# STUDIES ON THE MOLECULAR IMMUNOGENETICS OF CLASS II HISTOCOMPATIBILITY ANTIGENS IN MULTIPLE SCLEROSIS

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## ABSTRACT

Serological studies of HLA antigens in a number of different populations over many years have clearly demonstrated that MS is associated with particular products of the Major Histocompatibility Complex Class II region, particularly DR2/Dw2 and DQw1. It is widely accepted that immune mechanisms are responsible for the demyelination seen in MS, and there is a good deal of circumstantial evidence in favour of an autoimmune pathogenesis. Furthermore, it is now well established that MHC gene products play a pivotal rôle in T cell activation, and this raises the possibility that a true disease susceptibility gene might be encoded in the MHC. It has also been suggested that the rate of disease progression might be influenced by the MHC in a similar way. Since the genes encoded by the MHC exhibit at the genetic level an extraordinary degree of polymorphism, much of which is not detectable using serological techniques, genetic methods have been employed to investigate further the association between MS and the Class II region.

An extensive analysis of restriction fragment length polymorphism of the Class II region was carried out in an attempt to identify genetic markers more strongly associated with MS than the classical serological markers. Initial studies using pooled DNA samples from patients and controls from the Grampian region of Northeast Scotland enabled the screening of 14 restriction endonucleases with five HLA-D region probes (DPA, DPB, DQA, DQB and DRB). A small number of discriminatory polymorphisms were observed with the DQ probes, although none with DP or DR.

Based on these data, further studies were carried out on individual samples of DNA from 33 MS patients and 48 controls from the Grampian region. By identifying a number of DQA and DQB fragments of known specificity, it could be demonstrated that almost all of the antigens that typed serologically as DR2 and DQw1 were encoded by the Dw2 DQw6 alleles of DR and DQ, and also that these alleles were represented equally in both patients and controls. However, following Msp1 digestion and hybridization to a DQA1 probe a cluster of fragments was observed significantly more frequently in the patients ($p<0.001$), and furthermore, this association was found to be independent of DR2. These findings were confirmed in an independently conducted collaborative study in Northern Ireland.

The DQA restriction fragment cluster was further characterized by means of established RFLP allogenotyping systems. The cluster was seen in all DRw8 (DQw4) homozygous cell lines included in the Tenth International Histocompatibility Workshop, and in some DR4 and DR7 lines, but not in caucasoid DR2 lines. In a panel of healthy British donors, the cluster was again seen most commonly in association with DQw4 or DQw8. The apparent allelism thus demonstrated by the cluster raised the possibility that disease susceptibility was associated with transcomplementation between the DR2-positive and DQA cluster-positive haplotypes.

Since the putative allele marked by the DQA cluster possessed many of the features of DQw4, an allele which is thought to be uncommon in caucasoids, an attempt to demonstrate this directly using monoclonal serology was made. A number of lymphoblastoid cell lines were raised from Scottish MS patients and their families, and studied using the DQw4 β chain-specific antibody HU46. However, the relation between the cluster and HU46 reactivity, though close, was not exact. Although 5 out of 6 HU46-reactive cell lines were cluster-positive, 3 out of 8 cluster-positive lines were HU46 non-reactive.

In order to demonstrate that the DQA cluster corresponded to an expressed DQ α polymorphism, attempts were made to raise alloreactive T cell clones against cluster-positive cell lines. Using cloning strategies designed to eliminate the possibility of generating clones against the 'cluster-negative' haplotype, a number of CD4-positive clones were raised and maintained in stable culture. However, analysis of the fine specificities of these clones failed to demonstrate reactivity corresponding exclusively to the DQA cluster.

DQα1-encoding exons from 3 cluster-positive Scottish MS patients, 2 unaffected siblings from multiple case families and 2 cluster-positive homozygous cell lines were amplified enzymatically by the polymerase chain reaction, and probed with allele-specific oligonucleotides. The data obtained suggested that in each case, the cluster-positive haplotype contained the DQA allele predicted by the linked DR genotype, and thus that the DQA cluster did not correspond to an expressed member of the DQA allelic series. This conclusion was confirmed by analysing the nucleotide sequences of PCR-amplified DQA α1-encoding exons from selected cluster-positive haplotypes. Taken together, these data suggest that

an MS-associated polymorphism, detectable by RFLP analysis, lies in non-coding regions adjacent to the DQA gene.

## CONTENTS

## Abbreviations

| | |
|---|---|
| HCL | homozygous cell line |
| HLA | human leucocyte antigen |
| LCL | lymphoblastoid cell line |
| mAb | monoclonal antibody |
| MHC | major histocompatibility complex |
| MLR | mixed lymphocyte reaction |
| MS | multiple sclerosis |
| PBL | peripheral blood lymphocyte |
| PBMC | peripheral blood mononuclear cell |
| PCP MS | primarily chronic progressive MS |
| PCR | polymerase chain reaction |
| PFGE | pulsed field gel electrophoresis |
| RE | restriction endonuclease |
| RFLP | restriction fragment length polymorphism |
| R/R MS | relapsing/remitting MS |
| rIL-2 | recombinant human interleukin-2 |
| TcR | T cell antigen receptor |
| XIHWS | Tenth International Histocompatibility Workshop |

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# PART 1
# REVIEW OF LITERATURE

## 1.1 THE GENETICS OF MULTIPLE SCLEROSIS

### 1.1.1 Genes versus environment

Although it is now widely accepted that both environmental influences (Kurtzke, 1980; Spielman, Nathanson, 1982) and genetic susceptibility (Ebers, 1983) play equally important rôles in the development of MS, it has required a sustained effort over several decades to prove this beyond doubt. Even now, 121 years after Charcot first established diagnostic criteria for MS and described familial clustering (Charcot, 1868), and 86 years after attention was first drawn to the unequal geographic distribution of the disease (Bramwell, 1903), those genetic and environmental factors responsible remain unidentified. Furthermore, possible mechanisms whereby they might interact are matters only for speculation.

In the most fundamental sense, any disease is the result of a failure of the normal homeostasis existing between a host and its environment. The host phenotype is the result of a complex genotype and thus all diseases can be regarded as polygenic. It should not therefore be surprising that most common diseases of obscure aetiology, of which MS is an excellent example, are now regarded as both multifactorial and polygenic. A simple model demonstrating how the interaction of independant risk factors, genetic and environmental for instance, may govern the expression of a multifactorial disease has been described by Professor John Edwards of Oxford University (personal communication). In this model,

| | | Genetic | | | |
|---|---|---|---|---|---|
| | | 20 | 40 | 80 | |
| | 10 | 30 | 50 | 90 | - |
| Environmental | 30 | 50 | 70 | **110** | + |
| | 50 | 70 | 90 | **130** | + |
| | | - | - | ++ | |

**Figure 1.** Model to demonstrate how the expression of a multifactorial disease can be governed by the interaction of independent genetic and environmental risk factors. Risks may be additive, as in this example, or complementary.

individuals possess one of three levels of genetic susceptibility, and may be subjected to one of three levels of environmental risk, as shown in Figure 1. Expression of the disease will result, in the example shown, when the cumulative risk from genetic and environmental factors exceeds 100. It can be seen that individuals with genetic risks of 20 or 40 will never develop the disease, but that those with a risk of 80 will develop it even when the environmental risk is only moderate.

The difficulties associated with identifying the separate contributions of genetic and environmental influences in such a disease are well illustrated by considering the phenomenon of familial clustering. This might variously be ascribed to a shared environment, or to inherited biological factors, or to the cultural inheritance of risk factors. These individual influences will only become separable, and thus available for further investigation, if epidemiologic studies are designed with the highest standards of controls (Ebers et al.1986). A recent example of this is the important paper by Hammond et al. in which the relationship between prevalence of MS and latitude in Australia was studied (Hammond et al.1988a). Although it is well established that MS is most common between latitudes $45^{o}$ and $65^{o}$, and less common at equatorial and Mediterranean latitudes, this observation could be accounted for to a large degree by the global distribution of populations of northern European ancestry. However, a rigorous study of European immigrants to Perth ($32^{o}$S) and Hobart ($43^{o}$S) has clearly shown that the north-south gradient in Australia is not genetically determined, and that environmental factors have modified disease expression.

Epidemiologic methods for investigating the aetiology of polygenic diseases depend upon accurate case ascertainment, and in the case of MS there are two unique problems which have hampered progress. Firstly, there is still no diagnostic test for the disease, the clinician relying on clinical, laboratory, electrophysiological and MRI criteria to differing extent in each new case. In the pre-MRI era, it was estimated that up to 20% of cases were not recognized during life and were therefore not included in epidemiological studies (Mackay, Hirano, 1967). More recently, several other studies have reported the finding of unsuspected plaques of demyelination at autopsy in individuals who during life had no symptoms suggestive of neurologic dysfunction (Gilbert, Sadler, 1983; Phadke, Best, 1983). The other important difficulty is in deciding whether or not to include those patients who have suffered a single episode of

demyelination. It is known that there is an increased incidence of mono-symptomatic demyelination, especially optic neuritis, in the families of individuals with MS (Ebers et al.1981), and also that multiple lesions are frequently revealed by MRI at presentation in patients with optic neuritis (Ormerod et al.1985). In view of this, the importance of including mono-symptomatic patients in epidemiologic and genetic studies is generally now recognized.

The eventual goal of epidemiologic studies must be to identify the individual genetic factors that explain the cause of susceptibility, for all forms of a disease, and in all environments in which it occurs. In the case of MS, a genetic explanation of susceptibility will have to encompass a great many apparently diverse observations. For instance, the frequency of MS may differ within one ethnic group depending on latitude, both north and south of the equator (Downie, Phadke, 1984; Poskanzer et al.1980b; Hornabrook, 1975; Fawcett, Skegg, 1988; McCall et al.1969; Hammond et al.1988a; Kuroiwa et al.1983), and geographical location during childhood (Dean, 1967; Dean et al.1976). There may be focal changes in incidence within a geographically stable population over time (Kurtzke, Hyllested, 1979; Cook et al.1985), and focal differences in frequency within small areas where the disease has been surveyed serially using standardized methods in an ethnically stable population (Caruso et al.1968; Midgard et al.1987; Granieri et al.1985; Hoffman et al.1981). A genetic theory should eventually be able to explain why plaque formation in MS has a marked predilection for certain regions within the central nervous system, and should also account for the spectrum of different clinical pictures and rates of disease progression that occur. Finally, it should prove possible to explain the consistently observed patterns of racial susceptibility and resistance described below.

### 1.1.2 Evidence for significant genetic effects in multiple sclerosis

The evidence that there is a major genetic contribution to MS susceptibility has come from five main areas of study. These are 1) racial susceptibility; 2) familial MS; 3) concordance rates in twins; 4) association with gender, and 5) association with polymorphic genetic markers, particularly HLA loci. These studies have led to the concept of an MS susceptibility gene, or genes, at least one of which is HLA-linked.

### 1.1.2.1 Racial Susceptibility

Some racial groups are susceptible and others resistant to MS. The first convincing data to suggest a genetic influence on the distribution of MS was a comparison of the prevalence in individuals from northern Scotland of Nordic (103/100,000) and Celtic (50/10,000) descent (Sutherland, 1956). Since then the racial distribution of MS has been studied using both mortality data and prevalence data. Goldberg and Kurland (McGowan, 1903) published international death rates for a number of neurologic diseases between 1951 and 1958. Without exception, the highest annual death rates (between 2 and 3.5 per 100,000 population per year) were found in the countries of northern Western Europe, especially Scotland, N. Ireland and Eire, France, Germany and Scandinavia. Countries from the Mediterranean basin, Australia and New Zealand, and the Americas showed intermediate rates (0.5 to 1.5/100,000 per year) and the lowest rates were seen in Japan and the Philippines (less than 0.1/100,000 per year). It was noted that the disease was less common in US non-whites by a factor of two. Therefore MS is predominantly a disease affecting individuals of Fenno-Scandian origin, and has been described by Kurtzke as "the white man's burden".

Only limited conclusions can be drawn from death rates, and prevalence studies provide more information. The highest prevalence rates are seen in the Shetlands and Orkneys (309 cases per 100,000 population) (Poskanzer et al.1980b) closely followed by northeast mainland Scotland (178/100,000) (Downie, Phadke, 1984). The high prevalence rates found in the northern British Isles and Scandinavia have led some observers to propose that genetic susceptibility originated in the Vikings, becoming disseminated by migration since their time. In contrast, other races appear to be protected from developing MS. It has never been observed in the Bantu (G. Dean, personal communication), and is extremely rare in Eskimos (Kurtzke, 1975). Hungary has a large number of Gypsies, who are genetically distinct from the indigenous caucasoid population, having migrated to Europe from northern India in the 15th century (Gyodi et al.1981). Whereas the prevalence of MS in Hungarian caucasoids is 37/100,000 only 2 cases have yet been identified in the Gypsy population which totals 110,000. A number of other races appear to be similarly protected. In a careful analysis of US Army veterans, Kurtzke showed that blacks or Negroes had only half the risk of white males (Kurtzke et al.1979). MS is reported to be uncommon in North American

Indian peoples (Cosgrove, Tourillon, 1983) and Hutterites (Hader, 1982), both of which tend to live in isolated communities within a high-risk zone. There are unfortunately very few epidemiologic studies from South America or Africa.

The best studied non-caucasoid population is the Japanese, in whom consistently low prevalence and annual incidence rates have been found. The first study in Asia was carried out in 4 Japanese cities in 1958-1960, and showed a prevalence rate of 1.6 to 3.9/100,000. The survey was repeated in 1972-1978, when rates of 0.8 to 3.9/100,000 were found (Kuroiwa et al.1975). It is significant that the prevalence rate did not change in the interval despite rapid westernization of life styles in many parts of Japan. Although a significant north-south gradient exists in Japan (Kuroiwa et al.1983), suggesting an environmental contribution to susceptibility, Japan lies in a high-risk zone in terms of latitude ($30^{o}$ - $40^{o}$N), thus arguing strongly for genetically determined protection from MS. This hypothesis is supported by studies of migrant orientals. The prevalence rate in Japanese living in California is 6/100,000, whereas in caucasoids it is between 21.6 and 68.7/100,000, with a marked north-south gradient (Detels et al.1982). MS is also less common than expected in Japanese immigrants to Hawaii (Shibasaki et al.1978), London (Dean et al.1976) and France (Kurtzke, 1976). It is well established that optic neuritis and spinal cord involvement occur more frequently in Japanese patients than in Europeans, and are also often unusually severe. This has led some observers to question whether Japanese MS is a different disease, and one obvious possibility is that it is related to HTLV-1-associated myelopathy (HAM), which is common in Japan. However, recent studies of sera and CSF samples from patients and controls retrospectively over the last 20 years have shown that HAM has not had a significant effect on the accurate diagnosis of MS in the Japanese epidemiological surveys (Shibasaki, 1989).

#### 1.1.2.2 Familial MS

That MS may develop in more than one member of a family was intimated in the earliest descriptions of the disease (Charcot, 1868; Gowers, 1893; Gowers, 1908). In 1903, McGowan described the disease in two brothers, in both of whom the diagnosis was confirmed at autopsy (McGowan, 1903). When all the available data were reviewed in 1950 (Mackay, 1950), it was found that there were 188 cases belonging to 84

families. It was shown that the commonest relationship between cases was sibship (70%), with parental (14%) and more distant (16%) relationships occurring less frequently. There were a small number of families containing more than two cases.

A number of studies of the prevalence of familial MS have been published over the last few decades. The earliest work was carried out by Curtius (Curtius, 1933) and Curtius and Speer (Curtius, Speer, 1937), who determined the prevalence of MS among 3129 relatives of 106 patients in Bonn and Heidelberg. They found secondary cases in 9.4% of the families, and calculated the prevalence in siblings of patients as 900/100,000 (42 times that of the general population). These early studies aroused great interest in the genetic epidemiology of MS and led to several other large studies of systematically collected data. The most important of these are summarized in Table 1. Estimates of the prevalence of familial MS vary depending on the method of case ascertainment, population-based studies being the most reliable. Particular care was taken in the British Columbia study (Sadovnick, McLeod, 1981), to avoid case ascertainment bias. Of 416 patients with definite MS, it was found that 73 (17.5%) had at least one relative with definite MS, and that an additional 21 (5%) had at least one relative with possible MS. Among first degree relatives 28 of 1179 siblings were affected (40 times the population prevalence), and 11 of 826 parents were affected (23 times the population prevalence). In a population-based survey in the Grampian region of Northeast Scotland, secondary cases were found in first degree relatives of 67 of 840 patients, the calculated prevalence in these relatives being 809/100,000 (4.5 times the population prevalence) (Francis et al.1987a).

Using data collected from many sources, it would appear that the overall incidence of a positive family history in patients with MS is approximately 15-20% (Spielman, Nathanson, 1982; Compston, 1986). The commonest relationship between affected individuals is sibship (31%); parent-child combinations occur in 20% of multiplex families, uncles/aunts share the disease in a further 22%, cousins in 23% and more distant relationships account for the remainder.

In their large study of MS in British Columbia, Sadovnick and McLeod have calculated empiric recurrence risks for first, second and third degree relatives. These figures are shown in Table 2, in which the first figure is the risk for definite MS, and the second is for definite and

**Table 1** Frequency of familial occurrence and affected relatives in MS.
(See text, page 21, for explanation)

| Author | Area | Prevalence per 100,000 population | Familial occurrence (%) | No. | % | Risk | No. | % | Risk |
|---|---|---|---|---|---|---|---|---|---|
| Curtius (1933) | Germany | 23 | 9.4 | 4/414 | 1.0 | 42.2 | 1/212 | 0.5 | 21.7 |
| Curtius & Speer (1937) | | | | | | | | | |
| Pratt et al. (1951) | London | 50 | 6.5 | 6/538 | 1.1 | 22.0 | 3/465 | 0.6 | 12.0 |
| Müller (1953) | Stockholm | 70 | 3.6 | 22/2815 | 0.8 | 12.0 | 5/1493 | 0.3 | 5.0 |
| Millar & Allison (1954) | N.Ireland | 58 | 6.6 | 34/2939 | 1.2 | 20.7 | 11/1336 | 0.8 | 13.8 |
| Sutherland (1956) | N.Scotland | 67 | 11-18.9 | 7/545 | 1.3 | 19.4 | 2/254 | 0.8 | 11.9 |
| Schapira et al. (1963) | NE.England | 50 | 5.8 | 25/2151 | 1.2 | 24.0 | 7/1206 | 0.6 | 12.0 |
| Mackay & Myrianthopoulos (1958, 1966) | USA/Canada | 50 | 22 | 14/236 | 5.9 | 118.0 | 2/115 | 1.7 | 34.0 |
| Roberts et al. (1979) | Orkneys | 153 | 11.6 | 1/164 | 0.6 | 3.9 | 2/94 | 2.1 | 13.7 |
| Sadovnick & McLeod (1981) | W.Canada | 57 | 17.5-22.5 | 28/1179 | 2.4 | 40.3 | 11/826 | 1.3 | 22.8 |
| Roberts & Bates (1982) | NE.England | 50 | 10.8 | 12/1290 1st degree relatives | | | | 0.9 | 18.0 |
| Francis et al. (1987) | NE.Scotland | 17.8 | 8 | 34/4200 1st degree relatives | | | | 0.8 | 4.5 |

**Table 2** Empiric risk figures for relatives of patients with MS. (Adapted from Sadovnick and McLeod, 1981)

| Relationship to index patient | Number | Risk (%) |
|---|---|---|
| Mothers | 413 | 1.5-2.4 |
| Fathers | 413 | 1.2-2.2 |
| Sons | 349 | 0.6-1.1 |
| Daughters | 318 | 0.3-0.9 |
| Brothers | 575 | 2.6-2.8 |
| Sisters | 604 | 2.0-2.8 |
| Aunts | 676 | 2.5-2.8 |
| Uncles | 738 | 0.5-0.9 |
| Nieces | 889 | 0.2-0.3 |
| Nephews | 764 | 0.1-0.1 |
| Grandsons | 46 | 0 |
| Granddaughters | 36 | 0 |
| First cousins | 2124 | 1.1-1.4 |
| **Total** | **7945** | **1.1-1.5** |

probable MS combined. There are no significant differences in risk between the relatives of male and female index patients.

Attempts to determine the mode of inheritance of MS susceptibility from family studies have not been successful. The first attempt at segregation analysis concluded that an autosomal recessive pattern was possible, but only if there is assumed to be reduced penetrance (Mackay, Myrianthopoulos, 1966). More recently, investigators have tried to fit family data to a polygenic model of inheritance. Roberts et al. (Roberts et al.1979) felt that a single locus model of inheritance could be excluded simply from inspection of their MS pedigrees, and instead favoured a polygenic model; however, they did not formally test this. In the one study in which a polygenic model was tested, it was rejected (Sadovnick et al.1981). In an analysis of 364 families, Sadovnick et al. concluded that the mode of observed inheritance was most closely satisfied by a single major locus, but also that some genetic heterogeneity was possible.

#### 1.1.2.3 Multiple sclerosis in twins

The observation of familial clustering again brings up the question of whether heredity or environment is responsible. Indeed, the further affected persons in such clusters diverge from the patterns of simple Mendelian inheritance the greater the chance that an environmental agent is the sole explanation for the clustering (Ford, 1978). By 1979, before unbiased twin study data were available (see below), it was an increasingly expressed opinion that in a complex aetiology, "...the genetic contribution (is) polygenic and possibly subordinate to the environmental..." (Roberts et al.1979). Some went even further, and Kurtzke wrote "My own view has been that a genetic factor is unnecessary to consider in seeking an explanation for the cause [of MS] (not that there cannot be one), which cause to me lies in the environment" (Kurtzke, 1977).

Studies of twins have been a classic way of distinguishing between environmental and inherited determinants of susceptibility to disease (Galton, 1875), and recent rigorous studies of MS in twin pairs have provided some of the most convincing evidence yet that there is a major genetic contribution to susceptibility. The first studies, by Thums (Thums, 1936), were of an unbiased series of twin pairs from a hospital series. He followed up 13 monozygotic (MZ) and 30 dizygotic (DZ) pairs, finding one concordant pair of MZ twins; all the DZ twins were discordant. Unfortunately, no evidence of the zygosity of the pairs was presented, and

experience has shown that errors can easily be made on the basis of appearance alone. In more recent studies, zygosity has been established by comparison of major and minor blood groups, fingerprinting and, most recently, "DNA fingerprinting" (Jeffreys et al.1985).

Mackay and Myrianthopoulos (Mackay, Myrianthopoulos, 1958; Mackay, Myrianthopoulos, 1966) collected 60 twin pairs with at least one twin affected with MS, through advertising, and followed this group up after 8 years. They found no difference in the concordance rate for MS between MZ and DZ twins (23.1% vs. 20.7%). The results of this and four later studies are summarized in Table 3 (Cendrowski, 1968; Bobowick et al.1978; Williams et al.1980; Currier, Eldridge, 1982). It can be seen that the data of Mackay and Myrianthopoulos are alone in failing to show an increased concordancy in MZ pairs. However, all of these studies are subject to bias resulting from the greater likelihood of identifying concordant than discordant pairs. This bias is reflected in the MZ:DZ ratios shown in Table 3. The expected MZ:DZ ratio in the general population is 1:2 (Thompson, Thompson, 1980), and assuming that twinning does not affect susceptibility to MS, a similar ratio should be expected for a population-based sample of twins.

The most important studies so far reported are those of Ebers and his colleagues in Canada (Ebers et al.1984; Ebers et al.1986), which have largely overcome the problems of pair ascertainment bias and zygosity determination associated with these earlier studies. A total of 5463 patients attending 10 MS clinics across Canada were surveyed. 27 MZ and 43 DZ twin pairs were identified, the MZ:DZ ratio being 1:1.60. Zygosity was determined by both erythrocyte antigen typing and questionnaire/subjective assessment, and the diagnosis of MS confirmed by examination and laboratory testing. The frequency of MZ pairs concordant for MS was 7/27 (25.9%) compared to only 1/43 (2.3%) DZ pairs, and 87/4582 (1.9%) non-twin siblings of patients at two MS clinics.

As discussed previously, evidence of demyelination may be revealed by MRI in asymptomatic individuals, including relatives of patients. In a systematic study of MRI in the unaffected members of twin pairs, Ebers has recently shown that inclusion of asymptomatic, but MRI-positive, pair members further increases the MZ concordance rate (see Table 4; G. Ebers, unpublished data).

A number of important conclusions can be drawn from these studies of MS in twins. Most significantly, they have been able to confirm

Table 3 Summary of important recent studies of MS in twins

| Author | No. of twin pairs identified | Monozygotic Twin Pairs | | Dizygotic Twin Pairs | | MZ:DZ Ratio |
|---|---|---|---|---|---|---|
| | | Total | % concordance | Total | % concordance | |
| Mackay & Myrianthopoulos | 68 | 39 | 23.1 | 29 | 20.7 | 1:0.74 |
| Cendrowski | 107 | 51 | 29.4 | 56 | 8.9 | 1:1.10 |
| Bobowick et al. | 9 | 5 | 20.0 | 4 | 0 | 1:0.80 |
| Williams et al. | 24 | 12 | 50.0 | 12 | 16.7 | 1:1.00 |
| Currier & Eldridge | 51 | 22 | 36.3 | 29 | 10.3 | 1:1.32 |
| Ebers et al. | 70 | 27 | 25.9 | 43 | 2.3 | 1:1.60 |

**Table 4**
Concordance rates for MZ and DZ twin pairs, showing the effect of including MRI-positive unaffected twin pair members
(from: G. Ebers, unpublished data, 1989)

| | Concordance (%) | |
|---|---|---|
| | Without MRI data | With MRI data |
| MZ pairs | 10/34 (30.3) | 13/34 (38.2) |
| DZ pairs | 1/46 (2.2) | 1/46 (2.2) |

beyond doubt that there is a major genetic component in susceptibility to MS. However, the observation that a majority of MZ twin pairs are discordant by all criteria, including MRI and neuropathological examination at autopsy, also serves to emphasize the importance of an environmental contribution. Most studies, including the Canadian ones, have demonstrated that the DZ concordance rate is not significantly greater than that for siblings, and thus it can be stated that an environmental effect is not affecting the DZ and sibling concordance rates differently. There is no current explanation for the observation that female twins are more likely than male to be concordant.

1.1.2.4 Associations with genetic markers

The most direct evidence for genetic control of susceptibility to MS is provided by associations between MS and polymorphic genetic markers. By far the most important of these is the Major Histocompatibility Complex, which lies on the short arm of human chromosome 6. The MHC is at present divided into three regions (Class I, Class II and Class III), and associations with MS have been established with each of these, although most significantly with the Class II region. Other polymorphic genetic systems have been examined, and there is some evidence of associations with immunoglobulin heavy chain allotypes (Gm), α-1-antitrypsin (Pi), red blood cell antigens (ABO system) and, most recently, T cell antigen receptor genes. The well known association of MS with gender, females being affected twice as frequently as males, provides further evidence of a genetic influence on disease expression. These associations will be reviewed in Chapters 1.3 and 1.4.

## 1.2 THE CLASS II REGION OF THE MHC

### 1.2.1 Position, size and number of genes; evolution and polymorphism

The ability to mount an antigen-specific immune response and to regulate this response at a cellular level is an important characteristic of all higher animals. This ability is mediated by cell-surface recognition systems that allow the distinction between self and non-self. A major histocompatibility gene complex which encodes such molecules was first identified in mice through the discovery of a genetic control of graft rejection, and is now known to exist in all vertebrates examined. The human Major Histocompatibility Complex (MHC) is located on the short arm of chromosome 6, and spans approximately 4000 kilobases of the genome. The MHC is conventionally divided into three regions, Class I, Class II and Class III, each of which encodes a different family of molecules. The organization of the MHC has recently become clearer as a result of large fragment mapping using pulsed field gel electrophoresis (Hardy et al.1986; Dunham et al.1987). The Class II region lies at the centromeric end of the complex and is approximately 1000 kb in size (see Figure 2). Telomeric to Class II is the Class III region, spanning approximately 1100 kb. It encodes a number of components of the complement pathways (C4A, C4B, 21-hydroxylase A and B, factor B and C2) as well as at least one Heat Shock Protein (HSP70), and Tumour Necrosis Factors (TNF). The Class I region is 1900 kb in size, and encodes the HLA-A, -B and -C genes (as well as the more recently identified loci HLA-E, -F and -G). Recent work has demonstrated that there are a great many further genes in the MHC still to be located and characterized; Campbell et al. have found evidence of at least 40 previously unknown loci between TNF and the Class I region (Proc. Natl. Acad. Sci. USA; September 1989, in press).

The Class II region of the human MHC consists of at least 14 loci clustered together. At least 9 of the loci are translated and expressed, and these encode either an α or a β chain. The Class II region has been divided into three subregions, the DR subregion composed of one A (α chain-encoding) and three B (β chain-encoding) genes (Spies et al.1985), the DQ subregion composed of two A and two B genes (Okada et al.1985a), and the DP subregion, which contains two A and two B genes (Trowsdale et al.1985; Okada et al.1985b). Two additional loci have been described, DOB and DNA (see Chapter 1.2.3 for current nomenclature). The DQ and DR α and β genes are oriented in a 'head-to-head' configuration, so that the

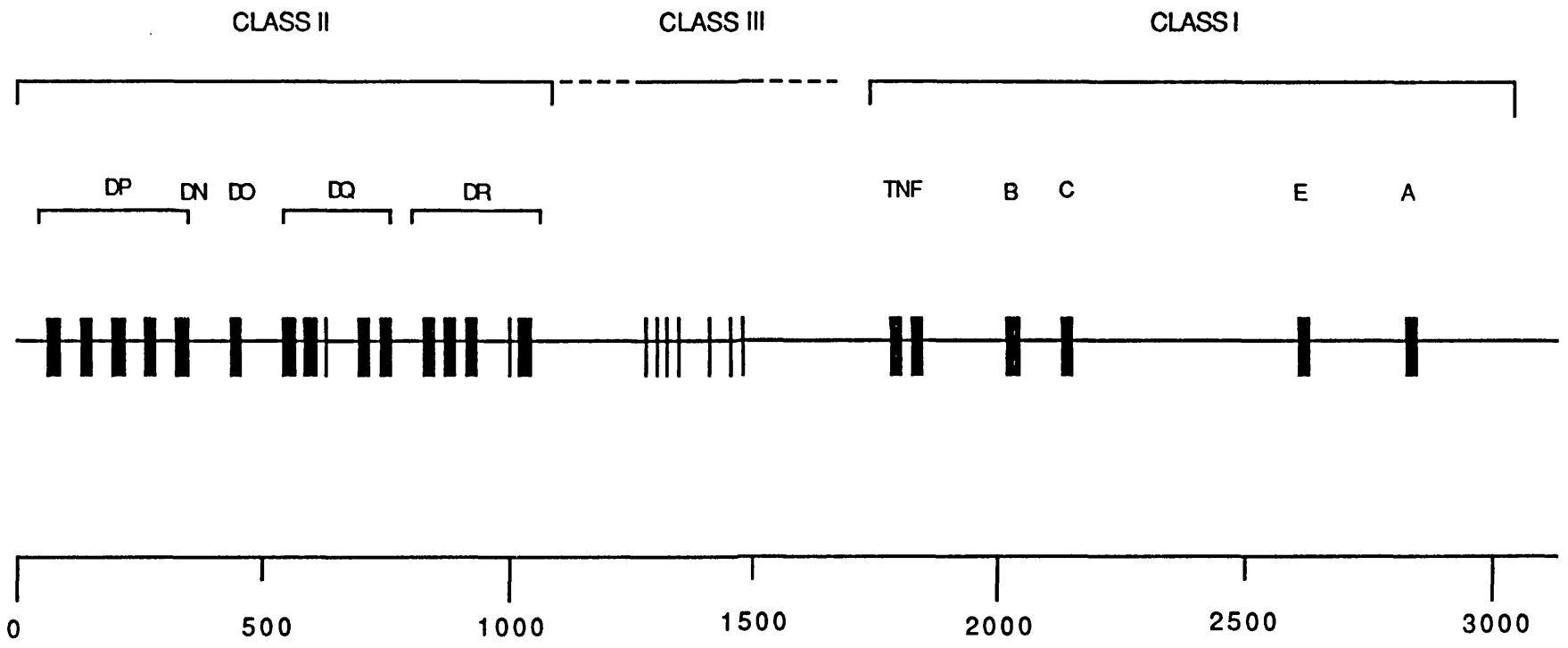



**Figure 2**

A physical map of the human Major Hispocompatibility Complex. The extent of the Class III region has not yet been fully determined, but it is known that there are many additional loci between the Class II and the Class III regions. (Adapted from Trowsdale and Campbell, Immunology Today (1988) 9(2):34-35)

transcription of a β gene starts at its centromeric end, whereas the transcription of an α gene starts at its telomeric end. The DP α and β genes are oriented 'tail-to-tail'. Figure 3 shows a map of the Class II region constructed using recent data from PFGE studies.

In common with other multigene complexes, the MHC has almost certainly evolved from a single primordial 'proto-gene', by successive gene duplication events. The complexity of the region has further been modified by the loss of certain loci, and by the mutation of other loci into inactive pseudogenes. Finally, the majority of the loci are polymorphic, encoding a number of different alleles. The nucleotide sequences of a large number of Class I and Class II alleles, both human and murine, have now been determined, and on the basis of these sequences an evolutionary scheme for the Class I and Class II regions can be postulated (reviewed in references (Klein, Figuero, 1985; Figuero, Klein, 1986)). Klein has proposed that a single ancestral Class II gene duplicated very early on in the evolutionary process (he estimates that this separation occurred more than 370 million years ago) and the two resulting loci became the ancestors of the α and β genes. The duplication may have occurred twice, the first time giving rise to DP ancestral genes and the second time to DN/DZ-DQ-DR ancestral genes. The former duplication arranged the β–α genes in a tail-to-tail relationship, and the latter arranged them in a head-to-head configuration. From then on, further evolution occurred by the duplication of the β–α modules and deletion of some genes from these modules. In man, the DP module duplicated once at a fairly early stage to produce the four contemporary DP genes. The DZ module either did not duplicate at all or the duplication was followed by a deletion of one locus. Duplication of the DQ module is thought to have occurred only fairly recently, accounting for the extremely high degree of homology observed between the DQ1 and DQ2 (DQ and DX) loci (Jonsson et al.1987; Auffray et al.1987). The present organization of the DR region suggests that there has been a double duplication of the DR module followed by the deletion of two α genes. Recent studies of the DQα gene in a variety of primate species indicate that the allelic diversity of this gene preceded the evolutionary split into humans, chimpanzees, and gorillas that occurred about 5 million years ago (Gyllensten, Erlich, 1988). A possible evolutionary tree for the Class II region is shown in Figure 4.

Several of these Class II genes exhibit considerable polymorphism, each locus having a large number of alleles (Korman et al.1985; Trowsdale

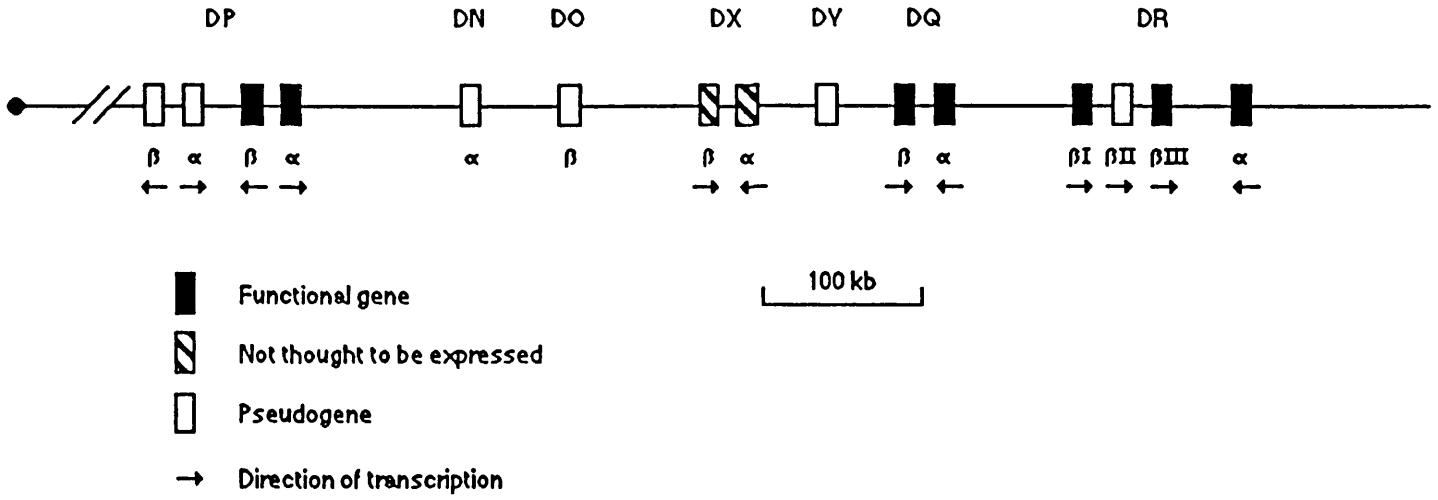



**Figure 3**

Scale map of the human Class II region showing the order of the known loci and approximate distances between them

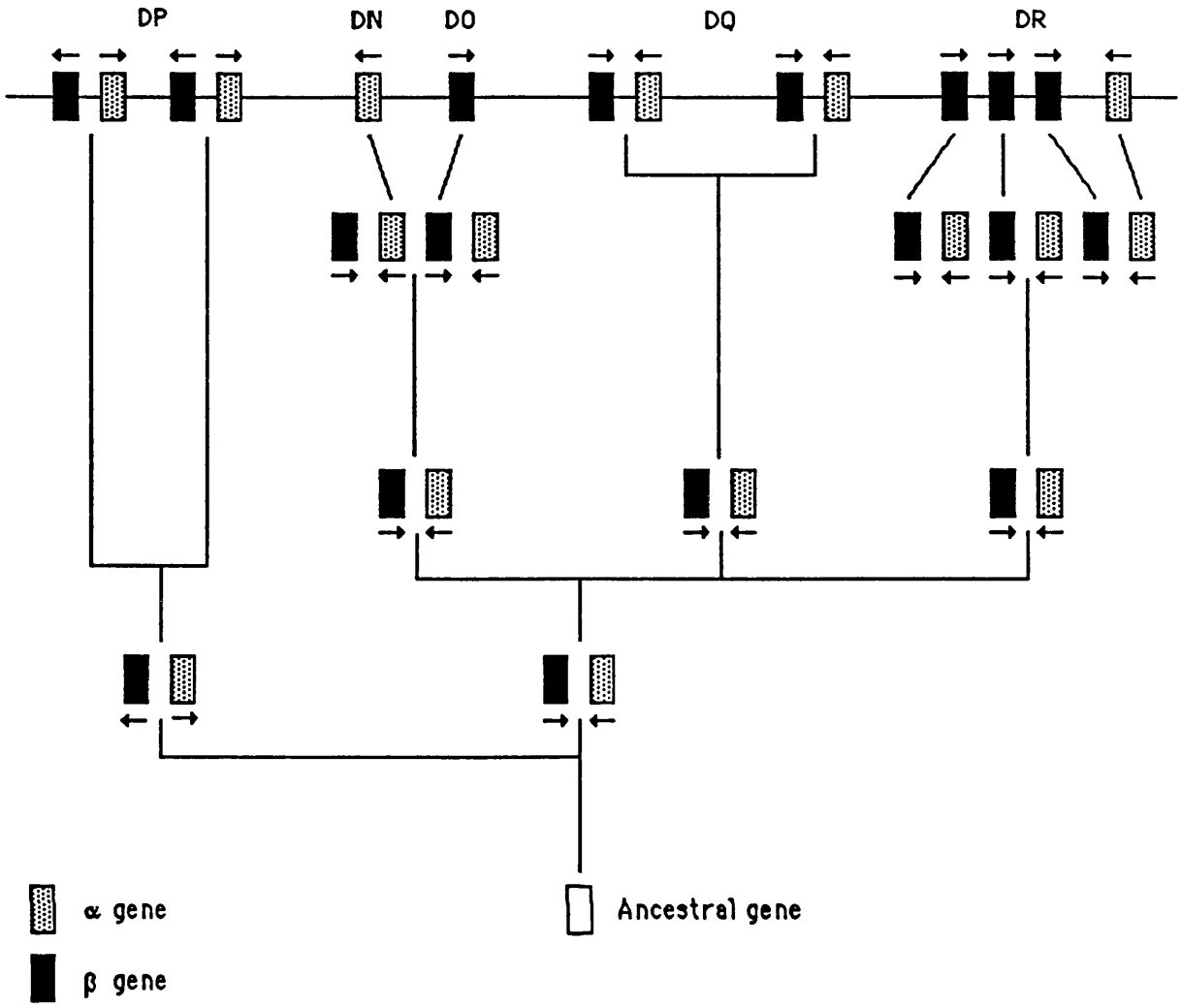



**Figure 4.**

Evolutionary tree showing the postulated evolution of Class II MHC genes. (adapted from J. Klein, Natural History of the Major Histocompatibility Complex, Wiley, New York, 1986)

et al.1985). In the DR region, DRBI is the most polymorphic locus. It is this gene which encodes the DR specificity detected by allospecific antisera. The DRBIII exhibits considerably less polymorphism, and encodes the minor DRw52 serospecificity and its cellularly defined subtypes Dw24, Dw25 and Dw26. On certain haplotypes DRBIII is not expressed and a fourth DRB gene encodes the DRw53 allele. DRBII is an inactive pseudogene. DRA codes for the α chain with which the β chain is co-expressed, is constant and exhibits no polymorphism (and this is also true for the equivalent murine locus Eα). In the DQ subregion, both the DQA1 and DQB1 genes display extensive genetic polymorphism. However, despite there being no obvious pseudogene criteria present in the DQA2 and DQB2 genes, it has not proved possible to demonstrate DQ2 gene transcription or an expressed DQ2 product. The reason for this is not yet clear, although it has recently been demonstrated that the DQA2 gene is accurately spliced when introduced into a retroviral vector, suggesting that its lack of expression is not due to aberrant splice signals (Auffray et al.1987). In the DP subregion only one pair of genes, DPA1 and DPB1, are expressed, the others being pseudogenes (Servenius et al.1984; Kappes et al.1984).

The Class II region genes are highly polymorphic. In both human and murine Class II genes, the genetic variability exists primarily in the α1 and β1 exons, which code for the membrane distal domains of the molecule. Furthermore, the derived amino acid sequences of a large number of murine and human genes reveal three regions of variability in the first domain in most cases. These variable regions are surrounded by regions of conserved sequence. The mechanisms are not fully understood whereby polymorphism has been generated in these areas while other regions have remained conserved. However, this presumably reflects the functional requirements of the α and β gene products to become paired in a consistent manner, and at the same time for the resulting dimer to restrict an immune response to a large number of antigens. From the recently elucidated 3-dimensional structure of an HLA heterodimer (see below), it can be seen that almost all the sequence polymorphism of both the A and B genes is located within regions encoding the antigen-binding groove of the expressed heterodimer. It is likely that this is a reflection of evolutionary pressure, enabling the species to combat a wide range of pathogens.

### 1.2.2 History and development of the Class II region

Whereas the HLA-A, -B and -C antigens encoded by the Class I region of the human MHC were identified and characterized initially using polyclonal alloantisera (Payne, 1957; Dausset, 1958; van Rood, van Leeuwen, 1963), the HLA-D region antigens were first detected using cellular techniques. The mixed lymphocyte reaction (MLR) was discovered by Bain (Bain et al.1963; Bain et al.1964) and later refined by Bach and Voynow (Bach, Voynow, 1966), who found that it was closely associated with the HLA type, and was thus genetically regulated. A separate locus was detected (Yunis et al.1971) and subsequently clarified and named HLA-D by the Sixth International Histocompatibility Workshop (Thorsby, Piazza, 1975). The cumbersome nature of the MLR made serological detection of the antigens of this series desirable. It was observed (Ceppellini et al.1971) that the MLR was inhibited by certain HLA typing sera, and that this blocking activity was not removed by absorption with platelets (Revillard et al.1972), which carry HLA-A, -B and -C antigens. The reaction of certain HLA-typing sera on lymphoid cell lines showed variations in relation to their known HLA-A, -B and -C antigens. It was realized that an antigen series distinct from HLA-A, -B and -C, and only present on a subset of normal lymphocytes, was being detected. Several groups (Jones et al.1975; Mann et al.1975; Winchester et al.1975b) proposed that this antigen series was the human equivalent of murine Ia expressed on B cells, and the correlation of the serological B cell alloantigens with MLR-defined antigens (Bodmer et al.1977) led to the definition of the D-related or HLA-DR antigen series (Bodmer, 1977).

As a result of biochemical, serological and cellular studies, further complexity in the Class II region became known. A series of cellularly defined specificities, strongly linked to DR and known as Dw, were detected. These cellular designations are not detectable serologically, and are assigned by reactions of bulk T cell cultures to allogeneic cell lines. The "w" designation has been maintained in order to distinguish these MLR-defined specificities from the DR series. It is now thought that Dw is a compound epitope to which both DRβ and DQβ chains contribute (Font et al.1986).

DC or MT (homologous to murine I-A) was first defined on the basis of serological cross reaction between DR1, DR2 and DRw6 cell lines (Tosi et al.1978). Biochemical studies later showed that MT epitopes were expressed independently of DR epitopes and that at least two structurally

distinct Class II moleculed are expressed on DR-homozygous cells (Karr et al.1982). A further region was identified by primed lymphocyte typing, and was initially known as SB ("secondary B cell antigens"). These loci were renamed DQ and DP following the 8th Workshop. The structural organization of the DR, DQ and DP subregions has been studied by many groups using RFLP, cosmid mapping and PFGE methods. These efforts have demonstrated that the DR subregion is composed of (on the majority of haplotypes) one α and three β genes (Spies et al.1985), the DQ subregion two α and two β genes (Okada et al.1985a), and the DP subregion two α and two β genes (Trowsdale et al.1984; Okada et al.1985b). The two most recently identified loci, DNA (previously DZα) and DOB (DOβ) were discovered from their unique nonpolymorphic restriction fragment patterns on Southern blots (Spielman et al.1984; Trowsdale, Kelly, 1985; Tonnelle et al.1985).

### 1.2.3 XIHWS nomenclature

The Tenth International Histocompatibility Workshop was convened in Princeton, NJ., in November 1987, and involved 362 laboratories. There were eight different collaborative research projects, and these centered around a core reference panel of 107 B-lymphoblastoid cell lines. In this way a comprehensive cross-analysis of data from each of the Workshop components was possible (DuPont, 1989a).

HLA Class II specificities were studied by 2 dimensional gel electrophoresis (2DGE), by alloreactivity of T cell clones to Class II epitopes, by monoclonal serology, and by RFLP analysis. In the 2DGE studies there were 19 DR β chain patterns associated with the DRBI locus and 5 additional patterns associated with a second expressed DRB gene. There were 10 DQ β chain patterns, 9 DQ α chain patterns, and 2 DP α chain patterns (Knowles, 1989). Seven new T-cell defined HLA-Dw specificities were recognized following the Workshop. The Dw20 - Dw23 specificities were identified as major stimulating determinants in primary MLC. The Dw24 - Dw26 specificities are related to DRw52. A further 34 T-cell defined specificities were detected, of which 18 were associated with DR and 16 with DQ (Flomenberg, 1989). Using monoclonal antibodies, 10 new Class II serological specificities were defined, including 4 new DR specificities, two splits of DRw5, a split of DQw2 and a variant of DRw53. The most extensive part of the Workshop was the RFLP analysis, by Southern blotting, of 70 of the B-cell lines (Marcadet et al.1989). A large number of

unique RFLPs could be correlated with the serologically defined Class II alleles with sufficient accuracy to form the basis of a highly specific DNA typing system of the Class II region (Simons et al.1989; Martell et al.1989). These data from the XIHWS will be further discussed with other non-Workshop data in Chapters 1.2.4 - 1.2.6.

Following the XIHWS, the Nomenclature Committee met to consider revisions and additions to the nomenclature of specificities defined in the Workshop. The naming of genes and alleles in the HLA-D region had previously been confused, and was greatly simplified. The agreed current nomenclature is shown in Table 5. Table 6 provides a summary of all the common HLA Class II haplotypes as determined from a synthesis of data from cellular, serological and RFLP studies. The studies reported in this thesis were conducted between February 1986 and April 1989, and thus span the XIHWS and the introduction of the new nomenclature arising from it. This new nomenclature (as shown in Table 5) will be used throughout the thesis, with the additional use of earlier names wherever these might be helpful in lessening confusion.

### 1.2.4 Definition of expressed products of the Class II region

The Class II region A and B genes encode α and β chain glycoproteins which are co-expressed at the cell surface. There are three available techniques which can be used to detect these gene products - serology, cellular typing methods and 2-dimensional gel electrophoresis of immunoprecipitated α and β chains.

Serological detection of the dimeric molecule is performed with either polyclonal antisera or with monoclonal antibodies, and monoclonal antibodies can also be raised against monomeric α or β chains. Antibodies to Class II antigens may be directed either against monomorphic determinants ("anti-framework"), or against polymorphic determinants which may be either public, shared by more than one allele, or private, in which case they may be allele-specific to varying degrees. There are now sufficient monoclonal antibodies available to type the majority of Class II antigens. Most polyclonal antisera contain more than one specificity, and give rise to a complex pattern of reactivity to a panel of homozygous cells. Typing is therefore carried out using a large number of antisera with overlapping specificities.

Many gene products of the Class II region can be detected by cellular typing methods. The most important of these has been the mixed

Table 5

TIHWS NOMENCLATURE FOR GENES IN THE HLA-D REGION

| Name | Previous equivalents | Molecular characteristics |
|---|---|---|
| HLA-E | E, "6.2" | Associated with Class I 6.2 kb HindIII fragment |
| HLA-DRA | DRα | DR α chain |
| HLA-DRB1 | DRβI, DR1B | DR β1 chain determining specificities DR1, DR3, DR4, DR5 etc |
| HLA-DRB2 | DRβII, DRB2 | Pseudogene with DRβ-like sequences |
| HLA-DRB3 | DRβIII, DR3B | DR β3 chain determining DRw52 and Dw24, Dw25, Dw26 specifities |
| HLA-DRB4 | DRβIV, DR4B | DR β4 chain determinig DRw53 |
| HLA-DQA1 | DQα1, DQ1A | DQ α chain as expressed |
| HLA-DQB1 | DQβ1, DQ1B | DQ β chain as expressed |
| HLA-DQA2 | DXα, DQ2A | DQ α chain related sequence, not expressed |
| HLA-DQB2 | DXβ, DQ2B | DQ β chain related sequence, not expressed |
| HLA-DOB | DOβ | DO β chain |
| HLA-DNA | DZα, DOα | DN α chain |
| HLA-DPA1 | DPα1, DP1A | DP α chain as expressed |
| HLA-DPB1 | DPβ1, DP1B | DP β chain as expressed |
| HLA-DPA2 | DPα2, DP2A | DP α chain related pseudogene |
| HLA-DPB2 | DPβ2, DP2B | DP β chain related pseudogene |

**Table 6** SUMMARY OF HLA CLASS II HAPLOTYPES (TIHWS).

| HLA DR | HLA-Dw | HLA-DRw52/53/Dw | HLA-DQ | Ethnic origin |
|---|---|---|---|---|
| DR1 | Dw1 | | DQw5 (DQw1) | |
| | Dw20 | | DQw5 (DQw1) | Jewish Black |
| DR2 | Dw2 | | DQw6 (DQw1) | |
| | Dw12 | | DQw6 (DQw1) | Japanese |
| | Dw21 | | DQw5 (DQw1) | |
| | Dw22 | | DQw7 (DQw3) | Warao Indian |
| DR3 | Dw3 | DRw52a, Dw24 | DQw2 | |
| | Dw3.1 | DRw52b, Dw25 | DQw2 | |
| | Dw new | DRw52a, Dw24 | DQw4 | Black |
| DR4 | Dw4.1 | DRw53 | DQw7 (DQw3) | |
| | Dw4.2 | DRw53 | DQw8 (DQw3) | |
| | Dw10 | DRw53 | DQw8 (DQw3) | Jewish |
| | Dw13 | DRw53 | DQw7 (DQw3) | |
| | Dw13 | DRw53 | DQw8 (DQw3) | |
| | Dw14 | DRw53 | DQw8 (DQw3) | |
| | Dw15 | DRw53 | DQw4 | Japanese |
| DR5 | Dw5 | DRw52b, Dw25 | DQw7 (DQw3) | |
| | DB2 | DRw52b, Dw25 | DQw1 | |
| | DB6 | DRw52b, Dw25 | DQw7 (DQw3) | |
| DRw6 | Dw18 | DRw52a, Dw24 | DQw6 (DQw1) | |
| | Dw18 | DRw52b, Dw25 | DQw1 | |
| | Dw19 | DRw52c, Dw26 | DQw6 (DQw1) | |
| | Dw9 | DRw52b, Dw25 | DQw5 (DQw1) | |
| | Dw16 | DRw52a, Dw24 | DQw7 (DQw3) | American Indian |
| DR7 | Dw7 | DRw53 | DQw2 | |
| | Dw17 | DRw53 | DQw2 | |
| | Dw11 | blank | DQw9 (DQw3) | |
| | Dw11 | DQw53 | DQw9 (DQw3) | |
| | DB1 | DRw53 | DQw2 | |
| DRw8 | Dw8.1 | | DQw4 | Caucasian |
| | Dw8.2 | | DQw4 | American Indian |
| | Dw8.3 | | DQw1 | Oriental |
| | Dw8.3 | | DQw7 (DQw3) | Dutch (LUY) |
| DR9 | Dw23 | DRw53 | DQw9 (DQw3) | Oriental |
| DRw10 | Dw new | | DQw5 (DQw1) | Jewish |
| DR Bon | Dw new | | DQw1 | |

lymphocyte culture (MLC), in which T cell alloreactivity in bulk culture results in blast cell transformation. The response is quantitated by assaying the incorporation of a label such as $^{3}$H-thymidine into the proliferating cells. One-way MLCs may be performed by first irradiating the stimulator cells, which lose their ability to transform whilst retaining their stimulatory capacity. For typing purposes, large numbers of homozygous cell lines (HTCs) have been developed in many different laboratories, and are widely available. Another important technique has been the secondary mixed lymphocyte culture, or primed lymphocyte test (PLT). The stimulus for secondary responses *in vitro* appears to be mainly HLA-DR, although Dw subtypes are also detectable, and as previously mentioned, products of the HLA-DP region were first identified in this way. The development of methods for producing and maintaining in culture individual clones of alloreactive T cells has led to the generation of cellular reagents which can be highly specific, in a manner analogous to monoclonal antibodies. It has been shown recently that, unlike monoclonal antibodies, alloreactive T cell clones can be capable of distinguishing between MHC molecules that differ only in their antigen-binding residues. It is currently thought that these allospecific T cells recognize MHC molecules complexed with a peptide derived from processed autologous MHC (Lombardi et al.1989). Clonal T cell recognition of Class II molecules was included for the first time in the XIHWS. The analysis of the reactivity of alloreactive T cell clones on the B cell reference panel demonstrated clear patterns of clustering which segregated with each of the serologically defined DR and DQ specificities. Within these large groups, a number of sub-clusters were seen. These studies resulted in a number of new splits, such as that of DRw52 into Dw24, Dw25 and Dw26 subtypes (Flomenberg, 1989).

### 1.2.5 Definition of the Class II region by DNA analysis

Molecular genetic methods have greatly increased the precision with which the complexity of the HLA-D region loci can be dissected. The techniques which are currently being employed include restriction fragment length polymorphism analysis, allele-specific oligonucleotide (ASO) hybridization, nucleotide sequencing and large fragment mapping by cosmid cloning and pulsed field gel electrophoresis (see (Trowsdale et al.1985) for review).

RFLP analysis is a powerful method of investigating the Class II region, but it is a relatively imprecise and indirect method for identifying

gene sequence variation. Restriction sites do occur within exons, but the great majority of RFLP cutting sites occur in non-coding regions, either introns or non-translated flanking sequences. This means that the relationship between coding sequence variation (correlating with an individual allele) and RFLP is a function of linkage disequilibrium between intron and flanking region sequences containing polymorphic restriction sites and exon sequences encoding allelic variability. Unlike serology, there are no "blank" alleles in Southern blot data. The gene dosage of a given allele (i.e. homo- or heterozygous) can often be estimated from the intensity of hybridization of the fragments detected. RFLP typing is likely to be incorporated as a standard method for histocompatibility testing and has already been in use in many laboratories for some time.

Although more specific than RFLP analysis, ASO hybridization and sequence analysis have not until recently been suitable for studying large numbers of subjects, as for instance in population studies of disease associations. However, with the development of a technique (PCR) for the rapid and economical enzymatic amplification of DNA or mRNA, these methods are now becoming more widely applicable (Saiki et al.1986).

### 1.2.6 Definition of HLA-DR2 and -DQw1

In 1975 several laboratories detected a B cell antigen in a high proportion of MS patients (see Chapter 1.3.1), and each of them gave that antigen a local name, i.e. Ag7a (Winchester et al.1975a), BT101 (Compston et al.1976), MS-B (Terasaki et al.1976). At the 7th Workshop in Oxford in 1977 it was determined that each laboratory was detecting the same gene product which then became known as HLA-DRw2. The "w" was dropped in 1980 and the MS-associated antigen became known as DR2. In 1986, Francis and his colleagues found that DQw1, which is in strong linkage disequilibrium with DR2, was more strongly associated with MS than was DR2, and thus might be a closer marker for susceptibility (Francis et al.1986b; Francis et al.1987b). Until then, population studies of HLA antigens had been carried out exclusively using polyclonal tissue typing antisera. However, data from monoclonal serological and molecular genetic studies had already demonstrated that there was a great deal of additional polymorphism in the DR and DQ subregions which could not be detected by routine serological techniques. To illustrate this, it can now be stated that whereas only three DQ specificities are detectable using 9th Workshop antisera (1984), at least 6 alleles can be detected by RFLP analysis

(Bidwell et al.1988) and nucleotide sequence analysis has so far revealed 8 allelic variants of DQα and 13 variants of DQβ (Horn et al.1988).

HLA-DR2, Dw2 and DQw1 are identifiable by a variety of methods and criteria. Serological definition of DR2 is well established, and has been in routine use in all tissue typing laboratories following the 8th Workshop (Trucco, Duquesnoy, 1986). Since then heterogeneity of DR2 has been demonstrated by serological, cellular and genetic methods. 9th Workshop antisera revealed that DR2 could be split into "long" and "short" (DR2S) variants (Claesson et al.1983; Freidel et al.1987), which have now been renamed DRw15 and DRw16 respectively. No further serologically defined heterogeneity of DR2 has been identified. However, the DR2 haplotype is now known to exhibit considerable polymorphism at both the DRB and DQB loci (and probably also DQA), and a number of HTC-defined specificities (Dw subtypes) associated with DR2 have been found. The DRw15, DR2S ("short") serological type includes Dw2 and Dw12. The DRw16, DR2L ("long") serotype includes a number of less common specificities including AZH, FJO, REM, LD-MN2, UCA, LD-5a and DB9 (Lee et al.1987; Yabe et al.1987). Following the XIHWS, the DRw16 type has been split into Dw21 and Dw22, being found in linkage with respectively DQw5 and DQw7 (XIHWS Newsletter, vol.2, no. 1, p.38). The molecular basis for these Dw subtypes has recently been elucidated by various groups. Analysis of Dw2, Dw12 and non-Dw2/non-Dw12 HCLs by 2DGE, using the anti-DRβ monomorphic monoclonal antibody SG171, showed that in each case 2 DRB genes were expressed (Lee et al.1987). One of these DRβ chains was non-polymorphic, but the second differed between each subtype. This is a similar situation to that of the supertypic families DRw52 and DRw53, in which one of the expressed DRβ chains is also conserved between haplotypes (Gorski, Mach, 1986). Yabe et al. (Yabe et al.1987), using the anti-DR framework monoclonal antibody L243, found that the Dw21 and Dw22 cell lines differed from Dw2 and Dw12 cell lines in that they expressed three DRβ chains, two of which differed from those expressed on the Dw2 and Dw12 cell lines. They also showed that the electrophoretic mobilities of the DQβ chains are different for Dw2, Dw12 and non-Dw2/non-Dw12 cell lines. Differences in the expressed DQα chains between these cell lines have also been demonstrated (Nakatsuji et al.1986).

DR2 and its Dw subtypes can readily be distinguished by RFLP data. Many different groups have utilized a variety of different DRB-, DQB- and DQA-hybridizing fragments in their studies of the DR2 haplotype genes.

The large number of different probes in use in different laboratories, the different enzyme/probe combinations used, and inaccuracies in the measurements of fragment sizes, all led to considerable confusion in the literature. Furthermore, the inaccurate assignment of specificities to fragments resulted in erroneous conclusions being drawn from population studies of diseases. An example of this is the study by Cohen (Cohen et al.1984a), in which it was found that an EcoR1-DQB 2.2 kb restriction fragment was associated with MS independently of DR2. However, it was later established that this fragment is characteristic of the Dw2 subtype, and that this was under-represented in the control sample in the study. In a study of Japanese narcoleptics, this same fragment was used to identify the presence of a Dw2 haplotype, uncommon in that population, in the patients, but the fragment was on this occasion measured as 2.4 kb (Inoko et al.1986). A number of other studies also attempted to define DR2 haplotypes by RFLP geno-allotyping but a universally recognized typing system did not at first emerge (Cohen et al.1984b; Segall et al.1986; Singal et al.1986; Cohen et al.1986; Jacobson et al.1986; Trucco, Duquesnoy, 1986). One of the more successful attempts to establish a common typing system was described by Bidwell and his colleagues (Bidwell et al.1988). Using just a single restriction endonuclease (Taq1) with three cDNA probes (DQA, DQB and DRB), he was able to positively identify the majority of common HLA-DR alleles, as well as the three major members of the DQ allelic series and certain splits of DR2, DRw6 and DR7. The most important attempt yet to standardize allogenotyping has been the RFLP component of the XIHWS. A large number of unique restriction fragments have been correlated with the DR and DQ alleles defined by serological and other methods. Fragments which are uniquely characteristic of DR2 haplotype genes are shown in Table 7.

The most recent developments in the definition of DR2 have been the use of allele-specific amplification by the polymerase chain reaction (Saiki et al.1986), followed by either oligonucleotide hybridization (Angelini et al.1986) or nucleotide sequence analysis (Horn et al.1988), as mentioned above. Allele-specific oligonucleotide (ASO) hybridization is a simple and rapid technique which enables DR and DQ alleles to be identified with considerable accuracy, and recently this method has been applied to disease studies (Vartdal et al.1989).

| | Enzyme | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| HLA | ECV | BAM | BGL | ECI | HID | MSP | PVU | SST | TAQ | KPN | PST |
| DR2 | | | RB1455 | RB628 | RB332 | | RB447 | RB1319 | | | |
| DR2.Dw2 | | | | QB220 | | | | | | | |
| DR15.Dw2 | | | | | | RB381 | | RB615 | RB142 | | |
| DR15.Dw12 | | | | | | | | | RB212 | | |
| DRw15.Dw2 | | | | | | | | | RB123 | | |
| DRw15.Dw12 | | | | | | | | | | | |
| DRw16.Dw21 | | | | | | | | RB1030 | RB169 | | |
| DRw16.Dw22 | | | QA382 | | | | | | | | |
| DQw1 | | | | QB383 | QB644 | QA287 | | | | QB515 | |
| DQw5 | QB262 | QB576 | | | | | | | QA245 | QA1041 | QB136 |
| DQw6 | QB524 | | QB420 | QB226 | | | | | | | |

**Table 7** Unique fragments which define DR2 and DQw1 and their subtypes, as identified in the XIHWS. The column entries show the probe and the fragment size, for instance QB576 indicates a 5.76 kb DQB-hybridizing fragment. The assignment of BGL QB420 to DQw6 (previously DQw1.2) was made by A.Bushell (personal communication).

### 1.2.7 Organization of the Class II genes

A schematic diagram of the organization of typical Class II A and B genes (still known as α and β genes in the mouse) is shown in Figure 5. Apart from minor differences at the 3' end, all human and murine genes have a similar intron/exon structure. The α genes comprise 5 exons, including a 5'UT region and leader sequence, α1 and α2 domain exons, an exon encoding the connecting peptide, transmembrane region (TM) and cytoplasmic portion (CY) together with part of the 3'UT region, and a fifth exon containing the remainder of the 3'UT region. The DQA allelic gene products differ widely in the length of their mRNA molecules and their 3'UT regions. This is probably the result of alternative splicing. The clone pDCH1 has a 3'UT region of 130 bp in length (Auffray et al.1984) while that of p-II-α-5 is about 500 bp long (Schenning et al.1984). Other minor variations in the Class II α-chain genes include the position of the stop codon, which is on exon 5 in DNA (DZα) (Trowsdale, Kelly, 1985). The β genes share the common feature of at least one extra intron in the region from the connecting peptide to the C terminus and, in this respect, are more similar to the Class I genes than the Class II α chain genes.

### 1.2.8 Structure of the Class II molecule

All Class II antigens are heterodimers consisting of an α chain of 33 to 35 kD and a β chain of 26 to 29 kD. These chains are tightly but non-covalently associated. Each chain consists of four domains, a membrane distal (N-terminal) domain, a membrane proximal domain, a connecting peptide and transmembrane region, and a small intracytoplasmic domain. The N-terminal domains (α1 and β1) consist of between 85 and 95 aminoacids, are analogous to immunoglobulin variable regions, and are in most cases highly polymorphic. The membrane proximal domains (α2 and β2) contain approximately 95 aminoacids and are analogous to the constant regions of immunoglobulins (see (Korman et al.1985) for review).

Class II antigens undergo several post-translational modifications. All α chains possess two carbohydrate moieties: one containing a high proportion of mannose and the other a complex oligosaccharide. All β chains contain one complex carbohydrate at position 19 (Shackelford, Strominger, 1983). The carbohydrate does not influence the surface expression or the binding of alloantisera to DR molecules.

Class II antigens are associated with a third polypeptide of 31 kD known as γ or invariant chain. Both α and β chains interact with invariant

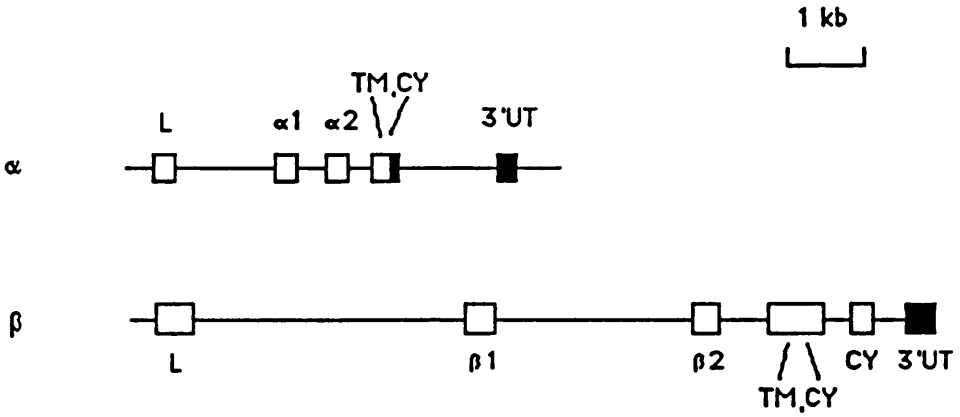



**Figure 5.**
Exon-intron organization of human class II genes. The open boxes represent translated sequence and the solid boxes untranslated sequence.

chains during biosynthesis prior to formation of the heterodimer (Kvist et al.1982). The invariant chain remains associated with the Class II dimer until its appearance at the cell surface. The invariant chain is a transmembrane protein which lacks a signal sequence. The location of the hydrophobic region and two N-glycosylation sites suggest that its N-terminus may be intracellular and its C-terminus extracellular (Claesson et al.1983). It has long been thought likely that the invariant chain is involved in the assembly and intracellular transport of α and β chains to the cell surface, and recently it has been shown that it may play an important rôle in antigen processing (Stockinger, 1989).

Recently the three dimensional structure of the human Class I antigen HLA-A2 has been determined from X ray crystallographic analysis (Bjorkman et al.1987a; Bjorkman et al.1987b). The membrane proximal domains (α3 and β2-microglobulin) are closely associated and support the membrane distal portion of the glycoprotein (α2 and α1), which forms a platform of eight antiparallel strands which together form a β-pleated sheet topped by two α-helices. This arrangement has been descriptively likened to two sausages lying on a barbecue grid. A large groove between the α-helices creates a binding site for processed antigen fragments. The size of the groove is approximately 25 Å long, 10 Å wide and 11 Å deep, enabling it to accommodate peptides about 8-20 aminoacids in length, depending on their secondary structure. Attempts to crystallize and determine the structure of a Class II molecule have not yet been successfully completed. However, great similarity in structure between Class I and Class II antigens is suggested by (i) their similar genomic organization (Kaufman et al.1984), (ii) their close equivalence in the C-terminal domain sequences, (iii) the observation that some T cells specific for either class of molecule use the same receptor (Rupp 1985, Marrack 1986), and (iv) a large body of structure/function analyses (Braunstein, Germain 1986). A four-domain model of a Class II molecule can easily be deduced by imagining that the Class I α1 domain and β2-microglobulin are joined to one another and that the α1 and α2 domains are non-contiguous (Bjorkman et al.1987a). Like a Class I molecule, the hypothetical Class II molecule formed in this way would have a cleft between the C-terminal α-helices of its polymorphic α1 and β1 domains, with the floor of the cleft being formed by the N-terminal β-pleated sheets of each domain (Bjorkman et al.1987b). The distribution of polymorphic residues in both α and β regions of Class II sequences closely matches those in Class I

sequences, enabling a large number of sequences of both classes to be aligned. On the basis of such an alignment, a hypothetical structure of the antigen binding site of a Class II molecule has been proposed (Brown et al.1988). From this it becomes obvious that antigen recognition by Ia-restricted T cells depends upon allele-specific contributions from both the α and the β chains, and that each α-β combination will function as a unique restriction element. This conclusion accords well with predictions made from a large number of earlier functional studies of transfected α and β genes (reviewed by (Lechler, 1988)).

### 1.2.9 Functions of Class II molecules

It has become clear that the fundamental property of HLA molecules is their ability to bind peptide fragments and present these to antigen receptors on the surface of T cells. The most important immunological functions of Class II molecules derive from this property, and are (i) co-recognition with foreign antigen by CD4+ve T cells; (ii) determination of immune response phenotype to a large number of antigens; (iii) stimulation of auto- and allo-mixed lymphocyte reactions, and MHC Class II incompatible graft rejection; and (iv) ontogenetic selection in the thymus of the repertoire of mature CD4+ve T cells (reviewed by (Paul, 1984)). It also appears necessary that the Class II molecule subserves a transmembrane signalling function, but there is little direct evidence for this at present. These functions account for the pivotal rôle played by MHC Class II molecules in T cell dependent immune responses. This raises the possibility that Class II alleles might be true susceptibility genes for autoimmune diseases, and has provided a major impetus for studies of Class II genes in autoimmunity.

### 1.2.10 Class II genes and autoimmunity

Recent advances in understanding the restriction by MHC molecules of $T_H$ cell responses to foreign and allo-antigens have provided a theoretical basis for equivalent MHC restriction in the case of auto-antigens. Present concepts of the rôle that MHC Class II antigens are likely to play in autoimmune diseases have been derived from three main areas of study. Firstly, a great many diseases have been found to be associated with HLA alleles, but it is significant that these associations are especially strong in the case of Class II antigens and diseases with a definite or likely autoimmune pathogenesis (Tiwari, Terasaki, 1985). Whilst the majority of

these data have associated disease susceptibility with individual alleles, some studies have raised the possibility that it is the combination of alleles on a given extended haplotype which may be important, and there are also instances where risk is further increased by heterozygosity (see below). Secondly, the analysis of human and murine Class II gene sequences have led to the identification of nucleotide substitutions which are tightly linked to susceptibility and resistance to disease (Todd et al.1988a). Lastly, data from experimental animal models of autoimmune diseases have confirmed that Class II antigens can alone determine susceptibility and resistance to the disease in particular strains (Reich et al.1989).

The first recorded associations of human disease with an HLA antigen were those of Hodgkin's lymphoma with a cross-reactive group of antigens, HLA-B5, B35, B15 and B18 (Amiel, 1967), and acute lymphoblastic leukaemia with HLA-A2 (Walford et al.1970). Although these particular associations have not since been consistently observed, the extremely strong association between ankylosing spondylitis and HLA-B27 was discovered soon afterwards (Brewerton et al.1973; Schlosstein et al.1973). This proved a powerful early impetus for further research into the disease associations of both Class I and Class II antigens, and there is now an enormous literature describing these studies. The known HLA disease associations have been comprehensively reviewed by Tiwari and Terasaki (Tiwari, Terasaki, 1985). Some of the more important associations of disease with Class II antigens are shown in Table 8, and the published data on the Class II associations of MS are reviewed in more detail in Chapter 1.3. The HLA-associated diseases can be divided into two broad groups: (i) those involving probable immune (including autoimmune) mechanisms, and (ii) those thought not to involve immune mechanisms. It can be seen that almost without exception, the strongest Class II associations are with autoimmune diseases. In a few cases, there is no evidence of an immunological process, and yet the strength of the association argues strongly in favour of the HLA antigen itself being the risk factor for disease occurrence, and of it being intimately involved in the pathogenetic process. This has led to speculation that Class II antigens may possess important non-immune functions as well, for instance as differentiation antigens during embryogenesis (R. Heard, Cairns Society Lecture, September 1988). The most notable example of this is narcolepsy, which is associated with DR2 in virtually 100% of patients (Juji et al.1984).

**Table 8**
A summary of the more important HLA-DR and disease associations. Diseases which are thought to have an autoimmune pathogenesis are marked with an asterisk *. Adapted from Tiwari & Terasaki, 1985.

| Disease | Race | N | Patients | | Controls | RR |
|---|---|---|---|---|---|---|
| | | | No. | %+ | %+ | |
| *Rheumatoid Arthritis | | | | | | |
| DR4 | C | 17 | 1127 | 68 | 25 | 2.0 |
| DR4 | O | 5 | 348 | 66 | 39 | 2.8 |
| DR4 | N | 3 | 109 | 40 | 10 | 5.4 |
| *Juvenile rheumatoid arthritis | | | | | | |
| DR5 | C | 5 | 422 | 34 | 15 | 3.3 |
| *IDDM | | | | | | |
| DR3 | C | 13 | 1174 | 46 | 22 | 3.3 |
| DR4 | C | 12 | 1051 | 51 | 25 | 3.6 |
| DR3 | O | 4 | 139 | 38 | 14 | 4.8 |
| DR4 | O | 4 | 139 | 49 | 25 | 2.6 |
| DR3 | N | 3 | 135 | 57 | 28 | 3.2 |
| DR4 | N | 3 | 135 | 46 | 11 | 6.7 |
| *Coeliac disease | | | | | | |
| DR3 | C | 5 | 194 | 79 | 22 | 11.6 |
| DR7 | C | 4 | 137 | 60 | 15 | 7.7 |
| *Dermatitis herpetiformis | | | | | | |
| DR3 | C | 4 | 126 | 82 | 20 | 17.3 |
| *Psoriasis vulgaris | | | | | | |
| DR7 | C | 5 | 296 | 48 | 23 | 3.2 |
| DR7 | O | 2 | 148 | 10 | 1 | 7.6 |
| *Pemphigus vulgaris | | | | | | |
| DR4 | CJ | 3 | 62 | 91 | 32 | 14.6 |
| *Goodpasture's syndrome | | | | | | |
| DR2 | C | 2 | 25 | 88 | 27 | 13.8 |
| *Multiple sclerosis | | | | | | |
| DR2 | C | 13 | 1051 | 51 | 27 | 2.7 |
| Narcolepsy | | | | | | |
| DR2 | C | 2 | 45 | 100 | 22 | 129.8 |
| DR2 | O | 1 | 92 | 100 | 34 | 358.1 |

N = no. of studies; No. = no. of patients; C = Caucasian; O = Oriental; N = Negro; J = Jewish; %+ = percent positive; RR = Relative Risk

Insulin dependent diabetes mellitus (IDDM) is caused by the autoimmune destruction of insulin-producing β-cells in the pancreatic islets. There is a 50% concordance rate among monozygotic twins, similar to that seen in MS, and there is strong evidence to indicate that both genetic and environmental factors are necessary to produce the disease. Although the disease is polygenic in inheritance, it has been estimated that the HLA-D region contributes over 50% of the heritability. 95% of Caucasoid patients possess either HLA-DR3 or DR4, compared to 45% - 54% of healthy individuals. However, the individual risks of DR3 and DR4 are not just additive, and the risk of developing IDDM is further increased in DR3/4 heterozygotes (Wolf et al.1983). Deschamps, in a large study of IDDM patients and controls (Deschamps et al.1988), has investigated the distortions in the expected gene frequencies on extended haplotypes, for both *cis* (same haplotype) and *trans* (opposite haplotype) loci. It was found that in the trans situation there was a large excess of DR3/4 heterozygotes (RR=51, $p<10^{-6}$), and a deficit of DRx/x (RR=0.12, $p<10^{-6}$). DR3 and DR4 homozygosity did not differ between patients and controls. More recently, a similar increased risk has been demonstrated in DR4/DRw8 heterozygotes (Rønningen et al.1989). Convincing data for similar transcomplementation in other diseases is sparse. However, a two-haplotype effect is also implicated in coeliac disease, which is associated with DR3 and DR7, and data will be presented in this thesis which raise the possibility that transcomplementation also operates to influence susceptibility to MS.

One of the most interesting ways in which transcomplementation can occur is by the expression of hybrid Class II dimers, consisting of α and β chains encoded by different haplotypes. These hybrids have been detected on a number of occasions, and can function effectively in antigen presentation (Kimoto, Fathman, 1980; Charron et al.1984; Giles et al.1985; Kolstad et al.1989). This is an important observation in that it provides an explanation of transcomplementation at a cellular level. Of equal interest is the possibility of cross-isotype α-β pairing. Isotype-mismatched heterodimers were first reported in the mouse after transfection of Class II genes (Germain & Quill 1986; Germain & Malissen 1986; Malissen et al. 1986). Further transfection studies established the rules governing the permissiveness of various interspecies α-β combinations (Lechler 1990, in press). The first evidence that a human isotype-mismatched heterodimer could be expressed naturally came from immunoprecipitation studies

with the DR2 cell line PGF. Using anti-DR and anti-DQ monoclonal antibodies, Lotteau et al. demonstrated the presence of the hybrid molecule DRα-DQβ (Lotteau et al.1987). The significance of mixed isotype pairing in human cells is two-fold. Firstly, since it has been established that aberrant pairing is highly influenced by the allelic polymorphism of the I-Aβ chain, similarly DQβ chain polymorphism could influence the pairing with the monomorphic DRα chain. This in turn would indicate a greater or lesser potential for aberrant pairing depending on the haplotype. Secondly, in permissive haplotypes, mixed isotype heterodimers could be the result of unfavourable pairing occurring after the potential for favoured isotype-matched association was exhausted. One very interesting situation in which a mixed isotype Ia molecule is incriminated occurs in experimental allergic encephalomyelitis (EAE) in the Lewis rat. After inducing EAE by immunization with the encephalitogenic peptide MBP 68-88, encephalitogenic T cells can be recovered and used to passively transfer the disease to a second animal. Although these T cells are CD4+ and Class II-restricted, they can be completely blocked by both anti-I-A and anti-I-E monoclonal antibodies (Offner et al., Cell. Immunol. 1986, 100:364), thus suggesting these T cells may be recognizing a hybrid Ia molecule. It remains an interesting but unexplored possibility that the amount of aberrant pairing may vary during the upregulation of Class II expression by lymphokines. A further theoretical possibility is isotype-mismatched, trans-haplotype α-β pairing, although only DQβ-DRα, and its murine homologue Aβ-Eα, have been documented.

The most direct evidence yet for autoimmune disease susceptibility mapping to the HLA D-region has come from sequencing DQ genes in IDDM. Todd et al. cloned and sequenced the gene segments encoding the first domains of DRβ, DQα and DQβ chains from a number of common haplotypes (Todd et al.1987). The DR4/DQw8 haplotype confers the highest risk, irrespective of racial group, followed closely by the DR3/DQw2 haplotype. Two other haplotypes are also positively associated with IDDM, DR1/DQw5 and DR2/DQw5/DwAZH. Haplotypes which are associated with negative risk are DR4/DQw7, DR5/DQw7 and particularly DR2/DQw6. Other haplotypes are neutral and confer no risk. When the translated DQβ sequences from 17 haplotypes were examined it was found that in almost every case, disease risk was associated with the presence of a non-charged amino acid at position 57, whereas haplotypes associated with neutral or negative risk encoded aspartate at that position (Asp-57).

Remarkably this association extends to the NOD mouse, which possesses an unique Aβ chain in having a serine at position 57, other non-diabetic strains having Asp-57 (see Table 9). Since the NOD mouse does not express I-E, these data suggest that in this model, it is the Aβ chain which is responsible for the autoimmune process (Todd et al.1988a). (It should be noted however that in the Bio-Breeding (BB) rat, Class II β-chains from both IDDM-sensitive and -resistant strains have identical first domain sequences with Ser at position 57, and this cannot therefore be regarded as a universal rule in all species).

The strength of the DQβ-IDDM correlation in humans raises the possibility that IDDM autoreactive T cells are recognizing an islet-cell antigen in association with a limited number of DQ molecules (particularly DQw8 and DQw2). From the predicted structure of a Class II molecule discussed earlier (Brown et al.1988) it appears that amino acid 57 is directed inwards to the antigen-binding cleft. It is a further possibility that Asp-57 may form a salt bridge with a conserved Arg residue at position 79 of the DQα chain, occluding one end of the cleft. It therefore seems reasonable to predict that an Ala-Asp substitution at position 57 might alter the structure of the DQαβ dimer in such a way as to affect antigen binding and/or T cell recognition.

Despite this compelling evidence, a number of observations challenge this simple position-57 model of susceptibility to IDDM. Certain haplotypes, particularly DR7/DQw2 in caucasoids, confer neutral or negative risk despite having a neutral residue (Ala) at position 57; and others with Asp-57 which seem to be disease-associated (for instance DR4 and DRw9 in Japanese; H. Erlich, unpublished observations). Another problem is how to explain the increased risk of DR3/4 individuals relative to those who are DR3/3 and DR4/4, as they are all Asp-57 negative homozygotes. However, there is recent evidence that the neutral risk of the Asp-57 negative DR7 haplotype is a function of the DQα chain which, in contrast to the DQβ chain, differs between DR3/DQw2 and DR7/DQw2 haplotypes. By studying the association with IDDM of rare haplotypes in black subjects, Todd et al. have demonstrated that both DQα and DQβ chains (encoded by DQA1 and DQB1) influence susceptibility (Todd et al.1989).

As has been stated, the NOD mouse strain expresses I-A but not I-E Class II molecules due to a deletion in the I-Eα promoter (Acha-Orbea, McDevitt, 1987). In order to investigate whether this abnormality may be

| DR | DQ | IDDM association | 3 | 9 | 13 | 14 | 26 | 28 | 30 | 37 | 38 | 45 | 46 | 47 | 52 | 53 | 55 | 56 | 57 | 66 | 67 | 70 | 71 | 74 | 75 | 77 | 84 | 85 | 86 | 87 | 89 | 90 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DR4 | DQw8 | Positive | S | Y | G | M | L | T | Y | Y | A | G | V | Y | P | L | P | P | A | E | V | R | T | E | L | T | Q | L | E | L | T | T |
| DR3 | DQw2 | Positive | - | - | - | - | - | S | S | I | V | - | E | F | L | - | L | - | - | D | L | - | K | A | V | R | - | - | - | - | - | - |
| DR1 | DQw5 | Positive | - | - | - | L | G | - | H | - | V | - | - | - | - | Q | R | - | V | - | - | G | A | S | V | R | E | V | A | Y | G | I |
| DR2 | DQw5 | Positive | - | - | - | L | G | - | H | - | V | - | - | - | - | Q | R | - | S | - | - | G | A | S | V | R | E | V | A | Y | G | I |
| DR4 | DQw7 | Neutral | - | - | A | - | Y | - | - | - | - | E | - | - | - | - | - | - | D | - | - | - | - | - | - | - | - | - | - | - | - | - |
| DR5 | DQw7 | Neutral | - | - | A | - | Y | - | - | - | - | E | - | - | - | - | - | - | D | - | - | - | - | - | - | - | - | - | - | - | - | - |
| DR2 | DQw6 | Negative | - | F | - | - | - | - | - | - | - | - | - | - | - | Q | R | - | D | - | - | G | - | - | - | - | E | V | A | F | G | I |
| | Mouse | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| NOD | Aβ NOD | Positive | - | H | - | E | - | - | - | - | L | - | E | - | E | - | R | H | S | Y | * | - | - | - | - | - | E | E | T | E | P | - |
| B10 | Aβ (b) | Negative | - | Y | - | E | Y | - | - | - | V | - | E | H | E | - | R | - | D | E | I | - | - | - | - | - | E | G | P | E | H | - |

**Table 9** The association of IDDM with DQβ polymorphic residues. (From Todd et al., Nature, 1987).

responsible for the development of autoimmune insulitis, an I-E positive NOD strain was made by the insertion of an I-Eα transgene into the germline (Nishimoto et al.1987). It was found that the introduction of I-E protected mice from insulitis and diabetes. This could be explained either by the altered expression of Class II on the surface of the target tissue or by a change in the thymically selected T cell repertoire. Reich et al. investigated these possibilities by cloning CD4+ve and CD8+ve T cells from the islets of recently diabetic NOD mice (Reich et al.1989). These clones were islet-specific and pathogenic in both I-E+ve and I-E-ve strains. It was found that 4/4 CD4+ve and 7/13 CD8+ve clones expressed TcR that had β-chain variable regions encoded by Vβ5. Recently, several studies have shown that I-E molecules are associated with ontogenetic deletion of T cells carrying T cell receptors encoded in part by certain TcR Vβ gene segments (Kappler et al.1987; Kappler et al.1988; MacDonald et al.1988; Bill et al.1989), and in particular it has been shown that T cells expressing Vβ5 are deleted in I-E expressing mice (Bill et al.1988). Furthermore, it could be shown that NOD mice express Vβ5 on 5% of their T cells, whereas (NOD x BALB/c)$F_1$ mice express Vβ5 on <1% of T cells. Thus, the development of diabetes in I-E negative NOD mice can be explained by the persistence of autoreactive Vβ5+ve T cells.

By extending this argument to human IDDM, these data raise the possibility that the protective effect of DR2/DQw6 may also be due to the deletion of potentially autoreactive T cell clones, even though current evidence suggests that Vβ deletion is not a common event in man (Kappler et al.1989). Since, as we have seen, disease susceptibility and resistance may be so closely linked to allelic sequence variation, it is an intriguing possibility that the selective pressure responsible for linkage disequilibrium between HLA alleles results from the need to balance positive and negative selective effects on the T cell repertoire. In other words, the deletion of certain TcRs by each extended haplotype leaves a residual T cell repertoire which can interact effectively with the linked HLA molecules to generate protective immunity and avoid autoimmunity.

### 1.3.1 Population studies

The first reports of an association between MS and the HLA system were published in 1972 (Bertrams et al.1972; Naito et al.1972), and it was soon established that there was a reproducible association in both North America and Europe with HLA-A3 and -B7. The first report of a stronger association with an HLA-D locus product (then termed LD-7a) came soon after (Jersild et al.1973). By 1977, results from all centres demonstrated a significant increase in the antigen Dw2, varying from 47 to 75% (Fog et al.1977; Thorsby et al.1977; Opelz et al.1977; Stewart et al.1977). Early serological studies showed similar associations with antigens closely related to Dw2, notably MS-B (84%) (Terasaki et al.1976) and BT101 (83%) (Compston et al.1976). Since then, a great many further studies of HLA-D antigens in various populations have appeared, and these data are presented in Table 10.

In MS patients living in northern Europe there is a constantly observed association with HLA-A3, -B7, -Dw2 and -DR2. The strongest association is with DR2 and the Relative Risk varies between 1.4 and 2.9. A similar association is observed in migrant populations in North America (Opelz et al.1977) and Australasia (Stewart et al.1977) which originated in northern Europe. In Europe, the DR2 association is strongest in Scandinavia and the other northern countries, and less strong in more southerly countries. This led to the concept of a causal relationship between MS and latitude which has been discussed previously, and which has now been lent further weight by recent studies from Australia (Hammond et al.1988a; Hammond et al.1988b).

Although this association between MS and DR2 is observed in most Caucasoid populations, it is a puzzling fact that there is no significant association in patients from the Orkney Islands (Poskanzer et al.1980a), despite this region having the highest prevalence of MS yet described. It has been suggested that the lack of any association has arisen as a result of the extremely high background frequency of DR2 in the Orcadian population (Compston, 1981). This explanation is not entirely satisfactory however, and is inconsistent with the hypothesis that there is a single MS susceptibility locus in linkage with DR2. The simplest explanation for this finding is that a DR2-linked gene is necessary for the development of MS, but not sufficient by itself, and that another genetic factor is also required

**Table 10**

HLA-D associations in population studies of patients with multiple sclerosis. (Compiled from multiple sources; see text for references)

| Allele<br>Population | No. patients studied | Frequency (%)<br>MS | <br>Control | RR |
|---|---|---|---|---|
| **DR2** | | | | |
| NE Scotland | 178 | 49 | 41 | 1.4 |
| England | 100 | 72 | 59 | 1.8 |
| France | 94 | 53 | 35 | 2.1 |
| N Italy | 176 | 37 | 23 | 2.0 |
| S Italy | 68 | 45 | 29 | 2.2 |
| Hungary (gypsy) | 2 | 2/2 | 56 | 1.8 |
| Switzerland | 60 | 60 | 34 | 2.9 |
| USA (caucasoid) | 194 | 61 | 29 | 3.8 |
| USA (black) | 35 | 35 | 0 | 17.0 |
| Australia (N European) | 70 | 70 | | |
| Australia (S European) | 14 | 7 | | |
| Japan | 29 | 55 | 33 | 2.5 |
| Israel (Jewish) | 47 | 12 | 16 | 0.7 |
| **DR3** | | | | |
| N Europe | 60 | 35 | 12 | 2.9 |
| **DR4** | | | | |
| S Italy | 29 | 31 | 12 | 9.9 |
| Jordan (Arab) | 32 | 88 | 35 | 12.1 |
| Japan | 29 | 52 | 31 | 2.3 |
| **DRw6** | | | | |
| Japan | 11 | 55 | 18 | 5.4 |
| Mexico (Mestizo) | 52 | 16 | 5 | 4.7 |
| **DQw1** | | | | |
| NE Scotland | 178 | 77.5 | 62.5 | 2.1 |
| **DPw4** | | | | |
| N Europe | 45 | 93.3 | 72.3 | 5.4 |

for the development of the disease in most cases. As has been already discussed (in Chapters 1.1.1 and 1.2.10), this additional inherited factor may lie anywhere in the genome, but there are good reasons for believing that a second MHC Class II gene is involved. Further evidence that DR2 alone may be insufficient for the development of MS comes from studies of the gipsy community of Hungary (Gyodi et al.1981; Palffy, 1982; Palffy et al.1986). Central European gipsies have remained socially isolated from the indigenous caucasoid population and have a number of immunogenetic differences, including an absence of HLA-B7 and a high frequency of DR2 (56%). Despite this, MS is extremely uncommon, and it is estimated to occur more than ten times less frequently among gipsies than in caucasoid Hungarians.

The effect of racial origin on the HLA association of MS was first highlighted by Batchelor et al. (Batchelor et al.1978). A study from southern Italy failed to show an association with DR2 but instead demonstrated an increased frequency of DR4 (Compston, 1978). The population of this region of Italy is genetically heterogeneous, with historical racial admixture from North Africa, Iberia, the Eastern Mediterranean and the North. In contrast, the more homogeneous north of Italy shows the same DR2 association seen in central and northern European countries (L. Cazullo, personal communication). In Jordanian arabs, a strong association with an antigen closely related to DR4 was observed (Kurdi et al.1977). No association has been seen in Israelis (Brautbar et al.1982). Data from Japan are conflicting. Initially an association with DRw6 was reported (Batchelor, Morris, 1978), but more recent studies have not confirmed this in a population in which MS is rare. Naito et al. found DR2 in 55.2% of 29 patients with definite MS and in only 33.3% of controls (Naito et al.1978; Naito et al.1982). MS is also uncommon in American blacks, but an association with Dw2 was observed in a single study (DuPont et al.1977).

### 1.3.2 Multiple case family studies

Since an association between a disease and a genetic marker can sometimes result from selection artefacts such as stratification of the population being sampled, further evidence to support the HLA associations described above has been sought from studies of multiple case families. Chromosomal recombination occurs at an unusually high frequency in the HLA-D region; co-segregation of MS and, for instance,

DR2 in such families would constitute convincing evidence in favour of DR2 being linked to susceptibility. Several groups have attempted to establish linkage between HLA and MS in multiple case families, but have encountered a number of problems, particularly the unavailability of large pedigrees for study.

Compston and Howard summarized published HLA data from 145 families, finding that haplotype sharing occurred in 83% of siblings, 100% of parent/child pairs and 50% of cousins (Compston, Howard, 1982). These rates do not differ significantly from the expected rates (75%, 100% and 25% respectively). However, in those families with three or more affected members, a common haplotype was shared by all cases in 68% of families compared with an expected rate of 35%. Stewart (Stewart, Kirk, 1983) analysed data from 100 pairs and found that haplotype sharing occurred in 87% of them (expected 75%, $p<0.002$). In multiplex families with affected cousins, in whom chance haplotype sharing is less likely, he found sharing in 10/17 (59%) pairs (expected 25%, $p<0.001$). However, many of these families were drawn from the literature and were not systematically collected, thus introducing the possibility of bias. From a population-based sample of 611 patients, Ebers identified 40 sibling pairs, and found that the rate of haplotype sharing did not differ from that expected (Ebers et al.1982). Also in a population based study, Govaerts et al. analysed extended haplotype sharing in155 patients (Govaerts et al.1985). Although the expected weak association between MS and DR2 was present, there was no haplotype or allelic recombination significantly linked to disease. Twenty sibpairs and two trios of MS were also studied, but again there was no distortion of the random distribution of haplotypes.

Similarly, Francis et al. performed HLA typing of Class I, II and III alleles in 14 multiplex MS families, 9 single case families and 11 normal families from the Grampian region of northeast Scotland (Francis et al.1987a), and found that the distribution of shared haplotypes in 12 affected and 19 unaffected sibpairs did not differ significantly from that expected. However, in this study it was found that the extended haplotype HLA-B7, C4B*3, C4B*1, Bf*S, DR2, DQw1 accounted for 18.9% and 24.2% of parental haplotypes from multiplex and single case families respectively, in contrast to only 2.3% of parental haplotypes from control families ($p<0.05$, $p<0.01$). These data add significantly to the evidence from pedigree analysis indicating that the MHC contributes to susceptibility to MS.

### 1.3.3 RFLP studies

Only limited data on RFLP analyses of Class II genes in MS have been published. Early on it was found that haplotypes carrying the same serologically defined DR specificities can be differentiated on the basis of their different patterns of DQB restriction fragments (Cohen et al.1984b); DR2 haplotypes for instance were associated with two different DQβ fragment clusters. This finding was utilized in the first RFLP study of Class II genes in MS (Cohen et al.1984a). 24 MS patients, 24 HLA-DR matched controls and 24 random controls were studied by digestion of genomic DNA with the restriction endonucleases EcoRI and BamHI followed by hybridization with a full length DQB probe. A 2.2 kb EcoRI fragment was observed in 17/24 (79%) patients, and in only 8/24 (33%) and 7/24 (29%) random and matched controls respectively. A 12 kb BamHI fragment was seen more frequently in patients (33%) than in random (24%) and matched (16%) controls. The following year, the same group reported a similar significant MS association in the case of an EcoRV DQβ polymorphism (Marcadet et al.1985). It was found that in 44 patients plus HLA-DR matched controls, a 5.2 kb fragment was present in 84% patients and 55% controls ($p<0.005$), and that a 2.6 kb fragment was present in 18% patients and 45% controls ($p<0.01$). Of potentially greater interest was the observation that when the analysis was limited to only DR2 individuals (32/44 in each group) the differences were more significant. The 5.2 kb fragment was seen in 97% patients and 63% controls ($p<0.001$) and the 2.6 kb fragment in 16% patients and 56% controls ($p<0.001$).

From both these studies it was concluded that a particular subset of DR2 haplotypes was involved in susceptibility to MS. However, it has since emerged that these findings were distorted by bias in the selection of the control groups. The EcoRI 2.2 kb fragment and the EcoRV 5.2 kb fragment are now known to be specific for the Dw2 subtype of DR2, and the EcoRV 2.6 kb fragment for DwAZH (Segall et al.1986). The patient group was selected from a caucasoid French population, in which Dw2 is the most common subtype and DwAZH is extremely rare; the control groups were selected from an Israeli population in which DwAZH is much more common. From this can be seen the importance of comparing disease and control groups taken from the same population.

Limited panels of MS patients and controls have also been studied by Jacobson (Jacobson et al.1986) and Serjeantson (Serjeantson et al.1986). In the first of these studies, PstI and AvaII DRβ RF patterns in 6 DR2 MS

patients were identical to that obtained in a DR2.Dw2 cell line; PstI DQβ RF patterns were identical to that of the DR2.Dw2 cell line in 4/6 MS patients. The remaining two patients typed serologically as DR2,blank and had an additional doublet of fragments. Serjeantson has also showed by DRβ and DQβ allogenotyping that the dominant caucasoid DR2 subtype Dw2 is present in most cases of DR2-positive MS. Recently, in a study of 45 Swedish MS patients and 166 Danish controls, a significant association with DPw4 has been described by allogenotyping with DPα and DPβ probes (Ødum et al.1988). There have been no previous RFLP studies of DQα in MS.

### 1.3.4 Disease severity and rate of progression

Most patients with MS have a disease course characterized by relapses and remissions (R/R MS). Many will, however, eventually enter a secondary phase in which their symptoms evolve progressively. Between 9% and 37% of patients have an illness which is progressive from onset (primarily chronic progressive, PCP MS) (reviewed by (Matthews, 1985)). It has been proposed that R/R MS and PCP MS may be separate disease entities, since they differ with respect to epidemiology, age at onset, initial symptoms, prognosis with regard to both degree of disability and mortality, and response to immunosuppression (Larsen, 1985). However, efforts to differentiate between these two forms of the disease by serological HLA Class II typing have yielded inconclusive results.

Jersild et al. first correlated more severe disease with the presence of Ld7a (equivalent to Dw2) (Jersild et al.1973), and this later appeared to be confirmed by Stendahl-Brodin et al. who found a significant association between Dw2 and the development of moderately severe disability within 5 years of onset (Stendahl-Brodin et al.1979). Many other studies have also suggested a relationship between disease severity and rate of progression, and the presence of DR2 or Dw2, but confusingly, others have found both positive (Madigand et al.1982) and negative (Engell et al.1982) correlations between severity and DR3. Many other groups however have been unable to confirm these associations (Ebers, Paty, 1979; Poser et al.1981; Wentzel et al.1984), and this area remains contentious. Recently, Olerup et al. have reported a strong correlations between R/R and PCP MS and DQβ restriction fragment polymorphisms (Olerup et al.1987; Olerup et al.1989a; Olerup, 1989b). A TaqI DQβ RF pattern seen in DR4/DQw8, DR7/DQw9 and DRw8/DQw4 haplotypes was found in 65% of PCP MS and only 27%

of R/R MS ($p<0.001$). A DQβ allelic pattern seen in DR3-DRw17/DQw2 haplotypes was found in 32% of R/R MS and only 4% of PCP MS ($p<0.005$). These data indicating immunogenetic heterogeneity between two clinical forms of MS strongly support the hypothesis that MS may be aetiologically heterogeneous.

## 1.4 MULTIPLE SCLEROSIS AND NON-MHC MARKERS

The major problem limiting the usefulness of many human genetic markers other than HLA is their relative lack of polymorphism. Many other genetic systems have been studied however, although often without marked success (eg. erythrocyte surface antigens, alkaline phosphatase, α-1 antitrypsin inhibitor Pi). Apart from MHC Class I, II and III genes, each of which may directly influence the specificity or effectiveness of an immune response, the polymorphic immunoglobulin and T cell antigen receptor genes are also involved in immune responsiveness. There is some evidence that both of these classes of genes can in part confer susceptibility to MS. The effect of gender on the development of MS, as also on that of many other diseases, has been recognized for many years and it is unfortunate that there is at present no good explanation for this.

### 1.4.1 Gender

It is well recognized that MS is unequally distributed between women and men. Based upon 29 epidemiological studies reported in the literature between 1949 and 1984, the sex ratio (F:M) is 1.7 (10555/6118) (Duquette, 1989). The average age of onset is lower in women (28 years) than in men (29 years), and women are significantly more common among patients with early onset disease. In a group of 500 cases with the appearance of first symptoms before the age of 20, the sex ratio was 3.2 (382 women, 118 men).

Weitcamp (Weitkamp, 1983) compared pairs of affected relatives from published families, and found that the number of same-sex pairs was significantly increased above that expected by chance in families with 2 affected siblings, but not in families with 2 cases in 2 generations. He also found that a sex ratio of nearly 2 in sporadic, non-familial MS and in parent-child cases, decreased to 1.5 in sibships with 2 affected individuals, and to 1.0 in sibships with 3 affected individuals. These data were interpreted as indicating two types of genetic susceptibility to MS, one type being sex-related with low sibling recurrence risk and expressed predominantly in females, the other being sex-independent and having a higher sibling recurrence risk.

Duquette et al. (1986) have examined the pedigrees of 9 multiplex families in which there is parent-to-child transmission, and analysed a further 27 families from published literature for evidence of a mitochondrial pattern of inheritance. They found that 10 fathers

transmitted the disease to 5 boys and 6 girls, and that 26 mothers transmitted the disease to 14 boys and 21 girls. These figures are not in keeping with mitochondrial inheritance.

### 1.4.2 Immunoglobulin allotypes

The human immunoglobulin heavy chain gene complex (IGHC) maps to chromosome 14q32.3 and is comprised of approximately 200 variable (V) gene segments, at least 20 diversity (D) elements, and 6 joining (J) segments, a constant (C) region of nine genes, and two pseudogenes. The IGHC has recently been mapped by cosmid cloning and PFGE and found to span 350 kb (Cox, 1989; Hofker et al.1989). The immunoglobulin class is determined by the C genes, which in the case of IgG are termed Cγ. There are four IgG1, one IgG2 and 13 IgG3 Gm allotypes, which are markers of the Cγ3 and Cγ1 genes of the IGHC. Gm allotypes can be determined by haemagglutination inhibition techniques, and their distribution varies between populations (Johnson et al.1977).

Several studies have reported associations between Gm allotypes and a number of autoimmune diseases. In the case of MS, Pandey et al. in the USA found an association with the Gm haplotype 1,17,21 (RR=3.6) (Pandey et al.1981), and this observation was later confirmed and strengthened in Australia by Stewart, who reported a highly significant increase in patients of the phenotype Gm 1,3,5,10,11,13,14,17,21,26 ($p<0.0005$) (Stewart, Kirk, 1983). Blanc et al. have also showed a significant increase of Gm 3,5,10,11,13,14 in the Hautes-Pyrénées, but were unable to find a significant association with the Ig light chain (kappa) system (Blanc et al.1986). However, it must be noted that in two further groups of patients and controls studied by Stewart an association was not seen, and in yet another study it was reported that Gm 3,5,13,14 was associated with reduced susceptibility (Propert et al.1982).

Most recently RFLP techniques have been used to clarify the situation. Using a Igγ1 probe, which detects polymorphisms in the Cγ1, Cγ2, Cγ3 and pseudo-γ genes, a significant negative association was found between a 5.9 kb BstEII Cγ3 fragment and MS (Gaiser et al.1987). Southern blot analysis of genomic DNA revealed the presence of this fragment in 84/140 (60.0%) controls but in only 17/59 (28.8%) patients. This association was not seen in the case of either myasthenia gravis or Graves' disease.

### 1.4.3 T cell antigen receptor genes

The nature of the T cell antigen receptor (TcR) has been established at both genomic and protein level (Acuto et al.1983; Kronenberg et al.1986). The molecule is a heterodimeric cell surface glycoprotein consisting of disulphide linked α and β chains of 50 kd and 40 kd. Each chain is separately encoded by variable (V), diversity (D) (in the case of the β chain), joining (J) and constant (C) region gene segments which rearrange to generate functional genes in the differentiated T cell (Yoshikai et al.1984). Two further gene clusters encoding a γ-δ heterodimer have been identified more recently (Brenner et al.1986). The α and β chain genes are highly polymorphic, and in view of the central rôle the TcR plays in immune recognition and T cell activation (see previously), it has been a matter of considerable interest recently to investigate the possibility that particular TcR gene polymorphisms are involved in the development of autoimmune disease. There is evidence now that this is the case in both EAE and MS.

The first indication of limited idiotypy in the encephalitogenic response to MBP came from studies of EAE in the Lewis rat (Ben-Nun et al.1981). It was found that pre-immunization with an MBP-specific T cell line (or clone) that is itself encephalitogenic can prevent another animal from developing EAE upon immunization with MBP. This ability of a single T cell clone to block the onset of EAE, presumably by the induction of a regulatory population of anti-clonotypic cells, therefore suggests that the T cells used in generating this disease may be highly restricted in their TcR usage. This observation has been extended by finding that there is restricted heterogeneity of TcR α and β gene usage both by encephalitogenic T cells in EAE and by T cells isolated from the CSF of MS patients, and that the development of EAE can be blocked by monoclonal antibodies specific for the TcR. In the Lewis rat model of EAE, an analysis of TcR β gene usage by 25 Tcell clones showed that only two major rearrangements were used (Happ & Heber-Katz, JEM 167:502,1988), and the same was found in the SJL mouse model of EAE (Zamvil et al.1988). Sequence analysis of MBP-specific T cell clones in PL/J mice confirmed this limited heterogeneity, by showing that 8/8 clones tested shared usage of $V_{\alpha PJR\text{-}25}$, a new member of the $V_{\alpha 4}$ family, and 78% of the T cell clones expressed $V_{\beta 8.2}$ (Acha-Orbea et al.1988). Similar experiments in B10.PL mice have again shown a strong association with $V_{\beta 8.2}$ usage, and also with $J_{\beta 2.7}$, although $V_{\alpha}$ usage was more heterogeneous (Urban et al.1988). Data from several groups now demonstrate that in EAE, in two species

and in several different strains, encephalitogenic T cells very frequently use TcR $V_{\alpha 2/4}$ and $V_{\beta 8.2}$ rearrangements (Heber-Katz, Acha-Orbea, 1989).

There have been three published studies of the TcR germline repertoire in MS. Oksenberg et al. (Oksenberg et al.1988) found no significant differences between MS patients and controls in the frequencies of polymorphic TcRα and TcRβ restriction fragments. However, in a second report, the same authors amplified several selected sequences from a full length TcRα cDNA by PCR, which were specific for $V_\alpha$, $J_\alpha$, $C_\alpha$ and $TM_\alpha$ (Oksenberg et al.1989). Using these as probes, they found a significant difference in the frequencies of a $V_\alpha$ 6.3 kb fragment (65% v. 33%) and a $C_\alpha$ 2.0 kb fragment (91% v. 49%) between patients and controls respectively ($p<0.0001$). Beall et al. used $V_{\beta 8}$, $V_{\beta 11}$ and $C_\beta$ probes to define eight different β chain haplotypes in 40 MS patients and 100 controls (Biddison, 1989) (J Beall, personal communication). It was found that there were significant differences in the frequencies of these haplotypes between patients and controls ($p=0.012$), and also between HLA-DR2 positive patients and controls ($p=0.015$).

Few data on TcR gene rearrangements in CSF T cells from MS patients are available yet, although this is now an area of considerable interest. Hafler et al. analyzed the clonality of T cells derived from CSF and blood by means of TcR β and γ gene RFLPs, finding evidence for oligoclonality in CSF and blood T cells from patients with chronic MS, but not in healthy controls or in patients with other neurologic diseases (Hafler et al.1988). However, T cells from a single patient with acute fatal MS were not oligoclonal. Finally, using a different approach to examine the T cell repertoire at the site of the lesion in MS, Oksenberg and Steinman have detected the presence of rearranged $V_{\alpha 12.1}$ by enzymatic amplification of TcR-specific brain mRNA obtained from a MS patient (personal communication).

## 1.5 EVIDENCE THAT MS MAY BE AN AUTOIMMUNE DISEASE

The presence of mononuclear inflammatory cells within the CNS of patients with MS has led to the hypothesis that immunological mechanisms are important in the pathogenesis and aetiology of the disease. However, although it appears inescapable that MS is immunologically mediated, it has not yet proved possible to establish whether there is a primary abnormality of immune regulation in these patients. There is a body of somewhat indirect evidence that MS is a true autoimmune disease, caused by auto-aggressive T cells directed against a CNS antigen. The association between MS and HLA-DR2 is itself a factor in support of the autoimmune argument, since other diseases, which are more obviously autoimmune, are usually also associated most strongly with MHC Class II antigens. Despite this however, and in contrast to, for instance, myasthenia gravis and insulin dependent diabetes, other autoimmune diseases do not appear to be more common in MS patients (de Keyser, 1988). Several other hypotheses have been proposed to explain the immunological abnormalities that are seen in MS, such as an immune response against a persistent viral infection of the CNS, or an immune response involving primarily the CNS microvasculature, in which case the primary insult might be immune complex deposition, for instance. Furthermore, there is continuing uncertainty over whether the primary immunological processes involved in the development of MS are localized to the CNS or whether MS is a systemic disease. This controversy has been reviewed recently (Calder et al.1989; Hafler, Weiner, 1989).

A large number of immune abnormalities have been described in MS, in both peripheral blood and the CNS. The first described abnormality in blood was a decrease of ConA-induced T cell-mediated suppression (Antel et al.1979). The autologous mixed lymphocyte reaction (AMLR) is also depressed in patients with chronic MS, as it is also in SLE, rheumatoid arthritis and Sjögren's syndrome (Hafler et al, 1985). Other blood abnormalities that have been described include a reduction in the frequency of $CD4^+CD45R^+$ Tcells, and increased numbers of activated T cells. It has been argued that the clinical amelioration seen in response to systemic immunosuppressive therapy (ACTH, cyclophosphamide and total lymphoid irradiation) suggests that a major part of the immunopathogenesis is peripheral (Hafler, Weiner, 1989).

However, the most consistently observed abnormalities are seen within the CNS. Plasma cells are present within MS plaques, and also appear in normal looking white matter in more advanced cases (Adams, 1983). The opersistence of small numbers of plasma cells may thus explain the presence, and continual synthesis, of oligoclonal bands in the CSF. In addition to B cells, T cells and macrophages are found in the initial acute lesions (Traugott et al.1983). There is a corresponding increase in the number of cells seen in the CSF during the acute phase of MS, and this rise comprises mainly T cells. Phenotypic analysis of these cells shows an increased ratio of $CD4^+$ to $CD8^+$ cells. Activated lymphocytes ($IL\text{-}2R^+$) are found in perivascular areas of the brain in some patients with MS (Woodroofe et al, 1986). The expression of IL-2R is greater in lymphocytes from the CSF compared with those from peripheral blood (Bellamy et al, 1985).

Assuming that MS is a T cell-mediated (or incited) disease, the antigen(s) to which the T cells are directed remains unknown. Attempts to determine the specificity of the oligoclonal immunoglobulins in CSF by adsorption have not been successful. Experimental allergic demyelination (EAE) has served as the primary animal model for MS, particularly a chronic relapsing form of the disease (CREAE, reviewed by (Lassman, 1983). There is considerable evidence now that CREAE is a T cell-mediated disease, and that immune reactivity to major basic protein (MBP) is an important factor in its pathogenesis. The most striking evidence for the rôle of the T cell in EAE is afforded by adoptive transfer experiments (Ben-Nun et al.1981). CREAE can be induced by MBP-specific T cell lines or clones of the helper/inducer phenotype ($Lyt\text{-}1^+2^-$, $L3T4^+$). The resulting demyelination is more pronounced in syngeneic or MHC Class II (Ia)-compatible animals. The recent studies of Steinman, in which EAE can be completely prevented in susceptible animals by the administration of a monoclonal antibody against either the T cell receptor, the Class II molecule, or CD4, firmly establish the pivotal rôle played by the T cell in EAE (Steinman, 1989). Accordingly, there have been a number of attempts to elucidate the rôle of MBP-reactive cells in MS. The levels of MBP and anti-MBP antibodies in the CSF correlate with the course of demyelination and this suggests that there is localized sensitization of T cells to MBP within the CNS (Patterson et al, 1981). However, it can not be stated from these, and other, studies whether or not MBP-specific T cells are a

secondary phenomenon, resulting from the release of myelin breakdown products.

In summary, a large number of central and peripheral immunological abnormalities may be observed in MS. It has been clearly established that EAE, an animal model with many similarities to MS, is T cell-mediated and is a genuine autoimmune disease. Attempts to identify a potential autoantigen in MS have not been successful, and it must be remembered also that epidemiological observations indicate a strong likelihood that an antigen derived from an infectious agent may be involved. However, at present, the autoimmune hypothesis would appear to provide the most satisfactory explanation, and a possible scheme for this is described by (Calder et al.1989). She suggests that clonal expansion within the CNS is a result of the interaction of a small resident population of lymphocytes with antigen presenting cells (APC). Both astrocytes and microglial cells are inducible to express Class II molecules, and either of these may function as APC. Although myelin is relatively inert, there is a low rate of natural degradation and replacement in the CNS; many of the constituents of myelin are highly encephalitogenic, and might thus serve as the sensitizing antigen. The process may be triggered, either by infection or stress, in genetically susceptible individuals. Activated cytotoxic cells may themselves attack the myelin sheath, and may also recruit inflammatory peripheral mononuclear cells into the developing lesion.

# PART 2

## SUBJECTS, MATERIALS AND METHODS

## 2.1 SUBJECTS

### 2.1.1 Patients

33 caucasian MS patients indigenous to the Grampian region of northeast Scotland were studied. All of the patients had previously attended the Department of Neurology, Aberdeen Royal Infirmary and had been identified by reviewing accessible notes or computerized data. This work was carried out by Francis as part of his comprehensive study of MHC antigens in MS (Francis, 1986a), which provided the direct impetus for the subsequent studies reported in this thesis. All patients were contacted and examined by Dr Francis, who confirmed the diagnosis of MS in each case according to the clinical criteria of the Poser Committee (Poser et al.1983). Patients were categorized according to clinical course, either relapsing/remitting (24 patients) or progressive (9 patients). Relapsing/remitting MS was defined as having a course in which there were distinct attacks with stable periods between. Progressive MS was defined as progressive evolution of disability either from onset or following an initial relapsing phase of the illness. There were 24 females (73%) and 9 males (27%).

### 2.1.2 Controls

A total of 130 local healthy controls from the Grampian region were available for comparison. From these, 58 were selected for further study, selection being made so as to maximize matching of HLA-DR alleles in the patient and control groups (see Chapter 3.1.1). In view of the high prevalence of MS in the region, subjects with a positive family history of MS or of any previous neurological symptoms were excluded. Studies of the relationship between HLA-DR haplotypes and the presence of an HLA-DQA1 related RFLP cluster, described in Chapter 3.1.7, were carried out using samples from 33 healthy caucasian volunteers. These were either colleagues in the Royal Postgraduate Medical School or were potential bone marrow donors contacted through the Anthony Nolan Appeal Trust, and were selected by their known HLA-DR phenotype.

### 2.1.3 Subjects from Northern Ireland

A further 50 unrelated patients were identified through the Department of Neurology, Queen's University, Belfast, by the same criteria as above, all patients being examined by Dr S A Hawkins. 76 healthy controls were also

identified by the Tissue Typing Laboratory, Belfast City Hospital. These subjects were investigated by Cullen and Middleton as part of a collaborative study, as reported previously (Heard et al.1989) and described here in Chapter 3.1.6.

### 2.1.4 HLA typing of subjects

All the Scottish patients and controls were HLA typed in the Department of Immunology, Royal Postgraduate Medical School. Subjects were initially typed serologically, using polyclonal antisera validated by the Ninth International Histocompatibility Workshop, but many of them were later allogenotyped by HLA DR and DQ RFLP methods for greater accuracy and to resolve uncertainty about zygosity in selected cases. The Northern Ireland subjects were HLA typed in the Tissue Typing Laboratory, Belfast City Hospital, again by means of both conventional serology and Class II RFLP analysis. Normal volunteers selected from the Anthony Nolan Appeal Trust had been typed in the Trust's laboratories. The frequencies of DR phenotypes in the Scottish and Northern Irish subjects are shown in Table 11.

### 2.1.5 Samples

The majority of the blood samples, used for DNA preparation, were collected by standard venepuncture and transferred to sterile 20ml containers containing preservative-free heparin (20 i.u. per ml). The samples were separated by centrifugation at 2000 rpm for 10 min and the buffy coat layer was stored at -20$^{o}$. Cycles of thawing and re-freezing were avoided in order to prevent degradation of the DNA.

For the T cell cloning studies, fresh blood samples were necessary. Approximately 500ml of blood was collected into sterile transfusion packs with preservative-free heparin as before. The samples were obtained in the subjects' homes during a single day, before being flown from Aberdeen to London the same evening for separation of lymphocytes and setting up of bulk cultures prior to cloning (see Chapter 2.3.6).

A number of lymphoblastoid cell lines were also investigated, many of which were established in the Department of Immunology, RPMS. However, several other homozygous cell lines were obtained through the XIHWS, and were expanded in the Department.

**Table 11** Showing the frequencies of HLA-DR phenotypes in patients and controls from the Grampian region of Scotland and Northern Ireland, expressed as a proportion of the total number of haplotypes. These figures are based upon conventional serotyping; here only a single phenotype was detected, homozygosity has been assumed, although it is possible that a small number of these may be due to the presence of a DR'blank' phenotype.

| | | Patients | | Controls | |
|---|---|---|---|---|---|
| | HLA-DR | no. | % | no. | % |
| Grampian | 1 | 7 | 10 | 4 | 3 |
| | 2 | 35 | 51 | 42 | 36 |
| | 3 | 12 | 18 | 22 | 19 |
| | 4 | 8 | 12 | 20 | 17 |
| | 5 | 1 | 1 | 11 | 9 |
| | w6 | 0 | 0 | 6 | 5 |
| | 7 | 4 | 6 | 11 | 9 |
| | w8 | 1 | 1 | 0 | 0 |
| | 9 | 0 | 0 | 1 | 1 |
| | 10 | 0 | 0 | 1 | 1 |
| N. Ireland | 1 | 7 | 7 | 14 | 9 |
| | 2 | 37 | 37 | 39 | 26 |
| | 3 | 16 | 16 | 18 | 12 |
| | 4 | 11 | 11 | 27 | 18 |
| | 5 | 2 | 2 | 7 | 5 |
| | w6 | 11 | 11 | 13 | 9 |
| | 7 | 14 | 14 | 26 | 17 |
| | w8 | 1 | 1 | 3 | 2 |
| | 9 | 0 | 0 | 0 | 0 |
| | 10 | 1 | 1 | 5 | 3 |

## 2.2 METHODS

### 2.2.1 RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

#### 2.2.1.1 Materials and reagents

UNC PHENOL (University of North Carolina)

| | |
|---|---|
| phenol | 500g |
| 1M Tris-HCl (pH 7.8) | 220ml |
| $ddH_2O$ | 33ml |
| M-cresol | 27.5ml |
| 2-mercaptoethanol | 1.1ml |
| 8-hydroxyquinoline | 550mg |

Dissolve phenol crystals in water and Tris-HCl and add M-cresol, 2-ME and 8-OH quinoline. Shake well and stand until phases separate. If the phenol is uppermost, add enough $ddH_2O$ to invert the phases. Store (indefinitely) in airtight container at room temperature.

3M SODIUM ACETATE PH 5.2

| | |
|---|---|
| $NaOAc.3H_2O$ | 408.1g in 1000ml (adjust pH with acetic acid) |

5M POTASSIUM ACETATE

| | |
|---|---|
| KOAc | 147.2g |
| glacial acetic acid | 57.5ml |
| $ddH_2O$ | to 300ml |

4M SODIUM HYDROXIDE

| | |
|---|---|
| NaOH | 160g in 1000ml |

HYBRIDIZATION BUFFER (XIHWS METHOD)

| | volume | final conc. |
|---|---|---|
| 33% dextran sulphate | 60ml | 10% |
| formamide | 80ml | 40% |
| 20x SSC | 40ml | 4x |
| 2M Tris pH 7.5 | 1ml | 10mM |
| 100x Denhart's | 2ml | 1x |
| Salmon sperm DNA | | 50µg/ml |
| $H_2O$ | to 200ml | |

100x DENHART'S SOLUTION

| | |
|---|---|
| Ficoll 400 | 1g |
| polyvinyl pyrrolidone | 1g |
| BSA (Pentax fraction V) | 1g |
| $dH_2O$ | to 50ml |

Filter and sore at -20C.

4M SODIUM CHLORIDE

| | |
|---|---|
| NaCl | 233.8g in 1000ml |

0.5M SODIUM EDTA PH 8.0

| | |
|---|---|
| $Na_4EDTA$ | 186.1g in 1000ml (adjust pH with NaOH pellets) |

BACTERIAL GROWTH MEDIA

| | LB | YT | NZ amine |
|---|---|---|---|
| Bactotryptone | 10g | 5g | 2g |
| yeast extract | 5g | 5g | 10g |
| NaCl | 5g | 5g | 5g |
| NZ amine | - | - | 10g |

$H_20$ to 1000ml and autoclave.

BACTERIAL GLYCEROL STOCKS

Add 250µl of bacterial culture to 250µl sterile glycerol in a 1.8ml freezing (Nunc) vial. Mix by vortexing and freeze at -70°.

OVERNIGHT BACTERIAL CULTURES

Inoculate 5ml sterile LB medium with 50µl glycerol stock. Add appropriate antibiotic (5µl of 50mg/ml stock). Incubate overnight at 37° with vigorous shaking.

LARGE SCALE PLASMID PREPARATION

| Solution I (GTE) | | Final conc. |
|---|---|---|
| Dextrose | 4.5g | 50mM |
| 1m Tris-HCl pH 8.0 | 12.5ml | 25mM |
| 0.5M $Na_4EDTA$ pH 8.0 | 10ml | 10mM |
| $ddH_2O$ | to 500ml | |

Sterilize by filtration and store at RT.

| Solution II | | Final conc. |
|---|---|---|
| 10% SDS | 20ml | 1% |
| 10M NaOH | 4ml | 0.2M |
| $H_20$ | to 200ml | |

DE52 ELUTION BUFFER

| | |
|---|---|
| NaCl | 1M |
| Tris-HCl | 20mM |
| urea | 6.5M |

RSB BUFFER

| | |
|---|---|
| 1M Tris-HCl pH 8.0 | 2.5ml |
| 5M NaCl | 0.5ml |
| 0.5M EDTA | 12.5ml |
| $ddH_2O$ | to 250ml |

PROTEINASE K SOLUTION

| | |
|---|---|
| NaCl | 10mM |
| Tris-HCl pH 7.5 | |
| EDTA | 10mM |
| SDS | 0.5% |
| proteinase K | 100µg/ml |

Make fresh and prewarm to 37° before use.

20X PHOSPHATE BUFFERED SALINE (PBS)

| | |
|---|---|
| NaCl | 320g |
| KCl | 8g |
| $Na_2HPO_4$ (anhydr.) | 46g |
| $KH_2PO_4$ | 8g |
| $ddH_2O$ | to 2000ml |

1M TRIS

121.1g Tris base in 800ml $ddH_2O$.
Adjust to pH 7.8 by adding concentrated HCl (approx. 50ml) and make volume up to 1000ml.

20X SSC

| | |
|---|---|
| NaCl | 3M |
| trisodium citrate | 0.3M |
| NaOH | 0.6M |

30X SSPE

| | |
|---|---|
| NaCl | 4.5M |
| $NaH_2PO_4$ | 0.3M |
| $Na_4EDTA$ | 30mM |

40X TAE BUFFER (HOWLEY'S)

| | |
|---|---|
| Tris base | 193.6g |
| Na acetate | 108.9g |
| $Na_4$ EDTA | 17.1g |
| glacial acetic acid | ~90ml |

Make up to 1000ml with $ddH_2O$, titrating to pH 7.4 with acetic acid.

20X TBE BUFFER

| | |
|---|---|
| Tris base | 216g |
| boric acid | 110g |
| $Na_4$ EDTA | 18.6g |

Make up to 1000ml with $ddH_2O$.

ETHIDIUM BROMIDE 10MG/ML

10X BUFFERS FOR RESTRICTION ENZYME DIGESTION REACTION

| | Stock reagent | amount | final conc. |
|---|---|---|---|
| LOW | 1M Tris pH 7.5 | 100µl | 10mM |
| | 1M $MgCl_2$ | 100µl | 10mM |
| | 1M DTT | 10µl | 1mM |
| | $ddH_2O$ | 790µl | |
| MEDIUM | 4M NaCl | 125 µl | 50mM |
| | 1M Tris pH 7.5 | 100µl | 10mM |
| | 1M $MgCl_2$ | 100µl | 10mM |
| | 1M DTT | 10µl | 1mM |
| | $ddH_2O$ | 665µl | |
| HIGH | 4M NaCl | 250µl | 100mM |
| | 1M Tris pH 7.5 | 500µl | 50mM |
| | 1M $MgCl_2$ | 100µl | 10mM |
| | 1M DTT | 10µl | 1mM |
| | $ddH_2O$ | 150µl | |

| | | | |
|---|---|---|---|
| S (for Sma 1) | 1M KCl | 200µl | 20mM |
| | 1M Tris pH 7.5 | 100µl | 10mM |
| | 1M $MgCl_2$ | 100µl | 10mM |
| | 1M DTT | 10µl | 1mM |

5X OLIGOMER LABELLING BUFFER (OLB)

Solution "O"

| | |
|---|---|
| Tris-HCl pH 8.0 | 1.25M |
| $MgCl_2$ | 0.125M |

Store at +4C

Solution "A"

| | |
|---|---|
| Solution "O" | 1ml |
| 2-mercaptoethanol | 18µl |
| 0.1M dATP | 5µl |
| 0.1M dTTP | 5µl |
| 0.1M dGTP | 5µl |

Store at -20C

Solution "B"

2M HEPES, titrated to pH 6.6 with 4M NaOH

Solution"C"

Hexadeoxynucleotides (Pharmacia/PL #2166) evenly suspended in 3mM Tris pH 7.0, 0.2mM EDTA at 90 OD units/ml (555µl for 50 OD units). Store at -20C.

To make 5x OLB, mix solutions A:B:C in a ratio of 100:250:150 and store at -20C.

5X LOADING BUFFER

| | |
|---|---|
| Tris-HCl | 50mM |
| EDTA | 50mM |
| SDS | 0.5% |
| bromphenol blue | 0.1% |
| sucrose | 40% |

$CHCl_3$-ISOPROPYL ALCOHOL (24:1)

| | |
|---|---|
| $CHCl_3$ | 480ml |
| isopropyl alcohol | 20ml |

TE BUFFER

(10 mM Tris-HCl + 1mM EDTA)

| | |
|---|---|
| 1M Tris | 10ml |
| 0.5M EDTA | 2ml |
| $ddH_2O$ | to 1000ml |

DEPURINATION SOLUTION

| | |
|---|---|
| HCl | 0.25M |

AGAROSE GEL WASHING SOLUTION 1

| | |
|---|---|
| NaOH | 0.4M |
| NaCl | 0.6M |

AGAROSE GEL WASHING SOLUTION 2

| | |
|---|---|
| NaCl | 1.5M |
| Tris-HCl pH 7.5 | 0.5M |

SOUTHERN BLOTTING SOLUTION: NEUTRAL TRANSFER
10x SSC

SOUTHERN BLOTTING SOLUTION: ALKALINE TRANSFER

| | |
|---|---|
| NaOH | 0.4M |
| NaCl | 0.8M |

HYBRIDIZATION BUFFER (FOR GENE SCREEN PLUS)

| | | |
|---|---|---|
| 50% dextran sulphate | 20ml | 10% |
| formamide | 50ml | 50% |
| 4M NaCl | 25ml | 1M |
| 20% SDS | 5ml | 1% |

Add SDS last after warming to $40^{o}$. Store at $-20^{o}$.

100X DENHART'S

| | | |
|---|---|---|
| Ficoll 400 | 1g | |
| polyvinyl pyrrolidone | 1g | |
| BSA | 1g | (Pentax fraction V, Sigma) |
| $H_2O$ | to 50ml | |

Filter and store in 10ml aliquots at $-20^{o}$.

DENATURED SALMON SPERM DNA

Dissolve ssDNA in dd$H_2O$ at a concentration of 10µg/ml. Pass through an 18 gauge needle several times to shear the DNA. Boil for 10 minutes. Store at $-20^{o}$ in 200ml aliquots.

### 2.2.1.2 Gene probes

The MHC Class II and TcR gene probes used in these studies, which were obtained from several sources, are listed in Table 12 below. The Class II region probes were all full length cDNAs cloned into suitable plasmid vectors, and the probes were prepared for use by the methods given in Chapters 2.2.1.4 and 2.2.1.5. Three different DQA1 probes were used at various times, although comparable results were obtained in each case. p11-α-5, which was used for all the Southern blot studies of the Scottish subjects, is a 1261 bp fragment derived from the heterozygous cell line Raji (DR3, w6) (Schenning et al.1984). pDCH1 (797 bp) is another widely used DQA1 probe which was originally cloned from the cell line JY (DR4,w6) (Auffray et al.1982), and this probe was used in collaborative studies of subjects from Northern Ireland (Heard et al.1989). The difference in their lengths is mainly due to different splice sites in the 3' untranslated regions, as shown in Figure 6. The 3'UT region of DQA1 (Raji) contains intronic sequence and is about 500 bp long, whereas that of DQA1 (JY) is only about 130 bp. A third DQA1 probe was used in the XIHWS and was found to give results identical to those obtained with p11-α-5.

| gene | name | size (kb) | digest | vector | antibiotic | reference |
|---|---|---|---|---|---|---|
| DPA | | 1150 | Eco R1 | pBR 328 | Amp | Erlich 1984 |
| DPB | p11-β-7 | 844 | Pst1 | pBR 322 | Tet | Gustaffson 1984 |
| DQA1(i) | p11-α-5 | 1261 | Pst1 | pBR 322 | Tet | Schenning 1984 |
| DQA1(ii) | pDCH1 | 797 | Pst1 | pBR 322 | Tet | Auffray 1982 |
| DQA1(iii) | | 1600 | Bam H1 - Hind111 | pUC 9 | Amp | Auffray (NP) |
| DQB1 | p11-β-2 | 993 | Pst1 | pBR 322 | Tet | Schenning 1984 |
| DRB | pDR-β-1 | 1080 | Pst1 | pBR 322 | Tet | Larhammer 1982 |
| TcRα | pY14 | 1101 | Pst1 | pBR 322 | Tet | Yanagi 1985 |
| TcRβ (Cβ2) | | JURβ2 | 380 | Bgl 11 | pBR 322 | Tet Yoshikai 1984 |

**Table 12**

MHC Class II and T cell receptor probes used in these studies, showing the vector, cloning site and insert size.

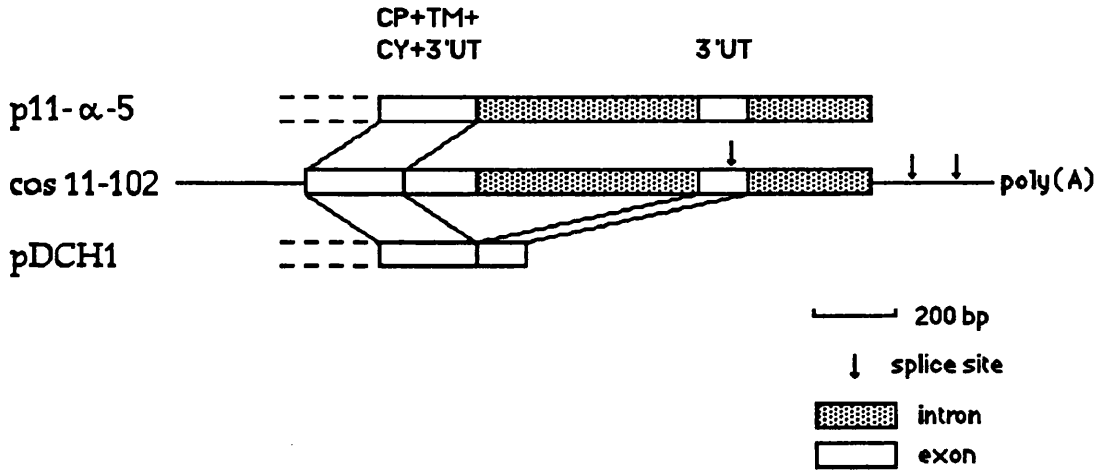



**Figure 6** Diagrammatic representation of the 3'UT regions of the DQA1 probes p11-α-5 and pDCH1, together with the genomic clone cos 11-102, showing their different splice arrangements

### 2.2.1.3 Preparation of genomic DNA

DNA for restriction enzyme digestion

High molecular weight DNA suitable for restriction enzyme digestion was prepared either from frozen buffy coat cells, or from suspensions of lymphoblastoid cells or cloned T lymphocytes numbering $5x10^6$ - $2x10^8$. Cells were washed in PBS and resuspended in 9.5ml of RSB. 0.5ml of freshly made Proteinase K (1mg/ml in RSB) warmed to 37C was added, followed by 1ml of 10% SDS. The tube was shaken gently at 37C for 4-6 hours. 5ml of 0.5M NaCl was added and the mixture extracted with UNC phenol and $CHCl_3$ 3 times, the phases being separated by centrifugation at 2500rpm for 5 minutes at RT. After the third extraction, the aqueous phase was extracted with ether until no further clearing was observed (at least 3 times). 2.5 volumes of ice cold ethanol were added and the DNA allowed to precipitate overnight at -20C, before being removed by twirling onto a flame-sealed pipette. The DNA was carefully washed in 70% EtOH and air dried, and resuspended in 10ml 0.1X SSC. The RNA was digested by incubating with 50µl 10mg/ml DNase-free RNase at 37C for 1 hour. 5ml of 0.5M NaCl was added, and the DNA was extracted with phenol/$CHCl_3$ and ether as before. The DNA was resuspended in 100-200µl TE to give a final

concentration of approximately 1μg/μl. The concentration was then determined by measuring its optical density at 260nm (A260), and adjusted by adding more TE if necessary. The purity of the DNA sample was checked by calculating the OD ratio A260/A280, which should be more than 1.7. DNA samples were stored at +4C.

Creation of DNA pools

As discussed later in Chapter 3.1.1, in order to detect a discriminatory polymorphism by RFLP analysis it may be necessary to cleave a large number of DNA samples with a wide range of restriction enzymes, and to use several different locus-specific probes. A previously described technique of pooling DNA samples in order to compare patients and controls provides a rapid and economical method for detecting association between polymorphic restriction fragments and HLA-linked diseases (Arnheim et al.1985). The initial RFLP analysis of DNA from patients and controls from the Grampian region of Northeast Scotland was performed using samples of DNA pooled in this manner. In order that differences between patients and controls which were independent of HLA-DR2, DR2-positive and DR2-negative pools were included in the analysis. Four pools (MS DR2+ve, MS DR2-ve, Control DR2+ve and Control DR2-ve) were constructed by mixing equal quantities of individual DNA samples and allowing these to equilibrate at +4C for several days. The composition of these pools in terms of DR alleles is shown in Table 13.

DNA for PCR amplification

Two methods were used to obtain relatively crude preparations of genomic DNA suitable for amplification in the polymerase chain reaction. In the first method, a frozen pellet of peripheral blood lymphocyte nucleii was buffered in 0.1M NaCl/0.05M EDTA (SEDTA) and lysed by the addition of 60μl of 20% SDS and 150μl of 5M Na perchlorate, followed by 500μl each of phenol and $CHCl_3$. The DNA was extracted with phenol and $CHCl_3$ in the usual way and resuspended in 100μl TE. In the second method, cells were washed in PBS and resuspended in TE at a concentration of $5x10^5$ cells per 100μl. The cells were then boiled in a sealed tube for 10 minutes and spun hard for 10 minutes to pellet the denatured proteins. The DNA in the supernatant was quantitated by electrophoresing 10% of the sample on an analytical minigel. The theoretical yield is 5pg DNA per cell and $2x10^5$ cells should give about 1μg.

This method gave high molecular weight DNA comparable to that obtained by the other methods, as shown in Figure 7.

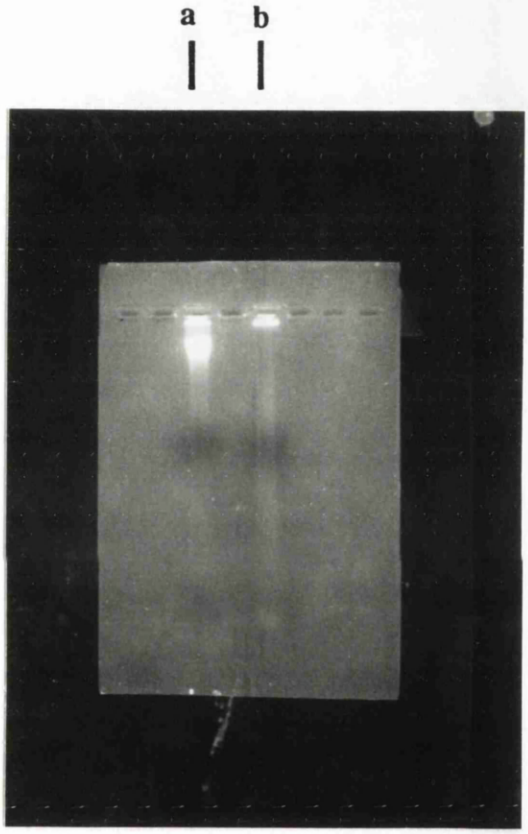



**Figure 7**

Photograph of mini 0.6% acrylamide gel showing the electrophoretic appearance of genomic DNA prepared by (a) the standard method, including digestion of RNA, and (b) rapid boiling of cells. Both methods resulted in DNA of high molecular weight

| | Pools | | | |
|---|---|---|---|---|
| Allele | MS DR2+ | Control DR2+ | MS DR2- | Control DR2- |
| DR1 | 2 | 0 | 28 | 5 |
| DR2 | 71 | 65 | - | - |
| DR3 | 10 | 10 | 39 | 29 |
| DR4 | 13 | 12 | 11 | 22 |
| DR5 | 0 | 3 | 6 | 42 |
| DRw6 | 0 | 0 | 0 | 10 |
| DR7 | 4 | 10 | 11 | 15 |
| DRw8 | 0 | 0 | 6 | 3 |

**Table 13**
The HLA-DR allele composition (percent) of the DNA pools constructed from individuals from the Grampian region.

### 2.2.1.4 Preparation of probe DNA

All the probes used in these studies were cDNA fragments cloned into suitable plasmid vectors in E. coli. These bacterial stocks were stored in glycerol at -20C.

Miniprep

5ml of LB medium with an appropriate antibiotic (50mg/µl ampicillin or 15µg/ml tetracycline) was inoculated with a single bacterial colony and grown overnight with shaking at 37C. 1.5ml of this culture was transferred to an Eppendorf tube and the bacteria pelleted (the remainder of the culture was stored at +4C). The supernatant medium was aspirated, the pellet resuspended in 100µl ice cold GTE buffer and stood for 7 minutes. After the addition of 200µl freshly prepared 0.2M NaOH/1% SDS, the contents were mixed by rapidly inverting the tube a few times, and then stored on ice for 5 minutes. 150ml ice cold 5M potassium acetate was added, the tube vortexed gently for 10 seconds, and stored on ice again. The denatured protein was pelleted by centrifugation and the supernatant transferred to a fresh tube before being extracted once with phenol:$CHCl_3$ and once with $CHCl_3$. Plasmid DNA was precipitated by adding 2 volumes of 100% ethanol at RT and standing for 5 minutes. The tube was centrifuged for 10 minutes, the supernatant carefully poured off, and the precipitate washed once with 1ml 70% ethanol. The pellet was air dried and resuspended in 25µl TE buffer. 10µl of this solution was sufficient for an analytical restriction enzyme digestion.

Single banded plasmid preparation

A single bacterial colony was picked into 5ml LB medium with antibiotic and cultured overnight as before. 250ml LB medium was inoculated with 0.5ml of this culture and shaken overnight at 37C (or until A550 > 0.7). The bacteria were pelleted by centrifugation at 5000 rpm, resuspended in 5ml ice cold GTE and transferred to a 50ml tube. The culture flask was rinsed with a further 2ml GTE containing 18mg/ml lysozyme which was added to the 5ml bacterial suspension, and the mixture stood on ice for 10 minutes. 14ml 0.2M NaOH/1% SDS was added, the tube inverted several times, and stored on ice for 10 minutes. Then 10.5ml 5M potassium acetate was added, the contents mixed by vigorous shaking, and stored on ice for

15 minutes. The tube was spun at 7000 rpm for 30 minutes at +4C to remove the denatured protein, and the supernatant containing the plasmid DNA transferred to a clean 50ml tube. The DNA was precipitated by adding 18ml isopropanol and spinning at 7000 rpm for 30 minutes at RT. The isopropanol was poured off and the pellet resuspended in 10ml TE buffer, warming to 37C if necessary. The RNA was removed by incubating with 500µg DNase-free RNase at 65C for 15 minutes. The plasmid solution was then extracted twice with phenol:$CHCl_3$ and once with $CHCl_3$, and the DNA precipitated by the addition of 1ml 3M sodium acetate and 2.5 volumes 100% ethanol, and standing for 15 minutes at RT. The DNA was pelleted by spinning at 7000 rpm for 30 minutes and resuspended in 3.5ml TE. To this solution in a 205g tube, and in a darkened room, was added 3.7g cesium chloride and 0.4ml ethidium bromide. The OD was adjusted to 1.3860, and the solution pre-spun at 10000 rpm to remove any precipitate. The supernatant was then transferred to a small Quickseal tube (Beckman), topped up with extra CsCl/EtBr mix, and sealed. The tube was then spun overnight at 54000 rpm in a Beckman VTi65 rotor for approximately 18 hours at +20C. The following day, the plasmid band was visualized under UVA illumination and removed by puncturing the side of the Quickseal tube immediately below the band with a 20G needle attached to a 2ml syringe. The EtBr was removed by extraction with butanol until no further clearing of the sample was seen. The CsCl and other salts were removed by dialysing the sample against 3x1 litre changes of TE over 48 hours at +4C. Finally the plasmid concentration was estimated (by A260 and polyacrylamide gel electrophoresis) and the solution stored at -20C.

Isolation and purification of DNA fragments for use as probes

The cloned insert was removed from its plasmid vector by digesting to completion with the appropriate restriction enzyme or enzymes, as shown in Table 12. Methods and conditions for performing restriction enzyme digestion of DNA are described in Chapter 2.2.1.6 below, and the restriction enzymes used in these studies , together with the appropriate buffers, shown in Table 14. After digestion, 10% of the digestion reaction mixture was separated on a 0.9 - 1.2% agarose gel, stained with EtBr and examined under UVA light to check that digestion was complete. In the case of incomplete digestion, more enzyme was added; however, when incomplete digestion was thought to be the result of the presence of

enzyme inhibitors, the plasmid was further purified by phenol:$CHCl_3$ extraction and ethanol precipitation as before. Sometimes, digestion could be enhanced by the addition of spermidine to a final concentration of 1.0mM. When digestion was complete, the DNA was separated by electrophoresing in a 1.2% preparative agarose gel. After separation, the fragment band was removed from the gel by cutting with a scalpel blade and inserted lengthwise into a short piece of buffered dialysis tubing containing 1-2ml filtered TBE buffer. The tubing was sealed at each end, with the gel slice positioned along one side, and placed in an electrophoresis tank containing 1x TBE with the gel slice towards the negative electrode. The tubing was electrophoresed at 100V for 30-45 minutes (according to the fragment size). Under UVA light the DNA can be seen to pass out of the gel and lie along the +ve side of the tubing. The polarity of the tank was then reversed for 30-60 seconds and the buffer in the tube removed by pipetting. The tubing was then rinsed with a further 1ml TBE and the gel slice checked under UV light for complete elution of the fragment.

After electroelution the fragment was purified by DE52 ion exchange chromatography. A 2ml syringe barrel was plugged at the narrow end with siliconized glass wool and filled with 0.5ml DE52 resin suspension. The column was washed with 10ml TBE and the DNA solution then applied to the column. The column was then washed as before with TBE and the purified DNA eluted from the column with 2 x 0.5ml volumes of DE52 elution buffer. The DNA in the eluted samples was ethanol precipitated (following the addition of yeast tRNA in the case of fragments smaller than 500bp) and resuspended in 200µl TE buffer. It was then phenol:CHCl3 extracted in the usual manner and precipitated with 95% ethanol with 0.3M Na acetate, spun, washed with 70% ethanol and resuspended in TE (approximately 20µl) to give an estimated final concentration of 100ng/µl. Finally, the fragments were quantitated by electrophoresis (PAGE for fragments smaller than 1kb or agarose gel electrophoresis for larger fragments), and stored at -20C.

### 2.2.1.5 Radiolabelling of probes

DNA fragments were radiolabelled to high specific activity by random hexamer substitution, using a modification of a previously described method (Feinberg, Vogelstein, 1983). 25ng of DNA fragment is sufficient for probing a single 20cm x 20cm nitrocellulose membrane. 250pg of

Lambda phage (λ) DNA per membrane was also included in the reaction in order to provide accurate molecular weight markers (see below). In a 250µl Eppendorf tube with a perforated cap, 25ng of fragment and 250pg λ DNA was denatured by boiling for 2 minutes, stood on ice for 5 minutes and spun down. To the side of the tube was added 5µl 5x OLB buffer, 8.7µl $ddH_2O$ and 4µl $\alpha^{32}$- dCTP at 10µCi/µl (>3000Ci/mM; Amersham), followed by 1.3µl Klenow polymerase (2units/µl; BRL). The labelling reaction was carried out at RT for at least 3 hours. It was found that this method labelled probes to a specific activity of $10^8$ - $2x10^9$ cpm/µg. The entire reaction mixture was used for probing the membrane since it was found that removal of excess unincorporated nucleotides was not necessary.

### 2.2.1.6 Restriction enzyme digestion of DNA

The restriction endonucleases used in these studies are shown in Table 14, which also indicates the most suitable standard buffer for each enzyme. The digestion reaction was set up as follows: 10-15µg genomic DNA, 5µl of the appropriate restriction endonuclease buffer, $ddH_2O$ to 50µl (see below), 2 units of restriction enzyme per µg DNA. The enzyme was added last, and the contents mixed by vortexing for a few seconds and then spun down. If very viscous, genomic DNA was pipetted using a cut-off plastic tip. Spermidine was not used in restriction digests of genomic DNA. The reaction was incubated for 90 minutes at 37C (65C in the case of Taq 1), after which a second aliquot of enzyme was added (1.5 units per µg DNA) and digestion continued for at least a further hour. The complete digestion of the DNA was confirmed by running 10% of the reaction on a 0.8% agarose analytical minigel for approximately 60 volt hours. To any incompletely digested samples a further 2 units enzyme per µg DNA was added and incubated for 60 minutes. The digested samples were either electrophoresed immediately or stored at -20C for later use.

The capacity of wells in the agarose gel resulting from use of the comb and volume of agarose employed here is approximately 20µl, and the digested samples were therefore concentrated prior to loading using a speed vacuum centrifuge (Savant) to a final volume of 16µl.

### 2.2.1.7 Electrophoresis and Southern blotting

The digested DNA samples were separated by electrophoresis in 0.7% agarose gels in TAE buffer. 2.1g agarose was dissolved in 300ml 1xTAE by boiling and EtBr was added to a concentration of 0.5µg/ml. The open ends

| | | | |
|---|---|---|---|
| Bam H1 | M | Nci 1 | L |
| Bgl 11 | M | Pst 1 | M |
| Eco R1 | H | Pvu 11 | M |
| Eco RV | * | Sac 1 | L |
| Hinf 1 | M | Sau96 1 | M |
| Kpn 1 | L | Sma 1 | S |
| Msp 1 | M | Taq 1 | H |

**Table 14**
The 14 restriction enzymes used in these studies with their recommended buffers. Eco RV does not cut well in any of these standard buffers and a buffer provided by the manufacturer (BRL) was used.

of the gel-forming tray were closed by sealing tape, the comb placed in position and the gel cast. Polymerization was complete after 60 minutes at RT, and the gel placed in position in an electrophoresis tank (BRL) containing 2 litres 1xTAE. 4µl loading buffer was added to each concentrated DNA digest and these samples heated to 80C in a water bath for 5 minutes. They were then spun in a microfuge for a few seconds, remixed by pipetting and stood on ice before being loaded. The outermost wells in the gel were loaded with 10ng of a suitable molecular weight marker (λ phage digested with either Hind111 or BstE11/Kpn1). These λ fragments are detected by hybridization with radio-labelled λ DNA mixed with the probe. The samples were separated by electrophoresing for 800-1000 volt hours (typically 40v for 20 hours).

At the end of this time, the DNA was transferred to nylon membranes according to the method described by Southern (Southern, 1975). The gel was carefully removed from the tank, cut at the level of the wells under UV light, and photographed. It was then gently agitated in 0.25M HCl/0.6M NaCl for 10 minutes to "nick" the DNA and facilitate the transfer of the larger fragments to the membrane. The acid was removed by rinsing in $dH_2O$, and the DNA denatured by incubating the gel for 1 hour in 0.4M NaOH/0.8M NaCl, and then neutralized in 0.5M Tris-HCl(7.6)/1M NaCl. A piece of Gene Screen Plus (NEN) membrane was cut to exactly the same size as the gel and prewetted first in $ddH_2O$ and then in 10xSSC. Several (10) sheets of Whatman 3MM paper were cut to 25x25cm and soaked in 10xSSC. These were placed on the benchtop to act as a reservoir of buffer for the DNA transfer. The neutralized gel was carefully laid on the reservoir and any bubbles removed by rolling with a glass pipette. The wetted membrane was then laid on top and bubbles eliminated as before. Two sheets of dry 3MM paper were placed on the membrane, followed by a stack of absorbent paper towels, a glass plate, and a 0.5kg weight. The protruding edges of the reservoir were covered with Parafilm. This arrangement is shown diagrammatically in Figure 8. In some experiments, Hybond-N (Amersham) membranes were used. Due to its different properties, DNA can be efficiently transferred to this material by alkaline transfer without prior denaturation. After electrophoresis, the gel was depurinated in 0.25M HCl as before, neutralized and blotted directly with 0.4M NaOH. In each case, the DNA was allowed to transfer overnight. Figure 9 demonstrates that the transfer of DNA by this method is virtually complete. After transfer, the stack was disassembled, incubated

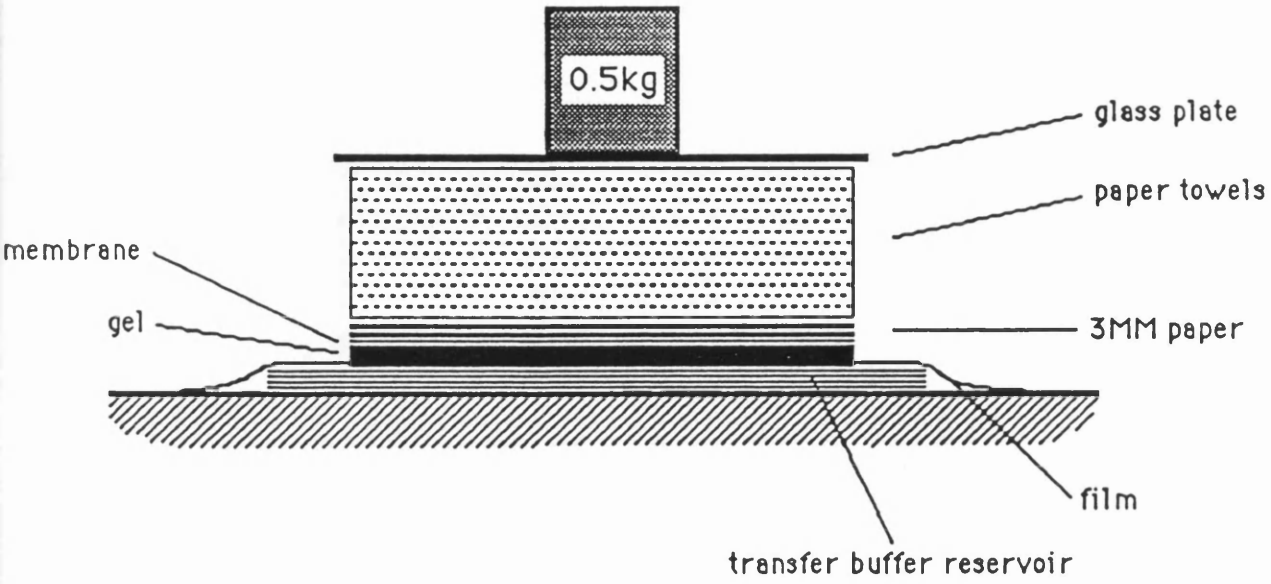
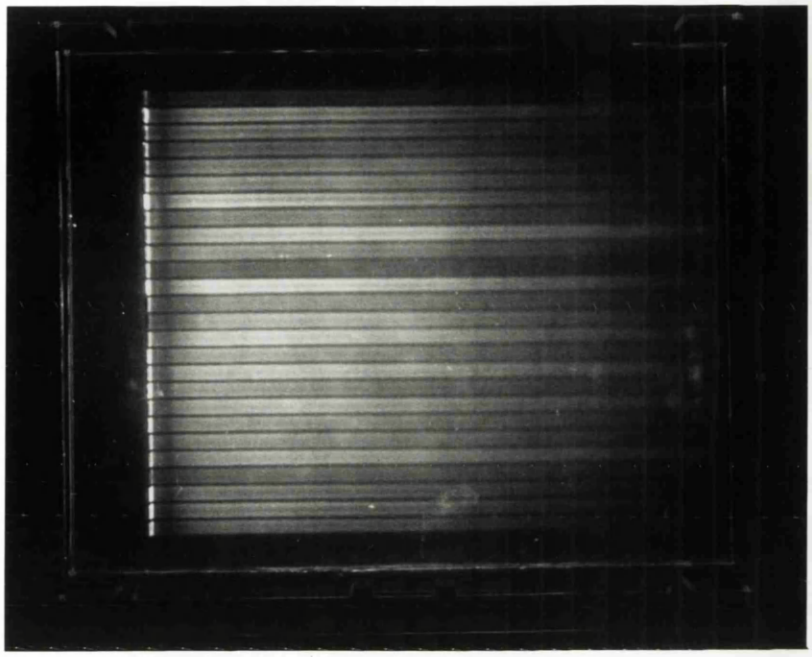



**Figure 8**
Diagram showing the method for transferring DNA fragments to a nylon membrane by Southern blotting

(a)



(b)

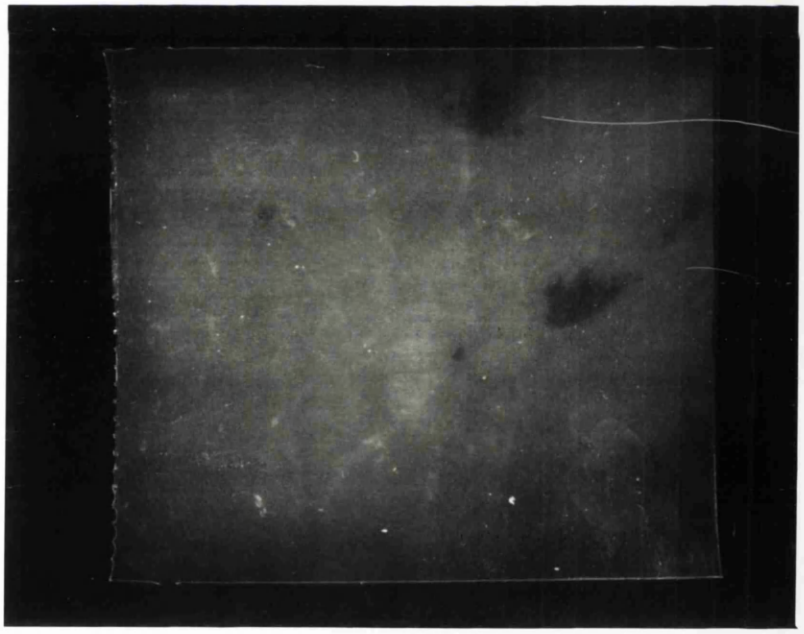


**Figure 9**

Showing the electrophoretic separation of EcoR1-digested DNA in 0.7% agarose, stained with ethidium bromide and photographed under UV light, (a) before Southern blotting, and (b) after transfer.

in 0.4M NaOH for 30-60 seconds (Gene Screen Plus), rinsed in 0.2M Tris-HCl (7.5)/2xSSC (Gene Screen Plus and Hybond-N) and allowed to air dry. Neither Gene Screen Plus nor Hybond-N membranes require the DNA to be fixed by baking or UV light exposure. The membranes were stored dry and in the dark in protective plastic envelopes.

**2.2.1.8 Hybridization, washing and autoradiography; rehybridization**

The membrane was placed flat into a plastic envelope into which 20ml hybridization buffer (per membrane) was poured, and the envelope sealed. The membrane was incubated at 37C for 1-2 hours ("prehybridization"). At the end of this time, 25ng of the labelled double stranded probe to be used (and containing 250pg radiolabelled λ phage)was added to 500ml ssDNA (10mg/ml), boiled for 10 minutes, and immediately put on ice. It was then mixed with 20ml fresh hybridization buffer. The prehybridization buffer was removed from the envelope and replaced with the buffer containing the labelled probe, any bubbles were expelled, the envelope was re-sealed and the contents evenly distributed by gentle rolling. Hybridization was carried out in a shaking water bath at 42C overnight. Up to 6 membranes could be hybridized simultaneously in a single envelope in this way.

The envelope was opened by cutting, and the buffer poured back into a screw-cap tube and stored at -20C. The membrane was removed and rinsed twice, with gentle rubbing, in 2x SSC at RT to remove remaining buffer containing unincorporated nucleotide. It was then washed twice in low stringency conditions (2x SSC/0.5% SDS for 15 minutes at 65C), followed by 2 washes in high stringency conditions (0.2x SSC/0.5% SDS for 15 minutes at 65C). The membrane was finally rinsed in 0.2x SSC at RT and laid on a sheet of Whatman paper to dry. It was covered with Saran wrap, placed in a suitably sized X-ray cassette with 2 $Li^+$ intensifying screens, and exposed to Kodak XAR-5 film at -80C overnight. Depending of the result of this, a longer exposure of between 2 and 5 days was then made. The exposed films were developed automatically.

After a satisfactory exposure had been obtained, the probe was stripped from the membrane in order that a second probe might be used. This was carried out by incubating the membrane with agitation in 0.4M NaOH for 30 minutes, followed by neutralization in 0.2M Tris-HCl(7.5)/0.5% SDS/0.1x SSC for 30 minutes. An overnight exposure was made to check complete removal of the previous probe. The membrane

was stored in a sealed plastic bag and was not allowed to dry out completely before the next hybridization.

### 2.2.1.9 Analysis of fragment patterns and frequencies

Fragment sizes

The migration of each fragment, corresponding to a dark band on the autoradiograph, was measured as its distance in cm from its origin at the well. The migration distances of the MW marker fragments were also recorded, and from them the sizes of the restriction fragments estimated. A visual correction was made for any distortions in the gel.

Fragment frequencies

In the studies of individual samples of DNA, large numbers of restriction fragments were analysed (Chapter 3.1.2). The complete repertoire of possible restriction fragments for a given enzyme-probe combination was determined by inspection, and the presence (1) or absence (0) of each fragment in each individual was recorded on a chart (see Fig. 2.2.1.9) and stored as a text file on a personal computer.

Statistical analysis

Stepwise logistic regression analysis of data was performed by the Department of Physics at the Royal Postgraduate Medical School, using BMDP Statistical Software. The significance of any deviations from chance were tested in goodness-of-fit Chi-square tests.

## 2.2.2 MONOCLONAL ANTIBODY STAINING AND EPICS ANALYSIS

### 2.2.2.1 Reagents

The monoclonal antibodies used in these studies are shown in Table 15. Two mouse anti-human framework antibodies, directed against monomorphic determinants of DR (L243) and DQ (Tü22), were used as positive controls. 3JP is an anti-murine ($A\alpha^d$) antibody which was included as a negative control antibody. HU46 (Ishikawa et al.1987) has been shown to react against the DQw4 α:β heterodimer (Endo et al.1987), and was the gift of H. Inoko. L243, 3JP and HU46 were hybridoma culture supernatants and were used undiluted. Tü22 was a commercial preparation and was diluted for use in PBS/2.5% FCS. The second layer antibody was fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin (FITC SaM; Amersham, UK) diluted 1:50 in PBS/2.5% FCS.

### 2.2.2.2 Methods

Approximately $10^6$ cells were used for each staining. The cells to be stained were harvested from culture, counted and divided as appropriate for staining with either the test antibody, control antibodies (positive and negative), or negative control (medium only). The cells were centrifuged at 1500rpm for 7 minutes and the pellets resuspended in 100µl of the appropriate mAb by vortexing. Cells were incubated at 4C for 30 minutes and washed twice in PBS/2.5% FCS. The pellets were then resuspended in 100µl 1:50 dilution sheep anti-mouse FITC and incubated for 30 minutes at 4C. The cells were then washed a further 3 times and resuspended in 500µl ice cold PBS/FCS before being analyzed immediately. If there was any delay before the cells could be analyzed, the final wash was performed using PBS without serum, and the cells were resuspended in 1% paraformaldehyde at 4C.

Phenotypic analysis of the stained cells by flow cytometry was carried out in the Department of Immunology, Royal Postgraduate Medical School by Miss Maria Daly, using an EPICS Profile Analyzer (Coulter Electronics Corporation). Cells were analyzed by size, granularity and antigen density by forward light scatter, $90^o$ light scatter and log fluorescence intensity respectively. Electronic gating was used to isolate individual cell populations for analysis. Negative controls (cells either stained with an irrelevant antibody or incubated with FITC-SaM alone)

| mAb | Specificity | Reference |
|---|---|---|
| L243 | anti HLA-DR monomorphic | Finn & Levy, 1982 |
| 3JP | anti murine $A\alpha^d$ | |
| Tü22 | anti HLA-DQ monomorphic | Pawelec et al., 1982 |
| Leu10 | anti HLA-DQ monomorphic | Chen et al., 1984 |
| HU46 | anti DQ'Wa' | Ishikawa, 1987 |
| anti-DP | anti HLA-DP monomorphic | Watson et al., 1983 |

**Table 15**

The monoclonal antibodies used in these studies. 3JP is a murine antibody included as a negative control; L243 and Tü22 are anti-framework antibodies and served as positive controls for the presence of DR and DQ. HU46 was originally raised against the homozygous cell line EBV Wa (HLA DR4, Dw15, DQ blank) and shown by 2D gel analysis to precipitate a DQ molecule. It was shown also to react against certain DRw8, DQ blank cell lines. This new DQ specificity was originally named DQ Wa but has been renamed DQw4 (XIHWS). Leu10 and an anti-DP monomorphic antibody were used in the mAb T cell clone inhibition studies described in Chapter 3.2.2.

were first analyzed and an electronic gate set above the level of the observed background count. Cells registering above this level were regarded as positively stained. Fluorescence histograms of the cell number (y-axis) versus log fluorescence intensity (x-axis) were generated by computer integration of the number of positive cells in the window. The antigen density was determined by calculating the mean cell fluorescence intensity (MFI).

An alternative method of staining was carried out using 96-well U-bottom plates. Cells were harvested and counted as before. They were spun down at 1600rpm for 7 minutes and resuspended in 200µl DMEM containing 5% FCS. This was divided into 2 x 100µl volumes in non-adjacent wells in a 96-well plate (Nunc). The plate was spun at 1100rpm for 2 minutes, the supernatant removed by inverting the plate and flicking sharply, and the pellets resuspended in either 100µl mAb or 100µl DMEM/5% FCS. The cells were incubated for 30 minutes on ice, spun, washed twice in 200µl PBS/5% FCS and resuspended in 100µl diluted FITC. After 30 minutes' incubation the cells were washed three times more and resuspended in 500µl PBS/FCS as before.

## 2.2.3 GENOMIC AMPLIFICATION BY THE POLYMERASE CHAIN REACTION

### 2.2.3.1 The polymerase chain reaction

A method for enzymatically amplifying double stranded DNA sequences was first described in 1985 (Saiki et al.1985). It has since been improved by the use of thermostable polymerase derived from the thermophilic organism *Thermus aquaticus* . The technique is simple and economical, and is highly specific for the intended target sequence. There are many ways in which the amplified sequence can be used. For instance, it can be labelled and used to probe other sequences; it can itself be probed with an oligonucleotide (Saiki et al.1986); it provides an elegant method for performing site-directed mutagenesis; and it can be cloned and sequenced (Scharf et al.1986).

The PCR reaction is shown schematically in Figure 10. It amplifies DNA *in vitro* by means of repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by the Klenow fragment of DNA polymerase, resulting in an exponential increase in copies of the region flanked by the primers. The method is particularly useful for amplifying genes which exhibit allelic polymorphism, and it is a precondition of the method that the sequence to be amplified is flanked by non-polymorphic regions. The target sequence can be between 90 and 1000 bp in length (Dr M Jones, personal communication) and preferably should not be C/G rich. Oligonucleotide primers which flank the target sequence are chosen, one being homologous to the 5' end of the single stranded target sequence and the other being homologous to the reversed and complementary 3' sequence, as shown for HLA-DQA1 in Figure 11. The primers should be 17-25 bases in length, should have a C/G content of 50-65%, and should not contain any internal complementary sequence which might lead to hairpin formation. The primers can be modified at their 5' ends to produce convenient restriction sites (linkers) for cloning directly into the M13 sequencing vector. A PCR reaction mixture contains a small quantity of double stranded DNA template (as little as 10ng is sufficient), an excess of each oligonucleotide primer, free nucleotides, a suitable buffer and Klenow. The mixture is subjected to repeated cycles of temperature variation, conveniently performed now by a microprocessor-controlled thermal cycler (Perkin-Elmer Cetus Corporation). The dsDNA template is

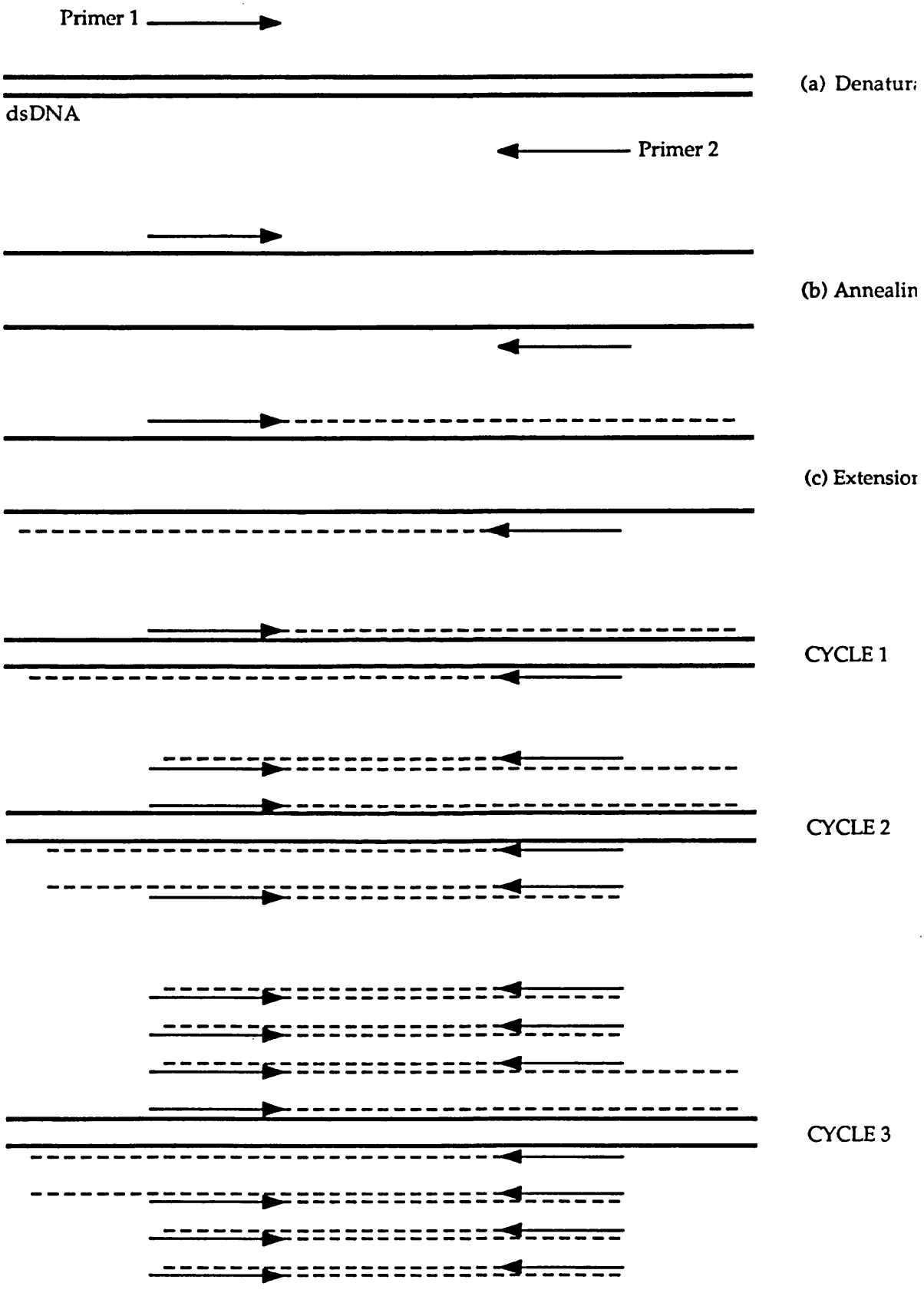



**Figure 10**

Showing the three stages of PCR i) denaturation, ii) oligonucleotide primer annealing and iii) primer extension; and three cycles of PCR showing the exponential amplification of the fragment spanned by the primers. See text for details.


**Figure 11**

Oligonucleotide primers for PCR amplification. The primers GH26 and GH27 are shown together with the sequence of the gene encoding the α2 exon of DQA1 in a DR3 subject, from Hurley et al. (1988). The primers are completely homologous to the target strand, but have been extended to produce restriction sites for Pst1 (GH26) and BamH1 (GH27) to facilitate directional cloning into M13. The homologous regions of the primers are shown in uppercase and the linkers in lowercase letters.

first denatured at 94C for 10 minutes. The primers are then allowed to anneal to the two single strands at a temperature below the $T_M$ of the primers (eg. 48C for 2 minutes), the primers are extended by the polymerase at 68-72C for 2-5 minutes, and the new dsDNA denatured once again at 94C. It has been shown that nearly 1 pM, or >1μg, of a 240 bp fragment is produced in this way after 25 cycles (equivalent to an enrichment of $>2x10^5$ over unamplified genomic DNA) (Gyllensten, Erlich, 1988).

### 2.2.3.2 Materials and reagents

| 10X PCR BUFFER | |
|---|---|
| KCl | 500mM |
| Tris-HCl (pH8.3) | 100mM |
| $MgCl_2$ | 15mM |
| gelatin | 0.1% (w/v) |

| dNTP MIXTURE | |
|---|---|
| dATP | 10mM |
| dCTP | 10mM |
| dTTP | 10mM |
| dGTP | 10mM |

### 2.2.3.3 Methods

For amplifying DQA1 α2 exon sequences, the oligonucleotide primers GH26 and GH27 were synthesized using a Cyclone DNA Synthesizer (Biosearch Inc.) in the Department of Clinical Chemistry, Royal Postgraduate Medical School. The oligonucleotide was removed from the solid phase by incubating with 1.5 ml "880" $NH_4OH$ (BDH) at RT for 2 hours. The $NH_4OH$ was then drawn off, sealed into an airtight 2 ml tube with screw cap, and incubated at 55C overnight. The sample was evaporated to dryness in a vacuum centrifuge and resuspended in 50μl TE8 buffer. Genomic DNA was prepared as described previously and diluted to a concentration of 500μg/ml. In an Eppendorf tube were mixed 2μl genomic DNA template, 16μl dNTP mix, 10μl PCR buffer, 69.5ml $ddH_2O$ and 1μl each of the two primers GH26 and GH27. The tube was heated to 94C for 10 minutes to denature the template. The tube was then centrifuged for 10 seconds to remove the condensate, and 0.5μl Taq polymerase (Cetus) was added to the side of the tube. It was then centrifuged again and 100μl mineral oil was carefully layered on top of the mixture. The tube was subjected to 35 cycles each comprising annealing (45C for 2 minutes), extension (70C for 3 minutes) and denaturation (94C

for 2 minutes) steps. The reaction mixture was then extracted once with phenol:$CHCl_3$ and once with $CHCl_3$, ethanol precipitated and resuspended in 100µl TE8. The concentration of the fragment was determined by running 5-10µl on an analytical minigel with a suitable marker (eg. Hae111ΦX). It was found that the conditions described gave up to 2.0µg of a 242 bp DQA1 fragment (see Figure 12).

## 2.2.4 OLIGONUCLEOTIDE PROBING

### 2.2.4.1 Reagents

PREHYBRIDIZATION BUFFER

| | |
|---|---|
| SSC | 6x |
| Denhardt's | 5x |
| SDS | 0.5% |
| salmon sperm DNA | 100µg/ml |
| Na pyrophosphate | 0.05% |

HYBRIDIZATION BUFFER

| | |
|---|---|
| SSC | 6x |
| Denhardt's | 5x |
| yeast tRNA | 20µg/ml |
| Na pyrophosphate | 0.05% |

OLIGONUCLEOTIDE WASHING BUFFER

| | |
|---|---|
| SSC | 6x |
| Na pyrophosphate | 0.05% |

### 2.2.4.2 Allele-specific oligonucleotide probes

By amplifying a fragment of genomic DNA with PCR and probing the product with a labelled allele-specific oligonucleotide (ASO), the presence of a particular sequence can be detected with extreme sensitivity and specificity (Saiki et al.1986; Baxter-Lowe et al.1988b). ASOs for detecting alleles of HLA-DQA1 were chosen from the known allelic sequences, as shown in Figure 13 (Horn et al.1988). Three ASOs were designed in order to detect DQA1, DQA3 and DQA4 alleles of DQA. Their sequences are shown below:

| | |
|---|---|
| RHA1 | 5'- TGAGTTCAGCAAATTTG -3' |
| RHA3 | 5'- TTCCGCAGATTTAGAAGATTT -3' |
| RHA4 | 5'- GTTTGCCTGTTCTCAGA -3' |

The melting temperatures ($T_M$) of the probes, calculated according to the formula $T_M = 2x[A+T] + 4x[G+C]$, were 46C (RHA1), 56C (RHA2) and 50C (RHA3).

#### 2.2.4.3 Methods

The ASOs were synthesized as described previously and stored at -20C in TE at a concentration of 500ng/µl. 400ng of the appropriate ASO was end-labelled by phosphorylation at the 5'-terminus with [$\gamma^{32}$P]-dATP (Amersham PBK8094; >5000 Ci/mmole) and T4 polynucleotide kinase (Anglia). The labelling reaction was set up as shown in a volume of 25µl and incubated at 37C for 1 hour:

| | |
|---|---|
| oligonucleotide | 400ng |
| 50mM $MgCl_2$ | 10µl |
| 1M Tris-HCl (7.6) | 5µl |
| 200mM 2-mercaptoethanol | 5µl |
| [$\gamma^{32}$P]-dATP | 100µCi |
| T4 polynucleotide kinase | 10 units |

The whole reaction mixture was used for hybridization without any purification to remove unincorporated [$\gamma^{32}$P]-dATP.

DQα2exon (DQα1 domain-encoding) gene sequences were amplified from genomic DNA as described in Chapter 2.2.3.3 using the primers GH26 and GH27. 100ng of the resulting fragment was electrophoresed in a 1.2% agarose gel in TAE buffer and transferred under alkaline conditions to Hybond-N membrane. The membrane was washed in 3xSSC at RT, sealed in a plastic envelope and prehybridized in 5-10ml pre-boiled prehybridization buffer at 37C for 1 hour. The prehybridization buffer was then removed and replaced by 10ml hybridization buffer containing the end-labelled ASO. The membrane was incubated with gentle agitation at the hybridization temperature ($T_H$) calculated as [$T_M$ - 5] degrees for at least 3 hours. At the end of this time the membrane was washed twice for 30 minutes in 6xSSC/0.05% Na pyrophosphate at exactly the melting temperature ($T_M$) of the oligonucleotide. The membrane was air dried and exposed to Kodak XAR-5 film, a satisfactory autoradiograph being achieved within 30-240 minutes.

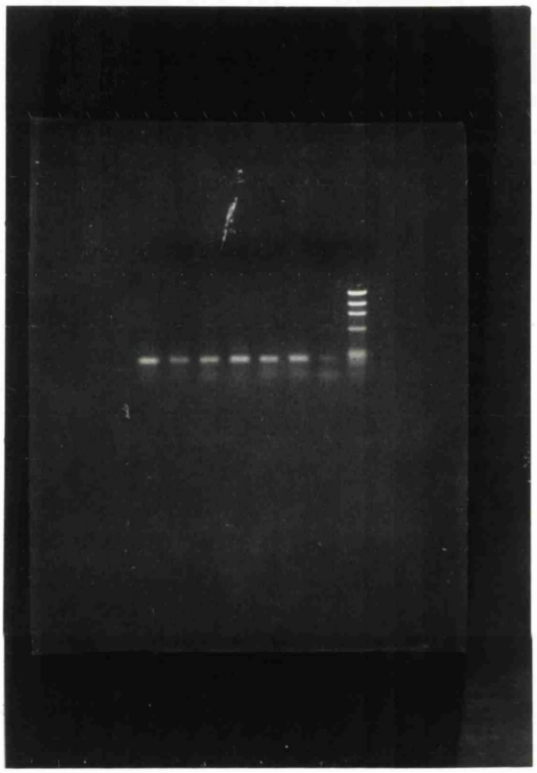



**Figure 12**
Showing the amplification of a 242 base pair DQA1 fragment by PCR from 6 heterozygous cell lines and one homozygous cell line. The marker lane contains ΦX174-Hae111

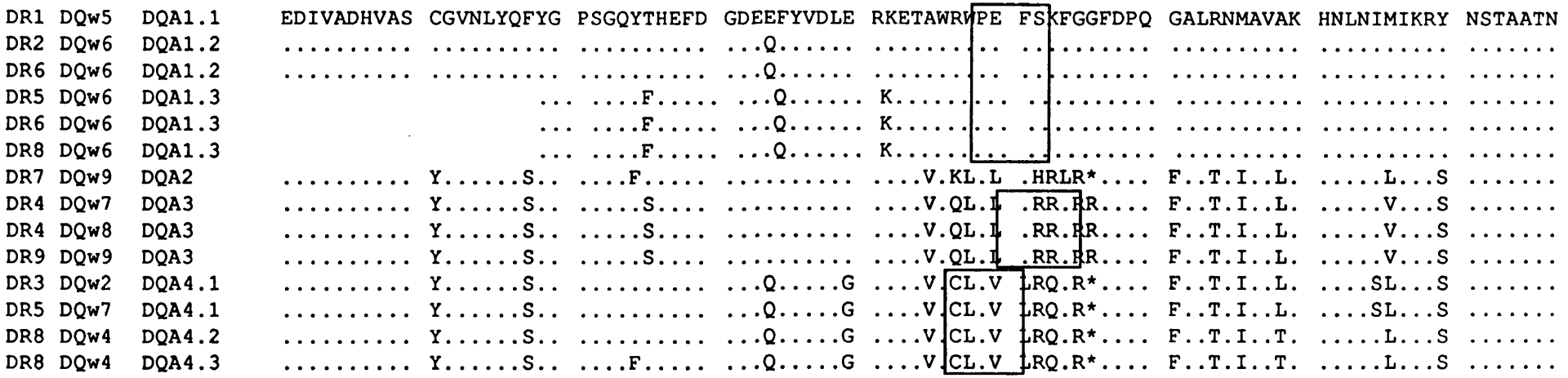





Figure 13

Alignment of HLA-DQA protein sequences showing the regions corresponding to the allele-specific oligonucleotide probes RHA1, RHA2 and RHA3. RHA1 is equivalent to DQA1 between positions 51-54, RHA3 to DQA3 (51-56), andRHA4 to DQA4 (48-52). See text for further details of ASO probes. (Sequences from Horn et al. (1988) Proc. Natl. Acad. Sci. USA, 85:6012-6016)

## 2.2.5 cDNA NUCLEOTIDE SEQUENCING

### 2.2.5.1 Summary

The nucleotide sequences of HLA-DQA1 α2 exons were determined in a number of heterozygous patients by cloning PCR-amplified gene fragments into the sequencing vectors M13 mp18 and mp19, and sequencing using the dideoxynucleoside method of Sanger. Briefly, PCR amplification reactions using the primers GH26 and GH27 were subjected to 35 cycles comprising 1 min denaturation at 94 C, 2mins annealing at 45 C and 3 mins primer extension at 70 C. The reaction mixture was then ethanol precipitated and resuspended in 10mM Tris/0.1mM EDTA, pH8.0. In later experiments it was found that overnight dialysis at this stage, presumably to remove remaining deoxynucleotides, increased the efficiency of ligation. The fragments were then quantitated by mini-acrylamide gel electrophoresis and digested with BamH1 and Pst1 under the appropriate conditions, extracted once with phenol, ethanol precipitated, resuspended and quantitated. The fragments were then ligated directionally into prepared M13 mp18 and mp19 phage (Norrander et al.1983), and transformed into *E. coli* TG2. After plating out, up to 50 plaques could be obtained, 70%-90% of which contained DQα sequences. Single-stranded templates were prepared and dideoxy sequencing was carried out using established protocols (Sanger et al.1977; Maniatis et al.1982). Sequencing reactions were electrophoresed in 0.4 mm 8% polyacrylamide gels with 8M urea at 80 watts for 1.5 and 2.5 hrs.

### 2.2.5.2 Materials and reagents

1M MAGNESIUM CHLORIDE

| | |
|---|---|
| $MgCl_2.6H_2O$ | 203.3g in 1000ml |

TE8

| | |
|---|---|
| Tris-HCl pH 8.0 | 10mM |
| $Na_2EDTA$ pH 8.0 | 0.1mM |

10x TM

| | |
|---|---|
| Tris-HCl pH 8.0 | 100mM |
| $MgCl_2$ | 100mM |

10x CIP BUFFER

| | |
|---|---|
| Tris-HCl pH 8.0 | 0.5M |
| EDTA | 1mM |

TRANSFORMATION SOLUTION I

| | |
|---|---|
| $MgCl_2$ | 0.1M |
| $MgSO_4$ | 0.05M |

TRANSFORMATION SOLUTION II

| | |
|---|---|
| $CaCl_2$ | 0.1M |

10x LIGATION BUFFER

| | |
|---|---|
| Tris-HCl pH 7.5 | 0.5M |
| $MgCl_2$ | 0.1M |
| DTT | 10mM |

PHAGE DIP BUFFER I

| | |
|---|---|
| NaOH | 0.2M |
| NaCl | 1.5M |

PHAGE DIP BUFFER II

| | |
|---|---|
| Tris-HCl pH 7.5 | 0.4M |
| SSC | 2x |

PHAGE DIP BUFFER III

| | |
|---|---|
| SSC | 2x |

ACRYLAMIDE STOCK

| | |
|---|---|
| acrylamide | 29% |
| bisacrylamide | 1% |

Dissolve in $ddH_2O$. Store at $4^o$.

6% ACRYLAMIDE GEL

| | |
|---|---|
| ddH2O | 37.5ml |
| 20x TBE | 2.5ml |
| 10% $NH_4$ persulphate | 0.5ml |
| TEMED | 25ml |
| 30% acrylamide stock | 10ml |

10X SEQUENCING BUFFER

| | |
|---|---|
| Tris-HCl pH 7.5 | 66mM |
| $MgCl_2$ | 66mM |
| DDT | 66mM |

LOADING BUFFER FOR SEQUENCING GELS

| | |
|---|---|
| bromphenol blue | 0.2% |
| EDTA pH 8.3 | 0.2M |
| glycerol | 50% |

REAGENTS FOR SEQUENCING REACTIONS

Nucleotide solutions

Store all dNTPs at $-20^o$ as 20mM stocks and ddNTPs as 10mM stocks.

| $N^o$ mixes | $A^o$ | $G^o$ | $C^o$ | $T^o$ |
|---|---|---|---|---|
| 0.5mM dGTP | 20 | 1 | 20 | 20 |
| 0.5mM dCTP | 20 | 20 | 1 | 20 |
| 0.5mM dTTP | 20 | 20 | 20 | 1 |
| TE8 | 5 | 5 | 5 | 5 |

Make up solutions as shown (in µl) and store at $-20^o$.

ddNTP stops

| | |
|---|---|
| ddATP | 0.1mM |
| ddGTP | 0.05mM |
| ddCTP | 0.1mM |
| ddTTP | 1.0mM |

Dilute ddNTPs in TE8 from 10mM stocks and store at -20$^{o}$. These concentrations may have to be adjusted periodically to maintain good sequencing throughout the gel.

"Cold chase"

| | |
|---|---|
| dATP | 0.5mM |
| dGTP | 0.5mM |
| dCTP | 0.5mM |
| dTTP | 0.5mM |

BUFFERS FOR SEQUENCING

Formamide dye/stop solution

| | |
|---|---|
| deionized formamide | 98% |
| bromphenol blue | 0.2% |
| xylene cyanol | 0.2% |

Dissolve dyes in EDTA 0.5mM pH 8.0. Store at -20$^{o}$.

### 2.2.5.3 Cloning DNA fragments into M13

Genomic DNA was subjected to PCR amplification as described in Chapter 2.2.3.3 and the entire reaction mixture extracted once with phenol:$CHCl_3$, ethanol precipitated and resuspended in 25µl 10mM Tris/0.1mM EDTA (pH8). The fragment was then sticky ended by digestion with BamH1 and Pst1 in the following reaction:

| | |
|---|---|
| fragment | 2µl |
| 10x buffer | 2µl ("React 3"; BRL). |
| BamH1 | 10 units |
| Pst1 | 10 units |
| $ddH_2O$ | to 20µl |

Digestion was carried out at 37C for 1 hour and the enzymes inactivated by heating to 65C for 10 minutes. 3µg tRNA was added and the reaction was diluted in 80µl TE buffer, extracted once with phenol:$CHCl_3$ and ethanol precipitated in the presence of 0.2M NaOAc by minifuging for 15 minutes at RT. The ethanol was poured off and the pellet air-dried before resuspension in 25µl TE. The concentration was estimated by electrophoresing 3µl on a 1.2% agarose gel with Hae111ΦX174 or λBstE11 markers, and if necessary was adjusted to approximately 20ng/µl.

The vector used was commercially prepared RF M13 mp18 and mp19 (BRL), and was digested and dephosphorylated as follows. 10µg phage was digested with BamH1 and Pst1 in React 3 buffer in the same way as for the fragment. It was then diluted to 180µl with $ddH_2O$ and 20µl 3M NaOAc was added. It was extracted with phenol:$CHCl_3$ and $CHCl_3$, ethanol

precipitated and resuspended in 20µl TE. 2µl of this was saved for later use as a ligation control, and to the remaining 18µl was added 2µl 10x CIP (calf intestinal phosphatase, Boehringer) buffer followed by 1µl of a 1:20 dilution of CIP in TE. The tube was vortexed and centrifuged, and incubated for 30 minutes at 37C and then for 15 minutes at 45C. A further 1µl enzyme was added and the incubation steps repeated. The DNA was finally extracted and precipitated as before and resuspended in TE at a concentration of 20ng/µl.

Because of the high efficiency of directional ligation of sticky-ended fragments, the ligations were carried out using vector:insert ratios of 5:1 and 1:1. The reaction was set up as follows:

| | |
|---|---|
| dephosphorylated vector | 20ng |
| DNA insert | 4ng or 20ng |
| dd$H_2O$ | to 14µl |
| 10x ligation buffer | 2µl |
| 10mM spermidine | 2µl |

The tube was heated to 65C for 5 minutes and cooled to RT. 1µl 5mM ATP was added followed by 0.3µl T4 DNA ligase (400 units/µl; BRL). The contents were thoroghly mixed by vortexing and centrifugation, and incubated at RT overnight. The following control ligations were also set up:

1. insert control - insert known to have worked previously (provided by Dr A So)
2. dephosphorylation control - dephosphorylated vector without insert
3. vector control - non-dephosphorylated vector without insert

Ligations were stored at -20C until used.

#### 2.2.5.4 Bacterial transformation of E. coli TG2

A 5ml overnight culture of TG2 was grown from a single colony. A 1/100 dilution of this culture (5ml per ligation) was grown in 2x TY medium with vigorous aeration until the $OD_{550}$ was 0.3-0.4. The remainder of the overnight culture was reserved at 4C for plating out later. The bacteria were centrifuged (4000rpm for 5 minutes at 4C), resuspended in 1/5th of their original volume of ice cold Transformation Solution I and incubated on ice for 15 minutes. The cells were pelleted, resuspended in 1/50th of their original volume of ice cold Transformation Solution II and incubated on ice for a further 30 minutes. 200µl aliquots of this cell suspension were transferred to Eppendorf tubes on ice and DNA added.

10µl of a ligation reaction were used for each transformation, and in addition the following transformation controls were treated in the same way:

1. 1ng supercoiled vector (efficiency control)
2. 25ng dephosphorylated non-ligated vector (negative control)
3. 25ng non-dephosphorylated non-ligated vector (negative control)

The contents were mixed by gentle pipetting and stood on ice for 30 minutes. The cells were heat shocked for 60 seconds at 42C and incubated on ice for a further 5 minutes. The cells were divided in the proportion 89:10:1 in three 10ml tubes, to each of which was then added at RT: 10µl 100mM IPTG[1], 25µl 2% Xgal[2], 200µl fresh growing TG2 cells and 4ml 0.7% TY top agar at 50C. The contents were rapidly mixed by inverting, spread onto 1.2% agar plates and incubated at 37C overnight.

#### 2.2.5.5 Plaque purification and screening

Since insertion of a foreign DNA fragment into the multiple cloning site of M13 mp18 and mp19 leads to inactivation or reduction of the β-galactosidase activity, successful ligations were suggested by the presence of clear plaques, in contrast to the blue plaques produced by wild-type M13.

The plaques were screened to confirm that they contained a DQA insert. The agar plate was cooled to 4C and an assymmetrically marked nitrocellulose filter was laid onto it. The agar was marked in the same orientation as the filter. The filter was allowed to thoroughly wet for about 2 minutes, and was then removed carefully. It was processed through denaturation and neutralizing steps by first floating (phage side up) then immersing in phage dip buffers I, II and III for 1 minute each. The filter was air-dried and baked for 2 hours at 80C in a vacuum oven. The filter was hybridized using a radio-labelled full length DQA1 probe (p11-α-5), using the same protocol as described in Chapter 2.2.1.8. Positive plaques were identified by aligning the agar plate with the autoradiograph. The numbers of clear and blue plaques, and those shown by autoradiography to contain a DQA insert, obtained in one representative experiment are shown here in Table 16.

---

[1] IPTG: a gratuitous inducer of β-galactosidase

[2] Xgal: a chromogenic susbstrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

**Table 16**

| Ligation | clear | blue | positive |
|---|---|---|---|
| +ve control | 50%* | 50%* | 45%* |
| -ve control | 1 | 42 | 0 |
| 1 DQA1 | 10 | 0 | 10 |
| 2 DQA1 | 13 | 6 | 8 |
| 3 DQA1 | 17 | 2 | 13 |
| 4 DQA1 | 26 | 4 | 19 |
| 5 DQA1 | 15 | 1 | 13 |

* of >300 plaques

All the positive plaques were picked with a sterile toothpick and inoculated into 200µl 2xTY medium. The tubes were minifuged for 10 minutes and the supernatant taken off and stored at +4C (when it would be stable for >1 year). This phage suspension contained an estimated $10^3$ pfu/µl.

2.2.5.6 Single stranded template preparation

1µl of the stored M13 phage was diluted 1:100 in 2xTY medium. 10µl of this solution was added to 300µl of an overnight culture of TG2 and 3ml 1% top agar/2xTY. The mixture was plated out and incubated overnight at 37C. The following day, a 1:20 dilution of TG2 in 2xTY was grown for 10 minutes. To 2ml of this culture in a 50ml Falcon tube was added a single well isolated plaque removed from the plate using a sterile Pasteur pipette. The cap of the tube was loosely taped and the tube was shaken vigorously for 4.5-5.5 hours. The culture was transferred to 2ml tube and minifuged for 5 minutes at RT. The supernatant was decanted and spun again to remove all bacteria. 200µl 20% PEG/2.5M NaCl was added, the contents mixed well and the tube incubated on ice for 60 minutes. The tube was minifuged for 10 minutes at RT, the supernatant removed completely and the phage pellet resuspended in TE (pH8). The phage was extracted once with 50ml freshly buffered phenol, once with phenol:$CHCl_3$ and finally with $CHCl_3$ alone. The DNA was precipitated with 10µl 3M NaOAc and 300µl absolute ethanol at -20C overnight. The tube was minifuged for 10 minutes , the supernatant decanted and the pellet washed with 1ml cold 95% ethanol, respun and air-dried. Finally the DNA was resuspended in 20µl TE (pH8) and stored at -20C. 3-5µl of this template were required for a sequencing reaction.

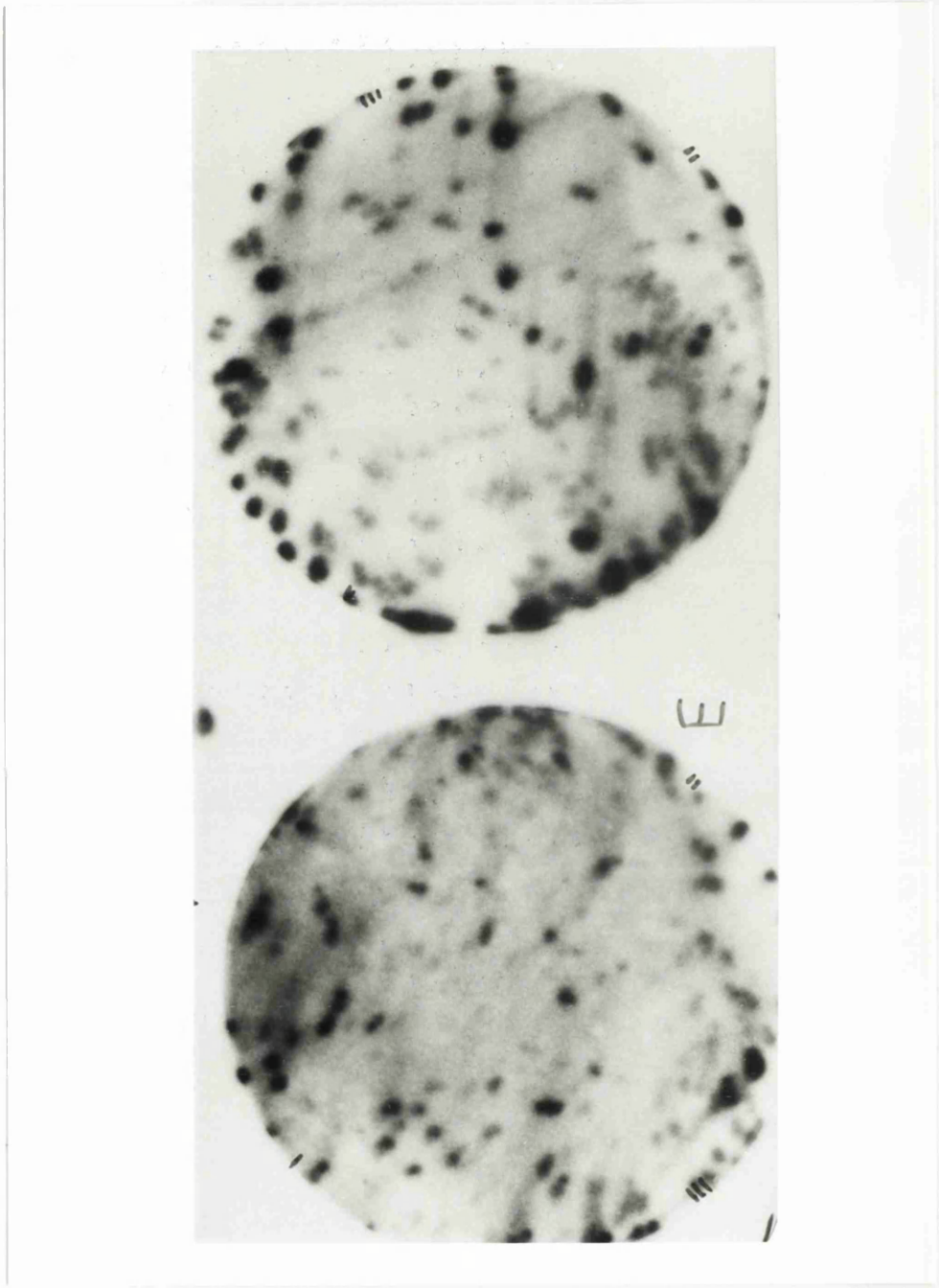


**Figure 14**
Autoradiographs of two filter lifts, hybridized with a DQA1 probe and washed under stringent conditions. Successful ligations into M13 are revealed by hybridization to the plaque. The upper filter shows a previous ligation (positive control) and "E" shows the ligation of the PCR fragment from DM. Both filters show >50 positive plaques.

### 2.2.5.7 Sequencing reactions

4ng of M13 sequencing primer (17-mer) was annealed to 3-5µl single stranded template in 1xTM buffer by heating to 80C and allowing to slowly cool to RT in a water bath. Simultaneously, four tubes were prepared as follows:

| | |
|---|---|
| Tube A | 1µl $A^o$ + 1µl 0.1mM ddATP |
| Tube G | 1µl $G^o$ + 1µl 0.05mM ddGTP |
| Tube C | 1µl $C^o$ + 1µl 0.1mM ddCTP |
| Tube T | 1µl $T^o$ + 1µl 1.0mM ddTTP |

After annealing was complete, 1µl 0.1M DTT, 1.5µl [$\alpha^{35}$S]-dATP[3] and 1µl large fragment DNA polymerase[4] were added directly to the hybridized primer/template and mixed gently by pipetting. A 3µl aliquot of this mixture was dispensed into each of the four tubes A, G, C and T which were spun to form a single drop. The tubes were incubated at 30C for 20 minutes. 2ml cold chase was added and the tubes incubated for a further 20 minutes. The reactions were stopped by the addition of 5µl formamide dye mix and stored on ice. ( If the samples were not to be sequenced immediately, the reactions were stopped using 0.2M EDTA and stored at -20C).

### 2.2.5.8 Polyacrylamide gel electrophoresis

Sequencing gel electrophoresis was carried out in 0.4mm thick 8% polyacrylamide gels containing 8M urea in TBE buffer using sharks' fin tooth combs. The glass plates were thoroughly cleaned and rinsed with acetone and ethanol. One plate was siliconized and the plates were assembled. Polyacrylamide was prepared as follows and poured between the plates: 46g urea, 15ml 40% acrylamide stock, 10ml 10xTBE, $dH_2O$ to 100ml, 800µl 10% ammonium persulphate and 55µl TEMED. After polymerization was complete, the combs were removed, the wells rinsed with water, the electrophoresis tank assembled and the gel pre-warmed by running at 100watts for 30 minutes. The sequencing reaction samples were denatured by heating to 90C for 5 minutes and stored on ice until loaded. 2-3µl of the samples AGCT were applied to the gel and electrophoresed at 80watts; by repeating this loading at intervals, the same samples could be separated for 5, 2.5 and 1.5 hours, thus giving readable sequence over a region of >400 nucleotides.

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[3] Amersham; 600Ci/mmole

[4] BRL; diluted to 1.5 units/µl with 1xTM

After electrophoresis was complete (typically the bromphenol blue marker, mobility equivalent to 26 bases in 6% PAG, was at the lower end of the gel), the gel was allowed to cool for 10 minutes and the equipment was disassembled. One glass plate was carefully removed leaving the gel adherent to the other. The gel was fixed in 10% acetic acid for 10 minutes and washed twice in water. It was then blotted dry and transferred to a sheet of Whatman 3M paper, coated with Saran wrap and dried on a gel drier. Autoradiography was carried out as before at -70C overnight without intensifying screens and the autoradiograph read manually.

## 2.2.6 ALLOREACTIVE T CELL CLONES

All T lymphocyte cloning and the testing of clones reported in this thesis were carried out in the Department of Immunology, Royal Postgraduate Medical School by Dr Giovanna Lombardi, Mr Sid Sidhu and Mr Stephen Man. Alloreactive T cell clones were raised from members of the Scottish family M4. As discussed in Chapter 3.2.2, the stimulators were DM and CG, and the responder was AM. All of these donors were DR2/2,DQw6/6, but RFLP analysis demonstrated that haplotype **a**, which was shared by CG and DM but not by AM, was associated with a cluster of Msp1 DQA1 restriction fragments. These cloning combinations were chosen in order to maximize the chance of obtaining clones specific for a Class II gene product associated with this cluster.

### 2.2.6.1 Preparation of T cell lines and clones

Alloreactive T cell lines and clones were isolated from AM, a 50 year old male MS patient from the Grampian region. Fresh blood samples (500ml in sterile transfer packs containing preservative-free heparin) were collected from this responder and from the two stimulators, DM and CG, and flown to London the same day for processing. Responder PBMC, enriched from whole blood on Ficoll-Hypaque gradients, were co-cultured with X-irradiated PBMC from the stimulators in RPMI 1640 supplemented with 10% human AB serum, 2mM glutamine, and penicillin-streptomycin. After 7 days of culture, the cells were then harvested and viable cells were maintained in 24-well plates (Flow Laboratories) and were restimulated every week with irradiated stimulator PBMC and rIL-2 (20 U/ml). T cell clones were obtained by limiting dilution in Terasaki plates (Sterilin) at a cell concentration of 0.3 cells per well in the presence of irradiated stimulator PBMC. The clones were maintained in culture by further stimulation at weekly intervals. They were then stored at -70C, and expanded prior to testing by culturing in RPMI 1640 with rIL-2. The CD4/CD8 phenotype of the clones was determined by staining with OKT4 and OKT8, using the methods described in Chapter 2.2.2, and analysing by flow cytometry (EPICS Profile Analyzer).

### 2.2.6.2 T cell proliferation assay

T cell clones were cultured ($10^4$ cells/well) in the presence of irradiated PBMC ($5x10^4$ cells/well), in 10% human serum, in flat-bottom microtitre

plates (Sterilin) in a total volume of 200µl. [$^3$H]TdR, 1µCi (Amercham International), was added after 48 hours and the cultures harvested onto glass fibre filters 18 hours later. Proliferation was measured as [$^3$H]TdR incorporation by liquid scintillation spectroscopy. The results are expressed as cpm ± SD of triplicate cultures. Monoclonal antibody inhibition studies were performed by preincubating the target (stimulator) PBMC with the mAb at the appropriate final concentration (described in Figure 34) for 30 minutes. The target PBMC were then added to the responder cells and the technique completed as above. The antibodies used are shown in Table 15.

# PART 3

# RESULTS

## 3.1 RESTRICTION FRAGMENT LENGTH POLYMORPHISM STUDIES

### 3.1.1 POOLED DNA SAMPLES

#### 3.1.1.1 Introduction

DNA polymorphisms tightly linked to loci at which mutations cause disease or disease susceptibility can serve as useful genetic markers. As discussed earlier, genetic susceptibility to a number of diseases, including MS, shows linkage as well as association in population studies with specific serologically defined variants of the HLA Class I and Class II loci. Accordingly efforts have been made to detect DNA markers in linkage disequilibrium with MS (Cohen et al.1984a; Marcadet et al.1985; Jacobson et al.1986; Serjeantson et al.1986; Ødum et al.1988). The usual procedure for identifying informative restriction fragment polymorphisms utilizes a cloned HLA sequence as a hybridization probe and compares the restriction fragment patterns obtained with a variety of restriction enzymes in Southern blots (Southern, 1975) of DNA samples from healthy subjects and MS patients. This analysis represents a search for (i) enzymes that reveal polymorphisms and (ii) polymorphic fragments whose frequency is increased in the patient population. This screening procedure may be extremely laborious, and an alternative strategy was described by Arnheim (Arnheim et al.1985), in which pooled samples of DNA derived from disease and control individuals were compared. If restriction digests of these pools are analyzed in adjacent lanes, a disease-linked RFLP will be revealed as a band which is more intense in the disease pool than in the control pool. Using this method, Arnheim was able to identify several DQB and DRB RFLP associated with IDDM. In theory the method is limited only by the ability to resolve different hybridization intensities of fragments, and although this may be improved by means of densitometry, the clearest results will be obtained in the case of fragments which are present at very low frequency in the control population and at a frequency at least 3 times greater in the disease population.

For the present studies, in order to identify possible genetic markers which were independent of HLA DR2, four DNA pools, [1] MS DR2+ve, [2] MS DR2-ve, [3] Control DR2+ve and [4] Control DR2-ve, were constructed as described in Chapter 2.2.1.3 and Table 13. A panel of 14 restriction enzymes was selected (BamH1, Bgl1, Bgl11, EcoR1, EcoRV, HinF1, Kpn1, Msp1, Pst1, Pvu11, Sac1, Sau96 1, Sma1 and Taq1). In order to maximize the chances of including those enzymes which were most likely to detect

polymorphisms within or immediately adjacent to the coding regions of the genes, computer-generated restriction maps of several Class II α and β cDNA clones were studied and those enzymes which recognized only a small number of restriction sites, and particularly within the hypervariable regions of the genes, were preferred. In general only restriction enzymes with recognition sequences of at least 5 bases were used. Southern blots were probed successively with each of the probes listed in Table 12, the previous probe being stripped before reprobing.

### 3.1.1.2 Results

The four DNA pools were digested with each of the 14 restriction enzymes and probed with 5 Class II probes (DPA, DPB, DQA1, DQB1, DRB) and 2 T cell antigen receptor probes (TcRα and TcRβ) representing 98 enzyme/probe combinations. Examples of the autoradiographs obtained by probing two of the Southern blots with DQA1 and DQB1 are shown in Figures 15-17.

DQA1 polymorphism between MS and Control pools

With the DQA1 probe p11-α-5, polymorphisms were detected with only one of the restriction enzymes used. Following digestion with Msp1, a 5.6kb fragment was visible in both MS pools (lanes 1 and 2) but not in the Control pools (lanes 3 and 4). Additionally a 6.1kb fragment was seen in the MS DR2-ve pool (lane 2). Also of interest was the finding that a 3.25kb Msp1 fragment was detected in the MS DR2+ve pool (lane 1) but not in the Control DR2+ve pool (lane 3), suggesting that this fragment was associated with MS independently of HLA DR2 (Figure 19).

DQB1 polymorphism between MS and Control DR2-ve pools

Once again the vast majority of the hybridizing fragments were invariant between the 4 pools. However, in three instances polymorphic restriction fragments were observed. Following cleavage with Bgl11 a 5.5kb fragment hybridized strongly with DQB1 in the MS DR2-ve pool but was only weakly present in the Control DR2-ve pool. In contrast this situation was reversed for a 3.8kb fragment. Whilst it is possible that these differences reflect bias in the distribution of non-DR2 alleles in the two DR2-ve pools, it must be pointed out that no restriction fragment pattern differences were seen with either the DQA1, DRB or DP probes. Thus it appeared most likely that these Bgl11/DQB1 RFLPs reflected polymorphisms in linkage

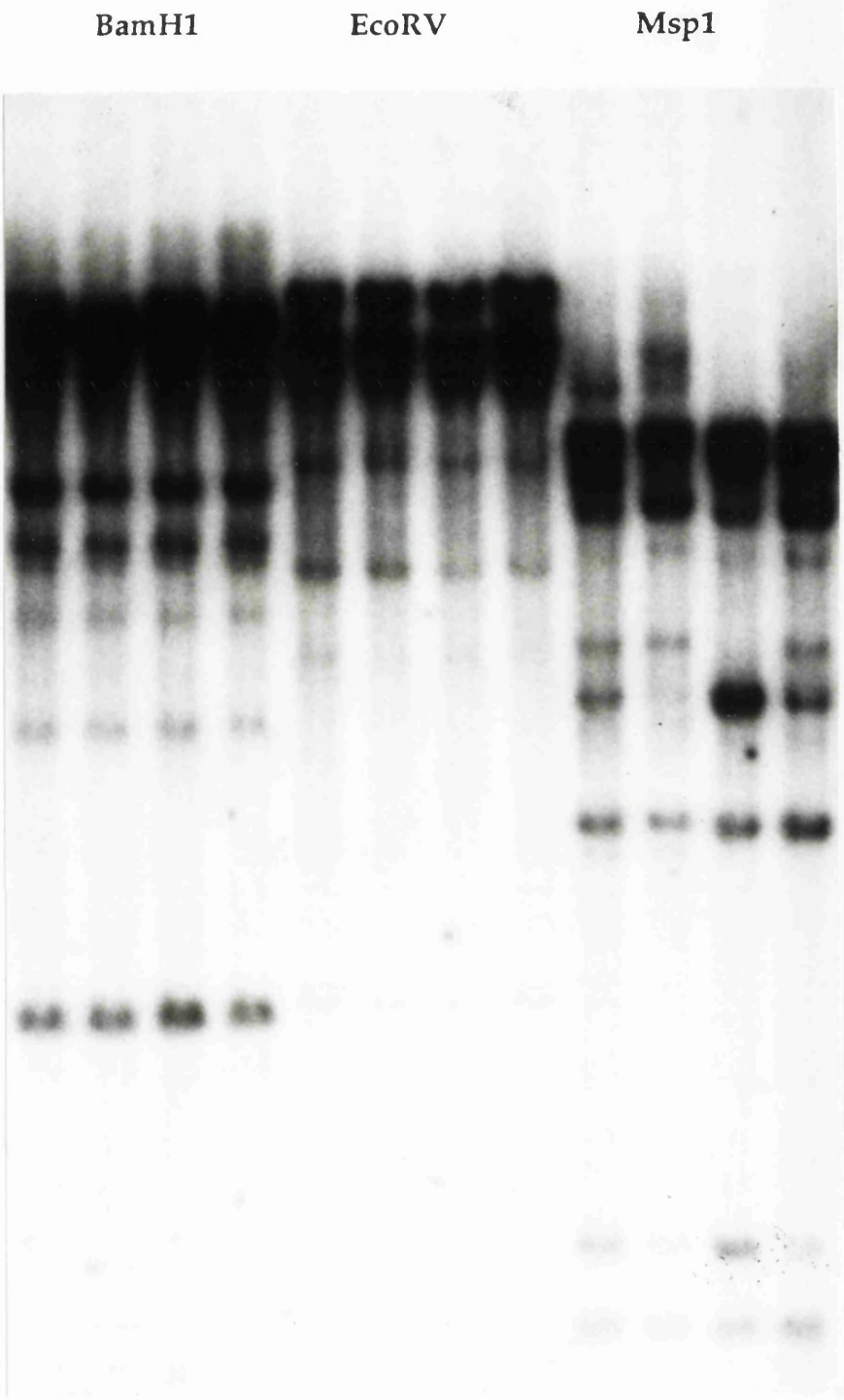


**Figure 15**
Autoradiograph showing four DNA pools digested with BamH1, EcoRV and Msp1, probed with DQA1. The pools are (from left to right) MS DR2+ve, MS DR2-ve, Control DR2+ve, Control DR2-ve. Most of the restriction fragments are represented equally in each of the pool, bu differences can be seen in the case of Msp1.

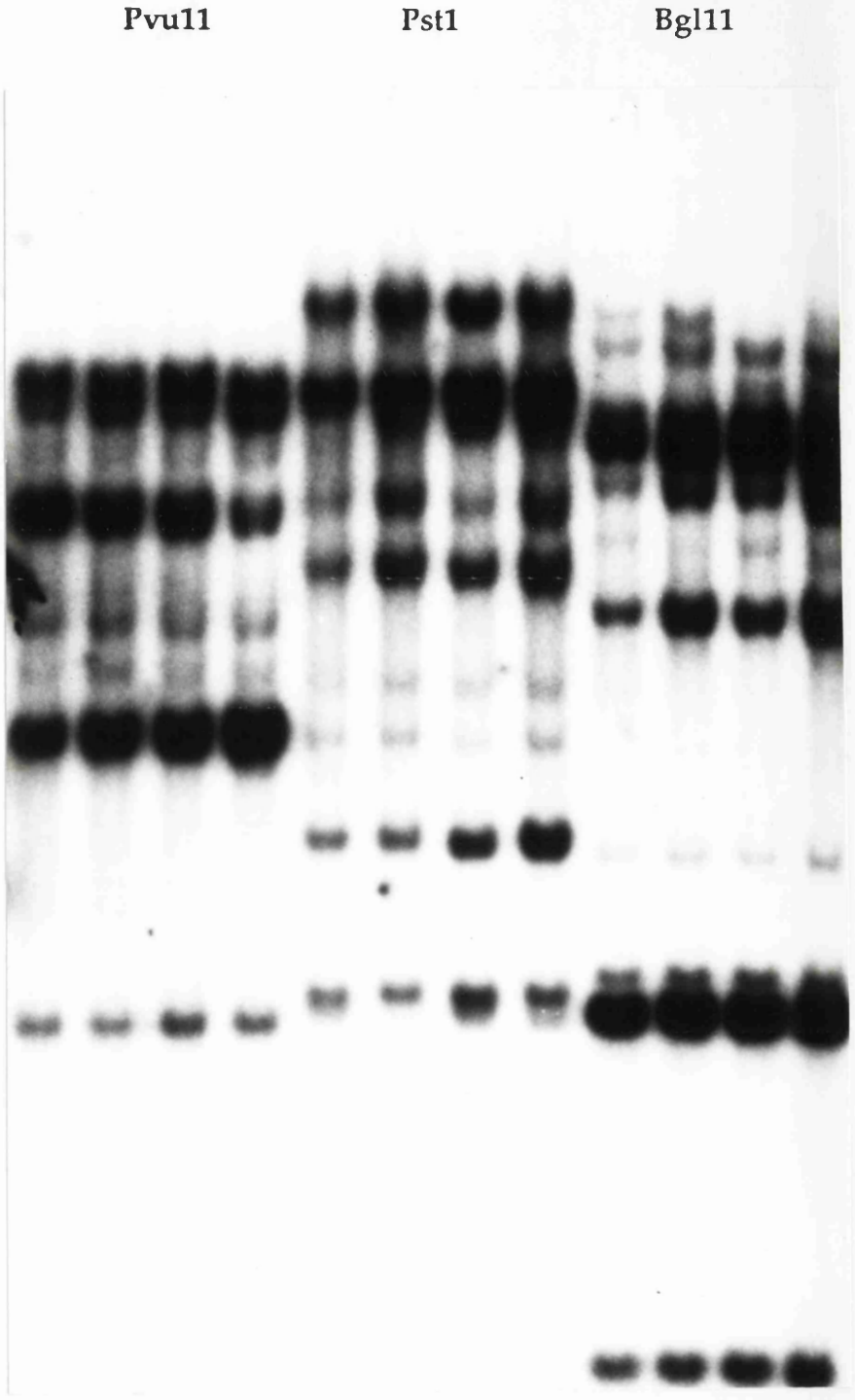


**Figure 16**
Autoradiograph showing four DNA pools digested with PVU11, Pst1 and Bgl11, probed with DQA1. No discriminatory polymorphism is seen.

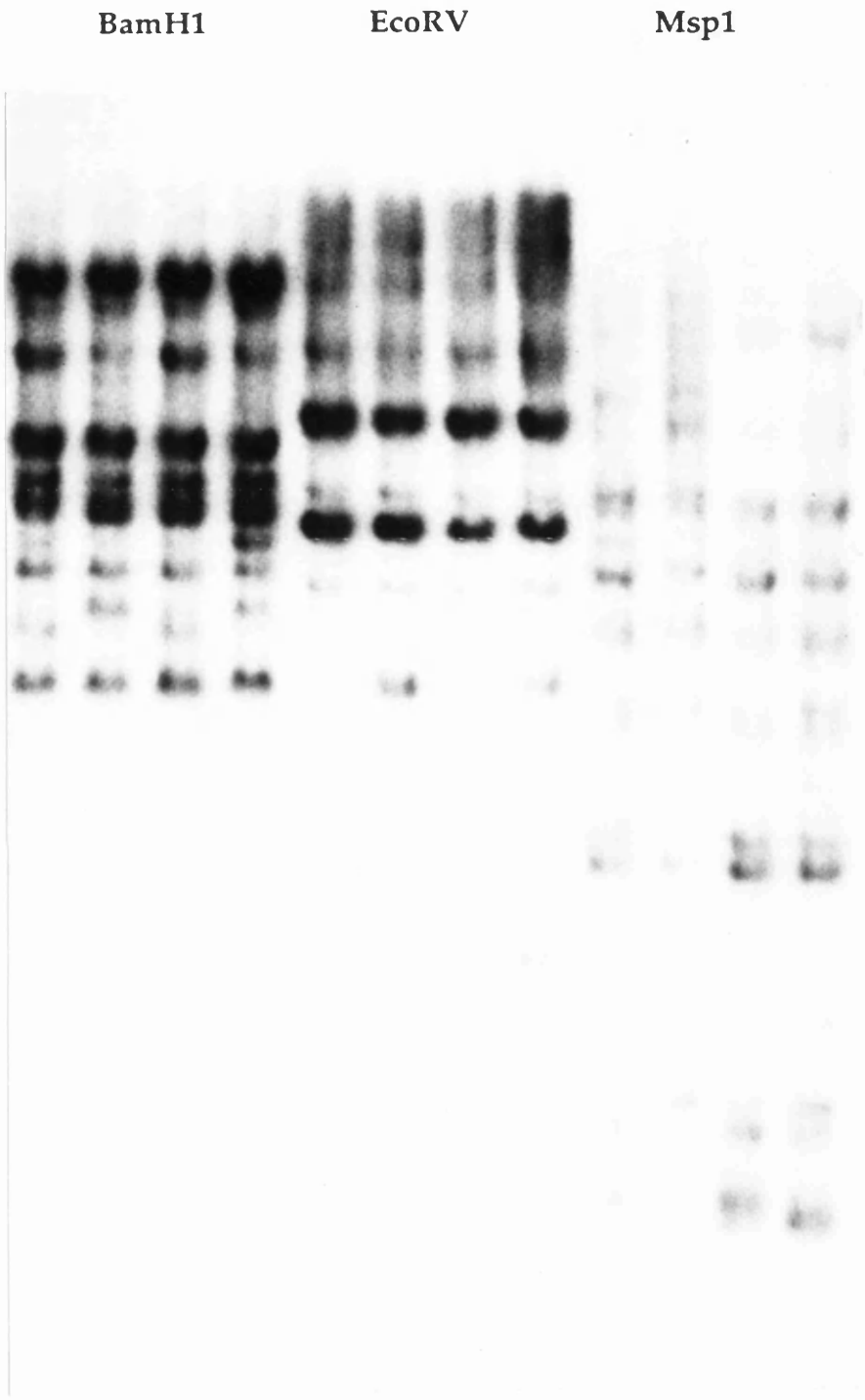


**Figure 17**
Autoradiograph showing four DNA pools digested with BamH1, EcoRV and Msp1, probed with DQB1. Faintly hybridizing DR2-specific fragments are seen with BamH1 and EcoRV, but there is no disease-associated polymorphism.

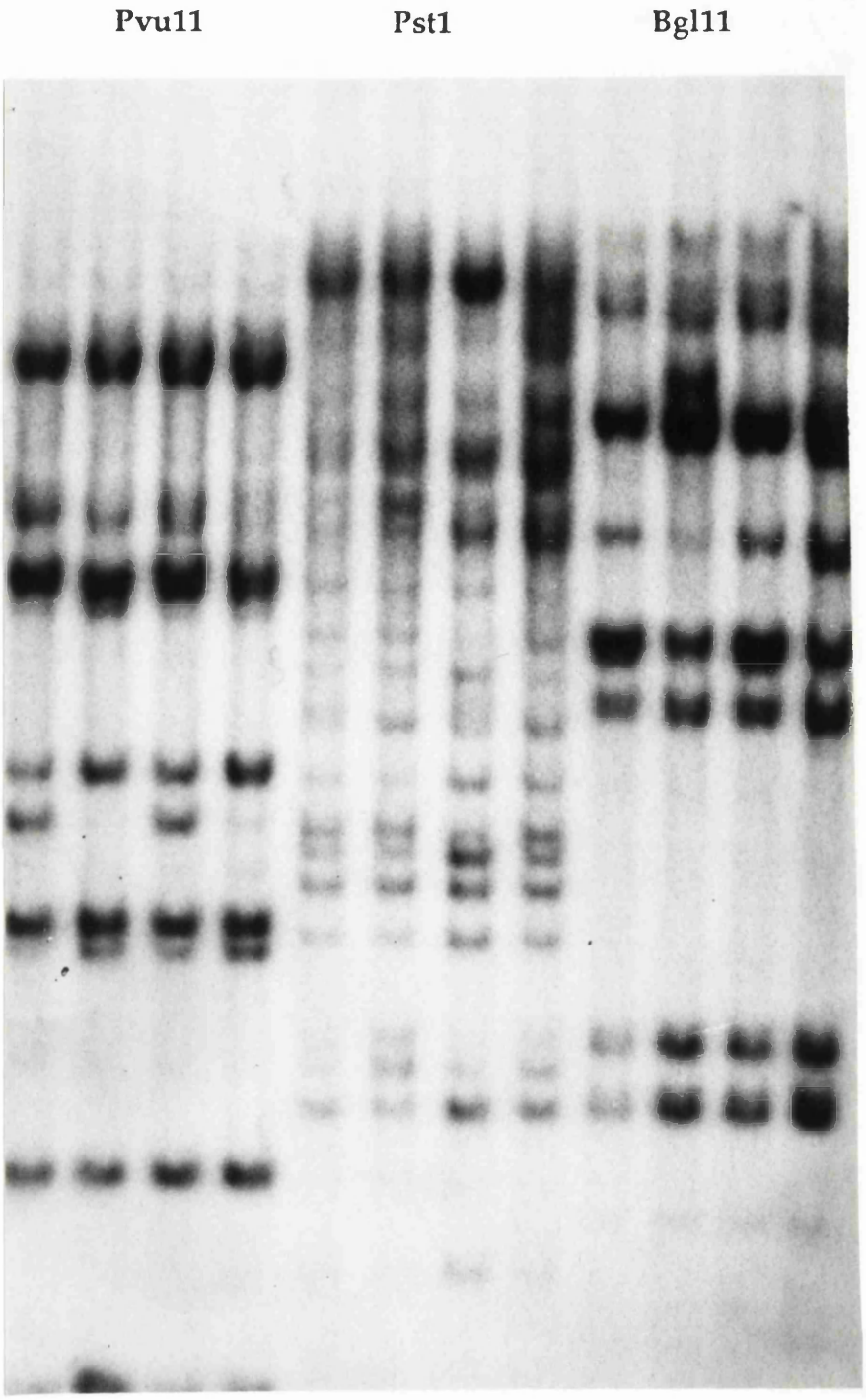


**Figure 18**
Autoradiograph showing four DNA pools digested with Pvu11, Pst1 and Bgl11, probed with DQB1. A 2.6kb Pvu11 DQB1 fragment is associated with DR2. No other discriminatory polymorphism is seen

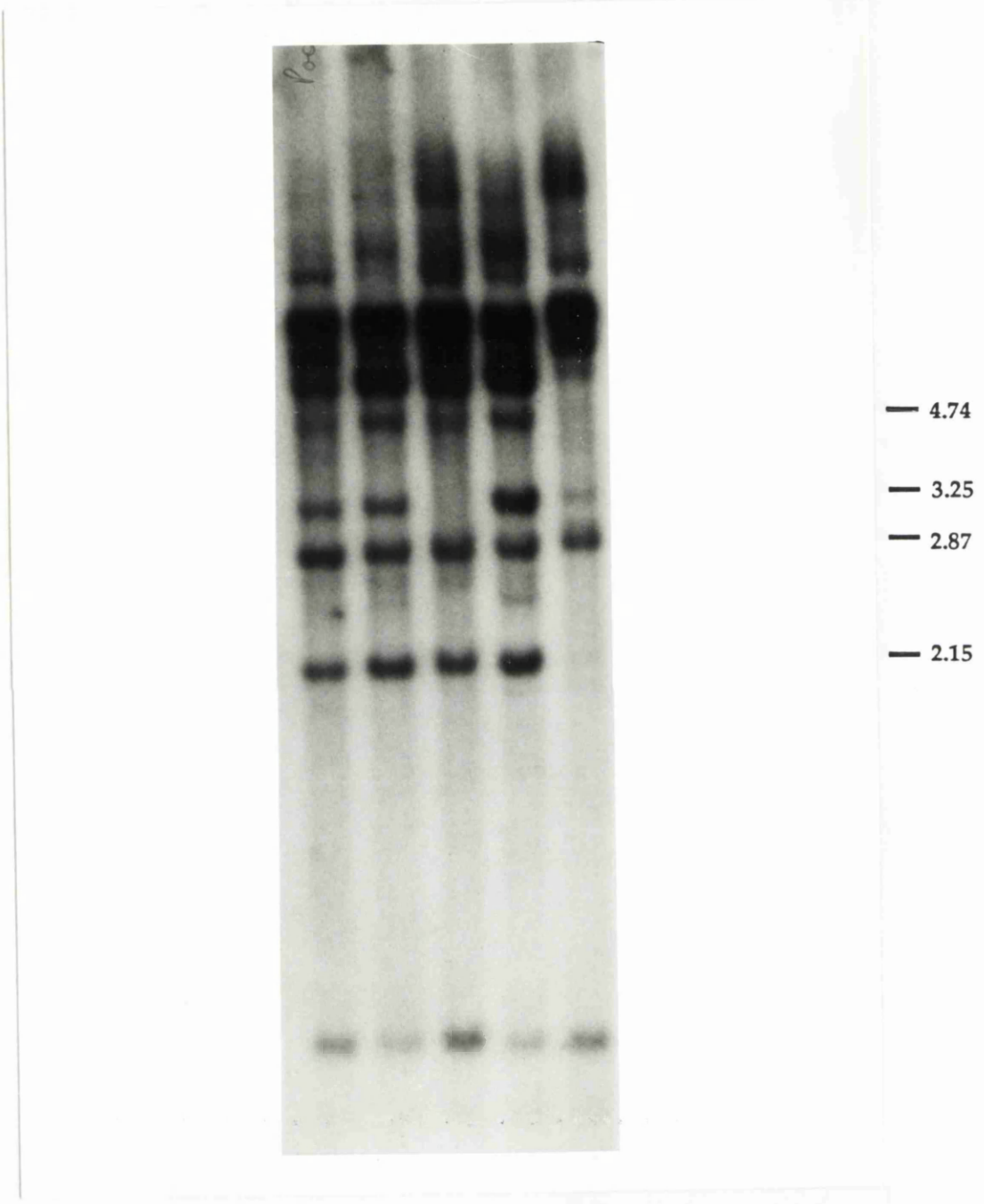


Figure 19

Autoradiograph showing four DNA pools digested with Msp1 and probed with DQA1. The far right lane contains DNA from a DR4/4 individual. All of the pools are characterized by a poorly resolved cluster of fragments between 6.05 and 5.12kb, and 4.74kb, 2.87kb and 2.15kb fragments. A 3.25kb fragment is clearly absent from the Control DR2+ve lane and present in the MS DR2+ve lane.

with a DQB gene and were not the result of cross-hybridization. Following digestion with Taq1 a number of RFLP were detected with DQB1. A 4.5kb fragment was only weakly present in the MS DR2+ve pool in contrast to the other 3 pools, and a 1.2kb fragment was unique to the Control DR2-ve pool. A cluster of fragments sized between 2.6kb and 2.15kb appeared to discriminate between the DR2-ve pools. The only other polymorphism detected with a DQB1 probe was a 3.7kb BamH1 fragment which discriminated between the DR2-ve pools.

No discriminatory polymorphism between patients and controls

The remaining probes, DPA, DPB, DRB and TcRα (all of which were full length) and TcRβ (which was an internal Cβ2 fragment), failed to reveal any differences between the disease and control preparations of pooled DNA with any of the 14 restriction enzymes. However, in many instances, fragments closely associated with the presence or absence of DR2 could be identified. The most notable examples of such fragments are EcoR1 2.2kb DQB1, which appears to be characteristic of the subtype Dw2 (Segall et al.1986), and Pvu11 2.6kb DQB1 (Figure 18).

### 3.1.1.3 Summary

In a well characterized, HLA-typed population from a single geographical area, a number of HLA-DQ RFLP differences between MS and control pools were observed (Heard et al.1988). A 5.6kb Msp1 fragment hybridizing to DQA1 was present in the MS pools but was not seen in the Control pools. A 3.25kb Msp1 DQA1 fragment clearly discriminated between the MS and Control DR2+ve pools. Hybridization with DQB1 suggested that in three instances there might be differences between DR2-ve patients and controls. These data appear to confirm the value of this method of studying pooled samples of DNA for the rapid screening of patients and controls with a large number of different enzyme/probe combinations. To perform this screening using individual samples would have required 70 Southern blots to be probed 7 times each. As a result of these preliminary studies, the subsequent analysis of individual DNA samples could be carried out using a considerably limited number of restriction enzymes, and with an enhanced likelihood of identifying MS-associated polymorphism.

### 3.1.2 INDIVIDUAL DNA SAMPLES

#### 3.1.2.1 Introduction

Based on the results obtained in these studies using pooled DNA, 33 MS patients and 48 controls from the Grampian region of northeast Scotland were studied individually. The distribution of HLA-DR alleles in these subjects is shown in Table 17. Eleven Class II probe/enzyme combinations were chosen for these studies: DQA1 with Msp1 and Taq1; DQB1 with BamH1, Bgl11, EcoR1 and Taq1; and DRB with BamH1, Bgl11, EcoR1, Msp1 and Taq1. In addition, since it is known that the TcR genes exhibit Bgl11 polymorphism (TcRα and TcRβ) and Taq1 polymorphism (TcRα) these combinations were also studied individually (Yanagi et al.1985; Yoshikai et al.1984). The individual DNA samples were digested, transferred to Gene Screen Plus membranes and hybridized successively to the appropriate probes as described previously.

#### 3.1.2.2 Results

DQA1 and DQB1 restriction fragments associated with MS

Representative autoradiographs of individually digested DNA samples are shown in Figures 20-26. A total of 127 Class II restriction fragments were analysed, 18 DQA1 fragments, 34 DQB1 fragments and 75 DRB fragments. The sizes of these fragments are given in Table 18. The occurrence of these fragments is shown in Tables 19 (DQA1), 20 (DQB1) and 21 (DRB). These fragments were tested separately and independently of DR2 for significant association with MS by stepwise logistic regression analysis and a series of Chi-square tests. Those fragments achieving a significance of $p \leq 0.05$ are shown in Table 22. It can be seen that a number of DQA1 and DQB1 fragments are significantly associated with MS. The most strongly associated fragments are the Msp1 3.25kb DQA1 fragment discussed in the previous section, and a similar Msp1 2.50kb DQA1 fragment which is seen in the same subjects as the 3.25kb fragment. A third Msp1 DQA1 fragment, 2.31kb, was also seen in 6 of the 10 individuals possessing these two fragments and thus completed a characteristic cluster of DQA1 restriction fragments. The 2.31kb fragment was itself significantly associated with MS when the DR2 subjects were analysed separately, as was a 5.91kb Msp1 DQA1 fragment also. None of these fragments were associated with MS in the non-DR2 group. A small number of DQB1 fragments were also associated with MS but at lower levels of significance in most cases.

| HLA-DR | MS patients n=33 | Controls n=48 |
|---|---|---|
| 1 | 10 | 6 |
| 2 | 52 | 51 |
| 3 | 18 | 14 |
| 4 | 12 | 13 |
| 5 | 1.5 | 3 |
| w6 | 0 | 0 |
| 7 | 6 | 13 |
| w8 | 1.5 | 0 |
| 9 | 0 | 0 |
| 10 | 0 | 0 |

**Table 17**

The distribution of HLA-DR alleles (%) in the individually studied Scottish MS patients and controls.

## Table 18

Showing the 18 DQA1, 34 DQB1 and 75 DRB restriction fragments analysed in studies of individuals from the Grampian region (sizes in kilobases).

| DQA1/Msp1 | DQA1/Taq1 |
|---|---|
| 6.05 | 6.42 |
| 5.91 | 6.08 |
| 5.68* | 5.53 |
| 5.48 | 4.84 |
| 5.12 | 2.45 |
| 4.74 | 2.09* |
| 3.25 | 2.00* |
| 2.87 | |
| 2.50 | |
| 2.31 | |
| 2.15 | |

| DQB1/BamH1 | DQB1/Bgl11 | DQB1/EcoR1 | DQB1/Taq1 |
|---|---|---|---|
| 10.26 | 9.38 | 14.45 | 6.05 |
| 8.72 | 8.27 | 10.59 | 4.81 |
| 6.36 | 6.93 | 9.77 | 4.68 |
| 5.76 | 5.61 | 8.71 | 3.00 |
| 5.27 | 4.23 | 6.10 | 1.97 |
| 4.62 | 4.12 | 5.57 | |
| 4.19 | 3.27 | 4.03 | |
| 3.88 | 3.10 | 2.20 | |
| 3.83 | 1.29 | | |
| 3.55 | | | |
| 3.03 | | | |
| 2.89 | | | |

| DRB/BamH1 | DRB/Bgl11 | DRB/EcoR1 | DRB/Msp1 | DRB/Taq1 |
|---|---|---|---|---|
| 15.49 | 17.76 | 15.13 | 8.93 | 11.48 |
| 12.45 | 16.70 | 14.30 | 8.08 | 11.11 |
| 11.33 | 14.55 | 12.55 | 5.62 | 9.79 |
| 8.41 | 11.74 | 11.52 | 5.24 | 8.66 |
| 6.59 | 11.14 | 10.06 | 4.99 | 7.23 |
| 5.26 | 10.27 | 9.46 | 4.17 | 7.00 |
| 4.78 | 9.77 | 9.15 | 3.81 | 6.08 |
| 4.63 | 9.34 | 6.28 | 3.68 | 5.80 |
| 4.28 | 9.16 | 5.58 | 3.51 | 4.40 |
| 4.17 | 5.64 | 4.72 | 3.15 | 4.12 |
| 4.05 | 3.96 | 4.56 | 2.53 | 3.15 |
| 3.92 | 3.09 | 4.40 | 2.43 | 2.60 |
| 3.80 | 2.60 | 3.98 | 1.93 | 2.14 |
| 2.78 | | 3.73 | 1.76 | 1.72 |
| | | 3.31 | 1.52 | 1.42 |
| | | | 1.36 | 1.23 |
| | | | | 1.08 |

* These fragments are now known to be associated with DQA2 (DXα)

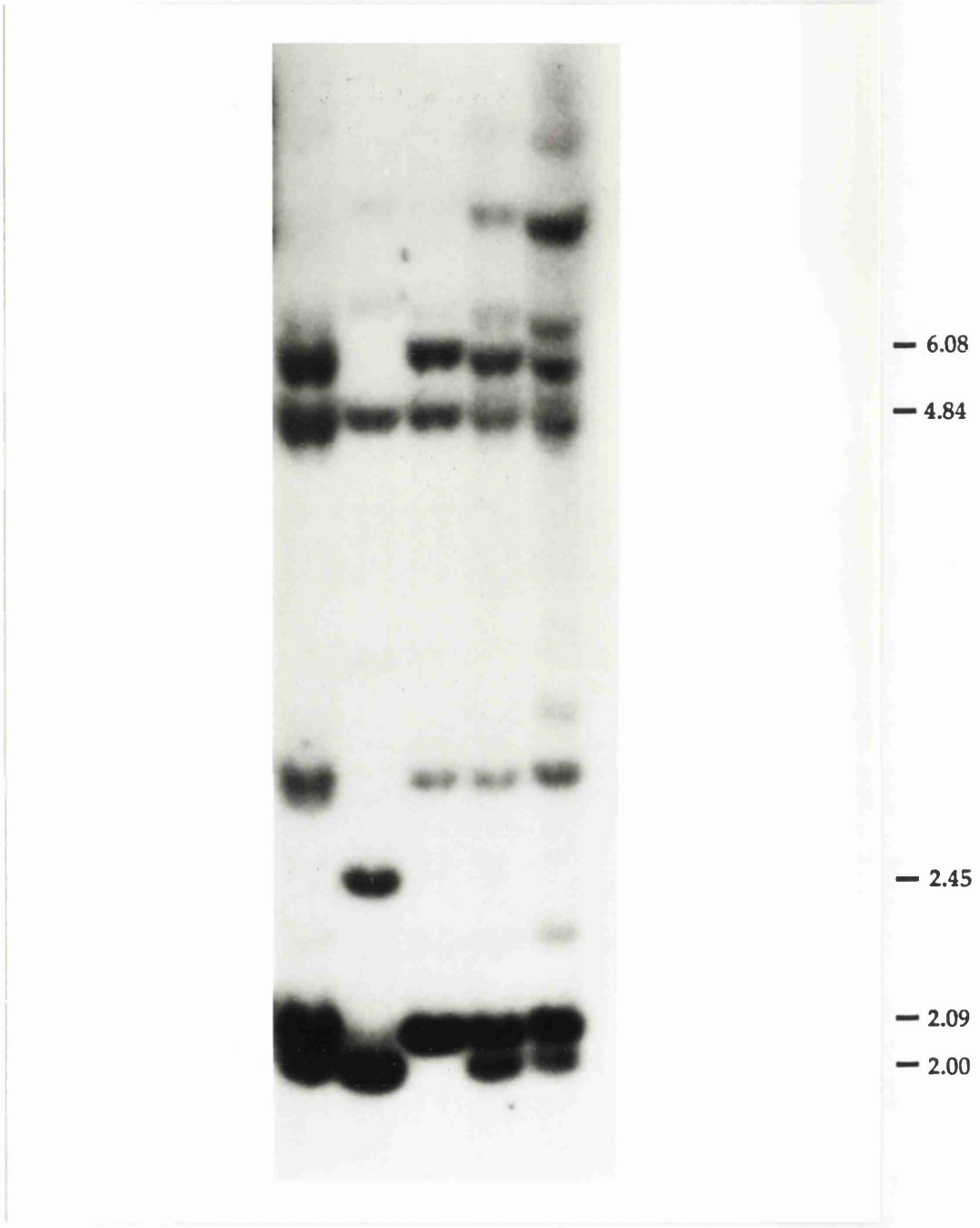


**Figure 20**
Autoradiograph showing individual samples of DNA digested with Taq1 and probed with DQA1. The 2.00kb and 2.09kb fragments are associated with the DQA2 locus and are revealed by cross hybridization. DQA2 is a diallelic locus, and thus the individuals in lanes 2 and 3 are homozygous.

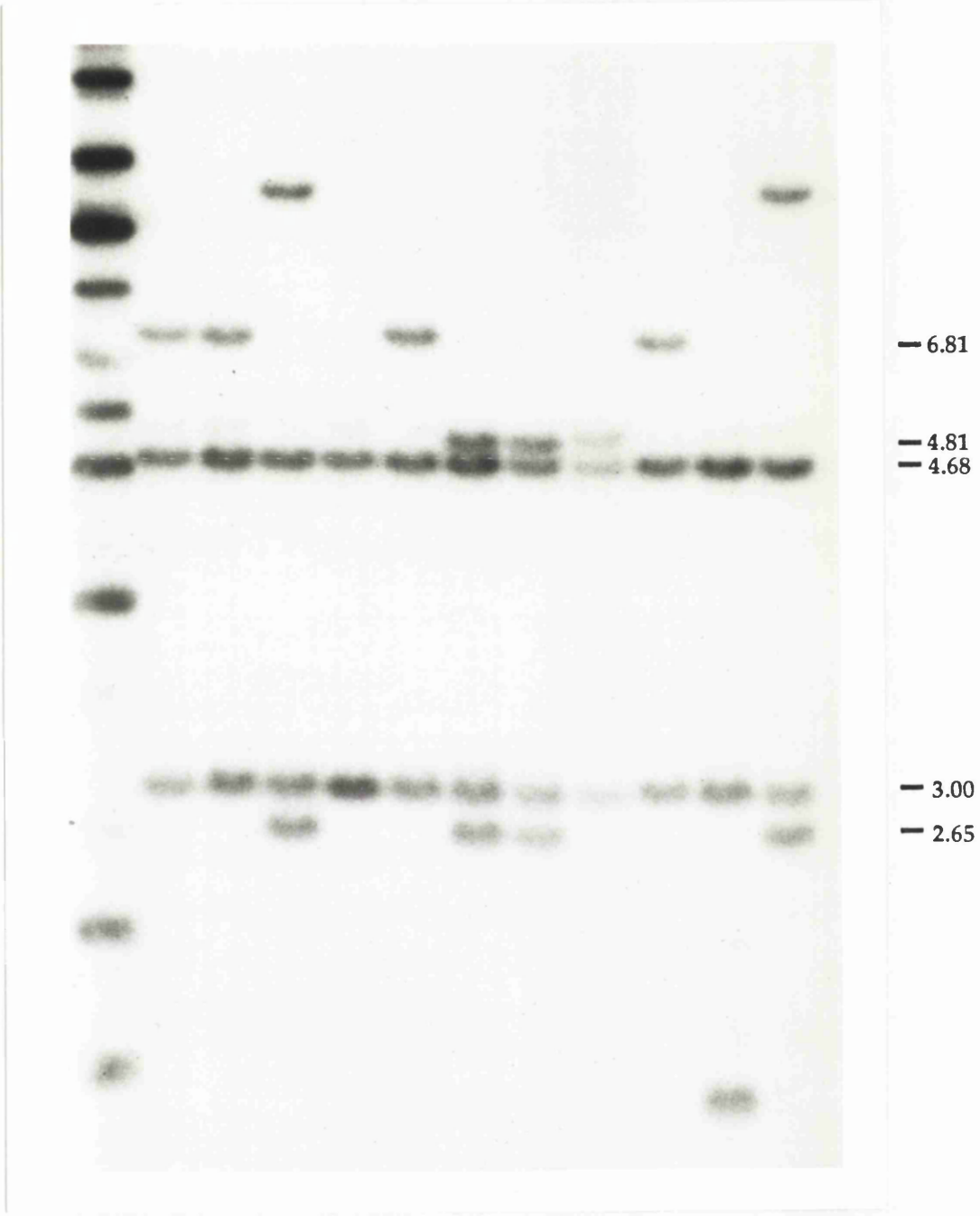


**Figure 21**
Autoradiograph showing individual samples of DNA digested with Taq1 and probed with DQB1. The left hand lane contains λKpn1/BstE11 marker. The 4.81kb and 4.68kb fragments are associated with DQA2.

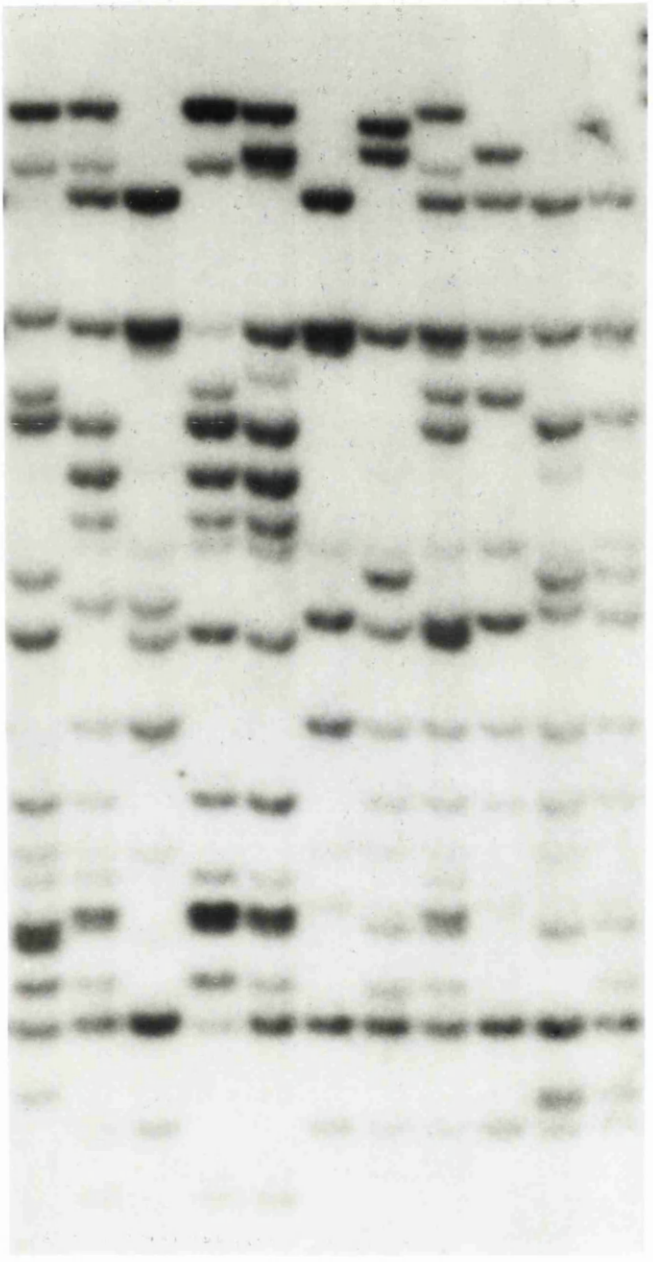

**Figure 22**
Autoradiograph showing individual samples of DNA digested with Taq1 and probed with DRB1. 17 restriction fragments were analyzed, as shown in Table 18.

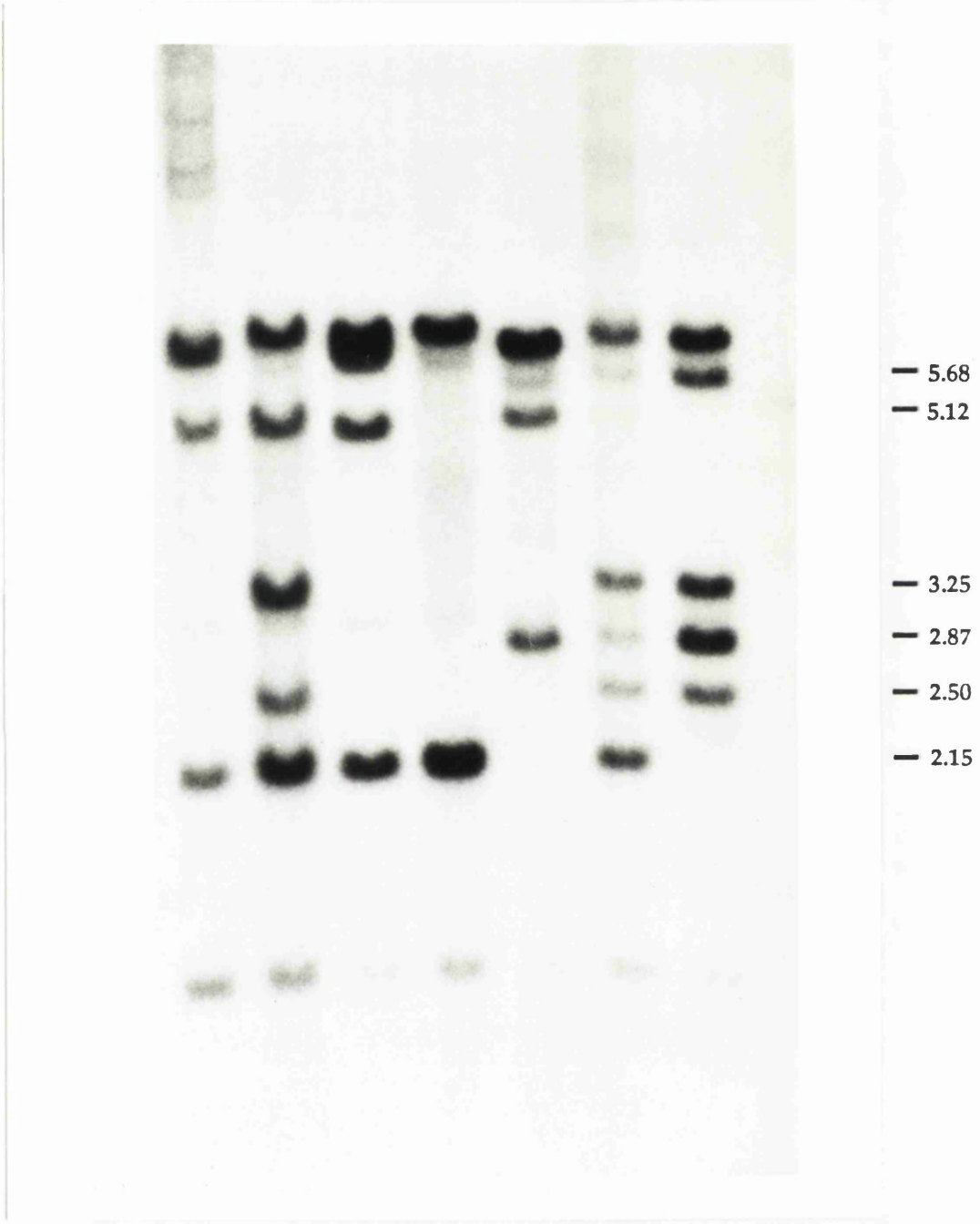


**Figure 23**
Autoradiograph showing individual samples of DNA digested with Msp1 and probed with DQA1. The DQA1 cluster described in the text* is seen in lanes 2, 6 and 7. The DQw1-associated 2.87kb fragment is seen in lanes 5, 6 and 7. (5.68kb is a DQA2 fragment).

*The faintly hybridizing 2.31kb fragment is not seen in this example.

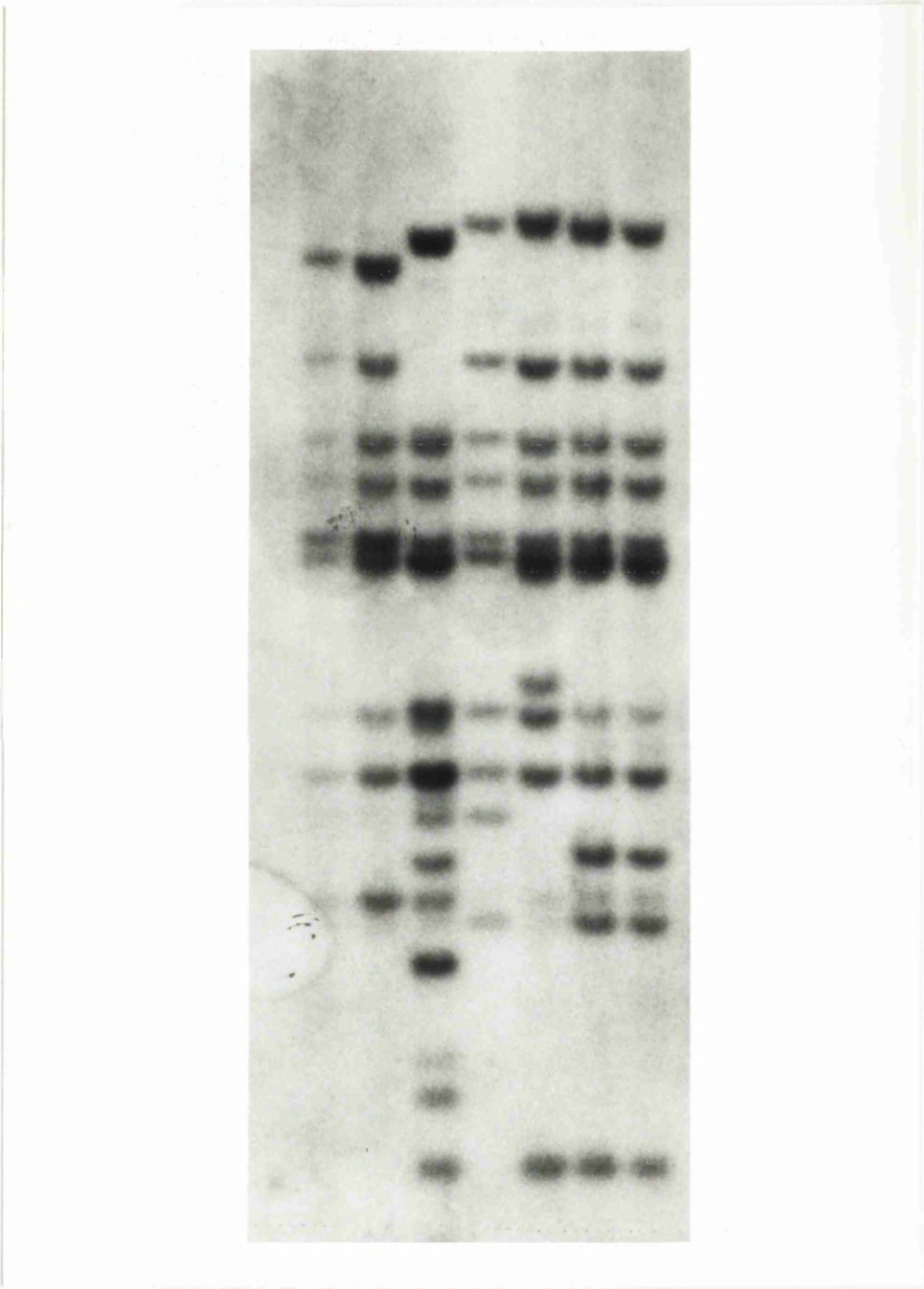

**Figure 24**
Autoradiograph showing individual samples of DNA digested with Msp1 and probed with DRB1.

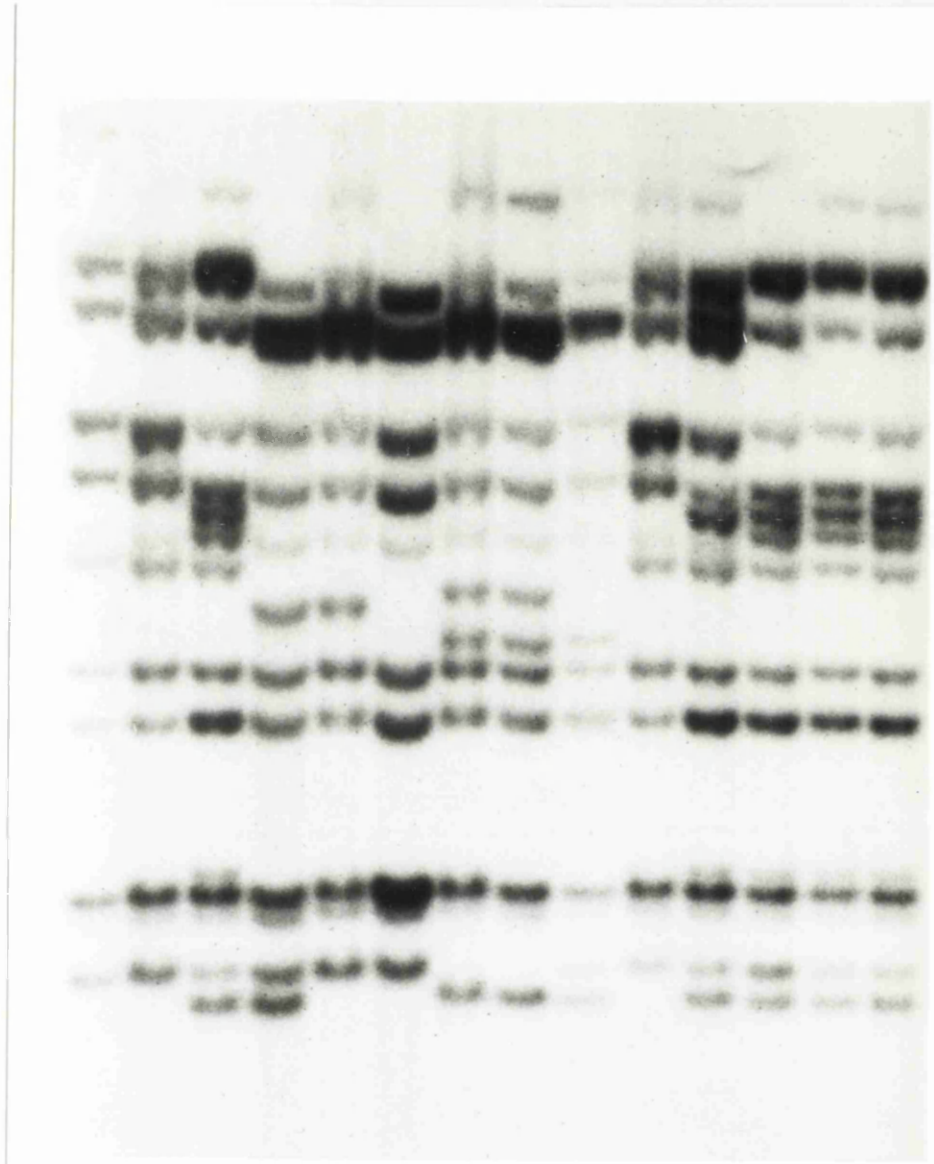

**Figure 25**
Autoradiograph showing individual samples of DNA digested with Bgl11 and probed with DRB1.

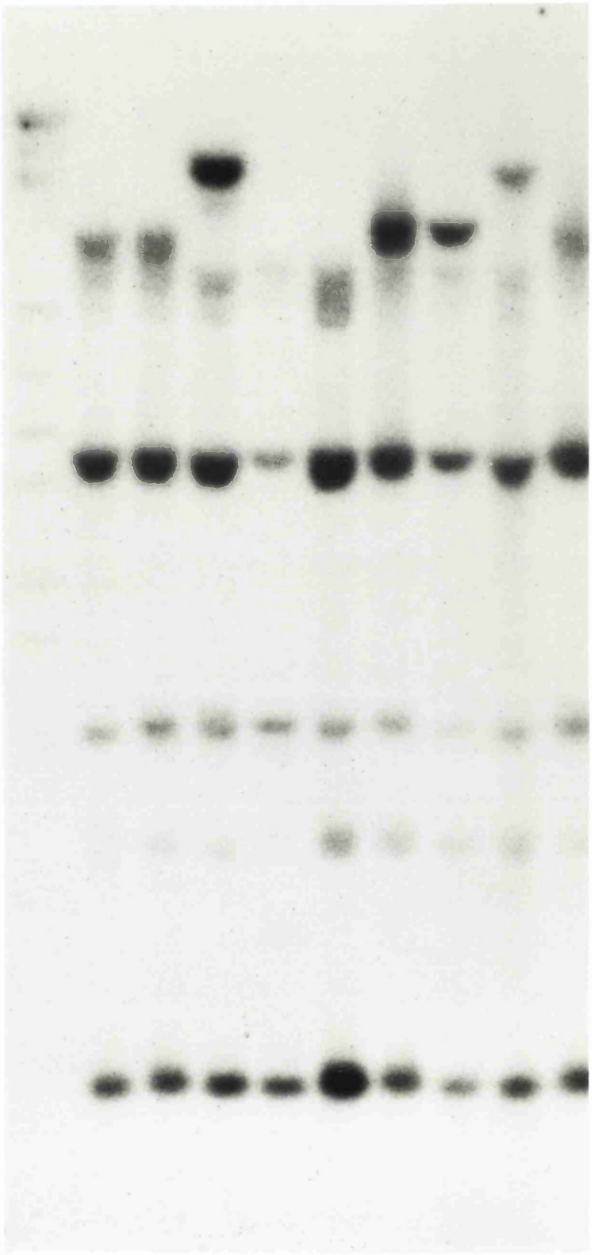

**Figure 26**
Autoradiograph of individual samples of DNA digested with EcoR1 and probed with DQB1.

Table 19

DQA restriction fragments

| | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 4 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 2 | 2 | 4 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 3 | 2 | 4 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 4 | 2 | 4 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 5 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 6 | 2 | 7 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 7 | 1 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 8 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| 1 | 9 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| 1 | 10 | 3 | 4 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| 1 | 11 | 2 | 3 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 12 | 2 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| 1 | 13 | 2 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| 1 | 14 | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 15 | 2 | 3 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 16 | 3 | 3 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 17 | 3 | 7 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| 1 | 18 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| 1 | 19 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| 1 | 20 | 3 | 7 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| 1 | 21 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| 1 | 22 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 23 | 2 | 2 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| 1 | 24 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 25 | 2 | 4 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| 1 | 26 | 2 | 7 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 27 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 1 | 28 | 3 | 5 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 1 | 29 | 2 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 31 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 32 | 2 | 2 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 33 | 2 | 4 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 1 | 34 | 1 | 4 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 1 | 35 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 36 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 37 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 1 | 38 | 2 | 7 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 39 | 2 | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 40 | 2 | 4 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 41 | 2 | 3 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 42 | 2 | 4 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 44 | 2 | 4 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 45 | 2 | 4 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 46 | 2 | 3 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| 1 | 47 | 2 | 3 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 1 | 48 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 1 | 49 | 2 | 3 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| 1 | 50 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 51 | 2 | 7 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 52 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 53 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
| 1 | 54 | 2 | 4 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 55 | 2 | 5 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 56 | 2 | 5 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| 1 | 57 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 65 | 1 | 7 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| 1 | 67 | 3 | 4 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 77 | 2 | 7 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 89 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| 1 | 90 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 91 | 3 | 3 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| 1 | 92 | 3 | 3 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 1 | 96 | 2 | 7 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 1 | 97 | 2 | 7 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 98 | 2 | 4 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 99 | 2 | 4 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 100 | 3 | 3 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| 1 | 101 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| 1 | 102 | 2 | 4 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 104 | 2 | 5 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 106 | 2 | 5 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| 1 | 110 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| 1 | 111 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 112 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 113 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 1 | 114 | 2 | 3 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| 1 | 117 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 1 | 121 | 2 | 7 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 1 | 122 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 1 | 125 | 2 | 7 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| 1 | 126 | 2 | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |

Key

Column 1 indicates origin of subject (1=Grampian region)
Column 2 is the assigned sample number
Columns 3 and 4 show the serologically typed HLA-DR alleles
Column 5 indicates whether subject is MS patient (1) or control (0)

Columns 6-23 represent restriction fragments: present (1), absent (0), uncertain (-1)

Columns 6-16 MspI fragments
Columns 17-23 TaqI fragments

Table 20

DQB restriction fragments

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 4 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 2 | 2 | 4 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 3 | 2 | 4 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 4 | 2 | 4 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 5 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 6 | 2 | 7 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 7 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 8 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 9 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 0 | 1 | 1 | 1 | |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 10 | | 3 | 4 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | | | | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 11 | | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 12 | | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 13 | | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 14 | | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 15 | | 2 | 3 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 16 | | 3 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 17 | | 3 | 7 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 18 | | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | | | | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 19 | | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | | | | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 20 | | 3 | 7 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| | | | | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 21 | | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 22 | | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | | | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 23 | | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 24 | | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | | | | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 25 | | 2 | 4 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 26 | | 2 | 7 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 27 | | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 28 | | 3 | 5 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | | | | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 29 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 31 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 32 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 33 | 2 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | | | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 34 | 1 | 4 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| | | | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 35 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 36 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 37 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 38 | 2 | 7 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | 1 |
| | | | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 39 | 2 | 5 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 40 | 2 | 4 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 41 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 42 | 2 | 4 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 44 | 2 | 4 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 45 | 2 | 4 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 46 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 47 | 2 | 3 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 48 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 49 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 50 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 51 | 2 | 7 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 52 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 53 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 0 | 1 |
| | | | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 54 | 2 | 4 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 55 | 2 | 5 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 56 | 2 | 5 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 0 | 0 | 1 | 1 |
| | | | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 57 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 65 | 1 | 7 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 0 | 1 |
| | | | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 67 | 3 | 4 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 77 | 2 | 7 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 89 | 1 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 90 | 1 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | | | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 91 | 3 | 3 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 92 | 3 | 3 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| | | | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 96 | 2 | 7 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 0 | 1 |
| | | | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 97 | 2 | 7 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 98 | 2 | 4 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 99 | 2 | 4 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | 1 |
| | | | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | |
| 1 | 100 | 3 | 3 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | | |
| | | | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | |
| 1 | 101 | 1 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | | |
| | | | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | | | | | | | | | | | | | | | | | | | | |
| 1 | 102 | 2 | 4 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | | |
| | | | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | | | | | | | | | | | | | | | | | | | | |
| 1 | 104 | 2 | 5 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 0 | 0 | | |
| | | | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 106 | 2 | 5 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 110 | 2 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 111 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 112 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 113 | 2 | 3 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | | |
| | | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 114 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 117 | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 121 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 0 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 122 | 2 | 7 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 0 | 1 | 1 | | |
| | | | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 125 | 2 | 7 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | |
| 1 | 126 | 2 | 5 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0 | 1 | 1 | 1 | | |
| | | | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | | | | | | | | | | | | | | | | | | | | |

Key

Column 1 indicates origin of subject (1=Grampian region)
Column 2 is the assigned sample number
Columns 3 and 4 show the serologically typed HLA-DR alleles
Column 5 indicates whether subject is MS patient (1) or control (0)

Columns 6-39 represent restriction fragments: present (1), absent (0), uncertain (-1)

| | |
|---|---|
| Columns 6-17 | BamH1 fragments |
| Columns 18-26 | Bgl11 fragments |
| Columns 27-34 | EcoR1 fragments |
| Columns 35-39 | Taq1 fragments |

## Table 21

DRB restriction fragments

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 4 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | |
| | | | | | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | |
| 1 | 2 | 2 | 4 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | | | | | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | |
| | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | | | | |
| 1 | 3 | 2 | 4 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | |
| | | | | | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | |
| 1 | 4 | 2 | 4 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | |
| | | | | | -1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | | | | |
| 1 | 5 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | | | | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | | | |
| 1 | 6 | 2 | 7 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | |
| | | | | | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | | | | |
| 1 | 7 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | |
| | | | | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | | | | |
| 1 | 8 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | | | | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | | | | |
| 1 | 9 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | |
| | | | | | -1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | | | | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | | | | |
| 1 | 10 | | 3 | 4 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | |
| | | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | | | | | | | |
| 1 | 11 | | 2 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | | | | | | | |
| 1 | 12 | | 2 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | 0 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 13 | | 2 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | | 0 | 1 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 14 | | 2 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | |
| 1 | 15 | | 2 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | |
| 1 | 16 | | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | |
| | | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | | | | | | |
| 1 | 17 | | 3 | 7 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | |
| | | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | | | | | | |
| 1 | 18 | | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | |
| | | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | | | | | | | |
| 1 | 19 | | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | |
| | | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | | | | | | | |
| 1 | 20 | | 3 | 7 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | |
| | | 1 | 1 | 0 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 21 | | 2 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 0 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 22 | | 2 | 2 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | | |
| | | 1 | 0 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 23 | | 2 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | | | | | | | | |
| 1 | 24 | | 2 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | | |
| | | 0 | 0 | 1 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 25 | | 2 | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | | |
| | | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | | | | | | | | |
| 1 | 26 | | 2 | 7 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | | |
| | | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | | | | | | | | |
| 1 | 27 | | 2 | 2 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | | | | | | | | |
| 1 | 28 | | 3 | 5 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | | |
| | | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | | | | | | | | |
| 1 | 29 | | 2 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | | | | | | | | |
| 1 | 31 | | 2 | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | | | | | | | | |
| 1 | 32 | | 2 | 2 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | | |
| | | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | | | | | | | | |
| 1 | 33 | | 2 | 4 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | | |
| | | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | | | | | | | |
| 1 | 34 | | 1 | 4 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | | |
| | | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | | | | | | | |
| 1 | 35 | | 2 | 2 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | | | | | |
| 1 | 36 | | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | | | | | | | | |
| 1 | 37 | | 2 | 2 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | | | | | |
| 1 | 38 | | 2 | 7 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| | | | | | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | | |
| | | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | | | | | | | | |
| 1 | 39 | | 2 | 5 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 |
| | | | | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | | |
| | | 0 | 0 | 1 | 0 | 0 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 40 | | 2 | 4 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | | | |
| 1 | 41 | | 2 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 0 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 42 | | 2 | 4 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 44 | | 2 | 4 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | | | |
| 1 | 45 | | 2 | 4 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | | | | | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
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```
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             -1  0  0  0  0  1  0  0  0  1  0  0  0  0  1  1  1  0  1  1  0  0  0  1  0  0
        0  1  1  0  0  1  0  0  1  0  1  0  0  0  0  0  1  0  1  0  1  1  0
1  92    3  3  0 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1
             -1  0  0  0  0  1  0  1  1  1  0  1  0  0  0  1  1  1  0  1  0  0  1  1  0  0
        1  1  1  0  0  1  0  0  0  0  1  0  0  1  0  0  1  1  1  1  1  0  0
1  96    2  7  0 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1
             -1  0  1  1  0  1  0  1  1  0  1  1  1  1  0  0  1  1  0  1  0  0  1  1  1  1
        1  1  1  1  1  1  1  1  0  0  1  1  1  0  0  1  1  1  0  1  1  1  0
1  97    2  7  0 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1
             -1  0  1  1  1  0  1  1  1  1  1  1  1  0  1  1  0  0  0  1  1  1  1  1  1
        1  1  1  1  1  1  1  1  1  0  1  0  0  0  1  1  1  0  1  1  1  1
1  98    2  4  0 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1
             -1  0  1  1  1  0  1  1  1  1  1  1  1  1  0  1  1  0  1  0  1  1  1  1  1  1
        1  1  1  1  1  1  1  1  1  0  1  0  1  1  1  1  1  1  0  1  1  1  1
1  99    2  4  0 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1 -1
             -1  0  1  1  1  0  1  1  1  1  1  1  1  1  0  1  1  0  1  0  1  1  1  1  1  1
        1  1  1  1  1  1  1  1  1  0  1  0  1  1  1  1  1  1  0  1  1  1  1
```

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 100 | 3 | 3 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 0 | 1 | 0 | 0 | 0 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | |
| 1 | 101 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | | | | | | |
| 1 | 102 | 2 | 4 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 104 | 2 | 5 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 106 | 2 | 5 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | | | |
| 1 | 110 | 2 | 2 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| | | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | | | |
| 1 | 111 | 2 | 7 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| | | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | |
| 1 | 112 | 2 | 7 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 113 | 2 | 3 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 114 | 2 | 3 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 117 | 2 | 3 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| | | -1 | -1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | | | | | |
| 1 | 121 | 2 | 7 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 122 | 2 | 7 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 125 | 2 | 7 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |
| 1 | 126 | 2 | 5 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | | |
| | 1 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | | | | | | |

Key

Column 1 indicates origin of subject (1=Grampian region)
Column 2 is the assigned sample number
Columns 3 and 4 show the serologically typed HLA-DR alleles
Column 5 indicates whether subject is MS patient (1) or control (0)

Columns 6-80 represent restriction fragments: present (1), absent (0), uncertain (-1)

| | |
|---|---|
| Columns 6-19 | BamH1 fragments |
| Columns 20-32 | Bgl11 fragments |
| Columns 33-47 | EcoR1 fragments |
| Columns 48-63 | Msp1 fragments |
| Columns 64-80 | Taq1 fragments |

| Enz. | frag. | probe | MS | control | $X^2$ | p |
|---|---|---|---|---|---|---|
| All subjects (MS=33, control=48) | | | | | | |
| MSP | 325 | DQA | 10 | 2 | 10.585 | <0.001 |
| MSP | 250 | DQA | 10 | 2 | 10.585 | <0.001 |
| TAQ | 605 | DQB | 18 | 12 | 7.320 | <0.01 |
| BAM | 355 | DQB | 21 | 18 | 5.351 | <0.05 |
| DR2 subjects (MS=25, control=34) | | | | | | |
| MSP | 325 | DQA | 7 | 1 | 7.718 | <0.01 |
| MSP | 250 | DQA | 7 | 1 | 7.718 | <0.01 |
| MSP | 231 | DQA | 6 | 0 | 9.084 | <0.01 |
| MSP | 591 | DQA | 12 | 7 | 4.958 | <0.05 |
| TAQ | 468 | DQB | 20 | 18 | 4.601 | <0.05 |
| non-DR2 subjects (MS=8, control=14) | | | | | | |
| TAQ | 481 | DQB | 3 | 12 | 5.455 | <0.05 |
| TAQ | 300 | DQB | 2 | 10 | 4.426 | <0.05 |
| BAM | 383 | DQB | 1 | 7 | 3.094 | <0.05 |
| MSP | 325 | DQA | 3 | 1 | | NS |
| MSP | 250 | DQA | 3 | 0 | | NS |

**Table 22**

Restriction fragments showing significant association with MS in individual studies of patients and controls from the Grampian region

Absence of T cell receptor polymorphism discriminating between patients and controls

Individual samples of DNA from the Scottish patients digested with Bgl11 and Taq1 were probed with the TcRα and TcRβ probes. Representative autoradiographs are shown in Figures 27, 28, and 29. Only very limited polymorphism was observed, and there were no significant differences between patients and controls.

Absence of MHC Class II polymorphism discriminating between patients with relapsing/remitting MS and those with primarily progressive MS

The 33 Scottish patients were identified as having either a relapsing/remitting (R/R) course to their illness (n=24), or primarily progressive (PCP) disability (n=9). This classification was made on the grounds of clinical history and serial examination findings (Dr Francis and Dr Downie, Aberdeen Royal Infirmary). No significant differences in the frequencies of any of the Class II restriction fragments between the two groups were observed.

### 3.1.2.3 Summary

Individual RFLP analysis of 33 patients and 48 controls with selected restriction enzyme/Class II probe combinations showed a significant association with MS in the case of two Msp1/DQA1 fragments. One of these was the same 3.25kb fragment identified as discriminatory in the earlier studies of DNA pools, thus confirming that finding. With individual analysis the 3.25kb fragment was seen to occur in every case with a 2.50kb fragment, and with a 2.31kb fragment in 60% of cases, these fragments therefore appearing as a "cluster" representing 8.06kb of genomic DNA. This cluster was seen in 10/33 (30.3%) patients and in only 2/48 (4.2%) controls ($p<0.001$). Of the DR2+ve subjects, it was present in 7/25 (28%) patients and in 1/34 (2.9%) controls ($p<0.01$). There were insufficient non-DR2 cases in this study to determine whether a similar association might exist in this group. A number of DQB1-hybridizing restriction fragments generated by Taq1 and BamH1 were also found to be associated with MS though less strongly ($p<0.01$ or 0.05). These studies do not provide evidence of any association between MS and non-rearranged (germ line) T cell antigen receptor α or β gene polymorphism. There were no significant differences, in the frequencies of any of the restriction

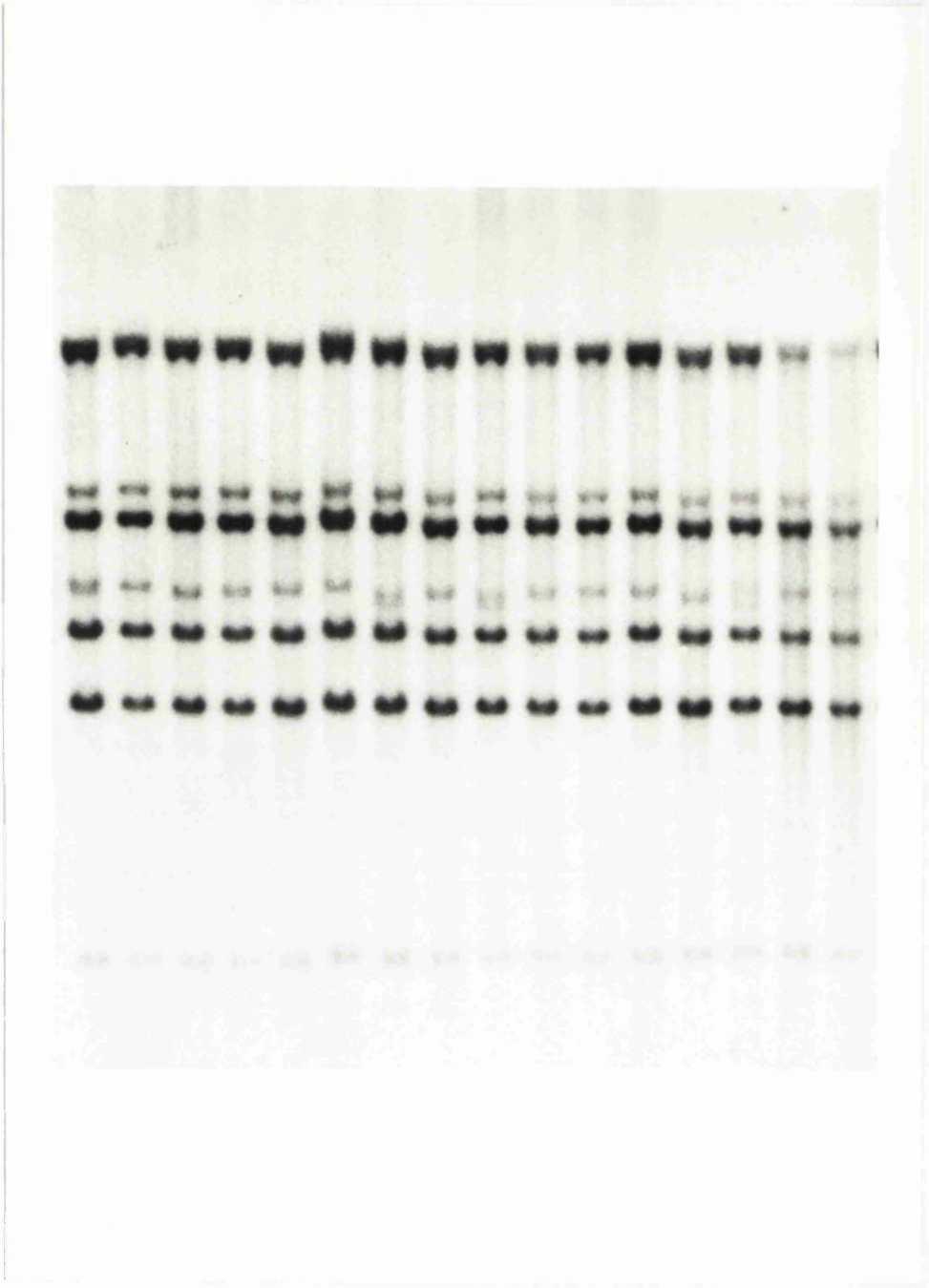


**Figure 27**
Autoradiograph of individual samples of DNA digested with Bgl11 and probed with a T cell receptor α gene probe. It can be seen that there is minimal germline polymorphism revealed by this enzyme.

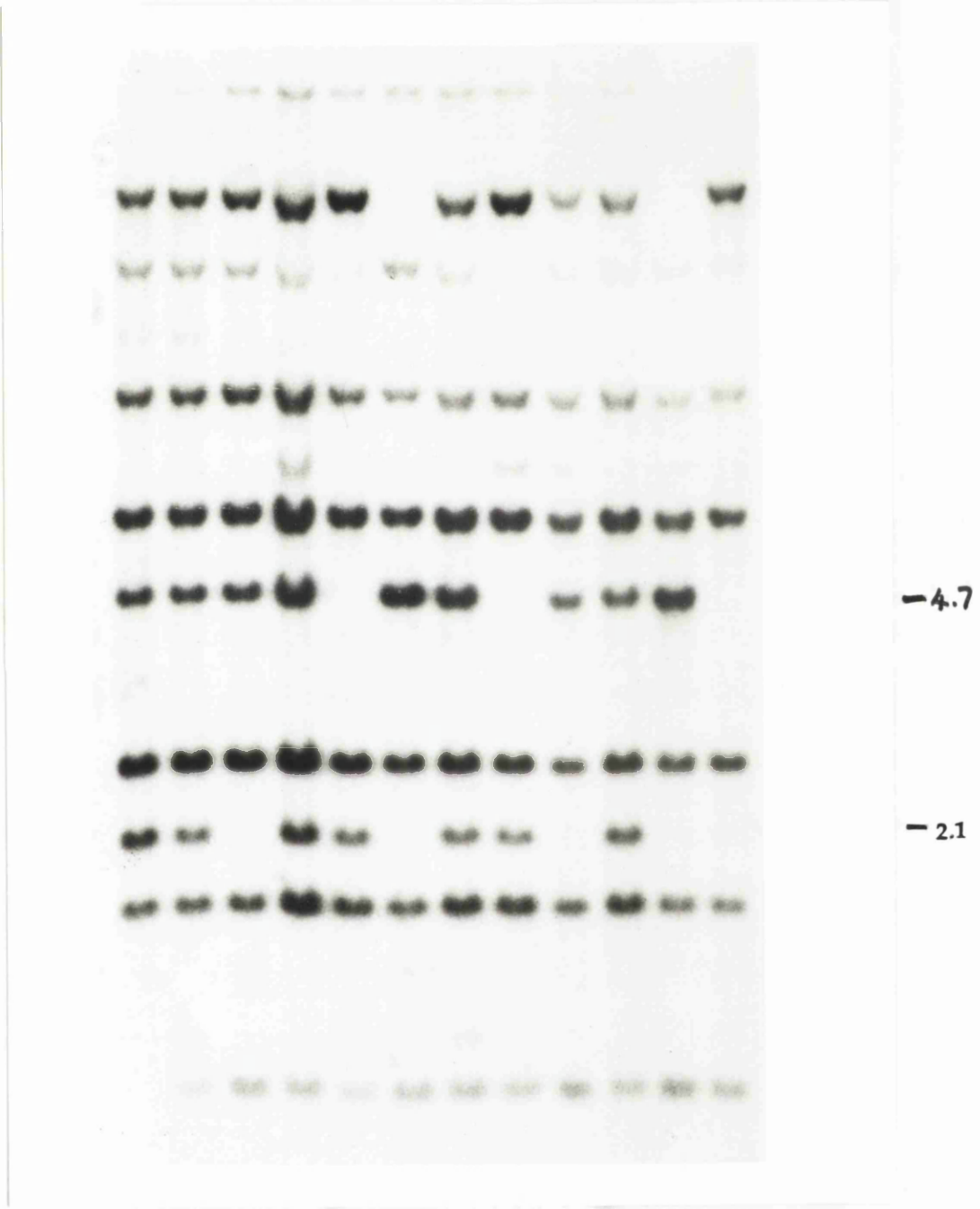



**Figure 28**
Autoradiograph of individual samples of DNA digested with Taq1 and probed with a T cell receptor α gene probe. Polymorphism was confined to fragments of 4.7 and 2.1 kilobases, and there was no significant association with MS.

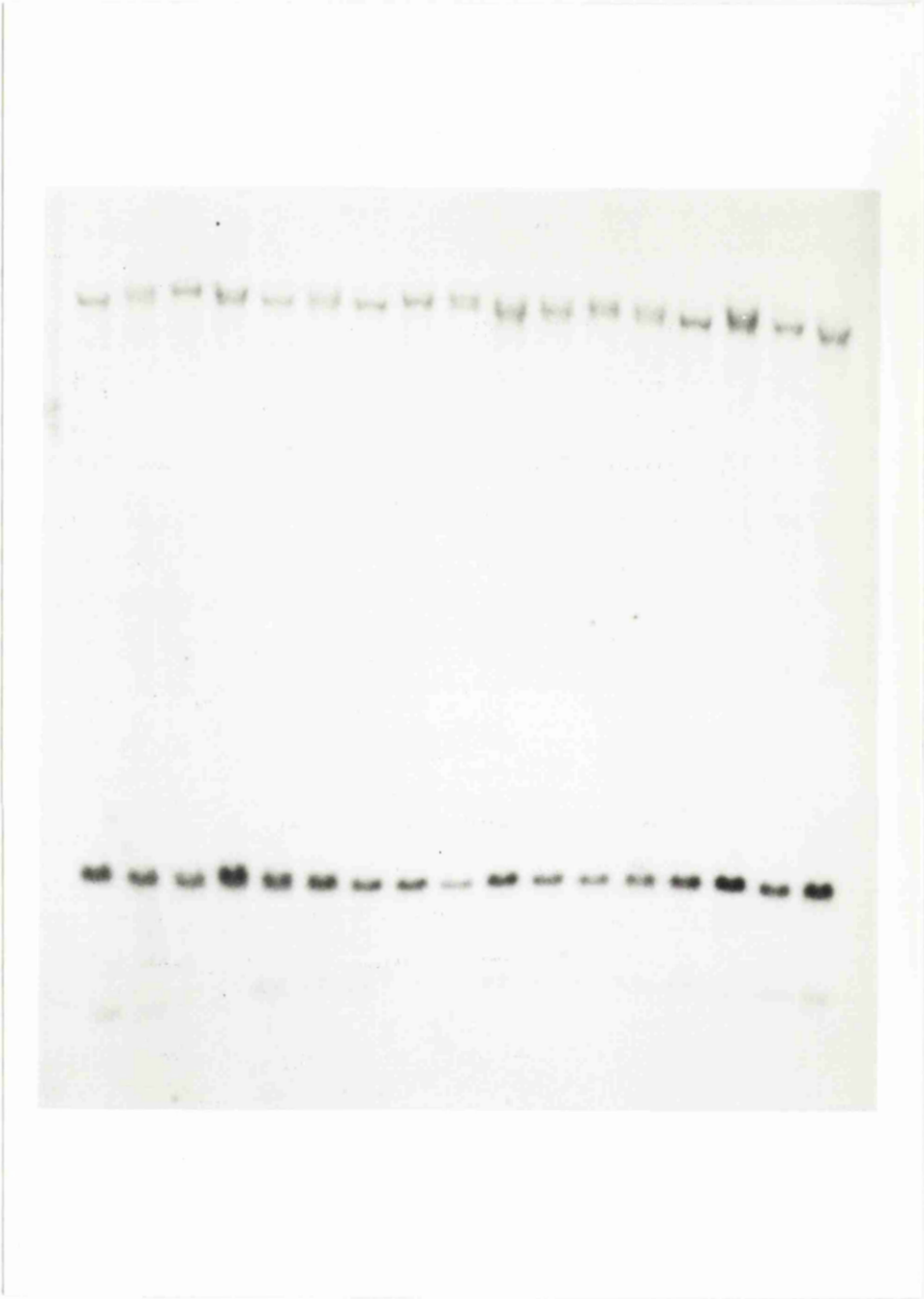


**Figure 29**
Autoradiograph showing individual samples of DNA digested with Bgl11 and probed with a T cell receptor β gene (Cβ2) probe. Only monomorphic restriction fragments are seen.

fragments studied, between patients with relapsing/remitting MS and those with primarily progressive disease. These data are therefore unable to confirm a recent report that R/R and PCP MS may be associated with different DQB1 RFLP (Olerup et al.1987; Olerup et al.1989a).

### 3.1.3 CHARACTERIZATION OF A DQA RESTRICTION FRAGMENT CLUSTER

#### 3.1.3.1 Introduction

The DQA1-hybridizing restriction fragment cluster described above had not previously been described. The Tenth International Histocompatibility Workshop (XIHWS) held in Princeton, NJ in November 1987 included a major systematic effort to evaluate the rôle of RFLP analysis in the characterization of loci and haplotypes comprising the HLA system. The data generated from the use of 12 restriction endonucleases on DNA from 70 cell lines were technically valid as revealed by fragment pattern consistency of hidden duplicates, and by the relationships between fragment patterns and known HLA specificities and haplotypes (DuPont, 1989b). More than 1100 fragments associated with 11 probes were analysed by computer. These data provided the opportunity for characterizing the DQA1 fragment cluster identified in the Scottish MS patients.

It has been suggested that 5-methyl cytosine (5-meCyt) in eukaryotic DNA regulates gene expression by altering protein-DNA interactions (Riggs, 1975). Many studies have shown a positive correlation between hypomethylation of a gene and its expression (for examples see (McGhee, Ginder, 1979; Mandel, Chambon, 1979; Gazit et al.1980; Compere, Palmiter, 1981)). Hypomethylation of Class II region genes might be expected therefore to have profound functional consequences. The restriction enzyme Msp1 recognizes and cleaves both the sequence 5'CCGG3' and the methylated sequence 5'C$^{m}$CGG3', whereas the isoschizomeric enzyme Hpa11 only cleaves the unmethylated sequence 5'CCGG3' and not 5'C$^{m}$CGG3'. Any restriction fragment differences betwen Hpa11 and Msp1 must be due methylation of the recognition sites. This provided the opportunity to investigate whether restriction sites giving rise to the DQA1 cluster were methylated.

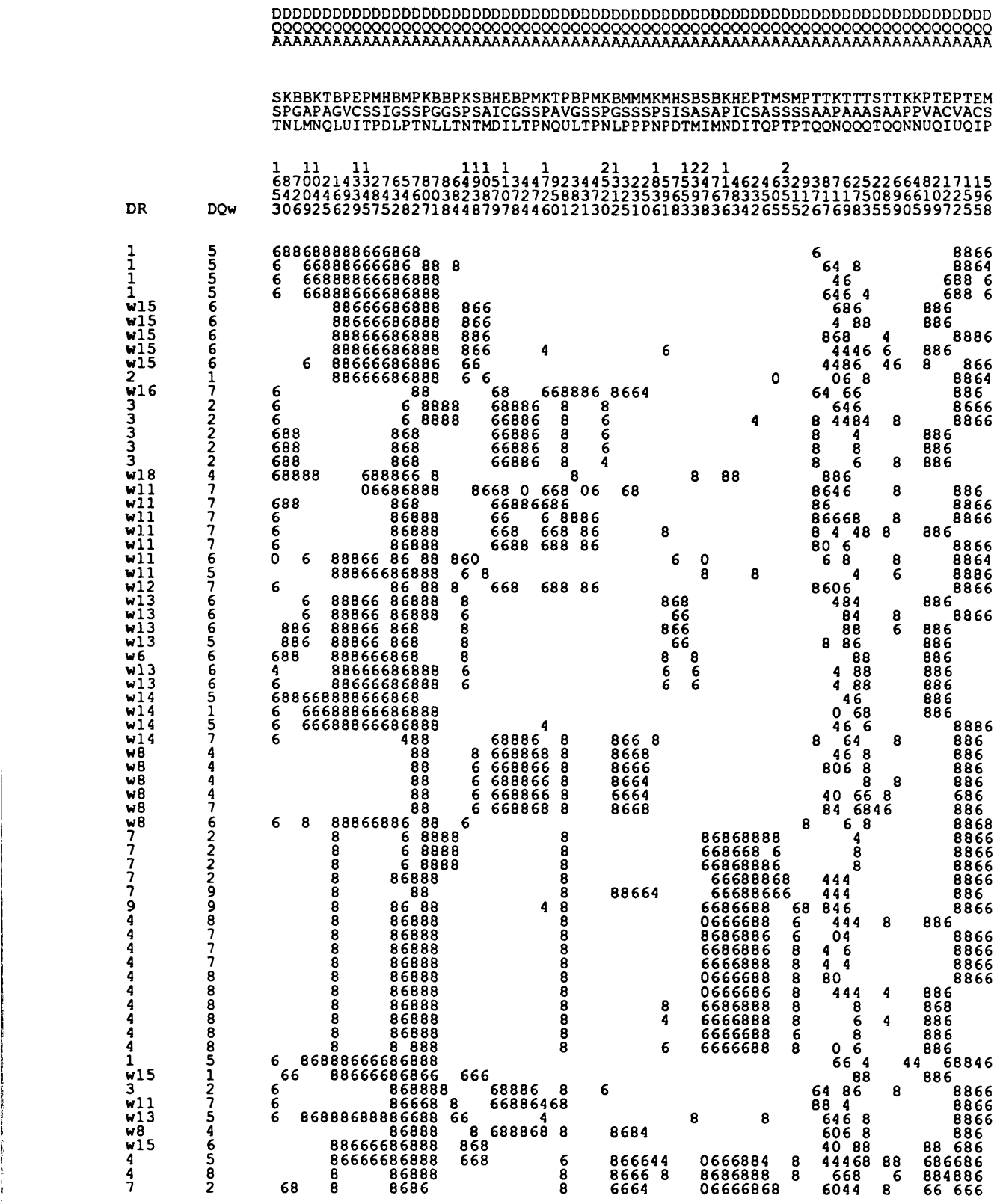
### 3.1.3.2 Results

The DQA1 restriction fragment cluster exhibits an allelic relationship to DQw1

Table 23 shows a cluster analysis of 72 DQA restriction fragments from the XIHWS grouped by the known DR, Dw and DQw allotypes of the core cell lines. The DR and DQw types are shown down the left side of the table, the rows at the top indicate the probe (DQA), enzyme (eg. TAQ = Taq1) and the fragment size (eg. 1345 = 13.45kb). DQA-associated fragments occupy the left two-thirds of the table (SST 1653 to PST 912). A number of block patterns of fragment presence and absence are apparent. Fragments associated with the DQA2 locus are shown at the extreme right of the table (PVU 819 to MSP 568).

The three Msp1 fragments described above are seen to lie adjacent to each other, and to form a cluster which is completed by a 13.12kb Bgl11 fragment. This cluster is present in 12 of the 70 cell lines, as shown in Table 24. It can be seen that it is present in all of the DRw8 cell lines, 2 of 11 DR4 cell lines and 2 of 6 DR7 cell lines. All of the DRw8 cell lines encode the recently recognized DQw4 specificity (previously DQ'blank'), and DQw7, w8 and w9 account for most of the other cluster-positive cell lines (Manika appears to be heterozygous at the DQ loci). Furthermore, the cluster is not seen in any of the DR2 cell lines, with the exception of RML which carries the rare Amerindian specificity Dw22 (previously known as DwLD5a). In contrast to the fragment cluster, a 2.87kb Msp1 DQA1 fragment appears to define a broad DQw1 specificity, being present exclusively in those cell lines which are DQw1, DQw5 or DQw6. It is of great interest that the 3.25/2.50/2.31kb fragment cluster and the 2.87kb fragment exhibit an allelic relationship and are never present together in a single homozygous cell line. Inspection of the XIHWS data failed to reveal a DQB1 restriction fragment or fragment cluster with the same haplotype distribution as the DQA1 cluster.

The sum of the Msp1 and Bgl11 restriction fragments in selected cluster-positive and cluster-negative cell lines is shown in Table 25. In most cases, the sum of the fragments in the cluster-positive line is slightly greater than that in the cluster-negative line of the same DR type. As discussed later, this raises the possibility that the cluster-positive haplotype contains extra genomic sequence in relation to the DQA1 locus, and would also be compatible with the presence of a third DQA region gene on these haplotypes.



## Table 23

Tenth International Histocompatibility Workshop DQA RFLP fragments, showing homozygous cell lines listed by DR and DQw specificities. Positive clusters.

| | | Phenotype | | |
|---|---|---|---|---|
| Name | No. | DR | Dw | DQw |
| RML | 9016 | 2 (w16) | 22 (LD5a) | 7 |
| PE117 | 9028 | 4 | 14 | 8 |
| LKT3 | 9107 | 4 | 15 | 8 |
| DBB | 9052 | 7 | 11 | 9 |
| Manika | 9106 | 7 | ? | 2,3 |
| BTB | 9067 | 8 | 8.1 | 4 |
| BM9 | 9068 | 8 | 8.1 | 4 |
| Madura | 9069 | 8 | 8.1 | 4 |
| Olga | 9071 | 8 | 8.2 | 4 |
| Spach | 9072 | 8 | 8.2 | 4 |
| Luy | 9070 | 8 | 8.3 | 7 |
| DHIF | 9104 | 11 | 5 | ? |
| Amala | 9064 | w14 | 16/24 | 7 |

**Table 24**

HLA-DR and -DQ phenotypes of the Xth Workshop core cell lines which have the Msp1/DQA1 3.25 kb restriction fragment polymorphism

| | W/S no. | 9016 | 9014 | 9106 | 9050 | 9028 | 9026 |
|---|---|---|---|---|---|---|---|
| | DR | 2 | 2 | 7 | 7 | 4 | 4 |
| | cluster | + | - | + | - | + | - |
| Bgl11 | | | | | | | |
| | kb | | | | | | |
| | 7.26 | - | - | + | - | + | - |
| | 13.12 | + | - | + | - | + | - |
| | 3.78 | - | - | - | - | - | - |
| | 3.82 | + | - | - | - | - | - |
| | 6.32 | - | + | + | - | + | + |
| | 1.66 | - | + | + | + | + | + |
| | 7.01 | - | + | - | + | + | + |
| | 8.38 | - | - | - | + | - | - |
| | 51.35 | 16.94 | 14.99 | 21.10 | 17.05 | 28.11 | 14.99 |
| Msp1 | | | | | | | |
| | kb | | | | | | |
| | 4.74 | - | - | - | - | - | - |
| | 2.87 | - | + | - | - | - | - |
| | 4.73 | + | - | - | - | - | - |
| | 5.48 | - | + | + | + | + | + |
| | 5.91 | - | - | - | - | + | - |
| | 5.68 | - | + | - | + | - | - |
| | 3.25 | + | - | + | - | + | - |
| | 2.50 | + | - | + | - | + | - |
| | 2.31 | + | - | + | - | + | - |
| | 6.05 | - | - | + | + | - | - |
| | 2.15 | - | - | - | - | + | + |
| | 45.67 | 12.79 | 14.03 | 19.59 | 17.21 | 21.60 | 7.63 |

**Table 25**

Showing the summation of the sizes of the Msp1 and Bgl11 restriction fragments observed in 3 cluster-positive and 3 cluster-negative cell lines. Comparing cell lines of the same DR phenotype, it can be seen that in 5 cases the sum of the fragments in the cluster-positive cell line is greater than that in the cluster-negative line. In the DR4 cell lines, the differences are >13kb.

Msp1 and Hpa11 give the same patterns of restriction fragments

A number of samples of DNA from subjects known to be cluster-positive were digested with Hpa11 and probed with p11-α-5 in the usual manner. The restriction fragment pattern obtained was identical to that given by Msp1 (see Figure 30).

### 3.1.3.3 Summary

Cluster analysis of data from the XIHWS confirms that the DQA1 Msp1 restriction fragments which are significantly associated with MS in a sample of individuals from the Grampian region form a DQA1-related cluster which also contains a single Bgl11 fragment. This cluster is strongly associated with DRw8/DQw4 haplotypes and is also seen in DR4 and DR7 Workshop cell lines. The cluster is never observed in a homozygous cell line with DQw1 and therefore exhibits an allelic relationship. These data raise the possibility that the fragment cluster defines an hitherto unrecognized member of the DQA1 allelic series.

By comparing the sums of the Bgl11 and Msp1 restriction fragments present in cluster-positive and cluster-negative cell lines, the possibility is raised that cluster-positive haplotypes may encode additional DQA locus-related sequence.

There was no difference in the restriction fragment patterns obtained in a cluster-positive cell line with the restriction enzymes Msp1 and Hpa11, indicating that the recognition sites responsible for the fragment cluster are not methylated.

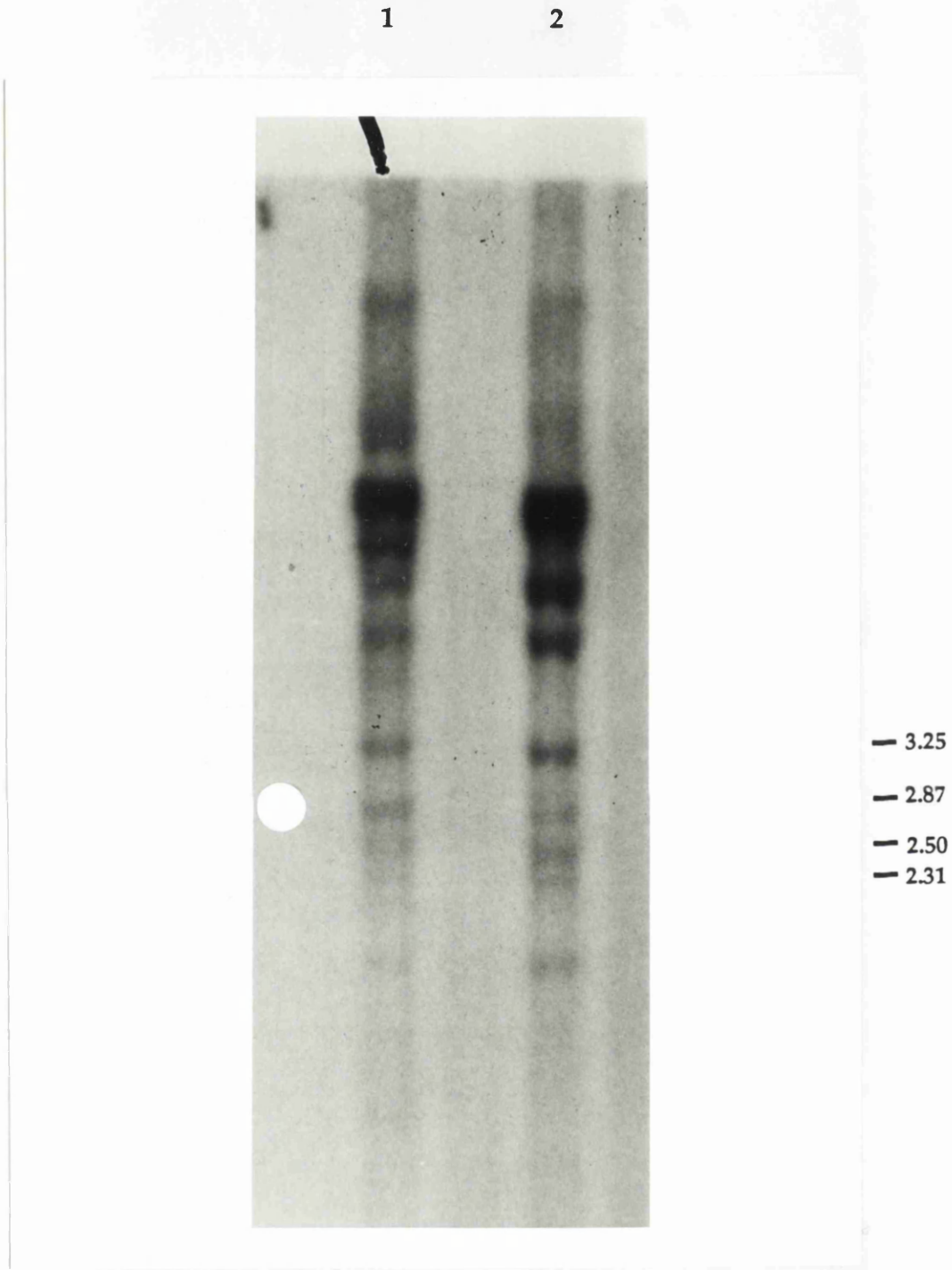



**Figure 30**
Autoradiograph showing a sample of 'cluster positive' DNA digested with Msp1 (lane 1) and Hpa11 (lane 2), and probed with DQA1. It can be seen that the cluster, comprising 3.25, 2.50 and 2.31kb fragments, is present in both lanes.

### 3.1.4 DEFINITION OF THE DR2 HAPLOTYPE IN MULTIPLE SCLEROSIS PATIENTS

#### 3.1.4.1 Introduction

As has been discussed previously, MS is strongly associated with the Class II allele HLA-DR2, and perhaps even more strongly with DQw1, with which it is tightly linked. Since these observations were first made, it has been hypothesized that DR2 might itself be a disease susceptibility gene. Furthermore, it has been speculated that the DR2 allele (or by inference the DQw1 allele) which is found in high MS-risk populations might differ in some subtle manner from that occurring more frequently in lower risk populations, such that the two alleles are indistinguishable serologically or with PLT typing. Allogenotyping by RFLP analysis provides a means for detecting allelic polymorphism not identifiable by these more established methods. The XIHWS has revealed a number of unique restriction fragments which can be used for accurate assignment of the majority of the Class II alleles, and those unique fragments relevant to alleles associated with DR2 haplotypes are shown in Table 26. By studying the frequencies of certain of these fragments in the subjects from the Grampian region, the alleles comprising the DR2 haplotype in this population could be characterized with greater confidence than has previously been possible.

#### 3.1.4.2 Results

<u>The predominant DR2 haplotype in subjects from the Grampian region is HLA-DR2,Dw2,DQw6.</u>

Four restriction fragments were analyzed enabling the DR and DQ alleles associated with the DR2 haplotypes of the Scottish subjects to be defined. A 2.2kb EcoR1 DQB1 fragment (in the XIHWS data this fragment was measured as 2.26kb) is known to characterize the subtype Dw2 (Segall et al.1986), and XIHWS data has shown a 3.81kb Msp1 DRB fragment to have the same specificity. The 2.87kb Msp1 DQA1 fragment associated with a broad DQw1 specificity has already been described. The fourth fragment studied was 3.10kb Bgl11 DQB1 which defines DQw6 (previously known as DQw1.2). The results are shown in Table 27. It can be seen that 96% of the DR2+ve patients and 91% of the DR2+ve controls have the typical DR2,Dw2,DQw6 haplotype common in northern European caucasoids, and that DQw6 is present in 100% of the DR2+ve patients. It should be noted that the frequency of DQw1 (DQw6) in these allogenotyped patients

| RFLP specificity | BAM | BGL | ECI | ECV | HIC | HID | MSP | PST | PVU | SST | TAQ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DR2 | | | RB628 | | | RB332 | | | RB447 | | |
| <DR2 | | RB1455 | | | | | | | | | |
| DR15, Dw2 | | | | | | | RB381 | | | | |
| DR15, Dw12 | | | | | | | | | | | RB212 |
| DQw1 | | | | | | QB644 | QA287 | | | | |
| <DQw1 | | | | QB673 | | | | | | | QB534 |
| DQw5 | QB576 | | | | | | | QB136 | | | |
| DQw6 | | QB310 | | | | | | | | | |
| DQw6, Dw2 | QB289 | | QB226<br>QB922 | QB524 | | | | | | | |
| DQw6, Dw12 | QB872 | | | QB1182 | QB185<br>QB1536 | | | | QB202 | | QB450 |

**Table 26**

Unique DR and DQ restriction fragments identified from XIHWS data which are suitable for defining alleles of the DR2.DQw1 haplotypes

| Fragment | Allele | All MS | All controls | | DR2+ MS |
|---|---|---|---|---|---|
| | DR2+ controls | | | | |
| | DR2 | 25/33 (75%) | 34/48 (71%) | | |
| DQB/EcoR1 2.20kb* | Dw2 | 24/33 (73%) | 31/48 (65%) | 24/25 (96%) | 31/34 (91%) |
| DRB/Msp1 3.81kb | Dw2 | 24/33 (73%) | 31/48 (65%) | 24/25 (96%) | 31/34 (91%) |
| DQA/Msp1 2.87kb | DQw1 | 27/33 (82%) | 34/48 (71%) | 24/25 (96%) | 32/34 (94%) |
| DQB/Bgl11 3.10kb | DQw6 | 25/33 (75%) | 38/48 (79%) | 25/25 (100%) | 33/34 (97%) |

**Table 27**

RFLP characterization of DR and DQ alleles on the DR2 haploptype in Scottish patients and controls

*2.26kb in XIHWS data

is similar to that found serologically in the same population (Francis et al.1987b), even though the proportion of HLA-DR2 was higher (see Table 28).

Heterozygosity in the Scottish subjects

Of the Scottish patients with the DQA1 restriction fragment cluster described above, 5/10 are DR2,x; 2/10 are DR2,2 and 3/10 are DRx,y. However, the two DR2,2 patients possess both DRB Msp1 3.81kb and DRB Taq1 2.12kb fragments, indicating the presence of both Dw2 and Dw12. Thus 7/10 cluster-positive patients have one DR2,Dw2,DQw6 haplotype. Since the DQA1 cluster appears to be allelic to DQw1, it must be contributed by the non-DR2 haplotype in these patients. Further evidence of heterozygosity will be presented in Chapter 3.1.6.

It has been reported recently that, using a sequence specific oligonucleotide probe, a DQB1 gene encoding polymorphic sequences in DR2, DR4 and DRw6 is found in 97% of Norwegian patients and in 70% of controls ($p<0.05$) (Vartdal et al.1989). Since the Scottish subjects investigated in the present studies had previously been serotyped within families, it was possible to assign both haploptyes in most cases (Francis et al.1987a). Using these data, it was found that DR2, DR4 and DRw6 serospecificities were present in 79% of patients and 68% of controls, the remaining 21% of the patients carrying predominantly either DR1, DR3 or DR7 haplotypes. While DR1 and DR3 haplotypes would not encode the DQB1 polymorphism described by the Norwegian group, this can not be ruled out in DR7 positive patients, since DR7 is associated with DQw9 in a small proportion (15-20%) of the population. However, it was found that a significantly larger number of Scottish MS patients possessed a double dose of DR2/4/w6 (eg. DR2,4 or DR2,6) compared to the controls ($p<0.01$) (see Table 29). The same also appears to be true in the Norwegian population (F. Vartdal, personal communication).

### 3.1.4.3 Summary

Analysis of a number of unique restriction fragments enabled the alleles comprising the DR2 haplotype in the Scottish subjects to be defined in terms of its DQA1, DQB1 and DRB alleles. These data show that the DR2 haplotype in this population sample is almost entirely DR2,Dw2,DQw6, and that DQw6 accounts exclusively for the DQ alleles associated with DR2. These data support a recent report from Australia in which it was

| | MS n=178 | Controls n=128 | $X^2$ | p |
|---|---|---|---|---|
| DR1 | 13.5 | 9.4 | 0.8 | |
| DR2 | 49.4 | 40.6 | 2.0 | |
| DR3 | 32.0 | 28.9 | 0.2 | |
| DR4 | 16.8 | 30.5 | 7.1 | <0.01 |
| DR5 | 6.2 | 14.1 | 4.5 | <0.05 |
| DRw6 | 21.3 | 10.9 | 5.0 | <0.03 |
| DR7 | 21.9 | 31.2 | 2.4 | |
| DRw8 | 0.6 | 0 | | |
| DRw9 | 2.2 | 2.3 | | |
| DRw10 | 0 | 0 | | |
| DR blank | 36.1 | 32.1 | | |
| DQw1 | 77.5 | 62.5 | 7.6 | <0.006 |

**Table 28**

HLA Class II antigen frequencies in northeast Scotland, from Francis et al. (1987b).

| | Single dose | Double dose dose | Single or double dose |
|---|---|---|---|
| Scotland | | | |
| MS | 20/63 (32%) | 30/63 (48%) * | 50/63 (79%) |
| Control | 19/37 (52%) | 6/37 (16%) * | 25/37 (68%) |
| Norway | | | |
| MS | | 32/61 (52%) † | |
| Control | | 14/56 (26%) † | |

* $X^2 = 9.98$ † $X^2 = 9.23$

Table 29

Heterozygosity in MS patients and controls. Showing the frequencies with which HLA-DR2, -DR4 and -DRw6 are present in single and double dosage in patients and controls from NE Scotland and Norway.

found that DR2,Dw2,DQw1 was the most common DR2 haplotype in MS patients and that the distribution of this and other DR2 haplotypes was similar in patients and healthy controls (Serjeantson et al.1986).

Seven of the ten DQA1 cluster-positive patients also have this typical DR2 haplotype, and 10/10 have the DQA1 Msp1 2.87kb fragment associated with DQw1. Since the cluster is allelic to DQw1, the cluster must be contributed by the non-DR2 haplotype in these cases. This finding raises the possibility that susceptibility to MS may be influenced by transcomplementation between genes of the DR2 haplotype and the cluster-positive haplotype. Further data in support of this hypothesis will be presented and discussed in Chapter 3.1.6.

Finally, examination of serological data from the Grampian population shows that a significantly higher proportion of MS patients possess a double dose of the alleles DR2, DR4 or DRw6. This finding is of greater interest in the light of the observation that the polymorphic amino-terminal amino acids shared by the DQβ chains encoded by the DQB1 gene on these haplotypes are all directed inwards to the antigen binding cleft of the heterodimer (Vartdal et al.1989).

## 3.1.5 TENTH INTERNATIONAL HISTOCOMPATIBILITY WORKSHOP STUDIES

### 3.1.5.1 Introduction

The RFLP studies on the Scottish population provided a basis for a larger multi-centre study, which was conducted within the framework of the XIHWS. All the individuals for this study were Northern European caucasoids and were drawn from five different centres. These centres and the list of participants are given in Appendix 1. The patients were all cases of clinically definite MS (Poser et al.1983) and the controls were healthy unrelated individuals from the same geographical location. The frequencies of DR2+ve and DR2-ve individuals in disease and control groups were matched. The proportions of DR alleles other than DR2 in the two groups was also matched as closely as possible. The enzyme/probe combinations studied were the same as those described above (DQA1:Msp1 and Taq1; DQB1:BamH1, Bgl11, EcoR1 and Taq1; DRB:BamH1, Bgl11, EcoR1, Msp1 and Taq1). Southern blot analysis was carried out in each centre and the marker and fragment mobilities recorded. The

autoradiographs were then sent to the Department of Immunology, RPMS, London for standardization and statistical analysis.

The films were read independently by three observers, and only those fragments which were present on films from all the centres were included. In case of unresolved doubts about interpreting a film, that film was excluded from analysis. The fragments were scored as either present or absent and analyzed in the same way as described in Chapter 3.1.2.

### 3.1.5.2 Results

Those fragments whose frequency was significantly different in the patients and the controls ($p \leq 0.05$) are shown in Table 30. There were significant differences in 7/18 DQA fragments (2 of these were DQA2 frament s), 3/34 DQB1 fragments and 6/75 DRB fragments. Once again it was the Msp1 DQA1 3.25kb fragment which was the most important finding, being present in 22.7% of patients and 6.4% of controls irrespective of DR type, and in 21.2% and 5.7% respectively of the DR2-positive subjects. Other fragments with a significantly distorted distribution were Bgl11 DQB1 4.23kb (69.6% vs. 40% in DR2+ individuals), and Taq1 DRB 7.23kb (14.1 vs. 33.8% in all individuals and 8.3% vs. 41.7% in DR2-ve individuals). The Msp1 DQA1 4.74kb fragment was also negatively correlated to disease (57.6% vs. 75.8%).

### 3.1.5.3 Summary

By means of a collaborative study involving groups in southern England, Northern Ireland, Brittany and Holland, in addition to the Grampian region of Scotland, the numbers of patients and controls were increased to 88 and 84 respectively (maximum). As a result of these larger numbers, statistical significance was achieved by several other fragments which had not been significantly associated with MS in the Grampian population alone. However, it was noteworthy that the distribution of the Msp1 DQA1 3.25kb fragment once again was significantly distorted ($p<0.01$). Probably as a result of imperfect standardization of the Southern blotting protocol between the different centres, and the use of different length DQA1 cDNA probes, the 2.50 and 2.31kb fragments which complete the cluster were not seen on some of the autoradiographs. Analysis of this larger sample again confirmed that the predominant DR2 haplotype in these northern European populations is DR2,Dw2,DQw6 (51/52 MS patients and 53/53 controls).

| | | Frequency | | Percent | | | |
|---|---|---|---|---|---|---|---|
| | Fragment | MS | Control | MS | Control | $X^2$ | p |
| DQA1 | | | | | | | |
| ALL | MSP325 | 18/79 | 5/78 | 22.7 | 6.4 | 8.42 | <0.01 |
| | MSP474 | 38/66 | 50/66 | 57.6 | 75.8 | 4.91 | <0.05 |
| | MSP250 | 14/53 | 6/53 | 26.4 | 11.3 | 3.94 | <0.05 |
| | TAQ642 | 31/88 | 18/84 | 35.2 | 21.4 | 4.02 | <0.05 |
| | TAQ200* | 55/88 | 64/84 | 62.5 | 76.2 | 3.78 | =0.05 |
| DR2+ | MSP325 | 11/52 | 3/53 | 21.2 | 5.7 | 7.46 | <0.01 |
| | MSP474 | 23/39 | 33/41 | 59.0 | 80.5 | 4.41 | <0.05 |
| | MSP250 | 9/39 | 4/41 | 23.1 | 9.8 | N.S. | |
| DR2- | none significant | | | | | | |
| DQB1 | | | | | | | |
| ALL | BGL423 | 47/83 | 19/53 | 56.6 | 35.8 | 5.59 | <0.05 |
| | BGL693 | 17/83 | 20/53 | 20.5 | 37.7 | 4.86 | <0.05 |
| | BGL561 | 22/83 | 6/53 | 26.5 | 11.3 | 4.56 | <0.05 |
| DR2+ | BGL423 | 39/56 | 12/30 | 69.6 | 40.0 | 7.11 | <0.01 |
| DR2- | none significant | | | | | | |
| DRB | | | | | | | |
| ALL | TAQ723 | 10/71 | 24/71 | 14.1 | 33.8 | 7.58 | <0.01 |
| DR2+ | ECI628 | 12/52 | 23/54 | 23.1 | 42.6 | 4.56 | <0.05 |
| | MSP315 | 41/52 | 50/53 | 78.8 | 94.3 | 5.45 | <0.05 |
| | TAQ440 | 21/47 | 31/47 | 44.7 | 66.0 | 4.30 | <0.05 |
| | TAQ123 | 28/47 | 37/47 | 59.6 | 78.7 | 4.04 | <0.05 |
| DR2- | TAQ193 | 2/24 | 10/24 | 8.3 | 41.7 | 7.11 | <0.01 |
| | MSP723 | 6/22 | 14/23 | 27.3 | 60.9 | 5.14 | <0.05 |

Table 30

Class II restriction fragments whose frequencies differed significantly between MS patients and controls in the collaborative North European study

* DQA2 associated fragment

### 3.1.6 COLLABORATIVE STUDIES ON THE NORTHERN IRELAND POPULATION

#### 3.1.6.1 Introduction

Following the XIHWS, a more extensive collaboration with the group from Northern Ireland was entered upon. The populations of Northern Ireland and northeast Scotland are genetically similar, and both have high prevalence rates for MS. Northern Ireland is populated by two genetic stocks, Irish Gaelic and Ulster Scots. The frequencies of HLA-DR alleles in Northern Ireland and mainland Scotland are similar (see Table 31), with DR2 occurring at a frequency of 31% in both Northern Ireland and in southeast Scotland ((Middleton et al.1985), C Cullen and D Middleton, unpublished results). It should be noted however that the frequency of DR2 in the Grampian region, 40.6% (Francis et al.1987b) is significantly higher, closer to that seen in the Orkneys, which higher frequency in the general population is thought to account for the failure of DR2 to associate strongly with MS in patients from this region (Compston, 1981). The prevalence rates of MS in Northern Ireland and NE Scotland are respectively $137/10^5$ and $178/10^5$ (Hawkins, Kee, 1988; Francis et al.1987a).

50 patients and 76 healthy unrelated controls from Northern Ireland were studied at Belfast City Hospital. 33 patients and 48 controls from the Grampian region were studied at the Royal Postgraduate Medical School. Patients and controls were HLA Class II typed in the respective centres either serologically, using standard antisera, or by an established RFLP allogenotyping system (Bidwell et al.1988). The methods used for Southern blot analysis were identical in the two centres except that the Belfast membranes were probed with the slightly shorter DQA1 clone pDCH1 (Auffray et al.1982), and high stringency washing of the membranes after hybridization was carried out using 0.2X SSPE (0.15M NaCl/0.01M $NaH_2PO_4$/1mM $Na_2EDTA$, pH7.4). Fragment analysis was carried out at the RPMS as before.

#### 3.1.6.2 Results

The only restriction fragments which were distributed unequally between the patients and controls in the Northern Ireland population samples were once again the three DQA1 fragments comprising the cluster described above. Although this cluster was seen with equal frequency in the Belfast patients and controls, when only the DR2-positive individuals

| DR | Northern Ireland | Edinburgh | Grampian region |
|---|---|---|---|
| 1 | 12.5 | 11.3 | 9.4 |
| 2 | 31.0 | 31.0 | 40.6 |
| 3 | 33.3 | 31.0 | 28.9 |
| 4 | 34.9 | 31.0 | 30.5 |
| 5 | 8.2 | 9.9 | 14.1 |
| w6 | 18.4 | 16.9 | 10.9 |
| 7 | 25.8 | 25.4 | 31.2 |
| w8 | 1.1 | NT | 0 |
| 9 | 1.6 | NT | 2.3 |

**Table 31**

HLA-DR antigen frequencies in Northern Ireland, southeast Scotland (Edinburgh) and the Grampian region of northeast Scotland. (Personal communication from D Middleton and modified from *Tissue Antigens (1985)* 25:220-221 ).

were examined it was unequally represented in the two groups, being seen in 6/30 (20%) patients and 1/29 (3.4%) controls (see Table 32). As in the Scottish studies, the Northern Ireland data did not reveal a DQB1 polymorphism which paralleled the presence of the DQA1 cluster.

The Msp1 DQA1 2.87kb (DQw1-associated) fragment was present in 17/20 (85%) cluster-positive patients and 6/18 (33%) cluster-positive controls ($p \approx 0.001$). The Relative Risk for possessing both the DQA1 Msp1 cluster and the 2.87kb fragment, calculated by the method of Woolf, is 11.33.

### 3.1.6.3 Summary

In contrast to the Scottish data, when all 126 subjects from Northern Ireland were examined, the DQA1 Msp1 cluster occurred with equal frequencies in the patients and the controls. However, analysis of the DR2-positive individuals revealed once again a significant excess of this polymorphism in the MS patients, the cluster being seen in 20% of patients and 3.4% of controls. From these data it can be concluded that the cluster occurs infrequently in healthy DR2-positive individuals, but is significantly more common in DR2-positive MS patients. The lower frequency with which the cluster is seen in DR2-negative subjects from Scotland might be explained by sampling bias, although a genetic difference between the two populations studied remains a possibility.

The DR and DQ alleles of the 20 MS patients with the DQA1 cluster are shown in Table 33. While the DR alleles include several specificities, the DQ allogenotypes in 14 cases are DQw3. Furthermore, a DQ'blank' allele was "detected" on one haplotype in 5 of the Scottish patients using 9th Workshop antisera. This is strongly reminiscent of the association seen in the XIHWS core cell RFLP analyses between the DQA1 cluster and the DRw8,DQw4 (blank) haplotype.

From Table 33 it can also be seen that 13/20 cluster-positive MS patients are DR2, and that 17/20 are DQw1. In contrast, only 6/18 controls show this same heterozygosity. Since the cluster exhibits a strong allelic relationship to DQw1, and is therefore being contributed by the non-DR2 haplotype in these subjects, these data therefore raise the possibility that disease susceptibility is enhanced by the co-inheritance of DR2,DQw1 alleles with another haplotype characterized by the DQA1 restriction fragment cluster.

| | | MS | Control | $X^2$ | p |
|---|---|---|---|---|---|
| ALL | NE Scotland | 10/33 (30.3%) | 2/48 (4.2%) | 10.585 | ≈0.001 |
| | N Ireland | 10/50 (20%) | 16/76 (21%) | | |
| DR2+ | NE Scotland | 7/25 (28%) | 1/34 (2.9%) | 7.718 | <0.01 |
| | N Ireland | 6/30 (20%) | 1/29 (3.4%) | 3.863 | <0.05 |
| | NE Scotland + N Ireland | 13/55 (23.6%) | 2/63 (3.2%) | 11.080 | <0.001 |
| DR2- | NE Scotland | 3/8 (37%) | 1/14 (7.1%) | | |
| | N Ireland | 4/20 (20%) | 15/47 (32%) | | |

**Table 32**

Significant differences in the frequency of the Msp1/DQA1 3.25 kb fragment in MS patients and controls from Northeast Scotland and Northern Ireland

| Patient | HLA-DR | HLA-DQ | |
|---|---|---|---|
| | | Serotype | Genotype |
| A1 | 2,4 | 1,3 | 1,3 |
| A3 | 2,4 | 1,3 | 1,3 |
| A4 | 2,4 | 1,3 | 1,3 |
| A6 | 2,7 | 1,blank | 1,3 |
| A8 | 2,2* | 1,1 | 1 |
| A18 | 1,1 | 1,blank | 1 |
| A19 | 1,1 | 1,blank | 1 |
| A32 | 2,2* | 1,1 | 1 |
| A33 | 2,4 | 1,blank | 1,3 |
| A34 | 1,4 | 1,blank | 1,3 |
| B1 | 2,7 | - | 1,2 |
| B2 | 2,6 | - | 1,3 |
| B3 | 2,6 | - | 1,3 |
| B4 | 2,7 | - | 1,3 |
| B5 | 2,4 | - | 1,3 |
| B6 | 2,2 | - | 1 |
| B7 | 4,7 | - | 3 |
| B8 | 3,7 | - | 2,3 |
| B9 | 6,7 | - | 2,3 |
| B10 | 6,6 | - | 1,3 |

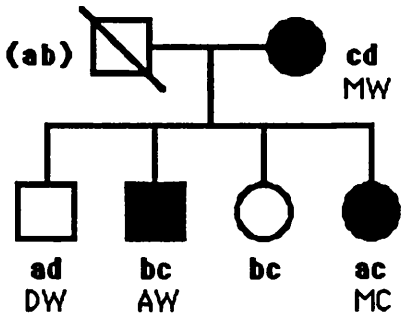
**Table 33**

The HLA-DR and -DQ alleles of the Scottish (A series) and Northern Irish (B series) MS patients with the Msp1/DQA1 3.25 kb restriction fragment polymorphism. Assignment of the DR and DQ alleles in the Scottish patients was made on the basis of family studies (by Dr DA Francis) and thus the DQ blank alleles are securely established. DQ allogenotyping was carried out as described by Bidwell et al. (1988).

### 3.1.7 STUDIES ON THE DQA1 POLYMORPHISM IN A NORMAL POPULATION

#### 3.1.7.1 Introduction

XIHWS data had shown the DQA1 restriction fragment cluster to be associated strongly with DRw8, and to be present also in certain of the DR4 and DR7 cell lines and a single DR2 cell line. The DRw8 cell lines were all DQw4, and the other cluster positive lines were either DQw7, w8 or w9. Studies of the Grampian population had showed the cluster to be present also in two patients who were homozygous for DR1. The 72 homozygous cell lines comprising the core panel of the XIHWS can be criticized for under-representing many of the more common haplotypes and for including a number of cell lines of various non-caucasoid origins without identifying these origins. For these reasons, it was considered important to investigate the relationship of the DQA1 cluster to DR and DQ haplotypes in a sample of caucasoid subjects. 34 heterozygous individuals were selected, comprising DR1 (5), DR4 (12), DR7 (7) and DRw8 (10). Also studied were three cluster-positive XIHWS cell lines, RML (DR2,Dw22,DQw7), BTB (DRw8,DQw4) and Amala (DRw14,DQw7), four EBV lymphoblastoid cell lines derived from a Scottish family, MW, DW, AW and MC (see Figure 31), and EBV Wa (DR4,Dw15,DQw4).




| | A | Cw | B | C4A | C4B | Bf | DR | DQw |
|---|---|---|---|---|---|---|---|---|
| a | 2 | 5 | 44 | 4 | 2 | S | 2 | 6 |
| b | 24 | 4 | 35 | 3/2 | Q0 | F | 1 | 5 |
| c | 24 | - | 7 | 3 | 1 | S | 2 | 6 |
| d | 1 | 6 | 17 | 6 | 1 | S | 7 | 2 |

**Figure 31**

Family M3, showing the extended haplotypes. Lymphoblastoid cell lines were raised from MW, DW, AW and MC.

All DNA samples were digested with Msp1 and probed with a DQA1 cDNA fragment, and additionally, DQA and DQB allogenotyping was performed using the method described by Bidwell et al. ((Bidwell et al.1988) and Appendix 2). The work described in this section was carried out largely by Dr CB Sanjeevi, and has formed the basis of a previous report*.

### 3.1.7.2 Results

The DQA1 cluster is not associated exclusively with any DQA1 or DQB1 allele

The results are summarized in Table 34. The DQA1 cluster was observed in 2/5 DR1 subjects, 0/11 caucasoid DR4 subjects, 4/7 DR7 subjects and 4/10 DRw8 subjects. It was seen in a single non-caucasian DR4 subject who has been shown previously to be DR4,5/Dw13/KT2. Its presence in the XIHWS lines Amala, RML and BTB, and also in EBV Wa, was confirmed. In the Scottish family M3, it was seen in MW (DR2,7) and DW (DR1,7), and from this it was clear that the cluster was contributed in this family by the DR7 haplotype (haplotype **d**). The 2.87kb DQw1-associated fragment was observed in 7/7 DR1 subjects, in 6/7 DR2 subjects (and was not determined in one further case), and in 6/8 DRw6 subjects (one of which was also DR1 (see below). The Scottish family members were all DR1 and/or DR2, and the 2.87kb fragment was present in each of them. The DQβ3.2 RFLP was present in 11/16 (69%) of cluster-positive cases, and the DQβ3.1 RFLP in 4/16 (25%) of cases (2 cases had both 3.1 and 3.2 alleles). The DQB1 allele associated with the DRw8,DQw4 haplotype was characterized by DQβ3.2 in 11/11 cases. However, the DQB1 gene associated with DQw4 is thought to be very closely related to that of the DQw8 (DQw3.2) sub-type of DQw3, and may be indistinguishable from it by DQB1 RFLP analysis (Bushell et al.1988).

It can be seen that a variety of DR and DQw haplotypes are represented in those individuals possessing the DQA1 cluster. The cluster and the 2.87kb fragments were observed to co-exist in 9 individuals, and in view of their allelic relationship to each other and the strong association of the 2.87kb fragment with DQw1, the haplotype contributing the cluster could be determined confidently in these cases as well as in the

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* "Correlations of the presence of DQ alpha RFLP cluster with DR and DQ alleles in healthy caucasians". Sanjeevi CB, Diploma in Immunology, University of London, 1988.

| | Phenotype | | Genotype | | | DQA1/Msp1 | |
|---|---|---|---|---|---|---|---|
| | DR | DQw | DQA1 | DQB1 | DQw | 2.87kb | Cluster |
| 1 | 1 | 1 | 1a | 1.1 | 5 | + | + |
| 2 | 1,2 | 1 | 1a,1b | 1.1,1.2 | 5,6 | + | - |
| 3 | 1,3 | 1,2 | 1a,2 | 1.1,2 | 5,6 | + | - |
| 4 | 1,5 | 1,3 | 1a,2 | 1.1,3.1 | 5,7 | + | - |
| 5 | 1,w6 | 1,3 | 1a,3 | 1.1,3.2 | 5,8 | + | + |
| 6 | 4 | 3 | 3 | 3.1,3.2 | 7,8 | - | - |
| 7 | 4,1 | 3,1 | 3,1a | 3.1,1.1 | 7,5 | + | - |
| 8 | 4,2 | 3,1 | 3,1b | 3.1,1.1 | 7,5 | - | - |
| 9 | 4,2 | 3,1 | 3,1b | 3.1,1.2 | 7,6 | + | - |
| 10 | 4,2 | 3,1 | 3,1b | 3.1,1.2 | 7,6 | + | - |
| 11 | 4,3 | 3,2 | 3,2 | 3.2 | 8,2 | - | - |
| 12 | 4,5 | 3 | 3 | 3.2 | 8 | - | - |
| 13 | 4,w6 | 3,1 | 3,1b | 3.1,1.1 | 7,5 | - | - |
| 14 | 4,w6 | 3,1 | 3,1c | 3.1,1.1 | 7,5 | + | - |
| 15 | 4,w6 | 3,1 | 3,1c | 3.1,1.2 | 7,6 | - | - |
| 16 | 4,w6 | 3,1 | 3,1c | 3.2,1.1 | 8,5 | + | - |
| 17 | 7 | 2,3 | 1b,( ) | 2,3.1 | 2,9 | - | - |
| 18 | 7,1 | 3,1 | ( ) | 3.2,1.1 | 9,5 | + | + |
| 19 | 7,2 | 2/3,1 | 3,1b | 3.2,1.2 | 9,6 | + | + |
| 20 | 7,2 | 3,1 | 3,1b | 3.2,(2) | 9,6 | ( ) | - |
| 21 | 7,3 | 3,2 | ( ) | 3.2,2 | 9,2 | - | + |
| 22 | 7,5 | 2,3 | 1b,1c | 2,3.1 | 2,7 | - | - |
| 23 | 7,5 | 3,1 | 3,1b | 3.2,1.1 | 9,1 | + | + |
| 24 | w8,2 | 1 | 1b | 3.2,1 | 4,6 | + | + |
| 25 | w8,2 | 1 | 1b | 3.2,1.2 | 4,6 | + | - |
| 26 | w8,2 | 1 | 1b,1c | 3.2,1.1 | 4,6 | + | - |
| 27 | w8,5 | 3 | 1b,2 | 3.2 | 4,7 | - | + |
| 28 | w8,5 | 3 | ( ) | 3.2,3.1 | 4,7 | - | + |
| 29 | w8,6 | 1 | 1b,1c | 3.2,1.1 | 4,6 | + | - |
| 30 | w8,6 | 1 | 1b,1c | 3.2,1.1 | 4,6 | + | - |
| 31 | w8,6 | 1 | ( ) | 3.2,1.2 | 4,6 | + | + |
| 32 | w8,9 | 3 | 1b,3 | 3.2 | 4,9 | - | - |
| 33 | w8,11 | 3 | 1b,2 | 3.2 | 4,7 | - | - |
| Amala | w6[16] | 3 | 1b | 3.1 | 7 | - | + |
| RML | 2[22] | 3 | 3 | 3.1 | 7 | - | + |
| BTB | w8[8.1] | Wa | 1c | 3.2 | 4 | - | + |
| MW | 2,7 | 1,2 | ( ) | 1.2,2 | 6,2 | + | + |
| DW | 2,7 | 1,2 | 1b,2 | 1.2,2 | 6,2 | + | + |
| AW | 1,2 | 1,1 | 1a,1c | 1.1,1.2 | 5,6 | + | - |
| MC | 2,2 | 1,1 | ( ),1c | 1.2,1.2 | 6,6 | + | - |
| SS | 4[13],5 | 3 | 3,2 | 3.1,3.2 | 7 | - | + |

**Table 34**
RFLP analysis of a panel of healthy caucasoid subjects and selected B lymphoblastoid cell lines, showing DQA1 and DQB1 allogenotypes and their relationship with the DQA1 cluster.

homozygous cell lines. These data are summarized in Table 35 below. It can be seen that the DQw allogenotype in 6 cases is DQw7, w8 or w9 (subtypes of DQw3), and in 3 cases is DQw4 (DQ Wa). The DQB1 allele is DQβ3.2 in 7 cases and 3.1 in 2 cases. The cluster appeared to be associated with a DR1,DQw5 haplotype in 1 case (subject #1). The cluster was observed in the presence of each of the five described DQA1 alleles 1a, 1b, 1c, 2 and 3, and thus no association is seen between the cluster and the DQA1 allele as determined by RFLP.

The results of DQ allogenotyping of the 10 cluster-positive Scottish MS patients described in Chapter 3.1.2 are shown in Table 36. Again, there is no association between the cluster and a single DQA1 allele, but the DQB1 allele is DQβ3.2 in 5/10 cases (and probably in one further case in which the restriction fragment pattern was ambiguous). It should be noted that 4/10 cases possessed two DQw1 haplotypes, and yet were cluster-positive. Two of these were DR1,DQw5 and two were DR2 homozygous. In each of these DR2,2 cases, both DQα1b and DQα1c RFLPs were present, suggesting that these subjects are Dw2,Dw12 heterozygous (see Appendix 2). It must be concluded therefore that although the cluster may occur in association with DQw1, and that its allelism is not absolute, it does not appear to be associated with the DR2.Dw2,DQw6 haplotype.

### 3.1.7.3 Summary

In summary, DQA1 and DQB1 allogenotyping of several subjects and three XIHWS HCLs was carried out in order to (i) confirm the previously observed relationship of the DQA1 cluster with DR1, DR4, DR7 and DRw8 haplotypes in a caucasoid population, and (ii) further investigate the relationship between the cluster and alleles of DQA1 and DQB1 as determined by DQ RFLP analysis. The association between the cluster and each of these haplotypes was confirmed, particularly with DR7 and DRw8. The cluster was not seen in caucasian DR4 individuals, but was present in a single non-caucasian DR4,Dw13 subject. The cluster may be associated with DQw1 on both DR1,DQw5 and DR2.Dw12,DQw6 haplotypes, but has not yet been observed associated with the more frequent DR2 haplotype DR2.Dw2,DQw6. No association was found between the cluster and the DQA1 allele. In view of the previously observed allelic relationship between the cluster and DQw1, this was an unexpected finding. In contrast however, the range of DQB1 alleles associated with the cluster was more limited. The cluster was associated with DQβ3.2 in 11/16 (69%) of cluster-

| | HLA-DR | HLA-DQw | DQA1 allele | DQB1 allele |
|---|---|---|---|---|
| 1 | 1 | 5 | 1a | 1.1 |
| 5 | w6 | 8 | 3 | 3.2 |
| 18 | 7 | 9 | | 3.2 |
| 19 | 7 | 9 | 3 | 3.2 |
| 23 | 7 | 9 | 3 | 3.2 |
| 24 | w8 | 4 | 1b | 3.2 |
| 31 | w8 | 4 | | 3.2 |
| Amala | w6[16] | 7 | 1b | 3.1 |
| RML | 2[22] | 7 | 3 | 3.1 |
| BTB | w8[8.1] | 4 | 1c | 3.2 |
| MW | 7 | 2 | | 2 |
| DW | 7 | 2 | 2 | 2 |

**Table 35**

Characterization of the DQA1 and DQB1 alleles, and DQw allogenotype, of the cluster positive haplotype in those individuals in which the presence of the DQA1/Msp1 2.87kb fragment allowed the accurate haplotype assignment of the cluster to be made, and in three homozygous cell lines.

| | Phenotype | | Genotype | | | DQA1/Msp1 | |
|---|---|---|---|---|---|---|---|
| Patient no. | DR | DQ | DQA1 | DQB1 | DQw | 2.87kb | Cluster |
| 129 | 2,4 | 1,3 | 1a,1b | 1.2,3.2 | 6,8 | + | + |
| 150 | 2,4 | 1,3 | 1b,3 | 1.2,3.2 | 6,8 | + | + |
| 142 | 2,4 | 1,3 | 1b,3 | 1.2 | 6,(3) | + | + |
| 135 | 2,7 | 1,BL | 1b,3 | 1.2,3.2 | 6,8 | + | + |
| 127 | 2,2 | 1,1 | 1b,1c | 1.2 | 6,6 | + | + |
| 132 | 1,1 | 1,BL | 1a | - | 5 | + | + |
| 133 | 1,1 | 1,BL | 1a | 1.1 | 5,5 | + | + |
| 147 | 2,2 | 1,1 | 1b,1c | - | 6 | + | + |
| 148 | 2,4 | 1,3 | 1a | 1.2,3.2 | 6,8 | + | + |
| 149 | 1,4 | 1,BL | 1a | 1.1,3.2 | 5,8 | + | + |

**Table 36**

The relationship between the DQA1 Msp1 restriction fragment cluster and the DQA1 and DQB1 allogenotypes in the 10 cluster-positive MS patients from northern Scotland.

positive cases and with DQβ3.1 or DQβ3.2 in 13/16 (81%). All of the DRw8 subjects and cell lines were allogenotyped as DQβ3.2; however, this allele may be indistinguishable by means of DQB1 RFLP from that of DQw4. Accordingly, it was not possible from these data to exclude the possibility that the DQB1 allele present on 69% of cluster-positive haplotypes was identical to that associated with DRw8,DQw4.

## 3.2 FURTHER DEFINITION OF THE DQA POLYMORPHISM

### 3.2.1 STUDIES WITH THE MONOCLONAL ANTIBODY HU-46

#### 3.2.1.1 Introduction

The striking relationship that exists between the Msp1/DQA1 restriction fragment cluster and HLA-DRw8 has been described above. The majority of caucasian DRw8 haplotypes are associated with DQw4. As discussed in Chapter 3.1.7, it may not be possible to differentiate between the DQB1 alleles associated with DQw8 and DQw4 by means of DQ RFLP analysis. Since allogenotyping of the cluster-positive subjects and cell lines described in Chapter 3.1.7 revealed the presence of DQβ3.2 in 69%, it clearly became important to determine whether the cluster was related to the DQw4 phenotype.

The monoclonal antibody HU-46 was produced by immunizing a C3H/He mouse with the human B lymphoblastoid cell line EBV-Wa (DR4,Dw15,DQ blank) (Ishikawa et al.1987). The antibody was found to react with B cells from not only DR4,Dw15 individuals but also from certain DRw8,Dw8 subjects whose DQ phenotypes had not yet been defined (DQ blank). From a selected panel of 22 human lymphoblastoid cell lines, HU-46 was found to react with 3/3 DR4,Dw15,DQ blank lines and with a DRw8,Dw8,DQ blank line (GI). No cell lines bearing the known specificities, DQw1, DQw2 or DQw3, reacted with HU-46. In studies of 105 healthy Japanese blood donors, the antibody was found to react with 41/44 (93%) of DR4,Dw15 individuals and 10/20 (50%) of DRw8 individuals. When caucasian subjects were studied, it was found that 8/11 (73%) of the DRw8 individuals, but none of the DR4 individuals were HU-46 reactive. This distribution is very similar to that observed for the Msp1/DQA1 cluster, which was found to be present in 5/10 DRw8 individuals, but in none of the caucasian DR4 subjects (see Chapter 3.1.7). Further studies of HU-46 have confirmed that it reacts with an HLA-DQ molecule, and that this specificity is in Hardy-Weinberg equilibrium with the other alleles of the DQ locus (Ishikawa et al.1987). N-terminal amino acid sequencing of the immunoprecipitated α and β chains revealed that the α chain is homologous to DQw3α but different from DQw1α at positions 11 and 18. The β chain differs from DQw1β and DQw3β in having a phenylalanine at position 9 (Endo et al.1987). More recently, nucleotide sequence analysis of the α1- and β1-encoding exons of DQA1 and DQB1 has demonstrated that

both both the α and β chains of DQw4 possess characteristic sequences (Horn et al.1988).

### 3.2.1.2 Results

Monoclonal serological analysis was carried out on 13 homozygous cell lines, one DRA:DRB1 L-cell transfectant, and 4 heterozygous lymphoblastoid cell lines derived from the Scottish family M3 (see Figure 31). In addition to HU-46, the antibodies used were 3JP, L243 and Tü22. These have specificity for murine Class II ($A\alpha^d$), HLA-DR and HLA-DQ respectively. The anti-murine antibody was included as a negative control, and the two anti-human Class II framework antibodies as positive controls.

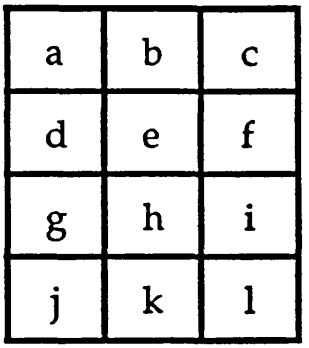
The results of the serological analyses are summarized in Table 37, and selected EPICS profiles are shown in Figures 3.2.1(a-l). It can be seen that none of the cells stained with 3JP, and that all reacted with L243 and Tü22, with the exception of R/RW1:1-L which expresses only HLA-DR and not HLA-DQ. HU-46 reactivity is observed in 4 homozygous cell lines: (a) EBV-Wa (DR4,DQw4), the line against which HU-46 was originally raised, (b) Olga and Madura (DRw8,DQw4), and (c) Luy (DRw8,DQw7). None of the caucasian DR4 cell lines reacted with HU-46, and together, these data agree closely with the findings reported by Ishikawa et al (Ishikawa et al.1987). Two heterozygous cell lines derived from family M3 also reacted with HU-46, DW (DR2/7,DQw6/2) and MC (DR2/2,DQw6/6).

It can also be seen that there may be some correlation between the Msp1/DQA1 cluster and HU-46 reactivity, but this relationship is not straightforward. The three DRw8 cell lines possess the cluster and are also HU-46 reactive, as is the DR4 cell line EBV-Wa. However, PE117 (DR4,DQw8) and DBB (DR7,DQw9) are cluster-positive and HU-46 non-reactive. The most interesting results come from the members of the family M3. As described above, the cluster is observed in both MW and DW, and can be assigned to the DR7,DQw2 haplotype in this family. Cells from two members of the family, DW and MC, stained strongly with HU-46, but this reactivity did not correspond with the cluster. The possibility that the cells being stained had become mis-labelled was excluded by repeating the analysis and obtaining the same results. None of the haplotypes present in these individuals, as determined by both serology and RFLP allogenotyping, has been associated with HU-46 reactivity previously.

| Cell line | DR | DQ | Cluster | 3JP | L243 | Tü22 | HU-46 |
|---|---|---|---|---|---|---|---|
| JESTHOM | 1 | 5 | - | - | + | + | - |
| JHAF | 4 | 7 | - | - | + | + | - |
| PE117 | 4 | 8 | + | - | + | + | - |
| KT2 | 4 | 8 | - | - | + | + | - |
| EBV-Wa | 4[15] | 4 | + | - | + | + | + |
| MOU | 7 | 2 | - | - | + | + | - |
| LBUF | 7 | 2 | - | - | + | + | - |
| DBB | 7 | 9 | + | - | + | + | - |
| SWEIG007 | 11 | 7 | - | - | + | + | - |
| D0208915 | 15 | 6 | - | - | + | + | - |
| Madura | w8 | 4 | + | - | + | + | + |
| Olga | w8 | 4 | + | - | + | + | + |
| Luy | w8 | 7 | + | - | + | + | + |
| R/RW1:1-L* | | 4 | - | | - | + | - - |
| MW | 2,7 | 6,2 | + | - | + | + | - |
| DW | 2,7 | 6,2 | + | - | + | + | + |
| MC | 2,2 | 6,6 | - | - | + | + | + |
| AW | 1,2 | 5,6 | - | - | + | + | - |

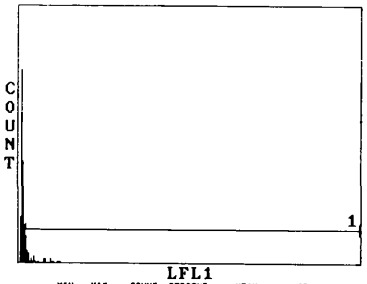
**Table 37**

HU-46 (anti-DQw4) reactivity of selected DQA-cluster positive and negative cell lines. The homozygous cell lines were obtained from the XIHWS core cell panel. R/RW1:1-L is an L-cell line transfected with the DRA and DRB1 genes of the DR4-DQw4 cell line EBV-Wa, and was a gift from H.Inoko (ICRF). MW, DW, AW and MC were lymphoblastoid cell lines derived from members of the Scottish family described in Chapter 3.1.7.1. It can be seen that HU-46 reactivity corresponds to DQw4 associated with both DR4 (EBV-Wa) and DRw8 (Madura and Olga). It is also seen with Luy (DRw8-Dw8.3-DQw7). Each of these cell lines is also cluster-positive; however, the remaining cluster-positive lines did not stain with HU-46. See text for further details.

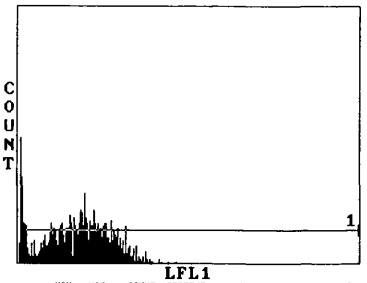



**Key to Figure 32**

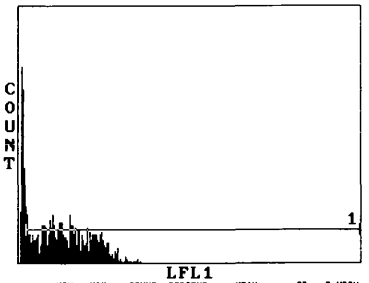
Monoclonal antibody staining of selected cell lines, expressed as log fluorescence intensity versus cell number. The cells shown here are: (a) EBV-Wa/medium alone, (b) EBV-Wa/Tü22, (c) EBV-Wa/HU46, (d) R/RW1:1-L/L243, (e) R/RW1:1-L /Tü22, (f) R/RW1:1-L/HU46, (g) Madura/medium alone, (h) Madura/Tü22, (i) Madura/HU46, (j) MW/HU46, (k) DW/HU46, (l) MC/HU46.



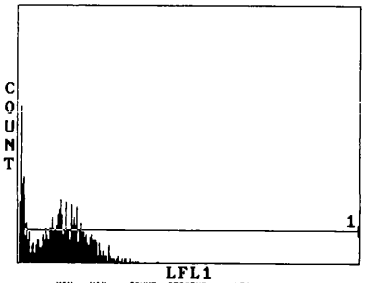

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 123 | 2.5 | 54.6 | 31.2 | |



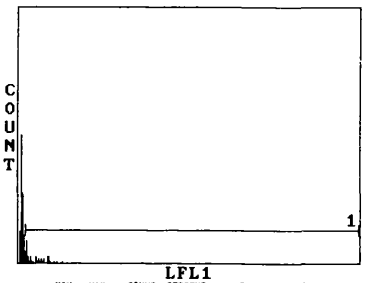

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 4075 | 81.5 | 199.7 | 88.3 | 0.52 |



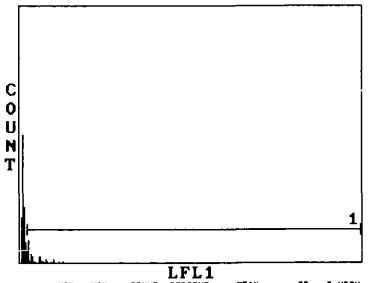

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 3104 | 62.1 | 148.4 | 77.5 | 3.38 |



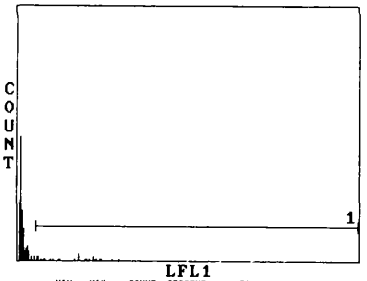

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 3204 | 64.0 | 144.1 | 68.7 | 1.52 |



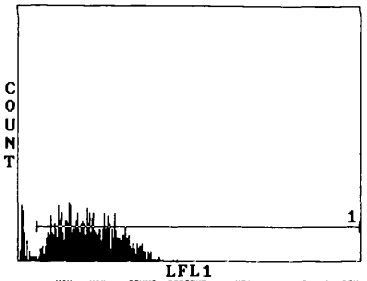

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 130 | 2.6 | 54.9 | 30.4 | 6.02 |



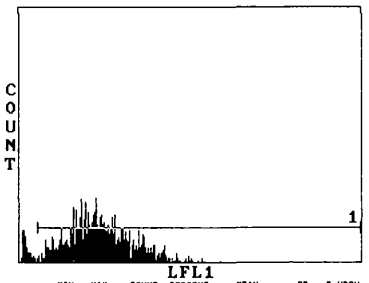

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 157 | 3.1 | 60.1 | 31.8 | |



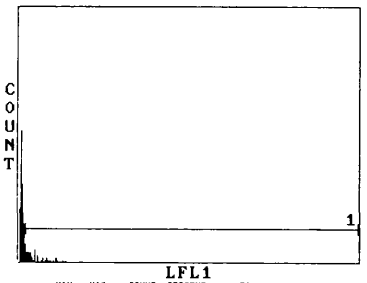

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 54 | 1022 | 95 | 1.9 | 180.5 | 90.3 | 0.30 |



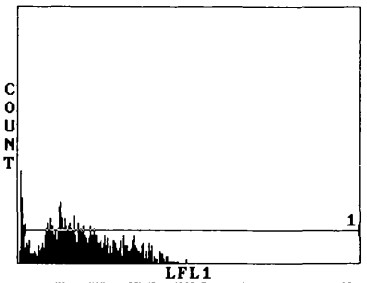

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 54 | 1022 | 4117 | 82.3 | 211.2 | 84.4 | 1.10 |



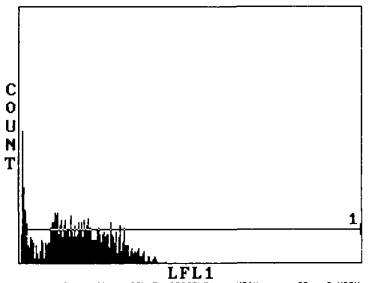

| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 54 | 1022 | 4240 | 84.8 | 245.8 | 97.6 | 0.53 |




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 201 | 4.0 | 68.5 | 39.1 | 2.15 |




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 4090 | 81.8 | 200.7 | 105.5 | 1.32 |




| | MIN | MAX | COUNT | PERCENT | MEAN | SD | % HPCV |
|---|---|---|---|---|---|---|---|
| 1 | 20 | 1022 | 3850 | 77.0 | 191.2 | 89.4 | 0.38 |

### 3.2.1.3 Summary

These studies were conducted in order to investigate the possible relationship between the Msp1/DQA1 restriction fragment cluster, and reactivity to the DQw4-specific monoclonal antibody HU-46. A similar situation has been described by Amar et al (Amar et al.1987), who found that DQA1 hybridization analysis of Bgl11-digested DNA revealed a genomic polymorphism characterized by two reciprocal patterns. One of these (4.0kb) was associated with DR3, 5 and w8, and the other (1.7kb) with DR1, 2, 4, 7 and 9. Furthermore, the distribution of the 4.0kb fragment in a panel of homozygous cell lines corresponded precisely to the reactivity of a monoclonal antibody, SFR20-DQα5. Western blotting data demonstrated that this antibody reacted with isolated α chains but not with β chains. It was concluded that this antibody defines a set of DQα allelic markers that are distinct from DQw1, 2, 3 or 4. In contrast to SFR20-DQα5, HU-46 is thought to be capable of reacting with isolated DQβ chains, but not with α chains (H Inoko, personal communication). Thus, the distribution of HU-46 reactivity in a given panel of cells might accurately reflect an association with the DQB1 allele, but only reflect the distribution of DQA1 alleles as a function of linkage disequilibrium between the α and β chain-encoding genes.

The data presented here confirm the reactivity of the monoclonal antibody HU-46 with HLA-DQw4, on both DR4 and DRw8 haplotypes. They also confirm the non-reactivity of caucasian DR1, DR4 and DR7 cell lines. The observation that EBV-Wa reacted with HU-46, whereas the transfectant line R/RW1:1-L did not, is consistent with the antibody being DQ-specific.

However, these data fail to substantiate a close relationship between the Msp1/DQA1 cluster and HU-46 reactivity. Although 5/6 HU-46 reactive cells were also cluster-positive, 3/8 cluster-positive cells were HU-46 non-reactive. It appears therefore as if the HU-46 reactive lines may represent a subset of those cell lines possessing the DQA1 restriction fragment cluster. The pattern of reactivity within the members of family M3 is extremely revealing. From an analysis of the extended haplotypes in the family (Figure 31), it is known that the cluster is contributed by the DR7,DQw2 haplotype. Two of the family members' cells also stain strongly with HU-46, DW ($DR2^a/7$, $DQw6^a/2$) and MC ($DR2^a/2^c$, $DQw6^a/6^c$)[5], and

[5] a and c are used here to distinguish between the two DR2 haplotypes in this family

from this it can be seen that HU-46 reactivity appears to be carried on one of the DR2,DQw6 haplotypes (haplotype **a**), as shown in Table 38. Although an unexpected result, HU-46 not having previously been reported to react with DR2 or DQw1 specificities, this observation is particularly helpful. In the case of subject DW, it can be seen that whereas the RFLP cluster is contributed by the DR7,DQw2 haplotype, HU-46 reactivity is associated with the $DR2^a$,$DQw6^a$ haplotype. Thus, it may be concluded that, although HU-46 reactivity may represent a subset of cluster-positive haplotypes (most obviously DRw8 haplotypes), there is no mandatory relationship between the cluster and the DQw4 phenotype.

**Table 38**

| | DR | DQ | HU-46 | cluster |
|---|---|---|---|---|
| MW | $2^b$,7 | $6^b$,2 | - | + |
| DW | $2^a$,7 | $6^a$,2 | + | + |
| AW | 1,$2^b$ | 5,$6^b$ | - | - |
| MC | $2^a$,$2^b$ | $6^a$,$6^b$ | + | - |

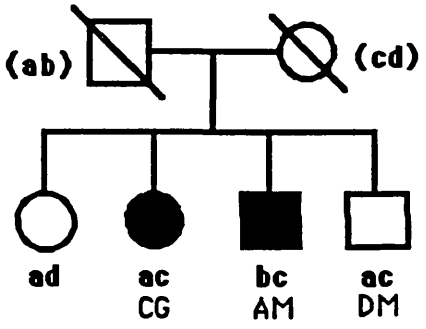
### 3.2.2 STUDIES WITH ALLOREACTIVE T CELL CLONES

#### 3.2.2.1 Introduction

MHC-incompatible cells induce vigorous primary immune responses when cultured together in vitro. This can largely be accounted for by a very high precursor frequency of T cells that are capable of recognizing and responding to foreign MHC molecules (Fischer Lindahl, Wilson, 1977). T cell clonal alloreactivity is now established as a powerful and sensitive means of detecting allelic heterogeneity of expressed MHC molecules. CD4-positive, Class II-restricted clones may recognize cellular determinants associated with DR, DRw52/53, DQ and DP molecules. Clonal T cell recognition of Class II molecules was included for the first time in an International Histocompatibility Workshop in the XIHWS (1987). The analysis of the reactivity of alloreactive T cell clones on the B cell reference panel demonstrated clear patterns of clustering which segregated with each of the serologically defined DR and DQ specificities. Within these large groups, a number of sub-clusters were seen. These studies resulted in a number of new splits, such as that of DRw52 into

Dw24, Dw25 and Dw26 subtypes (Flomenberg, 1989). Recently, it has been found that T cell clones can differentiate between specificities not distinguishable by other typing reagents, including monoclonal antibodies. It is currently believed that certain lymphocyte activating determinants may lie within the antigen binding groove of the heterodimer, where they are inaccessible to antibodies. An example of this is HLA-DR4, whose subtypes Dw4, Dw10, Dw13, Dw14 and Dw15 can be distinguished by means of genomic hybridization and alloreactivity, but cannot be differentiated serologically (Reinsmoen, Bach, 1986; Weyand et al.1986).

Thus it remained a possibility that the Msp1/DQA1 restriction fragment cluster might correspond to an expressed HLA-DQ molecule which was not detectable by means of serology. Therefore an attempt was made to obtain T cell clones whose reactivity might characterize this cluster. For this purpose, a Scottish family, M4, was chosen.




| | A | Cw | B | C4A | C4B | Bf | DR | DQw |
|---|---|---|---|---|---|---|---|---|
| a | 1 | - | 14 | 3 | 1 | S | 2 | 6 |
| b | 1 | - | 7 | 3 | 1 | F | 2 | 6 |
| c | 26 | - | 44 | 3 | Q0 | S | 2 | 6 |
| d | 1 | - | 7 | Q0 | 1 | S | 3 | 2 |

**Figure 33**
Family M4, showing the extended haplotypes. Clones reactive against products encoded by the cluster-positive haplotype **a** were generated by using DM and CG as stimulators and AM as the responder. Haplotypes **a** and **b** were originally thought to be Class I and Class II identical, but were subsequently shown to differ at the HLA-B locus. See text for details.

As shown in Figure 33 this family possesses three DR2,DQw6 haplotypes, and DQA1 hybridization analysis had previously demonstrated that the

cluster is contributed by haplotype **a**. Furthermore, haplotypes **a** and **b** were thought at first to be identical at each of the Class I and Class II loci (it was later found that haplotype **a** encoded HLA-B14 and not -B7). In this way it was hoped to raise clones against any products of haplotype **a** which were not encoded by haplotype **b**. The method used was to use PBMC from DM and CG (**a+c**) to stimulate cells from AM (**b+c**), thus eliminating the chance of of obtaining clones specific for products of haplotype **c**.

### 3.2.2.2 Results

Six stable T cell clones were obtained, and as shown in Table 39, four of these had a CD4-positive phenotype and were thus MHC Class II-restricted. The fine specificity of two of these clones, MS3 and MS4, was investigated using PBMC from a panel of healthy homozygous and heterozygous volunteers, and the results are shown in Table 40. MS3 and MS4 both proliferated in the presence of rIL-2, as well as in response to cells from the original stimulator DM, and there was no response to AM. Clone MS3 responded to only one other phenotype, AMa (DR5,w8, cluster +ve). MS4 responded to AMa, NF (DR1, cluster +ve), SF (DR3, cluster -ve), SS (DR4[Dw13]/5, cluster +ve) and EK (DR5/7, cluster +ve). Thus, MS3 responded to 2/6 cluster-positive cells. MS4 responded to 4/6 cluster-positive cells and to 2/8 cluster-negative cells.

In order to investigate the specificity of these clones further, blocking experiments were conducted. Figure 34 shows the effect of adding anti-Class II mAbs on the proliferative responses of MS3 (a) and MS4 (b) to AMa PBMC. There is dose-related inhibition of clone MS3 by anti-DP, less marked inhibition by L243 (anti-DR), and no inhibition by Tü22 (anti-DQ) or Leu10 (anti-DQ). In contrast, clone MS4 is blocked most strongly by L243 and is not inhibited by anti-DP.

### 3.2.2.3 Summary

Attempts to raise alloreactive T cell clones with specificity a Class II gene product associated with the DQA1 restriction fragment cluster are described. Six stable clones were obtained, of which four were CD4-positive and Class II-restricted. In analyses of the fine specificity of two of these clones, reactivity corresponding to the presence of the cluster was not seen. Monoclonal antibody blocking experiments have shown that the proliferative response of clone MS3 is inhibited by an anti-DP mAb, and that clone MS4 may be partially blocked by L243 (anti-DR). Since the HLA-

| Clone | Negative control | OKT4 (%) | OKT8 (%) |
|---|---|---|---|
| MS3 | 0.8 | 94.8 | 10.4 |
| MS4 | 0.4 | 91.9 | 3.8 |
| MS5 | 0.6 | 99.0 | 6.9 |
| MS11 | 2.2 | 2.6 | 67.7 |
| MS14 | 2.4 | 50.5 | 2.9 |
| MS15 | 1.4 | 11.7 | 88.3 |
| PBMC control | 0.4 | 56.3 | 27.4 |

**Table 39**

The CD4 and CD8 phenotypes of 6 T cell clones obtained from primary AM anti-DM MLR. $5x10^4$ cells were incubated with OKT4, OKT8 or medium alone, followed by FITC, and analyzed by flow cytometry. Results are expressed as the percentage of positive-staining cells. MS3, MS4 and MS5 are all strongly CD4+ (and MS14 less strongly), while MS11 and MS15 are CD8+.

| Stimulator PBMC | HLA -DR | DQA1 cluster | MS3 Δ cpm (± SD) | MS4 Δ cpm (± SD) |
|---|---|---|---|---|
| + medium | | | 89 (45) | 224 (56) |
| + rIL-2 | | | **15663** (1384) | **9241** (276) |
| DM | 2,2 | + | **2444** (113) | **4285** (898) |
| AM | 2,2 | - | -42 (31) | -34 (219) |
| NF | 1 | + | 160 (15) | **3645** (337) |
| PB | 1,2 | - | -62 (35) | 1416 (360) |
| DP | 2 | - | | 321 (23) |
| SM | 2 | - | | 836 (184) |
| ML | 2,4 | + | -11 (28) | 698 (37) |
| RL | 2,4 | - | 83 (48) | 568 (106) |
| DF | 2,5 | - | -236 (75) | 1218 (286) |
| RH | 2,7 | + | 71 (37) | 460 (221) |
| SF | 3 | - | 606 (452) | **7984** (975) |
| SS | 4,5 | + | 159 (212) | **3429** (27) |
| MK | 5,7 | - | 157 (69) | 1085 (254) |
| EK | 5,7 | - | | **3834** (126) |
| AMa | 5,8 | + | **1298** (518) | **11241** (1045) |

**Table 40**
Proliferative responses of AM anti-DM clones MS3 and MS4 to a selected panel of HLA-DR typed PBMC, showing the relationship with the DQA1 restriction fragment cluster. Both clones responded to rIL-2 alone and to the original stimulator cells, DM. MS3 also responded to AMa (DR5/8, cluster +ve); MS4 responded to AMa, NF (DR1, cluster +ve), SF (DR3, cluster -ve), SS (DR4[13]/5, cluster +ve) and EK (DR5/7, cluster -ve).

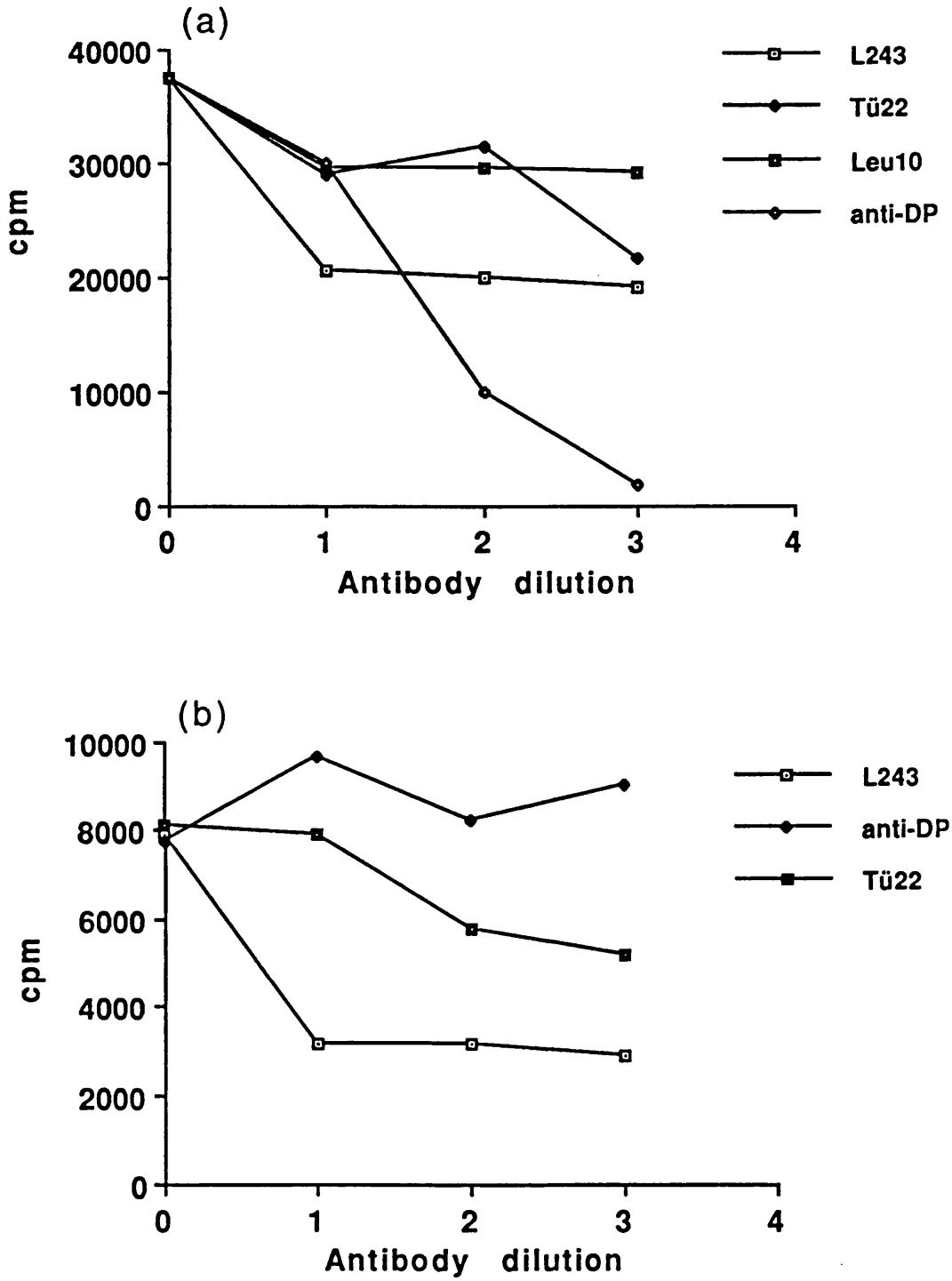



**Figure 34**
Antibody blocking curves, showing the effect of increasing concentrations of monoclonal antibodies on the proliferative responses of clones MS3 (a) and MS4 (b) to PBMC from AMa (DR5,w8, cluster+ve). The dilutions of each of the mAbs were: (i) L243 (supernatant) 1:100/20/10; (ii) Tü22 1:10000/1000/100; (iii) Leu10 (dialysate) 1:40/20/10; (iv) α-DP 1:1000/100/10. Proliferation of Clone MS3 is inhibited by L243 (α-DR) and Clone MS4 by α-DP. Neither of the clones is significantly blocked by Tü22 (α-DQ).

DP specificities of the cells used to generate these clones are not known, it is possible that differences at this locus might account for the production of a DP-reactive population of cells in the $1^{o}$ MLR. Another, untested, possibility is that the cloning strategy used resulted in the generation of CD4-positive, Class II-restricted anti-Class I (HLA-B14) clones. In conclusion therefore, although these studies are very limited, and the specificities of the clones have not been fully established, alloreactivity corresponding to the cluster was not seen. Therefore, these data provide further supportive evidence that the cluster does not parallel directly an expressed member of the DQA1 allelic series.

### 3.2.3 OLIGONUCLEOTIDE HYBRIDIZATION STUDIES

#### 3.2.3.1 Introduction

DQA1 genotyping of a number of selected cluster-positive and cluster-negative cell lines was carried out as described in Chapter 2.2.4. PCR-amplified DQA fragments were probed with labelled allele-specific oligonucleotides under stringent conditions (Saiki et al.1986; Todd et al.1987). The probes used are shown below:

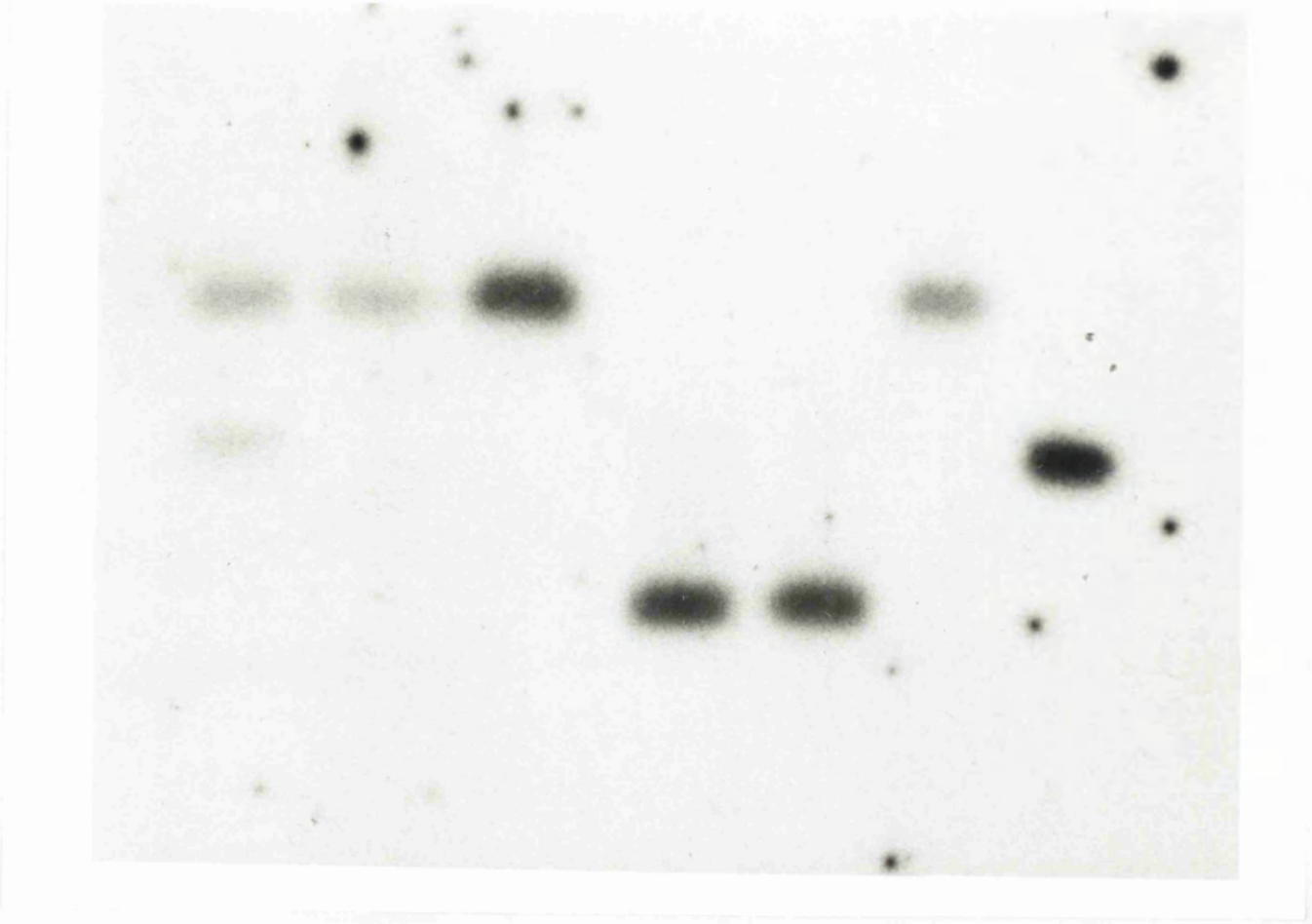
| | |
|---|---|
| RHA1 | 5'- TGAGTTCAGCAAATTTG -3' |
| RHA3 | 5'- TTCCGCAGATTTAGAAGATTT -3' |
| RHA4 | 5'- GTTTGCCTGTTCTCAGA -3' |

RHA1 is homologous to the sense strand coding for amino acids 50-54 of the α chains of DQw5 and DQw6. RHA3 is homologous to the sense strand coding for amino acids 51-56 of the α chains of DQw7(DR4), DQw8 and DQw9. RHA4 is homologous to the sense strand coding for amino acids 48-52 of the α chains of DQw2, DQw4 and DQw7(DR5). The relationship of these probes to the known allelic sequence variation of DQA1 is shown in Figure 13.

The seven cell lines chosen for ASO-hybridization analysis are listed in Table 41. Five of these lines were cluster-positive and 2 were cluster-negative. MW and DM are members of the Scottish families M3 and M4 (described previously). MG and JG are members of family M1. AMa is a healthy donor (DR5,w8) described in Chapter 3.2.1. BTB and KT2 are homozygous cell lines (respectively DRw8[8.2],DQw4 and DR4,DQw8).

| Cell | HLA-DR | HLA-DQw | Cluster |
|---|---|---|---|
| BTB | w8 | 4 | + |
| KT2 | 4 | 8 | - |
| AMa | 5,w8 | 7,4 | + |
| DM | 2,2 | 6,6 | + |
| MG | 2,2 | 6,6 | + |
| JG | 2,4 | 6,7 | - |
| MW | 2,7 | 6,2 | + |

**Table 41**
Cell lines studied by DQA1 allele-specific oligonucleotide hybridization. See text for details.




| | JG | MW | MG | BTB | AMa | DM | KT2 |
|---|---|---|---|---|---|---|---|
| DR | 2,4 | 2,7 | 2,2 | 8,8 | 5,8 | 2,2 | 4,4 |
| DQw | 1,3 | 1,(2) | 1,1 | 4,4 | 4,4 | 1,1 | 3,3 |
| Cluster | - | + | + | + | + | + | - |

**Figure 35**
Oligonucleotide hybridization analysis of DQA1 alleles in 5 subjects and 2 homozygous cell lines. The filter was probed successively with RHA1, RHA3 and RHA4. A single densely hybridizing band is seen with MG, BTB, AM, DM and KT2, indicating DQ homozygosity. Fainter bands are seen with JG (DR2,4) and MG (DR2,7), due to the presence of two DQ alleles. These data indicate the presence in all of the cells of the DQA1 alleles predicted from studies of cluster-negative cell lines.

#### 3.2.3.2 Results

The results are shown in Figure 35. It can be seen that a dense hybridization signal is obtained with RHA1 in the case of MG and DM, and a weaker signal in the case of JG and MW. These results are consistent with MG and DM being DQw6 homozygous (ie. having a "double dose" of the DQA1.2 allele), and JG and MW being DQw6 heterozygous. With the probe RHA3, a dense signal is obtained with KT2 and a weaker signal with JG, indicating the presence, respectively in these cells, of homozygous and heterozygous doses of the DQA3 allele. Finally, with the probe RHA4, dense hybridization signals were obtained with BTB and AMa. Once again, this is consistent with these cells having the predicted DQA1 alleles, DQA4.2 (BTB, homozygous) and DQA4.1/DQA4.2 (AMa).

#### 3.2.3.3 Summary

These studies indicate the presence, in all the cell lines studied, of the DQA1 alleles predicted from the large body of data derived from linkage studies and nucleotide sequence analyses. They have not provided evidence that any of these cluster-positive cell lines might possess a DQA1 allele hitherto unrecognized. The possibility of allelic variation in the DQα2-encoding gene segments has not been excluded by these studies. However, most of the sequence variation of DQα and DQβ is concentrated in their first domains, and all the alleles of DQA1 and DQB1 identified to date have unique differences in their DQα1- and DQβ1-encoding exons (Trowsdale et al.1985; Horn et al.1988).

### 3.2.4 DQA NUCLEOTIDE SEQUENCE ANALYSIS

#### 3.2.4.1 Introduction

The DQα-chain first domain-encoding gene segments from four individuals and from a single homozygous cell line were determined. The subjects were all from the Grampian region and were members of the families described previously: MW (MS patient, DR2/7, cluster +ve), DM (MS patient, DR2/2, cluster +ve), MG (MS patient, DR2/2, cluster +ve) and JG (healthy sib of MG, DR2/4, cluster -ve). The cell line studied was BTB (DRw8, DQw4, cluster +ve), whose DQA1 sequence has not previously been reported.

### 3.2.4.2 Results

The number of positive M13 plaques processed for preparation of the single-stranded sequencing templates were DM (12), MW (8), MG (8), JG (6) and BTB (6). Readable sequence information was realised in each case, and examples of the autoradiographs are shown in Figures 36 and 37. The nucleotide sequences obtained for each of the subjects are given in Figure 38 (a)-(j). The derived amino acid sequences are shown in Figure 39, aligned with the published DQα chain first domain sequences (Todd et al.1988b; Todd et al.1988a; Horn et al.1988). The DQA1.2 allele associated with DR2,DQw6 was obtained from each of the DR2-positive subjects, DM (DR2/2), MW (DR2/7), JG (DR2/4) and MG (DR2/2), and encoded a DQα first domain identical to those previously published. DQA1.2 was the only allelic sequence obtained from the two homozygous subjects. However, a DXα-encoding gene segment (now known as DQA2, but not to be confused with the DQA2 (DR7,DQw9-associated) allele of the DQA1 locus) was isolated from DM. A DQA4.2 sequence was obtained from the homozygous cell line BTB, and within the limits of this analysis, this sequence was identical to that obtained from the DRw8[8.1],DQw4 homozygous cell line Madura (Horn et al.1988). From subject MW, a second allelic sequence was obtained which translated to give a protein sequence identical to DQA2 (although there was a single silent nucleotide substitution at position 96). The second nucleotide sequence obtained from JG was identical to DQA3. All of these sequences were exactly the same as those predicted from the large body of linkage data, as well as from the oligonucleotide hybridization data discussed in the last chapter. Within the limits of this analysis, it has been shown that the presence of the Msp1/DQA1 cluster on DR2, DR7 and DRw8 haplotypes is associated with the same alleles of DQA1 as are found on cluster-negative haplotypes.

The methods used resulted also in the appearance of two hybrid sequences, as shown in Figure 36 and Figure 40 (DM9 and MW3). This phenomenon has been observed occasionally (J Todd and A So, personal communication), and is thought to be due to an artefact of PCR. This error can arise when two sequences with a high degree of homology are present in the genomic DNA being amplified. In the later stages of the PCR reaction, when the concentration of free nucleotides remaining may have fallen, or when the activity of the polymerase is reduced, chain extension may not have been completed before the temperature rises for the next cycle of denaturation. If this occurs, and if the two genomic sequences

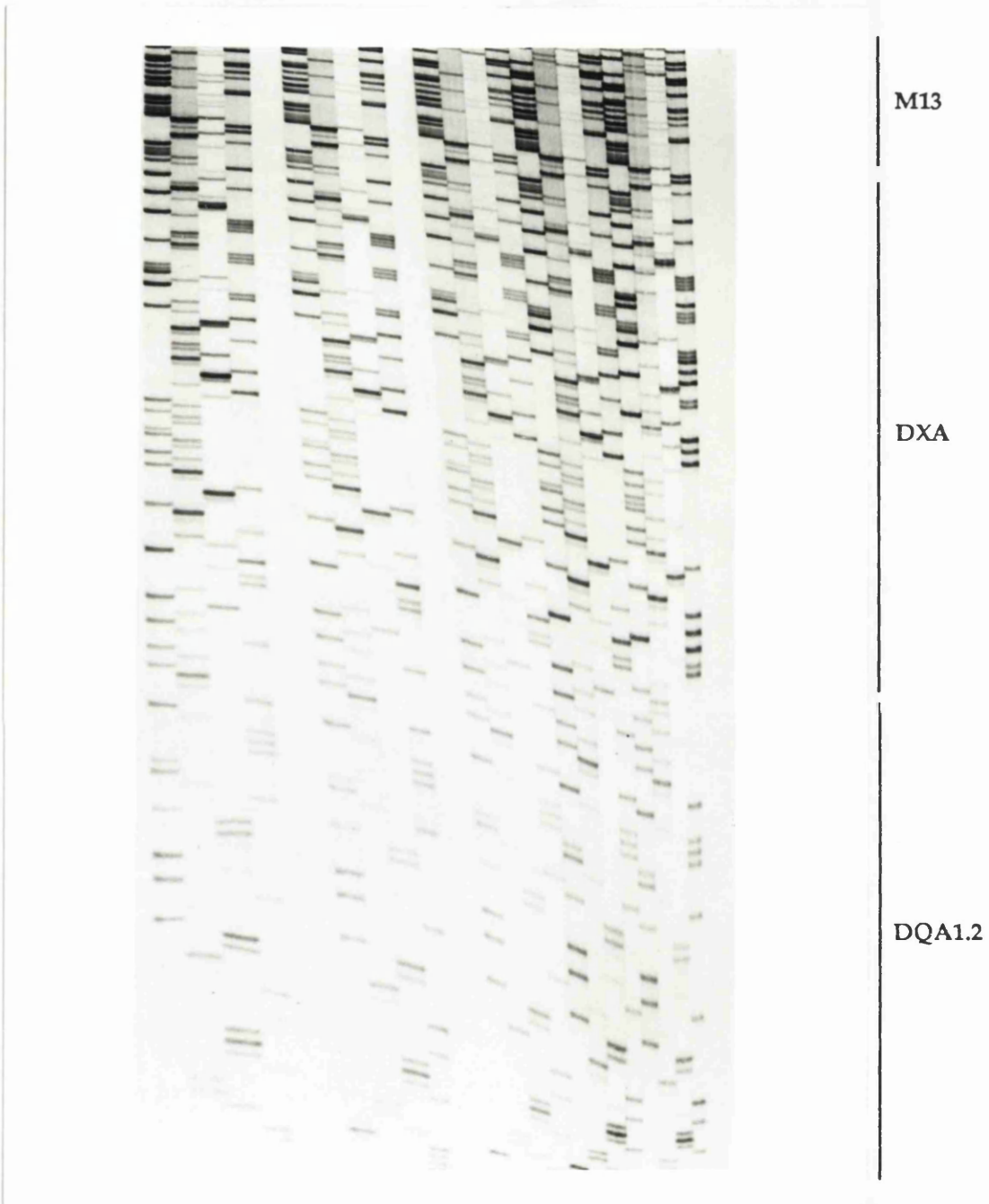



**Figure 36**
Sequencing autoradiograph, showing 5 templates from the subject DM (DR2). The lane order (L to R) for each template is AGCT. The sequences of templates 1 to 4 are identical to DQA1.2. The 5th sequence is a hybrid composed of DQA and DXA as indicated.


Figure 37
Sequencing autoradiograph showing the two DQA1 alleles identified in the subject MW. Template MW1 is identical to DQA2 and MW2 is identical to DQA1.2. The lane order is AGCT.

**Legend to Figure 38**

The nucleotide sequences of the DQα1-encodiıg first domains ampliied by PCR are shown in(a) - (j), together with ther derived amino acid sequences. The nucleotide numbering system i; based upon the bases(1-261) which encode the 87 amino acids of the DQα1 domain. See Appendix 3 for the single letter amino acid code. A stop codon in MW1 is indicated by [-]. [*] indicates that there is a single codondeletion, revealed by alignment of the DQα1 domains (see Figure 3.24.4). See text for a description of the alleles.

DM2

```
 60                                                          100
GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA GAC GAG GAG TTC
 G   P   S   G   Q   Y   T   H   E   F   D   G   D   E   E   F

                                                        150
TAT GTG GAC CTG GAG ACG AAA GAG ACT GTC TGG CAG TTG CCT ATG TTT
 Y   V   D   L   E   T   K   E   T   V   W   Q   L   P   M   F

                                                          200
AGC AAA TTT ATA AGT TTT GAC CCG CAG AGT GCA CTG AGA AAT ATG GCT
 S   K   F   I   S   F   D   P   Q   S   A   L   R   N   M   A

GTG GGA AAA CAC ACC TTG GAA TTC ATG ATG CGA CAA TCC AAC TCT ACT
 V   G   K   H   T   L   E   F   M   M   R   Q   S   N   S   T

                        266
GCT GCT ACC GGA TCC CG
 A   A   T   N   S
```

**Figure 38 (a)**
The nucleotide sequence of template DM2, and its derived amino acid sequence.

DM6

```
 54                                                                  99
TTT TAC GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA GAT GAG
 F   Y   G   P   S   G   Q   Y   T   H   E   F   D   G   D   E


CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT
 Q   F   Y   V   D   L   E   R   K   E   T   A   W   R   W   P

150
GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GGT GCA CTG AGA AAC
 E   F   S   K   F   G   G   F   D   P   Q   G   A   L   R   N

   200
ATG GCT GTG GCA AAA CAC AAC TTG AAC ATC ATG ATT AAA CGC TAC AAC
 M   A   V   A   K   H   N   L   N   I   M   I   K   R   Y   N

      250                                 271
TCT ACC GCT GCT ACC GGA TCC CCG GGT A
 S   T   A   A   T   G   S   P   G
```

**Figure 38 (b)**
The nucleotide sequence of template DM6, and its derived amino acid sequence.

DM9

```
 60                                                          100
GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA GAC GAG GAG TTC
 G   P   S   G   Q   Y   T   H   E   F   D   G   D   E   E   F

                                                         150
TAT GTG GAC CTG GAG ACG AAA GAG ACT GTC TGG CAG TTG CCT ATG TTT
 Y   V   D   L   E   T   K   E   T   V   W   Q   L   P   M   F

                                                             200
AGC AAA TTT ATA AGT TTT GAC CCG CAG GGT GCA CTG AGA AAC ATG GCT
 S   K   F   I   S   F   D   P   Q   G   A   L   R   N   M   A

GTG GCA AAA CAC AAC TTG AAC ATC ATG ATT AAA CGC TAC AAC TCT ACC
 V   A   K   H   N   L   N   I   M   I   K   R   Y   N   S   T

                                272
GCT GCT ACC GGA TCC CCG GGT AC
 A   A   T   G   S   P   G
```

**Figure 38 (c)**
The nucleotide sequence of template DM9, and its derived amino acid sequence.

MW1

```
46
TAC CAG TCT TAC GGT CCC TCT GGC CAG TTC ACC CAT GAA TTT GAT GGA
 Y   Q   S   Y   G   P   S   G   Q   F   T   H   E   F   D   G

  §      100
GAT GAG GAG TTC TAT GTG GAC CTG GAG AGG AAG GAG ACT GTC TGG AAG
 D   E   E   F   Y   V   D   L   E   R   K   T   E   V   W   K

           150                     [*]
TTG CCT CTG TTC CAC AGA CTT AGA TTT GAC CCG CAA TTT GCA CTG ACA
 L   P   L   F   H   R   L   R   F   D   P   Q   F   A   L   T

               200
AAC ATC GCT GTG CTA AAA CAT AAC TTG AAC ATC CTG ATT AAA CGC TCC
 N   I   A   V   L   K   H   N   L   N   I   L   I   K   R   S

                   250                             273
AAC TCT ACC GCT GCT ACG GAT CCG GGT ACG AGC TGA
 N   S   T   A   A   T   N   P   G   T   S  [-]
```

**Figure 38 (d)**
The nucleotide sequence of template MW1, and its derived amino acid sequence. § denotes a silent substitution at position 96 (C>T). [-] denotes the stop codon.

MW2

```
46
TAC CAG TTT TAC GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA
 Y   Q   F   Y   G   P   S   G   Q   Y   T   H   E   F   D   G

        100
GAT GAG CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG
 D   E   Q   F   Y   V   D   L   E   R   K   E   T   A   W   R

          150
TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GGT GCA CTG
 W   P   E   F   S   K   F   G   G   F   D   P   Q   G   A   L

              200
AGA AAC ATG GCT GTG GCA AAA CAC AAC TTG AAC ATC ATG ATT AAA CGC
 R   N   M   A   V   A   K   H   N   L   N   I   M   I   K   R

                  250                             273
TAC AAC TCT ACC GCT GCT ACG GAT CCG GAT CGA GCT
 Y   N   S   T   A   A   T   N   P   D   R   A
```

**Figure 38 (e)**
The nucleotide sequence of template MW2, and its derived amino acid sequence.

MW3

```
46
TAC CAG TCT TAC GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA
 Y   Q   S   Y   G   P   S   G   Q   Y   T   H   E   F   D   G

        100
GAT GAG CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG
 D   E   Q   F   Y   V   D   L   E   R   K   E   T   A   W   R

          150
TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GGT GCA CTG
 W   P   E   F   S   K   F   G   G   F   D   P   Q   G   A   L

              200
AGA AAC ATG GCT GTG GCA AAA CAC AAC TTG AAC ATC ATG ATT AAA CGC
 R   N   M   A   V   A   K   H   N   L   N   I   M   I   K   R

                  250                             273
TAC AAC TCT ACC GCT GCT ACG GAT CCG GAT CGA GCT
 Y   N   S   T   A   A   T   N   P   D   R   A
```

**Figure 38 (f)**
The nucleotide sequence of template MW3, and its derived amino acid sequence.

BTB

```
58                                                          100
GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA GAC GAG CAG TTC
 G   P   S   G   Q   Y   T   H   E   F   D   G   D   E   Q   F

                                                          150
TAC GTG GAC CTG GGG AGG AAG GAG ACC GTC TGG TGC TTG CCT GTT CTC
 Y   V   D   L   G   R   K   E   T   V   W   C   L   P   V   L

              [*]
AGA CAA TTT AGA TTT GAC CCG CAA TTT GCA CTG ACA AAC ATC GCT GTG
 R   Q   F   R   F   D   P   Q   F   A   L   T   N   I   A   V

                      218
ACA AAA CAC AAC TTG AA
 T   K   H   L   N
```

**Figure 38 (g)**
The nucleotide sequence of template BTB, and its derived amino acid sequence.

JG1

```
39
A AAC TTG TAC CAG TTT TAC GGT CCC TCT GGC CAG TAC ACC CAT GAA TTT
   N   L   Y   Q   F   Y   G   P   S   G   Q   Y   T   H   E   F

                100
GAT GGA GAT GAG CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC
 D   G   D   E   Q   F   Y   V   D   L   E   R   K   E   T   A

                  150
TGG CGG TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GGT
 W   R   W   P   E   F   S   K   F   G   G   F   D   P   Q   G

                      200
GCA CTG AGA AAC ATG GCT GTG GCA AAA CAC AAC TTG AAC ATC ATG ATT
 A   L   R   N   M   A   V   A   K   H   N   L   N   I   M   I

                          250                             273
AAA CGC TAC AAC TCT ACC GCT GCT ACG GAT CCG GAT CGA GCT
 K   R   Y   N   S   T   A   A   T   N   P   D   R   A
```

**Figure 38 (h)**
The nucleotide sequence of template JG1, and its derived amino acid sequence.

JG2

```
52
TCT TAC GGT CCC TCT GGC CAG TAC AGC CAT GAA TTT GAT GGA GAC GAG
 S   Y   G   P   S   G   Q   Y   S   H   E   F   D   G   D   E

100
GAG TTC TAT GTG GAC CTG GAG AGG AAG GAG ACT GTC TGG CAG TTG CCT
 E   F   Y   V   D   L   E   R   K   T   E   V   W   Q   L   P

  150
CTG TTC AGA AGA TTT AGA CGA TTT GAC CCG CAA TTT GCA CTG ACA AAC
 L   F   R   R   F   R   R   F   D   P   Q   F   A   L   T   N

     200                                                     243
ATC GCT GTG CTA AAA CAT AAC TTG AAC ATC GTG ATT AAA CGC TCC AA
 I   A   V   L   K   H   N   L   N   I   V   I   K   R   S
```

**Figure 38 (i)**
The nucleotide sequence of template JG2, and its derived amino acid sequence.

MG

```
61                                                          100
CCC TCT GGC CAG TAC ACC CAT GAA TTT GAT GGA GAT GAG CAG TTC TAC
 P   S   G   Q   Y   T   H   E   F   D   G   D   E   Q   F   Y

                                                        150
GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC
 V   D   L   E   R   K   E   T   A   W   R   W   P   E   F   S

                                                    200
AAA TTT GGA GGT TTT GAC CCG CAG GGT GCA CTG AGA AAC ATG GCT GTG
 K   F   G   G   F   D   P   Q   G   A   L   R   N   M   A   V

                                                        250
GCA AAA CAC AAC TTG AAC ATC ATG ATT AAA CGC TAC AAC TCT ACC GCT
 A   K   H   N   L   N   I   M   I   K   R   Y   N   S   T   A

                            273
GCT ACG GAT CCG GAT CGA GCT
 A   T   N   P   D   R   A
```

**Figure 38 (j)**
The nucleotide sequence of template MG, and its derived amino acid sequence.

## Figure 39

```
DR1 DQw5  DQA1.1    EDIVADHVAS CGVNLYQFYG PSGQYTHEFD GDEEFYVDLE RKETAWRWPE FSKFGGFDPQ GALRNMAVAK HNLNIMIKRY NSTAATN
DR2 DQw6  DQA1.2    .......... .......... .......... ...Q...... .......... .......... .......... .......... .......
DR6 DQw6  DQA1.2    .......... .......... .......... ...Q...... .......... .......... .......... .......... .......
DR5 DQw6  DQA1.3    .......... ...... ....F..... ...Q...... K......... .......... .......... .......... .......
DR6 DQw6  DQA1.3    .......... ...... ....F..... ...Q...... K......... .......... .......... .......... .......
DR8 DQw6  DQA1.3    .......... ...... ....F..... ...Q...... K......... .......... .......... .......... .......
DR7 DQw9  DQA2      .......... Y......S.. ....F..... .......... ....V.KL.L .HRLR*.... F..T.I..L. .....L...S .......
DR4 DQw7  DQA3      .......... Y......S.. .....S.... .......... ....V.QL.L .RR.RR.... F..T.I..L. .....V...S .......
DR4 DQw8  DQA3      .......... Y......S.. .....S.... .......... ....V.QL.L .RR.RR.... F..T.I..L. .....V...S .......
DR9 DQw9  DQA3      .......... Y......S.. .....S.... .......... ....V.QL.L .RR.RR.... F..T.I..L. .....V...S .......
DR3 DQw2  DQA4.1    .......... Y......S.. .......... ...Q.....G ....V.CL.V LRQ.R*.... F..T.I..L. ....SL...S .......
DR5 DQw7  DQA4.1    .......... Y......S.. .......... ...Q.....G ....V.CL.V LRQ.R*.... F..T.I..L. ....SL...S .......
DR8 DQw4  DQA4.2    .......... Y......S.. .......... ...Q.....G ....V.CL.V LRQ.R*.... F..T.I..T. .....L...S .......
DR8 DQw4  DQA4.3    .......... Y......S.. ....F..... ...Q.....G ....V.CL.V LRQ.R*.... F..T.I..T. .....L...S .......

DXα       DXA       .......... Y...F..SH. .......... .......... T...V.QL.M ....IS.... S.......G. .T.EF.MRQS .......

IA(B)               ...E....GT Y.ISV..SP. DI....F... ...L.....D K...V.ML.. .GQLAS.... .G.Q.I..V. ...GVLT..S ...P...
IA(D)               ...E....GF Y.TTV..SP. DI........ ...L.....D K.K.V..L.. .GQLIL.... .G.Q.I.AV. ...G.LT..S .F.P...

DM2       (DXA)           ... Y...F..SH. .......... .......... T...V.QL.M ....IS.... S.......G. .T.EF.MRQS .
DM6       (DQA1.2)            ...... .......... ...Q...... .......... .......... .......... .......... .......
DM9       (DQA/DXA)             . .......... .......... T...V.QL.M ....IS.... .......... .......
MW1       (DQA2)            ..S.. ....F..... .......... ....V.KL.L .HRLR*.... F..T.I..L. .....L...S .......
MW2       (DQA1.2)          ..... .......... ...Q...... .......... .......... .......... .......... .......
MW3       (DQA1.2/2)        ..S.. .......... ...Q...... .......... .......... .......... .......... .......
BTB       (DQA4.2)            . .......... ...Q.....G ....V.CL.V LRQ.R*.... F..T.I..T. ...
JG1       (DQA1.2)        ..... .......... ...Q...... .......... .......... .......... .......... .......
JG2       (DQA3)            S.. .....S.... .......... ....V.QL.L .RR.RR.... F..T.I..L. .....V...S
MG        (DQA1.2)              ......... ...Q...... .......... .......... .......... .......... .......
```

**Legend to Figure 39**

DQα-chain first domain amino acid sequences derived from the nucleotide sequences in Figure 38. These are aligned with the DQα1 protein sequences taken from *Horn et al (1988), Proc.Natl.Acad.Sci.USA 85:6012-6016,* a DXα (DQA2-encoded) first domain sequence, *Trowsdale et al (1988), Proc.Natl.Acad.Sci.USA 85:7652-7656,* and two murine Class II first domain sequences (from *Brown et al (1988), Nature 332:845-850*). The nomenclature used is that described by Horn et al, and the common relationships of each of the alleles to DR and DQ are shown in the upper part of the figure. To avoid confusion, the DQA2 gene, whose encoded product is DXα is here termed DXA. A single amino acid deletion at position 56 of the DQA2 and DQA4 alleles is denoted by an asterisk. Sequences identical to DQA1.2 were obtained from each of the DR2,DQw6 subjects (DM, MW, JG and MG). A DXA allele was sequenced from DM, a DQA2 allele from MW and a DQA3 allele from JG. All of these sequences are identical to those of previously published alleles, although occasional silent mutations were encountered (see text for details). DM9 and MW3 are hybrid sequences generated as an artefact of PCR (see text and Figure 3.2.4.5).

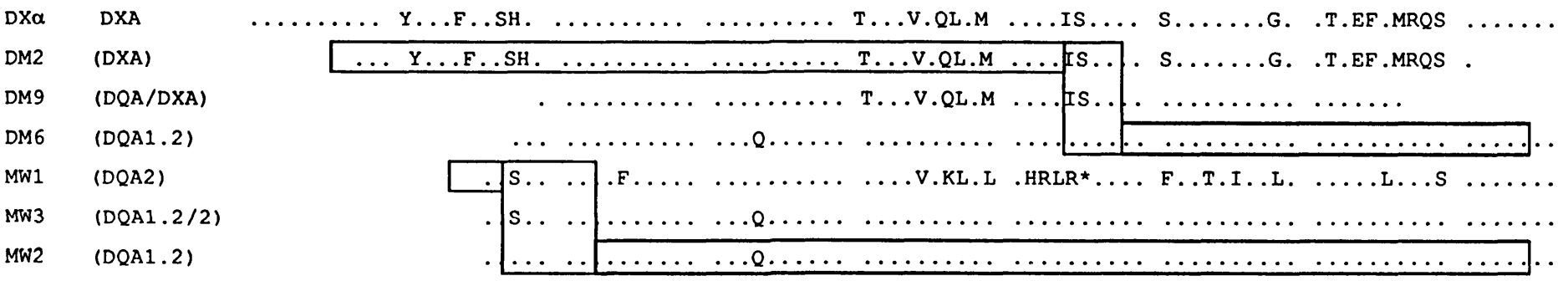





**Figure 40**

Alignment of DQα first domain amino acid sequences, showing the hybrid sequences generated as an artefactof PCR. DM9 is a hybrid composed of DXA and DQA1.2. MW3 is composed of DQA2 and DQA1.2.

share an homologous region spanning 4 or more bases, the incomplete strand may re-anneal to the "wrong" complementary strand. In the next cycle, it will then be extended in the usual way to produce a hybrid strand. This may then act as a template for further cycles of amplification. Thus, DM9 is composed of unique DXA sequence from positions 1-56, DQA1.2 sequence from positions 61-87, and shared sequence from positions 57-60. A similar event has occurred in the case of MW3, which is composed of sequence from two DQA alleles, DQA1.2 and DQA2. In this case, the shared region spans 18 bases (amino acids 19-24).

#### 3.2.4.3 Summary

Most of the allelic sequence variation of the DQA genes lies in the DQα1-encoding gene segments, and all alleles identified to date possess unique sequences within this region. Within the limits of this study, there were no differences between the DQα1-encoding sequences obtained from cluster-positive DR2, DR7 and DRw8 haplotypes, and those reported previously from cluster-negative haplotypes. Thus the cluster does not appear to be associated with a unique allele of DQA1. These data are consistent with the results of allogenotyping, mAb studies, T cell clonal analyses and oligonucleotide hybridization studies presented above. The possible interpretations and significance of these data will be discussed in Chapter 4.

# PART 4
# DISCUSSION

## 4.1 SUMMARY OF RESULTS

**Chapter 3.1.1**

1. Four DNA pools, MS DR2 +ve, MS DR2 -ve, Control DR2 +ve and Control DR2 -ve, were constructed with genomic DNA from 33 MS patients and 58 controls from the Grampian region of Northeast Scotland. These pools were subjected to restriction fragment analysis using 14 restriction endonucleases, 5 MHC Class II probes and 2 T cell antigen receptor probes.

2. Following digestion with Msp1 and hybridization with a DQA1 probe, differences were observed between the disease and control pools. A 5.6kb fragment was present in both MS pools but not in the Control pools. A 6.1kb fragment was seen in the MS DR2-ve pool. Of particular interest was the finding that a 3.25kb Msp1 fragment was present in the MS DR2+ve pool but absent from the Control DR2+ve pool, suggesting that this fragment was associated with MS independently of HLA DR2. Msp1 was the only restriction enzyme to differentiate between the MS and the control pools using the DQA1 probe.

3. A number of DQB1-hybridizing restriction fragments also appeared to be discriminatory, although less convincingly than the DQA1/Msp1 fragments. These fragments were generated particularly with the enzymes BamH1, Bgl11 and Taq1.

4. The remaining probes, DPA, DPB, DRB,TcRα and TcRβ, failed to reveal any differences between the MS and control DNA pools with any of the 14 restriction enzymes. However, in many instances, fragments closely associated with the presence or absence of DR2 could be identified. The most notable examples of such fragments are EcoR1 2.2kb DQB1, which appears to be characteristic of the subtype Dw2, and Pvu11 2.6kb DQB1.

**Chapter 3.1.2**

5. Based on the results obtained in these studies using pooled DNA, 33 MS patients and 48 controls from the Grampian region of northeast Scotland were studied individually.

6. A total of 127 Class II restriction fragments were analysed, 18 DQA1 fragments, 34 DQB1 fragments and 75 DRB fragments. These fragments were tested separately and independently of DR2 for significant association with MS by stepwise logistic regression analysis and a series of Chi-square tests.

7. The Msp1 3.25kb DQA1 fragment identified by studying the DNA pools, was observed in 10/33 patients but in only 2/48 controls ($p \approx 0.001$). An Msp1 2.50kb DQA1 fragment was seen in the same subjects as the 3.25kb fragment. A third Msp1 DQA1 fragment, 2.31kb, was also seen in 6 of the 10 individuals possessing these two fragments and thus completed a characteristic cluster of DQA1 restriction fragments. The 2.31kb fragment was itself significantly associated with MS when the DR2 subjects were analysed separately, as was a 5.91kb Msp1 DQA1 fragment also. None of these fragments were associated with MS in the non-DR2 group.

8. DQB1-hybridizing fragments were discriminatory at only low levels of significance.

9. With the TcRα and TcRβ gene probes, only very limited polymorphism was observed, and there were no significant differences between patients and controls.

10. The 33 Scottish patients were classified according to whether their illness had a relapsing/remitting (R/R) course (n=24), or was primarily progressive (PCP) (n=9). No significant differences in the frequencies of any of the Class II restriction fragments between the two groups were observed.

**Chapter 3.1.3**

11. Cluster analysis of data from the XIHWS established that the three Msp1 fragments described above form a cluster which is completed by a 13.12kb Bgl11 fragment.

12. This cluster was present in all of the DRw8 cell lines, 2 of 11 DR4 cell lines and 2 of 6 DR7 cell lines. All of the DRw8 cell lines encoded the DQw4 specificity, and DQw7, w8 and w9 accounted for most of the other cluster-positive cell lines. The cluster was not seen in any of the DR2 cell lines, with the exception of RML which carries the rare Amerindian specificity Dw22.

13. In contrast to the fragment cluster, a 2.87kb Msp1 DQA1 fragment defined a broad DQw1 specificity, being present exclusively in DQw1, DQw5 or DQw6 cell lines. The 3.25/2.50/2.31kb fragment cluster and the 2.87kb fragment exhibited an allelic relationship and were never present together in a single homozygous cell line.

14. Inspection of the XIHWS data failed to reveal a DQB1 restriction fragment or fragment cluster with the same haplotype distribution as the DQA1 cluster.

15. In selected cluster-positive and cluster-negative cell lines, the sum of the Msp1 and Bgl11 restriction fragments, in most cases, was slightly greater in the cluster-positive line than in a cluster-negative line of the same DR type. This raises the possibility that the cluster-positive haplotype contains extra genomic sequence in relation to the DQA1 locus, and would also be compatible with the presence of a third DQ region $\alpha$ gene on these haplotypes.

16. Identical DQA1 restriction fragment patterns were obtained with both Msp1 and its isoschizomer Hpa11, indicating that the recognition sites responsible for the fragment cluster are not methylated.

**Chapter 3.1.4**

17. Analysis of four restriction fragments enabled the DR and DQ alleles associated with the DR2 haplotypes of the Scottish subjects to be defined. A 2.2kb EcoR1 DQB1 fragment and a 3.81kb Msp1 DRB fragment characterize the subtype Dw2. 2.87kb Msp1 DQA1 is associated with DQw1, and 3.10kb Bgl11 DQB1 defines DQw6 (previously known as DQw1.2). It was found that 96% of the DR2+ve patients and 91% of the DR2+ve controls had the typical DR2,Dw2,DQw6 haplotype

common in northern European caucasoids, and that DQw6 was present in 100% of the DR2+ve patients.

18. 7/10 cluster-positive patients had a single DR2,Dw2,DQw6 haplotype. Since the DQA1 cluster appears to be allelic to DQw1, it must be contributed by the non-DR2,Dw2,DQw6 haplotype in these patients.

19. Since the Scottish subjects had previously been serotyped within families, it was possible to assign both haploptyes in most cases. Using these data, it was found that DR2, DR4 and DRw6 serospecificities were present in 79% of patients and 68% of controls. However, it was found that a significantly larger number of Scottish MS patients possessed a double dose of DR2/4/w6 (eg. DR2,4 or DR2,6) compared to the controls (48% vs 16%; $p<0.01$).

Chapter 3.1.5

20. A larger, multi-centre RFLP study, involving groups from Scotland, Northern Ireland, Holland and France, was organized within the framework of the XIHWS. The design of the study was based upon the results obtained in the Gramoiuan population, described above.

21. There were significant differences between patients and controls in the case of 7/18 DQA fragments (2 of these were DQA2 framents), 3/34 DQB1 fragments and 6/75 DRB fragments.

22. Msp1 DQA1 3.25kb was present in 22.7% of patients and 6.4% of controls irrespective of DR type, and in 21.2% and 5.7% respectively of the DR2-positive subjects. Other fragments with a significantly distorted distribution were Bgl11 DQB1 4.23kb (69.6% vs. 40% in DR2+ individuals), and Taq1 DRB 7.23kb (14.1 vs. 33.8% in all individuals and 8.3% vs. 41.7% in DR2-ve individuals). Msp1 DQA1 4.74kb was also negatively correlated to disease (57.6% vs. 75.8%).

23. Probably as a result of imperfect standardization of the Southern blotting protocol between the different centres, and the use of different length DQA1 cDNA probes, the 2.50 and 2.31kb fragments which complete the cluster were not seen on some of the autoradiographs.

24. Analysis of this larger sample confirmed that the predominant DR2 haplotype in these northern European populations is DR2,Dw2,DQw6 (51/52 DR2+ MS patients and 53/53 DR2+ controls).

**Chapter 3.1.6**

25. Collaborative studies of the Northern Ireland population, which is genetically similar to that of Northeast Scotland, were conducted. These involved 50 MS patients and 76 healthy controls.

26. The only restriction fragments which were distributed unequally between the patients and controls in the Northern Ireland population samples were once again the three DQA1 fragments comprising the cluster described above. This cluster was seen with equal frequency in the Belfast patients and controls, but was unequally distributed between the DR2-positive subjects, being seen in 6/30 (20%) patients and 1/29 (3.4%) controls. Combining the Scottish and Irish data, the cluster was present in 23.6% patients and in 3.2% controls ($p<0.001$).

27. The Msp1 DQA1 2.87kb (DQw1-associated) fragment was present in 17/20 (85%) cluster-positive patients and 6/18 (33%) cluster-positive controls ($p\approx0.001$). The Relative Risk for possessing both the DQA1 Msp1 cluster and the 2.87kb fragment, calculated by the method of Woolf, was 11.33. Since these restriction fragment patterns are allelic, these data indicate that disease risk may be associated with heterozygosity, and raise the possibility that transcomplementation contributes to susceptibility.

28. The DR and DQ alleles of the 20 MS patients with the DQA1 cluster were determined by allogenotyping. While the DR alleles included several specificities, the DQ allogenotypes in 14 cases were DQw3. Furthermore, a DQ'blank' allele was "detected" on one haplotype in 5 of the Scottish patients using 9th Workshop antisera. This is strongly reminiscent of the association seen in the XIHWS core cell RFLP analyses between the DQA1 cluster and the DRw8,DQw4 (blank) haplotype.

**Chapter 3.1.7**

29. The relationship of the DQA1 cluster to DR and DQ haplotypes in a selection of caucasoid subjects and cell lines was investigated.

30. The DQA1 cluster was observed in 2/5 DR1 subjects, 0/11 caucasoid DR4 subjects, 4/7 DR7 subjects and 4/10 DRw8 subjects. It was seen in a single non-caucasian DR4 subject who had been shown previously to be DR4,5/Dw13/KT2. Its presence in the XIHWS lines Amala, RML and BTB, and also in EBV Wa, was confirmed. In a Scottish family, it was seen in two members (DR2,7 and DR1,7), and from this it was clear that the cluster was contributed in this family by the DR7 haplotype.

31. The haplotype contributing the cluster could be determined confidently in 9 heterozygous individuals and in the homozygous cell lines. The DQw allogenotype in 6 cases was DQw7, w8 or w9, and in 3 cases was DQw4. The DQB1 allele was DQβ3.2 in 7 cases and 3.1 in 2 cases. The cluster appeared to be associated with a DR1,DQw5 haplotype in 1 case. The cluster was observed in the presence of each of the five described DQA1 alleles 1a, 1b, 1c, 2 and 3, and thus no association was seen between the cluster and the DQA1 allele as determined by RFLP.

32. The DQβ3.2 RFLP was present in 11/16 (69%) of cluster-positive cases, and the DQβ3.1 RFLP in 4/16 (25%) of cases (2 cases had both 3.1 and 3.2 alleles). The DQB1 allele associated with the DRw8,DQw4 haplotype was characterized by DQβ3.2 in 11/11 cases. Since the DQB1 gene associated with DQw4 is indistinguishable from the DQw8 (DQw3.2) sub-type of DQw3 by DQB1 RFLP analysis, it was not possible from these data to exclude the possibility that the DQB1 allele present on 69% of cluster-positive haplotypes was identical to that associated with DRw8,DQw4.

**Chapter 3.2.1**

33. Monoclonal serological analysis with the DQw4-specific mAb HU-46 was carried out on 13 homozygous cell lines, one DRA:DRB1 L-cell transfectant, and 4 heterozygous lymphoblastoid cell lines derived

from a Scottish family . In addition to HU-46, the antibodies 3JP, L243 and Tü22 were included as controls.

34. HU-46 reactivity was observed in 4 homozygous cell lines, EBV-Wa (DR4,DQw4), Olga and Madura (DRw8,DQw4), and Luy (DRw8,DQw7). None of the caucasian DR4 cell lines reacted with HU-46, and together, these data agree closely with previous reports of the specificity of HU-46. No staining was seen of the DR transfectant line R/RW1:1-L, with either HU-46 or Tü22, and this is consistent with the antibody being DQ-specific.

35. Two heterozygous cell lines derived from family M3 also reacted with HU-46, DW (DR2/7,DQw6/2) and MC (DR2/2,DQw6/6). HU-46 reactivity was associated with one of the DR2 haplotypes in this family, and this specificity has not been recorded in the past.

36. 5/6 HU-46 reactive cells were also cluster-positive, and 3/8 cluster-positive cells were HU-46 non-reactive. The pattern of HU-46 reactivity seen within family M3 demonstrated that the DQA1 cluster and reactivity to HU-46 were carried on different haplotypes. Thus, although the cluster and HU-46 reactivity are shared properties of most DRw8 cells, they are independent markers, and there is no binding relationship between them.

Chapter 3.2.2

37. Attempts were made to raise T cell clones with patterns of alloreactivity corresponding to the DQA1 cluster. For this purpose, members of a Scottish family were used, in which there were two DR2,DQw6 haplotypes, (a and b), one of which (a) was also cluster-positive. The method used was to use PBMC from DM and CG (a+c) to stimulate cells from AM (b+c), thus eliminating the chance of of obtaining clones specific for products of haplotype c.

38. Six stable T cell clones were obtained, and four of these had a CD4-positive phenotype and were thus MHC Class II-restricted.

39. The fine specificity of two of these clones, MS3 and MS4, was investigated using PBMC from a panel of healthy homozygous and heterozygous volunteers. MS3 and MS4 both proliferated in response to cells from the original stimulator DM. MS3 responded to only one other phenotype, AMa (DR5,w8, cluster +ve). MS4 responded to AMa, NF (DR1, cluster +ve), SF (DR3, cluster -ve), SS (DR4[Dw13]/5, cluster +ve) and EK (DR5/7, cluster +ve). Thus, MS3 responded to 2/6 cluster-positive cells, and MS4 responded to 4/6 cluster-positive cells and to 2/8 cluster-negative cells. Therefore, no relationship with the cluster could be established.

40. In monoclonal antibody blocking experiments, there was dose-related inhibition of clone MS3 by anti-DP, less marked inhibition by L243 (anti-DR), and no inhibition by Tü22 or Leu10 (anti-DQ). Clone MS4 was blocked most strongly by L243 and is not inhibited by anti-DP. Although the specificities of these clones were not investigated further, these data suggested that MS3 was DP-specific, and that MS4 had Class II-restricted anti-Class I activity.

**Chapter 3.2.3**

41. DQA1 genotyping of 5 cluster-positive and 2 cluster-negative cell lines was carried out, by probing PCR-amplified DQA fragments with labelled allele-specific oligonucleotides under stringent conditions.

42. The hybridization patterns obtained indicated the presence, in all of the cells studied, of exactly those DQA1 alleles predicted from known linkage relationships and recent studies of DQα1-encoding gene segment sequences. They did not provide evidence that any of the cluster-positive cell lines might possess an hitherto unrecognized DQA1 allele.

**Chapter 3.2.4**

43. The DQα-chain first domain-encoding gene segments from four individuals and from a single homozygous cell line were determined. A DQA1.2 allele, associated with DR2,DQw6 and identical to those previously published, was obtained from each of the DR2-positive

subjects. A DQA2 sequence was obtained from a DR7 subject, and this was unremarkable except for a single silent nucleotide substitution at position 96. A DQA3 sequence was obtained from a DR4 subject.

44. A DQA4.2 sequence was obtained from the homozygous cell line BTB, and within the limits of this analysis, this sequence was identical to that obtained from the DRw8[8.1],DQw4 homozygous cell line Madura.

45. The methods used resulted also in the appearance of two hybrid sequences, DQA1.2/DQA2 and DQA1.2/DXA. This was an artefact of the PCR process.

46. Within the limits of this study, there were no differences between the DQα1-encoding sequences obtained from cluster-positive DR2, DR7 and DRw8 haplotypes, and those reported previously from cluster-negative haplotypes. Thus the cluster does not appear to be associated with a unique allele of DQA1. These data are consistent with the results of allogenotyping, mAb studies, T cell clonal analyses and oligonucleotide hybridization studies presented above.

## 4.2 DISCUSSION

In this study of MHC Class II genes in patients with multiple sclerosis, discriminatory polymorphisms have been detected in the DQA1 gene following DNA digestion with the restriction enzyme Msp1. The initial investigations using pooled DNA samples enabled four groups of subjects to be screened with 14 restriction enzymes, 5 HLA-D region probes and 2 T cell antigen receptor probes. The sensitivity of Southern blot analysis of pooled DNA was verified by the detection of fragments which discriminated clearly between the DR2+ve and DR2-ve pools. There was no evidence from these studies to suggest that information was lost through fragments becoming obscured by co-migration when DNA samples were pooled.

Four of the enzyme/probe combinations revealed possible differences between the MS and control pools. All of these differences were seen using DQ gene probes. A 3.25kb Msp1 fragment hybridizing to DQA1 discriminated between the DR2+ve disease and control pools. Taq1, Bgl11 and BamH1 revealed possible further differences when hybridized to DQB1, but were only capable of differentiating between the DR2-ve pools. No differences were seen with DRB1, or with the DP or TcR probes. The hybridization intensity of a fragment in a sample of pooled DNA is proportional to its representation in the individual samples making up the pool. The DQB1 fragments generated by Taq1, Bgl11 and BamH1 were identified as being of possible discriminatory value by their moderate differences in hybridization intensities between disease and control pools, whereas the DQA1 differences between the pools were much more striking.

These findings were subsequently confirmed when individual samples were examined. In the Scottish subjects, the Msp1/DQA1 3.25 and 2.50kb fragments were seen in 30.3% of patients, but in only 4.2% of controls. When the DR2+ve individuals were analysed separately the relative frequencies of these fragments in patients and controls were unchanged (28% and 2.9%). Thus these fragments were associated with MS independently of DR2. There were no significant differences between patients and controls with any DQB1 or DRB1 restriction fragments.

Confirmation of these findings in a second population was sought, and a collaborative study was conducted with the Tissue Typing Laboratory, Belfast City Hospital. The Scottish and Northern Irish

populations are genetically similar, and both have high prevalence rates for MS. It should be noted however that the frequency of DR2 in the Grampian region is significantly higher, closer to that seen in the Orkneys, and this higher frequency in the background population is thought to account for the failure of DR2 to associate strongly with MS in patients from this region. When data from the Irish subjects were examined, there was no difference in the frequency of the Msp1/DQA1 polymorphism between the patients and the controls, in contrast to the Scottish data. However, amongst the DR2+ve cases, there was once again a significant excess in the MS patients, the cluster being present in 6/30 (20%) patients and 1/29 (3.4%) controls. From these data it was concluded that the cluster is rare in healthy DR2+ve individuals, but is significantly more common in DR2+ve MS patients. It is not infrequent among non-DR2 individuals, of whom there are more in N Ireland than in NE Scotland.

Analyses of associations between a disease and genetic markers can give rise to spurious conclusions for a number of reasons. One is that such analyses normally involve making comparisons of frequencies of many genetic markers in the "disease" and "control" groups. In these circumstances, an apparently significant difference is expected in 5% of all frequency comparisons. It is therefore conventional practice to apply a correction factor, multiplying the raw p value by the number of comparisons. However, if subsequently, a further independent study confirms the conclusions reached in the first study, application of a correction factor is not necessary. When the results presented here, from two independently conducted studies in two populations, were combined, they achieved a high degree of significance ($p<0.001$).

The XIHWS data included an analysis by Southern blotting of 72 homozygous cell lines (HCL). Cluster analysis of the DQA1 fragments generated by 12 restriction enzymes, including Msp1, revealed that Msp1 3.25kb belongs to a cluster which also contains Msp1 2.50 and 2.31kb, and Bgl11 13.12kb. In the 10th Workshop data, this fragment cluster was seen in all of the DRw8 cell lines and some of the DR4 and DR7 cell lines. It was not seen in the DR2 lines, with the exception of RML which carries the rare Amerindian specificity Dw22. (There is an intriguing relationship between Dw22 and DRw8, in that they are both associated with an increased risk of Chagas'disease (trypanosomiasis) in South America; the basis for this is unknown (Layrisse et al.1988)).

Cluster analysis of the XIHWS data showed that the Msp1/DQA1 3.25/2.50/2.31kb cluster and the Msp1/DQA1 2.87kb fragment failed to occur together in any of the HCLs, thus exhibiting apparent allelism. Whereas the 2.87kb fragment appeared to define a broad DQw1 specificity, the cluster was seen predominantly in HCL with a DQw3 or DQ "blank" phenotype. RFLP allogenotyping was employed in order to investigate the relationship between the DQA1 cluster and DQA and DQB alleles, both in the MS patients and in a panel of caucasoid cells. A DQB1 RFLP pattern characteristic of the DQw8 sub-type of DQw3 was found in 11/16 (69%) of the cluster positive cells. However, since the DQB1 gene associated with DQw4 is indistinguishable from DQw8 by DQB1 RFLP analysis, it was not possible from these data to exclude the possibility that the DQB1 allele present on 69% of cluster-positive haplotypes was identical to that associated with DRw8,DQw4. Additionally, it was observed that in the panel of cluster positive cells, all of the five alleles of DQA1 that are distinguishable by the typing system used, were present. Thus, no clear link could be established between the cluster and a recognized DQA1 allele, despite the cluster exhibiting an allelic relationship to DQw1. The possibility that it represented polymorphism related to the closely related DQA2 locus seemed very unlikely.

More precise definition of the DR2 and DQw1 alleles in the present study was achieved by examining the frequencies of four DQA1 and DQB1 fragments with known specificities. It was seen that almost all of the DR2+ve individuals had the typical DR2,Dw2,DQw1.2 alleles, and furthermore that these were present with equal frequencies in both patients and controls. These data were in agreement with similar work reported recently from Australia and European centres (Serjeantson et al.1986; Semana et al.1988). It was of considerable interest therefore that the DQA1 polymorphism described above appeared to be allelic to DQw1, and was associated with disease independently of DR2 in that it was equally represented in both DR2+ve and DR2-ve patients. Furthermore, of the 10 Scottish patients with this DQA1 cluster, all 10 also had the allelic 2.87kb DQw1-associated fragment. The cluster was therefore contributed by the non-DR2 haplotype in these cases, raising the possibility that disease susceptibility is enhanced by the coinheritance of a DR2,DQw6 haplotype with a second haplotype characterized by the DQA1 cluster. Establishing whether this cluster might be associated with a functional rôle became a matter of considerable interest.

A variety of methods were then employed in order to attempt to determine whether the cluster was associated with an expressed allele of DQ. Since the cluster had exhibited strong linkage with DRw8, and allogenotyping had confirmed an association with DQw4β or DQw8β, cluster-positive cells were studied with the DQw4-specific monoclonal antibody HU46. The relationship between the cluster and HU-46 reactivity was not strong, however, and in a family study it could be seen that the cluster and HU-46 reactivity were contributed by different haplotypes. Using a cloning combination expected to result in the generation of alloreactivity directed against the cluster-positive haplotype, four CD4+ve clones were obtained. However, the specificities of two of these were tested, and no relationship to the cluster was seen. DQA1 genotyping by means of allele-specific oligonucleotide hybridization of amplified DNA (ASO-PCR typing) demonstrated the presence in cluster positive cells of exactly those alleles which would be predicted from previous analyses of cluster-negative cells. Finally, the presence of the predicted consensus DQA1 genes (second exon) in 5 cluster-positive cells was confirmed by nucleotide sequence analysis. In summary therefore, genomic sequence variation identifiable only by RFLP, has been found to be associated with MS independently of DR2.

One of the conclusions to be drawn from these studies is that the effect of these two haplotypes on disease susceptibility may be more than merely additive. This effect, transcomplementation, is usually interpreted as indicating that there is functional interaction between haplotypes. This might occur at the nuclear or gene product level, and no universal mechanism has been identified. Although there has not previously been any evidence of transcomplementation in MS, it is suggested in the cases of certain other autoimmune diseases, in which associations with two Class II alleles are seen. The best examples identified by serological studies are insulin dependent diabetes (IDDM), associated with DR3 and DR4 (Wolf et al.1983), and coeliac disease (DR3 and DR7) (Tiwari et al.1984). Most of the susceptibility to IDDM encoded within the MHC is contributed by the DR4,DQw8 haplotype, but the RR of DR3,4 heterozygosity is considerably greater than the risk of either DR3 or DR4 alone (Deschamps et al.1988). Nucleotide sequence analysis of Class II genes in IDDM has highlighted the importance of the amino acid at position 57 of the DQβ chain (Todd et al.1987; Todd et al.1988a). Both the DQw8β allele and the DR3-associated DQw2β allele have alanine-57, whereas those alleles

associated with neutral or diminished risk of IDDM have aspartate-57. Further attention has been brought recently to the importance of heterozygosity in IDDM by the observation that the disease is significantly more frequent among DR4,DQw8/DRw8/DQw4 heterozygotes (Rønningen et al.1989). It was suggested by the authors that DR4,DQw8/DRw8/DQw4 and DR3,DQw2/DR4,DQw8 heterozygotes may share similar Class II epitopes expressed by transcomplementing hybrid DQα/DQβ dimers. This possibility has received support from Kolstad et al. who raised two human monoclonal antibodies specific for the transcomplementing molecule DQw7α/DQw4β (Kolstad et al.1989).

There is evidence also that transcomplementation might be important in coeliac disease. While the greatest risk is conferred by the DR3,DQw2 haplotype, DR7 in association with DR3 or DR5 greatly further increases the risk. The (DR3,) DQw2α chain sequence is identical to that of (DR5,)DQw7α, while (DR3,) DQw2β differs from (DR7,) DQw2β at only a single position (Karr et al.1986). Thus DR5,DQw7/DR7,DQw2 heterozygotes could, by transcomplementation, express the same (or almost the same) DQα/β heterodimers formed by DQA1 and DQB1 genes in *cis* position on the DR3,DQw2 haplotype (Sollid et al.1989).

In both these autoimmune diseases therefore, there is preliminary evidence that transcomplementation may increase susceptibility. Heterozygosity is normally advantageous to an organism, and it may be this heterozygote advantage (overdominant selection) which has been responsible for the generation and maintainance of the immense polymorphism of the MHC (Hughes, Nei, 1989). If this is the case, then susceptibility to autoimmune disease may be regarded as an unfortunate side effect of this tendency towards heterozygosity.

From the data described in this thesis, it can be proposed that heterozygosity also increases susceptibility to MS. Since the cluster does not appear to be associated with an expressible allele of DQA1, it must be concluded that either the cluster is in tight linkage with another, unidentified, locus whose expressed product might interact with products encoded by the DR2 haplotype; or alternatively, the cluster defines sequence variation which lies in a functionally important, but non-coding, region near the DQA loci. The possibility that regulatory polymorphism might be important in controlling the expression, and hence the function, of MHC molecules is frequently discussed, but there exists little data to suggest how it might operate (Mach et al.1986). The strongest evidence yet

that the relationship between autoimmunity and the Class II region is governed by micropolymorphism within the coding regions comes, as discussed above, from IDDM. However, the situation has not proved to be as clear in many other autoimmune diseases, and the probability that allelic differences affecting the regulation of Class II genes may also be involved is increased. Polymorphism affecting the promoter region could alter the expression of the gene in various ways. Sensitivity to γIFN could be increased or decreased, Class II expression could be induced on certain, normally non-inducible, cells (eg. oligodendrocytes), or the aberrant expression of inter-isotypic or transcomplementing α/β pairs could be promoted.

MHC Class II gene expression appears to be under the control of both *cis*- and *trans*-acting regulatory elements (reviewed in (Sullivan et al.1987)). An abnormality of the *cis* promoter is thought to account for the bare lymphocyte syndrome, in which the coding regions of the DR and DQ genes are unaffected (and can be typed by PCR and ASO-typing (Baxter-Lowe et al.1988a)), but the genes are not transcribed. Class II genes give rise to three distinct phenotypic states. Constitutive high expression is seen in B cells, human T-blasts and virally infected T cells. Class II expression is inducible by γIFN in APCs, macrophages, dendritic cells, and certain parenchymal cells including astrocytes and microglia. In many other cells, Class II genes are not expressed and their expression cannot be induced by conventional means. Clearly, any Class II dysregulation which resulted in a cell exchanging one of these phenotypes for another might have a profound effect on T cell activation.

In general, eukaryotic DNA is methylated at cytosine residues in CpG dinucleotides, and this correlates with transcriptional inactivity. In the DRA gene, a demethylated CpG-rich island flanks exon 1, whereas the 5' upstream and the coding and 3'UT flanking regions are methylated. The present studies have shown that identical DQA1 restriction fragment patterns are obtained with both Msp1 and Hpa11, indicating that the recognition sites responsible for the fragment cluster are not methylated. Thus, although the cluster cannot be accounted for by allele-specific differences in methylation, the DQA1 locus on cluster-positive haplotypes is associated with three additional non-methylated CpG dinucleotides, and it is possible that these might be functionally active.

Disease susceptibility has been associated with the DQA1 locus less frequently than with DQB1. However, there have been two recent reports

indicating that DQA1 may encode as much susceptibility as DQB1, in both IDDM (Todd et al.1989) and coeliac disease (Roep et al.1988). In the present studies, no significant DQB1 association with MS was found, and in contrast, an association with a DQA1 polymorphism was observed in independently conducted studies in two populations. It has recently been demonstrated that 59/61 (97%) of Norwegian MS patients possess at least one of the alleles DQB6, DQB8 or DQB9 (associated with DR2, DR4 and DRw6 respectively) (Vartdal et al.1989). Inspection of the protein sequences of these alleles reveals that they share sequences in the membrane-distal domain of the DQβ chain. Furthermore, these shared residues are clustered, and are thought to be directed in toward the antigen binding cleft. Thus it is hypothesized that these alleles might be capable of recognizing an MS-associated antigen. Since the DQA1 cluster is allelic to DQw6 present on DR2 and DRw6 haplotypes, it would be of great interest to investigate whether the cluster is more frequent in these Norwegian patients. It is also notable that DR4 has been implicated as a risk factor for MS in two separate studies, one in Jordanian arabs, the other in Italians.

## 4.3 CONCLUSIONS

Although the conclusions of these study should be interpreted cautiously at this stage, it is relevant to stress that 1) the DQA1/Msp1 fragment cluster has been detected at an increased frequency in DR2 positive MS patients in two independent studies; 2) the preliminary analysis of pooled DNA samples was confirmed as correct, when the individual DNA samples contributing to the pools were probed; 3) the data of the XIHWS validates this RFLP cluster as an hitherto undefined DQA1 polymorphism, with an allelic relationship to DQw1; 4) the cluster does not appear to correlate with an allele of DQA1 or with an expressed DQα product. Further studies of both caucasians and other ethnic groups will be required to confirm this disease association. However, the present data provide the first evidence that two haplotypes may be involved in determining susceptibility to MS.

## 4.4 RECOMMENDATIONS

1. The numbers of MS patients and controls examined for the presence of the DQA1 cluster should be increased by further studies.
2. Non-caucasoid populations should also be studied, for associations with alleles of both DQA1 and DQB1. An association with DR4 has been described in Jordanian arabs and Italians. It would be of great interest to determine the frequency of the DQA1 cluster in these patients, and its relationship to the DQB1 polymorphism reported by Vartdal et al (1989).
3. Further studies will be greatly facilitated by making a "cluster-specific" probe. This could be achieved most efficiently by making a size-fractionated genomic library from Msp1-digested DNA.
4. By cloning the three Msp1 fragments, or the single Bgl11 fragment, which comprise the cluster, the genomic region containing the sequence variation responsible for the cluster could be characterized, by both large fragment mapping and by sequence analysis. Two hypotheses which could be tested in this way are 1) that the cluster is related to regulatory polymorphism of the DQA1 gene, and 2) that cluster-positive haplotypes contain an additional DQA locus.

# PART 5

# APPENDICES

Appendix 1

Participants in the XIHWS multi-centre study of HLA-D gene polymorphism in Multiple Sclerosis. Reported in *Immunobiology of HLA. Volume 2: Immunogenetics and Histocompatibility*, edited by B. Dupont, Springer-Verlag, New York, 1989.

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| | | DQA1 RFLP | | | | |
|---|---|---|---|---|---|---|
| | | DQα1a | DQα1b | DQα1c | DQα2 | DQα3 |
| DQ serotype | DQB1 RFLP | | | | | |
| DQw1 | DQβ1a | Br,1,w10<br>w14[Dw9] | w13[Dw19]<br>2sh | | | |
| | DQβ1b | | 2[Dw2]<br>w13 | 5(11)<br>w13[Dw18] | | |
| | DQβx | | | 2[Dw12] | | |
| DQw2 | DQβ2a | | | | 3 | |
| | DQβ2b | | | | | 7[Dw7] |
| DQw3 | DQβ3a<br>(3.2) | | w8[Dw8] | | | 4[Dw4]<br>4[Dw10]<br>4[Dw14]<br>7[Dw11]<br>9 |
| | DQβ3b<br>(3.1) | | w8[Dw8.2] | 8 | 5(11)<br>5(12)<br>Br,13<br>w14[Dw16] | 4[Dw4]<br>4[Dw13] |

## Appendix 2

Correlation between DRB1, DQB1 and DQA1 RFLPs, based upon the analysis of over 1400 Caucasian haplotypes based upon the sequential hybridization of Taq1-digested DNA with DRB1, DQB1 and DQA1 (modified from *Bidwell et al. (1988), Immunology Today 9(1):18-23).* Using this system, the DRB hybridization signals reveal allele-specific patterns for most DR specificities. The exceptions are failures to discriminate between DR3 and DRw13-DQw6, and DR7-DQw9 and DRw9-DQw9. These alleles however, may be differentiated by the DQB1 and DQA1 alleles with which they are in strong linkage disequilibrium. Seven DQB RFLP patterns can be distinguished, and these correspond closely to splits of DQw1, w2 and w3 as shown above. The DQA RFLPs identify an allelic series which appears independent of DQ serologic specificity, but which is nonetheless in strong linkage disequilibrium with DRB and DQB RFLP series. In this way DRB, DQB and DQA RFLP data may be combined to identify most recognized DR, DQ and Dw genotypic specificities (although distinction between the DQw7- and DQw8-associated splits of DR4 may require analysis with additional restriction enzymes).

## Appendix 3

Single letter amino acid codes

| | | | |
|---|---|---|---|
| Phe: F | Leu: L | Ile: I | Met: M |
| Val: V | Ser: S | Pro: P | Thr: T |
| Ala: A | Tyr: Y | His: H | Gln: Q |
| Asn: N | Lys: K | Asp: D | Glu: E |
| Cys: C | Trp: W | Arg: R | Gly: G |

# PART 6

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