering of the reference sequence. Many polymorphisms were first described as RFLPs, and these are given where previously described in the literature. The location of several polymorphisms relative to the coding initiation site is not given where the reference sequence is divided into several discontinuous segments, each with its own numbering. Amino acids are denoted by capital letters, and nucleotides by lower case.

IL-1α genotyping was established by Dr Sara Marshall. TGFβ genotyping was developed by Dr Penny Lympany.

*This position was originally reported as nucleotide 188 of reference sequence X16150 (Wenzel et al, 1996), but is incorrect.

JRA = juvenile rheumatoid arthritis. IDDM = insulin-dependent diabetes mellitus, [IgE] = serum IgE concentration, MI = myocardial infarction, SBP = systolic blood pressure, IBD = inflammatory bowel disease, HITS = heparin-induced thrombocytopenia syndrome, nk = not known, na = not applicable.

†Unpublished sequence kindly provided by Dr Nigel Morrison.

Table 6.3: PCR-SSP primer specificities and sequences

Gene	Polymorphism	Primer no.	Sense (S) / antisense (AS)	Sequence accession number	Annealing position	Specificity	Sequence
IL-1α	-889 C/T	1164	S	X03833	524-549	-889 t	5' CTTTAATAATAgTAACCAggCAACAT
		1165	S		524-549	-889 c	5' CTTTAATAATAgTAACCAggCAACAC
		1166	AS		743-724	consensus	5' AAgTAgCCCTCTACCAAggA
IL-1β	+3962 C/T	1131	AS	X04500	5906-5887	+3962 t	5' CCTCgTTATCCCATgTgTCA
		1132	S		5570-5589	consensus	5' CATTgTCACCAgAggTTCgT
		1133	AS		5906-5887	+3962 c	5' CCTCgTTATCCCATgTgTCg
IL-4	-590 C/T	797	S	M23442	561-582	-590 c	5' CTAAACTTgggAgAACATTgTC
		798	S		561-582	-590 t	5' CTAAACTTgggAgAACATTgTT
		976	AS		1008-990	consensus	5' CACTTggggCCAATCAgCA
IL-6	+3247 A/G	778	AS	Y00081	4470-4490	+3247 a	5' CTgTTgAAAgACCACTgATCT
		779	AS		4470-4490	+3247 g	5' CTgTTgAAAgACCACTgATCC
		784	S		3438-3556	consensus	5' gAggAACAAgCCAgAgCTgT
IL-10	-1082 A/G	1030	S	Z30175	175-192	-1082 a	5' CTACTAAggCTTCTTTgggAA
		1031	S		175-192	-1082 g	5' CTACTAAggCTTCTTTgggAg
	-819 C/T	1032	S		435-455	-819 c	5' CCCTTgTACAggTgATgTAAC
		1033	S		435-455	-819 t	5' CCCTTgTACAggTgATgTAAT
		1034	AS		474-455	-819 c	5' CAAACTgAggCACAgAgATg
		1035	AS		474-455	-819 t	5' gCAAACTgAggCACAgAgATA
	-592 A/C	1038	AS		703-782	-592 c	5' CCAgAgACTggCTTCCTACAgg
		1039	AS		703-782	-592 a	5' CCAgAgACTggCTTCCTACAgT
TGFβ	-800 G/A	1070	S	J04431	545-563	-800 g	5' AgggACTCTgCCTCCAACg
		1071	S		545-563	-800 a	5' AgggACTCTgCCTCCAACA
	-509 C/T	1072	AS		872-854	-509 c	5' gggCAACAggACACCTgAg
		1073	AS		872-854	-509 t	5' gggCAACAggACACCTgAA
	10 T/C	1080	AS	X02812	887-870	aa 10 L	5' gCAgCggTAgCAgCAgCA
		1081	AS		887-870	aa 10 P	5' gCAgCggTAgCAgCAgCg
		1349	S		471-487	consensus	5' CCCCAgACCTCgggCgC
	263 C/T	1086	AS	X05844	359-342	aa 263 T	5' gggCCCTCTCCAgCgggg
		1087	AS		359-342	aa 263 I	5' gggCCCTCTCCAgCgggA
		1088	S		27-44	consensus	5' gCTTCCCTCTCgCCACTC

Table 6.3: PCR-SSP primer specificities and sequences (continued)

Gene	Polymorphism	Primer no.	Sense (S) / antisense (AS)	Sequence accession number	Annealing position	Specificity	Sequence
E-selectin	**aa 128 S/R**	907	S	M61888	134-153	aa 128 S	5' CTgTACCAATACATCCTgCA
		908	S		134-153	aa 128 R	5' CTgTACCAATACATCCTgCC
		909	AS		384-363	consensus	5' TCTgACTTCATAgTCTCAgCT
E-selectin	**aa 554 L/F**	910	S	M61892	368-388	aa 554 L	5' TCCTgACATTAgCACCATTTC
		911	S		368-388	aa 554 F	5' TCCTgACATTAgCACCATTTT
		912	AS		589-579	consensus	5' CCACACTgAgTTgTACTACTA
L-selectin	**aa 49 T/S**	901	S	M32408	75-94	49 T	5' TTCCTggCACATCATggAAC
		902	S		75-94	49 S	5' TTCCTggCACATCATggAAg
		903	AS		266-247	consensus	5' CTTCCggATTCCTATCCAgT
	aa 206 F/L	904	S	M32409	247-265	aa 206 F	5' TATgggCCCCAgTgTCAgT
		905	S		247-265	aa 206 L	5' ATgggCCCCAgTgTCAgC
		906	AS		585-565	consensus	5' CAAgCTCATTAgATCgTgAgC
ICAM-1	**aa 29 K/M**	940	S	X59287	102-120	aa 29 K	5' CCTCCTgTgACCAgCCCAA
		941	S		102-120	aa 29 M	5' CCTCCTgTgACCAgCCCAT
		942	AS		293-274	consensus	5' gTTACTCACAgTACACggTg
	aa 241 G/R	854	S	X59288	639-657	aa 241 G	5' gTggTCTgTTCCCTggACg
		855	S		639-657	aa 241 R	5' gTggTCTgTTCCCTggACA
	aa 469 E/K	856	AS		1566-1548	aa 469 E	5' gCACATTCACggTCACCTC
		857	AS		1566-1548	aa 469 K	5' gCACATTCACggTCACCTT
CTLA-4	**-308 C/T**	880	S	M74363	855-875	-308 C	5' CACTTAgTTATCCAgATCCTC
		881	S		855-875	-308 T	5' CACTTAgTTATCCAgATCCTT
		882	AS		1257-1241	aa 17 T	5' CAgggCCAggTCCTggT
		883	AS		1257-1241	aa17 A	5' CAgggCCAggTCCTggC
$Fc\gamma RIIA$	**aa 131 R/H**	747	S	M90724	74-94	consensus	5' CAAgTTCTgTgAgTAACgTAC
		748	AS		531-512	aa 131 R	5' ggAgAAggTgggATCCAAAC
		959	AS		531-512	aa 131 H	5' ggAgAAggTgggATCCAAAT

Table 6.3: PCR-SSP primer specificities and sequences (continued)

	Gene	Primer no.	Sense (S) / antisense (AS)	Sequence accession number	Annealing position	Specificity	Sequence
Vitamin D receptor	**exon 2 splice**	1025	S	unpublished (intron 1)	na	consensus	5' AgCTggCCCTggCACTgA
		1026	AS	J03258	134-117	*Fok*I f	5' TggCCgCCATTgCCTCCA
		1027	AS	J03258	134-117	*Fok*I F	5' TggCCgCCATTgCCTCCg
		1151	S	unpublished (intron 1)	na	*Apa*I A	5' gTgggATTgAgCAgTgAggT
		1152	S	unpublished (intron 1)	na	*Apa*I a	5' gTgggATTgAgCAgTgAggg
		965	AS	J03258	1188-1171	*Taq*I T	5' CggTCCTggATggCCTCA
		966	AS	J03258	1188-1171	*Taq*I t	5' CggTCCTggATggCCTCg
CCR5	**32bp deletion**	1020	AS	U54994	743-760	consensus	5' gCCTCACAgCCCTgTgCC
		1022	S		585-607/640-642	Δccr5	5' CTgCAgCTCTCATTTTCCATACATTA
		1023	S		591-612	CCR5	5' CTCTCATTTTCCATACAgTCAg
Control primers	**DRB1**	63	S	M17378	517-535	consensus	5' TgCCAAgTggAgCACCCAA
		64	AS		598-579	consensus	5' gCATCTTgCTCTgTgCAgAT
	APC	210	S	M74088	5627-5647	consensus	5' ATgATgTTgACCTTTCCAggg
		211	AS		5876-5854	consensus	5' TTCTgTAACTTTTCATCAgTTgC
	HGH	724	S	M13438	893-912	consensus	5' gCCTTCCCAACCATTCCCTT
		725	AS		1319-1298	consensus	5' TCACggATTTCTgTTgTgTTTC

Table 6.4: Primer mix details for genotyping of non-HLA immunogenetic polymorphisms

Volume refers to volume of 2000µg/ml primer stock per ml of primer mix. S = sense primer number, AS = antisense primer number. PM no. = primer mix number, a unique identifier used by the Oxford Tissue Typing Laboratory

PM no.	Specificity	S	volume	conc. (mM)	AS	volume	conc. (mM)	Control primers	Amplicon size (bp)
687	IL-1α -889 t	1164	40	13.6	1166	10	3.4	63+64	220
688	IL-1α -889 c	1165	40	13.6	1166	20	6.8	63+64	220
636	IL-1β +3962 c	1133	10	3.4	1132	10	3.4	63+64	336
637	IL-1β +3962 s	1131	10	3.4	1132	10	3.4	63+64	336
587	IL-4 -590 c	797	20	6.8	976	20	6.8	63+64	447
588	IL-4 -590 t	798	20	6.8	976	20	6.8	63+64	447
644	IL-6 +3247 a	778	10	3.4	784	10	3.4	210+211	952
645	IL-6 +3247 g	779	10	3.4	784	10	3.4	210+211	952
604	IL-10 -1082a to -592c	1030	10	3.4	1038	10	3.4	63+64	530
605	IL-10 -1082a to -592a	1030	10	3.4	1039	10	3.4	63+64	530
606	IL-10 -1082g to -592c	1031	10	3.4	1038	10	3.4	63+64	530
607	IL-10 -1082g to -592a	1031	10	3.4	1039	10	3.4	63+64	530
608	IL-10 -1082a to -819t	1030	10	3.4	1035	10	3.4	63+64	303
609	IL-10 -1082a to -819c	1030	10	3.4	1034	10	3.4	63+64	303
610	IL-10 -1082g to -819t	1031	10	3.4	1035	10	3.4	63+64	303
611	IL-10 -1082g to -819c	1031	10	3.4	1034	10	3.4	63+64	303
612	IL-10 -819c to -592c	1032	10	3.4	1038	10	3.4	63+64	257
613	IL-10 -819t to -592c	1033	10	3.4	1038	10	3.4	63+64	257
614	IL-10 -819c to -592a	1032	10	3.4	1039	10	3.4	63+64	257
615	IL-10 -819t to -592a	1033	10	3.4	1039	10	3.4	63+64	257
616	TGFβ -800g to -509c	1070	5	1.7	1072	5	1.7	63+64	291
617	TGFβ -800g to -509t	1070	5	1.7	1073	5	1.7	63+64	291
618	TGFβ -800a to -509c	1071	5	1.7	1072	5	1.7	63+64	291
619	TGFβ -800a to -509t	1071	5	1.7	1073	5	1.7	63+64	291
646	TGFβ aa 10 L	1118	2	0.68	1080	1.5	0.51	63+64	416
647	TGFβ aa 10 P	1118	2	0.68	1081	2.5	0.85	63+64	416
620	TGFβ aa 263 T	1086	5	1.7	1088	5	1.7	63+64	200
621	TGFβ aa 263 I	1087	5	1.7	1088	5	1.7	63+64	200
535	L-Selectin aa 49 T	901	10	3.4	903	10	3.4	63+64	191
536	L-Selectin aa 49 S	902	10	3.4	903	10	3.4	63+64	191
537	L-Selectin aa 206 F	904	10	3.4	906	10	3.4	63+64	338
538	L-Selectin aa 206 L	905	10	3.4	906	10	3.4	63+64	338
539	E-selectin aa 128 S	907	10	3.4	909	10	3.4	63+64	229
540	E-selectin aa 128 R	908	10	3.4	909	10	3.4	63+64	229
541	E-selectin aa 554 L	910	15	5.1	912	15	5.1	63+64	221
542	E-selectin aa 554 F	911	15	5.1	912	15	5.1	63+64	221
543	ICAM-1 G241-E469	854	5	1.7	856	5	1.7	725+726	927
544	ICAM-1 G241-K469	854	5	1.7	857	5	1.7	725+726	927
545	ICAM-1 R241-E469	855	5	1.7	856	5	1.7	725+726	927
546	ICAM-1 R241-K469	855	5	1.7	857	5	1.7	725+726	927
547	ICAM-1 aa 29 K	940	10	3.4	942	10	3.4	63+64	191
548	ICAM-1 aa 29 M	941	10	3.4	942	10	3.4	63+64	191
549	CTLA-4 -318c to +49a	880	20	6.8	882	20	6.8	63+64	405
550	CTLA-4 -318c to +49g	880	20	6.8	883	20	6.8	63+64	405
551	CTLA-4 -318t to +49a	881	20	6.8	882	20	6.8	63+64	405
552	CTLA-4 -318t to +49g	881	20	6.8	883	20	6.8	63+64	405
553	FcγRIIA aa 131 R	959	10	3.4	747	10	3.4	63+64	457
554	FcγRIIA aa 131 H	959	10	3.4	748	10	3.4	63+64	457
602	VDR *FokI* f	1025	10	3.4	1026	10	3.4	63+64	100
603	VDR *FokI* F	1025	10	3.4	1027	10	3.4	63+64	100
660	VDR *Apa-Taq* A-T	1151	10	3.4	965	10	3.4	63+64	116
661	VDR *Apa-Taq* a-T	1152	10	3.4	965	10	3.4	63+64	116
662	VDR *Apa-Taq* A-t	1151	10	3.4	966	10	3.4	63+64	116
663	VDR *Apa-Taq* a-t	1152	10	3.4	966	10	3.4	63+64	116
638	CKR5 wild type (*CCR5*)	1023	15	5.1	1020	15	5.1	63+64	169
639	CKR5 deletion (Δccr5)	1022	10	3.4	1020	10	3.4	63+64	143

Figure 6.2: Example of simultaneous genotyping of multiple DNA polymorphisms

One DNA sample has been subjected to 96 allele-or haplotype specific PCR-SSP reactions simultaneously. Reaction products have then been electrophoresed through an ethidium bromide impregnated 1% agarose gel, overlaid with a numbered template, illuminated under UV light and photographed.

The direction of electrophoresis is upwards. Each reaction incorporates a control amplicon (to verify successful amplification) in addition to an allele- or haplotype-specific band. The numbers at the base of each lane in the photograph correspond to the lane numbers in the chart above. In most lanes, the control band is below the specific amplicon. Where reactions generate a large amplicon, a smaller, uppermost control amplicon is utilised. These lanes are shaded on the chart. Amplicon size is checked against the chart, and positive reactions scored.

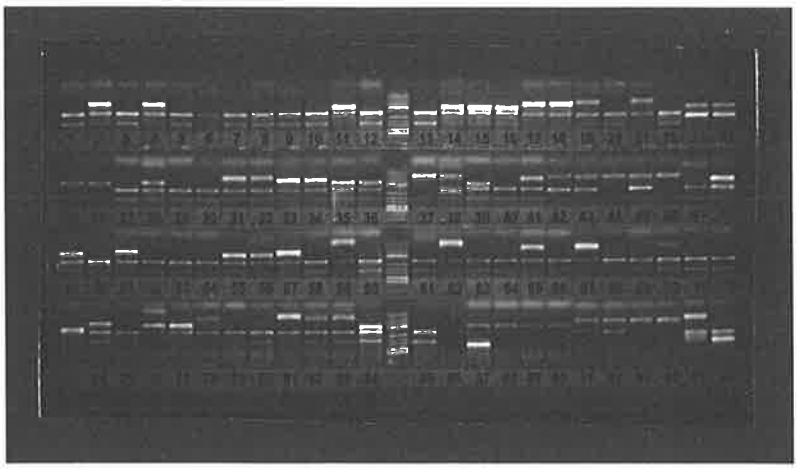
Haplotype-specific reactions are denoted by alleles separated by dashes. eg. reaction 1 is specific for the H allele at -550 of the mannose-binding lectin promoter in *cis* with allele X at -221. Polymorphisms known to produce changes in amino acid sequence are denoted by the amino acid in capitals preceding the polymorphic codon. eg reaction 17 is specific for phenylalanine (F) at codon 206 of L-selectin. Polymorphism corresponding to nucleotide substitutions but *not* amino acid changes are denoted by the nucleotide position (numbered relative to the transcription start site) followed by the nucleotide allele in lower case. eg lane 35 is specific for cytosine at position -590 of the IL-4 promoter.

Several polymorphisms genotyped by this method but not examined in CVID are shown to illustrate the broad applicability of this method. Several of these genes are detailed in the appendix (e.g. HLA-HFE, Duffy, Apo-E).

Figure 6.2. Example of simultaneous genotyping of multiple DNA polymorphisms

Lane	Positive?	Gene	Alleles	Primer mix no.	Product size (bp)	Control size (bp)
1		Mannose-binding	H — X	640	370	796
2	+	lectin	H — Y	641	370	796
3			L — X	642	370	796
4	+		L — Y	643	370	796
5			X — C52	511	481	796
6			X — D54	673	490	796
7			X — E57	513	497	796
8			X — R52	514	481	796
9			X — G54	515	490	796
10			X — G57	516	497	796
11	+		Y — C52	517	481	796
12			Y — D54	672	490	796
13			Y — E57	519	497	796
14	+		Y — R52	520	481	796
15	+		Y — G54	521	490	796
16	+		Y — G57	522	497	796
17	+	L-selectin	F206	537	338	796
18	+		L206	538	338	796
19	+	E-selectin	S128	539	229	796
20			R128	540	229	796
21	+		L554	541	221	796
22			F554	542	221	796
23	+	ICAM-1	G241 — E469	543	927	450
24	+		G241 — K469	544	927	450
25			R241 — E469	545	927	450
26			R241 — K469	546	927	450
27		CTLA-4	-318c — +49a	549	405	796
28	+		-318c — +49g	550	405	796
29			-318t — +49a	551	405	796
30			-318t — +49g	552	405	796
31	+	Fc gamma receptor IIA	R131	553	457	796
32	+		H131	554	457	796
33	+	Interleukin-1β	3954c	636	420	796
34	+		3954t	637	420	796
35	+	Interleukin-4	-590c	587	447	796
36	+		-590t	588	447	796
37		Interleukin-6	+3247 (intron 4) a	644	850	256
38	+		+3247 (intron 4) g	645	850	256
39	+	Interleukin-10	a — c — c	604	530	796
40		(-1082 — -819 — -592)	a — t — a	608	303	796
41	+		g — c — c	611	303	796
42	+	Tumour necrosis factor	g — g — g	589	835	256
43	+	(488 — -238 — -308)	g — g — a	590	835	256
44			g — a — g	591	763	256
45	+		gga and ggg	592	763	256
46			t — c — c	593	763	256
47		Lymphotoxin α	c — g — a	594	390	796
48	+	(720 — 365 — 249)	c — c — a	595	390	796

Lane	Positive?	Gene	Alleles	Primer mix no.	Product size (bp)	Control size (bp)
49	+		a — g — g	596	390	796
50			a — c — a	597	390	796
51	+	TGFβ 1	-800g — -509c	616	291	796
52			-800g — -509t	617	291	796
53			-800a — -509c	618	291	796
54			-800a — -509t	619	291	796
55	+		L10	646	c.400	796
56	+		P10	647	c.400	796
57	+		T263	620	200	796
58			I263	621	200	796
59	+	VDR	f	602	c.100	796
60			F	603	c.100	796
61			A — T	660	116	796
62	+		a — T	661	116	796
63			A — t	662	116	796
64			a — t	663	116	796
65	+	Factor V	normal	558	218	796
66			Leiden	559	218	796
67	+	gpBIIIa	Pl^{A1}	560	194	796
68			Pl^{A2}	561	194	796
69		Apo-E	ε2	555	173	796
70	+		ε3	556	173	796
71			ε4	557	173	796
72		N-acetyl	341t	579	717	796
73	+	transferase 2	341c	580	717	796
74	+		590g	581	469	796
75			590a	582	469	796
76	+	GSTM1, GSTT1	GSTM1A	562	276/380	796
77	+		GSTM1B	563	276/380	796
78	+	GSTM3	GSTM3A	564	215	796
79			GSTM3B	565	215	796
80		Paroxonase	54a	598	160	796
81	+		54t	599	160	796
82	+		191g	600	180	796
83	+		191a	601	180	796
84	+	Secretor	Se	576	519	796
85			se	577	520	796
86			se	578	212	796
87	+	HLA-HFE	H63 — C282	570	710	450
88			H63 — Y282	571	710	450
89			D63 — C282	572	710	450
90			H63 — Y282	573	710	450
91	+	Duffy	FY*exp — FY*B	570	710	256
92	+		FY*exp — FY*A	571	710	256
93			FY*null — FY*B	572	710	256
94			FY*null — FY*A	573	710	256
95	+	CKR5	CCR5	638	169	796
96			Δccr5	639	143	796



6.2.3. Phenotype, allele and genotype frequencies in CVID and controls.

24 polymorphisms were genotyped in 160 CVID patients and 101 Caucasoid controls. The cadaveric renal allograft Caucasoid control group was described in chapter 3. Frequencies were also examined in CVID patient subgroups stratified on the basis of clinical (autoimmunity, splenomegaly and granulomata) and immunophenotypic criteria as described in chapters 3-5. Patients were also subgrouped on the basis of HLA and MBL genotype.

A comprehensive listing of control phenotype, genotype and allele frequencies is given in table 8.10 (appendices). Where available, previously reported frequencies have also been listed for comparison. All control allele frequencies were in Hardy-Weinberg equilibrium. CVID and control phenotype and genotype frequencies are compared in tables 6.5 and 6.6.

The large number of polymorphisms examined in CVID raised the possibility that any associations detected would not remain significant once corrected for multiple comparisons. In anticipation of this potential problem, frequencies were first examined in a group of 90 CVID patients (the HLA allele frequencies of this group were first published in Mullighan et al, 1995). Any significant associations were re-examined in a subsequently recruited group of 70 patients. If confirmed in the second patient group, associations were said to be significant and data was pooled. Only those associations significant in both cohorts are discussed below.

6.2.4. CVID is significantly associated with IL-1α genotype

The IL-1α -889t allele was significantly less frequent in the entire CVID patient group than in controls (table 6.5). 74 (46%) of patients carried this allele, compared to 62 (61%) controls (χ^2=5.68, p=0.017; RR=0.54[8], CI=0.33-0.90; PF=0.28). This association is reflected in an increased frequency of the IL-1α c/c genotype (χ^2=5.68, p=0.017; RR=1.84, CI 1.11-3.07), a reduced frequency of the IL-1α c/t

[8] RR=relative risk, CI=95% confidence interval, AF=aetiologic fraction, PF=preventive fraction (derivation in chapter 2).

genotype (χ^2=6.42, p=0.01; RR=0.52; CI 0.31-0.86), and a significant difference in IL-1α genotype distribution between patients and controls (χ^2 6.63, p=0.036; table 6.6).

No other significant differences in phenotype or genotype frequencies were detected between the entire CVID patient group and the control group.

6.2.5. Vitamin D receptor and IL-6 polymorphisms are associated with immunophenotypic abnormalities in CVID

VDR genotype was significantly associated with variation in B lymphocyte and CD8+CD57+ cell number in CVID, both by comparison of means and by contingency table analysis of specific abnormalities (i.e. B lymphopenia and CD8+CD57+ lymphocytosis).

The VDR *Apa*I *a* allele, the *aT* haplotype were significantly associated with a higher mean B lymphocyte count. The mean B cell count of patients carrying the *a* allele (or *aT* haplotype) was $0.24x10^9$/l (SD 0.15) compared to $0.13x10^9$/l (SD 0.12) in *a* or *aT* negative patients (t test p=0.0049). Both the *a* allele and *aT* haplotype were also negatively associated with B lymphopenia (table 6.7). 54.3% of B lymphopenic patients *a* (or *aT*) positive, compared to 71.1% of patients with normal B lymphocyte counts (χ^2 p=0.002; RR=0.30). Conversely, the *AT* haplotype was positively associated with B lymphopenia. 41% of B lymphopenic patients were *AT* positive, compared with 16.9% of patients with normal B cell counts (χ^2 p=0.0019; RR=3.47).

Similar associations were observed with *Apa*I genotype: the *AA* genotype was also strongly positively associated with B lymphopenia (χ^2 p=0.002, RR=3.31). Conversely, heterozygosity at this polymorphism (*Aa*) was negatively associated with B lymphopenia (χ^2 p=0.004; RR 0.34). A trend towards a negative association between *aa* genotype and B lymphopenia was observed, but was not significant, due to the low numbers of *aa* positive patients with immunophenotypic data available. The *Aa* genotype was also negatively associated with other immunophenotypic abnormalities associated with B lymphopenia, namely CD3+

lymphopenia (χ^2=6.76, p=0.009; RR=0.23, CI 0.06-0.82; PF=0.37), CD4+ lymphopenia (χ^2=6.11, p=0.013; RR 0.37, CI 0.17-0.83; PF=0.33) and CD4+CD45RA+ lymphopenia (χ^2=4.29, p=0.038; RR 0.42, CI 0.17-0.97; PF=0.30).

Both VDR and IL-6 alleles were associated with CD8+CD57+ lymphocytosis. The VDR *AT* haplotype was associated with a higher percentage of CD8+ cells also CD57+ (mean 0.47x10^9/l in *AT* positive, 0.36 in *AT* negative, t test p=0.033). The *AT* haplotype was specifically associated with CD8+CD57+ lymphocytosis (<40% CD8+ cells also CD57+). 40.6% of patients with CD8+CD57+ lymphocytosis bore the *AT* haplotype, 16.7% of patients without elevation of this lymphocyte subset (χ^2 p=0.03; RR=3.42). Furthermore, the IL-6 +3247 t allele was also specifically associated with CD8+CD57+ lymphocytosis (χ^2=9.54, p=0.002).

VDR *Taq*I genotypes were also assessed for associations with immunophenotypic, or other abnormalities in CVID. No associations with the *TT*, *Tt* or *tt* haplotypes were detected, nor with positivity for the *T* or *t* alleles, irrespective of genotype. This would support the observations above that the *Apa*I polymorphism is primarily responsible for the associations with lymphocyte phenotype.

6.2.6. IL-10 polymorphisms are associated with granulomatous CVID

The IL-10 -1082a/-819t/-592a (a-t-a) haplotype was more frequent in CVID patients with granulomata than in those without granulomata (the definition of this subgroup was discussed in detail in chapters 3 and 4). 18 of 24 (75%) granulomatous patients were IL-10 a-t-a positive, compared to 34 of 81 (40.5%) patients without granulomata (χ^2=8.077, p=0.0045; RR=4.14, CI 1.49-11.55; AF=0.56). Identical associations were observed with the two alleles unique to this haplotype, -819t and -592a.

In chapter 4, a strong association was observed between granulomata in CVID and the TNF +488A allele. However, this allele was only present in 40% of granulomatous patients. It is possible that other alleles, such as in the IL-10 gene, confer susceptibility to the development of granulomata in the remaining patients. Thus, IL-10 allele and haplotype frequencies were also examined in

those patients with granulomata who were TNF +488 negative. The association with IL-10 a-t-a was even more striking in this group. 14 of 17 (82.4%) granulomatous, TNF +488A negative patients were a-t-a positive, compared to 31 of 77 (40.2%) non-granulomatous patients (χ^2=9.88, p=0.0016, RR=6.92, 95% CI 1.84-26.12; AF=0.69).

6.2.7. Stratification on the basis of HLA type

In chapter 3 CVID was shown to be positively associated with the HLA class I and II haplotypes A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 and B*14-DRB1*0701-DQB1*0201 haplotypes. These haplotypes share HLA class III polymorphisms, and it was suggested that the HLA allele primarily associated with CVID resides in the class III region. However only 37.5% of CVID patients carry either the B*08-DRB1*0301 or B*14-DRB1*0701 class III-spanning segments of these haplotypes. It is possible that susceptibility to CVID in the remaining 62.5% of patients is conferred by polymorphisms in one or more of the non-HLA genes examined. Thus allele frequencies were specifically compared between patients negative for one or both of these HLA haplotypes and controls. No significant associations were found.

6.2.8. Stratification on the basis of MBL and FcγRIIA genotype

In the previous chapter it was hypothesised that the integrity of compensatory immune mechanisms in the presence of hypogammaglobulinaemia may influence the CVID phenotype. This was supported by the finding of a high rate of deleterious mannose-binding lectin alleles in CVID patients with early age of onset of symptoms. The FcγRIIA R131H polymorphism is a similar candidate. This polymorphism influences the affinity of the FcγRIIA receptor with IgG2, and FcγRIIA genotype has been associated with meningococcal and respiratory tract infection in children (Bredius et al, 1994; Sanders et al, 1994), infections commonly observed in CVID. No associations with FcγRIIA phenotype or genotype frequencies were observed in the entire patient group. However, it is possible that FcγRIIA alleles might act synergistically with other polymorphisms, such as MBL mutations, and influence the CVID phenotype. Such an interaction has been previously suggested to influence susceptibility to infection in non-

hypogammaglobulinaemic individuals (Turner, 1996). Data was thus stratified on the basis of MBL coding genotype, promoter genotype and FcγRIIA genotype. For example, it was asked if the combination of low affinity FcγRIIA alleles or genotypes and MBL mutations conferred increased susceptibility to CVID, or specific features such as early age of onset. No such associations were detected.

Table 6.5: Comparison of patient and control phenotype frequencies

Phenotype frequencies refer to the proportion of individuals carrying an allele or haplotype (c.f. allele frequencies, the proportion of positive chromosomes). Amino acids are represented by capital letters, nucleotides by lower case. RR = relative risk, OR = odds ratio (derivation, see chapter 2). P values given are uncorrected. Several RR/OR/χ^2 statistics are missing (na), where low sample numbers prevented meaningful calculation.

		Controls		Patients					
Gene	Allele	count n=101	freq	count n=160	freq	RR	OR	χ^2	χ^2 p
CCR5	CCR5	100	0.99	158	0.99	0.95	1.00	0.04	ns
	Δccr5	14	0.14	28	0.18	1.30	1.26	0.61	ns
CTLA-4 -318/+49	c-a	77	0.76	118	0.74	0.88	0.97	0.20	ns
	c-g	59	0.58	102	0.64	1.25	1.09	0.75	ns
	t-a	14	0.14	20	0.13	0.88	0.90	0.10	ns
CTLA-4 -318	c	101	1.00	160	1.00	na	1.00	na	na
	t	14	0.14	20	0.13	0.88	0.90	0.10	ns
CTLA-4 +49	a	81	0.80	124	0.78	0.86	0.97	0.27	ns
	g	59	0.58	102	0.64	1.25	1.09	0.75	ns
E-selectin aa 128	S	99	0.98	157	0.98	1.13	1.00	0.00	ns
	R	23	0.23	24	0.15	0.60	0.66	2.53	ns
E-selectin aa 554	L	101	1.00	158	0.99	0.31	0.99	1.27	ns
	F	2	0.02	7	0.04	1.94	2.21	1.07	ns
L-selectin aa 206	F	95	0.94	145	0.91	0.64	0.96	0.99	ns
	L	42	0.42	76	0.48	1.27	1.14	0.87	ns
FcγRIIA aa 131	R	81	0.80	123	0.77	0.83	0.96	0.40	ns
	H	71	0.70	114	0.71	1.05	1.01	0.03	ns
ICAM-1 aa 241-469	G-E	60	0.59	91	0.57	0.90	0.96	0.16	ns
	G-K	80	0.79	120	0.75	0.79	0.95	0.61	ns
	R-E	19	0.19	27	0.17	0.87	0.90	0.16	ns
ICAM-1 aa 241	G	101	1.00	158	0.99	0.31	0.99	1.27	ns
	R	19	0.19	27	0.17	0.87	0.90	0.16	ns
ICAM-1 aa 469	E	74	0.73	106	0.66	0.72	0.90	1.42	ns
	K	80	0.79	120	0.75	0.79	0.95	0.61	ns
IL-1α -889	c	93	0.92	146	0.91	0.92	0.99	0.06	ns
	t	62	0.61	74	0.46	0.54	0.75	5.68	0.02
IL-1β +3962	g	98	0.97	152	0.95	0.64	0.98	0.63	ns
	a	46	0.46	60	0.38	0.72	0.82	1.66	ns
IL-4 -590	c	98	0.97	158	0.99	2.25	1.02	0.98	ns
	t	26	0.26	44	0.28	1.09	1.07	0.10	ns
IL-6 +3247	t	80	0.79	118	0.74	0.74	0.93	1.01	ns
	c	64	0.63	95	0.59	0.85	0.94	0.41	ns
IL-10 -1082/-819/-592	a-c-c	53	0.52	73	0.46	0.76	0.87	1.16	ns
	a-t-a	44	0.44	70	0.44	1.01	1.00	0.00	ns
	g-c-c	78	0.77	121	0.76	0.92	0.98	0.09	ns
IL-10 -1082	a	82	0.81	116	0.73	0.62	0.89	2.55	ns
	g	78	0.77	121	0.76	0.92	0.98	0.09	ns
IL-10 -819	t	44	0.44	70	0.44	1.01	1.00	0.00	ns
	c	95	0.94	155	0.97	1.92	1.03	1.22	ns
IL-10 -592	c	95	0.94	155	0.97	1.92	1.03	1.22	ns
	a	44	0.44	70	0.44	1.01	1.00	0.00	ns

Table 6.5: Comparison of patient and control phenotype frequencies (continued)

		Controls		Patients					
Gene	**Allele**	**count n=101**	**freq**	**count n=160**	**freq**	**RR**	**OR**	**χ^2**	**p**
TGFβ -800/-509	**g-c**	85	0.84	136	0.85	1.08	1.01	0.03	ns
	g-t	42	0.42	73	0.46	1.18	1.10	0.41	ns
	a-c	19	0.19	35	0.22	1.20	1.16	0.35	ns
TGFβ -800	**g**	100	0.99	159	0.99	1.59	1.00	0.11	ns
	a	19	0.19	35	0.22	1.20	1.16	0.35	ns
TGFβ -509	**c**	91	0.90	148	0.93	1.36	1.03	0.46	ns
	t	42	0.42	73	0.46	1.18	1.10	0.41	ns
TGFβ aa 10	**L**	86	0.85	137	0.86	1.05	1.01	0.01	ns
	P	53	0.52	96	0.60	1.36	1.14	1.43	ns
TGFβ aa 263	**T**	100	0.99	160	1.00	4.79	1.01	1.59	ns
	I	8	0.08	6	0.04	0.46	0.47	2.12	ns
VDR *ApaI-TaqI*	**AT**	29	0.29	37	0.23	0.75	0.81	1.02	ns
	aT	76	0.75	112	0.70	0.77	0.93	0.85	ns
	At	56	0.55	103	0.64	1.45	1.16	2.07	ns
VDR *ApaI*	**A**	72	0.71	121	0.76	1.25	1.06	0.60	ns
	a	76	0.75	112	0.70	0.77	0.93	0.85	ns
VDR *TaqI*	**T**	90	0.89	136	0.85	0.71	0.95	0.90	ns
	t	56	0.55	103	0.64	1.45	1.16	2.07	ns
VDR *FokI*	**F**	88	0.87	144	0.90	1.34	1.03	0.52	ns
	f	71	0.70	98	0.61	0.67	0.87	2.22	ns

Table 6.6: Comparison of patient and control genotype frequencies

Gene	Genotype	Controls		Patients		Each genotype				Genotype distribution	
		count n=101	%	count n=160	%	RR	OR	χ^2	χ^2 p	χ^2	χ^2 p
CCR5	CCR5/CCR5	87	86.1	132	82.5	0.77	0.96	0.607	ns	0.607	0.740
	CCR5/Δccr5	13	12.9	26	16.3	1.29	1.26	0.56	ns		
	Δccr5/Δccr5	1	1.0	2	1.3	1.06	1.26	0.04	ns		
CTLA-4 -318/+49	c-a/c-a	32	31.7	44	27.5	0.82	0.87	0.525	ns	0.816	0.936
	c-a/c-g	35	34.7	60	37.5	1.13	1.08	0.22	ns		
	c-a/t-a	10	9.9	14	8.8	0.86	0.88	0.10	ns		
	c-g/c-g	20	19.8	36	22.5	1.17	1.14	0.27	ns		
	c-g/t-a	4	4.0	6	3.8	0.91	0.95	0.01	ns		
CTLA-4 -318	c/c	87	86.1	140	87.5	1.14	1.02	0.101	ns	0.101	0.750
	c/t	14	13.9	20	12.5	0.88	0.90	0.10	ns		
CTLA-4 +49	a/a	42	41.6	58	36.3	0.80	0.87	0.745	ns	0.777	0.678
	a/g	39	38.6	66	41.3	1.11	1.07	0.18	ns		
	g/g	20	19.8	36	22.5	1.17	1.14	0.27	ns		
E selectin aa 128	S/S	78	77.2	136	85.0	1.67	1.10	2.533	ns	2.722	0.257
	S/R	21	20.8	21	13.1	0.58	0.63	2.70	ns		
	R/R	2	2.0	3	1.9	0.88	0.95	0.00	ns		
E selectin aa 554	L/L	99	98.0	153	95.6	0.51	0.98	1.067	ns	1.602	0.449
	L/F	2	2.0	5	3.1	1.41	1.58	0.31	ns		
	F/F	0	0.0	2	1.3	3.20	na	1.27	ns		
L-selectin aa 206	F/F	59	58.4	84	52.5	0.79	0.90	0.87	ns	1.41	0.50
	F/L	36	35.6	61	38.1	1.11	1.07	0.16	ns		
	L/L	6	5.9	15	9.4	1.57	1.58	0.99	ns		
FcγRIIA	R/R	30	30.0	46	28.8	0.95	0.97	0.027	ns	0.403	0.817
	R/H	51	51.0	77	48.1	0.91	0.95	0.14	ns		
	H/H	20	20.0	37	23.1	1.21	1.17	0.40	ns		
ICAM-1 aa 241-469	G-E/G-E	16	15.8	26	16.3	1.02	1.03	0.008	ns	5.368	0.373
	G-E/G-K	39	38.6	53	33.1	0.79	0.86	0.82	ns		
	G-E/R-E	5	5.0	12	7.5	1.48	1.52	0.66	ns		
	G-K/G-K	27	26.7	54	33.8	1.39	1.26	1.42	ns		
	G-K/R-E	14	13.9	13	8.1	0.55	0.59	2.20	ns		
	R-E/R-E	0	0.0	2	1.3	3.20		1.27	ns		
ICAM -1 aa 241	G/G	82	81.2	133	83.1	1.15	1.02	0.160	ns	1.664	0.435
	G/R	19	18.8	25	15.6	0.80	0.83	0.45	ns		
	R/R	0	0.0	2	1.3	3.20		1.27	ns		
ICAM -1 aa 469	E/E	21	20.8	40	25.0	1.26	1.20	0.612	ns	3.163	0.206
	E/K	53	52.5	66	41.3	0.64	0.79	3.15	0.08		
	K/K	27	26.7	54	33.8	1.39	1.26	1.42	ns		
IL-1α -889	c/c	39	38.6	86	53.8	1.84	1.39	5.684	0.017	6.626	0.036
	c/t	54	53.5	60	37.5	0.52	0.70	6.42	0.01		
	t/t	8	7.9	14	8.8	1.09	1.10	0.06	ns		
IL-1β +3962	g/g	55	54.5	100	62.5	1.39	1.15	1.66	ns	3.01	0.22
	g/a	43	42.6	52	32.5	0.65	0.76	2.71	0.10		
	a/a	3	3.0	8	5.0	1.57	1.68	0.63	ns		
IL-4 -590	c/c	75	74.3	116	72.5	0.92	0.98	0.10	ns	1.28	0.53
	c/t	23	22.8	42	26.3	1.20	1.15	0.40	ns		
	t/t	3	3.0	2	1.3	0.44	0.42	0.98	ns		
IL-6 +3247	t/t	37	36.6	65	40.6	1.18	1.11	0.41	ns	2.52	0.28
	t/c	43	42.6	53	33.1	0.67	0.78	2.38	ns		
	c/c	21	20.8	42	26.3	1.34	1.26	1.01	ns		

Table 6.6: Comparison of patient and control genotype frequencies (continued)

Gene	Genotype	Controls		Patients		Each genotype				Genotype distribution	
		count n=101	%	count n=160	%	RR	OR	χ^2	χ^2 p	χ^2	χ^2 p
IL-10 -1082/-819/-592	acc/acc	2	2.0	7	4.4	1.94	2.21	1.07	ns	7.05	0.22
	acc/ata	15	14.9	27	16.9	1.15	1.14	0.19	ns		
	acc/gcc	36	35.6	39	24.4	0.58	0.68	3.84	0.05		
	ata/ata	6	5.9	5	3.1	0.52	0.53	1.22	ns		
	ata/gcc	23	22.8	38	23.8	1.05	1.04	0.03	ns		
	gcc/gcc	19	18.8	44	27.5	1.62	1.46	2.55	ns		
IL-10 -1082	a/a	23	22.8	39	24.4	1.09	1.07	0.09	ns	3.26	0.20
	a/g	59	58.4	77	48.1	0.66	0.82	2.63	ns		
	g/g	19	18.8	44	27.5	1.62	1.46	2.55	ns		
IL-10 -819	t/t	6	5.9	5	3.1	0.52	0.53	1.22	ns	1.31	0.52
	t/c	38	37.6	65	40.6	1.13	1.08	0.23	ns		
	c/c	57	56.4	90	56.3	0.99	1.00	0.00	ns		
IL-10 -592	c/c	57	56.4	90	56.3	0.99	1.00	0.00	ns	1.31	0.52
	c/a	38	37.6	65	40.6	1.13	1.08	0.23	ns		
	a/a	6	5.9	5	3.1	0.52	0.53	1.22	ns		
TGFβ -800/-509	g-c/g-c	45	44.6	63	39.4	0.81	0.88	0.68	ns	1.84	0.87
	g-c/g-t	27	26.7	50	31.3	1.24	1.17	0.61	ns		
	g-c/a-c	13	12.9	23	14.4	1.12	1.12	0.12	ns		
	g-t/g-t	10	9.9	12	7.5	0.73	0.76	0.46	ns		
	g-t/a-c	5	5.0	11	6.9	1.35	1.39	0.40	ns		
	a-c/a-c	1	1.0	1	0.6	0.63	0.63	0.11	ns		
TGFβ -800	g/g	82	81.2	125	78.1	0.84	0.96	0.35	ns	0.55	0.76
	g/a	18	17.8	34	21.3	1.23	1.19	0.46	ns		
	a/a	1	1.0	1	0.6	0.63	0.63	0.11	ns		
TGFβ -509	c/c	59	58.4	87	54.4	0.85	0.93	0.41	ns	1.33	0.52
	c/t	32	31.7	61	38.1	1.32	1.20	1.12	ns		
	t/t	10	9.9	12	7.5	0.73	0.76	0.46	ns		
TGFβ aa 10	L/L	48	47.5	64	40.0	0.74	0.84	1.43	ns	1.76	0.42
	L/P	38	37.6	73	45.6	1.39	1.21	1.62	ns		
	P/P	15	14.9	23	14.4	0.95	0.97	0.01	ns		
TGFβ aa 263	T/T	93	92.1	154	96.3	2.16	1.05	2.12	ns	2.96	0.23
	T/I	7	6.9	6	3.8	0.53	0.54	1.32	ns		
	I/I	1	1.0	0	0.0	0.21	0.00	1.59	ns		
VDR Apa-Taq	AT/AT	1	1.0	5	3.1	2.37	3.16	1.26	ns	5.71	0.34
	AT/aT	15	14.9	13	8.1	0.51	0.55	2.93	0.09		
	AT/At	13	12.9	19	11.9	0.90	0.92	0.06	ns		
	aT/aT	29	28.7	39	24.4	0.80	0.85	0.60	ns		
	aT/At	32	31.7	60	37.5	1.29	1.18	0.92	ns		
	At/At	11	10.9	24	15.0	1.41	1.38	0.90	ns		
VDR Apa	A/A	25	24.8	48	30.0	1.29	1.21	0.85	ns	1.07	0.59
	A/a	47	46.5	73	45.6	0.96	0.98	0.02	ns		
	a/a	29	28.7	39	24.4	0.80	0.85	0.60	ns		
VDR Taq	T/T	45	44.6	57	35.6	0.69	0.80	2.07	ns	2.35	0.31
	T/t	45	44.6	79	49.4	1.21	1.11	0.58	ns		
	t/t	11	10.9	24	15.0	1.41	1.38	0.90	ns		
VDR Fok	F/F	30	29.7	62	38.8	1.49	1.30	2.22	ns	2.34	0.31
	F/f	58	57.4	82	51.3	0.78	0.89	0.95	ns		
	f/f	13	12.9	16	10.0	0.75	0.78	0.52	ns		

Table 6.7: Associations between VDR and IL-6 polymorphisms and immunophenotypic abnormalities in CVID

B lymphopenia is defined as <0.1x109 cells/l. CD8+CD57+ lymphocytosis is defined as >40% CD8+ cells also CD57+. RR = relative risk, AF = aetiologic fraction, PF = preventive fraction (derivation, see chapter 2).

Allele / haplotype / genotype	CVID patients with allele (n, %)		χ^2	χ^2 p	RR (95% CI)	AF/PF
	With immunophenotypic abnormality	Without immunophenotypic abnormality				
	B lymphopenia (n=46)					
VDR *a* (or *aT*)	25 (54.3)	71 (71.1)	8.35	0.002	0.30 (0.13-0.66)	0.55
VDR *AT*	19 (41.3)	15 (16.9)	9.62	0.0019	3.47 (1.55-7.79)	0.29
VDR *AA*	21 (45.7)	18 (20.2)	9.54	0.002	3.31 (1.52-7.21)	0.32
VDR *Aa*	14 (30.4)	50 (56.2)	8.06	0.004	0.34 (0.16-0.73)	0.36
	CD8+CD57+ lymphocytosis (n=32)					
VDR *AT*	13 (40.6)	5 (16.7)	4.31	0.03	3.42 (1.04-11.2)	0.38
IL-6 +3247t	21 (45.7)	18 (20.2)	9.54	0.002	3.31 (1.52-7.21)	0.32

6.3. DISCUSSION

This and the preceding two chapters have detailed a novel approach to the investigation of the genetic susceptibility to complex, heterogenous diseases. By genotyping polymorphisms in many theoretically pathogenetically relevant genes in a large group of well-characterised patients, genetic factors contributing to several features of CVID - granulomata, splenomegaly, immunophenotypic abnormalities, age of onset and autoimmunity - were elucidated.

The associations identified between vitamin D receptor *Apa*I alleles and peripheral blood lymphocyte phenotype reinforce the conclusions of chapters 3 and 4, that immunophenotypic abnormalities in CVID are genetically determined. In chapter 4, the uncommon TNF +488A allele was found to be associated with CD3+ lymphopenia, B lymphopenia, CD4+CD45RA+ lymphopenia and CD8+CD57+ lymphocytosis, in addition to granulomata and splenomegaly. Whilst VDR alleles were not associated with these clinical features, the positive associations of the VDR *A* allele, *AT* haplotype and *Aa* genotype with these multiple immunophenotypic perturbations suggests that VDR alleles contribute to the development of these abnormalities, perhaps in concert with TNF genotype.

This novel finding reinforces the large body of data implicating the vitamin D axis in regulation of T and B lymphocyte proliferation and immunoglobulin secretion. Exogenous calcitriol is known to suppress B and T lymphocyte proliferation (Lemire et al, 1984; Iho et al, 1985; Shiozawa et al, 1987; Haq, 1989; Muller et al, 1992), cytokine synthesis (IL-2, IFNγ) and immunoglobulin production (Lemire et al, 1984; Iho et al, 1986; Provvedini et al, 1986; Chong et al, 1989; Yang et al, 1993a). Less data is available concerning the relationship between vitamin D and lymphocyte populations, however one study has reported T (but not B) lymphopenia, in children with vitamin D deficient rickets (Yener et al, 1995); administration of vitamin D has also been associated with an increase in the CD4+:CD8+ ratio (Bargman et al, 1989).

If the VDR polymorphisms influence the level of expression of the receptor, it would seem logical to infer that they would thus modulate the potency of the immunoregulatory actions of calcitriol. One study using minigene constructs concluded that the *BAt* haplotype supported greater VDR expression than the baT haplotype (Morrison et al, 1994). Unfortunately this data did not permit conclusions regarding the effects of the *Apa*I polymorphism. However the VDR itself is also a *trans*-acting regulatory factor for the expression of osteocalcin, and it has been found that VDR *Apa*I *aa, aA* and *AA* genotypes are associated with ascending levels of osteocalcin expression (Morrison et al, 1992). This would suggest that the *A* allele is associated with higher VDR expression than the *a* allele, and thus would enhance the immunosuppressive actions of calcitriol. This may explain the association of *Apa*I *A* with CD3+ and B lymphopenia. The association with CD8+CD57+ lymphocytosis is more difficult to explain, but may result from a more complex interaction with alleles at the TNF and IL-6 loci. The IL-6 +3247 t allele was also found to be associated with CD8+CD57+ lymphocytosis, and it is possible that the combined action of IL-6, VDR and TNF alleles induces CD8+CD57+ lymphocytosis, either directly, or via a more profound immunoregulatory abnormality associated with these multiple immunophenotypic disturbances. Obviously, more work is required, both to replicate these associations, and also to assess the functional significance of these polymorphisms.

The finding of a second genetic association with granulomata in CVID provides compelling evidence that this feature is genetically determined. The attractiveness of this hypothesis is enhanced by the known interaction of the two associated cytokines - IL-10 and TNF - in macrophage activation and cytokine production. The IL-10 -1082/-819/-592 a-t-a haplotype was negatively associated with CVID. This association was particularly marked in granulomatous patients who did not carry the TNF +488 A allele found to be associated with granulomata in chapter 4.

Again, data regarding the functional importance of the IL-10 promoter polymorphisms is limited. However, the polymorphisms lie in a region of the

IL-10 promoter rich in regulatory sequences (Eskdale et al, 1997a). The -1082 polymorphism lies in a regulatory area known to enhance luciferase (and thus transcriptional) activity, and the -592 polymorphism lies in an area known to abolish transcriptional activity (Kube et al, 1995). Furthermore, the -1082a allele (which lies on the a-t-a haplotype associated with granulomata) is associated with significantly lower IL-10 production by ConA-stimulated control lymphocytes than -1082g (Turner et al, 1985). It is proposed that the presence of the a-t-a haplotype favours lower IL-10 production, and thus higher TNF production, and the formation of granulomata. IL-10 production in the granulomatous subset of patients has not been specifically investigated. Both this, and further work characterising the functional correlates of the IL-10 polymorphisms, will be of great interest.

Whilst multiple genetic associations were detected with disease subgroups, overall associations with CVID were few and relatively weak. The principle association was with IL-1α genotype. Although primarily considered to be a pro-inflammatory cytokine (indeed, polymorphisms have previously been associated with juvenile rheumatoid arthritis) (McDowell et al, 1995; Ploski et al, 1995), it is feasible that polymorphisms in this gene might contribute to the pathogenesis of hypogammaglobulinaemia. Confirmation of this hypothesis will require further examination of the currently poorly understood role of IL-1 in B lymphocyte differentiation. Furthermore, as stated earlier, the action of IL-1 is complex, involving two agonists (IL-1α and IL-1β), a specific antagonist (IL-1RA) and two receptors (IL-1RI and II), each of which are genetically complex and exist in multiple isoforms. The genes encoding each of these molecules is polymorphic, and these preliminary results behove further investigation of IL-1, IL-1RA and IL-1R polymorphisms in CVID. This is in progress.

The success of this approach in determining the genetic basis of complications of CVID suggests that investigation of other polymorphisms may be useful in determining the genetic factors responsible for hypogammaglobulinaemia. Previous genetic and functional studies suggest several candidates. The recent interesting finding of impaired T cell signal transduction in a proportion of

patients offers a potential explanation for the myriad T and B cell defects characteristic of CVID. If genetically determined, polymorphism in genes intimately involved in signal transduction may be responsible for impaired lymphocyte differentiation (and thus cytokine production), rather than polymorphism in cytokine genes. Several of the T cell receptor complex genes are known to be polymorphic and are under investigation.

This latter point illustrates the current limitation of the candidate gene studies. The identification of gene polymorphisms and the investigation of the functional importance of known polymorphisms is presently in its infancy. As is readily apparent from the introduction to this chapter, relatively few of the polymorphisms studied have been assessed for functional importance, and where available, functional data is limited. Polymorphisms in many interesting candidate genes are unidentified for want of thorough sequencing in multiple individuals. This is a lengthy process, and for this reason the current study was intentionally limited to the establishment of PCR-SSP genotyping of known polymorphisms, rather than an intensive effort to identify new ones. Obviously, ongoing intensive efforts in this area will further enhance the utility of the candidate gene approach.

7. Discussion

This thesis has described the largest reported case-control genetic association study in CVID. By examining polymorphisms in a large number of immunoregulatory genes, both within and outside the MHC, and collating this with extensive clinical and laboratory data, it has been possible to rigorously analyse the genetic predisposition to this complex condition.

Prior to this study, considerable evidence suggested that CVID and the clinically similar condition IgA deficiency (IgAD) share associations with major histocompatibility complex polymorphisms. Due to the dearth of studies specifically examining HLA polymorphisms in large groups of CVID patients using high-resolution molecular methods, it was impossible to conclude from the existing literature which, if any locus was responsible for the multiple observed HLA associations. Olerup et al suggested that both CVID and IgAD are primarily associated with non-aspartate residues at DQB1 codon 57 (Olerup et al, 1990; Olerup et al, 1991; Olerup et al, 1992). This was disputed by Wilton and French et al (Wilton et al, 1985; French et al, 1990) who argued that HLA class III associations explain all other observed HLA class I and II associations by linkage disequilibrium. This was based upon examination of C4, 21-hydroxylase and C2 polymorphism, and whilst significant associations were found, it was not clear whether one of these genes represented the primary association, or if another linked gene in close proximity was involved.

In this study, the initial approach taken to resolve these issues was genotyping of 8 HLA class I and II loci (HLA-A, -B, -Cw, -DRB1, -DRB3-5, and -DQB1) in 179 CVID patients and 605 controls. This incorporated the development of high-resolution genotyping of HLA-DQB1 (Mullighan et al, 1997b), in order to precisely evaluate the role of polymorphism at this locus. It was shown that three haplotypes were associated with CVID: A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 and B*14-DRB1*0701 (both positively associated), and A*02-Cw*0702-B*07-DRB1*1501-DQB1*0602 (negatively associated). Further stratification and

reanalysis of data, coupled with detailed amino acid frequency analysis at every polymorphic HLA-A, -B, -Cw -DRB1 and -DQB1 codon demonstrated two important findings: no single allele on any of these three haplotypes was primarily associated with CVID; and no single codon, including HLA-DQB1 codon 57, was responsible for the observed HLA associations with CVID. In contrast, the two positively associated haplotypes, but not the negatively associated haplotype share HLA class III polymorphisms. These findings disprove the primary involvement of HLA-DQB1 codon 57 asserted by Olerup *et al*, and in addition, support the 'HLA class III' hypothesis of Wilton and French. To directly assess the involvement of HLA class III polymorphism in CVID, two class III genes of major immunological importance, tumour necrosis factor (TNF) and lymphotoxin alpha (LTα) were examined.

In view of the extensive clinical heterogeneity of CVID, it has been suggested that CVID may represent several discrete, aetiologically distinct conditions (Spickett et al, 1997). Alternatively, if CVID represents a single or limited number of conditions, additional genetic factors might determine phenotypic variability. Whichever is correct, it is possible that different HLA associations might be observed if CVID patients were divided into subgroups. Although no single classification system has been validated, as much data as possible was collected in order to enable patient stratification according to criteria including sex, age of onset, age at diagnosis, autoimmunity, granulomata, splenomegaly, and peripheral blood immunophenotype. This examination of genetic associations with specific clinical and laboratory features in CVID, represents a major departure from previous studies.

Six biallelic polymorphisms in the gene encoding TNF, and the linked LTα gene were investigated. Both genes encode cytokines of fundamental importance in humoral and inflammatory immune responses. A highly significant association was detected between the TNF +488A allele and the presence of granulomata in CVID. This association was independent of linkage disequilibrium with HLA class I and II alleles. Furthermore, features such as splenomegaly, CD3+ lymphopenia, CD4+CD45RA+ lymphopenia, and CD8+CD57+ lymphocytosis

were associated with each other, with granulomata and with this TNF allele, suggesting that distinct subgroups of CVID do indeed exist, and that that they are genetically determined.

Associations between TNF -308A and LTα +365C alleles and the entire patient group were also observed. Unfortunately, it was impossible to determine if these were of primary importance due to the strong linkage disequilibrium between these alleles and the HLA class I and II haplotypes previously found to be associated with CVID. Thus whilst these findings were consistent with primary involvement of class III genes in CVID pathogenesis, this hypothesis was not proven. Further examination of these and other immunoregulatory genes in close proximity, is required in further cohorts.

These findings also suggested that further examination of other immunoregulatory genes outside the MHC in CVID was warranted. For example, the TNF +488A allele associated with granulomatous CVID was only present in 40% of granulomatous patients. Regulation of TNF gene expression is complex and regulated by multiple cytokines, several of which, such as IL-10, are known to be polymorphic. Genes encoding molecules mediating the effects of TNF, such as the TNF receptors, are also polymorphic. Thus whilst it is tempting to conclude that the TNF +488A association is of genuine biological significance, this data behoves thorough examination of polymorphisms in as many genes known to influence TNF production and function as possible.

To facilitate such an investigation, a PCR-SSP method was developed capable of genotyping large numbers of single nucleotide polymorphisms and deletions simultaneously. 30 polymorphisms in 14 immunoregulatory genes of theoretical relevance to the pathogenesis of CVID were genotyped, including cytokines (IL-1α, IL-1β, IL-4, -6, -10 and TGFβ), adhesion molecules (E- and L-selectin, ICAM-1 and CTLA-4), the complement component mannose binding lectin, the immunoglobulin receptor FcγRIIA, the vitamin D receptor, and the chemokine receptor CCR5.

A major association was detected that may explain one of the most enigmatic features of CVID: the variable age of onset of symptoms. Polymorphisms in the MBL gene were examined for two reasons: MBL can activate complement in an antibody-independent fashion, and thus is a potentially important innate host defence mechanism; and previous reports have detected associations between non-functional MBL alleles and infection in young children, at a time when antibody-mediated immunity is physiologically suboptimal. Six polymorphisms in the 5′ and coding regions of the gene were examined, five of which are unequivocally associated with variation in circulating levels of MBL. Both promoter and coding variants associated with low MBL levels were found to be associated with early age of onset of symptoms and early age at diagnosis, an effect particularly striking in patients carrying two copies of coding mutations. This suggests that innate host defence mechanisms play an important role in compensating for defective antigen-specific immunity.

Further analysis of cytokine polymorphisms in the granulomatous subgroup revealed an interesting interaction between the previously discussed TNF +488A allele and IL-10 polymorphisms. The IL-10 -1082a/-819t/-592a (a-t-a) haplotype was associated with granulomatous CVID, and was particularly frequent in those granulomatous patients not carrying the TNF +488A allele. This observation is important for several reasons. First, it further supports the hypothesis that granulomatous CVID is genetically determined, and that the previously detected TNF +488A association is not artefactual, or secondary to hitherto unrecognised linkage disequilibrium with other genes not involved in TNF function. Secondly, this interaction of IL-10 and TNF polymorphisms in the predisposition to inflammatory disease underscores the well-established functional interaction and counter-regulation of these two cytokines. Down-regulation of monocyte TNF production by IL-10 is well documented (de Waal Malefyt et al, 1991; Fiorentino et al, 1991a; Armstrong et al, 1996), and if high TNF levels are an essential prerequisite for the development of granulomata in CVID, it is likely that low producing IL-10 alleles (which IL-10 -1082a is known to be; Turner et al, 1997) will facilitate this process. Indeed, this appears to be the first reported example of reciprocal interaction of cytokine allelic variants in human disease.

Stratification of CVID patients by immunophenotypic criteria revealed associations between several other polymorphisms and immunophenotypic abnormalities. The vitamin D receptor gene (VDR) *Apa*I *A* and IL-6 +3247t alleles were associated with both B lymphopenia and CD8+CD57+ lymphocytosis, further supporting the hypothesis that these abnormalities are genetically determined. These findings reinforce the known role of the calcitriol axis in immunoregulation, and suggest that polymorphisms in the VDR gene influence the nature of immune responses.

Overall associations with the entire CVID patient group were weak. The only associations detected were with the IL-1α -889 promoter polymorphism: the IL-1α -889t allele was negatively associated with CVID, and the -889c/c genotype positively associated. It is possible that this association is biologically significant, but that the presence of alleles in other genes influencing the action of IL-1 is required, such as IL-1β, IL-RA and the IL-1 receptors, all of which are polymorphic. Thus further studies of these genes, in addition to confirmation if further cohorts is required.

This multiple candidate gene approach has not identified a genetic association likely to explain the genetic predisposition to CVID in all patients. Thus it may be asked, was this methodological approach valid, and was CVID an appropriate disease for its application? The answer is an unequivocal 'yes', and this assertion can be justified in several ways. The multiple associations identified with clinical and laboratory markers of more severe disease provide compelling evidence that phenotypic variability in CVID is genetically determined, and that this genetic predisposition is itself complex. Without the simultaneous analysis of multiple genes in large numbers of clinically well-stratified patients, the interaction of allelic cytokine variants would not have been detected. This analysis of CVID is thus a paradigm for the investigation of a genetically determined, clinically heterogeneous disease. This approach is also applicable to other diseases where previously identified associations are unable to explain the entire genetic diathesis, particularly where such diseases are characterised by a quantitatively or qualitatively abnormal immune response.

Indeed, the clinical heterogeneity of CVID, and the complex interaction of immunoregulatory molecules provides an explanation as to why the current study has not 'solved' the aetiology of CVID. The intricate regulation of the humoral immune response has been repeatedly stressed. Whilst this study has attempted to genotype as many known theoretically relevant polymorphisms as possible, comprehensive knowledge of DNA polymorphism, as well as the functional relevance of many known polymorphisms, is lacking. Further candidates will also be suggested by the results of the European collaborative genome screen of CVID/IgAD-multiplex families currently in progress. This investigation using hundreds microsatellite markers spread throughout the genome arose from a preliminary study, also using microsatellites, that examined linkage to the MHC in a 16 families with autosomal dominant transmission of IgAD or CVID (Vorechovsky et al, 1995b). No MHC linkage was detected, in contrast to previous linkage studies in IgAD using HLA class II alleles, rather than microsatellites as markers in a sib-pair analysis (Olerup et al, 1992). There are two possible explanations for this discrepancy. The first is that familial CVID/IgAD represents a distinct, non-HLA associated disorder. The second is that the MHC polymorphisms do confer susceptibility, but that this is only detected by population-based case-control association studies but not family linkage analysis. Resolution of this issue awaits the findings of the genome-wide study, and the investigation of any identified candidate regions of the genome in both families and sporadic cases of CVID. Thus this study, whilst of enormous interest in its own right, can also be viewed as a prelude to more comprehensive investigation of immunoregulatory gene polymorphism on a truly Wagnerian scale.

Investigation of this rare condition has also shed valuable light on the regulation of the immune response. For example, the interacting TNF and IL-10 associations reinforce the importance of cytokine balance in determining the outcome of cellular activation, and also highlight the regulatory role of these molecules in human disease. The associations of vitamin D receptor and mannose-binding lectin polymorphisms with phenotypic variability that have

been identified add credence to the ostensible role of these molecules in immunity and immunopathology.

The novel associations described in this thesis notwithstanding, it is impossible to propose hypotheses explaining the fundamental aetiology of CVID that are anything more than speculative. It is likely that the pathogenesis of this condition is multifactorial, and involves genetic, environmental and stochastic elements. A tripartite approach will be required to definitively unravel the pathogenesis of this elusive condition: thorough genome-wide examination of the familial form(s) of the condition; judicious examination of candidate gene polymorphisms suggested by genetic or functional studies; and very importantly, thorough and intensive collection of epidemiological and clinical information. Accurate retrospective data collection will be essential to evaluate the role, if any, of environmental agents in the aetiology of CVID. Without rigorous classification of patients based upon meticulous characterisation of disease phenotype, it will not be possible to determine whether the aetiology of hypogammaglobulinaemia in each subgroup is different, or if genetic factors influence phenotype but not antibody deficiency. However, this study provides compelling evidence that CVID does consist of multiple, genetically determined subgroups.

8. Appendices

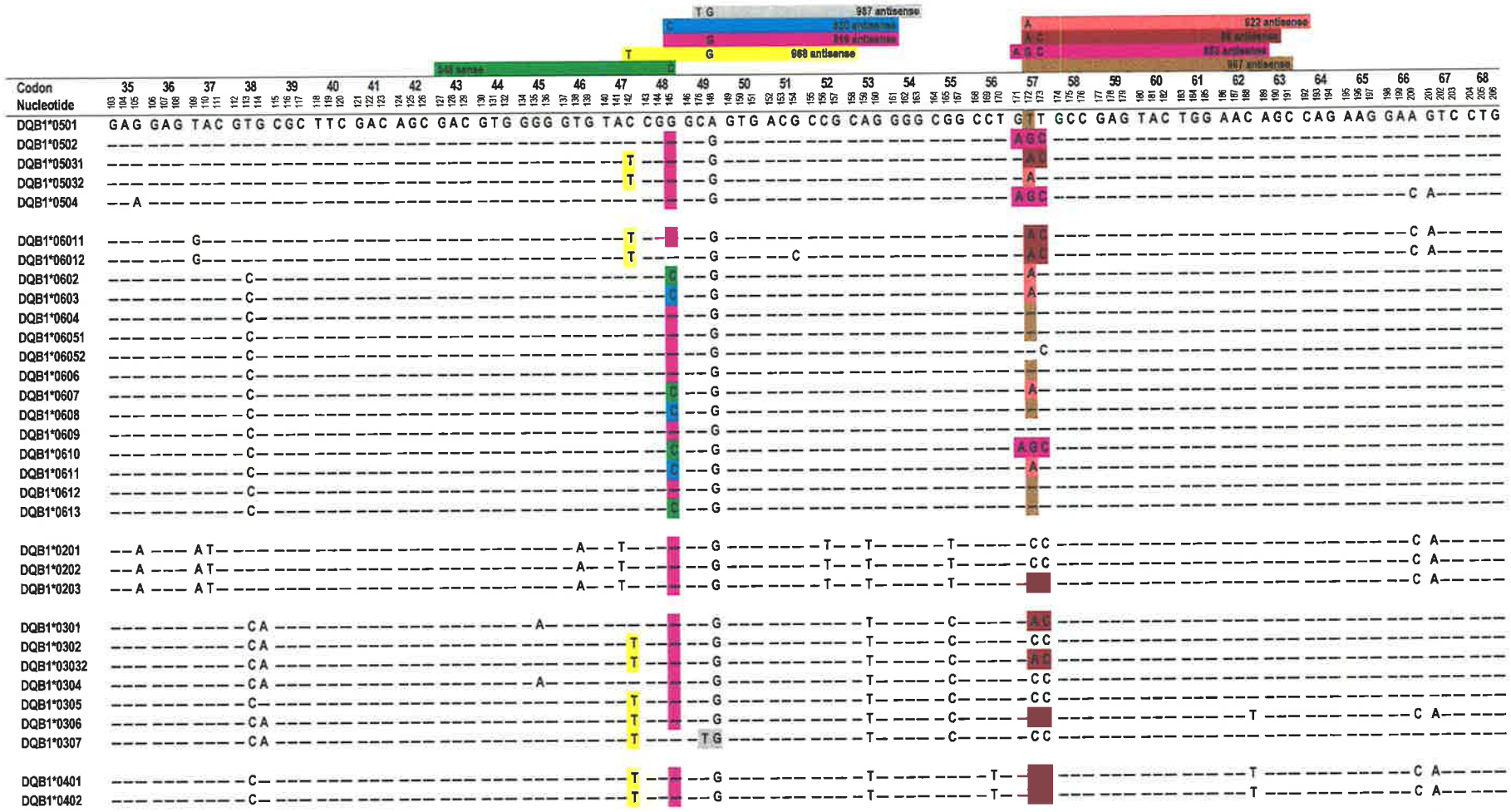
8.1. HLA-DQB1 nucleotide sequence alignment and primer annealing positions.

8.2. Stratification of the HLA class I and II haplotype associations with CVID.

8.3. Comprehensive listing of significant HLA-A, -B, -CW, -DRB1, and -DQB1 amino acid associations with CVID.

8.4. Correlations between immunophenotypic parameters.

8.5. Additional genes examined by PCR-SSP.



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C
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G
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T
G
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C
A
922 antisense
A C
A G C
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Codon
Nucleotide
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DQB1*05032
DQB1*0504
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DQB1*06012
DQB1*0602
DQB1*0603
DQB1*0604
DQB1*06051
DQB1*06052
DQB1*0606
DQB1*0607
DQB1*0608
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DQB1*0613
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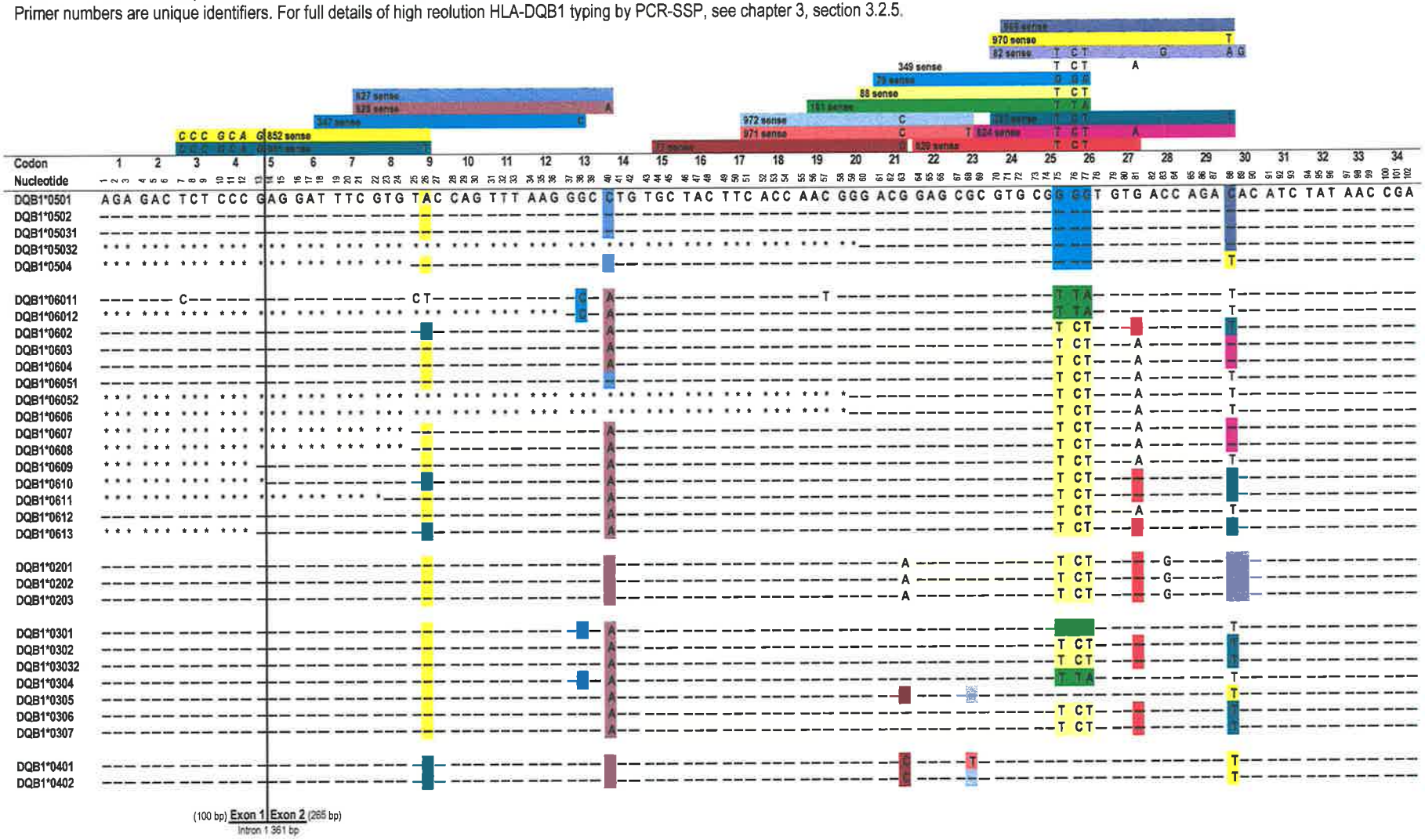
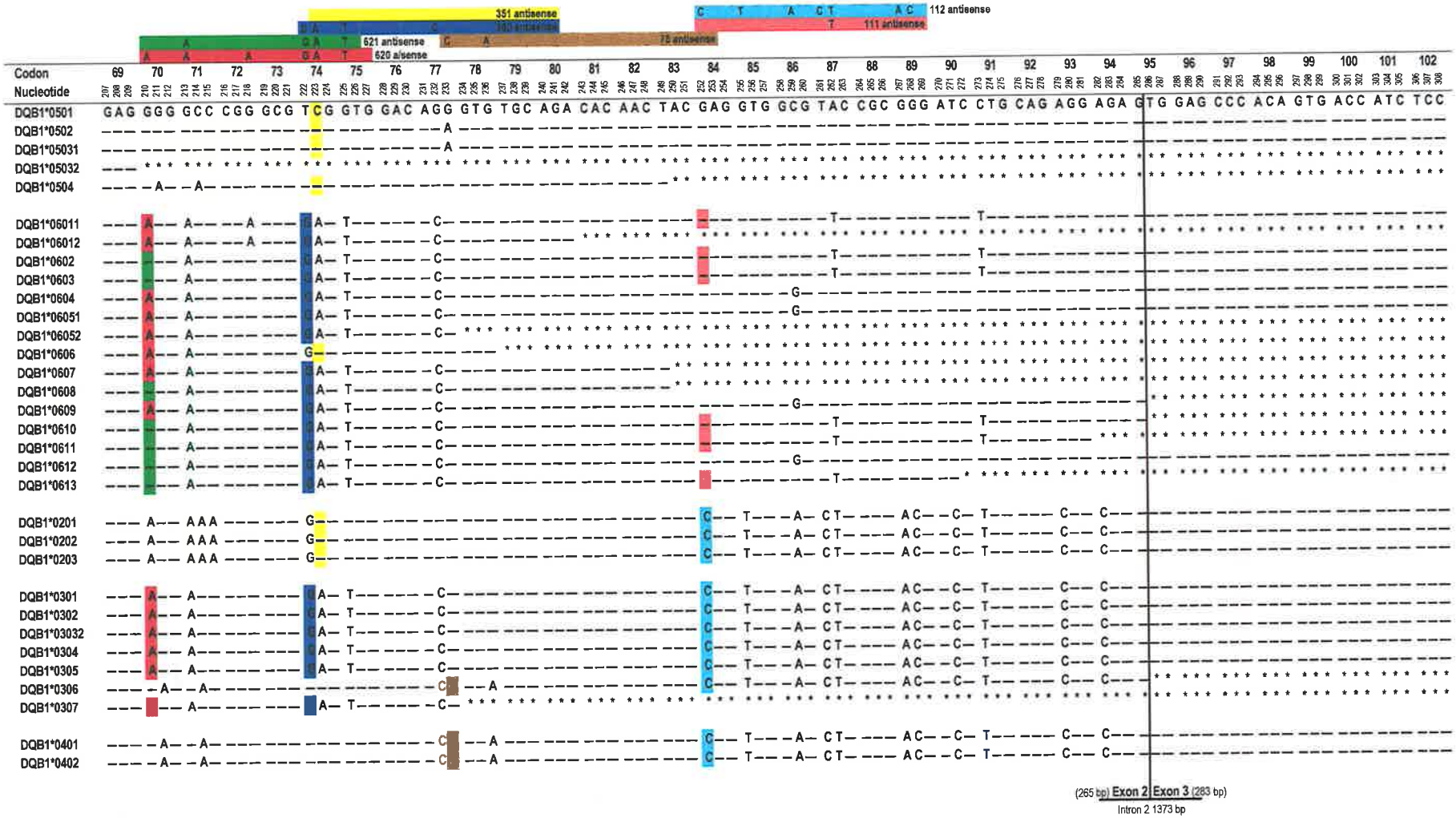
DQB1*0202
DQB1*0203
DQB1*0301
DQB1*0302
DQB1*03032
DQB1*0304
DQB1*0305
DQB1*0306
DQB1*0307
DQB1*0401
DQB1*0402

Appendix 8.1: HLA-DQB1 nucleotide sequence alignment and SSP primer annealing positions

Sequences are compared to the DQB1*0501 cDNA nucleotide sequence (identical bases are denoted by dashes).
Annealing positions are shown by coloured bars. Nucleotides determining primer specificity are highlighted in colours corresponding to the primer in question.
The first 7 bases of sense primers 851 and 852 anneal to intron 1, and are denoted by italics
Primer numbers are unique identifiers. For full details of high reolution HLA-DQB1 typing by PCR-SSP, see chapter 3, section 3.2.5.

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Nucleotide
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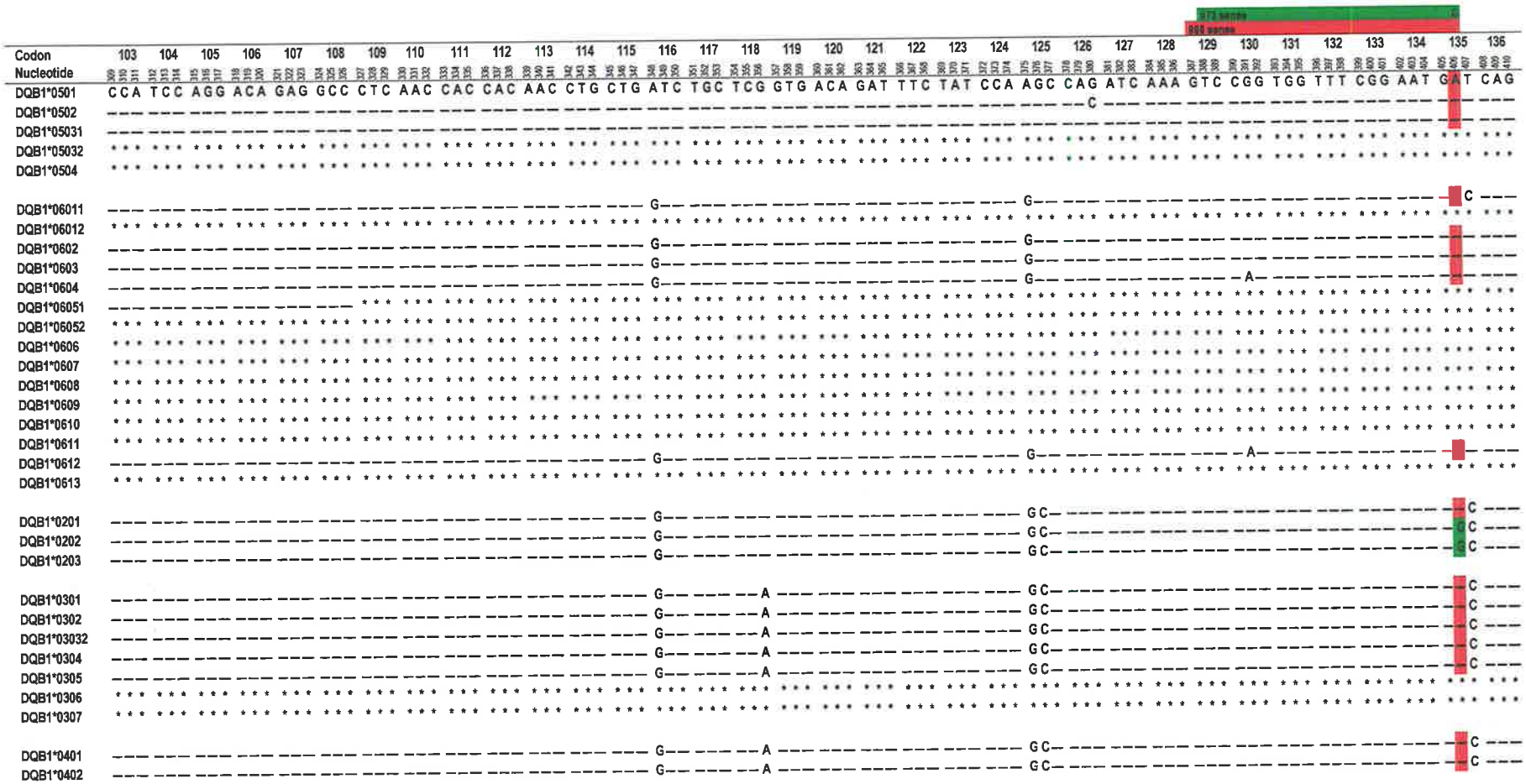
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DQB1*0501
DQB1*0502
DQB1*05031
DQB1*05032
DQB1*0504
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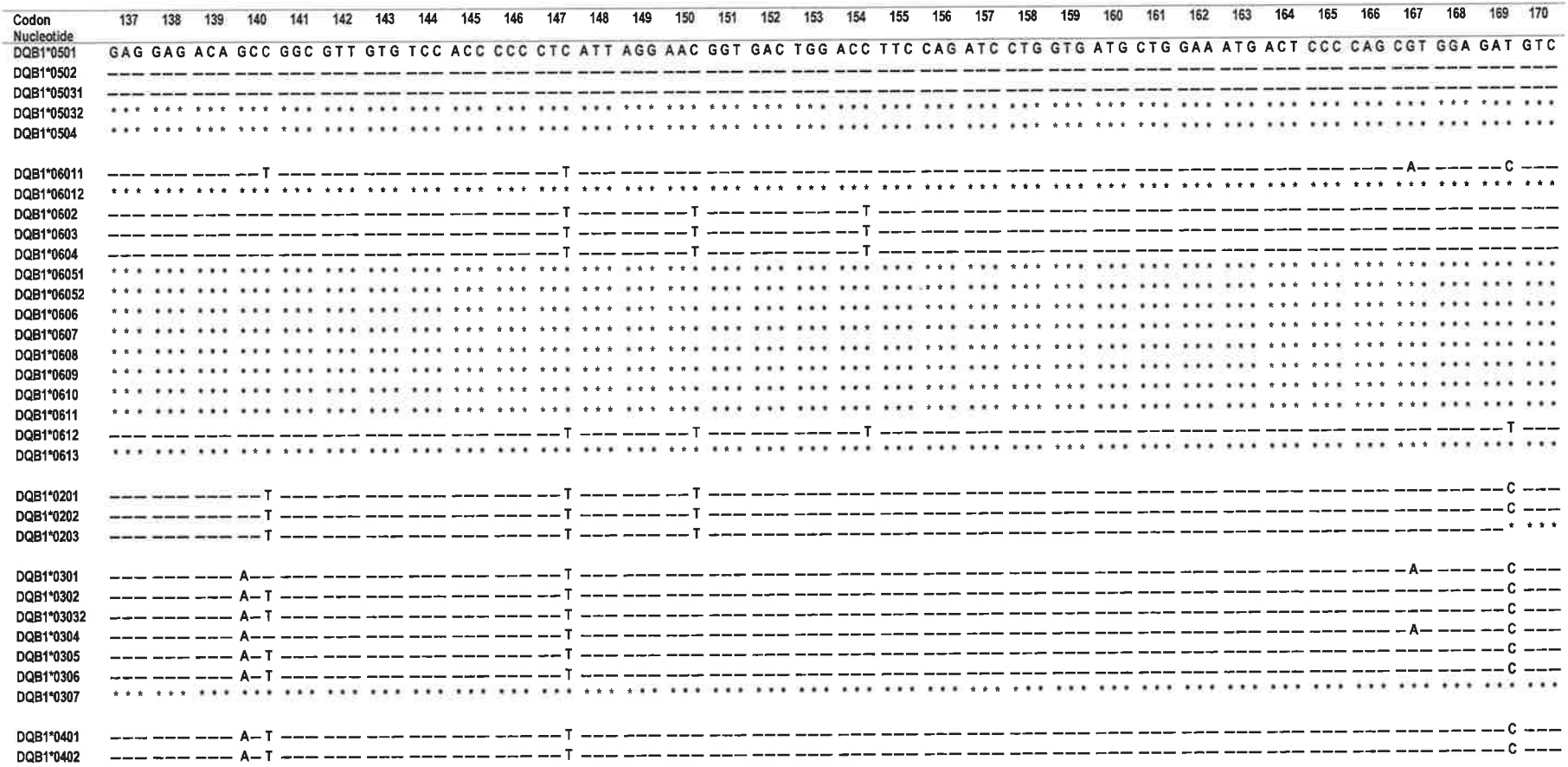
DQB1*0402


Codon
Nucleotide
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DQB1*05031
DQB1*05032
DQB1*0504
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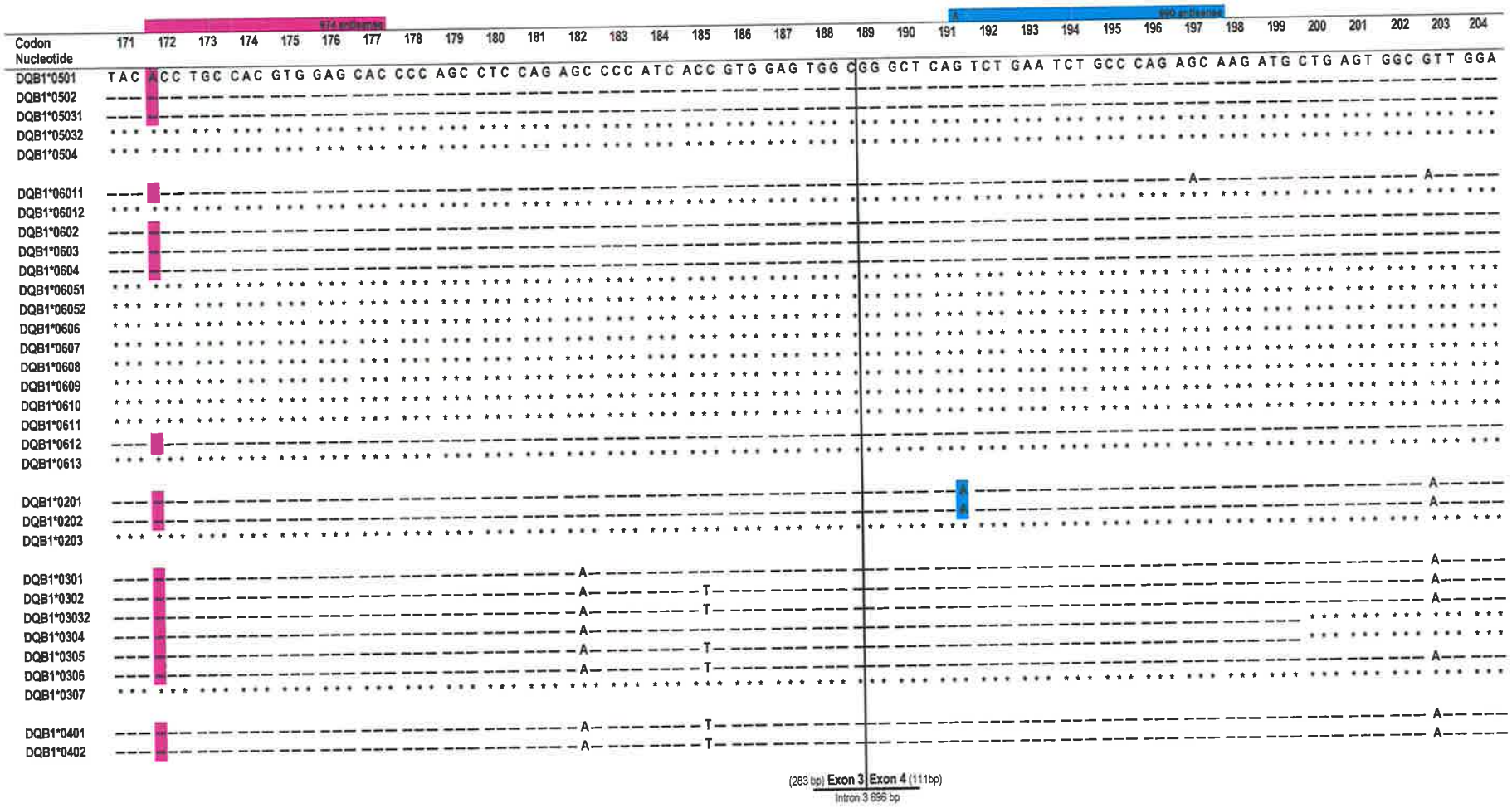
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890 endonuclease
Codon
Nucleotide
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DQB1*0305
DQB1*0306
DQB1*0307
DQB1*0401
DQB1*0402
(283 bp) Exon 3 | Exon 4 (111bp)
Intron 3 696 bp

8.2. STRATIFICATION OF THE HLA CLASS I AND II HAPLOTYPE ASSOCIATIONS WITH CVID

Table 8.1: Summary of analysis of the "A1-Cw7-B8-DR3(17)-DQ2" association with CVID

The comparisons in columns [1] to [10] are fully described in chapter 3. For example, the test in row 1, column[3], compares the frequency of A*01 in Cw*0701-positive patients and controls (hence the abbreviation +/+ v. -/+).

No allele is significantly associated with CVID in the absence of any other linked alleles (columns [3] - [6]). Consequently there are no detectable differences in strength of associations between alleles ([7]). This is at least partly due to the extremely strong linkage disequilibrium between alleles on this haplotype ([9] and [10]). ns = not significant.

Comparison			Individual association		Independent X-association		Independent Y-association		Difference between X & Y associations	Combined association	Association between X and Y	
Factor X	Factor Y		[1] X	[2] Y	[3] +/+ v. -/+	[4] +/- v. -/-	[5] +/+ v. +/-	[6] -/+ v. -/-	[7] +/- v. -/+	[8] +/+ v. -/-	[9] CVID	[10] controls
A1	Cw*0701	RR	1.45	1.66	2.4	0.7	2.7	0.8	0.9	2	18.60	5.70
		χ^2	4.20	7.97	6.74	1.73	11.23	0.70	0.23	11.14	67.58	88.10
		χ^2 p	0.04	0.005	0.009	ns	0.0008	ns	ns	0.0008	$2x10^{-16}$	$6x10^{-21}$
A1	B8	RR	1.45	1.69	2.00	0.94	2.04	0.96	0.98	1.91	20.73	9.71
		χ^2	4.20	7.82	2.70	0.18	6.30	0.16	0.04	9.69	65.98	128.29
		χ^2 p	0.04	0.005	ns	ns	0.01	ns	ns	0.002	$5x10^{-16}$	$1x10^{-29}$
A1	DR3	RR	1.45	1.48	2.66	0.95	2.05	0.73	1.30	1.95	11.00	3.92
		χ^2	4.20	4.15	7.26	0.14	6.83	1.37	0.33	9.22	45.38	51.71
		χ^2 p	0.04	0.04	0.007	ns	0.009	ns	ns	0.002	$2x10^{-11}$	$6x10^{-13}$
A1	DQ2	RR	1.45	1.57	1.58	1.07	1.87	1.26	0.85	1.99	3.39	2.29
		χ^2	4.20	6.70	3.22	0.00	4.64	0.76	0.55	9.58	14.42	22.55
		χ^2 p	0.04	0.01	0.07	ns	0.03	ns	ns	0.002	$1x10^{-04}$	$2x10^{-06}$
Cw*0701	B8	RR	1.66	1.69	0.77	1.47	1.19	2.27	0.65	1.74	189.81	363.32
		χ^2	7.97	7.82	0.41	0.79	0.13	0.10	0.76	8.27	120.19	421.22
		χ^2 p	0.005	0.005	0.52	ns	ns	ns	ns	0.004	$6x10^{-28}$	$1x10^{-93}$
Cw*0701	DR3	RR	1.66	1.48	2.10	1.37	1.31	0.86	1.60	1.79	26.99	17.56
		χ^2	7.97	4.15	2.91	0.93	0.66	0.47	0.77	7.95	74.15	199.83
		χ^2 p	0.005	0.04	0.09	ns	ns	ns	ns	0.005	$7x10^{-18}$	$2x10^{-45}$
Cw*0701	DQ2	RR	1.66	1.57	1.71	1.13	1.78	1.18	0.96	2.02	8.30	5.51
		χ^2	7.97	6.70	4.35	0.02	2.69	0.34	0.12	11.30	37.94	83.69
		χ^2 p	0.005	0.01	0.04	0.88	ns	ns	ns	0.001	$7x10^{-10}$	$6x10^{-20}$
B8	DR3	RR	1.69	1.48	1.90	1.46	1.20	0.92	1.59	1.74	47.08	35.98
		χ^2	7.82	4.15	2.32	0.66	0.12	0.27	0.51	7.27	95.43	277.60
		χ^2 p	0.005	0.04	0.13	ns	ns	ns	ns	0.007	$2x10^{-22}$	$3x10^{-62}$
B8	DQ2	RR	1.69	1.57	1.53	1.25	1.56	1.27	0.98	1.94	14.85	12.14
		χ^2	7.82	6.70	2.70	0.03	0.75	0.92	0.13	10.08	48.09	135.26
		χ^2 p	0.005	0.01	ns	ns	ns	ns	ns	0.001	$4x10^{-12}$	$3x10^{-31}$
DR3	DQ2	RR	1.48	1.57	1.06	4.22	0.39	1.53	2.75	1.63	91.16	361.79
		χ^2	4.15	6.70	0.02	0.04	2.21	3.00	0.00	5.86	71.74	279.55
		χ^2 p	0.04	0.01	0.88	ns	ns	0.08	ns	2E-02	$2x10^{-17}$	$9x10^{-63}$

Table 8.2: Summary of analysis of the "A2-Cw7-B7-DR15-DQ6" association

The first 4 pairs of analyses, with A*02, show that the alleles Cw*0702, B7, DRB1*1501 and DQB1*0602 are significantly associated with CVID in the absence of A*02 (columns [3] - [6]). Reflecting this, these alleles are more strongly associated with CVID than A*02 (column [7]). However it is not possible to determine which of the remaining alleles, if any, are more strongly associated with CVID; as with the A1-B8-DR3 haplotype, this is at partly due to the extremely strong linkage disequilibrium between alleles on this haplotype ([9] and [10]). ns = not significant.

Comparison			Individual association		Independent X-association		Independent Y-association		Difference between X & Y associations	Combined association	Association between X & Y	
Factor X	Factor Y		[1] X	[2] Y	[3] ++ v. -+	[4] +- v. --	[5] ++ v. +-	[6] -+ v. --	[7] +- v. -+	[8] ++ v. --	[9] CVID	[10] controls
A*02	Cw*0702	RR	0.72	0.39	0.71	0.69	0.39	0.38	1.81	0.27	0.77	0.74
		χ^2	4.01	17.51	1.03	4.42	7.24	12.02	3.42	14.44	0.73	3.10
		χ^2p	0.05	0.00003	ns	0.04	0.01	0.0005	0.06	0.0001	ns	0.08
A*02	B*07	RR	0.72	0.43	0.64	0.71	0.40	0.44	1.60	0.28	0.71	0.78
		χ^2	4.01	14.39	1.55	3.78	6.97	8.93	2.17	13.37	1.04	2.03
		χ^2p	0.05	0.0001	ns	0.05	0.01	0.003	ns	0.0003	ns	ns
A*02	DRB1*15	RR	0.72	0.39	1.38	0.65	0.59	0.28	2.34	0.38	2.05	0.96
		χ^2	4.01	17.51	0.28	5.77	3.34	16.51	5.49	10.84	2.01	0.09
		χ^2p	0.05	0.00003	ns	0.02	0.07	0.00005	0.02	0.001	ns	ns
A*02	DQB1*0602	RR	0.72	0.40	2.06	0.60	0.75	0.22	2.73	0.45	2.82	0.82
		χ^2	4.01	16.02	1.93	7.97	1.18	20.18	6.70	7.72	4.40	1.35
		χ^2p	0.05	0.00006	ns	0.005	ns	0.00001	0.01	0.005	0.04	ns
Cw*0702	B*07	RR	0.39	0.43	0.16	1.08	0.32	2.18	0.50	0.34	77.88	538.40
		χ^2	17.51	14.39	15.14	0.05	5.69	1.25	1.88	19.11	89.31	494.92
		χ^2p	0.00003	0.0001	0.0001	ns	0.02	ns	ns	0.00001	$3x10^{-21}$	$1x10^{-109}$
Cw*0702	DRB1*15	RR	0.39	0.39	0.55	0.47	0.55	0.47	1.00	0.26	6.44	5.49
		χ^2	17.51	17.51	2.49	7.30	2.49	7.30	0.04	17.55	13.70	78.99
		χ^2p	0.00003	0.00003	ns	0.01	ns	0.007	ns	0.00003	0.0002	$6x10^{-19}$
Cw*0702	DQB1*0602	RR	0.39	0.40	0.45	0.51	0.48	0.54	0.95	0.24	5.39	6.11
		χ^2	17.51	16.02	4.06	6.23	3.66	5.02	0.11	18.21	10.11	87.47
		χ^2p	0.00003	0.00006	0.04	0.01	0.06	0.03	ns	0.00002	0.001	$9x10^{-21}$
B*07	DRB1*15	RR	0.43	0.39	0.64	0.53	0.55	0.46	1.17	0.29	7.64	6.30
		χ^2	14.39	17.51	1.54	5.29	2.59	7.68	0.03	15.87	17.69	90.94
		χ^2p	0.0001	0.00003	ns	0.02	ns	0.006	ns	0.00007	0.00003	$1x10^{-21}$
B*07	DQB1*0602	RR	0.43	0.40	0.63	0.53	0.57	0.48	1.11	0.30	8.31	7.02
		χ^2	14.39	16.02	1.62	5.30	2.41	6.41	0.00	15.21	19.15	99.94
		χ^2p	0.0001	0.00006	ns	0.02	ns	0.01	ns	0.0001	0.00001	$2x10^{-23}$
DRB1*15	DQB1*0602	RR	0.39	0.40	0.14	0.67	0.57	2.81	0.24	0.38	891.53	4392.71
		χ^2	17.51	16.02	7.48	1.16	0.93	0.00	3.98	17.20	144.55	546.48
		χ^2p	0.00003	0.00006	0.006	ns	ns	ns	0.05	0.00003	$3x10^{-33}$	$7x10^{-121}$

Table 8.3: Summary of analysis of the B*14 containing haplotypic associations

Several alleles not associated individually (DR7 and DR1) have been included in the analysis as they are associated with CVID in combination with other alleles (eg. DR7-DQ2 is positively associated with CVID). From this data, it can be concluded that B*14 is more strongly associated with CVID than several linked alleles, such as A28, DR7 and DQ2 (although the p values for these latter 2 analyses in [7] do not quite reach significance). B14 is also associated with CVID in the absence of A1 (analysis 1, column [6]), but this is not reflected in column [7] as a significant difference between the 2 associations, as A1 is also associated with CVID as part of the A1-B8-DR3 haplotype. It is not possible to determine of B*14 or Cw*0802 is more strongly associated with CVID.

Comparison			Individual association		Independent X-association		Independent Y-association		Difference between X & Y associations	Combined association	Association between X & Y	
Factor X	Factor Y		[1] X	[2] Y	[3] ++ v. -+	[4] +- v. --	[5] ++ v. +-	[6] -+ v. --	[7] +- v. -+	[8] ++ v. --	[9] CVID	[10] controls
A*01	B*14	RR	1.45	2.03	1.54	1.48	2.18	2.10	0.71	3.24	0.67	0.65
		χ^2	4.20	6.46	0.30	4.20	2.11	4.30	1.40	5.73	1.34	2.25
		χ^2p	0.04	0.007	ns	0.04	ns	0.04	ns	0.02	ns	ns
A*28	B*14	RR	0.86	2.03	1.54	0.65	4.47	1.88	0.34	2.90	4.78	2.01
		χ^2	0.45	6.46	0.05	1.71	2.45	4.20	6.00	1.45	3.90	0.90
		χ^2p	ns	0.007	ns	ns	ns	0.04	0.01	ns	0.05	ns
Cw*0802	B*14	RR	2.03	2.03	0.55	3.64	0.55	3.64	1.00	2.01	1671.4	11065.7
		χ^2	6.46	6.46	1.38	0.01	1.38	0.01	1.0	6.0	154.90	562.21
		χ^2p	0.01	0.007	ns	ns	ns	ns	ns	0.01	$1x10^{-35}$	$3x10^{-124}$
Cw*0802	DR*0701	RR	2.03	1.29	1.90	2.03	1.15	1.23	1.65	2.34	2.23	2.38
		χ^2	6.46	1.63	1.86	3.10	0.001	0.83	1.17	4.11	2.68	6.96
		χ^2p	0.01	ns	ns	0.08	ns	ns	ns	0.04	ns	0.008
Cw*0802	DQB1*0201	RR	2.03	1.57	1.61	2.54	1.03	1.64	1.55	2.63	0.92	1.46
		χ^2	6.46	6.70	1.12	5.08	0.03	6.90	0.85	5.94	0.15	1.18
		χ^2p	0.01	0.01	ns	0.02	ns	0.009	ns	0.01	ns	ns
B*14	DRB1*01	RR	2.03	1.03	2.30	1.99	1.18	1.02	1.94	2.35	1.10	0.95
		χ^2	6.46	0.00	1.04	4.68	0.01	0.00	3.10	1.26	0.04	0.20
		χ^2p	0.007	ns	ns	0.03	ns	ns	0.08	ns	ns	ns
B*14	DR*0701	RR	2.03	1.29	2.09	1.86	1.34	1.19	1.56	2.50	2.68	2.38
		χ^2	6.46	1.63	2.77	2.12	0.13	0.56	0.81	5.24	4.41	6.96
		χ^2p	0.007	0.20	0.10	ns	ns	ns	ns	0.02	0.04	0.01
B*14	DQB1*0201	RR	2.03	1.57	1.75	2.31	1.21	1.60	1.45	2.79	1.11	1.46
		χ^2	6.46	6.70	1.77	3.66	0.02	6.17	0.48	7.17	0.00	1.18
		χ^2p	0.007	0.01	ns	0.06	ns	0.01	ns	0.01	ns	ns
DR*0701	DQB1*0201	RR	1.29	1.57	1.23	0.73	2.32	1.37	0.53	1.69	11.08	6.56
		χ^2	1.63	6.70	0.56	1.12	3.53	1.88	3.09	5.67	37.09	91.51
		χ^2p	ns	0.01	ns	ns	0.06	ns	0.08	0.02	$1x10^{-09}$	$1x10^{-21}$

8.3. COMPREHENSIVE LISTING OF SIGNIFICANT HLA-A, -B, -CW, -DRB1, AND -DQB1 AMINO ACID ASSOCIATIONS WITH CVID

Result are listed by locus, and are divided into 2 groups, each of which is preceded by a summary of codons involved in the most significant associations (in descending order of significance). The first group of results for each locus ("1. Individual amino acids") lists individual amino acid associations. For example, in HLA-DQB1, 124 of 358 CVID chromosomes have aspartate (D) at codon 57, compared with 552 control chromosomes. These frequencies differ significantly between patients and controls ($p<0.01$) (table 8.4).

Table 8.4: HLA-DQB1 codon 57 individual amino acid analysis

codon: 57	χ^2: 16.046	p<0.001
aa : D	CVID: 124 (35%)	cont: 552 (46%)
aa : A	CVID: 160 (45%)	cont: 414 (34%)
aa : V	CVID: 70 (19%)	cont: 232 (19%)

The second group of results ("2. Amino acid combinations") compares frequencies all possible combinations of amino acids at each polymophic locus. Each combination of amino acids is referred to as a "set". For example, again examining DQB1 codon 57:

Table 8.5: HLA-DQB1 codon 57 amino acid combination analysis

set A	χ^2: 4.974	p=0.02
	Controls: 312	CVID: 105
set D	χ^2: 11.055	p=0.0009
	Controls: 353	CVID: 78
set A D	χ^2: 0.673	p=0.42
set V	χ^2: 0.044	p=0.89
set A V	χ^2: 3.786	p=0.05
	Controls: 435	CVID: 135
set D V	χ^2: 9.301	p=0.002
	Controls: 450	CVID: 112

Thus, amino acids aspartate (D) OR valine (V) are significantly more frequent in controls than patients (p=0.002). Numbers of patients and controls positive for each amino acid or combination of amino acids are only given for significant associations ($p<0.05$). The amino acid code is provided below (table 8.6).

Table 8.6: The amino acid code

Amino acid	Single letter code
alanine	A
cysteine	C
aspartic acid	D
glutamic acid	E
phenylalanine	F
glycine	G
histidine	H
isoleucine	I
lysine	K
leucine	L
methionine	M
asparagine	N
proline	P
glutamine	Q
arginine	R
serine	S
threonine	T
valine	V
tryptophan	W
tyrosine	Y

HLA-A: all patients v. controls

1. Individual amino acids

Summary of most significant associations (p value codon)

0.009 166, 167
0.01 67, 150, 158
0.01 163
0.01 90
0.02 105
0.02 76
0.02 77
0.04 142, 145
0.04 156
0.05 74, 107
0.09 127
0.11 193, 194, 207, 253
0.14 95
0.14 184
0.15 66

Details of associations at all polymorphic codons

(counts are as number of chromosomes)

Codon 44 df: 1 χ^2: 6.509 p=0.01
aa: K CVID: 94 (26%), cont: 243 (20%)
aa: R CVID: 262 (73%), cont: 967 (79%)

Codon 67 df: 1 χ^2: 6.509 p=0.01
aa: V CVID: 262 (73%), cont: 967 (79%)
aa: M CVID: 94 (26%), cont: 243 (20%)

Codon 74 df: 1 χ^2: 3.831 p=0.05
aa: D CVID: 271 (76%), cont: 857 (70%)
aa: H CVID: 85 (23%), cont: 353 (29%)

Codon 76 df: 2 χ^2: 7.427 p=0.03
aa: A CVID: 117 (32%), cont: 312 (25%)
aa: V CVID: 191 (53%), cont: 736 (60%)
aa: E CVID: 48 (13%), cont: 162 (13%)

Codon 77 df: 2 χ^2: 7.68 p=0.02
aa: N CVID: 150 (42%), cont: 413 (34%)
aa: S CVID: 15 (4%), cont: 61 (5%)
aa: D CVID: 191 (53%), cont: 736 (60%)

Codon 90 df: 1 χ^2: 5.949 p=0.01
aa: D CVID: 133 (37%), cont: 369 (30%)
aa: A CVID: 223 (62%), cont: 841 (69%)

Codon 95 df: 2 χ^2: 3.862 p=0.15
aa: L CVID: 33 (9%), cont: 101 (8%)
aa: I CVID: 238 (66%), cont: 756 (62%)
aa: V CVID: 85 (23%), cont: 353 (29%)

Codon 105 df: 1 χ^2: 4.789 p=0.03
aa: P CVID: 144 (40%), cont: 413 (34%)
aa: S CVID: 212 (59%), cont: 797 (65%)

Codon 107 df: 1 χ^2: 3.831 p=0.05
aa: G CVID: 271 (76%), cont: 857 (70%)
aa: W CVID: 85 (23%), cont: 353 (29%)

Codon 127 df: 1 χ^2: 2.726 p=0.01
aa: N CVID: 228 (64%), cont: 716 (59%)
aa: K CVID: 128 (35%), cont: 494 (40%)

Codon 142 df: 1 χ^2: 4.304 p=0.04
aa: I CVID: 261 (73%), cont: 817 (67%)
aa: T CVID: 95 (26%), cont: 393 (32%)

Codon 145 df: 1 χ^2: 4.304 p=0.04
aa: R CVID: 261 (73%), cont: 817 (67%)
aa: H CVID: 95 (26%), cont: 393 (32%)

Codon 150 df: 1 χ^2: 6.509 p=0.01
aa: A CVID: 262 (73%), cont: 967 (79%)
aa: V CVID: 94 (26%), cont: 243 (20%)

Codon 156 df: 3 χ^2: 8.109 p=0.04
aa: L CVID: 184 (51%), cont: 711 (58%)
aa: Q CVID: 49 (13%), cont: 172 (14%)
aa: W CVID: 29 (8%), cont: 84 (6%)
aa: R CVID: 94 (26%), cont: 243 (20%)

Codon 158 df: 1 χ^2: 6.509 p=0.02
aa: A CVID: 262 (73%), cont: 967 (79%)
aa: V CVID: 94 (26%), cont: 243 (20%)

Codon 163 df: 1 χ^2: 5.622 p=0.02
aa: T CVID: 224 (62%), cont: 842 (69%)
aa: R CVID: 132 (37%), cont: 368 (30%)

Codon 166 df: 1 χ^2: 6.864 p=0.009
aa: D CVID: 127 (35%), cont: 344 (28%)
aa: E CVID: 229 (64%), cont: 866 (71%)

Codon 167 df: 1 χ^2: 6.864 p=0.009
aa: G CVID: 127 (35%), cont: 344 (28%)
aa: W CVID: 229 (64%), cont: 866 (71%)

Codon 184 df: 1 χ^2: 2.124 p=0.15
aa: S CVID: 217 (60%), cont: 685 (56%)
aa: A CVID: 139 (39%), cont: 525 (43%)

Codon 193 df: 1 χ^2: 2.45 p=0.12
aa: S CVID: 203 (57%), cont: 633 (52%)
aa: A CVID: 153 (42%), cont: 577 (47%)

Codon 194 df: 1 χ^2: 2.45 p=0.12
aa: I CVID: 203 (57%), cont: 633 (52%)
aa: V CVID: 153 (42%), cont: 577 (47%)

Codon 207 df: 1 χ^2: 2.45 p=0.12
aa: S CVID: 153 (42%), cont: 577 (47%)
aa: V CVID: 203 (57%), cont: 633 (52%)

Codon 236 df: 0 χ^2: 0.0 p=1.0
Codon 245 df: 1 χ^2: 0.219 p=0.64
Codon 246 df: 1 χ^2: 0.248 p=0.62

Codon 253 df: 1 χ^2: 2.45 p=0.11
aa: Q CVID: 153 (42%), cont: 577 (47%)
aa: T CVID: 203 (57%), cont: 633 (52%)

2. Amino acid combinations

Summary of most significant associations (p value, codon, amino acid combination)

0.003 77 set (D S)
0.004 152 set (V E)
0.004 90 set (A)
0.006 156 set (L)
0.006 163 set (T)
0.007 156 set (W L)
0.009 152 set (W V E)
0.009 166 set (E), 167 set (W)
0.01 152 set (V)
0.02 105 set (S)
0.02 76 set (V), 77 set (D)
0.02 152 set (W V)
0.02 76 set (A)
0.03 156 set (W R)
0.04 156 set (L Q)
0.04 44 set (K), 67 set (M), 150 set (V), 156 set (R), 158 set (V)
0.04 142 set (T), 145 set (H)
0.04 97 set (M R)

Details of associations at all polymorphic codons

Codon 44
set K χ^2: 4.434 p=0.04
Controls: 217 CVID: 80
set R χ^2: 2.912 p=0.09
Controls: 579 CVID: 164

Codon 62
set G χ^2: 3.147 p=0.07
Controls: 296 CVID: 73
set E G χ^2: 2.905 p=0.08
Controls: 365 CVID: 94
set G E L χ^2: 2.82 p=0.09
Controls: 394 CVID: 103

Codon 67
set M χ^2: 4.434 p=0.03
Controls: 217 CVID: 80
set V χ^2: 2.912 p=0.08
Controls: 579 CVID: 164

Codon 73
set T χ^2: 3.403 p=0.06
Controls: 605, CVID: 177

Codon 74
set H χ^2: 3.147 p=0.07
Controls: 296 CVID: 73

Codon 76
set A χ^2: 5.101 p=0.02
Controls: 266 CVID: 96
set V χ^2: 5.641 p=0.01
Controls: 508 CVID: 135

Codon 77
set D χ^2: 5.641 p=0.01
Controls: 508 CVID: 135
set D S χ^2: 8.729 p=0.004
Controls: 534 CVID: 141

Codon 90
set A χ^2: 8.218 p=0.004
Controls: 551 CVID: 148

Codon 95
set V χ^2: 3.147 p=0.07
Controls: 296 CVID: 73
set L V χ^2: 2.905 p=0.08
Controls: 365 CVID: 94

Codon 97
set M R χ^2: 4.363 p=0.03
Controls: 488 CVID: 130

Codon 105
set S χ^2: 5.889 p=0.01
Controls: 537 CVID: 145

Codon 107
set W χ^2: 3.147 p=0.07
Controls: 296 CVID: 73

Codon 114
set H χ^2: 2.905 p=0.08
Controls: 365 CVID: 94
set H Q χ^2: 3.213 p=0.07
Controls: 448 CVID: 119

Codon 127
set K χ^2: 3.725 p=0.05
Controls: 387 CVID: 99

Codon 142
set T χ^2: 4.333 p=0.03
Controls: 321 CVID: 78

Codon 145
set H χ^2: 4.333 p=0.03
Controls: 321 CVID: 78

Codon 149
set A χ^2: 3.403 p=0.06
Controls: 605, CVID: 177

Codon 150
set A χ^2: 2.912 p=0.08
Controls: 579 CVID: 164
set V χ^2: 4.434 p=0.03
Controls: 217 CVID: 80

Codon 152
set V χ^2: 6.743 p<0.001
Controls: 468 CVID: 120
set V E χ^2: 8.192 p=0.004
Controls: 556 CVID: 150
set W V χ^2: 5.274 p=0.02
Controls: 486 CVID: 128
set W V E χ^2: 6.799 p=0.009
Controls: 563 CVID: 154
set A V χ^2: 0.085 p=0.78

Codon 156
set R χ^2: 4.434 p=0.03
Controls: 217 CVID: 80
set W R χ^2: 4.6 p=0.03
Controls: 279 CVID: 99
set L χ^2: 7.48 p=0.006
Controls: 500 CVID: 130
set L Q χ^2: 4.429 p=0.04
Controls: 557 CVID: 154
set W L χ^2: 7.441 p=0.007
Controls: 536 CVID: 143
set Q W L χ^2: 2.912 p=0.09
Controls: 579 CVID: 164

Codon 158
set A χ^2: 2.912 p=0.09
Controls: 579 CVID: 164
set V χ^2: 4.434 p=0.04
Controls: 217 CVID: 80

Codon 163
set T χ^2: 7.537 p=0.007
Controls: 552 CVID: 149

Codon 166
set D χ^2: 2.922 p=0.09
Controls: 297 CVID: 101
set E χ^2: 6.819 p=0.009
Controls: 558 CVID: 152

Codon 167
set G χ^2: 2.922 p=0.09
Controls: 297 CVID: 101
set W χ^2: 6.819 p=0.009
Controls: 558 CVID: 152

Codon 193
set A χ^2: 3.458 p=0.06
Controls: 440 CVID: 116

Codon 194
set V χ^2: 3.458 p=0.06
Controls: 440 CVID: 116

Codon 207
set S χ^2: 3.458 p=0.06
Controls: 440 CVID: 116

Codon 253
set Q χ^2: 3.458 p=0.06
Controls: 440 CVID: 116

HLA-C: all patients v. controls

1. Individual amino acids

Summary of most significant associations (p value, codon)

0.11 143, 170, 270

Details of associations at all polymorphic codons

Codon 143 df: 1 χ^2: 2.532 p=0.11
aa: S CVID: 4 (1%) cont: 5 (0%)
aa: T CVID: 344 (98%) cont: 1201 (99%)

Codon 170 df: 1 χ^2: 2.532 p=0.11
aa: G CVID: 4 (1%) cont: 5 (0%)
aa: R CVID: 344 (98%) cont: 1201 (99%)

Codon 270 df: 1 χ^2: 2.532 p=0.11
aa: C CVID: 4 (1%) cont: 5 (0%)
aa: S CVID: 344 (98%) cont: 1201 (99%)

2. Amino acid combinations

Summary of most significant associations (p value, codon, combination of amino acids)

0.02 9 set (F S)
0.03 9 set (S)
0.06 73 set (A)
0.06 16 set (G), 163 set (T L), 211 set (D)
0.09 90 set (D)

Details of associations at all polymorphic codons

Codon 9
set C S χ^2: 4.525 p=0.03
Controls: 226 CVID: 49
set F C S χ^2: 5.007 p=0.02
Controls: 247 CVID: 54

Codon 16
set G χ^2: 3.517 p=0.06
Controls: 548, CVID: 155

Codon 73
set T χ^2: 0.0 p=1.0
set A χ^2: 3.396 p=0.06
Controls: 471, CVID: 124

Codon 90
set D χ^2: 2.864 p=0.09
Controls: 450, CVID: 118

Codon 211
set D χ^2: 3.517 p=0.06
Controls: 548, CVID: 155

HLA-B: all patients v. controls

1. Individual amino acids

Summary of most significant associations (p value codon)

0.0001 67
0.0001 163
0.0002 9
0.0009 156
0.001 178
0.002 71
0.005 114
0.006 11
0.007 70
0.03 74
0.04 12

Details of associations at all polymorphic codons

Codon 9 df: 2 χ^2: 16.869 p=0.0002
aa: Y CVID: 217 (61%), cont: 806 (67%)
aa: D CVID: 80 (22%), cont: 164 (13%)
aa: H CVID: 57 (16%), cont: 232 (19%)

Codon 11 df: 1 χ^2: 7.611 p=0.006
aa: S CVID: 77 (21%), cont: 351 (29%)
aa: A CVID: 277 (78%), cont: 851 (70%)

Codon 12 df: 1 χ^2: 4.232 p=0.04
aa: M CVID: 264 (74%), cont: 828 (68%)
aa: V CVID: 90 (25%), cont: 374 (31%)

Codon 67 df: 4 χ^2: 23.064 p=0.0001
aa: Y CVID: 36 (10%), cont: 202 (16%)
aa: S CVID: 129 (36%), cont: 497 (41%)
aa: F CVID: 128 (36%), cont: 319 (26%)
aa: M CVID: 13 (3%), cont: 64 (5%)
aa: C CVID: 48 (13%), cont: 120 (9%)

Codon 69 df: 1 χ^2: 9.555 p=0.002
aa: A CVID: 62 (17%), cont: 306 (25%)
aa: T CVID: 292 (82%), cont: 896 (74%)

Codon 70 df: 3 χ^2: 11.736 p=0.007
aa: N CVID: 292 (82%), cont: 896 (74%)
aa: S CVID: 13 (3%), cont: 64 (5%)
aa: Q CVID: 36 (10%), cont: 202 (16%)
aa: K CVID: 13 (3%), cont: 40 (3%)

Codon 71 df: 1 χ^2: 9.555 p=0.002
aa: A CVID: 62 (17%), cont: 306 (25%)
aa: T CVID: 292 (82%), cont: 896 (74%)

Codon 74 df: 1 χ^2: 4.421 p=0.04
aa: Y CVID: 205 (57%), cont: 770 (64%)
aa: D CVID: 149 (42%), cont: 432 (35%)

Codon 114 df: 2 χ^2: 10.684 p=0.005
aa: N CVID: 182 (51%), cont: 506 (42%)
aa: D CVID: 158 (44%), cont: 655 (54%)
aa: H CVID: 14 (3%), cont: 41 (3%)

Codon 156 df: 3 χ^2: 16.643 p=0.0009
aa: L CVID: 165 (46%), cont: 546 (45%)
aa: W CVID: 13 (3%), cont: 73 (6%)
aa: D CVID: 148 (41%), cont: 409 (34%)
aa: R CVID: 28 (7%), cont: 174 (14%)

Codon 163 df: 2 χ^2: 17.991 p=0.0001
aa: L CVID: 151 (42%), cont: 539 (44%)
aa: T CVID: 137 (38%), cont: 338 (28%)
aa: E CVID: 66 (18%), cont: 325 (27%)

Codon 178 df: 1 χ^2: 10.812 p=0.001
aa: K CVID: 46 (12%), cont: 250 (20%)
aa: T CVID: 308 (87%), cont: 952 (79%)

2. Amino acid combinations

Summary of most significant associations (p value, codon, amino acid combination)

0.000007 163 set (L E)
0.00006 156 set (W R)
0.0001 114 set (D H)
0.0001 9 set (Y H)
0.0002 114 set (D)
0.0003 178 set (K)
0.0004 163 set (E)
0.0004 67 set (Y M S)
0.0004 156 set (R)
0.0005 67 set (Y S)
0.0005 67 set (F C)
0.001 67 set (Y M), 70 set (S Q)
0.001 67 set (Y M C S)
0.001 74 set (Y)
0.004 9 set (D)
0.005 97 set (R V N)
0.007 69 set (A), 70 set (Q K S), 71 set (A)
0.006 70 set (K Q)
0.001 67 set (Y C S)
0.001 67 set (Y), 70 set (Q)

Details of associations at all polymorphic codons

Codon 9
set Y χ^2: 5.472 p=0.02
CVID: 143 cont: 529
set Y H χ^2: 14.845 p=0.0001
CVID: 161 cont: 587
set D χ^2: 8.048 p=0.005
CVID: 64 cont: 150

Codon 11
set S χ^2: 6.208 p=0.01
CVID: 68 cont: 297

Codon 12
set M χ^2: 5.035 p=0.02
CVID: 168, cont: 534

Codon 45
set M χ^2: 4.257 p=0.04
CVID: 28 cont: 141
set T M χ^2: 5.775 p=0.02
CVID: 76 cont: 322
set T K M χ^2: 6.142 p=0.01
CVID: 133 cont: 503

Codon 46
set A χ^2: 4.257 p=0.04
CVID: 28 cont: 141

Codon 67
set F χ^2: 6.157 p=0.01
CVID: 101 cont: 277
set Y χ^2: 10.347 p=0.001
CVID: 32 cont: 185
set Y M χ^2: 10.765 p=0.001
CVID: 45 cont: 236
set C χ^2: 4.342 p=0.03
CVID: 47 cont: 114
set F C χ^2: 12.308 p=0.0004
CVID: 132 cont: 359
set F M C χ^2: 6.873 p=0.008
CVID: 136 cont: 397
set F S χ^2: 5.512 p=0.01
CVID: 168, cont: 532
set Y S χ^2: 11.989 p=0.0005
CVID: 124 cont: 495
set Y M S χ^2: 12.76 p=0.0003
CVID: 133 cont: 521
set Y C S χ^2: 10.584 p=0.001
CVID: 143 cont: 542
set M C S χ^2: 4.126 p=0.04
CVID: 132 cont: 492
set Y M C S χ^2: 10.558 p=0.001
CVID: 150 cont: 559

Codon 69
set A χ^2: 7.374 p=0.006
CVID: 58 cont: 268

Codon 70
set Q χ^2: 10.347 p=0.001
CVID: 32 cont: 185
set K Q χ^2: 7.539 p=0.006
CVID: 45 cont: 222
set S Q χ^2: 10.765 p=0.001
CVID: 45 cont: 236
set Q K S χ^2: 7.374 p=0.006
CVID: 58 cont: 268

Codon 71
set A χ^2: 7.374 p=0.006
CVID: 58 cont: 268

Codon 74
set Y χ^2: 10.176 p=0.001
CVID: 136 cont: 523

Codon 97
set W χ^2: 6.567 p=0.01
CVID: 25 cont: 45
set V R χ^2: 5.735 p=0.01
CVID: 126 cont: 481
set T R V χ^2: 5.349 p=0.02
CVID: 141 cont: 523
set R W V χ^2: 3.927 p=0.04
CVID: 136 cont: 503
set T R W V χ^2: 4.373 p=0.03
CVID: 149 cont: 542
set N R χ^2: 5.726 p=0.01
CVID: 122 cont: 469
set T R N χ^2: 4.976 p=0.02
CVID: 139 cont: 516
set W N χ^2: 5.251 p=0.02
CVID: 38 cont: 84
set R V N χ^2: 8.031 p=0.005
CVID: 128 cont: 495
set T R V N χ^2: 7.167 p=0.008
CVID: 143 cont: 534
set R W V N χ^2: 5.779 p=0.01
CVID: 138 cont: 516
set T R W V N χ^2: 5.976 p=0.01
CVID: 151 cont: 552

Codon 113
set H χ^2: 3.86 p=0.04
CVID: 154 cont: 554

Codon 114
set D χ^2: 13.875 p=0.0001
CVID: 113 cont: 469
set D H χ^2: 14.749 p=0.0001
CVID: 119 cont: 488

Codon 116
set Y S χ^2: 5.546 p=0.01
CVID: 144 cont: 532

Codon 156
set R χ^2: 12.483 p=0.0004
CVID: 24 cont: 161
set W R χ^2: 15.917 p<0.0001
CVID: 35 cont: 217
set D χ^2: 6.458 p=0.01
CVID: 116 cont: 327

Codon 163
set E χ^2: 12.372 p<0.001
CVID: 58 cont: 289
set L E χ^2: 22.759 p<0.001
CVID: 142 cont: 558

Codon 178
set K χ^2: 13.023 p=0.0003
CVID: 40 cont: 226

HLA-DRB1: all patients v. controls

1. Individual amino acids

Summary of most significant associations
(p value codon)

0.00006	73
0.00006	133, 142
0.0002	96
0.0004	37
0.0004	71
0.0007	11
0.001	74
0.002	77
0.002	13
0.005	26
0.009	32
0.02	60
0.03	85, 161
0.04	57
0.04	149, 233

Details of associations at all polymorphic codons

(counts are as number of chromosomes)

Codon 4 df: 1 χ^2: 4.007 p=0.04
aa: Q CVID: 65 (18%), cont: 168 (13%)
aa: R CVID: 291 (81%), cont: 1036 (86%)

Codon 11 df: 4 χ^2: 19.474 p<0.001
aa: L CVID: 44 (12%), cont: 149 (12%)
aa: G CVID: 65 (18%), cont: 168 (13%)
aa: S CVID: 152 (42%), cont: 452 (37%)
aa: V CVID: 70 (19%), cont: 251 (20%)
aa: P CVID: 25 (7%), cont: 184 (15%)

Codon 13 df: 5 χ^2: 19.425 p<0.001
aa: Y CVID: 65 (18%), cont: 168 (13%)
aa: F CVID: 47 (13%), cont: 164 (13%)
aa: G CVID: 16 (4%), cont: 46 (3%)
aa: S CVID: 136 (38%), cont: 406 (33%)
aa: R CVID: 25 (7%), cont: 184 (15%)
aa: H CVID: 67 (18%), cont: 236 (19%)

Codon 14 df: 1 χ^2: 4.007 p=0.04
aa: K CVID: 65 (18%), cont: 168 (13%)
aa: E CVID: 291 (81%), cont: 1036 (86%)

Codon 25 df: 1 χ^2: 4.007 p=0.04
aa: Q CVID: 65 (18%), cont: 168 (13%)
aa: R CVID: 291 (81%), cont: 1036 (86%)

Codon 26 df: 2 χ^2: 10.732 p<0.001
aa: L CVID: 53 (14%), cont: 164 (13%)
aa: F CVID: 223 (62%), cont: 853 (70%)
aa: Y CVID: 80 (22%), cont: 187 (15%)

Codon 32 df: 1 χ^2: 6.85 p<0.001
aa: Y CVID: 225 (63%), cont: 849 (70%)
aa: H CVID: 131 (36%), cont: 355 (29%)

Codon 37 df: 4 χ^2: 20.821 p<0.001
aa: L CVID: 11 (3%), cont: 16 (1%)
aa: S CVID: 64 (17%), cont: 317 (26%)
aa: F CVID: 75 (21%), cont: 196 (16%)
aa: Y CVID: 94 (26%), cont: 363 (30%)
aa: N CVID: 112 (31%), cont: 312 (25%)

Codon 57 df: 3 χ^2: 8.554 p=0.03
aa: S CVID: 5 (1%), cont: 30 (2%)
aa: A CVID: 10 (2%), cont: 28 (2%)
aa: D CVID: 260 (73%), cont: 946 (78%)
aa: V CVID: 81 (22%), cont: 200 (16%)

Codon 60 df: 2 χ^2: 7.532 p<0.001
aa: Y CVID: 265 (74%), cont: 976 (81%)
aa: S CVID: 81 (22%), cont: 200 (16%)
aa: H CVID: 10 (2%), cont: 28 (2%)

Codon 71 df: 3 χ^2: 18.157 p<0.001
aa: A CVID: 24 (6%), cont: 177 (14%)
aa: K CVID: 142 (39%), cont: 407 (33%)
aa: R CVID: 156 (43%), cont: 483 (40%)
aa: E CVID: 34 (9%), cont: 137 (11%)

Codon 73 df: 1 χ^2: 16.111 p<0.001
aa: G CVID: 140 (39%), cont: 339 (28%)
aa: A CVID: 216 (60%), cont: 865 (71%)

Codon 74 df: 4 χ^2: 18.212 p<0.001
aa: L CVID: 5 (1%), cont: 30 (2%)
aa: Q CVID: 65 (18%), cont: 168 (13%)
aa: A CVID: 196 (55%), cont: 791 (65%)
aa: R CVID: 75 (21%), cont: 171 (14%)
aa: E CVID: 15 (4%), cont: 44 (3%)

Codon 77 df: 1 χ^2: 9.748 p<0.001
aa: N CVID: 75 (21%), cont: 171 (14%)
aa: T CVID: 281 (78%), cont: 1033 (85%)

Codon 78 df: 1 χ^2: 3.867 p=0.04
aa: Y CVID: 286 (80%), cont: 1020 (84%)
aa: V CVID: 70 (19%), cont: 184 (15%)

Codon 85 df: 1 χ^2: 5.009 p=0.02
aa: A CVID: 11 (3%), cont: 16 (1%)
aa: V CVID: 345 (96%), cont: 1188 (98%)

Codon 96 df: 3 χ^2: 19.955 p<0.001
aa: Y CVID: 67 (18%), cont: 236 (19%)
aa: Q CVID: 28 (7%), cont: 199 (16%)
aa: H CVID: 207 (58%), cont: 578 (48%)
aa: E CVID: 54 (15%), cont: 191 (15%)

Codon 133 df: 1 χ^2: 16.156 p<0.001
aa: L CVID: 25 (7%), cont: 184 (15%)
aa: R CVID: 331 (92%), cont: 1020 (84%)

Codon 142 df: 1 χ^2: 16.156 p<0.001
aa: V CVID: 331 (92%), cont: 1020 (84%)
aa: M CVID: 25 (7%), cont: 184 (15%)

Codon 149 df: 1 χ^2: 4.058 p=0.04
aa: Q CVID: 219 (61%), cont: 810 (67%)
aa: H CVID: 137 (38%), cont: 394 (32%)

Codon 161 df: 1 χ^2: 5.009 p=0.02
aa: P CVID: 11 (3%), cont: 16 (1%)
aa: L CVID: 345 (96%), cont: 1188 (98%)

Codon 233 df: 1 χ^2: 4.058 p=0.04
aa: T CVID: 219 (61%), cont: 810 (67%)
aa: R CVID: 137 (38%), cont: 394 (32%)

2. Amino acid combinations

Summary of most significant associations (p value, codon, amino acid combination)

<0.000001	37 set (Y S)
0.000002	37 set (Y L S)
0.000006	73 set (A), 74 set (A E L)
0.000008	11 set (P V L), 13 set (H F R),
0.000009	96 set (Q Y E)
0.00001	13 set (H F R G) 74 set (A E), 74 set (A L)
0.00002	13 set (R H)
0.00002	74 set (A)
0.00002	11 set (P V), 96 set (Y Q)
0.00004	11 set (P), 13 set (R)
0.00005	32 set (Y), 96 set (Q)
0.00007	71 set (A) (L), 142 set (M)
0.00008	13 set (H R G)
0.0002	13 set (G R)
0.0005	96 set (E Q)

Details of associations at all polymorphic codons

Codon 4
set R χ^2: 6.414 p=0.01
Controls: 595, CVID: 170

Codon 10
set Q χ^2: 7.207 p<0.001
Controls: 508, CVID: 134

set E Q χ^2: 8.276 p<0.001
Controls: 514, CVID: 135

Codon 11
set P χ^2: 16.751 p<0.001
Controls: 175, CVID: 24
set P G χ^2: 4.094 p=0.04
Controls: 301, CVID: 73
set P V χ^2: 18.291 p<0.001
Controls: 368, CVID: 76
set P G V χ^2: 5.852 p=0.01
Controls: 453, CVID: 117
set P L χ^2: 10.913 p<0.001
Controls: 286, CVID: 59
set P V L χ^2: 21.975 p<0.001
Controls: 453, CVID: 101
set P G V L χ^2: 8.276 p<0.001
Controls: 514, CVID: 135
set P L V S χ^2: 6.414 p=0.01
Controls: 595, CVID: 170

Codon 12
set K χ^2: 8.276 p<0.001
Controls: 514, CVID: 135

Codon 13
set R χ^2: 16.751 p<0.001
Controls: 175, CVID: 24
set R H χ^2: 18.885 p<0.001
Controls: 360, CVID: 73
set F R χ^2: 11.958 p<0.001
Controls: 297, CVID: 61
set H F R χ^2: 21.975 p<0.001
Controls: 453, CVID: 101
set Y R χ^2: 4.094 p=0.04
Controls: 301, CVID: 73
set H F Y R χ^2: 8.276 p<0.001
Controls: 514, CVID: 135
set G R χ^2: 13.881 p<0.001
Controls: 217, CVID: 37
set H R G χ^2: 15.69 p<0.001
Controls: 389, CVID: 85
set F R G χ^2: 11.308 p<0.001
Controls: 332, CVID: 72
set H F R G χ^2: 19.247 p<0.001
Controls: 475, CVID: 111
set Y R G χ^2: 5.835 p=0.01
Controls: 338, CVID: 81
set H Y R G χ^2: 6.314 p=0.01
Controls: 470, CVID: 122
set F Y R G χ^2: 6.465 p=0.01
Controls: 428, CVID: 108
set H F Y R G χ^2: 9.652 p=0.001
Controls: 531, CVID: 140
set H R S χ^2: 4.294 p=0.03
Controls: 543, CVID: 150
set H F R S χ^2: 11.73 p<0.001
Controls: 589, CVID: 164
set F Y R G S χ^2: 4.317 p=0.03
Controls: 577, CVID: 163

Codon 14
set E χ^2: 6.414 p=0.01
Controls: 595, CVID: 170

Codon 25
set R χ^2: 6.414 p=0.01
Controls: 595, CVID: 170

Codon 26
set F χ^2: 4.015 p=0.04
Controls: 553, CVID: 154
set Y χ^2: 5.304 p=0.02
Controls: 170, CVID: 67
set F L χ^2: 6.291 p=0.01
Controls: 585, CVID: 165
set Y L χ^2: 6.316 p=0.01
Controls: 302, CVID: 109

Codon 28
set D H χ^2: 3.895 p=0.04
Controls: 558, CVID: 156

Codon 30
set H χ^2: 4.099 p=0.04
Controls: 16, CVID: 11
set Y G χ^2: 3.895 p=0.04
Controls: 558, CVID: 156
set Y C χ^2: 7.0 p<0.001
Controls: 588, CVID: 166
set H Y C χ^2: 5.328 p=0.02
Controls: 590, CVID: 168
set Y R C χ^2: 7.014 p<0.001
Controls: 590, CVID: 167
set H Y R C χ^2: 5.31 p=0.02
Controls: 592, CVID: 169
set Y G C χ^2: 7.973 p<0.001
Controls: 591, CVID: 167
set H Y G C χ^2: 6.229 p=0.01
Controls: 593, CVID: 169
set Y R G C χ^2: 8.168 p<0.001
Controls: 593, CVID: 168
set H Y R G C χ^2: 6.414 p=0.01
Controls: 595, CVID: 170
set Y χ^2: 16.614 p<0.001
Controls: 549, CVID: 142

Codon 33
set N χ^2: 4.317 p=0.03
Controls: 577, CVID: 163

Codon 37
set S χ^2: 11.049 p<0.001
Controls: 276, CVID: 56
set Y S χ^2: 32.779 p<0.001
Controls: 499, CVID: 111
set F Y S χ^2: 11.854 p<0.001
Controls: 553, CVID: 147
set Y S N χ^2: 7.0 p<0.001
Controls: 588, CVID: 166
set L χ^2: 4.099 p=0.04
Controls: 16, CVID: 11
set F L χ^2: 4.185 p=0.04
Controls: 198, CVID: 74
set L S χ^2: 7.887 p<0.001
Controls: 287, CVID: 63
set Y L S χ^2: 27.873 p<0.001
Controls: 504, CVID: 116
set F Y L S χ^2: 10.302 p<0.001
Controls: 555, CVID: 149
set Y L N S χ^2: 4.513 p=0.03
Controls: 591, CVID: 169

Codon 38
set L χ^2: 4.099 p=0.04
Controls: 16, CVID: 11

Codon 57
set D χ^2: 6.832 p<0.001
Controls: 577, CVID: 161
set D A χ^2: 9.584 p<0.001
Controls: 585, CVID: 163
set A V χ^2: 4.334 p=0.03
Controls: 210, CVID: 78
set D S χ^2: 5.614 p=0.01
Controls: 584, CVID: 165
set A D S χ^2: 7.014 p<0.001
Controls: 590, CVID: 167

Codon 60
set Y χ^2: 5.614 p=0.01
Controls: 584, CVID: 165
set Y H χ^2: 7.014 p<0.001
Controls: 590, CVID: 167
set S H χ^2: 4.334 p=0.03
Controls: 210, CVID: 78
set F I χ^2: 7.65 p=0.002
Controls: 475, CVID: 122

Codon 71
set K R χ^2: 6.348 p=0.01
Controls: 554, CVID: 174
set A χ^2: 15.883 p<0.001
Controls: 168, CVID: 23
set A R χ^2: 6.83 p<0.001
Controls: 472, CVID: 122
set A E χ^2: 10.325 p<0.001
Controls: 266, CVID: 54
set R A E χ^2: 6.836 p<0.001
Controls: 529, CVID: 142

Codon 73
set G χ^2: 4.512 p=0.03
Controls: 295, CVID: 104
set A χ^2: 23.514 p<0.001
Controls: 558, CVID: 142

Codon 74
set R χ^2: 4.786 p=0.02
Controls: 157, CVID: 62
set R Q χ^2: 4.512 p=0.03
Controls: 295, CVID: 104
set A χ^2: 18.434 p<0.001

Controls: 533, CVID: 134
set A R χ^2: 4.617 p=0.03
Controls: 580, CVID: 164
set E R χ^2: 5.142 p=0.02
Controls: 196, CVID: 75
set Q R E χ^2: 5.33 p=0.02
Controls: 328, CVID: 115
set A E χ^2: 21.611 p<0.001
Controls: 547, CVID: 138
set Q A E χ^2: 4.617 p=0.03
Controls: 580, CVID: 164
set R A E χ^2: 9.939 p<0.001
Controls: 591, CVID: 166
set A L χ^2: 21.611 p<0.001
Controls: 547, CVID: 138
set Q A L χ^2: 7.934 p<0.001
Controls: 583, CVID: 163
set R E L χ^2: 4.033 p=0.04
Controls: 215, CVID: 79
set A E L χ^2: 23.514 p<0.001
Controls: 558, CVID: 142
set Q A E L χ^2: 8.751 p<0.001
Controls: 588, CVID: 165
set R A E L χ^2: 6.414 p=0.01
Controls: 595, CVID: 170

Codon 77
set T χ^2: 8.751 p<0.001
Controls: 588, CVID: 165
set N χ^2: 4.786 p=0.02
Controls: 157, CVID: 62

Codon 78
set Y χ^2: 5.31 p=0.02
Controls: 592, CVID: 169
set V χ^2: 1.632 p=0.2

Codon 85
set A χ^2: 4.099 p=0.04
Controls: 16, CVID: 11

Codon 96
set Q χ^2: 16.644 p<0.001
Controls: 187, CVID: 27
set Y Q χ^2: 18.291 p<0.001
Controls: 368, CVID: 76
set E Q χ^2: 12.033 p<0.001
Controls: 328, CVID: 70
set Q H E χ^2: 4.317 p=0.03
Controls: 577, CVID: 163
set Q Y E χ^2: 20.891 p<0.001
Controls: 473, CVID: 109

Codon 133
set L χ^2: 16.751 p<0.001
Controls: 175, CVID: 24

Codon 140
set A χ^2: 4.38 p=0.03
Controls: 421, CVID: 109

Codon 142
set M χ^2: 16.751 p<0.001
Controls: 175, CVID: 24

Codon 149
set Q χ^2: 9.393 p<0.001
Controls: 533, CVID: 141
Codon 161
set P χ^2: 4.099 p=0.04
Controls: 16, CVID: 11

Codon 180
set V χ^2: 4.317 p=0.03
Controls: 577, CVID: 163

Codon 181
set T χ^2: 5.328 p=0.02
Controls: 590, CVID: 168

Codon 233
set T χ^2: 9.393 p<0.001
Controls: 533, CVID: 141

HLA-DQB1: All patients v. controls

1. Individual amino acids

Summary of most significant results (p value codon)

0.00003 87
0.00004 52
0.00005 55
0.0001 74
0.0001 77
0.0002 75
0.0002 30
0.0002 67
0.0003 220, 221
0.0003 57
0.0003 71
0.0003 38
0.0005 203
0.0006 53, 84, 85, 89, 90
0.0008 125

Details of associations at each polymorphic codon

codon: 28 df: 1 χ^2: 16.91 p<0.0001
aa : S CVID: 126 (35%) cont: 294 (24%)
aa : T CVID: 228 (64%) cont: 904 (75%)

codon: 30 df: 2 χ^2: 17.123 p<0.001
aa : Y CVID: 149 (42%) cont: 606 (50%)
aa : S CVID: 126 (35%) cont: 294 (24%)
aa : H CVID: 79 (22%) cont: 298 (24%)

codon: 38 df: 1 χ^2: 13.434 p<0.001
aa : A CVID: 170 (48%) cont: 707 (59%)
aa : V CVID: 184 (51%) cont: 491 (40%)

codon: 46 df: 1 χ^2: 16.91 p<0.0001
aa : V CVID: 228 (64%) cont: 904 (75%)
aa : E CVID: 126 (35%) cont: 294 (24%)

codon: 47 df: 1 χ^2: 16.91 p<0.0001
aa : Y CVID: 228 (64%) cont: 904 (75%)
aa : F CVID: 126 (35%) cont: 294 (24%)

codon: 52 df: 1 χ^2: 16.91 p<0.0001
aa : P CVID: 228 (64%) cont: 904 (75%)
aa : L CVID: 126 (35%) cont: 294 (24%)

codon: 53 df: 1 χ^2: 11.915 p=<0.0001
aa : L CVID: 248 (70%) cont: 718 (59%)
aa : Q CVID: 106 (29%) cont: 480 (40%)

codon: 55 df: 2 χ^2: 19.958 p<0.0001
aa : P CVID: 116 (32%) cont: 399 (33.3%)
aa : R CVID: 112 (31%) cont: 505 (42%)
aa : L CVID: 126 (35%) cont: 294 (24%)

codon: 57 df: 2 χ^2: 16.046 p<0.001
aa : D CVID: 124 (35%) cont: 552 (46%)
aa : A CVID: 160 (45%) cont: 414 (34%)
aa : V CVID: 70 (19%) cont: 232 (19%)

codon: 66 df: 1 χ^2: 13.576 p<0.001
aa : D CVID: 132 (37%) cont: 325 (27%)
aa : E CVID: 222 (62%) cont: 873 (72%)

codon: 67 df: 1 χ^2: 13.576 p<0.001
aa : I CVID: 132 (37%) cont: 325 (27%)
aa : V CVID: 222 (62%) cont: 873 (72%)

codon: 70 df: 2 χ^2: 11.861 p<0.01
aa : G CVID: 94 (26%) cont: 433 (36%)
aa : E CVID: 6 (1%) cont: 25 (2%)
aa : R CVID: 254 (71%) cont: 740 (61%)

codon: 71 df: 3 χ^2: 18.712 p<0.001
aa : A CVID: 58 (16%) cont: 191 (15%)
aa : K CVID: 126 (35%) cont: 294 (24%)
aa : D CVID: 6 (1%) cont: 25 (2%)
aa : T CVID: 164 (46%) cont: 688 (57%)

codon: 74 df: 2 χ^2: 18.469 p<0.001
aa : S CVID: 64 (18%) cont: 216 (18%)
aa : A CVID: 126 (35%) cont: 294 (24%)
aa : E CVID: 164 (46%) cont: 688 (57%)

codon: 75 df: 1 χ^2: 13.6 p<0.001
aa : L CVID: 164 (46%) cont: 688 (57%)
aa : V CVID: 190 (53%) cont: 510 (42%)

codon: 77 df: 1 χ^2: 14.718 p<0.001
aa : T CVID: 170 (48%) cont: 713 (59%)
aa : R CVID: 184 (51%) cont: 485 (40%)

codon: 84 df: 1 χ^2: 11.915 p<0.001
aa : Q CVID: 248 (70%) cont: 718 (59%)
aa : E CVID: 106 (29%) cont: 480 (40%)

codon: 85 df: 1 χ^2: 11.915 p=0.001
aa : L CVID: 248 (70%) cont: 718 (59%)
aa : V CVID: 106 (29%) cont: 480 (40%)

codon: 86 df: 2 χ^2: 12.601 p<0.01
aa : G CVID: 12 (3%) cont: 41 (3%)
aa : A CVID: 94 (26%) cont: 439 (36%)
aa : E CVID: 248 (70%) cont: 718 (59%)

codon: 87 df: 2 χ^2: 21.085 p<0.0001
aa : L CVID: 248 (70%) cont: 718 (59%)
aa : F CVID: 36 (10%) cont: 248 (20.7%)
aa : Y CVID: 70 (19%) cont: 232 (19%)

codon: 89 df: 1 χ^2: 11.915 p=0.001
aa : G CVID: 106 (29%) cont: 480 (40%)
aa : T CVID: 248 (70%) cont: 718 (59%)

codon: 90 df: 1 χ^2: 11.915 p=0.001
aa : I CVID: 106 (29%) cont: 480 (40%)
aa : T CVID: 248 (70%) cont: 718 (59%)

codon: 125 df: 2 χ^2: 14.419 p<0.001
aa : S CVID: 98 (27%) cont: 369 (30.8%)
aa : G CVID: 35 (9%) cont: 201 (16%)
aa : A CVID: 221 (62%) cont: 628 (52%)

codon: 203 df: 1 χ^2: 12.002 p<0.001
aa : I CVID: 160 (45%) cont: 420 (35%)
aa : V CVID: 194 (54%) cont: 778 (64%)

codon: 220 df: 1 χ^2: 13.273 p<0.001
aa : H CVID: 160 (45%) cont: 414 (34%)
aa : R CVID: 194 (54%) cont: 784 (65%)

codon: 221 df: 1 χ^2: 13.273 p<0.001
aa : Q CVID: 194 (54%) cont: 784 (65%)
aa : H CVID: 160 (45%) cont: 414 (34%)

2. Amino acid combinations

Summary of most significant associations (p value, codon, amino acid combination)

0.000008 87 set (F)
0.00001 71 set (T A)
0.00001 28 set (T), 30 set (Y H), 37 set (Y D), 46 set (V), 47 set (Y), 52 set (P), 55 set (P R), 71 set (T A D), 74 set (S E)
0.00002 37 set (Y)
0.00003 66 set (E), 67 set (V)
0.0001 86 set (A)

0.0003 70 set (G)
0.0003 9 set (F L)
0.0004 70 set (E G)
0.0006 9 set (F)
0.0006 71 set (T D), 77 set (T)
0.0007 71 set (T), 74 set (E), 75 set (L)

Details of associations at each polymorphic codon

Codon 9
set F χ^2: 11.898 p=0.0006
Controls: 161 CVID: 24
set F L χ^2: 13.319 p=0.0003
Controls: 166 CVID: 24

Codon 28
set T χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 30
set Y χ^2: 7.591 p=0.006
Controls: 379 CVID: 90
set Y H χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 37
set Y χ^2: 17.88 p<0.0001
Controls: 488 CVID: 121
set Y D χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 38
set A χ^2: 10.962 p=0.0009
Controls: 426 CVID: 102
set V χ^2: 4.192 p=0.04
Controls: 369 CVID: 119

Codon 46
set V χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 47
set Y χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 52
set P χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 53
set Q χ^2: 10.894 p=0.001
Controls: 346 CVID: 76

Codon 55
set R χ^2: 10.395 p=0.001
Controls: 354 CVID: 79
set P R χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121
set P L χ^2: 4.263 p=0.04
Controls: 416 CVID: 131

Codon 57
set A χ^2: 4.974 p=0.02
Controls: 312 CVID: 105
set D χ^2: 11.055 p=0.0009
Controls: 353 CVID: 78
set D V χ^2: 9.301 p=0.002
Controls: 450 CVID: 112

Codon 66
set E χ^2: 17.727 p<0.0001
Controls: 481 CVID: 118

Codon 67
set V χ^2: 17.727 p<0.0001
Controls: 481 CVID: 118

Codon 70
set G χ^2: 13.094 p=0.0003
Controls: 327 CVID: 68
set E G χ^2: 12.505 p=0.0004
Controls: 335 CVID: 71

Codon 71
set T χ^2: 11.598 p=0.0007
Controls: 419 CVID: 99
set T D χ^2: 11.737 p=0.0006
Controls: 428 CVID: 102
set T A χ^2: 19.234 p<0.0001
Controls: 483 CVID: 118
set K A χ^2: 4.911 p=0.03
Controls: 365 CVID: 119
set T A D χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121
set K A D χ^2: 4.545 p=0.03
Controls: 367 CVID: 119

Codon 74
set E χ^2: 11.598 p=0.0007
Controls: 419 CVID: 99
set A S χ^2: 4.545 p=0.03
Controls: 367 CVID: 119
set S E χ^2: 19.535 p<0.0001
Controls: 490 CVID: 121

Codon 75
set V χ^2: 4.545 p=0.03
Controls: 367 CVID: 119
set L χ^2: 11.598 p=0.0007
Controls: 419 CVID: 99

Codon 77
set T χ^2: 11.737 p=0.0006
Controls: 428 CVID: 102
set R χ^2: 4.911 p=0.03
Controls: 365 CVID: 119

Codon 84
set E χ^2: 10.894 p=0.001
Controls: 346 CVID: 76

Codon 85
set V χ^2: 10.894 p=0.001
Controls: 346 CVID: 76

Codon 86
set A χ^2: 14.386 p=0.0002
Controls: 331 CVID: 68
set A G χ^2: 10.894 p=0.001
Controls: 346 CVID: 76

Codon 87
set F χ^2: 21.595 p<0.001
Controls: 212 CVID: 29
set F Y χ^2: 10.894 p=0.001
Controls: 346 CVID: 76

Codon 89
set G χ^2: 10.894 p<0.001
Controls: 346 CVID: 76

Codon 90
set I χ^2: 10.894 p<0.001
Controls: 346 CVID: 76

Codon 125
set G χ^2: 10.201 p=0.001
Controls: 175 CVID: 29
set A χ^2: 4.263 p=0.04
Controls: 416 CVID: 131
set S G χ^2: 10.395 p=0.001
Controls: 354 CVID: 79

Codon 203
set I χ^2: 4.275 p=0.03
Controls: 316 CVID: 105
set V χ^2: 8.535 p=0.004
Controls: 448 CVID: 112

Codon 220
set H χ^2: 4.974 p=0.03
Controls: 312 CVID: 105
set R χ^2: 9.301 p=0.002
Controls: 450 CVID: 112

Codon 221
set Q χ^2: 9.301 p=0.002
Controls: 450 CVID: 112
set H χ^2: 4.974 p=0.03
Controls: 312 CVID: 105

DQB1 codon 57 analysis

Amino Acid Frequencies :
aa: D CVID: 124 (35%) cont: 552 (46%)
aa: A CVID: 160 (45%) cont: 414 (34%)
aa: V CVID: 70 (19%) cont: 232 (19%)

Chi Squared on overall aa frequency
χ^2: 16.046 (df=2) p=0.0003
aa: D CVID: 124 (35%) cont: 552 (46%)
aa: A CVID: 160 (45%) cont: 414 (34%)
aa: V CVID: 70 (19%) cont: 232 (19%)

Chi Squared on pairs of alleles by subset
Set A χ^2: 7.99 p=0.005
cont: 336 CVID: 121
Set D χ^2: 9.37 p=0.002
cont: 424 CVID: 103
Set A D χ^2: 0.53 p=0.46
Set V χ^2: 0.012 p=0.96
Set A V χ^2: 7.35 p=0.007
cont: 471 CVID: 156
Set D V χ^2: 7.97 p=0.005
cont: 521 CVID: 138

DQB1 alleles with D (Asp) at codon 57
DQB1*0203
DQB1*0301
DQB1*0303
DQB1*0306
DQB1*0401
DQB1*0402
DQB1*0503
DQB1*0601
DQB1*0602
DQB1*0603
DQB1*0607
DQB1*0611

DQB1 alleles with V (Val) at codon 57
DQB1*0501
DQB1*0604
DQB1*0605
DQB1*0606
DQB1*0608
DQB1*0609
DQB1*0612
DQB1*0613

DQB1 alleles with A (Ala) at codon 57
DQB1*0201
DQB1*0202
DQB1*0302
DQB1*0304
DQB1*0305
DQB1*0307

8.4. CORRELATIONS BETWEEN IMMUNOPHENOTYPIC PARAMETERS

Table 8.7: Lymphocyte subset correlations

Only significant correlations are shown. Pairs of correlated variables are listed in the first column separated by a comma. The further the deviation of the (Pearson's) correlation coefficient from zero, the stronger the correlation. Negative r_p values represent inverse correlations. eg. rising CD3+ counts correlate strongly with rising CD4+ counts (r_p=0.79, Z=13.12, p<0.0001). In contrast, low CD4+:CD8+ ratio value significantly correlate with high CD8+ counts (r_p=-0.42, Z=-5.57, p<0.0001). The $100r_p^2$ statistic estimates the percentage of data variability due to the correlation between two variables. Deviation from the null hypothesis (that there is no correlation between variables) is assessed by Fisher's r to Z test, generating Z- and p-values. ns = not significant.

						95% r_p confidence intervals	
	(r_p)	no. examined	$100r_p^2$	Z	p	lower	upper
CD3, CD4	0.79	157	61.8	13.12	<.0001	0.72	0.84
CD3, CD8	0.85	157	71.4	15.31	<.0001	0.79	0.88
CD3, B cells	0.61	157	37.0	6.95	<.0001	0.47	0.72
CD3, CD4CD45RA	0.59	71	35.3	5.64	<.0001	0.42	0.73
CD3, CD4CD45RO	0.60	71	36.2	5.74	<.0001	0.43	0.73
CD3, CD8CD45RA	0.83	71	68.2	9.63	<.0001	0.73	0.89
CD3, CD8CD45RO	0.58	71	34.1	5.43	<.0001	0.40	0.72
CD3, CD8CD57	0.45	71	20.0	3.88	0.0001	0.23	0.62
CD4, CD8	0.45	157	19.9	5.95	<.0001	0.31	0.56
CD4, CD4:CD8	0.24	157	6.0	3.10	0.002	0.09	0.39
CD4, B cells	0.71	157	49.7	8.64	<.0001	0.59	0.79
CD4, CD4CD45RA	0.73	71	53.6	7.69	<.0001	0.60	0.82
CD4, CD4CD45RO	0.62	71	38.1	5.94	<.0001	0.45	0.74
CD4, CD8CD45RA	0.61	71	36.8	5.77	<.0001	0.43	0.74
CD4, CD8CD45RO	0.32	71	10.2	2.68	0.007	0.09	0.52
CD4, %CD8 also CD57	-0.27	71	7.5	-2.26	0.02	-0.48	-0.04
CD8, CD4:CD8	-0.42	157	17.7	-5.57	<.0001	-0.54	-0.28
CD8, B cells	0.44	157	19.6	4.69	<.0001	0.27	0.59
CD8, CD4CD45RA	0.34	71	11.2	2.87	0.004	0.11	0.53
CD8, CD4CD45RO	0.49	71	23.8	4.39	<.0001	0.29	0.65
CD8, CD8CD45RA	0.89	71	79.9	11.81	<.0001	0.84	0.93
CD8, CD8CD45RO	0.74	71	54.5	7.68	<.0001	0.61	0.83
CD8, CD8CD57	0.69	71	47.1	6.78	<.0001	0.54	0.80
CD4:CD8, CD8CD45RA	-0.36	71	13.2	-3.12	0.002	-0.55	-0.14
CD4:CD8, CD8CD45RO	-0.39	71	15.3	-3.35	0.0008	-0.57	-0.17
CD4:CD8, CD8CD57	-0.36	71	13.0	-3.04	0.002	-0.55	-0.13
B cells, CD4CD45RA	0.55	71	30.6	5.10	<.0001	0.37	0.70
B cells, CD4CD45RO	0.53	71	28.4	4.86	<.0001	0.34	0.68
B cells, CD8CD45RA	0.51	71	26.3	4.64	<.0001	0.32	0.67
B cells, CD8CD45RO	0.26	71	6.8	2.17	0.03	0.03	0.47
B cells, %CD8 also CD57	-0.33	68	10.9	-2.76	0.0057	-0.53	-0.10
CD4CD45RA, CD8CD45RA	0.50	71	25.3	4.53	<.0001	0.30	0.66
CD4CD45RA, %CD8 also CD57	-0.35	71	12.0	-2.86	0.004	-0.54	-0.11
CD4CD45RO, CD8CD45RA	0.42	71	17.3	3.62	0.0003	0.20	0.59
CD4CD45RO, CD8CD45RO	0.56	71	31.7	5.18	<.0001	0.38	0.71
CD4CD45RO, CD8CD57	0.38	71	14.5	3.19	0.001	0.15	0.57
CD8CD45RA, CD8CD45RO	0.61	71	37.1	5.74	<.0001	0.43	0.74
CD8CD45RA, CD8CD57	0.58	71	33.5	5.25	<.0001	0.39	0.72
CD8CD45RO, CD8CD57	0.82	71	66.7	9.10	<.0001	0.72	0.88
CD8CD57, %CD8 also CD57	0.45	71	19.9	3.87	0.0001	0.23	0.62

8.5. ADDITIONAL GENES EXAMINED BY PCR-SSP

Assays for a several polymorphisms not directly relevant to CVID were developed as part of other disease studies performed by the immunogenetics group in the Nuffield Department of Surgery. Methodological details of these polymorphisms are appended, as they are of considerable interest to a wide range of inflammatory and acquired immunodeficiency conditions, including inflammatory bowel disease, solid organ transplantation, IDDM, coeliac disease and systemic sclerosis:

- the codon 506 Arg→Glu (Leiden) polymorphism of the blood coagulation factor, factor V (Bertina et al, 1994);
- the codon 33 Leu→Pro polymorphism of the platelet integrin fibrinogen receptor gpBIIIa, that determines the HPA-1 blood group antigens, Pl^{A1} and Pl^{A2} (Newman et al, 1989);
- the codon 112 and 158 Cys→Arg polymorphisms determining the e2, e3 and e4 variants of apolipoprotein E (Zannis et al, 1981);
- the codon 63 His→Asp and 282 Cys→Tyr polymorphisms in *HLA-HFE*, the recently identified candidate gene for hereditary haemochromatosis (Feder et al, 1996; Mullighan et al, 1997a);
- the promoter (FY*exp/FY*null) and exonic (FY*A/FY*B) variants of the Duffy antigen receptor (DARC) gene (Iwamoto et al, 1995; Tournamille et al, 1995a; Tournamille et al, 1995b; Mullighan et al, 1997c).

Polymorphism details, primer sequences and reaction mix composition are listed below.

Table 8.8: PCR-SSP primer specificities and sequences

Gene	Polymorphism	Primer no.	Sense (S) / antisense (AS)	Sequence accession number	Annealing position	Specificity	Sequence
Factor V	**aa 506 R/Q**	919	S	L32764	69-88	consensus	5' CAACACCATgATCAgAgCAg
		920	AS		266-287	normal (506 R)	5' ggACAAAATACCTgTATTCCTC
		921	AS		266-288	Leiden (506 Q)	5' ggACAAAATACCTgTATTCCTT
HPA-1 (gpBIIIa)	**aa 33 L/P**	956	S	M32672	1481-1498	PI^{A1} (33 L)	5' CTTACAggCCCTgCCTCT
		957	S		1481-1498	PI^{A2} (33 P)	5' CTTACAggCCCTgCCTCC
		958	AS		1703-1721	consensus	5' TTCAggTCTCTCCCCATgg
Apolipoprotein E	**aa 112 C/R**	884	S	M10065	3915-3934	ε2, ε3	5' CggACATggAggACgTgT
		885	S		3915-3934	ε4	5' CggACATggAggACgTgC
	aa 158 C/R	886	AS		4087-4070	ε2	5' CTggTACACTgCCAggCg
		887	AS		4087-4070	ε3, ε4	5' CTggTACACTgCCAggCA
HLA-HFE	**aa 63 H/D**	805	S	U60319	388-408	63 H	5' AgCTgTTCgTgTTCTATgATC
		806	S		388-408	63 D	5' AgCTgTTCgTgTTCTATgATg
	aa 282 C/Y	807	AS		1083-1066	282 C	5' CTgggTgCTCCACCTggC
		808	AS		1083-1066	282 Y	5' CTgggTgCTCCACCTggT
Duffy	**-540 t/c**	839	S	X85785	418-439	FY*exp	5' TCATTAgTCCTTggCTCTTAT
		840	S		418-439	FY*null	5' TCATTAgTCCTTggCTCTTAC
	aa 44 G/D	841	AS		1127-1109	FY*A	5' gCTgCTTCCAggTTggCAT
		842	AS		1127-1109	FY*B	5' gCTgCTTCCAggTTggCAC
Control primers	**DRB1**	63	S	M17378	517-535	consensus	5' TgCCAAgTggAgCACCCAA
		64	AS		598-579	consensus	5' gCATCTTgCTCTgTgCAgAT
	APC	210	S	M74088	5627-5647	consensus	5' ATgATgTTgACCTTTCCAggg
		211	AS		5876-5854	consensus	5' TTCTgTAACTTTTCATCAgTTgC
	HGH	724	S	M13438	893-912	consensus	5' gCCTTCCCAACCATTCCCTT
		725	AS		1319-1298	consensus	5' TCACggATTTCTgTTgTgTTTC

Table 8.9: Primer mix details for genotyping of non-HLA immunogenetic polymorphisms

Volume refers to volume of 2000µg/ml primer stock per ml of primer mix. S = sense primer number, AS = antisense primer number

Primer mix	Specificity	S	Volume	conc. (mM)	AS	Volume	conc (mM)	Control primers	Amplicon size (bp)
558	**FV normal**	919	20	6.8	920	20	6.8	63+64	218
559	**FV Leiden**	919	20	6.8	921	20	6.8	63+64	218
560	**PI^{A1}**	956	5	1.7	958	5	1.7	63+64	240
561	**PI^{A2}**	957	5	1.7	958	5	1.7	63+64	240
555	**Apo-E ε2**	884	10	3.4	887	10	3.4	63+64	173
556	**Apo-E ε3**	884	10	3.4	886	10	3.4	63+64	173
557	**Apo-E ε4**	885	10	3.4	886	10	3.4	63+64	173
566	**HLA-H 63H-282C**	805	20	6.8	807	20	6.8	725+726	1460
567	**HLA-H 63H-282Y**	805	20	6.8	808	20	6.8	725+726	1460
568	**HLA-H 63D-282C**	806	20	6.8	807	20	6.8	725+726	1460
569	**HLA-H 63D-282Y**	806	20	6.8	808	20	6.8	725+726	1460
570	**Duffy FY*exp-FY*B**	839	10	3.4	841	10	3.4	210+211	710
571	**Duffy FY*exp-FY*A**	839	10	3.4	842	10	3.4	210+211	710
572	**Duffy FY*null-FY*B**	840	10	3.4	841	10	3.4	210+211	710
573	**Duffy FY*null-FY*A**	840	10	3.4	842	10	3.4	210+211	710

Table 8.10: Control phenotype, allele and genotype frequencies

All frequencies are for Caucasoid control populations, except where otherwise stated. Italicised phenotype and allele frequencies have been calculated from published genotype frequencies. H-W = Hardy-Weinberg equilibrium; the H-W χ^2 p value refers to the measure of significance of deviation observed from expected genotype frequencies as calculated by the Hardy-Weinberg equilibrium equation. Previously reported frequencies are taken from: [1]Bickeboller et al, 1997; [2]Schächter et al, 1994; [3]Liu et al, 1996; [4]Samson et al, 1996b; [5]Nistico et al, 1996; [6]Deichmann et al, 1996; [7]Wenzel et al, 1994b; [8]Wenzel et al, 1996; [9]Yang et al, 1995; [10]Osborne et al, 1994; [11]Bredius et al, 1994; [12]McDowell et al, 1995; [13]Ploski et al, 1995; [14]Pociot et al, 1992; [15]Guash et al, 1996; [16]Borish et al, 1994; [17]Rosenwasser et al, 1995 atopic kindreds; [18]Walley et al, 1996; [19]Turner et al, 1997; [20]Eskdale et al, 1997a; [21]Cambien et al, 1996; [22]Morrison et al, 1992; [23]Gross et al, 1996; [24]Keen et al, 1995; [25]Feder et al, 1996; [26]Merryweather-Clarke et al, 1997; [27]Daniels, 1995 (the FY*B allele frequency incorporates Fy^x, a serologically weak Fy^b), [28]Marron et al, 1997.

Phenotype and allele frequencies

Gene	Allele / haplotype	Phenotype		Previously reported frequencies		Allele		Previously reported frequencies	
		count n=101	freq			count n=202	freq		
Apo E	ε2	17	0.17	*0.15*[1]	*0.135*[2]	17	0.08	0.068[1]	
	ε3	99	0.98	*0.95*[1]	*0.955*[2]	159	0.79	0.820[1]	
	ε4	25	0.25	*0.28*[1]	*0.205*[2]	26	0.13	0.112[1]	
							1.00		
CCR5	CCR5	100	0.99	*0.99*[3]		187	0.93	0.902[3]	0.908[4]
	Δccr5	14	0.14	*0.19*[3]		15	0.07	0.098[3]	0.092[4]
CTLA4 -318/+49	c-a	77	0.76			109	0.54		
	c-g	59	0.58			79	0.39		
	t-a	14	0.14			14	0.07		
CTLA4 -318	c	101	1.00			188	0.93		
	t	14	0.14			14	0.07	0.134[6]	
CTLA4 +49	a	81	0.80			123	0.61	0.68[5]	0.621[28]
	g	59	0.58	0.55[5]		79	0.39	0.32[5]	0.379[28]

Genotype frequencies

Genotype	Observed		Expected		H-W	Previously reported frequencies	
	count n=101	freq	count n=101	freq	χ^2 p		
ε2/ε2	0	0	1	0.01	0.77	0.01[1]	0.000[2]
ε2/ε3	16	0.16	13	0.13		0.12[1]	0.111[2]
ε2/ε4	1	0.01	2	0.02		0.03[1]	0.025[2]
ε3/ε3	60	0.59	63	0.62		0.59[1]	0.683[2]
ε3/ε4	23	0.23	20	0.20		0.24[1]	0.161[2]
ε4/ε4	1	0.01	2	0.02		0.01[1]	0.019[2]
wt/wt	87	0.86	86	0.86	0.81	0.81[3]	0.827[4]
wt/del	13	0.13	14	0.14		0.18[3]	0.162
del/del	1	0.01	1	0.01		0.01[3]	0.011
c-a/c-a	32	0.32	29	0.29	0.47		
c-a/c-g	35	0.35	43	0.42			
c-a/t-a	10	0.10	8	0.07			
c-g/c-g	20	0.20	15	0.15			
c-g/t-a	4	0.04	5	0.05			
t-a/t-a	0	0.00	0	0.00			
c/c	87	0.86	87	0.87	0.75		
c/t	14	0.14	13	0.13		0.251[6]	
t/t	0	0.00	0	0.00			
a/a	42	0.42	37	0.37	0.16	0.45[5]	0.363[28]
a/g	39	0.39	48	0.48		0.46[5]	0.518[28]
g/g	20	0.20	15	0.15		0.10[5]	0.121[28]

Table 8.10: Control phenotype, allele and genotype frequencies (continued)

Phenotype and allele frequencies

Gene	Allele / haplotype	Phenotype		Previously reported frequencies		Allele		Previously reported frequencies	
		count n=101	freq			count n=202	freq		
E-selectin aa 128	S	99	0.98			177	0.88	0.91[7]	
	R	23	0.23	0.155[8]		25	0.12	0.09	0.087[8]
E-selectin aa 554	L	101	1.00			200	0.99		
	F	2	0.02	0.030[8]		2	0.01	0.015[8]	
L-selectin aa 206	F	95	0.94			154	0.76		
	L	42	0.42	0.363[8]		48	0.24	0.211[8]	
Factor V	N	101	1.00			195	0.97		
	Leiden	7	0.07			7	0.03		
FcγRIIA aa131	R	81	0.80	*0.79*[10]	*0.77*[11]	111	0.55	0.50[10]	
	H	71	0.70	*0.71*[10]	*0.71*[11]	91	0.45	0.50[10]	
ICAM-1 aa 241-469	G-E	60	0.59			76	0.38		
	G-K	80	0.79			107	0.53		
	R-E	19	0.19			19	0.09		
ICAM-1 aa 241	G	101	1.00			183	0.91		0.921[9]
	R	19	0.19	0.228[8]	0.145[9]	19	0.09	0.114[8]	0.079[9]
ICAM-1 aa 469	E	74	0.73			95	0.47	0.448[9]	
	K	80	0.79	0.701[9]		107	0.53	0.552[9]	

Genotype frequencies

Genotype	Observed		Expected		H-W	Previously reported frequencies	
	count n=101	freq	count n=101	freq	χ^2 p		
S/S	78	0.77	77	0.77	0.91		
S/R	21	0.21	22	0.22			
R/R	2	0.02	2	0.02		0.019[8]	
L/L	99	0.98	98	0.98	0.99		
L/F	2	0.02	2	0.02			
F/F	0	0.00	0	0.00		0.0[8]	
F/F	59	0.58	58	0.58	0.98		
F/L	36	0.36	36	0.36			
L/L	6	0.06	6	0.06		0.059[8]	
N/N	94	0.94	93	0.93	0.93		
N/L	7	0.07	7	0.07			
L/L	0	0	0	0.00			
R/R	30	0.3	30	0.30	0.98	0.29[10]	0.29[11]
R/H	51	0.51	50	0.50		0.50[10]	0.48[11]
H/H	20	0.2	20	0.20		0.21[10]	0.23[11]
G-E/G-E	16	0.16	14	0.14	0.64		
G-E/G-K	39	0.39	40	0.40			
G-E/R-E	5	0.05	7	0.07			
G-K/G-K	27	0.27	28	0.28			
G-K/R-E	14	0.14	10	0.10			
R-E/R-E	0	0.00	1	0.01			
G/G	82	0.81	82	0.82	0.57		0.855[9]
G/R	19	0.19	17	0.17			0.132[9]
R/R	0	0.00	1	0.01		0.0[8]	0.013[9]
E/E	21	0.21	22	0.22	0.86	0.299[9]	
E/K	53	0.52	50	0.50		0.506[9]	
K/K	27	0.27	28	0.28		0.195[9]	

Table 8.10: Control phenotype, allele and genotype frequencies (continued)

Phenotype and allele frequencies										Genotype frequencies						
Gene	**Allele / haplotype**	**Phenotype**		**Previously reported frequencies**		**Allele**		**Previously reported frequencies**		**Genotype**	**Observed**		**Expected**		**H-W**	**Previously reported frequencies**
		count n=101	freq			count n=202	freq				count n=101	freq	count n=101	freq	χ^2 p	
IL-1α -511	**c**	93	0.92	*0.888*[12]		132	0.65	0.702[12]		**c/c**	39	0.39	43	0.43	0.19	0.515[12]
	t	62	0.61	0.485[12]	0.494[13]	70	0.35	0.298[12]		**c/t**	54	0.53	45	0.45		0.373[12]
										t/t	8	0.08	12	0.12		0.111[12]
IL-1β +3962	**g**	98	0.97	0.922[14]		153	0.76	0.74[14]	0.78[15]	**g/g**	55	0.54	57	0.57	0.28	0.566[14]
	a	46	0.46	0.434[14]		49	0.24	0.26[14]	0.22[15]	**g/a**	43	0.43	37	0.37		0.356[14]
										a/a	3	0.03	6	0.06		0.078[14]
IL-4 -590	**c**	98	0.97			173	0.86	*0.82*[16]		**c/c**	75	0.74	73	0.73	0.75	0.63[16]
	t	26	0.26	0.368[17]	0.27[18]	29	0.14	*0.20*[16]		**c/t**	23	0.23	25	0.25		0.37[16]
										t/t	3	0.03	2	0.02		0.02[16]
IL-6 +3247	**t**	80	0.79			117	0.58			**t/t**	37	0.37	34	0.34	0.44	
	c	64	0.63			85	0.42			**t/c**	43	0.43	49	0.49		
										c/c	21	0.21	18	0.18		
IL-10	**acc**	53	0.52			55	0.27	0.28[19]		**acc/acc**	2	0.02	7	0.07	0.13	
-1082/-819/-592	**ata**	44	0.44			50	0.25	0.21[19]		**acc/ata**	15	0.15	14	0.13		
	gcc	78	0.77			97	0.48	0.51[19]		**acc/gcc**	36	0.36	26	0.26		
										ata/ata	6	0.06	6	0.06		
										ata/gcc	23	0.23	24	0.24		
										gcc/gcc	19	0.19	23	0.23		
IL-10 -1082	**a**	82	0.81			105	0.52	0.51[19]		**a/a**	23	0.23	27	0.27	0.23	
	g	78	0.77			97	0.48	0.49[19]		**a/g**	59	0.58	50	0.50		
										g/g	19	0.19	23	0.23		
IL-10 -819	**t**	44	0.44			50	0.25	0.21[19]	0.233[20]	**t/t**	6	0.06	6	0.06	0.99	
	c	95	0.94			152	0.75	0.79[19]	0.767[20]	**t/c**	38	0.38	37	0.37		
										c/c	57	0.56	57	0.57		
IL-10 -592	**c**	95	0.94			152	0.75	0.79[19]	0.767[20]	**c/c**	57	0.56	57	0.57	0.99	
	a	44	0.44			50	0.25	0.21[19]	0.233[20]	**c/a**	38	0.38	37	0.37		
										a/a	6	0.06	6	0.06		

Table 8.10: Control phenotype, allele and genotype frequencies (continued)

Phenotype and allele frequencies								Genotype frequencies							
Gene	**Allele / haplotype**	**Phenotype**		**Previously reported frequencies**	**Allele**		**Previously reported frequencies**	**Genotype**	**Observed**		**Expected**		**H-W**	**Previously reported frequencies**
		count n=101	freq		count n=202	freq			count n=101	freq	count n=101	freq	χ^2 p	
TGFβ -800/-509	**g-c**	85	0.84		130	0.64		**g-c/g-c**	45	0.45	42	0.41	0.68	
	g-t	42	0.42		52	0.26		**g-c/g-t**	27	0.27	33	0.33		
	a-c	19	0.19		20	0.10		**g-c/a-c**	13	0.13	13	0.13		
								g-t/g-t	10	0.10	7	0.07		
								g-t/a-c	5	0.05	5	0.05		
								a-c/a-c	1	0.01	1	0.01		
TGFβ -800	**g**	100	0.99	*0.99*[21]	182	0.90	0.912[21]	**g/g**	82	0.81	81	0.81	0.99	0.849[21]
	a	19	0.19	*0.151*[21]	20	0.10	0.088[21]	**g/a**	18	0.18	18	0.18		0.141[21]
								a/a	1	0.01	1	0.01		0.010[21]
TGFβ 509	**c**	91	0.90	*0.890*[21]	150	0.74	0.657[21]	**c/c**	59	0.58	55	0.55	0.22	0.418[21]
	t	42	0.42	*0.582*[21]	52	0.26	0.343[21]	**c/t**	32	0.32	38	0.38		0.472[21]
								t/t	10	0.10	7	0.07		0.110[21]
TGFβ aa10	**L**	86	0.85	*0.830*[21]	134	0.66	0.584[21]	**L/L**	48	0.48	44	0.44	0.28	0.358[21]
	P	53	0.52	*0.642*[21]	68	0.34	0.416[21]	**L/P**	38	0.38	45	0.45		0.472[21]
								P/P	15	0.15	11	0.11		0.170[21]
TGFβ aa 263	**T**	100	0.99	*0.997*[21]	193	0.96	0.970[21]	**T/T**	93	0.92	91	0.91	0.17	0.930[21]
	I	8	0.08	*0.070*[21]	9	0.04	0.030[21]	**T/I**	7	0.07	9	0.09		0.067[21]
								I/I	1	0.01	0	0.00		0.030[21]

Table 8.10: Control phenotype, allele and genotype frequencies (continued)

Phenotype and allele frequencies								Genotype frequencies							
Gene	**Allele / haplotype**	**Phenotype**		**Previously reported frequencies**	**Allele**		**Previously reported frequencies**	**Genotype**	**Observed**		**Expected**		**H-W**	**Previously reported frequencies**
		count n=101	freq		count n=202	freq			count n=101	freq	count n=101	freq	χ^2 p	
VDR Apa-Taq	**AT**	29	0.29		30	0.15		**AT/AT**	1	0.01	2	0.02	0.85	
	aT	76	0.75		105	0.52		**AT/aT**	15	0.15	16	0.15		
	At	56	0.55		67	0.33		**AT/At**	13	0.13	10	0.10		
								aT/aT	29	0.29	27	0.27		
								aT/At	32	0.32	35	0.34		
								At/At	11	0.11	11	0.11		
VDR Apa	**A**	72	0.71	*0.71*[22]	97	0.48	0.494[22]	**A/A**	25	0.25	23	0.23	0.79	0.21[22]
	a	76	0.75	*0.79*	105	0.52	0.506	**A/a**	47	0.47	50	0.50		0.50
								a/a	29	0.29	27	0.27		0.29
VDR Taq	**T**	90	0.89	*0.85*[24]	135	0.67	*0.605*[24]	**T/T**	45	0.45	45	0.45	0.99	0.36[24]
	t	56	0.55	*0.63*	67	0.33	*0.385*	**T/t**	45	0.45	44	0.44		0.49
								t/t	11	0.11	11	0.11		0.14
VDR Fok	**F**	88	0.87	*0.85*[23]	118	0.58	*0.61*[23]	**F/F**	30	0.30	34	0.34	0.18	0.37[23]
	f	71	0.70	*0.63*	84	0.42	*0.39*	**F/f**	58	0.57	49	0.49		0.48
								f/f	13	0.13	17	0.17		0.15

Table 8.10: Control phenotype, allele and genotype frequencies (continued)

Phenotype and allele frequencies									Genotype frequencies						
Gene	**Allele / haplotype**	**Phenotype**		**Previously reported frequencies**		**Allele**		**Previously reported frequencies**		**Genotype**	**Observed**		**Expected**		**H-W**	**Previously reported frequencies**
		count	freq			count	freq				count	freq	count	freq	χ^2 p	
Duffy		n=100				n=200					n=100					
	FY*exp-FY*A	72	0.72			90	0.45			**FY*exp-FY*A homozygous**	18	0.18				
	FY*exp-FY*B	80	0.80			108	0.54			**FY*exp-FY*A/FY*exp-FY*B**	54	0.54				
	FY*null-FY*A	0	0.0			0	0			**FY*exp-FY*B homozygous**	26	0.26				
	FY*null-FY*B	2	0.02			2	0.01			**FY*exp-FY*B/FY*null-FY*B**	2	0.02				
	FY*exp	100	1.0			198	0.99			**FY*exp/exp**	98	0.98	98	0.98	1.0	
	FY*null	2	0.02			2	0.01			**FY*exp/null**	2	0.02	2	0.02		
										FY*null/null	0	0	0	0		
	FY*A	72	0.72			90	0.45	0.425[27]		**FY*A/A**	18	0.18	20	0.20	0.85	
	FY*B	82	0.82			110	0.55	0.573[27]		**FY*A/B**	54	0.54	50	0.50		
										FY*B/B	28	0.28	30	0.30		
HLA-HFE		n=330				n=660					n=330					
aa 63-282	**63H-282C**	314	0.95							**63H-282Y/63D-282C**	11	0.033				
	63D-282C	89	0.27													
	63H-282Y	65	0.20													
	63D-282Y	0	0													
aa 63	**63 H**	326	0.99		*1.000*[26]	568	0.86		*0.872*[26]	**63 H/H**	241	0.73	244	0.74	0.75	0.743[26]
	63 D	89	0.27		*0.257*[26]	92	0.14	0.17[25]	0.128[26]	**63 H/D**	85	0.26	80	0.24		0.257[26]
										63 D/D	4	0.01	6	0.02		0.0[26]
aa 282	**282 C**	329	0.997		*0.995*[26]	594	0.90			**282 C/C**	265	0.80	267	0.81	0.55	0.877[26]
	282 Y	65	0.197	0.064[25]	*0.123*[26]	66	0.10	0.032[25]	0.064[26]	**282 C/Y**	64	0.19	59	0.18		0.119[26]
										282 Y/Y	1	0.003	3	0.01		0.004[26]

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