CHAPTER 1
Introduction and Literature Review

This chapter presents an introduction and the literature review on the immunogenetics of multiple sclerosis. The sections of this chapter are presented as follows:

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Introduction and Literature Review

Multiple sclerosis (MS) is considered a debilitating auto-immune disease of the central nervous system (CNS) characterised by inflammation and demyelination of the nerve fibres (McFarland and Dhib-Jalbut, 1989; Olerup et al, 1989; Thorsby et al, 1989). The demyelination in the brain and spinal cord leads to an interruption in the transmission of nerve impulses resulting in a variety of manifestations, depending on which nerves are affected. In Australia, MS is considered one of the most common chronic neurological conditions among young adults and the female: male ratio in N.S.W. is 2.3 : 1 (Hammond et al, 1987; 1988; McLeod et al, 1994).

According to Larsen et al (1985) and Olerup et al. (1989), there are two distinct disease categories known as primary chronic progressive and relapsing/remitting multiple sclerosis. The primary chronic progressive form of the disease has a continuous course from the onset and approximately 15-40% of patients with MS have this form of the disease. In relapsing/remitting MS, the disease course is characterised by relapses and remissions and in many cases these sufferers will eventually enter a phase with secondary progressive symptoms.

The cause of MS is still unknown although an array of evidence exists supporting both genetic and environmental causative factors. This review will explore the clinical manifestations of the disease in an attempt for the author to better understand the disease under study. The proposed aetiology of the disease will be also be discussed. Particular attention will be paid to the evidence supporting an immunogenetic contribution as this forms the rationale for the approach taken in this study.
1.1 Clinical manifestations of MS

The clinical manifestations of MS are varied because the demyelination leading to loss of function can occur anywhere in the central nervous system. Complaints of both sensory and motor function disturbances are most common although the onset of these impairments may be monosymptomatic in the early stages of the disease (Hallpke, 1983). Below are some of the more common signs and symptoms of MS. Other manifestations are possible and their severity and type depends on the location of the lesions and extent of the damage due to the demyelination.

1.1.1 Prodromal symptoms

These symptoms described by patients are very similar to those found in rheumatic- and neurasthenic-like conditions. These non-specific symptoms include limb pains, fatigue, irritability, poor memory, and weight loss. They could be considered as insignificant and generalised if not for cerebral spinal fluid abnormalities which are found at this phase of the disease (Matthews, 1991; Hallpke, 1983).

1.1.2 Visual disturbances

A. Optic neuritis.

Optic neuritis, the sudden onset of pain and loss of vision in one or both eyes is frequently an early manifestation of myelin sheath damage in MS. It occurs twice as frequently in females as in males which parallels the distribution of the disease between the different sexes. The pain in the eye may persist for the duration of the visual impairment and until vision is gradually restored which is usually between two to eight weeks. Unilateral or generalised headache often accompanies the eye pain. Initial blurring of vision is reported with descriptions of a haze or mist effect. Complete loss in vision has been reported in 3% of cases (Perkin & Rose, 1979).
Most patients will present with a visual acuity of between 6/36 and will detect hand movement (Reagan et al, 1977). The status of the visual acuity is determined by the use of the Snellen chart and the Jaeger test or their equivalent. On examination, the optic disc appears normal in 50% of patients with complete loss of the disc margins found in 20% of cases. Linear haemorrhages radiating from the disc are also seen in some patients. The field defect is either central scotoma or over the whole field of vision (Lightman et al, 1987). The frequency of developing MS following an attack of acute optic neuritis ranges from 11.5% to 85% (Firth et al, 1992; Kurland et al, 1966; Hely et al, 1986; Francis et al, 1987; Kurtzke, 1985; Crompton et al, 1978; Matthews, 1991; and Cohen et al, 1979). Frith and colleagues (1992) in a lontitudinal study of 13 years have reported that 46% of Australian patients with optic neuritis developed MS whereas Kelly et al (1993) reported that 61% of optic neuritis patients developed MS in a five year follow-up study.

B. Other optical disturbances.

Abnormal visually evoked responses as a result of ocular nerve demyelination and plaque formation may manifest in a variety of ocular disturbances depending on the site of the damage. Subclinical abnormalities of ocular movements leading to diplopia, visual field disturbances and nystagmus are some of the clinical manifestation as a result of damage within the ocular system (Matthews, 1991).

1.1.3 Motor and sensory disturbances of the muscular system

Limb weakness is a common complaint. Both lower limbs are usually affected and weakness in one arm without weakness of the legs is uncommon (Hallpike, 1983). Progressive hemiparesis beginning with the lower limb and progressing to the upper limb on the same side is often seen in this condition. Progressive hemiplegia is seldom seen in the face and it is possible that the
lesion is usually found in the lateral column of the cervical spinal cord (Matthews, 1991). Respiratory weakness is common in relapses of the condition and is aggravated by energy spent in mobilisation with weak legs (Kurtzke, 1970).

Pyramidal signs of tiredness of the limbs and loss of reflexes occur early in the disease. Dragging of one leg after exercise and loss of abdominal reflexes are found in a high proportion of patients. In some cases, progressive spastic weakness of a lower limb is accompanied by an extensor plantar reflex on the other limb. Exaggerated tendon and extensor plantar reflexes are present in the more established disease (Ferrari et al, 1988).

Spasticity accounts for much of the disability as a result of upper motor neuron lesions in MS patients. Tonal spasms of the limbs account for many of the falls in MS. These spasms can occur without warning and flexor spasms are frequently painful. With prolonged flexor positions, contractures usually develop (Matthews, 1991).

In later stages of MS, muscle wasting occurs usually affecting the hands. Atrophy of the pectoral, gluteal, deltoid, sternomastoid muscles are also seen in patients (Davidson et al, 1934). Wasting of the lower limbs also occurs but to a lesser extent than that seen in the hand muscles (Fisher et al, 1985).

Excessive fatigue is often a complaint from patients. This is not associated with depression or disability but is aggravated by heat (Krupp et al, 1988).

Numbness described as a tingling and/or a "pins and needles" sensation is a common complaint. It usually begins in one foot spreading to the whole of both lower limbs, buttocks, perineum and other areas of the trunk. Numbness of the perineal area is upsetting to a lot of patients as the sensations of defaecating, micturition and sexual sensitivities are lost (Matthews, 1991). The restless leg syndrome, an unpleasant paraesthesiae (painful
burning, tingling sensation) of the leg at rest has also been noted (Sanders et al, 1986).

1.1.4 Pain

Pain occurs in the initial stage of the disease and persists throughout the course of the disease. Much of the pain can be linked to specific sources and causes: for example, pain in optic neuritis, in trigeminal neuralgia, or from muscle spasm. Pain that is well characterised (eg. linked to immobility) may be relieved by specific measures such as positioning but the diffuse pain in all parts of the body is more challenging to treat because the source and the cause of the pain is non-specific (Matthews, 1991).

1.1.5 Lhermitte's sign

This sensation is described as an electric feeling passing down the back to the legs when the neck is flexed. The unpleasant spread of sensation is usually downwards terminating at the base of the spine or down the lower limbs and lasts about two seconds. All four limbs may be affected and it occurs in at least one third of patients with MS (Kanchandani & Howe, 1982).

1.1.6 Mental changes

Until the 1950's, mental changes in MS have been difficult to document. Labile emotional expressions are almost universal and according to Ombredane (1929), intellectual deterioration is seen in 72% of patients studied. Intellectual impairment and deterioration is now acknowledged as characteristic of MS (Pratt, 1951). Poser (1978) found that mental changes correspond with severe disability and the long duration of the disease. Mental impairment was found by Truelle et al (1987) in 54% of patients graded 0-2 on the Kurtzke scale and similarly, Van den Burg et al (1987) reported 30% of patients graded a 1-4 on the Kurtzke scale.
1.1.7 Facial nerve weakness

Damage of the seventh nerve which controls facial expression and symmetry of facial movements results in weakness of the facial muscle. Bell's palsy is a common finding in MS and the onset is sudden but recovery is good with slight residual impairment (Kurtzke, 1970).

1.1.8 Hearing defects

Mild auditory impairment in MS has been observed and some patients complained of tinnitus and vertigo. Total deafness is rare and recovery from hearing loss is good (Daugherty et al, 1983).

1.1.9 Autonomic nervous system impairment

Loss of bladder and bowel control are most disturbing conditions faced by MS sufferers. Urgency of micturition, frequency, incontinence and recurrent retention of urine are some of the discomforts. Nocturia and nocturnal enuresis do occur in some patients (Miller et al, 1965; Anderson & Bradley, 1976; Kurtzke, 1970). With increasing disability, bladder control diminishes (Awad et al, 1984; Hallpike, 1983). Constipation and distension of the lower bowel are also common in MS. Faecal incontinence and urgency are known to occur albeit rarely (Glick et al, 1982).

Sexual functioning may be impaired. In males, erectile impotence was encountered in approximately 44% of the patients examined by Miller et al (1965). In females the usual complaints are loss of libido, alteration in genital sensation and lack of vaginal secretions (Lundberg, 1981).

Cardiovascular problems such as pulmonary oedema, hypertension, paroxysmal atrial fibrillation are some of the manifestations following damage to the autonomic nervous system (Pentland & Ewing, 1987; Chagnac et al, 1986).
1.1.10 Paroxysmal symptoms

There are a variety of paroxysmal symptoms recognised in MS. These clinical manifestations are brief episodes, occurring with differing frequencies and often triggered by motor or sensory stimuli.

A. Trigeminal neuralgia.

The signs and symptoms of trigeminal (fifth) nerve involvement includes pain, paraesthesia of the face and some unilateral loss of taste. The fifth nerve controls the muscles of mastication, and the sensory component detects facial sensations. Therefore trigeminal neuralgia will interfere with the above functions (Parker, 1928; Hallpike, 1983). Remission in trigeminal neuralgia is temporary and spontaneous recovery is seldom seen (Olafson et al, 1966).

B. Dysarthria and Ataxia.

Attacks of ataxia and dysarthria are sudden with slurring of speech, clumsy arm and leg movements and paraesthesia of the affected limbs or face. The ataxia affecting the legs is often bilateral and the attacks are brief in duration but occur frequently (Matthews, 1991).

C. Tonic seizures.

The tonic seizures of the limbs, side of the face and other muscle groups occur suddenly, frequently with differing degrees of severity of the spasm. Paraesthesia and pain in the affected muscle usually accompany the spasm. Motor activity appears to trigger an attack but sensory stimulation is also known to initiate the spasm (Joynt & Green, 1962; Matthews, 1991).
D. Itching.

The itching episodes may be spontaneous or induced by sensory or motor stimulation. The itching is usually described as intense and between attacks, feelings of dysaesthesia or lessening of cutaneous sensation are described (Osterman, 1976; Yamamoto et al, 1981; McAlpine, 1972).

E. Akinesia.

The paroxysmal loss of use in one or more limbs differs from ataxia or tonic attacks. The onset is sudden, frequent and the lower limbs are commonly affected (Matthews, 1991).

1.2. Diagnosis

Diagnosis of MS is based on clinical history and examination and on laboratory findings. The signs and symptoms of MS resemble many of those found in other neurological conditions and diagnosis is further complicated by the diversity of descriptions of the manifestations reported in the literature. As the clinical assessment is fundamental in understanding the nature of the disease, a number of researchers have attempted to develop clear guidelines for diagnostic categories (Allison & Millar 1954; McDonald & Halliday 1977; McAlpine 1972; Schumacher et al 1965; Rose et al 1976; and Poser et al 1983).

1.2.1 Diagnostic categories

Appropriate categorisation of MS is important to ensure that the same cohorts are included in studies around the world i.e. case ascertainment needs to be uniform and of high quality. The classifications most widely accepted were proposed by Poser et al (1983) as follows:
Category A. Clinically definite MS (CDMS).

The diagnosis requires:

1. either two attacks and clinical evidence of two separate lesions (the two attacks must involve different parts of the central nervous system and must be separated by a period of at least one month. Each of the attacks must last a minimum of 24 hours).

or

2. two attacks; clinical evidence of one lesion and paraclinical evidence of another separate lesion (paraclinical evidence is defined as the demonstration by means of various tests of the existence of a lesion of the CNS which has not produced signs of neurological dysfunction but may or may not have caused symptoms in the past).

Category B. Laboratory-supported definite MS (LSDMS).

The diagnosis is dependent on:

1. Two attacks; either clinical or paraclinical evidence of one lesion plus the presence of cerebral spinal fluid (CSF) oligoclonal bands (OB) and/or the increase in IgG synthesis.

or

2. One attack; clinical evidence of two separate lesions; and CSF OGIgG.

or

3. One attack; clinical evidence of one lesion and paraclinical evidence of another separate lesion; and CSF OGIgG.
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Category C. Clinically probable MS (CPMS).

This category is defined identically to LSDMS, except that the CSF changes are not required.

Category D. Laboratory-supported probable MS (L-SPMS).

In this category, two attacks and the characteristic CSF changes in the absence of clinical signs of the disease are required.

1.2.2 Laboratory tests

A variety of laboratory tests aimed at assessing motor and sensory functions are available. Electronic advances have enabled the recording of low amplitude potentials evoked (EP) by many forms of sensory stimulus. Visual (VEP), somatosensory (SEP) and auditory evoked potentials (AEP) and other EP testing assist in the diagnosis. Although optic nerve damage is better detected by VEP recordings, visual acuity with the use of the Snellen's Chart and visual field testing also aid in diagnosis. Brainstem function assessment and other neurological testing, eg myelograms, may be performed in order to give a cogent picture of the disease (Miller et al, 1993).

1.2.3 Computerised tomography scan

Computerised tomography (CT) scan greatly assists in the diagnosis of MS (Cala & Mastaglia, 1976; Gyldenstedt, 1976; Hallpike, 1983). Cerebral lesions and abnormalities of the ventricles can be detected with this method. Although plaques in the brainstem, cerebellum, optic nerve may not be resolved, many abnormalities can be seen with the CT scan without presentation of clinical signs and symptoms (Coin et al, 1979; Latack et al, 1984).
1.2.4 Magnetic resonance imaging (MRI)

The development of MRI for use in diagnosis and monitoring the progress of MS has significantly aided in diagnosis and is considered superior to CT scan (Lukes et al, 1983; Paty et al, 1988). One caution in the interpretation using MRI results is that the lesions detected may not correspond to the specific signs and symptoms, and many clinical manifestations cannot be attributed to focal MRI abnormalities (Jacobs et al, 1986; Matthews, 1991). However MRI can detect clinically silent multiple lesions which can be observed, measured and monitored (Raine, 1992).

1.2.5 Cerebral spinal fluid (CSF) analysis

Analysis of the CSF provides an insight into the events happening in the CNS. According to Allen (1991), about 65% of MS patients have excess of inflammatory cells in the CSF. The excess is due to an increase of T cells, plasma cells, macrophages and occasionally polymorphonuclear leucocytes. Webber et al (1987), have observed that although CD8 cytotoxic cells are present, the main cell type is a precursor cytotoxic cell of the CD4 helper phenotype. It has also been shown that plasma and B cells present in the CSF can synthesize immunoglobulin which is detected by electrophoresis and reported as the presence of oligoclonal bands. These bands are detected in approximately 90% of MS patients (Walsh and Tourtellotte, 1983; Thompson et al, 1979) but are not specific to MS and occur in a wide variety of other neurological conditions. It has been noted that higher rates of progression to MS have been found in patients with optic neuritis with oligoclonal bands than in those without (Allen, 1991).

1.3 Monitoring

Although MRI abnormalities and the presence of multiple lesions may not reflect clinical findings, there is significant correlation between numbers and size of lesions and the degree of disability.
The Kurtzke Disability Status Scale (DSS) and the extended form (EDSS) (Kurtzke, 1961; 1983) designed to assess response to treatment, together with MRI are used in monitoring progress of the disease.

1.4 Aetiology of MS

Numerous hypotheses have been proposed as to the cause of MS but as yet, no single hypothesis satisfactorily explains all aspects of the disease. Table 1.1 summarises the three major groups of these hypotheses. Each of these hypotheses will be discussed in the paragraphs following Table 1.1.
Table 1.1. Current aetiological hypotheses and supporting evidence in multiple sclerosis.

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Supportive evidence</th>
<th>Authors</th>
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<tr>
<td>Genetic factors</td>
<td>Racial, familial predisposition and the HLA associations with the disease</td>
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1.4.1 Environmental factors: latitude

One of the main evidence that environmental factors are involved in MS susceptibility comes from epidemiological studies which examined incidence rates of MS at different latitudes and the impact of migration from low to high risk areas.

In general, MS is more prevalent in the colder latitudes of northern Europe, north America and Australia but rare in the Asia, Africa, South America and the Orient. Variation in the distribution of the disease with latitude, suggests the involvement of an environmental factor in determining susceptibility to MS (Kurtzke, 1983).

The incidence of MS within tropical latitudes (about 23°N to 23°S) is rare with an increase in incidence rates above 50°N and below 50°S (Martyn, 1991). The most persuasive evidence that latitudinal factors are associated with MS was provided by McCall et al (1968) and later confirmed by Hammond et al (1988 & 1989) and McLeod et al (1994). McCall et al (1968) looked at the frequency of MS in three Australian cities: Perth, Newcastle and Hobart whilst McLeod et al (1994) included Queensland in their studies. These studies showed an increasing incidence, prevalence and mortality from MS as the latitude increases from north to south in Australia. There was a seven fold increase in the prevalence of MS in Hobart than tropical Queensland. Since the epidemiological studies surveyed a wide range of latitudes in a predominantly white population mainly of United Kingdom ancestry with no genetic differences in the marker HLA-DR2 found in the surveyed populations, it can be concluded that environmental factors associated with latitude make an important contribution to MS susceptibility (McLeod et al, 1994).

Similarly, the impact of migration from identified low to high risk areas had been studied and these findings suggest that other environmental factors play a part in MS aetiology (Allen, 1991). Dean (1967) looked at the incidence and prevalence of MS in white African-born individuals as compared to the immigrants to South
Africa. He showed that the age corrected frequency of MS was highest in European immigrants to South Africa compared to the lower incidence in Afrikaaners. He also concluded that migration before the age of 14 from a country of high prevalence to an area of low prevalence resulted in immigrants acquiring the low risk prevalence of the host country whereas individuals who migrate after the age of 15 retain the high risk of the disease associated with the country of origin. The table below shows the age corrected prevalence of MS in immigrants from Europe to South Africa.

<table>
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<th>Age</th>
<th>Prevalence rate of MS for European immigrants</th>
<th>Prevalence rate of MS for overall immigrants</th>
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<tr>
<td>&lt; than 14 years old</td>
<td>12.8/10^5</td>
<td>50.8/10^5</td>
</tr>
<tr>
<td>15 to 19 years old</td>
<td>66.1/10^5</td>
<td></td>
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</tbody>
</table>

Similarly, Elian et al (1987 & 1990) also studied the frequency of MS in the migrant population from low risk West Indies, Africa and the Indian subcontinent to a high risk country, United Kingdom, to see if there was an increase in the incidence of the disease associated with migration from low to high risk areas. They found that the prevalence of MS in the first generation children of immigrants from Africa, West Indies, and India to the United Kingdom approximates to that of native English populations. This rate differed markedly from the rates observed in the parental generation from the country of origin.

Other migration studies however have not yielded such clear cut changes in the pattern of MS susceptibility. Leibowitz et al (1983) found that the incidence rate of MS in the Jewish community was not altered by migration or geographical location.

The above results clearly implicate an association with an environmental component but despite a large amount of research,
the exact nature of this/these component/s remain unknown. Furthermore, lifestyle factors have been largely unexplored and warrant further study. The research that has been done has focused on the potential role of infectious agents associated with latitude as contributing to MS susceptibility.

1.4.2 Infective process

Overall convincing evidence for an infective contribution to the aetiology of MS is lacking. There have been a conflicting, confusing and controversial array of information about the possible microbial aetiological of MS. As early as the 1890's, the putative cause of MS was said to be due to an infectious episode. Bacteria, spirochaetes and, recently, viruses have been favoured as the candidate organisms (Brabic & Jewell, 1989; Poser, 1992) and many laboratory methods have been used to test the viral hypothesis (Allen, 1991). According to Arnason (1992), in 10% of cases or 3 times the expected frequency, a MS attack occurs approximately 5 weeks after a viral infection. The discovery of high titres of antibodies to the measles virus in MS patients blood and the CSF and the occurrence of autoimmune encephalomyelitis after measles infection has focused attention on the possible role of the measles virus, in MS aetiology (Adams & Imagawa, 1962; Johnson et al, 1984). Impaired T-cell responses to measles virus as measured by the monocyte migration inhibition test and by the generation of specific cytolytic T lymphocytes (CTL) have been observed in MS patients (Utermohlen & Zabriskie, 1973; Jacobson et al, 1985). Measles virus can also cause the fatal disease, subacute sclerosing panencephalitis (SSPE), in which inflammatory cells in the white matter are associated with myelin loss, and large quantities of measles virus RNA have been isolated from the CNS in SSPE infections (Haase et al, 1985). However, it should also be noted that antibody titres to other viruses namely herpes simplex, mumps, rubella were also found to be high (Norrby et al,1974; Brankin et al, 1989). Findings of human T-lymphotrophic virus type 1 (HTLV-1) antibodies in the serum and CSF of MS patients (Koprowski et al, 1985) has implicated retroviruses as possible agents of MS.
aetiology. However subsequent studies failed to confirm this observation (Madden et al, 1988; Kuroda et al, 1987). Several other researchers have isolated virus particles, viral antibodies and viral nucleic acids from the CSF, serum and CNS samples of MS patients (Brahic & Jewell, 1989; Jacobsen, et al, 1985; Johnson, 1984). Whilst viruses and virus-like particles have been isolated from tissues of MS patients (Johnson, 1985; Field, 1972; ter Meulen, 1972, 1978 & 1983; Padgett et al, 1977; Weiner et al, 1972; Haase et al, 1985), these particles isolated were small in numbers and from small tissue samples and these studies are difficult to repeat. In total, experiments concerning the identification and isolation of a single causative organism have been largely unsuccessful.

The concept of persistence, reactivation and latency of viruses has been demonstrated in many viral infections and this concept has been applied to MS and may explain the relapsing-remitting nature of the disease. Studies on the latency and persistence of viruses as well as presence of viral genome in MS patients have yielded mixed results, highlighting the difficulties with viral research in this area (Matthews, 1991; Allen, 1991). Cook et al (1986) and Martin et al (1988), in their search for viral persistence identified morbillivirus and herpes virus (HSV-2) components in the CNS plaques of MS patients. The search for viral genome sequences led Haase et al (1981) to discover measles virus genome in two of the eight MS patients. Similarly, Premkumar et al (1989) have identified sequences of retrovirus HTLV-1 in peripheral blood mononuclear cells of six MS patients.

The role of viruses in disease models of demyelination is well established (McFarland & Dhib-Jalbut, 1989). The association between a viral aetiology and MS has been supported by experimental models of virus-induced demyelination (McFarland & Dhib-Jalbut, 1989; Allen, 1991). In an animal model involving the Theiler's murine encephalomyelitis virus (TMEV) infection, inflammation and demyelination of the CNS occurred approximately one to several months after the injection of the virus (Dal Canto & Lipton, 1979; Clatch et al, 1986; Aubert et al, 1987; Cash et al,
1985). The subsequent development of demyelination and lesion formation in TMEV infection is thought to be immune-mediated. Induced demyelination in man by viruses has also been studied. For example papovavirus has been identified in progressive multifocal leucoencephalopathy (PML) (Johnson et al, 1977; Allen, 1991) whilst JC virus (Padgett et al, 1971) and SV40/PML (Weiner et al, 1972), two strains of papovavirus have been recovered from PML brain material. Although the evidence of virally-induced experimental models cannot fully support a role in MS susceptibility, there is much circumstantial evidence implicating the importance of an infectious agent (ter Meulen & Stephenson, 1983; McFarland & Dhib-Jalbut, 1989; Brahic & Jewell, 1989; Allen, 1991).

1.4.3 Genetic factors

In addition to the previous evidence implicating an environmental factor, three major lines of evidence support a genetic contribution to MS susceptibility. The genetic transmission of MS susceptibility is clearly indicated on the basis of increased risk of this disease occurring in monozygotic twins and to a lesser extent in dizygotic twins. Differences in racial susceptibility, and the increased frequencies of particular HLA antigens occurring in MS sufferers, are two other lines of evidence pointing to the involvement of a genetic factor in MS susceptibility.

These factors will be discussed below.

1.4.3.1 Genetic associations with MS

Support for a genetic role in MS has come from population, twin, and multiple case family studies. It has been suggested that there is a higher frequency and risk of contracting MS amongst close relatives of affected patients than the general population (Stewart et al, 1981). The closer the biological relationship the higher the risk of developing MS. Kuwert (1977) reported a seven times risk of developing MS in first cousins, 12 times for parents and 22 times
for siblings. In addition, approximately 15% of patients have an affected relative (Ebers, 1983) and the highest risk is also observed in siblings. In a population-based survey, Sadovnick et al (1988) found 19.9% of MS patients had an affected relative. In the same study, the age-adjusted risk of contracting MS was 1.5% in nephews & nieces, 2% in aunts, uncles & cousins, 2.5% in offspring, 3% in parents and the highest risk of 4% was in siblings. In another study, the risk of developing MS for siblings, parents and offspring of an affected patient was reported as 1.2%, 0.6% and 0.5% respectively (Spielman & Nathanson, 1982). It is therefore important to also consider the findings from twin studies to evaluate the role of genetic involvement.

An array of twin studies have implicated a genetic component but there has been variations in these observations. In twin studies, the higher concordance in monozygotic than dizygotic twins implies a genetic contribution to disease aetiology. McKay & Myrianthropoulous (1966) found that the monozygotic twin of an affected individual had a 20-25% chance of developing MS. Kuwert (1977) also found that the risk of developing MS in monozygotic twins was 533 times and in dizygotic twin was 259 times that of the normal population. Williams et al (1980) found a concordance rate for MS in monozygotic twins was 50% and 17% in dizygotic twins. In another study, Heltberg & Holm (1982) reported a 21% and 4% concordance rate for MS in monozygotic and dizygotic pairs respectively. However, Kinnunen et al (1988) found a lower concordance rate of 7% and 3% in monozygotic and dizygotic pairs respectively. Several of these studies have been criticised on the basis that the twin pairs selected favoured monozygotic pairs who were concordant. Perhaps the most careful twin study was conducted in Canada by Ebers et al (1986) who revealed a concordance rate for MS of 26% in monozygotic twins as compared with 2.3% in dizygotic twins and 1.9% in nontwin siblings. Even allowing for problems with bias in the research designs, there is a clear difference in concordance rates between monozygotic and dizygotic pairs. However as the concordance rate does not reach
100% in the monozygotic twins this suggests that either several
genes are involved or the genes are of low penetrance.

1.4.3.2 Racial susceptibility to MS

It has been suggested that MS occurs most frequently where
Vandals, Goths and Viking settled and this genetic legacy and
migratory patterns fit this ethnographic distribution (Matthews,
1991; Poser, 1992). This proposal is supported by the observation
that the incidence of MS in Palestine Arabs is 2.5 times that of the
Kuwaiti Arabs. It is presumed that the Viking-Norman ancestry is
reflected in the Palestine Arabs and absent in the Kuwaiti Arabs
(Al-Din et al, 1991). To substantiate their argument, the authors
reported that 16% of the Palestine Arabs had blue eyes, 46% had
hazel eyes and only 22% of the Kuwaiti Arabs had hazel eyes and
none had blue eyes.

No other racial group is more vulnerable to MS than the Northern
Europeans and the genes conferring susceptibility may be more
prevalent in the Shetland and the Orkney Islands (Matthews,
1991). The observation that the high frequency of Viking-Nordic
genetic legacy distributed throughout Europe and other places can
be supplemented by evidence that certain ethnic groups have lower
susceptibility to MS irrespective of their geographical locations.
MS in Hungarian gypsies is more than ten times less frequent than
the other Hungarians (Palffy, 1982; Crompton, 1991). Among the
American Indians, black Africans, Lapps, Hutterites, Asians and
Eskimos, MS is seldom seen (Crompton, 1991; Yu et al, 1989; Poser,

1.4.3.3 HLA associations with MS

The ability to recognise self and non-self is one of the fundamental
characteristics of the immune system. The mounting of an
appropriate response and the regulation of the response is the
result of such a recognition. The Major Histocompatibility Complex
(MHC) is acknowledged as an immunoglobulin-like supergene family which plays a major regulatory role in transplantation, disease association and the immune response during antigen presentation (Dupont, 1990). The ability of different MHC gene products to interact with a vast array of different protein antigens contributes to the genetic differences in immune responsiveness.

The immune response involves a series of events commencing with antigen presentation and recognition. There are two kinds of antigen recognition: by antibody idiootype (humoral or B cell response) and by antigen-specific T cell receptor (cellular or T cell response). The antigen-specific T cell receptor can recognise processed antigens only when physically associated with molecules encoded by the MHC (Dupont, 1990; Roitt et al, 1989). The trimolecular complex (consisting of the MHC Class II molecule and the T cell receptor together with the antigenic peptide fragment) is required for T cell activation and the subsequent initiation of specific immune response against either a foreign or self antigen (Figure 1).
Figure 1. A diagramatic representation of a tri-molecular complex structure involved in antigen presentation and recognition and the subsequent mounting of an immune response.

From crystallographic analysis of the three dimensional structure of the human Class I molecule (Bjorkman et al, 1987a, and 1987b), a four-domain model of the Class I molecule can be inferred. The cleft of the Class II molecule is formed by the C-terminal α-helices, with the floor formed by the N-terminal β-pleated sheets of the α1...
and $\beta_1$ domains and by analogy with Class I antigens, this cleft is the site of antigen recognition. From molecular modelling, it can be deduced that antigen recognition by MHC Class II restricted T cells depends upon the polymorphic allelic-specific contributions from both the $\alpha$ and the $\beta$ chains (Parham, 1992). According to Rothbard and Geffer (1991), sequence variation of one single amino acid at specific sites within the antigen-binding groove can alter the ability to present peptides to T cells. Hence, an individual's ability to respond to an antigen, whether foreign or self, is partly determined by the amino acid sequences of these polymorphic MHC and T cell receptor (TCR) molecules. Therefore, variations in the amino acid sequences of both the MHC and the TCR could potentially contribute to the development and inheritance of autoimmune diseases (Hillert et al, 1992; Hauser, 1989; Todd et al, 1988; Ødum et al, 1988; Olerup and Hillert, 1991; Haegert et al, 1990). The MHC region of man has a high degree of polymorphism and so considerable research effort has been directed at investigating any associations which may exist between a particular MHC haplotype and susceptibility to a specific disease. Therefore identification of specific gene sequences of the MHC, which is the focus of this study, will be examined.

In the early 1970's it was noticed that inheritance of certain MHC antigens was linked to immune responsiveness and with susceptibility to certain diseases, in particular autoimmune diseases. This is illustrated in Table 1.2 which documents specific human MHC - HLA Class I & II antigen polymorphisms that have been found to be associated with a range of autoimmune diseases (adapted from Tiwari & Terasaki, 1985; Todd et al, 1988).
Table 1.2. HLA class I & II associations with autoimmune diseases.

<table>
<thead>
<tr>
<th>Class 1 antigen</th>
<th>Class II antigen</th>
<th>Relative risk</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>B8</td>
<td>DR3</td>
<td>11.6</td>
<td>Celiac disease</td>
</tr>
<tr>
<td></td>
<td>DR7</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DR7</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>DR3</td>
<td>3.3</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td></td>
<td>DR4</td>
<td>2.6-6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B15</td>
<td>DR4</td>
<td>2.8-5.4</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
<td>28.9</td>
<td></td>
<td>Ankylosing Spondylitis</td>
</tr>
<tr>
<td>B27</td>
<td>DR3</td>
<td>2.5</td>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td></td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>DR2</td>
<td>2-5.5</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5-1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>DR4</td>
<td>14.6</td>
<td>Pemphigus vulgaris</td>
</tr>
<tr>
<td>A3</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A26</td>
<td>DR2</td>
<td>1-3</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>B38</td>
<td>DR3</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The support for immunological mechanisms in MS is primarily based on MHC studies and known pathological findings of abnormalities in macrophage and lymphocyte function and distribution in the CSF which ultimately lead to myelin and
oligodentrocyte destruction. The observation of T-lymphocytes in the CSF and the aberrant expression of Class 11 molecules in astrocytes and endothelial cells of the CNS suggest that the genetic predisposition to MS is linked to human leucocyte antigen (HLA) genes (Hafler et al, 1988; McFarland & Dhib-Jalbut, 1989).

Bertram et al (1972); and Maito etal (1972) first reported significant associations between MS and the human leucocyte antigen (HLA). In northern European caucasoids with MS ,the most common serologically detected antigen specificities encoded within the MHC is HLA Class 1 -A3,-B7; and the Class 11 -Dw2 and -DR2 haplotype (Bertram et al, 1972; Maito et al, 1972; Gogolin et al, 1989; Haegert et al, 1989; Haegert et al, 1990; Francis et al, 1991; Cullen et al, 1991). However the strongest association is with the DR2 with the relative risk of between 1.4 - 6.32 (see Table 1.3). The HLA Class II allelic nomenclature recognised by the WHO Nomenclature Committee used in this project is detailed by Bodmer et al, 1992 (see Appendix A).

The expected frequency of finding a given allele at any genetic HLA locus with a given allele of a second linked locus would be the product of the individual gene frequencies in the population. However certain combinations of alleles are found to occur at a higher frequency than the expected frequency. This phenomenon, known as linkage disequilibrium, is quantified as the difference between the observed and the expected frequencies (Roitt et al, 1989). In MS patients, linkage disequilibrium is seen between DR2 and DQw1 which correspond to the antigen Dw2 defined by cellular typing techniques. Earlier analysis of MHC associations using serological typing procedures confirmed an association between susceptibility to MS and the DR2, Dw2 and DQw1 haplotype. However, the advent of molecular typing techniques, such as restriction fragment length polymorphisms (RFLP), has permitted closer subtyping of the loci and subsequent studies have indicated that the MS association to be more specifically identified as the DR2, Dw2, DQw6 (which is a subtype of DQw1) haplotype. The use of RFLP has assisted in clarifying some of the anomalies reported
using serological typing. For example, the DR2 haplotype in the Hungarian gypsies contained the DQw5 alleles and not the DQw6. This appears to explain why MS is rare in the Hungarian gypsies although DR2 is very common in this population. Using RFLP, the DR2 locus can be divided into subtypes of DRB1-1501, -1502, -1503, -1601, -1602 and similarly the DQw6 is separated into subtypes of DQB1-0601, -0602, -0603, -0604, -0605 (Bodmer et al, 1992). Lately, the association between the DRB1-1501, DQB1-0602, DQA1-0102 haplotype and MS has been established by various authors (Francis et al, 1987 & 1991; Gogolin et al, 1989; Haegert et al, 1989; Haegert et al, 1990; Cullen et al, 1991; Spurkland et al, 1991; Olerup & Hillert, 1991; Haegert & Francis, 1992; Kelly et al, 1993; Haegert & Francis, 1993; Allen et al, 1994). The investigation into association between MS and the HLA-DR & DQ loci will be examined in detail in section 1.5.

1.4.3.4 HLA and racial associations with MS

Until recently, the association between MS and HLA alleles has been defined largely by serologic studies. The incidence of MS among particular racial groups has been investigated and is suggestive of a genetic legacy left by the Vandals, Goths and Vikings. The association between MS and the HLA-A3, B7 & DR2 haplotype is seen in the northern European populations and this association is also seen in populations in other parts of the world with the Viking-Nordic ancestry. The strongest association is with DR2(Dw2) and is observed with the migrant populations in North America and Australasia (Jersild et al, 1973 and Tiwari & Terasaki, 1985). In Europe, the DR2 association is strongest in the Scandinavian and other northern countries with a weakening association with populations towards the equator. The DR2 association with MS is weak in the Orkney Islands where MS is highly prevalent, and this may be due to the very high frequency of DR2 in that population (Compton, 1981). Populations such as the blue- and hazel-eyed Palestinian Arab patients have a higher DR2 frequency than the non-blue-eyed Kuwaiti Arabs where DR2 is absent (Ali-Din et al, 1991).
These population studies indicate that the relative risk of MS in DR2 candidates ranges from 1.4 to 6.32 and are summarised in Table 1.3.
Table 1.3. The relative risks of HLA-Class I and Class II antigens and MS in different racial groups.

<table>
<thead>
<tr>
<th>Racial group</th>
<th>Class I</th>
<th>Class II</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen</td>
<td>Relative</td>
<td>Antigen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>risk</td>
<td></td>
</tr>
<tr>
<td>N. European USA (White)</td>
<td>A3</td>
<td>2.9</td>
<td>DR/Dw2</td>
</tr>
<tr>
<td>French Canadians</td>
<td>B7</td>
<td>3.7-4.1</td>
<td>DR3</td>
</tr>
<tr>
<td>N. Ireland</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>SE Wales</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>NE Scotland</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>Australia</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>New Zealand</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>Orkney Islanders</td>
<td>A3</td>
<td>0.8</td>
<td>Dw2</td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>1.0</td>
<td>DR2</td>
</tr>
<tr>
<td>USA (black)</td>
<td>A3</td>
<td>4.9</td>
<td>DR2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dw2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>N. Italy</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>S. Italy</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DR4</td>
</tr>
<tr>
<td>Sardinia</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DR4</td>
</tr>
<tr>
<td>Malta</td>
<td></td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>Greece</td>
<td>A3</td>
<td>2.2</td>
<td>DR4</td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Jordan (Arab)</td>
<td></td>
<td></td>
<td>DR4</td>
</tr>
<tr>
<td>Iran</td>
<td>A11</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Hungary (caucasion)</td>
<td>B7</td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>Hungary (gypsy)</td>
<td>B7</td>
<td></td>
<td>DR2</td>
</tr>
<tr>
<td>Spain</td>
<td>B18</td>
<td>2.5</td>
<td>DR2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DQw1</td>
</tr>
<tr>
<td>Mexico (mestizo)</td>
<td></td>
<td></td>
<td>DRw6</td>
</tr>
<tr>
<td>Israel</td>
<td>A1</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>A31</td>
<td>2.5</td>
<td>DR2</td>
</tr>
<tr>
<td></td>
<td>Cw3</td>
<td>3.2</td>
<td>DR4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D Rw6</td>
</tr>
<tr>
<td>China (Hong Kong)</td>
<td></td>
<td></td>
<td>DR2/DQw</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>India (Asian)</td>
<td>A9, B5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B12</td>
<td></td>
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</tr>
</tbody>
</table>
Although an increased frequency of MS patients with DR2 is seen in the northern European caucasians, approximately one third of the patients are DR2 negative (Batchelor, 1985; Tiwari & Terasaki, 1985). This relatively weak association with DR2 indicates that this allele is neither necessary nor sufficient for the disease expression and in other populations the incidence of MS is associated with other HLA genes. Italians, Sardinians, Jordanian Arabs with MS have an increased frequency with DR4 than DR2 and the DR6 is frequently found in the Japanese and Mexican MS patients (Naito et al, 1978; Gorodezky et al, 1986; Compston, 1978 & 1991; Marrosu et al, 1988; Kurdi et al, 1977; Allen, 1991).

1.4.3.5 HLA and family studies

As indicated previously, members of affected families have a higher risk of developing MS. Estimates of expected frequency can be made on the basis of the amount of HLA haplotype sharing between affected sib-pairs and cousin pairs. However, there are intrinsic difficulties in deciding who is "normal" within families when unaffected sibs may develop MS later in life, or MS may be clinically undetected throughout life. Furthermore, if environmental factors are at play, family members who would inherit the genetic blueprint for MS may not develop the disease unless triggered by those environmental factors (Stewart & Kirk, 1981). To overcome this problem of ascertainment, Stewart et al (1981) examined the disordered segregation of HLA genes in 100 published sibling pairs. In a normal population the chance of randomly selected siblings sharing both haplotypes is 25%, sharing one haplotype 50% and no haplotype 25%. Similarly there is a 25% chance that cousins share one haplotype and share none 75% of the time. Disordered segregation analysis by Stewart et al (1981), found a 2, 1, 0 haplotype sharing of 37%, 50% and 13% respectively. Compston & Howard (1982) looked at 145 affected families and concluded that sharing of one haplotype occurs in 83% of siblings, 100% of parent/child pairs (as would be expected) and 50% of cousins as compared to the expected 75%, 100%, 25% respectively. Where more than one relative is affected the sharing of one
haplotype is 68% compared to the expected 35%. However there are several studies which found that the rate of haplotype sharing did not differ from the expected rates (Ebers et al, 1982 and Govaerts et al, 1985). This apparent lack of consistency between the different studies would appear to suggest that the role of the MHC in determining susceptibility to MS is limited or that the MHC encoded gene/s responsible for MS expression are relatively common in the general population and hence the affected members in the family may inherit the MS susceptibility gene but on a different haplotype from the same or different ancestors.

1.5 Why study the HLA DQB1 locus?

Since the late 1980's, it has been demonstrated that MS susceptibility is related to the presence of the DRB1-1501, DQB1-0602, DQA1-0102 haplotype. There is debate among the researchers as to which of these genes contribute to MS susceptibility ie does DR or DQ play a primary role in susceptibility. Several authors have reported that it is difficult to assess whether the DRB1- (a subtype of DR2) or the DQB1 (a subtype of DQw1) genes play the primary role in MS susceptibility. Francis et al (1991) studied 71 MS patients and 100 controls and found that there were no significant differences in the frequency of the DR2 and the DQBI alleles (63% of patients compared to 32% of controls for the DR2, p=0.001; 65% of patients compared to 42% of controls for the DQBI alleles, p=0.001). In the French Canadian population, Haegert & Francis (1992) confirmed that the DQB1-0602 was the major MS-associated allele (the corrected p value, ie. pc=0.000004, the relative risk, RRr=6.51) and that the DR2 allele also showed strong disease association ( pc=0.0004, RR=4.33). A study of MS patients and controls from Hong Kong by Serjeantson et al (1992) revealed that the DQB1-0602 allele is associated with MS susceptibility although the role of DRB1-1501 allele in MS pathogenesis is less certain. Allen et al (1994) in a Swedish study, found that the DRB1-1501, DQA1-0102, DQB1-0602 haplotype is positively associated with MS (p<0.0001, RR=3.48). They were
unable to distinguish the contribution of DRBI from that of DQBI genes in MS susceptibility. However, several authors have argued that susceptibility to MS is more strongly associated with the HLA-DQ than with -DR2 (Vartdal et al, 1989; Marcadet et al, 1985; Heard et al, 1989; Cohen et al, 1984; Jacobson et al, 1986; Serjeantson et al, 1986; Spurkland et al, 1991). Thus, the experimental rationale underlying this project is to determine whether the DQB1 or the DRB1 loci play a primary role in MS association.

1.5.1 Associations between MS susceptibility and the HLA-DR locus

Miller et al (1989) using populations of 144 caucasoid MS patients, 117 caucasoid controls and 82 Maori controls in Wellington, New Zealand, found a stronger association between MS and HLA-DR2 than with -DQw1. Similarly using polymerase chain reaction-sequence specific oligonucleotide probes (PCR-SSO), Gogolin et al (1989) found that there was an increase in the DR2 haplotype in MS patients (26% in MS, 12% in controls; p<0.01). This study however found no significant difference in the frequency of DQB1 alleles between MS patients and the controls. Using RFLP- and DR, DQ cDNA probes, Olerup et al (1987) initially showed a closer association between MS susceptibility and the DR locus than the DQ locus. In a later Swedish study, Olerup et al (1991) found that the MS association corresponded to the DRw15 (DRw15 and DRw16 are subtypes of DR2), DQw6 (a subtype of DQw1), Dw2 i.e. according to the genomic nomenclature, the DRB1-1501, DQA1-0102, DQB1-0602 haplotype (p<10^{-10}, RR = 3.9) (please refer to Appendix A for the nomenclature used). They also found that the DQw6 association was secondary to the DRw15, DQw6, Dw2 haplotype (p<10^{-7}). Hillert & Olerup (1993) also confirmed in a Swedish study that the DRw15, DQw6 and Dw2 haplotype is positively associated with MS.
1.5.2 Associations between MS susceptibility and the HLA-DQ locus

A number of RFLP studies have indicated that MS susceptibility is more closely associated to the DQ allele than the DR2 specificity (Vartdal et al, 1989, Marcadet et al, 1985, Heard et al, 1989, Cohen et al, 1984, Haegert et al, 1989). Evidence for a DQ association with MS was provided by Heard et al (1989) in a study using pooled DNA from patients and controls from northeast Scotland. After digestion with the enzyme Msp I and hybridization to the probe DQα, a 3.25 kilobase fragment was present in the group of patients with DR2 which was absent in the corresponding group of controls. Independent studies were also conducted in the Grampian region of northeast Scotland and Northern Ireland. In the Scottish study, this 3.25 kb fragment was found in 30% of patients and 4% of controls (p=0.001) and in equal frequency in the Northern Ireland control and test populations. When only the DR2 positive individuals were analysed the unequal frequency in patients and controls were found in both areas. This fragment was associated with MS independently of DR2 and was associated with DRw8 and DR7. The fragment appeared to exhibit apparent allelism to DQw1 independently of DR2. They concluded that the DQAI1 fragment may be inherited on another haplotype than the DR2/DQw1 implicating a second susceptibility gene. Kolstad et al, (1989) found that MS patients have a higher frequency of an HLA-DQB1 epitope in MS patients than controls (97% patients compared to 75% controls, p<0.005, RR=11.6). Only 76% of the patients were DR2 and they concluded that the DQB1 variants are more strongly associated with MS than is DR2.

It has also been suggested that susceptibility to MS may be associated with several alleles in the DQ region with shared common amino acid sequences and/or shared common residues. Vartdal et al (1989) found that 97% of Norwegian MS patients carry the DQB1 genes which encode shared polymorphic amino acid sequences in the membrane-distal domain of the DQB chain (p< 0.0001, RR=10.5). The DQB1 alleles encoding the shared amino acid
sequences are in linkage disequilibrium with the DRB1 alleles of DR2, DR4, and DRw6 which is associated with MS susceptibility in different ethnic populations. In another Norwegian study, Spurkland et al (1991) found that 99% MS patients compared to 79% of the controls carried the DQA1 alleles which encode glutamine at residue 34 (p<0.001, RR=12). They also found that 97% of patients compared to 72% of controls have the DQBI alleles encoding DQB1 chains sharing long polymorphic epitopes thought to be involved in peptide binding (p<0.001, RR=10). Combining the two sets of data, 96% of patients as compared to 60% of controls (RR=13) have both one of the DQAI and the DQBI alleles which led them to conclude that predisposition to MS may be influenced by a particular combination of DQ α and β chains, encoded either in cis or trans configuration.

The presence of Aspartate at residue 57 of the DQB chain carries an increased risk of insulin dependent diabetes mellitus (IDDM) providing evidence that susceptibility to autoimmune disease is associated with genes located in the HLA D region (Todd et al, 1987). Similarly, Leucine at amino acid residue 26 can be plotted to the floor of the antigen binding cleft, under the α-helix, which may influence antigen binding and the initiation of an immune response (Brown et al, 1988 & 1993). Haegert & Francis (1992), in a French Canadian study concluded that the DQBI alleles encoding leucine at residue 26 contribute positively to MS association (Pc<0.0001, RR=11.98) and in 1993 affirmed their earlier findings that DQBI alleles encoding leucine at residue 26 was not secondary to a primary association with the DRBI-1501 haplotype (p<0.05). However, Francis et al (1991) failed to confirm that shared hypervariable region sequences confer susceptibility. In another study, Haegert & Francis (1993) found that the DRBI-1501 bearing haplotype was positively associated with MS in the French Canadians but the DQBI alleles which share long stretches of nucleotides were secondary to the DRBI-1501 haplotype. Allen et al (1994) also failed to confirm that the putative DQ- α β heterodimer, encoded for by certain DQAI and DQBI alleles confers MS susceptibility.
It would seem that the strong linkage disequilibrium which exists across the DR-DQ region has prevented a clear resolution of the precise contribution of each of these genes in MS susceptibility. However, Altmann et al (1991) in their review concluded that the arguments for a DQ association with MS looked compelling.

1.5.3 Aims of the study

The aim of this study is to examine an Australian population in the hope to answer the following questions:

1. Is the primary MHC factor(s) in MS susceptibility encoded by the DQBI or the DRBI loci? Although this project focuses only on the DQBI polymorphisms, data on the DR polymorphisms have also been investigated to assist in comparative analysis.

2. Do the DQBI alleles which encode shared polymorphic amino acid sequences contribute to MS susceptibility?

3. Do the DQBI alleles encoding leucine at position 26 contribute to MS susceptibility?

4. Is the DQBI locus:

   A) necessary and/or sufficient for disease expression? The necessary disease locus means that disease alleles must be present in all affected persons and is necessary for disease expression. Sufficient disease loci implies that other factors eg. environmental and/or infective factors together with the disease gene is required for disease expression (Greenberg, 1993).

   or

   B) the susceptibility locus? The susceptibility locus is not the determining factor for disease expression but an allele at that locus will increase the risk for the disease. The true
determining factor could be environmental or genetic in nature (Greengerg, 1993).

or

C) a marker locus that increases susceptibility or in linkage disequilibrium with the susceptibility gene?

In order to achieve the above aims, detection of the allelic variations of the second exon of the DQB1 gene will be conducted by PCR-RFLP genotyping of 100 MS patients and 100 controls in an Australian population.
## Appendix A.

Nomenclature of the HLA system (adapted from Bodmer et al, 1992)

**Designations of HLA-DR alleles**

<table>
<thead>
<tr>
<th>HLA alleles</th>
<th>HLA-DR serological specificities</th>
<th>HLA-D-associated (T-cell-defined) specificities</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1-0101</td>
<td>DR1</td>
<td>Dw1</td>
</tr>
<tr>
<td>DRB1-0102</td>
<td>DR1</td>
<td>Dw20</td>
</tr>
<tr>
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<td>DR15 (DR2)</td>
<td>Dw2</td>
</tr>
<tr>
<td>DRB1-1502</td>
<td>DR15 (DR2)</td>
<td>Dw12</td>
</tr>
<tr>
<td>DRB1-1601</td>
<td>DR16 (DR2)</td>
<td>Dw21</td>
</tr>
<tr>
<td>DRB1-1602</td>
<td>DR16 (DR2)</td>
<td>Dw22</td>
</tr>
<tr>
<td>DRB1-0301</td>
<td>DR17 (DR3)</td>
<td>Dw3</td>
</tr>
<tr>
<td>DRB1-0302</td>
<td>DR18 (DR3)</td>
<td>Dw'RSH'</td>
</tr>
<tr>
<td>DRB1-0401</td>
<td>DR4</td>
<td>Dw4</td>
</tr>
<tr>
<td>DRB1-0402</td>
<td>DR4</td>
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Note: 'w' denotes provisional status
### Designations of HLA-DQ alleles

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Note: 'w' denotes provisional status
CHAPTER 2
Materials and Methods

This chapter describes the materials used and methods developed to genotype the second exon of the HLA DQB1 region. It is divided into the following sections:

2.1 Introduction
2.2 Sample collection from whole blood and cell lines
2.3 DNA extraction
   2.3.1 Isolation of nuclear pellet from whole blood and from EBV transformed B cell lines
   2.3.2 DNA extraction from nuclear pellets obtained from nuclear pellets obtained from whole blood or from EBV transformed B cell lines using rapid phenol-free method
2.4 DNA amplification
   2.4.1 Selection of primers
   2.4.2 Optimization of conditions for PCR
   2.4.2.1 Preparation for the amplification
   2.4.2.2 Reagent concentrations
   2.4.2.3 PCR amplification conditions
2.4.3 PCR-reaction protocol using primers, GH29 & DB130
2.4.4 PCR-reaction protocol using primers, GH28NL & QB202; GH28NL & QB204; and MCBB & QB202
2.5 Digestion
   2.5.1 Digestion of DNAs amplified with primers GH29 & QB130 using the five restriction endonucleases Fok1, HaeI1, Rsal, Sau3A1 and HhaI (Uryu, 1990)
   2.5.2 Digestion of DNAs amplified with:
      a) DQw1 group specific primers, GH28NL & QB202 using 6 restriction enzymes, Fok1, ApaI, HaeI1, BssHII HhaI and HphI
      b) DQw2,3,4 group specific primers, GH28NL & QB204 using restriction enzymes, Fok1, BglI
Sac1, BsaH1(ACY1) and Msp1 (HpaII).

**c)** primers, MCBB & QB202.

2.6 Analysis

2.6.1 Data analysis
2.1 Introduction

The key objective of this work was to examine DQB1 polymorphism in MS affected and control populations. The second exon of the DQB1 gene was selected because of the polymorphic nature of this region. There are several methods available to distinguish HLA allelic variation. These are allele specific oligonucleotide probing of polymerase chain reaction fragments (PCR-SSO); PCR amplification with sequence-specific primers (PCR-SSP); digestion of polymerase chain reaction-amplified DNA with restriction endonucleases (PCR-RFLP); and amplified refractory mutation system (ARMS). Due to the small number of samples available for typing at one particular time (an average of about 5 samples per week), the PCR-RFLP method has been chosen because it appeared to provide a simple, rapid and economical technique for accurate analysis of HLA-DQ allelic variations compared to the other techniques.

The technique for PCR-RFLP typing of HLA-DQBI alleles was first reported by Trucco et al, (1989) followed by Uryu et al, (1990); Nomura et al, (1991); Salazar et al, (1992); Hao et al, (1992). The method described by Uryu et al, (1990) was at first followed and adapted for use in this project; however the concurrent amplification of the DQB2 pseudogene led to complex digest patterns which were difficult to interpret. Subsequently the method by Nomura et al, 1991 was used and modified. This resulted in the amplification of DQBI alleles only. Finally to allow for the clear discrimination of the 17 DQB1 alleles further modifications with respect to primer design and endonuclease selection were developed (Teutsch et al, 1995).

2.2 Sample collection from whole blood and cell lines

Peripheral blood samples were collected from 100 clinically defined MS patients and 100 control subjects residing in NSW, Australia. The MS patients were identified either through the Austim register,
and had participated in a clinical trial of alpha interferon and
transfer factors (Austims, 1989, Basten et al, 1980), or from
referrals from the various MS clinics and hospitals in NSW. The
control samples came from staff at Westmead Hospital, Sydney and
spouses and friends of the MS patients. Care was taken to match
the ethnic origins of the MS patients and the control populations to
the Northern European stock. In addition, 8 HLA-D homozygous
reference cell lines used by the NSW Red Cross Blood Transfusion
Tissue Typing Laboratory were also included in the study. All
samples used in this study were DR typed by the NSW Red Cross
Blood Bank to enable comparative analysis.

2.3 DNA extraction

2.3.1 Isolation of nuclear pellet from whole blood
and from EBV transformed B lymphoblastoid
cell lines (John et al, 1991)

5 ml of whole blood was collected in a tube containing 100 μl of
15% Ethylenediaminetetraacetate (EDTA) as an anti-coagulant. The
5 ml of blood was transferred into a 10 ml centrifuge tube and 5 ml
of buffer containing 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM
MgCl₂ was added. 120 μl of Nonidet P-40 was added to lyse the
cells and the contents were mixed well by inverting the tube
several times. The sample was centrifuged at 2000 rpm for 10
minutes in a Beckman Microfuge E, 15,000 rpm centrifuge and the
supernatant was slowly poured off and the nuclear pellet at the
bottom of the tube was retained. Similarly, a nuclear pellet was
prepared from EBV transformed cell lines by addition of 125 μl of
Nonidet P-40 for every 5 x 10⁶ cells to lyse the cells, and the
contents were mixed well by inversion. The nuclei were pelleted
by centrifugation at 2,200 rpm for 10 minutes at room temperature
in a Beckman centrifuge. The pellet from both preparations was
washed with 5 mls of buffer containing 10mM Tris-HCl pH 7.6, 10
mM KCl, 10 mM MgCl₂, 2mM EDTA and centrifuged and retained.
2.3.2 DNA extraction from nuclear pellets obtained from whole blood or from EBV transformed B cell lines using rapid phenol-free method

The nuclear pellet obtained from either whole blood or from the EBV transformed B cells was gently resuspend in 0.8 ml of high salt buffer containing 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM MgCl₂, 0.4 M NaCl and 2 mM EDTA (Lahiri & Nurnberger, 1991). 50 μl of 10% SDS (sodium dodecyl sulfate) was added and the whole suspension was mixed thoroughly. The sample was then incubated at 55°C for 10 mins. 0.30 ml of 6M NaCl was added to the tube and centrifuged at 12,000 rpm for 15 min in a microcentrifuge. The supernatant containing the DNA was transferred to clean tube and the precipitated protein pellet at the bottom of the tube was discarded. 0.5 ml of the supernatant containing DNA was added to each of two tubes containing 1 ml of cold 100% ethanol. The samples were inverted several times until the DNA precipitated and the DNA strands were removed from the two tubes and placed in two fresh tubes, each containing 1 ml of ice-cold 70% ethanol. The samples were centrifuged at 12,000 rpm for 5 min at 4°C. The ethanol was gently removed and the pellet drained and dried for 30 minutes at room temperature. The pellet was then resuspended with 250 μl of 10 mM Tris-HCl, 1 mM EDTA pH 8.0 and heated for 10 min at 65°C. The presence of the DNA was confirmed by agarose gel electrophoresis. The samples were then stored at -20°C.

2.4 DNA amplification

Multiple copies of the target genes were obtained using the techniques of polymerase chain reaction (PCR) developed by Kary Mullis (Saiki, et al, 1985; Erlich, 1990). The second exon of the DQB1 genes were chosen for amplification because most of the sequence-defined polymorphism is localized in this region (Sengar & Goldstein, 1994). Figure 2.1 shows the schematic representation of the exon-intron structure of a Class II β chain gene. Figure 2.2 shows the allelic nucleotide variations of the 17 DQB1 alleles. The
primers and their sequences are also shown in Figure 2.2. The HLA Class II nucleotide sequences shown were obtained from Marsh and Bodmer (1991).
Figure 2.1. A schematic representation of the exon-intron structure of a Class II HLA β chain gene. The exon-intron structure corresponds to the domains on the Class II structure.

29 kD

2nd exon of the HLA-DQB1 alleles

amplified with primers
DB130 & GH29;
GH28NL & QB202;
GH28NL & QB204;
MCBB & QB202

2nd exon of alleles DQB1-0501, -0502, -05031, -05032, -0504,
-0601, -0602, -0603, -0604, -0605, -0201, -0301, -0302,
-03031, -03032, -0401, -0402 were amplified with primers, DB130 & GH29.

2nd exon of alleles DQB1-0501, -0502, -05031, -05032, -0504,
-0601, -0602, -0603, -0604, -0605 were amplified with DQw1 group-specific primers, GH28NL & QB202.

2nd exon of alleles DQB1-0201, -0301, -0302, -03031, -03032,
-0401, -0402 were amplified with DQw2,3,4 group-specific primers, GH28NL & QB204.

2nd exon of alleles DQB1-0602 & -0603 were amplified with primers MCBB & QB202. (please see text for all the details)
Figure 2.2. Alignment of the nucleotide sequences of the exon 2 region of the DQB1 genes. The complimentary alignment of the primers selected is also shown.

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Note: 0503.1 and 0503.2 are variants of DQB1-0503 allele where the completed sequences of the two variants are not known at time of report. Similarly, 0303.1 and 0303.2 are variants of DQB1-0303 allele.

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<th>TCT</th>
<th>GCA</th>
<th>CAC</th>
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<tr>
<td>(QB 202) 3'</td>
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<td>GIC</td>
<td>AAG</td>
<td>CIG</td>
<td>CAC</td>
<td>TAC</td>
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<tr>
<td>(QB 204) 3'</td>
<td>ATG</td>
<td>GIC</td>
<td>AAG</td>
<td>CIG</td>
<td>CAC</td>
<td>TAC</td>
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| 0501 | CAC | AAC | TAC | GAG | GTG | GOG | GGC | GCG | TGC | GIG | CAG | AGG | AGA | GIG | GAG |
| 0502 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0503 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0504 | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| 0601 | T   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0602 | T   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0603 | T   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0604 | G   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0605 | G   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 0606 | C   | T   | A   | CT  | AC  | C   | T   | C   | C   | C   | C   | C   | C   | C   | C   | C   |
| 0607 | C   | T   | A   | CT  | AC  | C   | T   | C   | C   | C   | C   | C   | C   | C   | C   | C   |
| 0608 | C   | T   | A   | CT  | AC  | C   | T   | C   | C   | C   | C   | C   | C   | C   | C   | C   |
| 0609 | C   | T   | A   | CT  | AC  | C   | T   | C   | C   | C   | C   | C   | C   | C   | C   | C   |
| 0610 | C   | T   | A   | CT  | AC  | C   | T   | C   | C   | C   | C   | C   | C   | C   | C   | C   |

* Sequences unavailable.

(Marsh & Bodmer, 1991)
2.4.1 Selection of primers

PCR primers are a pair of single-stranded oligonucleotides which hybridize to specific sites flanking the DNA region to be amplified (Erlich, 1989). Primers selected in this study were between 20 to 30 base pairs in length as recommended by Saiki (1990). As recommended, primers should contain equal percentage of ATs and GCs with no internal secondary structures (Anderson, 1990). A concentration of 100 ng/ml of each primer was used in this study.

At the commencement of the study, two DQBI primers, DB103 & GH29 (see Table 2.1), were used to amplify the DNA of the 17 alleles encoded by the second exon of the DQBI genes (Uryu, et al, 1990; Perkins Elmer Cetus Corporation, 1991). The concomitant amplification of the DQB2 pseudogenes using primers DB103 and GH29 made interpretation of the RFLP patterns difficult. To avoid this problem, a new set of primers designated DH28NL & QB202 (see Table 2.1) were developed to amplify the second exon of the DQB1 genes with the DQw1 specificities, DQB1-0501, -0502, -0503, -0503, -0602, -0603, -0603, -0604 (Nomura et al,1991). Similarly, the DQB1 genes with the DQw2, 3, 4 specificities, DQB1-0201, -0301, -0302, -0303, -0401, -0402, were amplified using primers, GH28NL and QB204 (see Table 2.1). Finally to identify the DQB1-0602 allele, a specifically designed primer, MCBB, with a mutation, which in combination with allele DQBI-0602 generate a new Nde I restriction site was used (see below).

Primer MCBB 5'-GGA CGG AGC GCG TGC GTC ATA T-3'.
DQB1-0602 5'-GGA CGG AGC GCG TGC GTC TTG TG-3'.
DQB1-0603 5'-GGA CGG AGC GCG TGC GTC TTG TA-3'.
DQB10602/PCR 5'-GGA CGG AGC GCG TGC GTC ATA TG-3'.

NdeI

DQB10603/PCR 5'-GGA CGG AGC GCG TGC GTC ATA TA-3'.

The groups of oligonucleotides used and the alleles they amplify are detailed in Table 2.1. Table 2.2 details the base sequence, the
sequence size and the size of the amplification products of these primers.

Table 2.1. The PCR primers and their DQB1 specificity

<table>
<thead>
<tr>
<th>Group</th>
<th>Oligonucleotide</th>
<th>Alleles amplified</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>GH28NL &amp; QB202</td>
<td>DQw1 specificity- (DQBI-0501, -0502, -0503, -0504, -0601, -0602, -0603, -0604, -0605) DQw2,3,4 specificities- (DQBI-0201, -0301, -0302, -0303, -0401, -0402</td>
<td>Nomura et al, (1991)</td>
</tr>
<tr>
<td>3</td>
<td>MCBB &amp; QB202</td>
<td>DQBI-0602, -0603</td>
<td>designed and developed in the present study</td>
</tr>
</tbody>
</table>
The sequences of the primers are presented in Table 2.2.

<table>
<thead>
<tr>
<th>HLA-DQBI Primer</th>
<th>Sequence</th>
<th>Size (base pairs)</th>
<th>PCR Product Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH29</td>
<td>5'-GAG CTG CAG GTA GTT GTG TCT GCA CAC-3'</td>
<td>27 bp</td>
<td>259 bp (with DB130)</td>
<td>Perkins-Elmer Cetus</td>
</tr>
<tr>
<td>DB130</td>
<td>5'-AGG GAT CCC CGC AGA GGA TTT CGT GTA CC-3'</td>
<td>29 bp</td>
<td>259 bp (with GH 29)</td>
<td>Perkins-Elmer Cetus</td>
</tr>
<tr>
<td>GH28NL</td>
<td>5'-GCA TGT GCT ACT TCA CCA ACG-3'</td>
<td>21 bp</td>
<td>241 bp (with QB 202)</td>
<td>Nomura et al (1991)</td>
</tr>
<tr>
<td>QB 204</td>
<td>3'-ATG GTC AAC CTC GAG GCG TGA CGT CCA C-5'</td>
<td>28 bp</td>
<td>237 bp (with GH28NL)</td>
<td>Nomura et al (1991)</td>
</tr>
<tr>
<td>MCBB</td>
<td>5'-GGA CGG AGC GCG TGC GTC ATA T-3'</td>
<td>22 bp</td>
<td>220 bp (with QB 202)</td>
<td>designed and developed in the present study</td>
</tr>
</tbody>
</table>

Primer GH29 and DB130 were purchased from Macquarie University whilst the others were obtained from DNA Express, California, U.S.A..
2.4.2 Optimization of conditions for PCR

In order to minimise such problems as no detectable products or low yield of the desired product; the presence of nonspecific background bands due to mispriming or misextension of the primers; the formation of primer-dimers which compete for amplification; and mutation or heterogeneity due to misincorporation (Innis & Gelfand, 1990), the following steps were taken.

2.4.2.1 Preparation for the amplification

To prevent contamination with "alien" DNA, RNA, proteases, nucleases and other enzyme inhibitors, the preparation for amplification was conducted in a designated laboratory room separate from the area where the rest of the PCR-RFLP procedures were conducted. Equipment used in each of the rooms were non-transferable. In addition, gloves were always worn and the preparation of the samples are conducted in a laminar flow cabinet (UV hood). On conclusion of the preparative procedures, the UV lamp was switched on for 1 hr to degrade any contaminating DNA. The 'master cocktail' mixes of reaction reagents were prepared to cut down the number of handling steps and inaccuracies when pipetting minute volumes (Anderson, 1990). Filter pipette tips were used for non-DNA reagents and positive replacement pipettes were used to draw up samples of DNA. To minimise contamination of equipment and samples, adherence to internal laboratory rules were followed.

2.4.2.2 Reagent concentrations

A. *Taq* polymerase.

The standard *Taq* DNA polymerase concentration is between 1-2.5 units per 100 μl reaction (Innis & Gelfand, 1990).
Increasing the concentration beyond 1-4 units per 100 μl can result in non-specific products and a reduction in yield (Saiki, 1990). In this study, after experimenting with a range of different units, 1.5 units of Taq polymerase (Promega Corporation) per 50 μl reaction were used with good results.

B. Deoxynucleotide triphosphates (dNTPs).

Equivalent concentrations of the four dNTPs (dATPs, dTTPs, dGTPs, dCTPs) are used to minimise misincorporation (Innes & Gelfand, 1990). High concentrations, 4-6mM, of dNTPs may inhibit polymerase by as much as 20-30% and tend to promote misincorporations by the polymerase. It is recommended that the PCR reaction contain 200 μM of each dNTP (Saiki, 1990; Anderson, 1990). In this study, 200 μM dNTP in 50 μl reaction were used. The stock solution of 2mM dNTP were prepared by adding 30 μl of 100mM dATP, 30 μl of 100mM dTTPs, 30 μl of 100mM dGTP, 30 μl of 100mM dCTP and 1380 μl of milli-Q dH2O.

C. Magnesium.

Taq polymerase is highly sensitive to the concentration of magnesium ion and as dNTPs can bind to the magnesium ions, it is recommended that the PCR reaction mix contain 0.5 to 2.5 mM magnesium over the total dNTP concentration (Anderson, 1990; Innis & Gelfand, 1990). In this study, 1.5 mM MgCl₂ was used in method 1 (Uryu et al., 1990) and 4mM MgCl₂ was used in method 2 (Nomura et al., 1991). Experiments with concentrations higher and lower than 1.5 mM MgCl₂ (in method 1) and 4 mM MgCl₂ (in method 2) produced primer dimers and non-specific background bands.

D. Buffers.

The buffer used was recommended by the manufacturers (Promega Corp.). It consisted of 50% glycerol, 50mM Tris-HCl
(pH 8.0). 100mM NaCl, 0.1 nanomoles EDTA, and 1.0% Triton X-100.

E. Water.

MilliQ water was used in all preparations.

To minimise contamination, aliquots of the dNTPs, buffer solutions, primers and MgCl₂ were prepared and all reagents were stored at -20°C. Negative and positive controls were used to check for contamination and experimental faults.

### 2.4.2.3 PCR amplification conditions

In this study, the PCR amplification required 40 cycles with repeats of three temperature conditions. Different denaturation and annealing temperatures were trialed to obtain the best protocol. At denaturation temperatures higher than 95°C and annealing temperatures higher than 55°C, less specific bands were observed. The optimal conditions eventually chosen were the denaturation temperature of 95°C, the annealing temperature of 55°C and the extension temperature of 72°C (Saiki, 1990; Anderson, 1990; Innes & Gelfand, 1990; Erlich, 1990).

Two sets of cycling conditions were used in this study. The first was followed when primers GH19 & DB130 were used in the thermocycler (Corbett Thermal Sequencer) and consisted of:

40 cycles of:

- 95°C x 1 minute
- 55°C x 1 minute
- 72°C x 1 minute
followed by 1 cycle of:

\[ 72^\circ C \times 5 \text{ minutes} \]

finishing with 1 cycle of:

\[ 25^\circ C \times 1 \text{ minute} \]

When primers GH28NL, QB202 & QB204 were used in the thermocycler (Corbett Research-Fast Thermal Sequencer), the conditions consisted of:

1 cycle of:

\[ 95^\circ C \times 3 \text{ min} \]
\[ 55^\circ C \times 15 \text{ sec} \]
\[ 72^\circ C \times 15 \text{ sec} \]

then by 4 cycles of:

\[ 95^\circ C \times 15 \text{ sec} \]
\[ 55^\circ C \times 15 \text{ sec} \]
\[ 72^\circ C \times 15 \text{ sec} \]

followed by 35 cycles of:

\[ 95^\circ C \times 3 \text{ sec} \]
\[ 55^\circ C \times 3 \text{ sec} \]
\[ 72^\circ C \times 15 \text{ sec} \]
finishing with 1 cycle of

25°C x 10 sec.

2.4.3. PCR-reaction protocol using primers GH29 &
DB130 (Uryu et al., 1990)

Genomic DNA (100 ng/μg) was amplified using a 50 μl reaction mix
containing 100 ng each of primers GH29 and DB130, 2 mM dNTPs,
1.5 mM MgCl₂, 1.5 units Taq DNA polymerase (Promega). 1 μl of
genomic DNA was added to the 49 μl of the reaction mix. For the
negative control, 1 μl of milli-Q dH₂O was added instead of the DNA.
3 drops of mineral oil was added to each sample to prevent
evaporation of the samples during the amplification in the
thermocycler (Corbett Thermal Sequencer). On completion,
amplification of the target DNA was checked by 10%
polyacrylamide gel electrophoresis to detect bands of 259 base
pairs (see electrophoresis section).

2.4.4 PCR protocol using primers GH28NL & QB
202; GH28NL & QB204 (Nomura et al., 1991);
and MCBB & QB202.

In the previous method, the PCR amplification was achieved using a
50 μl reaction mix in a 0.5 ml microfuge tube. In this method, a 20
μl reaction mix in a capillary tube was used. The reaction mix was
identical to the previous protocol except a final concentration 4mM
MgCl₂ was used. 1.0 μl of DNA (0.1 to 0.5 μg) was added to 19.0 μl
of the PCR cocktail mix and to the negative control sample, 1 μl of
milli Q water was added instead of 1 μl of DNA. 20 μl of the sample
was drawn up in a capillary tube followed by 2.5 μl air with an
automatic pipetter. This provides an air gap at the tip of the
capillary to prevent contamination. The tip of the capillary tube
was wiped with a clean lint-free tissue and heat sealed to prevent
drying during the thermal cycling and to prevent contamination.
The amplification was conducted in a thermocycler (Corbett
Research - Fast Thermal Sequencer FTS I-S). On completion of the thermal cycling program, the tips of the capillary was clipped and the contents were emptied into the microcentrifuge tubes. Amplification of the target DNA was checked by using 10% polyacrylamide gel electrophoresis. The gel stained with ethidium bromide was placed under the UV transilluminator to check for the expected band sizes of:

241bp using DQW1 group specific primers, GH28NL & QB202

237bp using DQW2,3,4 group specific primers, GH28NL & QB204

220bp using primers, MCBB & QB202

2.4.5 Polyacrylamide gel electrophoresis (PAGE).

The electrophoresis apparatus was set up as follows:

A. clean glass plates, combs and spacers in alcohol and dry well.

B. assemble the apparatus (Mini Protean II, Bio-Rad) ensuring that the glass plates sit perfectly at the base so that there is no leakage of polyacrylamide.

The necessary buffers were made up as follows:

5x TBE

To a litre beaker, add 54 grams of Tris, 27.5 grams of Boric Acid, 3.72 grams of EDTA, 900ml of milli-Q dH2O and stir until dissolved. The pH was adjusted to 8.0.
10% ammonium persulfate

10 mg of ammonium persulfate was added to 100 µl of milli-Q dH₂O and the solution was mixed well.

The polyacrylamide solution for two 0.75 mm gels was prepared as follows:

2.5 ml of 40% acrylamide (acrylamide and bisacrylamide are in a ratio of 29:1) was added to 2 ml of 5 x TBE and 5.5 ml dH₂O to make 10 ml of solution.

This solution was then degassed in a vacuum desiccator for 20 minutes. 63 µl of freshly made ammonium persulfate was added to the acrylamide solution and the solution was mixed well quickly by swirling. To the solution, 6 µl of Tetramethylethylenediamine (TEMED) was added and the solution was again mixed. The solution was pipetted into the assembled apparatus and the combs were placed into the gel ensuring that no bubbles were trapped within the well spaces. The gel was allowed to polymerise for 1 hour when the combs were removed and the wells washed with distilled water followed by 1 x TBE. The gel was then placed into the running tank apparatus and the inner and outer chambers were filled with 1 x TBE. The gel was pre-electrophoresed for 10-20 minutes at 200V to remove any unpolymerised products. The samples were prepared by adding 2.0 µl of 5 x ficoll stop mix (bromophenol blue and xylene cyanol dye) to 8.0 µl of amplified DNA samples. The size marker was prepared by adding 1 µl 5 x ficoll stop mix to 1 µl of size marker, pUC19 restricted with Hpa II (500 ng/ml, Bresatec), plus 3 µl of milli-Q dH₂O. The samples and markers were loaded into the pre-run gel and electrophoresed at 200V until the bromophenol blue dye front reached the bottom of the gel. The gel was removed and stained in 0.5 µg /ml ethidium bromide solution for 20 minutes. Amplified DNA bands were visualised with the UV transilluminator (Ultra-Lum). The gel was
then photographed with a DS-34 Polaroid-direct screen instant camera using polaroid film (Polaroid Type 667, Coatless Black & White Land Film) and an orange filter.

2.5 Digestion

The amplified DNA was digested with allele specific restriction endonucleases which cut the DNA at specific restriction sites. The DNA amplified with primers, GH28 & QB130 was digested with five restriction endonucleases Fok1, Hae111, Rsa1, Sau3A1 and Hha1. The DQw1 specific DNA amplified with primers GH28NL and QB202 was digested with six restriction endonucleases, Fok1, Apa1, Hae111, BssH11 Hha1 and Hph1. Similarly, the DQw2,3,4 specific DNA amplified using primers GH28NL and QB204 was digested by five enzymes, Fok1, BglI SacI, BsaHI(AcyI) and Msp1 (Hpa11). DNA amplified with primers MCBB and QB204 was digested with Nde1. Product details of the endonucleases used are detailed in Table 2.3 whilst the base specificities of these endonucleases are given in Table 2.4:
Table 2.3. Product details of the restriction endonucleases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>10 x Buffer</th>
<th>Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FokI</td>
<td>NE4</td>
<td>4 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>HaeIII</td>
<td>M</td>
<td>11 U/μl</td>
<td>Boeringer Manheim</td>
</tr>
<tr>
<td>RsaI</td>
<td>RsaI</td>
<td>20 U/μl</td>
<td>Promega</td>
</tr>
<tr>
<td>Sau3A1</td>
<td>B</td>
<td>5,000 U/ml</td>
<td>Promega</td>
</tr>
<tr>
<td>ApaI</td>
<td>NE4</td>
<td>20 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>HaeII</td>
<td>HaeII</td>
<td>10 U/ml</td>
<td>Progen</td>
</tr>
<tr>
<td>BssH11</td>
<td>BssH11</td>
<td>4 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>HphI</td>
<td>NE4</td>
<td>5 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>HhaI</td>
<td>HhaI</td>
<td>20 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>BglI</td>
<td>BglI</td>
<td>7 U/ml</td>
<td>Progen</td>
</tr>
<tr>
<td>SacI</td>
<td>SacI</td>
<td>20 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>BsaH1 (Acy1)</td>
<td>BsaH1</td>
<td>10 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>Msp1 (HpaII)</td>
<td>Msp1</td>
<td>11 U/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NdeI</td>
<td>NE4</td>
<td>20 U/ml</td>
<td>New England Biolabs</td>
</tr>
</tbody>
</table>

NB - all the 10 x buffers were supplied by the manufacturers.
Table 2.4. Base specificities of the endonucleases used in digestion of amplified DNA fragment

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Base recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fok1</td>
<td>GGATG(N)9*</td>
</tr>
<tr>
<td>Hae11I</td>
<td>GG•CC</td>
</tr>
<tr>
<td>RsaI</td>
<td>GT•AC</td>
</tr>
<tr>
<td>Sau3A1</td>
<td>•GATC</td>
</tr>
<tr>
<td>ApaI</td>
<td>GGGCC•C</td>
</tr>
<tr>
<td>Hae11I</td>
<td>RGCGR•Y (Where R= A or G; Y= C or T)</td>
</tr>
<tr>
<td>BssH1I</td>
<td>G•CGGC</td>
</tr>
<tr>
<td>HphI</td>
<td>GGTGA(N)8*</td>
</tr>
<tr>
<td>HhaI</td>
<td>GCG•C</td>
</tr>
<tr>
<td>BglI</td>
<td>GCC(N)4•NGGC</td>
</tr>
<tr>
<td>SacI</td>
<td>GAGCT•C</td>
</tr>
<tr>
<td>BsaH1 (AcyI)</td>
<td>GA•CGCC and/or GA•CGTC and/or GG•CGCC and/or GG•CGTC</td>
</tr>
<tr>
<td>MspI (HpaI)</td>
<td>C•CGG</td>
</tr>
<tr>
<td>NdeI</td>
<td>CT•TATG</td>
</tr>
</tbody>
</table>

2.5.1 Digestion of DNA amplified with primers GH29 & QB130 using the five restriction endonucleases Fok1, Hae11I, RsaI, Sau3A1 and HhaI (Uryu et al., 1990).

An 8 μl restriction enzyme reaction mix containing 5 μl of between 2-5U of restriction enzyme and 3 μl of amplified DNA was prepared and incubated in a water bath for 3 hrs at 37°C. The resultant
fragments were analysed after electrophoresis on a 10% polyacrylamide gel followed by ethidium bromide staining.

2.5.2 Digestion of DNAs amplified with:

a) DQw1 group specific primers, GH28NL & QB202 using 6 restriction enzymes, Fok1, Apa1, Hae11, BssH11 Hha1 and Hph1 (Nomura et al, 1991).

b) DQw2,3,4 group specific primers, GH28NL & QB204 using restriction enzymes, Fok1, BglII SacI, BsaH1(ACY1) and MspI (HpaII) (Nomura et al, 1991).

c) primers, MCBB & QB202.

The digestion procedure was essentially the same as previously described in 2.5.1. All the restriction fragments (except Nde1) digests were best resolved on the gel when the bromophenol blue dye front is just at the edge of the bottom of the gel (approximately 20 to 25 minutes) and the xylene cyanol dye front was just past the mid point of the gel.

For the Nde1 restriction fragments, the resolution was best seen when the bromophenol blue dye front had run off the gel and the xylene cyanol dye front was about 5 cm from the bottom edge of the gel (approximately 35 to 40 mins).

2.6 Analysis

The identity of the DQB1 alleles was determined by comparing the digestion patterns of patients and controls subjected to allele-specific restriction endonucleases with the known restriction patterns. These predicted patterns are presented in Tables 3, 3.1, 3.2 in Chapter 3. Reference patterns were determined by subjecting 8 HLA-D homozygous cell lines used by the Red Cross
Blood Transfusion Tissue Typing Laboratory as reference cells for
tissue typing to the process of PCR amplification and digestion with
restriction endonucleases as described.

2.6.1 Data analysis

In this study, the phenotype frequencies (antigen frequencies) are
simple counts of each antigen and is presented as a percentage.
The gene frequencies (allele frequencies) are calculated by the
following formula:

\[ 1 - \frac{1}{1 - \text{antigen frequency}} \]

In this study, each individual patient and control was tested for
several antigens and the same data were used to compare the
frequency of all the typed antigens. Because of the multiple
comparisons, any significant increase or decrease in an antigen
frequency at a p value of 0.05 may be due to chance alone. Hence
to accept a frequency deviation may lead to spurious conclusions of
HLA antigen and disease associations. Therefore, where necessary,
the p values were corrected by the Bonferoni inequality method ie.
multiplying by the number of comparisons made (Tiwari &
Terasaki, 1985).

The relative risk (RR) which estimates the likelihood of the disease in
an individual positive for an antigen compared to that in
individuals negative for the antigen, was calculated by the formula
(Haldane, 1956):

\[ \frac{\text{no. of patients with allele}}{\text{no. of patients without allele}} \]
\[ \frac{\text{no. of controls with allele}}{\text{no. of controls without allele}} \]

Two-locus linkage analysis was performed to determine whether
observed MS associations with the shared DQB1 sequences and for
the DQB1 alleles encoding for Leucine at residue 26 (DQB1-0602,
-0603, -0604, -0302, -0303, -0201) were secondary to the
association with the DRB1-1501-bearing haplotype. The strength of
association of the various haplotypes was analysed by the $\chi^2$ (chi-squared) method with Yates correction in $2 \times 2$ tables. Fishers' exact test was used when the cell values in a $2 \times 2$ table were less than five (Tiwari & Terasaki, 1985).

The relative predispositional effect method (RPE) which sequentially compares allele frequency in patients and controls to determine whether associations with a second allele are true or whether they are due to the increase or decrease of a primary associated allele (Payami et al, 1989) was used. Comparison of the DQB1 allelic frequency distribution of MS patients and controls was first performed using the $\chi^2$ test. When the overall $\chi^2$ was statistically significant, the allele with the largest contribution was removed from the patient and control groups and the test repeated with the allele with the next highest value until the overall deviation was found to be not significant.
CHAPTER 3
Results of PCR-amplification with specific primers and digestion of the amplified products with selected endonucleases

This chapter presents the results associated with the development of the PCR-RFLP methodology. The sections of the chapter are presented as follows:

3.1. Selection of appropriate primers-results of the PCR-amplification using four sets of primers.
   3.1.1 Amplification using primers GH28 & GH29; DB130 & GH29; and GH26 & GH27.
   3.1.2 Amplification using primer sets, GH28NL & QB202; GH28NL & QB204; and MCBB & QB202.
   3.1.3 Optimization of the PCR protocol for Mg CL2 concentration.

3.2. Results of the digestion of the PCR-amplified products with restriction endonucleases.
   3.2.1 Results of digestion of DNA amplified with primers GH19 & DB130 with five endonucleases (Uryu et al,1990).
   3.2.2 Results of digestion with eleven endonucleases to detect allelic variations in the DQB1 genes (Nomura et al,1991).
   3.2.2.1 Results of digestion of DNA amplified with primers GH28NL & QB202 with six endonucleases to detect DQw1 group-specific allelic variations.
   3.2.2.2 Results of digestion of DNA amplified with primers GH28NL & QB204 with five endonucleases to detect DQw2,3,4 group-specific allelic variations.
   3.2.3 Results of digestion with Nde 1.
3.1 Selection of appropriate primers - results of the PCR-amplification using four sets of primers

As indicated in the methods chapter, four sets of primers were used in this study. The first set of primers DB130 and GH29, as recommended by Horn et al (1988) & Perkin Elmer Cetus (1991) were initially used to amplify the second exon DQB1 alleles. The amplification process using the primers DB130 & GH29 did not pose any significant problems. It was the digestion process of the amplified products using these primers which proved problematic. The concomitant amplification of the DQB2 pseudogenes led to RFLP patterns which were difficult to resolve. Subsequently, in order to amplify all the DQB1 alleles without concurrent amplification of the DQB2 pseudogene, a further three sets of primers, GH28NL & QB202; GH28NL & QB204; and MCBB & QB202 were selected. The use of these three sets of primers led to clear amplified DNA products which on digestion with restriction endonucleases gave RFLP patterns which were easy to interpret.

3.1.1 Amplification using primers GH28 & GH29; DB130 & GH29; and GH26 & GH27

Before primers DB130 & GH29 were selected, a comparison of the amplification products was obtained using the primers, GH28 and GH29; DB130 and GH29; and GH26 and GH27. Primers GH28 & GH29 were used by Uryu et al (1989) to amplify the second exon of the DQB1 alleles. The primers GH26 & GH27 were used successfully by another member of the research team to amplify the DQA1 alleles. Hence, it was used as a positive control in this experiment. Figure 3.1 shows the amplified products using the three sets of primers. The DNA used came from 3 individual MS patients. Lanes 2-4 show the DNA from three MS patients amplified with primers GH28 & GH29; Lanes 5-7 show the DNA from the same three individuals amplified with primers GH29 & DB130; and similarly, Lanes 8-10 show the DNA from the same individuals amplified with primers GH26 & GH27. Lane 1 shows the marker (pUC 19 digested with Hpa
11) with a range of band sizes from 34-501bp, and Lanes 2 and 4 show the expected 230 bp DNA amplification products using primers GH28 and GH29 which hybridize to specific sites flanking the DNA region of the DQB1 gene to be amplified. Lanes 5 and 7 show the products of PCR amplification by the primers DB130 and GH 29 to produce a band of 259 bp. In lanes 8 and 10, the 242bp amplified DNA products using primers GH26 and GH27 are clearly seen. The two sets of primers, GH28 & GH 29 and DB130 & GH29, could potentially be used to amplify the second exon of the DQB1 alleles. However difficulties with the digestion patterns of the amplified products using primers GH28 & GH29 were reported (Olerup, 1990). As a result, the primers DB130 & GH29 were selected to amplify all the MS patient and control DNA samples.

As indicated above, the primers GH29 & DB130 were used to successfully amplify the DNA from the MS patients and control samples. Figure 3.2 shows the PCR amplification product of eight MS patients using primers DB130 and GH29. Lanes 1-8 show clearly the expected 259bp bands of amplified DNA products from the eight MS individuals. However the bands in Lanes 1, 2, & 7 were brighter than those in Lanes 3, 4, 5, 6 & 8. This may be due to the different initial concentration of the DNA used prior to the amplification. Multiple bands just above the 259bp band were observed in Lanes 1 & 2. This multiple banding pattern may be the DNA heteroduplex (HD) electrophoretic pattern observed in most of the heterozygotes (Tong et al, 1994). Most of the homozygotes gave patterns of a single band as seen in Lanes 3-8. The negative control is in Lane 9 and Lane 10 is the marker (pUC 19 digested with Hpa 11). The temperatures selected for the amplification were successful in eliminating non specific background bands and the formation of primer-dimers.

3.1.2 Amplification using primer sets, GH28NL & QB202; GH28NL & QB204; and MCBB & QB202

As indicated, Figures in 3.1 & 3.2 show the successful amplification of DNA using an array of primer pairs. However as indicated
previously, upon digestion of these amplified fragments with site specific endonucleases, there was no clear definition of the DQB1 alleles and the precise discrimination of heterozygotes could not be resolved because of concurrent amplification of the DQB2 pseudogenes. As a result, the choice of using two group-specific primers which did not concurrently amplify the DQB2 pseudogenes was made and tested (Nomura et al, 1991). The amplification of the group specific DQw1 alleles-0501, -0502, -05031, -05032, -0504, -0601, 0602, -0603, -0604, -0605 and the group-specific DQw2,3,4 alleles -0201, -0301, -0302, -03031, -03032, -0401, -0402 of the DQB1 genes were performed with the primers GH28NL and QB202, and GH28NL and QB204 repectively. The primers GH28NL & QB202 which hybridize to specific sites flanking the DNA region of the DQw1 alleles to be amplified produced bands of 241bp and similarly, the primers GH28NL & QB204 produced bands of 237bp.

Figure 3.3 shows the products of PCR amplification of the DQw1 group-specific DQB1 alleles using primers GH28NL & QB202. The DNA used in Lanes 2, 8, 9, 10 & 11 were from MS patients; Lanes 1, 3, 5, 6 & 12 were from controls; and Lanes 4 & 7 were from homozygous reference cell lines, all amplified with primers GH28NL & QB202 which hybridize to specific sites flanking the DNA region of the DQB1 gene to be amplified. As mentioned earlier, the use of these two primers produced an amplified DNA product of size 241bp. The size marker was in Lane 13. All the expected bands of 241bp were clearly resolved. Because all the bands were detected by the 10% polyacrylamide gel electrophoresis, it is assumed that all the samples must belong to the group specific DQw1 alleles. This was found to be true when the digested fragment patterns were matched with the predicted patterns belonging to group specific DQw1 alleles (-0501, -0502, -05031, -05032, -0504, -0601, 0602, -0603, -0604, -0605) (see the section on results of digestion).

Figure 3.4 shows the products of PCR amplification of the DQw2,3,4 group-specific DQB1 alleles using primers GH28NL & QB204. As stated earlier, the use of these two primers produced bands of 237bp. The DNA used in Lanes 1-12 came from the same
individuals as in Figure 3.3. No amplification was observed in Lanes 4, 7 & 10 as these homozygote individuals carry the DQw1 group specific alleles. The negative control was in Lane 13 and the marker in Lane 14. Clear expected bands of 237bp were observed in Lanes 1, 2, 3, 5, 6, 8, 9, 11 & 12.

Figure 3.5 shows the amplification of the DQB-0602, -0603 alleles. For the discrimination of the DQB1-0602 and -0603 alleles, the amplification was achieved with a fourth primer MCBB with a specially designed mutation at the 3' end to create a NdeI restriction site when -0602 allele was amplified. The use of these primers produced bands of 220bp. The DNA were from six MS individuals. Lanes 1-5 show the expected 220bp band of PCR-amplified products produced using primers MCBB and QB202. The positive and negative controls were in Lanes 6 & 7 respectively and the marker was in Lane 8. A non specific band of approximately 170bp and of unknown origin was also visualised in Lanes 1, 3, 4 & 5 but this did not interfere with the interpretation of the RFLP pattern after digestion with NdeI.

3.1.3 Optimization of MgCl₂ concentration

In order to optimize PCR protocol for MgCl₂ concentration, a series of trials using different concentrations with each primer set was conducted. Figure 3.6 shows the comparison of the different concentrations of MgCl₂ used in the amplification of DNA. In Figure 3.6 the results using two concentrations of MgCl₂ were shown. The DNA in Lanes 1-4 was from one MS patient. In Lanes 1 & 2 a concentration of 4mM MgCl₂ was used with 40 and 30 amplification cycles respectively. Bands in Lanes 3 & 4 were produced using 3mM MgCl₂ at 40 and 30 amplification cycles respectively. The band shown in Lane 1 had the best resolution and as a result, the concentration of 4mM MgCl₂ and 40 amplification cycles was selected. These conditions of amplification reduced the non specific background and primer-dimer bands to a minimum and those present did not interfere with the resolution of the RFLP patterns (see Figure 3.5).
3.2 Results of the digestion of the PCR-amplified products with restriction endonucleases

3.2.1 Result of digestion of DNA amplified with primers GH29 & DB130 with five endonucleases (Uryu et al, 1990)

Sequence data predicts an array of fragment sizes when DNA amplified using primers DB130 and GH29 is digested with five restriction endonucleases, Hae III, Hha I, Rsa I, Sau3AI and Fok I. Seven polymorphic patterns of the restriction fragments of the amplified DQB1 alleles are expected with Hae111 digestion; six with Hha1; three with Rsa1; two with Sau3A1; and two with Fok1. However, in practice, some fragments with 1 to 3 bp differences from each other were difficult to resolve using 10% polyacrylamide gel electrophoresis. For example, the 125bp, 127bp and 128bp fragments from Hae 111 digestion could not be discriminated from each other. Similarly, the 46bp and 47bp fragments, and the 163bp and 166bp fragments produced by digestion with Hae111; the 46bp and 48bp fragments produced by Hha1; and the 80bp and 83bp fragments produced by Rsa1 were also difficult to resolve. The 30bp and 39bp fragments produced by Rsa I and the 38bp and 39bp fragments produced by Hae III were too small to be detected by gel electrophoresis. As a result, four restriction patterns (A, B, C are similar to each other; D, E, G, H, L are similar to each other; F, I, K are similar to each other; and M & N are the same) using Hae 111, six with Hha1 and three with Rsa I were used in the analysis (Table 3.1). Upon digestion with the range of five endonucleases, all these patterns could be combined and classified into fourteen patterns (A-N) as shown in Table 3.1. From the restriction pattern shown in Table 3.1, the DQB1-0602 and -0603; -05031 & -05032; and -0302 and -0303 gave the same RFLP pattern and separation of these alleles were not possible using this PCR-RFLP method.

The RFLP patterns as a result of Hae111, Hha1, Rsa1, Sau3A1 and Fok1 digestion of the DQB1 alleles are shown in Figures 3.7-3.11. The DNA in each of the Figures 3.7-3.11 were obtained from the
same 12 MS patients digested with Hae111, Hha1, Rsal, Sau3A1 and Fok1 respectively. The observed RFLP patterns from Figures 3.7-3.11 were matched with the predicted RFLP patterns (Table 3.1). Lane 1 of Figures 3.7-3.11 shows the bands of the fragments of DNA of one MS patient digested with the restriction enzymes Hae111, Hha1, Rsal, Sau3A1 and Fok1 respectively. From Lane 1 in Figures 3.7-3.11, the following bands were observed:

259bp, 163/6bp, 125/7/8bp, 93/6bp (digested with Hae111, Figure 3.7);

190bp, 113/5bp, 69bp (digested with Hha1, Figure 3.8);

80/3bp (digested with Rsal, Figure 3.9);

259bp (digested with Sau3A1, Figure 3.10); and

259bp (digested with Fok1, Figure 3.11).

When these observed band patterns were matched with the predicted pattern (Table 3.1), this MS patient could be assigned to the alleles DQB1-0501, -0502, -05031, -05032, -0602, -0603, -0604, -0605, -0301, -0302, -0303 in Table 3.1.

Similarly from Lane 2 in Figures 3.7-3.11, the following bands were observed:

163/6bp, 93/6bp (digested with Hae111, Figure 3.7);

113/5bp, 69bp (digested with Hha1, Figure 3.8);

80/3bp (digested with Rsal, Figure 3.9);

259bp (digested with Sau3A1, Figure 3.10); and

259bp (digested with Fok1, Figure 3.11).
Again, when the observed band patterns were matched with the predicted pattern (Table 3.1), this other MS patient could be assigned to the allele DQB1-0501, -0502, -05031, -05032, -0602, -0603, -0604, -0605, -0301, -0302, -0303 in Table 3.1.

As can be seen with the two examples above, the RFLP patterns in Figures 3.7-3.11 presented multiple complex bands which were difficult to interpret. The concomitant amplification of the DQB2 genes made it difficult to discriminate heterozygotes from each other. The use of endonucleases which have multiple cleavage sites produced some digested fragments of similar sizes. This made analysis of RFLP pattern difficult to interpret. As a result, this method was abandoned and replaced by another PCR-RFLP method described by Nomura et al (1991).
Table 3.1. The predicted cleavage pattern of bands for the HLA-DQB1 alleles

<table>
<thead>
<tr>
<th>DQB1-alleles</th>
<th>Pattern</th>
<th>Fragment size (bp) digested with restriction endonucleases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hae III</td>
</tr>
<tr>
<td>0501 A</td>
<td></td>
<td>9 9 0 1 0 0</td>
</tr>
<tr>
<td>0502 B</td>
<td></td>
<td>9 9 0 1 0 0</td>
</tr>
<tr>
<td>0503.1 C</td>
<td></td>
<td>9 9 0 1 0 0</td>
</tr>
<tr>
<td>0503.2 D</td>
<td></td>
<td>0 0 1 0 1 0</td>
</tr>
<tr>
<td>0504 E</td>
<td></td>
<td>0 0 1 0 1 0</td>
</tr>
<tr>
<td>0601 F</td>
<td></td>
<td>9 0 1 1 0 0</td>
</tr>
<tr>
<td>0602/0603 G</td>
<td></td>
<td>0 0 1 0 1 0</td>
</tr>
<tr>
<td>0604 H</td>
<td></td>
<td>0 0 1 0 1 0</td>
</tr>
<tr>
<td>0605 I</td>
<td></td>
<td>9 0 1 1 0 0</td>
</tr>
<tr>
<td>0201 J</td>
<td></td>
<td>0 0 0 0 1 1</td>
</tr>
<tr>
<td>0301 K</td>
<td></td>
<td>9 0 1 1 0 0</td>
</tr>
<tr>
<td>0302/0303 L</td>
<td></td>
<td>0 0 1 0 1 0</td>
</tr>
<tr>
<td>0401 M</td>
<td></td>
<td>0 0 0 0 0 1</td>
</tr>
<tr>
<td>0402 N</td>
<td></td>
<td>0 0 0 0 0 1</td>
</tr>
</tbody>
</table>

Note: 0- fragment (bp) not present; 1- fragment (bp) present, 9- fragment present but were too small to be defined.
3.2.2 Result of digestion with eleven endonucleases to detect allelic variations in the DQB1 genes (Nomura et al, 1991)

3.2.2.1 Results of digestion of DNA amplified with primers GH28NL & QB202 with six endonucleases to detect DQw1 group-specific allelic variations

Six endonucleases, Fok I, Apa I, Hae II, BssH II, Hph I and Hha I, were selected for digestion to detect the DQw1 allele-specific cleavage patterns after PCR amplification using primers GH28NL and QB202. Five of the restriction endonucleases, Fok I, Apa I, Hae II, BssH II and Hph I produced two predicted polymorphic restriction fragment patterns and Hha I produced three. When all the predicted restriction patterns were combined, ten (10) polymorphic patterns (A-J) emerged, and these are shown in Table 3.2a. As can be seen, five of the endonucleases selected produced either a single cleavage site in some alleles or no cleavage sites in others. Only Hha I cleaved at three or less restriction sites in some alleles (Table 3.2a). All the alleles would be expected to produce unique patterns except the DQB1-0602 & -0603 alleles, the -05031 & -05032 and the -0604 & -0605 alleles. These alleles produced the same restriction patterns and so discrimination between these alleles was not possible with the use of the six endonucleases. The six restriction endonucleases discriminated seven out of the ten DQw1 group-specific alleles. Figures 3.12-3.17 shows the observed RFLP patterns of the six endonuclease digesta of the ten DQw1 group-specific DQB1 alleles (0501, 0502, 05031, 05032, 0504, 0601, 0602, 0603, 0604 & 0605) in an Australian population. These observed patterns were easy to interpret and matched the predicted RFLP patterns. The DNA in each of the Figures 3.12-3.17 were obtained from the same 12 individuals digested with Fok1, Apa1, Hae11, BssH11, Hph1 & Hha1 respectively.
Figure 3.12 shows the DQw1 group-specific DQB1 RFLP pattern for Fok1-digested DNA amplified with GH28NL & QB202. The DNA in Lanes 7-9 were obtained from MS patients and the rest were from the control population. All the amplified DNA in Lanes 1-12 were digested with Fok1. The marker was in Lane 13. Uncut bands of size 241bp were observed in Lanes 1-10 and 12. In Lane 11, bands of sizes 241bp & 149bp were observed. Faint non-specific primer dimer bands of approximately 50bp were observed in Lanes 1-5 and these did not interfere with the interpretation of the RFLP pattern. These non-specific bands were observed in all Figures 3.12-3.17.

Figure 3.13 shows the RFLP pattern of Apa1-digested DNA amplified with GH28NL & QB202. The DNA was obtained from the same individuals as described in Figure 3.12. All the 12 samples were digested with Apa1. Fragments of sizes 241bp only were observed in Lanes 1 & Lanes 6-9. In Lanes 2-5, bands of sizes 176bp & 65bp were observed and in Lanes 10-12, the bands of sizes 241bp, 176bp & 65bp were seen.

Figure 3.14 shows the RFLP pattern of Hae11-digested DNA amplified with GH28NL & QB202. The DNA was obtained from the same individuals as mentioned in Figure 3.12. All samples were digested with Hae11. Lanes 1-10 shows the fragment of 241bp whereas in Lane 11 shows the bands of 241bp, 105bp & 136bp, and in Lane 12, only the bands of 105bp & 136bp were observed.

Figure 3.15 shows the RFLP pattern of BssH11-digested DNA amplified with GH28NL & QB202. The DNA were obtained from the same individuals as mentioned in Figure 3.12. All samples were digested with BssH11. Lanes 1 & 6-9 shows the 166bp & 75bp fragments. In Lanes 2-5 & 10-12, bands of 241bp were observed.

Figure 3.16 shows the RFLP pattern of Hph1-digested DNA amplified with GH28NL & QB202. The DNA was obtained from the same individuals as mentioned in Figure 3.12. All samples were digested with Hph1. Bands of 236bp were observed in Lanes 2-5 &
& 10, and bands of 119bp & 122 bp were also observed in Lanes 1, 4 & 6-12.

Figure 3.17 shows the RFLP pattern of Hha1-digested DNA amplified with GH28NL & QB202. The DNA was obtained from the same individuals as mentioned in Figure 3.12. All samples were digested with Hha1. In Lanes 1 & 6-8, bands of 133bp & 49bp were observed and similarly, in Lanes 2-5, 9 & 10, bands of 162bp & 49bp were shown. Fragments of sizes 162bp, 106bp, 56bp & 49bp were observed in Lane 11 and only 106bp & 56bp & 49bp were seen in Lane 12.

The amplified DNA of an individual from the control population digested with the six restriction endonucleases is represented in Lane 1 of Figures 3.12-3.17. From Lane 1 in Figures 3.12-3.17 the following bands were observed:

- 241bp (digested with Fok1, Figure 3.12);
- 241bp (digested with Apa1, Figure 3.13);
- 241bp (digested with Hae11, Figure 3.14);
- 166bp & 75bp (digested with BssH11, Figure 3.15);
- 119bp & 122bp (digested with Hph1, Figure 3.16); and
- 133bp & 49bp (digested with Hha1, Figure 3.17).

When these observed RFLP band patterns were matched with the predicted patterns, this control individual could be assigned to pattern G or H in Table 3.2a. Pattern G & H correspond to the DQB1-0602 & 0603 respectively. Similarly, all the other eleven individuals in Figures 3.12-3.17 were assigned to the respective alleles from matching the observed to the predicted patterns.
Table 3.2a. The predicted cleavage fragment pattern obtained by the six restriction endonucleases in PCR-amplified DQB1 genes with the DQw1 specificity.

<table>
<thead>
<tr>
<th>DQB1-Pattern</th>
<th>Fragment size (bp) digested with restriction endonucleases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fok I</td>
</tr>
<tr>
<td>0501 A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0502 B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0503.1 C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0503.2 D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0504 E</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0601 F</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0602 G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0603 H</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0604 I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0605 J</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Note: 0- fragment (bp) not present, 1- fragment (bp) present, 9- fragment present but were too small to be defined.
3.2.2.2 Results of digestion of DNA amplified with primers GH28NL & QB204 with five endonucleases to detect DQw2,3,4 group-specific allelic variations

The PCR-amplified DQw2,3,4 group-specific alleles were cleaved with five (5) restriction endonucleases, Fok I, Bgl I, Sac I, BsaH I (Acy I), and Msp I (Hpa II). All the endonucleases except Msp I were predicted to have a single or no cleavage sites (Table 3.2b). The five endonucleases selected enabled six out of the seven DQw2,3,4 group-specific alleles (0201, 0301, 0302, 03031, 03032, 0401, 0402) to be discriminated. The two variants of the DQB1-0303 allele, -03031 and -03032 that differ only by a silent substitution in the nucleotide sequence were not able to be discriminated from each other by this method. In combination, the five endonucleases produced seven polymorphic patterns (A-G) of the restriction fragments as shown in Table 3.2b. The RFLP patterns of the DQw2,3,4 group-specific DQB1 alleles from MS patients and controls digested with the five endonucleases are presented in Figures 3.18-3.22. The observed RFLP digest patterns matched predicted patterns and were easy to interpret. The DNA in each of the Figures 3.18-3.22 were obtained from the same 12 individuals digested with Fok1, Apal, Bgl 1, Sac1, BsaH1, Msp1 respectively.

Figure 3.18 shows the DQw2,3,4 group-specific DQB1 RFLP pattern of Fok1-digested DNA amplified with GH28NL & QB204. The DNA in Lanes 1-3 & 7 was obtained from the control population and the rest was from MS patients. All the samples were digested with Fok1. Fragments of expected sizes 237bp were observed in Lanes 1-7 & 11. Lanes 8, 9 & 12 showed bands of 149bp & 88bp. All the observed bands matched the predicted bands as shown in Table 3.2b. The DNA in Lane 10 in Figure 3.18-3.22 was not digested by any of the five restriction enzymes. Non-specific primer dimer bands of approximately 50bp were observed but it did not interfere with the interpretation of the RFLP patterns. These primer dimer bands occur in all the figures from 3.18-3.22.
Figure 3.19 shows the DQw2,3,4 group-specific DQB1 RFLP pattern of Bgl 1-digested DNA amplified with GH28NL & QB204. The DNA was obtained from the same individuals as those in Figure 3.18. All samples were digested with Bgl 1. Lanes 1-7 & 11 represent the fragments of 115bp & 122bp. However the resolution of the two bands was not clear. This however did not interfere with the interpretation of the RFLP patterns. Bands of expected 237bp were observed in Lanes 8, 9 & 12.

Figure 3.20 shows the DQw2,3,4 group-specific DQB1 RFLP pattern of Sac 1-digested DNA amplified with GH28NL & QB204. The DNA was obtained from the same individuals as those in Figure 3.18. All samples were digested with Sac1. All the lanes show the fragment of 223bp except Lane 10 as previously explained.

Figure 3.21 shows the DQw2,3,4 group-specific DQB1 RFLP pattern of BsaH1 1-digested DNA amplified with GH28NL & QB204. The DNA was obtained from the same individuals as those in Figure 3.18. All samples were digested with BsaH1. Lanes 1-7 & 11 represent the fragments of 104bp & 114bp. As previously mentioned, the resolution of the two bands was not possible and this did not interfere with the interpretation of the RFLP patterns. In Lanes 8, 9 & 12, bands of 237bp were seen.

Figure 3.22 shows the DQw2,3,4 group-specific DQB1 RFLP pattern of Msp 1-digested DNA amplified with GH28NL & QB204. The DNA was obtained from the same individuals as those in Figure 3.18. All samples were digested with Msp1. Bands of 61bp, 72bp & 104bp were resolved in Lanes 1, 2, 5, 6 & 7. Lane 6 had an additional band of 176bp. Fragments of 176bp & 61bp were seen in Lanes 3, 4 & 11 and in Lanes 8, 9 & 12 the bands of 104bp & 133bp were clearly seen.

As with the previous example, the control sample in Lane 1 of Figures 3.18-3.22 gave a restriction pattern which was easy to interpret. The bands observed in Lane 1 of Figures 3.18-3.22. were as follows:
237bp (digested with Fok1, Figure 3.18);

115bp & 122bp (digested with BglI, Figure 3.19);

223bp (digested with SacI, Figure 3.20);

104bp & 114bp (digested with BsaH1, Figure 3.21); and

61bp, 72bp & 104bp (digested with Msp1, Figure 3.22).

Matching these observed band pattern with the predicted RFLP pattern, this individual was assigned to Pattern B of Table 3.2b which correspond to the DQB1-0301 allele. In this way all the other observed band patterns were matched to the predicted pattern and were easy to interpret.
Table 3.2b. The predicted cleavage fragment pattern obtained by five restriction endonucleases in the PCR-amplified DQBI genes with the DQw2,3,4 specificities

<table>
<thead>
<tr>
<th>DQBI</th>
<th>Pattern</th>
<th>Fragment size (bp) digested with restriction endonucleases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fok I</td>
</tr>
<tr>
<td>0201</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>0301</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>0302</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>0303.1</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>0303.2</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>0401</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>0402</td>
<td>G</td>
<td></td>
</tr>
</tbody>
</table>

Note: 0- fragment (bp) not present, 1- fragment (bp) present, 9- fragment (bp) present but too small to be defined.

3.2.3 Results of digestion with Nde I

Once the assignment of the DQBI-0602 & -0603 alleles to individuals was confirmed by matching the observed RFLP pattern to the predicted pattern, the DNA of these individuals was further digested with NdeI. This digestion process allowed the possible discrimination of the DQBI-0602 from the -0603 alleles. The restriction endonucleases Nde I was used to digest the 220bp PCR-amplified DNA identified as belonging to the DQBI-0602 and the -0603 alleles only. The Nde I had a restriction site at the 3' end of the primer MCBB designed with a mutation to create the restriction site required. The enzyme Nde I cleaved at only one site to produce two fragment sizes, 19bp and 201bp, in the PCR-amplified
DNA of the DQB1-0602 allele only. There is no cleavage site in the amplified PCR-DNA of the DBQ1-0603 allele. Table 3.3 shows the predicted restriction pattern produced with Nde I digestion. The PCR- amplified DNA of the DQB1-0602 and -0603 positive MS patient and controls were digested with Nde I and the RFLP pattern is shown in Figure 3.23.

Figure 3.23 shows the observed restriction pattern of Nde I-cleaved DNA amplified with primers MCBB & QB202. Lanes 1-5 showed the DNA obtained from five MS patients digested with Nde1. The fragments of 201bp were observed in Lane 1 & 2 and in Lanes 3 & 4, bands of 220bp were seen. Both fragments of 220bp & 201bp were observed in Lane 5. Non-specific bands of approximately 147bp of unknown origin were seen in Lanes 1, 3, 4 & 5, but this did not interfere with the RFLP analysis. By matching the observed band pattern to the predicted pattern in Table 3.3, the MS patients in Lanes 1 & 2 carried the DQB1-0602 allele, those in Lanes 3 & 4 had the DQB1-0603 and the individual in Lane 5 had both.

Table 3.3. Cleavage fragment patterns produced by the digestion with the restriction endonuclease, Nde I, of the PCR-amplified DNA of the DQB1-0602 and -0603 alleles only.

<table>
<thead>
<tr>
<th>DQB1 alleles</th>
<th>Pattern</th>
<th>Fragment sizes (bp) digested with restriction endonuclease, Nde I.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>0602</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>0603</td>
<td>B</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: 0 - no fragment, 1- fragment present
As indicated from the complex RFLP digestion patterns of amplified products, the use of the primes DB130 & GH29 for amplification of the DBQ1 alleles was abandoned. The selection of the three sets of primers, GH28NL & QB202; GH28NL & QB204; and MCBB & QB202 produced amplified products which on digestion with the 12 endonucleases produced RFLP digestion patterns which were easy to interpret. As a result, these procedures were used to successfully type the 100 MS patients and 100 controls.
Figure 3.1. PCR-amplification pattern of the second exon of the DQB1 alleles of three MS individuals using three sets of primers. The DNA from the three MS patients was amplified with three set of primers: GH28 & GH29 in Lanes (L) 2-4; the same DNA with GH29 & DB130 in Lanes 5-7; and similarly with GH26 & GH27 in Lanes 8-10.

L 1: MW marker pUC19 digested with HpaII (marker sizes range from 34bp-501bp).
L 2: primer pair GH28 & GH29. 230bp fragment visible.
L 4: primer pair GH28 & GH29. 230bp fragment visible.
L 5: primer pair GH29 & DB130. 259bp fragment visible.
L 7: primer pair GH29 & DB130. 259bp fragment visible.
L 8: primer pair GH26 & Gh 27. 242bp fragment visible. These primers amplify the DQA1 alleles and were used as a positive control.
L 10: primer pair GH26 & GH 27. 242bp fragment visible.
L 3, 6, 9: The DNA did not amplify.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.2. PCR-amplified products with primers DB130 & GH29. The DNA was obtained from eight MS patients and all were amplified with the two primers to produce a band of 259bp.

L 1-8: Expected fragment of 259bp detected.
L 1 & 2: The DNA heteroduplex (HD) electrophoretic patterns (doublets or more bands) detected.
L 9: Negative control.
L 10: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.3. Amplification of the DQw1 group-specific DBQ1 alleles with primers GH28NL & QB202. Expected bands of 241bp were clearly seen in L 1-12.

L 1, 3, 5, 6, 12: DNA extracted from the control population.
L 2, 8, 9, 10, 11: DNA extracted from the MS patient population.
L 4 & 7: DNA extracted from the two homozygous reference cell lines.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 µg/ml ethidium bromide.

Two parallel bands at the top of the picture showed the residual DNA in the wells of the polymerised gel.

Figure 3.4. Amplification of the DQw2,3,4 group-specific DQB1 alleles with primers GH28NL & QB204.

L 1, 3, 5, 6, 12: DNA extracted from the control population.
L 2, 8, 9, 10, 11: DNA extracted from the MS patient population.
L 4 & 7: DNA extracted from the two homozygous reference cell lines.
L 13: Negative control.
L 14: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 µg/ml ethidium bromide.

Fragments of 237 bp were seen in L 1, 2, 3, 5, 6, 8, 9, 11 & 12. No amplification was observed in L 4, 7 & 10 as these individuals carry the DQw1 group-specific alleles.
Figure 3.5. The PCR-amplification of the DQB1-0602 and -0603 alleles with primers MCBB & QB202. L 1-5 showed the expected bands of 220bp of five MS patients.

L 1-5: The DNA was from five different MS patients. 220bp fragment detected.
L 1, 3, 4, 5: Non-specific band of approximately 170bp was also amplified but the presence of this band did not interfere with the interpretation of the RFLP patterns.
L 6: Positive control: the MS sample used was serologically typed to the DQw 2,3,4 specificities, therefore no amplification was expected and observed.
L 7: Negative control.
L 8: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.6. Comparison of the different concentrations of MgCl₂ used in the amplification of DNA. The DNA used was from one MS patient.

L 1: 4mM Mg Cl₂ with 40 amplification cycles.
L 2: 4mM Mg Cl₂ with 30 amplification cycles.
L 3: 3mM Mg Cl₂ with 40 amplification cycles.
L 4: 3mM Mg Cl₂ with 30 amplification cycles.
L 5: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.7. RFLP pattern of HaeII-digested DNA of 12 individual MS patients, amplified with DB130 & GH29.

L 1, 8, 10: Fragments of 259bp, 163/6, 125/7/8bp, 93/6bp were observed. Separation of the bands 163bp from 166bp; 125bp from 127bp & from 128bp; and 93bp from 96bp was not possible. Some of the expected bands were too small (eg. 38/39bp) to be detected.
L 2, 3, 4: Fragments of 163/6bp, 93/6bp were observed.
L 5, 6, 9, 12: Fragments of 259bp, 163/6bp, and 93/6bp were observed.
L 7: Fragments of 163/6bp, 125/7/8bp, 93/6bp, and 46/7bp were observed.
L 11: Fragments of 259bp, 125/7/8bp, 93/6bp were observed.
L 13: MW marker pUC19 digested with HpaII.

The heteroduplex electrophoretic pattern was also observed in L 3, 4, 6, 8, 9, 10, 11.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.8. RFLP pattern of HhaI-digested DNA amplified with DB130 & GH29. The DNA samples used were from the same 12 MS patients as in Figure 3.7.

L 1: Fragments of 190bp, 142bp (faint), 113/5bp, 69bp, 46bp, 48bp visible. Separation of the 113bp from the 115bp were not possible.
L 2: Fragments of 113/5bp, 69bp, 46bp, 48bp visible.
L 3, 4, 6: Fragments of 142bp, 113/5bp, 69bp, 46bp, 48bp visible.
L 5: Fragments of 259bp, 142bp, 113/5bp, 69bp, 46bp, 48bp visible.
L 7: Fragments of 142bp, 86bp, 69bp, 56bp, 46bp, 48bp visible.
L 8, 9: Fragments of 142bp, 69bp, 46bp, 48bp visible.
L 10, 11: Fragments of 142bp, 69bp, 48bp visible.
L 12: Fragments of 190bp, 142bp (faint), 113/5bp (faint), 69bp, 48bp (faint) visible.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.9. RFLP pattern of Rsal-digested DNA amplified with DB130 & GH29. The DNA samples used were from the same 12 MS patients as in Figure 3.7.

L 1,2: Fragments of 80/3bp detected. Separation of the 80bp from the 83bp was not possible. The two smaller bands at the bottom of the picture correspond to the 30bp & the 39bp.
L 3, 4: Fragments of 259bp, 80/3bp, 69bp (faint), 30bp, 39bp detected. Separation of the 80bp from the 83bp was not possible.
L 5, 9: Fragments of 259bp, 152bp, 80/3bp & 69bp (faint) detected. Again the separation of the 80bp from the 83bp was not possible.
L 6, 10,11: Fragments of 152bp, 80/3bp, 30bp & 39bp (both faint) detected. The separation of the 80bp from the 83bp was not possible.
L 7: Fragments of 152bp, 80/3bp, 69bp, 30bp & 39bp detected.
L 8: Fragments of 259bp, 152bp, 80/3bp, 30bp & 39bp (both faint) detected. Again the separation of the 80bp from the 83bp was not possible.
L 12: Fragments of 80/3bp, 69bp, 30bp & 39bp (both faint) detected.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 µg/ml ethidium bromide.

Multiple unpredicted bands of approximately 153bp-180bp were seen in L 6, 8, 9, 10, 11.

Figure 3.10. RFLP pattern of Sau3A1-digested DNA amplified with DB130 & GH29. The DNA samples used were from the same 12 MS patients as in Figure 3.7.

L 1-7, 10: Fragments of 259bp and multiple bands of sizes larger than the expected band of 259bp visible.
L 8: Fragments of 259bp, 154bp (faint), and a faint smaller unexpected band of approximately 69bp visible.
L 9, 11, 12: Fragments of 259bp, 154bp & 105bp visible.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 µg/ml ethidium bromide.
Figure 3.11. The RFLP pattern of FokI-cleaved DNA of 12 individual MS patients, amplified with primers DB130 & GH29. All the bands were clearly resolved.

L 1, 2, 3, 4, 12: Fragment of 259bp detected.
L 5, 6, 7, 8, 9, 10, 11: Fragments of 259bp & 187bp detected.
L 13: MW marker pUC19 digested with HpaII.

The heteroduplex electrophoretic pattern were also observed in L 3, 4, 6, 8, 9, 10, 11.

Gel: 10% polycarylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.12. The DQw1 group specific DQB1 RFLP pattern of FokI-digested DNA amplified with GH28NL & QB202. The DNA used in L 7-9 were obtained from MS patients and the rest were from the control population.

L 1, 2, 3, 4, 5, 6, 7, 8, 9, 19, 12: Fragments of 241bp visible.
L 11: Fragments of 241bp & 149bp visible.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polycarylamide stained with 0.5 ug/ml ethidium bromide.

Non-specific bands of approximately 50bp were seen at the bottom of the gel in all the Figures 3.12-3.17 and this did not interfere with the RFLP analysis.
Figure 3.13. RFLP pattern of Apal-digested DNA amplified with GH28NL & QB202. The DNA used was from the same individuals as in Figure 3.12.

L 1, 6, 7, 8, 9: The fragments of 241bp detected.
L 2, 3, 4, 5: Fragments of 176bp & 65bp (faint) detected.
L 10, 11, 12: Fragments of 241bp, 176bp & 65bp detected.
L 13: MW marker pUC19 digested with HpalI.

Gel: 10% polycarylamide stained with 0.5 ug/ml ethidium bromide.

As indicated earlier, the non-specific bands of approximately 50bp were also seen.

Figure 3.14. RFLP pattern of Haell-digested DNA amplified with GH28NL & QB202. The DNA used was from the same individuals as in Figure 3.12.

L 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: Fragment of 241bp visible.
L 13: MW marker pUC19 digested with HpalI.

Gel: 10% polycarylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.15. RFLP pattern of BssH II-digested DNA amplified with GH28NL & QB202. The DNA used was from the same individuals as in Figure 3.12.

L 1, 6, 7, 8, 9: Fragments of 166bp & 75bp detected.
L 2, 3, 4, 5, 10, 11, 12: Fragment of 241bp detected.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.16. RFLP pattern of Hph I-digested DNA amplified with GH28NL & QB202. The DNA used was from the same individuals as in Figure 3.12.

L 1, 6, 7, 8, 9, 11, 12: Fragments of 119bp & 122bp visible. Although the separation of the two bands was not resolved, this did not interfere with the RFLP interpretation.
L 2, 3, 5: Fragment of 236bp visible.
L 4, 10: Fragments of 236bp, 119bp & 122bp visible.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.17. RFLP pattern of HhaI-digested DNA amplified with GH28NL & QB202. The DNA used was from the same individuals as in Figure 3.12.

L 1, 6, 7, 8: Fragments of 133bp & 49bp detected.
L 2, 3, 4, 5, 9, 10: Fragments of 162bp & 49bp detected.
L 11: Fragments of 162bp, 106bp 56bp & 49bp detected.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polycarylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.18. The DQw2,3,4 group specific DQB1 RFLP pattern of FokI-digested DNA amplified with GH28NL & QB204. The DNA used in L 4, 5, 6, 8, 9, 10, 11, 12 was obtained from MS patients and the rest was from the control population (L 1-3 & 7). The DNA in L 10 remained uncut in all the Figures 3.18-3.22.

L 1, 2, 3, 4, 5, 6, 7, 11: Fragment of 237bp visible.
L 8, 9, 12: Fragments of 149bp & 88bp (faint) detected. Other non-expected band of approximately 60bp was visible but this did not interfere with the RFLP analysis.
L 13: MW marker pUC19 digested with HpaII.

Gel: 10% polycarylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.19. RFLP pattern of BglII-digested DNA amplified with GH28NL & QB204. The DNA used in L 4, 5, 6, 8, 9, 10, 11, 12 was obtained from MS patients and the rest was from the control population (L 1-3 & 7). The DNA in L 10 remained uncut in all the Figures 3.18-3.22.

L 1, 2, 3, 4, 5, 6, 7, 11: Fragments of 115bp & 122bp detected. The resolution of the two bands was not very clear but this did not interfere with the RFLP interpretation.
L 8, 9, 12: Fragment of 237bp detected.
L 13: MW marker pUC19 digested with HpaII.

Other non-expected band of approximately 60bp was visible but this did not interfere with the RFLP analysis.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.

Figure 3.20. RFLP pattern of Sac I - digested DNA amplified with GH28NL & QB204. The DNA used in L 4, 5, 6, 8, 9, 10, 11, 12 was obtained from MS patients and the rest was from the control population (L 1-3 & 7). The DNA in L 10 remained uncut in all the Figures 3.18-3.22.

L 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12: Fragment of 223bp visible.
L 13: MW marker pUC19 digested with HpaII.

Other non-expected band of approximately 60bp was seen but this did not interfere with the RFLP analysis.

Gel: 10% polyacrylamide stained with 0.5 ug/ml ethidium bromide.
Figure 3.21. RFLP pattern of BsaHI-digested DNA amplified with GH28NL & QB204. The DNA used in L 4, 5, 6, 8, 9, 10, 11, 12 was obtained from MS patients and the rest was from the control population (L 1-3 & 7). The DNA in L 10 remained uncut in all the Figures 3.18-3.22.

L 1, 2, 3, 4, 5, 6, 7, 11: Fragments of 104bp & 114bp detected. The resolution of these two bands was not clear but this did not interfere with the RFLP analysis.
L 8, 9, 12: Fragment of 237bp detected.
L 13: MW marker pUC19 digested with HpalI.

Other non-expected band of approximately 60bp was visible but this did not interfere with the RFLP analysis.

Gel: 10% polycrylamide stained with 0.5ug/ml ethidium bromide.

Figure 3.22. RFLP pattern of MspI-digested DNA amplified with GH28NL & QB204. The DNA used in L 4, 5, 6, 8, 9, 10, 11, 12 was obtained from MS patients and the rest was from the control population (L 1-3 & 7). The DNA in L 10 remained uncut in all the Figures 3.18-3.22.

L 1, 2, 5, 7: Fragments of 104bp, 72bp & 61bp visible.
L 3, 4, 11: Fragments of 176bp & 61bp visible.
L 6: Fragments of 176bp, 104bp, 72bp, 61bp visible.
L 8, 9, 12: Fragments of 104bp & 133bp visible.
L 13: MW marker pUC19 digested with HpalI.

Other non-expected band of approximately 60bp was seen but this did not interfere with the RFLP analysis.

Gel: 10% polycrylamide stained with 0.5ug/ml ethidium bromide.
Figure 3.23. Restriction fragment patterns of Nco I-cleaved DNA amplified with primer MCBB & QB202. The DNA used was obtained from five MS patients.

L 1, 2: Fragment of 201bp visible.
L 3, 4: Fragment of 220bp visible.
L 5: Fragments of 201bp & 220bp visible.
L 6: MW marker pUC19 digested with HpaII.

Non-specific band of approximately 147bp was seen in L 1, 3, 4, 5 but this did not interfere with the RFLP analysis.
Gel: 10% polycrylamide stained with 0.5 ug/ml ethidium bromide.
Results of HLA-DQB typing of MS & control populations

This chapter presents the statistical results associated with the genotyping of the MS patient and control population. The sections of the chapter are as follows:

4 Results of HLA-DQB1 typing of MS & control populations.
4.1 Frequencies of the HLA-DQB1 phenotype.
4.2 HLA-DQB1 gene frequency
4.3 HLA-DQB1 phenotype frequencies (%) of DRB1-1501 positive Australian MS patients and DRB1-1501 positive Australian controls.
4.4 HLA-DQB1 phenotype frequencies (%) of DRB1-1501 negative Australian MS patients and DRB1-1501 negative Australian controls.
4.5 The phenotype frequency (%) of the second HLA-DQB1 allele in the DQB1-0602 positive Australian MS patients and the DQB1-0602 positive Australian controls.
4.6 MS association with shared DQB1 sequences.
4.7 MS association with DQB1 alleles encoding Leucine at residue 26.
4.8 MS associations with the DRB1-1501, DQA1-0102, DQB1-0602 haplotype.
4.1 Frequencies of the HLA-DQB1 phenotype

The DQB1 phenotype and gene frequencies of the MS patients and control populations were calculated to find if there were any positive and/or negative associations of the alleles with MS. The HLA-DQB1 phenotype frequencies of the MS and control individuals are presented in Table 4.1a.

Table 4.1a. HLA-DQB1 phenotype frequencies (%) of 100 Australian MS patients and 100 Australian controls

<table>
<thead>
<tr>
<th>DQB1 alleles</th>
<th>MS n=100</th>
<th>Control n=100</th>
<th>P value (not corrected)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0602</td>
<td>54</td>
<td>18</td>
<td>&lt;0.0001</td>
<td>5.35</td>
</tr>
<tr>
<td>0603</td>
<td>15</td>
<td>6</td>
<td>0.038</td>
<td>2.76</td>
</tr>
<tr>
<td>0604/5</td>
<td>6</td>
<td>16</td>
<td>0.024</td>
<td>0.34</td>
</tr>
<tr>
<td>0601</td>
<td>1</td>
<td>3</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0501</td>
<td>15</td>
<td>25</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0502</td>
<td>1</td>
<td>1</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0503</td>
<td>1</td>
<td>6</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0201</td>
<td>40</td>
<td>42</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0301</td>
<td>23</td>
<td>32</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0302</td>
<td>17</td>
<td>12</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0303</td>
<td>5</td>
<td>10</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0401</td>
<td>0</td>
<td>0</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0402</td>
<td>2</td>
<td>4</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Note: not significant (ns) where p values were > 0.05.

RR= relative risk

From this data, there was a positive association between MS and the HLA-DQB1-0602 allele in MS patients as compared to the control population (54% MS, 18% control, p<0.0001, RR 5.35). There was also an increase in the DQB1-0603 allele in the MS group (15% MS, 6% control, p=0.038; RR 2.76; Table 4.1a) but when the p value was corrected the result was not significant (p=0.494).

Similarly, a negative association with the DQB1-0604/5 and MS was also observed (16% control, 6% MS, p=0.024, RR=0.34; Table 4.1a) but on correction of the p value the result was not significant.
(p<sub>c</sub>=4.4). Although the p values were not significant, it was noted that there appeared to be a negative association between MS and the DQB1-0301 (32% control, 23% MS) & the DQB1-0501 (25% control, 15% MS) alleles (Table 4.1a). None of the other alleles showed significant association with the disease (Table 4.1a).

### 4.2 HLA-DQB1 gene frequencies

The HLA-DQB1 gene frequencies of the MS patients and controls are presented in Table 4.1b.
Table 4.1b. HLA-DQB1 gene frequencies amongst 100 Australian MS patients and 100 Australian controls

<table>
<thead>
<tr>
<th>DQB1 alleles</th>
<th>MS n=200</th>
<th>Control n=200</th>
<th>P value (not corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0602</td>
<td>0.3100</td>
<td>0.0950</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0603</td>
<td>0.0750</td>
<td>0.0300</td>
<td>0.044</td>
</tr>
<tr>
<td>0604/5</td>
<td>0.0300</td>
<td>0.0850</td>
<td>0.018</td>
</tr>
<tr>
<td>0601</td>
<td>0.0050</td>
<td>0.0200</td>
<td>ns</td>
</tr>
<tr>
<td>0501</td>
<td>0.0750</td>
<td>0.1350</td>
<td>ns</td>
</tr>
<tr>
<td>0502</td>
<td>0.0050</td>
<td>0.0050</td>
<td>ns</td>
</tr>
<tr>
<td>0503</td>
<td>0.0050</td>
<td>0.0300</td>
<td>ns</td>
</tr>
<tr>
<td>0201</td>
<td>0.2350</td>
<td>0.2550</td>
<td>ns</td>
</tr>
<tr>
<td>0301</td>
<td>0.1200</td>
<td>0.0200</td>
<td>ns</td>
</tr>
<tr>
<td>0302</td>
<td>0.1000</td>
<td>0.0650</td>
<td>ns</td>
</tr>
<tr>
<td>0303</td>
<td>0.0250</td>
<td>0.0500</td>
<td>ns</td>
</tr>
<tr>
<td>0402</td>
<td>0.0100</td>
<td>0.0200</td>
<td>ns</td>
</tr>
</tbody>
</table>

Note: not significant (ns) where p values were > 0.05.

RPE=relative dispositional effect statistic was performed to test if the secondary associations (with alleles -0604/5, -0301, -0603, and -0501) were truly independent of the primary association (with -0602 allele) and not just a misleading adjustment due to the increase or decrease of the primary association.

\[
\text{RPE } \chi^2 \text{ overall } = 48.72, \ p < 0.0001 \\
\text{RPE } \chi^2 \text{ (without 0602) } = 20.68, \ p = 0.023 \\
\text{RPE } \chi^2 \text{ (without 0604/5) } = 17.56, \ p = 0.041 \\
\text{RPE } \chi^2 \text{ (without 0301) } = 15.72, \ p = 0.047 \\
\text{RPE } \chi^2 \text{ (without 0603) } = 10.15, \ p = 0.18 \ . \ ns
\]

These results are consistent with the phenotype frequency data where an increase in the DQB1-0602 gene frequency of 31% in patients versus 10% in controls was noted (Table 4.1b: p<0.0001). Increased gene frequency of DQB1-0603 and -0301 and decrease in -0604/5 and -0501 alleles in patients were also noted. The relative dispositional effect method was used to test if the secondary associations (with alleles -0604/5, -0301, -0603, and -0501) were
truly independent of the primary association (-0602 allele) and not just a misleading adjustment due to an increase or decrease in the primary association. The increased DQB1-0301 and -0603, and the decreased DQB1-0604/5 gene frequencies were not secondary to the primary increased in DQB1-0602 gene frequency ($\chi^2=17.56$, $p=0.041$; $\chi^2=15.72$, $p=0.047$; $\chi^2=20.68$, $p=0.023$ respectively). However when the p values were corrected, there were no significant association.

4.3 HLA-DQB1 phenotype frequencies (%) of DRB1-1501 positive Australian MS patients and DRB1-1501 positive Australian controls

The phenotype frequencies of the DQB1 allele in the DRB1-1501 (the subtype of DR2) positive individuals are presented in Table 4.2. The DQB1 phenotype frequencies were matched to the DRB1-1501 phenotype frequencies to show if there were any linkage patterns and any possible association with MS susceptibility.
Table 4.2. HLA-DQB1 phenotype frequencies (%) of 57 DRB1-1501 positive Australian MS patients and 17 DRB1-1501 positive Australian controls

<table>
<thead>
<tr>
<th>DQB1 alleles</th>
<th>MS n=57</th>
<th>Control n=17</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0602</td>
<td>95</td>
<td>100</td>
<td>ns</td>
</tr>
<tr>
<td>0603</td>
<td>11</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>0604/5</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0601</td>
<td>0</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0501</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0503</td>
<td>0</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0201</td>
<td>35</td>
<td>29</td>
<td>ns</td>
</tr>
<tr>
<td>0301</td>
<td>18</td>
<td>24</td>
<td>ns</td>
</tr>
<tr>
<td>0302</td>
<td>14</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>0303</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0402</td>
<td>4</td>
<td>0</td>
<td>ns</td>
</tr>
</tbody>
</table>

Note: not significant (ns) where p values were > 0.05.

In the DRB1-1501 positive individuals, all controls and 95% of the MS patients also carry the DQB1-0602 alleles (Table 4.2). The DQB1-0602 allele was found in all but three of the DRB1-1501 positive MS patients (Table 4.2).

In order to show the strength of the linkage disequilibrium between DQB1 & DRB1-1501 alleles, a two-locus linkage analysis was performed and presented in Table 4.3.
Table 4.3. Two-locus linkage analysis in Australians of the association between MS and the DRB1-1501 phenotype in the presence & absence of the DQB1-0602 allele

<table>
<thead>
<tr>
<th></th>
<th>DQB1 allele +</th>
<th>DQB1 allele -</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1-1501+</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>DRB1-1501-</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>MS</td>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>82</td>
</tr>
</tbody>
</table>

$\chi^2$ for significance = 8.501; p<0.005

$\chi^2$ for heterogeneity = 0.031; p=not significant (ns)

Two-locus linkage analysis in Australians of the association between MS & the DQB1 allele in the presence & absence of the DRB1-1501 phenotype.

<table>
<thead>
<tr>
<th></th>
<th>DRB1-1501 +</th>
<th>DRB1-1501 -</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQB1 alleles +</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>DQB1 alleles -</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>MS</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>82</td>
</tr>
</tbody>
</table>

$\chi^2$ for significance = 0.539; p=not significant (ns)

$\chi^2$ for heterogeneity = 0.030; p=ns.

From Table 4.3, it was found that the MS association with DQB1-0602 was secondary to the DRB1-1501 association.

4.4 HLA-DQB1 phenotype frequencies (%) of DRB1-1501 negative Australian MS patients and DRB1-1501 negative Australian controls

The phenotype frequencies of the DQB1 alleles in the DRB1-1501 negative individuals are presented in Table 4.4.
Table 4.4. HLA-DQB1 phenotype frequencies (%) of 43 DRB1-1501 negative Australian MS patients and 83 DRB1-1501 negative Australian controls

<table>
<thead>
<tr>
<th>DQB1 alleles</th>
<th>MS n=43</th>
<th>Control n=83</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0602</td>
<td>0</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>0603</td>
<td>21</td>
<td>7</td>
<td>0.025</td>
</tr>
<tr>
<td>0604/5</td>
<td>10</td>
<td>18</td>
<td>ns</td>
</tr>
<tr>
<td>0501</td>
<td>30</td>
<td>29</td>
<td>ns</td>
</tr>
<tr>
<td>0502</td>
<td>2</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>0503</td>
<td>2</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0201</td>
<td>47</td>
<td>45</td>
<td>ns</td>
</tr>
<tr>
<td>0301</td>
<td>33</td>
<td>34</td>
<td>ns</td>
</tr>
<tr>
<td>0302</td>
<td>21</td>
<td>12</td>
<td>ns</td>
</tr>
<tr>
<td>0303</td>
<td>7</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td>0402</td>
<td>0</td>
<td>5</td>
<td>ns</td>
</tr>
<tr>
<td>0601</td>
<td>2</td>
<td>2</td>
<td>ns</td>
</tr>
</tbody>
</table>

Note: not significant (ns) where p values were > 0.05.

As shown in Table 4.4, none of the DRB1-1501 negative MS patients carried the DQB1-0602 allele. Only one DRB1-1501 negative control individual carried the DQB1-0602 allele. This makes it difficult to assess whether the DQB1-0602 allele is the susceptibility allele independent of DRB1-1501. In the DRB1-1501 negative individuals, there was an increase in MS patients carrying the DBQ1-0603 allele (21% MS, 7% control, p=0.025, RR=3.40) but when the p value was corrected, the result was not significant (p=0.3).

4.5 The phenotype frequencies (%) of the second HLA-DQB1 allele in the DQB1-0602 positive Australian MS patients and the DQB1-0602 positive Australian controls

The phenotype frequencies of second DQB1 allele, other than the DQB1-0602 allele, was also calculated to determine its role in MS association. The phenotype frequencies of the second DQB1 alleles are presented in Table 4.5.
Table 4.5. The phenotype frequencies (%) of the second HLA-DQB1 allele in 54 DQB1-0602 positive Australian MS patients and 18 DQB1-0602 positive Australian controls

<table>
<thead>
<tr>
<th>DQB1 alleles</th>
<th>MS n=54</th>
<th>Control n=18</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0603</td>
<td>6</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>0604/5</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0501</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0503</td>
<td>0</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0201</td>
<td>35</td>
<td>28</td>
<td>ns</td>
</tr>
<tr>
<td>0301</td>
<td>15</td>
<td>22</td>
<td>ns</td>
</tr>
<tr>
<td>0302</td>
<td>15</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td>0303</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0402</td>
<td>4</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>0601</td>
<td>0</td>
<td>6</td>
<td>ns</td>
</tr>
</tbody>
</table>

Note: not significant (ns) where p values were > 0.05.

In the DQB1-0602 positive individuals in both the MS and control populations, there was no significant association with MS and the second DQB1 phenotype (Table 4.5).

4.6 MS association with shared DQB1 sequences

MS susceptibility has been reported to be encoded by multiple alleles sharing common sequence motifs (Vartdal et al, 1989; Spurkland et al, 1991). The DQB1-0602, -0603, -0604, -0302, and -0303 alleles show extensive sharing of nucleotide sequences. The frequencies of the shared DQB1 amino acid sequences of MS patients and control populations are presented in Table 4.6.
Table 4.6. The phenotype frequencies of the shared DQB1 amino acid sequences of MS and control populations

<table>
<thead>
<tr>
<th>Shared DQB1 sequences</th>
<th>Phenotype frequency</th>
<th>P value</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQB1-0602, -0603, -0604, -0302, -0303</td>
<td>MS 77% Control 54%</td>
<td>0.001</td>
<td>2.85</td>
</tr>
</tbody>
</table>

As shown in Table 4.6, there was an increase in the MS patients with phenotypes carrying alleles with shared sequences (77% MS, 54% control, P=0.001, RR=2.85, Table 4.6). However, this result was found to be secondary to the DQB1-1501 association when a two-locus linkage analysis was performed (Table 4.7: $\chi^2 =0.289$, p=ns).
Table 4.7. Two-locus linkage analysis in Australians of the association between MS and the DRB1-1501 phenotype in the presence & absence of the shared DQB1 sequences (DQB1-0602, -0603, -0604, -0302, -0303)

<table>
<thead>
<tr>
<th></th>
<th>Shared DQB1 sequences +</th>
<th>Shared DQB1 sequences -</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRB1-1501+</td>
<td>DRB1-1501-</td>
</tr>
<tr>
<td>MS</td>
<td>57</td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>36</td>
</tr>
</tbody>
</table>

$\chi^2$ for significance = 20.80; p<0.0005
$\chi^2$ for heterogeneity = 0.538; p=not significant (ns)

Two-locus linkage analysis in Australians of the association between MS & shared DQB1 sequences in the presence & absence of the DRB1-1501 phenotype.

<table>
<thead>
<tr>
<th></th>
<th>DRB1-1501+</th>
<th>DRB1-1501-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shared DQB1 sequences +</td>
<td>Shared DQB1 sequences -</td>
</tr>
<tr>
<td>MS</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

$\chi^2$ for significance = 0.266; p=not significant (ns)
$\chi^2$ for heterogeneity = 0.535; p=ns.

4.7 MS association with DQB1 alleles encoding Leucine at residue 26

It has been reported that DQB1 alleles (-0602, -0603, -0604, -0302, -0303, -0201) encoding Leucine at amino acid position 26 contribute positively to MS association (Haegert & Francis, 1992&
1993; Haegert et al 1993). The phenotype frequencies of DQB1 alleles encoding Leucine 26 are presented in Table 4.8.

Table 4.8. The phenotype frequencies of the DQB1 alleles encoding Leucine at residue 26

<table>
<thead>
<tr>
<th>DQB1 alleles encoding Leucine at residue 26</th>
<th>Phenotype frequency</th>
<th>P value</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQB1-0602, -0603, -0604, -0302, -0303, -0201</td>
<td>MS 90%</td>
<td>0.013</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>Control 77%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An increase in the phenotypes encoding Leucine at position 26 was observed in this study. Table 4.8 shows 90% of MS patients, 77% controls (p=0.013, RR=2.69) with phenotypes encoding Leucine 26 alleles. When a two-locus linkage analysis was conducted, the result showed that this association was secondary to the DRB1-1501 association (Table 4.9: \( \chi^2 = 0.521, p=\text{ns} \)).
Table 4.9. Two-locus linkage analysis in Australians of the association between MS and the DRB1-1501 phenotype in the presence & absence of the DQB1 alleles encoding for Leucine at residue 26 (DQB1-0602, -0603, -0604, -0302, -0303, -0201)

<table>
<thead>
<tr>
<th></th>
<th>Leucine 26+</th>
<th>Leucine 26-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRB1-1501+</td>
<td>DRB1-1501-</td>
</tr>
<tr>
<td>MS</td>
<td>57</td>
<td>33</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>60</td>
</tr>
</tbody>
</table>

χ² for significance = 26.629; p<0.0005
χ² for heterogeneity = 0.422; p=not significant (ns)

Two-locus linkage analysis in Australians of the association between MS & DQB1 alleles encoding for Leucine at residue 26 in the presence & absence of the DRB1-1501 phenotype.

<table>
<thead>
<tr>
<th></th>
<th>DRB1-1501+</th>
<th>DRB1-1501-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leucine 26+</td>
<td>Leucine 26-</td>
</tr>
<tr>
<td>MS</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

χ² for significance = 0.520; p=not significant (ns)
χ² for heterogeneity = 0.422; p=ns.

4.8 MS associations with the DRB1-1501, DQA1-0102, DQB1-0602 haplotype

Because of the known linkage disequilibrium between the DRB1-1501 and the DQA1-0102 and DQB1-0602 alleles and its association with MS, the frequency of the combined occurrence of these alleles was calculated and presented in Table 4.10.
Table 4.10. The DRB1-1501, DQB1-0602, and DQA1-0102 haplotype frequency

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Sample</th>
<th>P value</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1-1501, DQB1-0602 &amp; DQA1-0102</td>
<td>MS 53%</td>
<td>0.0001</td>
<td>5.51</td>
</tr>
<tr>
<td></td>
<td>Control 17%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data for the DQA1 frequency was obtained from S. Teutsch.

The frequency of the DRB1-1501 extended haplotype was significantly increased to 53% in patients as compared to 17% in controls (Table 4.10: p<0.0001, RR=5.51).
CHAPTER 5
Discussions and Conclusions

This chapter presents the discussion and conclusions of the DQB1 typing of 100 MS patients and 100 controls. The sections of the chapter are as follows:

5.1 Development of PCR-RFLP methodology
5.2 MS association with the HLA-D region genes
5.1 Development of PCR-RFLP methodology

This study represents a technical extension of previous reports by various authors who utilised PCR-RFLP for typing of DQB1 alleles (Uryu et al, 1989; Nomura et al, 1991; Sengar & Goldstein, 1994). The primers DB130 & GH29 were first used to amplified the second exon of the DQB1 locus (Uryu et al, 1989). However, concurrent amplification of the DQB2 pseudogene led to complex digest patterns which were difficult to interpret. As a result, two new sets of primers were selected which avoided the amplification of the DQB2 pseudogene. In this study, the two set of DQB1 primers GH28NL & QB202 and GH28NL & QB204 were used to amplify alleles encoding the DQw1 specificity (DQB1-0501-0503, 0601-0605) and DQw 2, 3, 4 specificity (DQB1-0201, 0301-3, 0401-2) respectively. In addition, a new primer, MCBB 1, was designed to contain a mutation, which in combination with DQB1-0602 generated a new Nde1 restriction enzyme site. This new designer PCR-RFLP technique using primers MCBB 1 & QB202 was successful in resolving the DQB1-0602 and the -0603 alleles which is crucial to this study since MS is strongly associated with the DQB1-0602 allele. The use of designer PCR-RFLP techniques to resolve the DQB1-0602 & -0603 alleles counters the criticism that PCR-RFLP methods are restricted to genotyping alleles with suitable polymorphic restriction digest patterns. As shown in this study, PCR-RFLP techniques can be used to resolve any allelic sequence when no informative restriction patterns can be found. Sengar & Goldstein (1994) have devised a similar technique for resolving the DQB1-0602 and 0603 alleles using a designer primer SDQ-01, a modified DB130 primer.

The specific selection of the restriction endonucleases with a single or no restriction sites in the amplified DNA region enabled accurate analysis of the restriction patterns. The DQB1 (DQw1) PCR products were successfully digested with: Fok 1, Apa 1, Hae 11, BssH 11, Hph 1 & Hha 1, and DQB1 (DQw 2, 3, 4) PCR products were digested with : Fok 1, Bgl I, Sac 1, BsaH 1 & Msp 1. This panel of restriction
enzymes was different to those used by Uryu et al (1989) and by Nomura et al (1991). The enzyme Hha 1 was substituted for SfaN 1 as it was a less expensive enzyme and was just as successful in resolving the DQw1 group alleles. The amplified products using primers MCBB & QB202 were digested with Nde 1. All the endonucleases gave clear banding patterns which matched the predicted cleavage patterns. The twelve endonucleases used for typing the DQw 1, 2, 3, and 4 group-specific alleles were successful in resolving the DQB1 alleles except the DQB1-0604 from -0605. Sengar & Goldstein (1994) reported that alleles -0604 and -0605 can be resolved with other endonucleases, Sau961 and Hae 111.

During the period of the study, new sequences were discovered. Sequences of the new DQB1 alleles, -06052, -0606, -0607, -0608, -0609 were reported (Meyer & Spauke, 1994; Fenske & Baxter-Lowe, 1992; Aldener & Olerup, 1993). DQB1-0607 and -0608 are identical to allele-0603 except for a single nucleotide substitution at codon 70 and 57 respectively (Sengar & Goldstein, 1994). Alleles DQB1-0606 and -0609 have restriction patterns similar to the -0604 allele. The DQB1-0606 and -0607 alleles can be resolved by digestion with Fnu4HI and SfaNI respectively. Cucca et al, (1993) have also reported a new allele, DQB1-0305. Although the resolution of the new DQB1 alleles with the suggested endonucleases have not been individually verified in this study, the sensitivity of the PCR-RFLP method and the strict enzyme-substrate specificity would yield similar reliable results as shown in this study.

There are several PCR-SSO (PCR-sequence-specific oligonucleotide probes (Haegert & Francis, 1992; Allen et al, 1993) and PCR-SSP (PCR - sequence-specific primers) methods used for the typing of DQB1 alleles (Aldener & Olerup, 1993; Bunce et al, 1993; Salazar et al, 1993). Although PCR-SSO and PCR-SSP typing will not be replaced in all situations by PCR-RFLP typing, it has a role in HLA typing. Where the HLA typing involves infrequent and small numbers of samples, as in the case of this study, the PCR-RFLP
method is both time and cost effective reducing the high start-up costs associated with the other methods.

In conclusion, the PCR-RFLP typing offers a simple, fast and reliable way of DNA typing. This method could be used independently or in conjunction with other typing methods such as PCR-SSO and PCR-SSP methods as confirmatory measures or to delineate new alleles.

5.2 MS association with the HLA-D region genes

In this study, the Class 11 typing of 100 MS patients and 100 controls showed an expected association between MS and the HLA-D region genes, an association first reported by Jersild et al (1973). Progressive refinement of the technical capacity to identify and type for an expanded number of loci and alleles over the past 20 years has confirmed that the susceptibility to MS has been associated specifically with the DR2, DQ6 haplotype or using the genomic nomenclature, the DRB1-1501, DQA1-0102, DQB1-0602 haplotype. It has also enabled comparisons of MS associations with the various haplotypes in different ethnic populations. This Australian study contributes to this worldwide comparison. Table 5.1 shows the MS associations with the various haplotype in different ethnic groups.
Table 5.1. Studies of MS associations with the various haplotype in different ethnic groups

<table>
<thead>
<tr>
<th>Nationality</th>
<th>Authors</th>
<th>No. typed</th>
<th>Haplotype</th>
<th>Additional points of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norwegian</td>
<td>Spurkland et al 1991 (d)</td>
<td>69 MS</td>
<td>72% MS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>181 control</td>
<td>33% control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.0001</td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert &amp; Francis 1992 (a)</td>
<td>78 MS</td>
<td>50% MS</td>
<td>RR=6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>79 control</td>
<td>13% control</td>
<td></td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert et al 1993 (b)</td>
<td>75 MS</td>
<td>29% MS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>74 control</td>
<td>10% control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.00006</td>
</tr>
<tr>
<td>Canadian (mixed</td>
<td>Haegert &amp; Francis 1993 (c)</td>
<td>62 MS</td>
<td>52% MS</td>
<td></td>
</tr>
<tr>
<td>ethnic white</td>
<td></td>
<td>69 control</td>
<td>32% control</td>
<td>no other haplotype was assoc with MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.03</td>
</tr>
<tr>
<td>Australian</td>
<td>current data 1994 (c)</td>
<td>100 MS</td>
<td>53% MS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 control</td>
<td>17% control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RR=5.51 p&lt;0.0001</td>
</tr>
</tbody>
</table>

Note:  

a) DRB5-0101, DQB1-0602, and DQA1-0102 haplotype.  
b) DQA1-0102, DQB1-0602 haplotype.  
c) DRB1-1501, DQB1-0602 and DQA1-0102 haplotype.  
d) DR2, DQA1-0102, DQB1-0602 haplotype

In this study, the positive association between the DRB1-1501, DQB1-0602 and the DQA1-0102 (Stewart et al, in press) haplotype and MS in the Australian population is confirmed (Table 5.1) and appears to be similar to those found in caucasians from Northern Europe and North America (Olertup & Hillert, 1991; Spurkland et al, 1991; Haegert & Francis, 1992, &1993).

There are relatively few studies of DQB1 associations with MS. The current Australian data appear to add confirmation that MS is significantly associated with HLA DQB1-0602. A comparison of the various studies of the HLA-DQB1 association with MS in different ethnic groups which are presented in Table 5.2 & Table 5.3, shows a comparison of the DQB1 phenotype frequencies, in particular the DQB1-0602 frequency, in different ethnic groups.
Table 5.2. Comparison of studies of DQB1 associations with MS in different ethnic groups

<table>
<thead>
<tr>
<th>Nationality</th>
<th>Authors</th>
<th>No. typed</th>
<th>Positive assoc. with phenotype</th>
<th>Positive assoc. with genotype</th>
<th>Additional points of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norwegian</td>
<td>Spurkland et al., 1991</td>
<td>69 MS 181 controls</td>
<td>0602: 72% MS 33% control RR=5, p=0.0001</td>
<td></td>
<td>neg assoc with 0301 RR=0.31 p&lt;0.03</td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert &amp; Francis, 1992</td>
<td>78 MS 79 controls</td>
<td>0602: 54% MS 15% control RR=6.51 p=0.000004</td>
<td></td>
<td>no other assoc (esp 0301) after removal of 0602 by RPE +ve assoc with DQA1 0102-DQB 0602 haplotype</td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert et al., 1993</td>
<td>75 MS(150 alleles) 76 control</td>
<td>0602: 29% MS 10% control p=0.000006</td>
<td></td>
<td>+ve assoc with 0201 after removal of 0302 by RPE(87 alleles exp 58.03) p&lt;0.01 stronger assoc with 0302 than 0201 p=0.04 no assoc with 0602</td>
</tr>
<tr>
<td>Sardinian</td>
<td>Haegert et al., 1993</td>
<td>116 MS(232 alleles) 86 control</td>
<td>0302: 14% MS (32 alleles exp 13.49) 6% control p&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swedish</td>
<td>Olerup &amp; Hillert 1991</td>
<td>179 MS 250 control</td>
<td>DRw15, DQw6: 63% MS 30% control RR=3.9 p&lt;10-10</td>
<td></td>
<td>+ve assoc with DQw6 (subtype of DQw1): 72% MS 54% control p&lt;0.0001 p&lt;0.005 DQw6 (RR=2.2) assoc weaker than DRw15 (RR=3.3) DQw6 secondary to DRw15, DQw6, Dw2 hap. p&lt;10-7</td>
</tr>
<tr>
<td>Australian</td>
<td>Current data 1994</td>
<td>100 MS 100 control</td>
<td>0602: 54% MS 18% control RR=5.35 p&lt;0.0001</td>
<td>0602: 31% MS (200 allele) 9.5% control (200 allele) p&lt;0.0001</td>
<td>after RPE, -ve assoc with 0604/5, p=0.041, &amp; 0301, p=0.047</td>
</tr>
</tbody>
</table>

RPE-relative dispositional effect
<table>
<thead>
<tr>
<th>Alleles</th>
<th>NSW Control</th>
<th>NSW MS</th>
<th>Finnish</th>
<th>Swedish (C)</th>
<th>Norwegian (standard clin.)</th>
<th>Australian (control clin.)</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.024</td>
<td>0.002</td>
<td>0.15</td>
<td>0.18</td>
<td>0.18</td>
<td>0.15</td>
<td>0.031</td>
</tr>
<tr>
<td>1</td>
<td>0.038</td>
<td>0.003</td>
<td>0.16</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
<tr>
<td>2</td>
<td>0.035</td>
<td>0.003</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
<tr>
<td>3</td>
<td>0.032</td>
<td>0.003</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
<tr>
<td>4</td>
<td>0.030</td>
<td>0.003</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
<tr>
<td>5</td>
<td>0.028</td>
<td>0.003</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
<tr>
<td>6</td>
<td>0.026</td>
<td>0.003</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
<tr>
<td>7</td>
<td>0.024</td>
<td>0.003</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.14</td>
<td>0.031</td>
</tr>
</tbody>
</table>

Table 5.3: Comparison of DORI phenotype frequencies with different population groups.
It has been suggested that the putative MHC encoded susceptibility gene(s) in MS is associated more strongly with the DQ locus (Vartdal et al, 1989, Heard et al, 1989, Spurkland et al, 1991). In this study, the phenotype frequency of the DQB1-0602 is positively associated with MS (54%MS, 18% control, p<0.0001, RR 5.35, Table 5.3). This observation was consistent with results of the various ethnic caucasian studies identified in Tables 5.2 & 5.3. Among the DRB1-1501 positive MS patients, 95% were positive for the DQB1-0602 allele (Table 4.2). In the DRB1-1501 negative individuals, none of the MS patients and only one control individual had the DQB1-0602 allele which makes it difficult to exclude the DQB1-0602 as a independent susceptibility allele (Table 4.4). The DQB1-0602 may be the susceptibility gene but is in linkage disequilibrium with DRB1-1501. However, the DQB1-0602 association was weaker than the DRB1-1501 association (RR= 5.35 and 6.47 respectively) and the two-locus linkage analyses suggests that the association between MS and the DQB1-0602 appears to be secondary to the DRB1-1501 association (Table 4.3). The above data appears to provide support for a stronger association of MS with the DRB1 than the DQB1, but this conclusion is based on the DRB1-DQB1 genotypes of three individuals. Thus, it is difficult to distinguish the primary contribution of DRB1 from that of DQB1 in MS association.

Several studies have reported that MS is associated with a shared HLA polymorphism found within the associated haplotype. Table 5.4 presents the various studies of MS associations with shared DQB1 amino acid sequences in different ethnic groups.
### Table 5.4. Studies of MS associations with shared DQB1 amino acid sequences in different ethnic groups

<table>
<thead>
<tr>
<th>Nationality</th>
<th>Authors</th>
<th>No. typed</th>
<th>Shared sequences</th>
<th>Additional points of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norwegian</td>
<td>Spurkland et al., 1991</td>
<td>a) 69 MS 181 control</td>
<td>a) 97% MS, 72% control RR=10, p=0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) 96% MS, 60% control RR=13, p=0.0001</td>
<td></td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert &amp; Francis, 1992</td>
<td>a) 78 MS 79 control</td>
<td>a) 81% MS, 40% control RR=6.3, p-not given</td>
<td>+ve assoc with DRB5-0101 RR=4.33, p=0.0004;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) 98% MS, 73% control RR=11.98, p=0.0001</td>
<td>-ve assoc with 0301 RR=0.31, p=0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c) 96% MS, 70% control RR=9.37, p=0.0001</td>
<td></td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert et al, 1993</td>
<td>a) 75 MS 60% RR 76 control</td>
<td>a) 80% MS, 47% control RR=4.4, p=0.00006</td>
<td>shared DQB1 sequences sec. to DQA1-0101-DQB1-0602 hap.;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) 99% MS, 75% control RR=24.7, p=0.00005</td>
<td>Leu 26 +ve assoc.</td>
</tr>
<tr>
<td>Norwegian</td>
<td>Vartdal et al, 1989</td>
<td>a) 61% MS 56 control</td>
<td>a) 97% MS, 70% control RR=10.5, p=0.0011</td>
<td></td>
</tr>
<tr>
<td>French Canadian</td>
<td>Haegert &amp; Francis, 1993</td>
<td>a) &amp; b) 79 MS 72 control</td>
<td>a)78% MS, 47% control p&lt;0.05</td>
<td>shared DQB1 sequences is sec. to DRB1-1501 bearing hap., p&lt;0.05;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b)79% MS, 76% control p=0.01</td>
<td>Leu 26 is NOT sec. to DQB1-1501 hap.</td>
</tr>
<tr>
<td>Canadian mixed</td>
<td>Haegert &amp; Francis, 1993</td>
<td>62 MS 65 control</td>
<td>a)68% MS,52% control p-not sig.</td>
<td>no assoc with Leu 26</td>
</tr>
<tr>
<td>ethnic</td>
<td></td>
<td>58 RR</td>
<td>b)84% MS,74% control</td>
<td></td>
</tr>
<tr>
<td>whites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swedish</td>
<td>Olerup &amp; Hillert, 1991</td>
<td>179 MS 250 control</td>
<td>a)84% MS, 74% control RR=19, p&lt;0.01</td>
<td>DRw15, DQw6 Dw2 -ve individuals:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>143 RR</td>
<td>d)79% MS, 60% control RR=2, p&lt;0.0001</td>
<td>no assoc with shared DQB1 sequences; shared DQB1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sequence sec. to DRw15, DQw6, Dw2 haplootype,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p&lt;10-9; DQo&amp;Binner sec. to DRw15, DQw6 Dw2</td>
</tr>
<tr>
<td>Australia</td>
<td>present study, 1993</td>
<td>100 MS 100 control</td>
<td>a)77% MS, 54% control RR=2.85, p=0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b)90% MS,77% control RR=2.69, p=0.013</td>
<td></td>
</tr>
</tbody>
</table>

Note:  
- a) shared amino acid sequences, DQB1-0602, -0603, -0604, -0302 and/or -0303.  
- b) DQB1 alleles encoding Leucine 26, -0602, -0603, -0604, -0302, -0303, and -0201.  
- c) DQB1 alleles encoding Leucine 26 using Leu 26 and DQw2 probes.  
- d) including one of the DQA1-(0102, 0103, 0401, 0501) and one of the DQB1-(0602, 0603, 0604, 0302, or 0303) alleles.  
- pc refers to corrected p value; sec.=secondary; hap.=haplotype; RR=relative risk

The results of this study are consistent with those of the Norwegian, Swedish and French Canadian MS populations (Vartdal et al, 1989; Olerup & Hillert, 1991; Spurkland et al, 1991; Haegert & Francis, 1992 & 1993; Haegert et al, 1993). In this study, the DQB1 alleles encoding shared amino acid sequences showed a positive association with MS (p=0.001, RR2.85, Table 4.6 & Table 5.4). However, two-locus linkage analysis found that this association was
secondary to the DRB1-1501 haplotype association (Table 4.7). Thus, it is concluded that shared DQB1 chain amino acid sequences do not play a primary role in MS association.

From the Class II crystal structure, Leucine at amino acid residue 26 can be plotted onto to the floor of the antigen binding cleft, under the α-helix. Therefore DQB1 chain polymorphisms at this residue may influence antigen binding and the mounting of an immune response in MS (Brown et al, 1988 & 1993). In this study, there was a positive MS association with DQB1 alleles encoding Leucine at residue 26 (p=0.013, RR=2.69, Table 4.8 & Table 5.4). Similar positive MS associations with DQB1 alleles encoding Leucine 26 were reported by Spurkland et al (1991); Olerup & Hillert (1991); Vahtio et al (1989); Haegert & Francis (1992 & 1993); Haegert et al (1993); and Allen et al (1994). However, two-locus linkage analysis found that this association in the present study was secondary to the DRB1-1501 association (Table 4.9). This finding concurs with those found by Olerup & Hillert (1991) but not those reported in the French Canadians by Haegert & Francis (1993) and Haegert et al (1993). An explanation for the discrepancy in the association is that the DRB1-1501-bearing haplotype is a predispositional haplotype for most caucasian populations but in French Canadians, DQB1 Leucine 26 is an additional predispositional factor (Haegert & Francis, 1993).

It has been suggested that the DQB1-0301 allele is an MS resistance gene amongst French Canadian patients (Haegert et al, 1990). Several studies have also reported a decrease frequency of the -0301 allele (Olerup et al, 1989; Spurkland et al, 1991; Cullen et al, 1991). In the present study, there was no significant decrease in the DQB1-0301 allele (Table 4.1a). There are no supporting data to support the claim that DQB1-0301 is associated with resistance to MS in the Australian population.

The DQB1-0602 allele, which is in strong linkage disequilibrium with the DRB1-1501 haplotype is associated with susceptibility to MS but which is neither necessary nor sufficient for the
development of the disease. It is likely that the low penetrance of the disease in the individuals carrying the MS-associated DR-DQ haplotype may be related to polygenetic and environmental factors. Many researchers have come to a conclusion that the pathogenesis of MS is multifactorial and it involves both genetic and environmental factors such as a infective episode prevalent in certain latitudes (McFarland & Dhib-Jalbut, 1989; Olerup & Hillert, 1991; Francis et al, 1991; Poser, 1992; Hao et al, 1992; Oksenberg et al 1993).

Therefore in conclusion,

1. due to the strong linkage disequilibrium, it is difficult to assess whether DRB1 or DQB1 plays the primary role in association with MS susceptibility,

2. there is a positive association with MS susceptibility and the DQB1-0602 alleles encoding shared nucleotide sequences and Leucine at residue 26 but these associations are secondary to the DRB1-1501, DQA1-0102, DQB1-0602 haplotype association,

3. the DQB1-0602 allele is neither necessary nor sufficient for MS disease expression. DQB1-0602 may be the marker gene in linkage with the true susceptibility DRB1-1501 gene or that these two loci could be attributed to linkage disequilibrium with the true susceptibility gene/s located in the Class I and Class II areas of the HLA region,

4. No HLA-DQB-region associated resistance alleles have been identified.
References


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IMMUNOGENETICS OF MULTIPLE SCLEROSIS:

An investigation of the alleles of the HLA-D region

A thesis submitted to the
Faculty of Science and Technology
University of Western Sydney, Nepean
as fulfilment of requirements for the degree of
Master of Science
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
by

Marianne Q.L. Castle

(B. Sc.) Macquarie

1995
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Summary

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system and the etiology is generally believed to be multifactorial. Possible factors, which include exposure to certain infective agent(s) in the genetically susceptible individuals, may lead to the development of MS. Since the 1970's, MS has been known to be associated with the HLA-DQ and DR2 specificities in European and North American Caucasian populations. In particular, several authors have reported that MS susceptibility has been more closely associated with the DQB1 than the DR2 alleles. This study examines the association of MS susceptibility with the DQB1 alleles.

The study has two focuses. The first focus explores the PCR-RFLP methodology which is used in the characterization and detection of HLA variation and the second is on the polymorphism of the DQB1 alleles and its association with MS susceptibility.

The PCR-RFLP technique involves the analysis of electrophoretically separated restriction fragments which were produced by the digestion of the amplified products with restriction endonucleases at allele specific sites. Four sets of primers were used in this study. The first set of primers, DB130 & GH29 were initially used to amplify the DNA from 100 MS patients and 100 controls. However the RFLP pattern obtained by digestion of the amplified product with the primers DB130 & GH29 were difficult to resolve because of the concomitant amplification of the DQB2 pseudogenes. Subsequently, two different sets of primers, GH28NL & QB202 and GH28NL & QB204, were chosen for selective amplification of the second exon of the DQB1 gene. These, together with the selection of the twelve restriction endonucleases which have either a single or no cleavage site in the amplified DNA region, made the reading of the RFLP band pattern easier. However the DQB1-0602 and -0603 alleles could not be discriminated from each other because of the unavailability of suitable enzymes. To overcome this problem, a primer MCBB was specifically designed with a base mutation at the 3' end to create a new restriction site which distinguished the two
alleles. The PCR-RFLP technique was successful in typing the DQB1 alleles except the DQB1-0604 and the -0605. This technique does not require high start-up costs associated with other typing methods such as PCR hybridisation with sequence-specific oligonucleotide probes (PCR-SSO) and PCR amplification with sequence-specific primers (PCR-SSP) and is both time and cost effective for infrequent and small numbers of samples.

The association of MS with the genes of the DR and DQ region loci was determined by PCR-RFLP typing of 100 Australian MS patients and 100 Australian controls. There was a positive MS association with the DQB1 alleles encoding shared amino acid sequences and those encoding Leucine at amino acid residue 26. However these associations were secondary to that of the DRB1-1501, DQA1-0102, DQB1-0602 haplotype. No protective association with MS and the DQB1-0301 allele was found in this study. The positive association between MS and the DQB1-0602 allele was confirmed in this study. However, due to linkage disequilibrium, it was difficult to assess whether the DQB1-0602 or the DRB1-1501 is the primary contributor to MS association.
CERTIFICATE

I hereby certify that this work has not been submitted for a higher degree to any other university or institution, and, except where acknowledged, is all my own work.

Signed

....................................
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Dedication

This work is dedicated to my mother, Y.Y and to my husband, Allan, for the unselfish support and encouragement throughout this project.