THE GENETICS OF TYPE 1 DIABETES:

FAMILY AND POPULATION STUDIES

by

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SYNOPSIS

Type 1 (insulin-dependent)diabetes mellitus is caused by autoimmune destruction of insulin-producing pancreatic ß-cells. Genetic susceptibility is necessary for the development of type 1 diabetes but inheritance does not follow simple Mendelian rules and environmental factors are also involved. Identification of the genes which mediate disease susceptibility should allow recognition of individuals at high risk of disease; this will facilitate the study of environmental factors and possibly the development of strategies for prevention. Further, the characterisation of susceptibility genes will allow a greater understanding of the aetiopathogenesis of type 1 diabetes and may lead to new therapeutic approaches. A major limiting factor, hindering the genetic dissection of type 1 diabetes, has been the lack of a large well-characterised clinical resource for study. This thesis describes how I have established a Repository of immortalised cell lines from large numbers of ethnically matched controls, sporadic type 1 diabetics and type 1 diabetic multiply affected families. The family resource is now the largest single collection of it's kind and is used by research groups throughout the world. Using this resource, I have further characterised HLA-mediated susceptibility to Type 1 diabetes, particularly with regard to age-related heterogeneity and inherited susceptibility. In addition, I have confirmedthe existence of linkage of type 1 diabetes to a gene (or genes) within the insulin gene region on chromosome 11p.I have also performed association studies using other candidate genes. These studies have led to a significant advance in the field of the genetics of type 1 diabetes and bequeath a permanent resource for future research.

DEDICATION

This thesis **is** dedicated to Beth **who saved me** from **General** Practice.

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CHAPTER 1

BACKGROUND AND STRATEGY

1. Introduction

Diabetes mellitus is a clinical syndrome characterised by hyperglycaemia. The fundamental defect in diabetes is a deficiency of insulin action. In the classical, juvenile-onset form of diabetes mellitus (type 1 diabetes), there is an absolute deficiency of insulin. In late-onset, non-insulin-dependent diabetes, there is a variable combination of diminished insulin secretion and tissue resistance to insulin activity (peripheral insulin resistance). The conventional diagnosis of diabetes mellitus is defined in terms of blood glucose levels at the beginning and end of a standard oral glucose tolerance test ***(OGTT)*** (WHO Expert Committee on Diabetes Mellitus ***1980).*** These criteria are, however, of little relevance to type 1 diabetes where the majority of patients present with severe symptoms of lethargy, polydipsia, polyuria and weight loss and the diagnosis is confirmed by an elevated random blood glucose. If treatment with insulin is delayed, fatal diabetic ketoacidosis may ensue.

The peak age-at-onset of type 1 diabetes is 12 years and the incidence of disease appears to be increasing rapidly in the United Kingdom, where it is now the second most common chronic disease of childhood (Metcalfe *und* Baum 1991). Affected individuals require life-long insulin therapy and are prone to disabling complications such as retinopathy, nephropathy and neuropathy.

1.2. Pathogenesis

The pathological process culminating in type 1 diabetes involves the chronic autoimmune destruction of insulin-producing pancreatic β-cells ***(Todd*** 1990). The histological hallmark of disease is 'insulitis', a mononuclear cell infiltration of the

pancreatic islets of Langerhans. Insulitis is accompanied by selective loss of the β-cells of the islets, leaving other endocrine cells intact. ß-cell loss appears to be a protracted process; sensitive measures of ß-cell function, such as the first-phase insulin response (FPIR) to intravenous glucose, have defined a period of up to 7 years before the need for insulin where the FPIR is reduced (Section 1.4.2.ii)(Srikanta, Ganda et al. 1985)(McCulloch, Klaff et al. 1990). Antibodies to several different islet cell components, present in 70-80% of new cases, can also be detected up to 5-10 years before diagnosis (Section 1.4.1). Eisenbarth's group have proposed a linear model of progressive loss of insulin secretion during the pre-diabetic period(Srikanta, Ganda et al. 1984). Other authors, however, find that β-cell function may fluctuate and does not invariably progress to overt disease(Palmer and McCulloch 1991)(Kobayashi, Itoh et al. 1987)(McCulloch, Klaget al. 1990). Whichever model is more accurate, it is likely that clinical presentation of type 1 diabetes occurs after several years of β-cell dysfunction during which up to 90% of the pancreatic β-cells may be destroyed(Gepts 1965).

The cells infiltrating human islets in type 1 diabetes are morphologically lymphocytes, staining positive for T200 leukocyte common antigen(Foulis, Liddle et al. 1986). In a single autopsy report, these cells were phenotypically characterised and appeared to be predominantly cytotoxic/suppressor T-cells (Bottazzo, Dean et al. 1985). In that study, Botazzo et al. reported increased class I MHC expression (HLA-A, -B and -C) on affected islet cells and found that some insulin-producing β-cells (but not α- or δ-cells) were class II (HLA-DR) positive (Section 1.3.1). Foulis et al. also noted abundant HLA-DR expression on insulin-secreting islet cells(Foulis, Liddle et al. 1986). The finding of class II HLA expression in affected islets is significant for two reasons; β-cells do not normally express class II antigens and cells expressing class II antigens can present antigen

to CD4-positive T-lymphocytes (T-helper cells). It has been proposed that aberrant class II expression by β-cells facilitates the presentation of antigen to T-lymphocytes which then initiate an immune response(Bottazzo, Pujol-Borrell et al. *1983).* Alternatively, class II expression may be a consequence of ongoing inflammation, rather than an initiating event (Dean, Walker et *al. 1985).*

Experimental work on MHC expression in animal models of disease may help to resolve this issue. As in the human, (3-cells from diabetic rats show increased expression of class I MHC antigens. Electron microscopy studies, however, suggest that class II expression in rat islets is limited to lymphoid cells, dendritic cells and macrophages(Farr and Anderson *1985).* Other studies have addressed the importance of class I and class II expression on β-cells in transgenic mouse models(Allison, Campbell et al. *1988)(Lo,* Burkly et al. *1988)*(Sawetnick, Liggitt et al. *1988).* In all cases, overexpression of MHC antigens caused the development of diabetes. However, ß-cell destruction did not occur. Histopathological findings were not typical of type 1 disease and it appeared that diabetes was caused by interference with insulin secretion. These findings suggest that MHC expression in islets is unlikely to initiate autoimmune ß-cell destruction. Species differences between human and rodent, however, cannot be excluded; for example, human T-cells are MHC-class II-positive whereas mouse T-cells, in general, are not.

Two other lines of investigation provide further support for an involvement of T-cells in the aetiology (or maintenance) of type 1 diabetes. First, a study of peripheral blood lymphocytes in pre-diabetics by Faustman et al. showed an increase in a subset of CD4-positive cells in 22 pre-diabetics compared to age-matched controls; a subset of CDw29 (helper-inducer) cells was reciprocally reduced in the same group(Faustman, Eisenbarth et al. *1989).* Second, there are

studies demonstrating an effect of cyclosporin A (CY-A) in early disease. CY-A inhibits early events in T-cell activation, at least in part by binding the protein cyclophilin (Krensky, Weiss et al. 1990). In a double-blind, placebo-controlled trial of CY-A in recently diagnosed diabetes, CY-A increased the rate and duration of insulin-free remissions; after 9 months treatment, 24.1% of the CY-A group were in complete remission, compared with 5.8% of the placebo-treated patients (Feutren, Papoz et al. 1986). Unfortunately, despite continued CY-A treatment, the majority of patients only have short remissions and ultimately require re-introduction of insulin treatment (Assan, Feutren et al. 1990)(Bougneres, Landais et al. *1990)*.

In summary, type 1 diabetes is preceded by invasion of the pancreatic islets by immune cells, including T-lymphocytes and macrophages, that cause specific lysis of β-cells. This process, known as insulitis, takes place over a period of months or years, during which 80-90% of the β-cells are destroyed. Only at this stage do the typical symptoms develop, allowing the clinical diagnosis of type 1 diabetes to be made. Intervention aimed at preserving ß-cell function is, therefore, more likely to succeed if started before the time of clinical presentation, when a larger proportion of β-cells are still viable.

1.3. Aetiology

The life-time risk of type 1 diabetes is approximately 0.4% in Caucasians; the average risk for siblings of type 1 diabetics is 6% and the concordance rate for genetically identical monozygotic (MZ) twins is reported to be 36% (Risch 1987)(Olmos, A'Hern et al. 1988). Although the MZ twin concordance figures are probably an overestimate due to ascertainment bias, these findings indicate significant familial clustering of disease, consistent with a genetic input. The large

discordance rates in MZ twins, however, indicate that susceptibility genes have low penetrance i.e., an individual who is 'genetically programmed' for disease rarely develops the clinical condition. The penetrance of an allele (the probability that it causes disease) is dependent upon three factors: (1) its interaction with alleles of additional unlinked loci, (2) random factors within the individual (e.g., T-lymphocyte antigen receptor and immunoglobulin somatic gene rearrangements) and **(3)** environmental agents. Only (2) and **(3)** can apply to MZ twins and whilst random factors could theoretically explain the low twin concordance rates, this would not be consistent with the observed risk among siblings. It is likely, therefore, that environmental factors are of great importance.

In the following sections, I describe the current knowledge of genetic susceptibility to type 1 diabetes and the evidence supporting environmental influences in disease aetiology.

1.3.1. HLA-encoded genetic susceptibility to type 1 diabetes

1.3.1.i. HLA; from B15 to Asp 57.

The best evidence for a genetic component to susceptibility to type 1 diabetes comes from studies of the Human Leukocyte Antigen (HLA) region. The HLA region is a cluster of genes located within the major histocompatibility complex (MHC) on chromosome 6p (figure 1.1). Hence, in human studies, the terms HLA and MHC tend to be interchangeable. In the 1960's, the HLA region was identified as the site of the Major Transplantation Antigen Complex of genes in humans, and was originally named because the serologically defined antigens which it encodes are absent on red blood cells. The search for associations between antigens of the HLA system and disease was stimulated by the discovery of genetic linkage between the

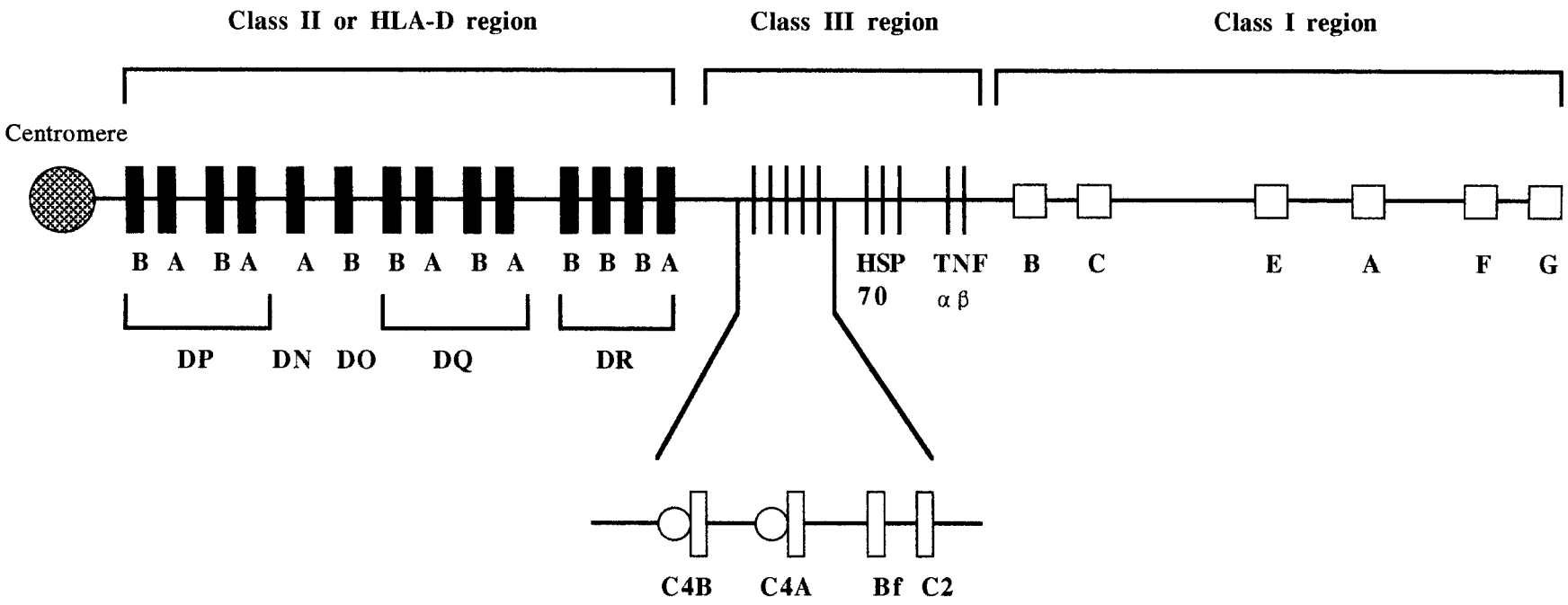


Figure 1.1. Schematic representation of the genes of the HLA complex.

mouse Major Transplantation Complex (H-2) and resistance to virus-induced leukaemia (McDevitt and Bodmer 1974). Further impetus to the search for autoimmune associations came when immune response (Ir) genes were found to be linked to H-2.

In 1973, Singal et al. described an association between HLA-B15 and type 1 diabetes, which was absent in non-insulin-requiringdiabetics(Singal and Blajchman 1973). This finding, and a further association with HLA-B8, was confirmed by Nerup et al. and Cudworth and Woodrow (Nerup, Platz *et* al. 1974)(Cudworth and Woodrow 1974). These contributions were important since they led to a formal split of the clinical syndrome of diabetes into type 1 and type 2 (maturity-onset, non-insulin-dependent) diabetes. Two points concerning this advance are worthy of note. First, an almost identical delineation of diabetes, made using clinical criteria had been reported 20 years earlier by R.D. Lawrence(Lawrence 1951). This had not, however, been accepted by geneticists who continued to analyse diabetes as one condition (with predictably poor results). Second, the study of Singal et al. was not the first to investigate an association between type 1 diabetes and HLA antigens. Finkelstein et al. had found no association in a study of juvenile-onset patients, a failure due to racial admixture of the population under study (23 Caucasians, 10 Hispanics and 6 Negroids) (Finlzelstein, Zeller et al. 1972). Racial differences in HLA disease associations have subsequently been exploited in fine mapping of disease susceptibility loci (Section 1.3.2).

The original studies detected associations with HLA class I gene polymorphisms by serological typing (the use of antibodies which recognise characteristic epitopes of HLA-encoded molecules). Class I genes encode alpha chains which combine with β2 microglobulin to form glycoproteins found on all nucleated cells. These

molecules are involved in the restriction of T-lymphocyte activity. Even at the time of discovery, it was not thought that class I genes were primary susceptibility determinants for type 1 diabetes; rather it was suggested that these plymorphisms were in linkage disequilibrium with true susceptibility alleles within the **HLA** region. Linkage disequilibrium describes the co-occurrence of two or more separate alleles on the same chromosome more frequently than expected by chance. This phenomenon is particularly strong across the 2,500 kilobase pairs of the **HLA** region, with few recombination events occurring during meioses; this leads to combinations of alleles at different loci (haplotypes) being inherited en bloc.

With the development of cellular typing and isolation of class II antisera, it emerged that the strongest population associations are with class **II** antigens (Nerup, Cathelineau et al. 1977); over 95% **of** Caucasian type 1 diabetics have **HLA-DR3** and **-DR4** antigens, compared with 50% of controls(Svejgaard and Ryder ***1989).*** **HLA-DR3** and **-DR4** are allelic products of the DRB1and **DRA** loci of the **HLA-D** or class **II** region of the MHC (figure 1.1). These molecules are also cell-surface glycoproteins but are made up of both α- and β-chains. Each class **II** gene is subdivided into at least one **A** and one **B** gene, alleles of which encode the α- and β-chains respectively. Class **II** molecules are found on antigen presenting cells such as macrophages, **B** lymphocytes and activated T-lymphocytes. They bind foreign (and self) antigen and present it to CD4-positive T-cells. Many of the class II genes are highly polymorphic, resulting in great variation in the class **JI** molecules possessed by different individuals.

Study of the segregation of **HLA** haplotypes in families with type 1 diabetes also implicates a disease susceptibility gene (or genes) closely linked to the class **II** region (Thomson, Robinson et al. ***1988)***(Spielman, ***Baur et*** al. ***1989).*** Thomson et

al., however, pointed out that the HLA-DRB1 alleles encoding DR3 and DR4 are too common in the general population to be susceptibility determinants; alternative loci within the class II region were, therefore, considered(Thomson, Robinson et al. ***1988).*** Linkage disequilibrium is strong between the HLA-DR and -DQ subregions and, as a result, little familial recombination between DQA1 and DRB1 is observed. This prevents detailed linkage analysis in families and creates difficulties in fine mapping of primary susceptibility genes. Owerbach et al. were the first to discover an HLA-DQB1 DNA polymorphism, based on restriction enzyme digestion of genomic DNA, that subdivided HLA-DR4 haplotypes into high and low risk(Owerbach, Lernmark et al. ***1983).*** This important result demonstrated that HLA-DQB1 is more closely associated with disease susceptibility than the HLA-DRB1 locus. Nepom et al. subsequently showed that this restriction fragment length polymorphism (RFLP) corresponded to a recognised serological specificity (TA10) and that two alleles of the HLA-DQB1 locus were associated with DR4(Kim ***1985).*** One of these, HLA-DQ8 (TA10-) is increased in Caucasian patients (90%) whilst the other, HLA-DQ7 (TA10+) is decreased (10%); the alleles are equally represented in HLA-DR4-positive controls(Nepom, Palmer et al. ***1986).***

In 1987, Michelsen and Lernmark reported the sequence of the HLA-DQB1 DQ7 allele (now known as ***HLA-DQB1*0301)(Michelsen and*** Lernmark ***1987).*** This showed only 6 amino acid differences from the sequence of the DQ8 allele (HLA-DQB1*0302); four of these differences are in the N-terminal polymorphic domain, at residues 13, 26, 45 and 57. The polymerase chain reaction (PCR) was used to sequence these alleles in type 1 diabetics and controls, to determine whether there was additional polymorphism between the coding sequences. The sequences of DQB1*0301 and DQB1*0302 were, however, identical in the two groups and there

was no sequence unique to type 1 diabetes. The associated DQA1 alleles were also found to be identical.

This suggested that one, or more, of the known amino acid changes might be responsible for the contrasting associations of HLA-DR4,-DQ8 and -DR4,-DQ7 haplotypes with type 1 diabetes. In addition, two other HLA-DR alleles, DR2(Dw15 and 16) and DR6 (DRw13 and 14), were associated with more than one DQB1 allele, posing the same question; did the frequencies of DQB1 alleles (on HLA-DR2 and -DR6 haplotypes) differ in patients versus controls? For both HLA-DR2(DRw16) and -DRw13, the predisposing haplotypes carried a DQB1 allele encoding a non-charged amino acid at position 57 (of the β-chain). Haplotypes which did not predispose to disease, including HLA-DQ7 haplotypes, had DQB1 alleles encoding the charged amino acid aspartic acid at this position (Asp-57). Thus, in the three cases where the distribution of DQB1 alleles could be compared in HLA-DR-matched diabetics and controls, there was a significant difference and this correlated with polymorphism of HLA-DQB1 codon 57(Todd, Bell *et* al. *1987)(Horn,* Bugawan *et* al. *1988).* Consistent with this finding, predisposing HLA-DR3 and -DR1 haplotypes have DQB1 alleles encoding alanine and valine, respectively, at position 57.

Further support for an important role for position 57 in diabetes susceptibility comes from animal models of disease. The non-obese diabetic (NOD) mouse is a strain which spontaneously develops diabetes with many histological, immunological and pathological features of human type 1 disease. The murine homologue of HLA-DQB1 is known as I-Aβ and the sequence of the NOD I-Aß allele is unique in having a Ser-57 codon; all other, diabetes-resistant, strains possess an I-Aβ gene with an Asp-57 codon(Acha-Orbeaand *McDevitt 1987).* The

spontaneously diabetic BB rat also encodes Ser-57 in its homologous class II β-chains.

In 1988, Brown et al. proposed a hypothetical structure for class II molecules(Brown, Jardetzky et al. 1988). In this, Asp-57 forms a salt bridge with a conserved arginine residue at position 79 of the DQ a-chain. This suggests that the β-chain residue 57 may be in a position on the surface of the antigen-binding site where it can interact with the T-cell receptor and/or peptides(Todd, Acha-Orbea et al. 1988). The functional importance of this position is emphasised by studies showing that alteration of residue 57 can affect the recognition of both alloreactive and antigen-specific T-cell clones(Lundin, Gauderernack et al. 1988)(Sterkers, Zeliszewski et al. 1988)(Kwok, Mickelson et al. 1990). Further, transgenic data in the mouse indicate that amino acid polymorphisms at positions 56 and 57 in the I-A molecule influence the development of overt diabetes(Miyazaki, ***Uno*** et al. ***1990)***(Slattery, Kjer-Nielson et al. 1990)*(Lund,* O'Reilly et al. 1990).

1.3.1.ii. HLA; trans-racial studies.

Recombination events during evolution, including those between the HLA-DQ and -DR loci, have created population-specific combinations of HLA alleles known as 'extended haplotypes'. If an allele of an HLA locus is directly involved in disease susceptibility, then its effects should be evident irrespective of the other alleles present on the haplotype. Extended haplotypes allow stringent testing of HLA associations with type 1 diabetes and are the basis of trans-racial studies.

Using RFLP analysis, Fletcher et al. described a race-specific HLA-DR7 haplotype that, in contrast to Caucasian DR7 haplotypes, is diabetogenic(Fletcher, Mijovic et al. *1988).* The amino-terminal domainencoding sequences of the HLA-DRB 1 and

-DQB1 alleles on this haplotype are identical to those on Caucasian DR7 haplotypes and the only coding sequence differences are at the HLA-DQA1 locus(Todd, Mijovic et al. 1989). The DQA1*0201 allele of the neutral Caucasian HLA-DR7 haplotype is replaced by DQA1*0301, an allele which is also found in predisposing Caucasian HLA-DR4 haplotypes. The DQA1*0301-DQB1*0201 allelic pair, present on this haplotype, also accounts for the susceptibility of different HLA-DR9 haplotypes: Caucasian DR9 haplotypes are neutral with respect to type 1 diabetes, in accord with the presence of DQB1*0303 which encodes Asp-57; Negro HLA-DR9 haplotypes are positively associated with disease and carry DQB1*0201 which encodes Ala-57(Todd 1990). The combination of DQA1*0301 and DQB1*0201 alleles on the same chromosome is rare in Caucasians; the A3/DQ2 molecule, however, is common in diabetics due to the high frequency of HLA-DR3/4 heterozygotes (DR3 and DR4 haplotypes carry DQB1*0201 and DQA1*0301 alleles respectively). This so-called transcomplementation of HLA-DQ alleles on different chromosomes is one explanation for the increased risk of HLA-DR3/4 heterozygotes(Nepom, Schwarz et al. 1987).

The frequency of HLA-DR2(w15) is decreased in Caucasian type 1 diabetics, indicating that HLA-DR2 haplotypes provide resistance to disease (particularly to susceptibility encoded by HLA-DR4 haplotypes)(Thomson, Robinson et al. 1988). Resistance correlates with a particular allele of HLA-DQB1, designated DQB1*0602, and this allele is associated with protection in all races that have been studied(Khalil,d'Auriol et al. 1990)(Jenkins, Mijovic et al. 1991)(Mijovic, Jenkins et al. 1991)(Cavan, Jacobs et al. 1993). Diabetogenic HLA-DR2 haplotypes have been identified but these show plymorphism at the HLA-DQB1 locus compared with resistance haplotypes(Kohonen-Corish, Serjeantson et al. 1987)(Bach,Rich et al. 1985)(Bohme, Carlsson et al. 1986)(Lundin, Gauderernack et al. 1988). The

Caucasian AZH haplotype, for example, which is positively associated with disease, is the only HLA-DR2 (DRw16) haplotype to have a HLA-DQB1 β-chain allele encoding a non-charged amino acid at position 57.

HLA-DRw6 also correlates with resistance to type 1 diabetes in Caucasians. This haplotype carries the HLA-DQB1 allele, DQB1*0603 and is associated with protection in other populations(Todd, Bell et al. 1987)(Fletcher, Mijovic et al. *1988)*(Fletcher, Odugbesan et al. 1988)(Todd, Mijovic et al. 1989)(Jenkins, Mijovic et al. 1991)(Mijovic, Jenkins et al. 1991)(Cavan, Jacobs et al. 1993). Three HLA-DQA1 alleles have negative associations with type 1 diabetes in Caucasians; HLA-DQA 1*0102 (found on HLA-DR2 and some -DRw6 haplotypes), HLA-DQA 1*0103 (HLA-DRw6 haplotypes) and HLA-DQA 1*0201. The HLA-DQA1*0201 allele occurs on HLA-DR7 haplotypes; this may explain the negative association of HLA-DR7,-DQB1*0303 haplotypes and the neutral effect of HLA-DR7,-DQB1*0201(Cavan, Bain et ***al.*** 1992).

There are two caveats to the interpretation of trans-racial analyses. First, the test assumes that the effect of an allele on disease susceptibility is independent and not influenced by other loci. This assumption has important consequences; for example, studies in Caucasian, North Indian Asian, Negroid and Japanese populations support the contention that HLA-DQA1*0301 is a primary disease susceptibility determinant. A recent report, however, shows no disease association in Southern Chinese(Cavan, Jacobs et al. 1993). If susceptibility alleles are truly independent, this one negative study theoretically excludes HLA-DQA1*0301 from involvement in disease. I have described, however, that the interaction of HLA-DQα- and β-chains is critical in determining antigen presentation by HLA molecules and this indicates that HLA-DQA1 and -DQB1 alleles are not

independent. Indeed, it would be surprising if other modifying factors were not also involved. It is possible that there are non-coding (and perhaps coding) differences between DQA1 and DQB1 alleles on different haplotypes; such polymorphisms might affect gene expression and, therefore, influence susceptibility to type 1 diabetes. The second assumption of trans-racial analysis is that the identical diagnostic criteria are used in the races under study. This requires rigorous attention to clinical detail which is not readily apparent in some studies.

1.3.1.iii. HLA; susceptibility loci other than HLA-DR and -DQ.

The HLA-DPB1 locus, centromeric to HLA-DQ (figure 1.1), is also highly polymorphic. Although disease associations have been reported, there is little evidence that susceptibility to type 1 diabetes is influenced by this locus(Easteal, Kohonen-Cotish et al. ***1990).*** The genes which encode tumour necrosis factor lie telomeric to the HLA-DR genes. ***An*** association between disease and heterozygosity for a TNF-β gene RFLP has been reported in Caucasians but not in other races; it is likely that this association is secondary to linkage disequilibrium with alleles in the class II region(Jenkins, Penny et al. ***1991).***

A number of reports suggest that the disease associations with HLA-B and -DR alleles are more than simply a reflection of linkage disequilibrium (with HLA-DQA1 and -DQB1 loci). When HLA-DR3,-DQB1*0201 haplotypes are sub-divided according to HLA-B alleles, HLA-B8 and -B18 haplotypes are generally found to be most predisposing (Thomson, Robinson et al. ***1988)***(Risch ***1989).*** Since the sequences of HLA-DR3 and DQB1*0201 are the same on all Caucasian haplotypes (although there is some variation at the DRB3 locus), this implies that other loci outside the HLA-DQ region influence susceptibility(Field ***1989)*** (Todd, ***Bell*** et al. ***1987)(Horn,***Bugawan et al. ***1988).*** Similar findings have been reported for HLA-

DR4; haplotypes, such as DR4(Dw14)DQ8 and DR4(Dw4)DQ8 which differ only at the DRB1 locus (Dw subtypes), do not confer the same risk of type 1 diabetes *(Bach, Rich et al. 1985)(Sheehy, Scharf et al. 1989).* Tuomilehto-Wolf et al. have recently claimed that polymorphism at the HLA-A locus also affects haplotypic *risk(Tuomilehto-Wolf, Tuomilehto et al. 1989).*

To summarise these data, certain alleles of HLA-DQA1, -DQB1 and DRB1 correlate directly with susceptibility to type 1 diabetes(Todd *1990)(Sheehy, Scharf et al. 1989)(Caillat-Zwman, Garchon et al. 1992).* The structure of the amino acid at position 57 of the HLA-DQ 0-chain provides a partial molecular basis for this correlation, however, this residue is not sufficient by itself to account for the observed effects and other HLA loci are involved. It is of note, however, that this specific amino acid 'defect' in the HLA-DQ molecule is conserved in the NOD mouse, unique among mouse strains in that it spontaneously develops insulin-dependent diabetes. The precise mechanism by which HLA polymorphisms predispose to disease is not known but probably involves the presentation of self-antigens, in the form of peptides, by class II molecules to T-cells. Certain alleles of HLA-DQ, for example, may present pathogenic epitopes of 0-cell associated proteins whereas other alleles of HLA-DR and -DQ may present nonpathogenic peptides. The balance of these molecular interactions might cause a reduction in susceptibility, or even active protection as observed for HLA-DQB1 **0602* haplotypes in human and for the I-E molecule in NOD.

1.3.2. Non-HLA-encoded genetic susceptibility to type 1 diabetes.

Risch has assessed the contribution of HLA-linked loci to the familial clustering of type 1 diabetes(Risch *1987).* This was done by examining the relationship between

the increased risk of disease in relatives of type 1 diabetics over the population prevalence and the probability of sharing HLA haplotypes identical-by-descent (IBD) (Section 1.5.2). The expected proportion of affected sib pairs sharing zero HLA haplotypes IBD under a single locus model is significantly less than the observed frequency. Risch concluded that the effect of HLA-linked loci on familial clustering of disease is minor and that additional familial factors exist; these may be genetic (non-HIA linked loci) or environmental.

1.3.2.i. The insulin gene (INS) region.

The autoimmune process leading to type 1 diabetes is highly specific to pancreatic β-cells, the only cells which produce insulin. The insulin gene is, therefore, a plausible candidate susceptibility locus since insulin (or insulin precursors) may act as an autoantigen. Alternatively, levels of insulin could modulate the interaction between the immune system and β-cells and ultimately determine whether these cells become a target for autoimmune destruction. It is of note that intensive insulin therapy given at the time of diagnosis can reduce ß-cell loss, possibly by reducing endogenous insulin secretion(Shah, Malone et al. 1989). Further, treatment of prediabetic NOD mice with insulin prevents the onset of disease, further suggesting that exogenous insulin protects β-cells from autoimmune damage(Atkinson, Maclaren et *al.* 1990).

Population studies of type 1 diabetics and non-diabetic controls have shown a positive association between alleles within the INS region and disease(Bell, Horita et al. 1984)(Hitman, Tarn et al. 1985). Linkage analyses in multiplex families, however, have failed to confirm this finding(Field *1991).* This conflict between association and linkage studies has puzzled investigators for some years. Although some were willing to accept the possibility of hidden stratification producing an

anomalous association, others suggested that small susceptibility effects (in comparison to HLA) may be easier to detect by association than by linkage analysis. Possible explanations for the latter hypothesis are that several haplotypes carry diabetogenic mutations(Thomson, Robinson ***et*** al. ***1989)*** and/or the frequencies of these susceptibility alleles in the generai population is high(Todd and Bain ***1992).***

In 1991, Julier et al. claimed to have explained the apparent paradox between association and linkage studies(Julier, ***Hyer et*** al. ***1991).*** They analysed a large group of unrelated subjects with type 1 diabetes and confirmed an association between disease and newly defined polymorphisms across the **INS** region. Direct evidence for linkage of **INS** was then obtained by analysis of affected sibling pairs whose parents were heterozygous for the disease-associated **INS** polymorphisms. Interestingly, in that study, linkage was observed only in male meioses, suggesting involvement of maternal imprinting. In addition, the **INS** association was significant only in HLA-DR4-positive diabetics, suggesting interaction between the HLA and **INS** susceptibility loci.

1.3.2.ii. T-cell receptor *a* and ß chain regions.

T-cells recognise antigen when it is in combination with HLA molecules on the surface of antigen-presenting cells. This recognition is facilitated by the T-cell receptor (TCR), a heterodimeric cell surface molecule made up of an a and ß chain, both with constant and variable regions. Since type 1 diabetes appears to be a T-cell mediated disease, the genes coding for TCR are plausible candidates for disease susceptibility. The loci for TCR a (TCRA) and ß (TCRB) chains are located on chromosome 14(14q11) and **7(7q35),** respectively. Association and linkage analyses of polymorphisms of the gene coding for the TCRA constant region and

type 1 diabetes have been negative(Hoover, Black et al. 1989)(Concannon, Wright et al. 1990). Early association studies of polymorphisms within the TCRB constant region suggested that patients were more likely to be heterozygous at this locus than controls(Hoover, Angelini et al. 1986)(Millward, Welsh et al. 1987). Recent studies, however, using larger populations and linkage studies in families have failed to confirm this observation(Hoover, Black et al. 1989)(Concannon, Wright et al. 1990). It should be noted that these studies are concerned only with susceptibility due to variation in germ-line TCR genes. Somatic diversification mechanisms allow the random addition or deletion of nucleotides at junctions between TCR gene segments, hence the TCR products expressed on the surface of peripheral T-cells are not entirely encoded in the germ line; even monozygotic twins may differ in their repertoire of TCR specificities.

1.3.2.iii. Immunoglobulin gene regions.

The immunoglobulin κ light chain (Km) locus is located on the short arm of chromosome 2(2p12). Early reports that Km(1) was increased in HLA-DR4 diabetics have not been confirmed(Field and McArthur 1987)(Field 1991). The immunoglobulin heavy chain region (Gm) is found on the long arm of chromosome 14(14q32.3). Gm allotypes are serologically defined antigens, located in the constant portion of the y heavy chains of IgG immunoglobulins. There is a good deal of evidence to support associations between Gm allotypes and susceptibility to autoimmune disease, hence the Gm region is a candidate for genetic susceptibility to type 1 diabetes. Studies searching for a direct association between Gm and type 1 diabetes have produced negative results, as have linkage studies(Field 1991). Field, however, has proposed that genes encoding Gm allotypes, or in linkage disequilibrium with them, may contribute to susceptibility through interactions with HLA, TCRB and INS(Field 1991). Her findings can be summarised thus:

i) HLA-Gm: HLA-DR3/4 diabetics have an increased frequency of phenotypes or haplotypes negative for Gm(23) compared with non-DR3/4 diabetics or controls.
ii) TCRB-Gm: Diabetics who are negative for Gm(23) have a higher frequency of the TCRB 9.3 kilobase fragment, compared with those diabetics who are positive for Gm(23) or controls (an association independent of HLA).
iii) INS-Gm: Diabetics who are not Class 1/1 homozygotes at the **INS** VNTR have decreased frequency of G2m(23) compared with Class 1/1 homozygote diabetics and non-diabetic siblings (an effect which is stronger in DR3/4 individuals).

The evidence for these interactions is often based on small numbers of subjects taken from pooled data sets. In addition, the number of tests performed (without a priori reason) leads to questions concerning an acceptable level of significance.

1.3.2.i~.Candidate genes from comparative mapping.

The best evidence for the existence of susceptibility genes outside HLA and **INS** comes from genetic analysis of the NOD mouse(Todd, ***Aitman et al. 1991).*** The NOD mouse is an inbred strain (i.e, individuals within a colony are essentially genetically identical) that spontaneously develops diabetes which is very similar to human type 1 disease. Autoimmune islet-cell destruction is **a** shared characteristic, as are the appearance of autoantibodies to β-cell components and other endocrine tissues, defects in T-cell activity, sensitivity to immunosuppression and the presence of a susceptibility locus within the MHC. Comparative mapping of the mouse and human has revealed extensive regions of homology. Prediction of the location of disease loci in man, given their location in mouse, is a potentially powerful application of the mouse/human comparative map ***(Nadeau 1989).***

A linkage map of the mouse genome has been generated using the polymerase chain reaction (PCR) to amplify microsatellite DNA markers(Love, ***Knight et al. 1990).*** Microsatellites are blocks of simple repetitive **DNA** (di-, tri- and tetranucleotide repeats) possessing length polymorphism that can be resolved on agarose or acrylamide gels, without the need for restriction enzyme digestion or radio-labelling ***(Weber and May 1989)(Love, Knight et al. 1990).*** Analysis of the eo-segregation of these randomly dispersed markers and disease in outcross and backcross experiments has led to the discovery of three significant linkages in the NOD mouse(Todd, ***Aitman et al. 1991)(Cornall, Prim et al. 1991).*** Idd-3 is located on mouse chromosome **3** (in mouse, the **MHC** susceptibility gene complex is Idd-1 and a putative locus on chromosome 9 has been designated Idd-2), Idd-4 on chromosome 11 and Idd-5 on chromosome **1.** Syntenous conserved regions in the human are located on chromosomes 1 or **4, 17** and **2** respectively and are candidate susceptibility regions for human type 1 diabetes/(Todd ***and Bain 1992).*** Of these, the region of chromosome **2q** homologous with the location of Idd-5 is of specific interest since two potential candidate genes reside within this area; the Interleukin-1 receptor gene ***(Il-1r1)*** and ***Lsh/Lty/Bcg*** which influence macrophage activation and resistance to infection(Cornall, Prim ***et al. 1991).***

The comparative mapping strategy has already been used to investigate the human homologue of Idd-2 ***(Prochazka, Leiter et al. 1987).*** Data from the NOD mouse suggest that the Thy-1 gene on mouse chromosome 9 is linked to diabetes susceptibility at a recombination distance of **10-**15%. In humans, the Thy-1'locus lies in a syntenic conserved linkage group on chromosome **11q.** Eighty one affected sib-pair families (40 from the collection described in this thesis) were analysed with 17 marker loci for linkage to type 1 diabetes(Hyer, ***Julier et al. 1991).*** There was no evidence to support the existence of a susceptibility locus within the region and it

was possible to exclude more than 90% of the region, assuming the probability of sharing two alleles IBD at a susceptibility locus was at least 50%.In the same year as this study, Wong et al. published an RFLP analysis of the CD3 epsilon *(E)* locus(Wong, ***Moore et al. 1991).*** This gene codes for the TCR-CD3 complex E chain and is located on chromosome 11q23, "in the vicinity" of the human Thy-1 locus. Although the authors found no association between this locus and type 1 diabetes overall, they did report a significant difference between the frequency of the CD3ε 8 kilobase allele in male and female subjects (0.268 vs 0.43; $p_c<0.02$) and between female diabetics and controls (0.43 vs 0.267; $p<0.015$). This led to the suggestion that a gene residing on chromosome 11q23 might cause susceptibility to type 1 diabetes in women and subsequently led to the designation of CD3ε as human 'IDDM2' by the Eleventh International Workshop on Human Gene Mapping ***(Junien and van Heyningen 1991).*** This assignment was particularly surprising given the conflicting results concerning this chromosomal region, the lack of a priori reason for a sex difference in disease association and the well-described association between **INS** and type 1 diabetes, first reported in 1984 ***(Bell, Horita et al. 1984).***

In summary, there is prodigious evidence that HLA genes are associated and linked to type 1 diabetes although the precise localisation of all of these susceptibility loci has yet to be defined. Irrespective of the number of HLA loci, family studies indicate that genes within the HLA region account for only a (minor) part of the disease clustering; other unlinked loci and/or environmental factors are implicated The **INS** region has been consistently associated with disease but only one family study has shown evidence for linkage; in that study maternal imprinting and an interaction with HLA(-DR4) had to be invoked to provide evidence for **INS-**

encoded susceptibility. Evidence supporting other candidate loci is weak, requiring confirmation in independent data sets.

1.3.3. Environmental factors.

Environmental factors may act at any time from conception until the moment of diabetes-onset and could both promote and prevent disease. Apart from twin studies, evidence that the environment plays an important role in the development of human diabetes includes reports describing a seasonal pattern of disease-onset (Ludvigsson and Afoke 1989) and the increasing incidence of type 1 diabetes in several countries (Joner and Sovik 1991)(Tuomilehto, Rewers et al. 1991)(Metcalfe and Baum 1991). Studies perporting to show effects of the environment, however, require close scrutiny. For example, geographical variation in the frequency of type 1 diabetes correlates more convincingly with HLA-linked determinants than average yearly temperature (Diabetes Epidemiology Research International Group 1988)(Dorman, LaPorte et al. 1990). Similarly, caffeine exposure is only one of a multitude of environmental and genetic differences between high risk Scandinavian populations and low risk groups elsewhere (Tuomilehto, Tuomilehto-Wolf et al. 1990)(Bain, Rowe et al. 1990). These concerns emphasize the likely interactive relationship between the environment and genetic factors. This contention is supported by reports that ethnic groups living in the same area, with similar healthcare and diet, may have significantly different frequencies of diabetes (Gay, Hamman et al. 1989). Also, the frequency of type 1 diabetes can increase in immigrants migrating from low risk countries to high risk ones (Diabetes Epidemiology Research International 1987).

1.3.3.i. Viruses.

A great deal of interest has focused on viral infections as "triggers" of the autoimmune process that ultimately leads to ß-cell destruction and there is now a large body of **data** concerning this topic(Todd *1991).* Viral infections can cause mononuclear infiltration of islets and be directly cytotoxic to β-cells, especially in the setting of overwhelming neonatal infections ***(Jenson, Rosenberg et al. 1980).*** In a case described by Yoon et al ***(Yoon, Austin et al. 1989),*** Coxsackie **B4** virus isolated from a child's pancreas at postmortem produced insulitis and diabetes when inoculated into mice, implying that the child's diabetes was virally induced.

Post-mortem histological studies of recently diagnosed cases, however, do not support the contention that rapid cytopathic viral infection is a major cause of ß-cell destruction. Persistent viral infections are more likely to be involved, given the long prodromal phase of type 1 diabetes. Viruses known to infect β-cells include rubella, cytomegalovirus (CMV) and Coxsackie virus ***(Jenson, Rosenberg et al. 1980).*** Congenital rubella is the foremost example of an intra-uterine infection causing diabetes indistinguishable from the sporadic type 1 disease; insulin-dependent diabetes is seen in 12-20% of cases of congenital rubella, has an age-at-onset in the mid-teens and **is** associated with HLA-DR3 and -DR4 ***(Ginsberg-Fellner, Witt et al. 1984)(Mensen, Forrest et al. 1978).*** Congenital rubella, however, cannot be involved in many sporadic cases since vaccination regimes in the UK, and elsewhere, have dramatically reduced the incidence of intra-uterine exposure.

Recently, two reports described an experimental model of diabetes in mice which offers a plausible mechanism for viral-related autoimmune destruction of β-cells in

humans ***(Ohashi, Oehen et al. 1991) (Oldstone, Nerenberg et al. 1991).*** Mice, which were not diabetes-prone, were made transgenic for a lymphocytic choriomeningitis virus (LCMV) gene, under control of the β-cell specific promoter of the rat insulin II gene. As a consequence, viral protein was selectively expressed by β-cells, however, the mice did not become diabetic unless they were infected with LCMV. The conclusions were that β-cells could express an exogenous antigen but were not a target for T cell cytolytic activity because other 'immune signals' were lacking. LCMV infection provided these signals, resulting in autoimmune destruction of β-cells and diabetes. In man, one could postulate that individuals become infected with a ß-cell tropic virus which persists without substantial viral or immune cell-mediated cytopathic damage. In the presence of type 1 diabetes susceptibility HLA molecules, immune cells may slowly infiltrate the islets, producing a low-grade insulitis which may resolve over time. Overt disease will only be precipitated if there is an additional viral infection during a critical phase or "window" in this process. This proposal is consistent with experimental evidence that viruses encode protein epitopes that are immunologically cross-reactive with ß-cell epitopes and reports that ***MZ*** twins who are discordant for disease after 5 years are likely to remain ***so.***

Hypotheses concerning persistent viral infection of β-cells *can* be rigidly tested by using highly sensitive PCR methodology. CMV infection is persistent, involves β-cells, and the presence of the virus has been associated with type 1 diabetes ***(Bantvala, Bryant et al. 1985).*** Foulis et al. tested the hypothesis that a persistent non-cytopathic infection of β-cells by CMV is involved in disease aetiology using PCR ***(Foulis 1989).*** They dissected individual β-cells from paraffin wax-embedded specimens taken from recently diagnosed cases: a PCR assay, specific for the

intermediate-early gene of CMV, showed no evidence of infection in any of the 47 diabetics tested(Foulis, McGill et al. *1992).*

It is equally, if not more likely that no single virus accounts for the majority of cases of type 1 diabetes and the effects of viral infection may be non-specific. ß-cell stress, in genetically susceptible individuals with early insulitis ('pre-diabetes'), could precipitate immune destruction of large numbers of β-cells and hence hasten the onset of disease. Any infection could have this effect and there are published reports of increases in mumps, chickenpox, rubella and other common viral and bacterial infections just prior to disease onset (Oldstone, Nerenberg et al. *1991).* Bantvala et al. found an increase in Coxsackie B-specific IgM titres in recently diagnosed type 1 diabetics whilst Pak et ai. have detected CMV genomic DNA more frequently in diabetics compared with controls(Bantvala, Bryant et al. *1985)(Pak,* Chu et *al. 1990).* Puberty and the concomitant increase in peripheral insulin resistance could also have a similar effect on the function of β-cells(Smith, Archibald et al. *1988).* It is, of course, difficult to prove that infections accelerate ß-cell destruction and it is possible that their apparent increase in the pre-diabetic period reflects the immunocompromised state (a consequence of mild to moderate hyperglycaemia).

The hypothesis that environmental factors (particularly infections) protect against the development of type 1 diabetes is novel and has a number of attractive features *(Todd 1991).* The increased incidence of childhood-onset disease in many countries has occurred against a background of widespread vaccination programmes and improved health care. Paralytic poliomyelitis affords a precedent for the serious long-term consequences of delayed exposure to an common infectious agent. The most powerful support, however, for a protective role of the environment comes

from the NOD mouse. The cumulative frequency of murine type 1 diabetes in inbred, genetically identical NOD mice is 50-90% for females and 10-50% for males, when housed under specific-pathogen free (SPF) conditions. Therefore, as in human MZ twins, possession of all the susceptibility alleles necessary for type 1 diabetes is not sufficient to cause overt disease in all animals; agents present in the animal house must affect the penetrance of susceptibility determinants. Two groups have shown, however, that if SPF NOD mice are Caesarean-derived, and then bred under germ-free conditions, the frequency of diabetes increases to 100% for females and 70% for males(Wicker, Miller et al. 1987)(Suzuki, Yamada et al. 1987). Similar results are also seen in caesarean derived BB/Wor diabetic rats(Like, Guberski et al. 1991).

Conversely, deliberate and accidental viral infections have been shown to reduce disease frequency. Most strikingly, in one colony, diabetes resistance correlated with high titres of antibodies to mouse hepatitis virus (MHV): after exposure to MHV, the disease frequencies were 36% and 9% (females and males respectively) compared to 95% and 60% in the MHV-negative progeny of caesarian-derived mice(Wilbertz, Partke et al. 1991). The implications for murine type 1 diabetes are that endogenous factors are sufficient to cause disease. Any causative role for viruses in this setting would, therefore, be restricted to endogenous, inherited retroviruses. To date, there is no firm evidence of the participation of a retroviral sequences in diabetes aetiology, however, endogenous retrovirues are common, stably integrated in both mouse (and human genomes) and can express proteins. Furthermore, immunological cross reactivity has been demonstrated between the retroviral antigen p73 and insulin (a candidate ß-cell autoantigen) and retroviruses have been shown to influence the T-lymphocyte activity(Serreze, Leiter et al. 1988)(Choi, Kappler et al. 1991).

Other environmental manipulations also protect against diabetes in the NOD mouse and BB rat; for example, a single injection of Freund's adjuvant or BCG given early in life(Sadelain, Quin et ***al.*** 1990), immunoregulation by cytokines such as interleukin 1 (Il-1) and Tumour necrosis factor (TNF-α)(Jacob, Aiso et *al*, 1990), neonatal stimulation of β-cells(Buschard, Jorgensen et *al.* 1990) and subcutaneous or oral insulin(Atkinson, Maduren et *al.* 1990)(***Zhang,*** Davidson et ***al.*** 1991).

There is little direct evidence to support a protective role for the environment in human type 1 diabetes. Indeed, the studies which point to such an effect can be interpreted in a number of ways. For example, a Finnish study of newly diagnosed type 1 diabetics found significantly lower levels of IgG antibodies to mumps virus in patients compared to non-diabetic siblings and controls(Hiltunen, Hyoty et *al.* 1991). Antibodies to CMV, Coxsackie **B4** and rubella viruses were equally distributed in the three groups. One interpretation of this result is that IgG antibodies to mumps protect against diabetes, however, an alternative explanation is that pre-diabetic individuals may be unable to mount an appropriate antibody response, permitting persistence of the virus and hence, accelerated ß-cell destruction.

1.3.3.ii. Diet.

Reports of social risk determinants for childhood diabetes(Christau, Kromann et *al.* 1977)(Blom, Dahlquist et ***al.*** 1989) indicate that lifestyle behaviour, such as eating habits, may contribute to disease onset. In one study, mean consumption of milk was correlated with the yearly incidence of type 1 diabetes (Scott 1990). Others have shown an association between short duration of breast-feeding and type 1 diabetes (Blom, Dahlquist et *al.* 1989)(Borsch-Johnsen, ***Mandrup-Poulson*** et ***al.*** 1984)(Mayer, Hammar et ***al.*** 1988) suggesting that the early introduction of cow's

milk proteins may be a **risk** factor for disease. This hypothesis is supported by reports that diabetes does not occur in diabetes-prone Bio Breeding (BB) rats reared on a diet free of cow's milk for the first two-to-three months of life (Elliott and Martin 1984)(Daneman, Fishman et al. 1987).

The whey protein, bovine serum albumin (BSA), is a candidate molecule for the 'environmental trigger' which leads to type 1 diabetes for the following reasons: early induction of tolerance to BSA prevents diabetes and immunisation accelerates it in the BB rat(Martin, Trink et al. 199l)(Glemm, ***Robinson*** et al. 1989)(Dusch, Karjalainen et al. 1992); serum concentrations of anti-BSA antibodies are significantly higher in diabetic rats than in controls(Beppu, Winter et al. 1987); anti-BSA antibodies are present in patients with type 1 diabetes(Martin, Trink et al. 1991). Anti-BSA antibodies bind to p69, a pancreatic ß-cell surface protein that is inducible by interferon gamma; this led Martin and colleagues to propose that a BSA-induced, p69 cross-reactive immune response could be the link between milk protein and ß-cell autoimmunity (Martin, Trink ***et*** al. 1991). BSA differs from human albumin in a 17 amino acid sequence extending from position 152 to 168 of the BSA molecule, termed ABBOS. Karjalainen et al. have analysed anti-BSA and anti-ABBOS antibodies in 44 type 1 diabetics at diagnosis, after 3-4 months and again at 1-2 years(Karjalainen, Martin et al. 1992). All the diabetics had elevated concentrations of **IgG** (and IgA) antibodies to BSA compared with controls, the majority of which were specific for ABBOS. Antibodies to other milk proteins were not increased. Antibody levels declined after diagnosis (especially those with anti-ABBOS specificity), reaching normal levels within two years. The authors concluded that patients with type 1 diabetes have immunity to cow's milk albumin, with an antibody capable of cross-reaction with a β-cell-specific surface protein. Whilst it is possible that such antibodies could be central in ß-cell destruction, an

alternative hypothesis is that there are multiple antibodies to β-cell antigens present at the onset of type 1 diabetes (a consequence of β-cell destruction, rather than it's cause) and that homology of the p69 antigen and BSA is clinically irrelevant(Chideckel 1992).The 'diabetes-triggering role' of BSA in animal models suggests that such scepticism is not warranted, however, a recent study does not support the earlier findings.

Dahlquist et al examined sera from 116 recent-onset diabetic children and 112 age- and sex-matched controls for islet cell antibodies (ICA), cow's milk protein IgA, IgG and IgM antibodies and β-lactoglobulin IgA and IgM antibodies (β-lactoblobulin is another protein present in cow's milk but not human, which has been shown to be highly antigenic) (Dahlquist, Savilahti et al. *1992).* Antibody titres were compared to questionnaire data on the duration of breast-feeding and introduction of formula feeds. They found that most antibody levels were increased in the diabetics and that the differences in both cow's milk and β-lactoglobulin antibodies were most pronounced in early onset disease. If these antibodies are a non-specific reaction to β-cell destruction, then one would expect a correlation with titres of ICA. This was indeed the case for cow's milk proteins, however, β-lactoglobulin IgA antibodies were independently related to risk, favouring a more specific and independent relationship between these antibodies and type 1 diabetes.

Given the central tenet of the above studies, one might expect milk intake to be high in children who go on to develop diabetes. In fact, there is a report of a decreased frequency of milk intake among diabetic compared to control children who were questioned about frequent intake of food and milk prior to disease onset (Dahlquist, ***Blom*** et al. *1990).*

1.4. Prospects for prevention of type 1 diabetes.

The study of type 1 diabetes is directed towards two major goals; better treatment for those with overt disease and prevention of type 1 diabetes in those at **risk.** Although improvements in treatment have been made in recent years (for example, methods of insulin delivery, glucose monitoring, retinal laser therapy), diabetics continue to be at risk of disabling and life-threatening complications. In addition, the incidence of new cases of disease appears to be increasing. In this setting, the prevention of type 1 diabetes would have a major impact in both human and economic terms.

As described in Section **1.3,** the aetiology of type 1 diabetes is incompletely understood. Genetic susceptibility is necessary for the development of disease but **MZ** twin studies indicate that overt disease is unlikely, even in the presence of a full complement of susceptibility alleles. Environmental factors are, therefore, required for the phenotype to be expressed and these have yet to be identified. It is also likely that the process which culminates in type 1 diabetes is protracted and may remit without causing overt disease. Given this background, it is feasible that type 1 diabetes could be prevented by manipulation of the environment before ß-cell damage has occurred or by therapeutic intervention at an early stage of ß-cell dysfunction. This will require both a knowledge of the environmental factors important in disease aetiology and an ability to detect those at high **risk** of disease.

Whilst it is possible that large epidemiological studies alone will detect important environmental influences, the odds are heavily stacked against such an outcome. Even in those countries with the highest incidence of disease, massive numbers of

individuals need to be prospectively studied for each new case of type 1 diabetes. Furthermore many, if not all, of the study population will have been exposed to the 'diabetogenic' environment and yet will not develop disease since they lack the necessary high-risk genotype. **If** the environmental influences are multiple, protective, intra-uterine (or a combination of all three) then the order of difficulty increases by many magnitudes.

This highlights the importance of identification of individuals at high risk of disease. **A** cohort of high-risk individuals, identified before any indication of disease pathogenesis (ideally at birth), would facilitate the prospective study of environmental influences on diabetes development. in such a group, there exists the potential for early diagnosis of type 1 diabetes, hence this cohort would also be a reagent for testing new therapeutic approaches. To date, strategies of risk assessment have used serological markers and metabolic tests in first-degree relatives of known diabetics; this combination has been employed to attain a high specificity of prediction, so as to limit the use of potentially harmful interventions to those at highest risk. Since over 90% of new type 1 diabetics have no immediate family history of disease, screening measures will have to be successfully applied to the general population to make a significant impact.

1.4.1. Humoral markers of type 1 diabetes.

Antibodies to islet cells were first detected in patients with polyendocrine disease(Bottazzo, Florin-Christensen et al. ***1974)***(MacCuish, Irvine et al. ***1974).*** Subsequently, it was found that antibodies to several islet cell components are present in 70-80% of newly diagnosed cases of **type** 1 diabetes(Landin-Olsson, Karlsson et al. ***1989).*** Islet cell antibodies **(ICA)** are detected by an indirect

immunofluorescencetechnique and the antigen to which they are directed remains unknown. Studies of fist-degree relatives of type 1 diabetics have shown that ICA can be present several years before disease-onset, however, antibody levels may fluctuate and are not an absolute indicator of future disease.

Despite technical problems with ICA measurement(Bonifacio,Boitard et al. 1990), current models of diabetes prediction are based on this marker. ICAs are detectable in 5-8% of first-degree relatives and new familial cases of diabetes are concentrated in this ICA-positive group. The risk of disease is approximately proportional to the ICA titre, hence 5% of first-degree relatives with ICA between 4-19 Juvenile Diabetes Foundation Units (JDFU) require insulin within 5 years, compared with 35% of those with ICA >20 JDFU(Bonifacio,Bingley et al. 1990)(Riley,Maclaren et al. 1990). In this latter group, approximately 60-70% will require insulin within ten years although progression to diabetes is not inevitable in the remainder ***(McCulloch,*** Klaff et al. 1990).

The predictive value of ICA can be further refined by the staining pattern of the antibodies(Gianani, Pugliese et al. 1992)(Genovese, Bonifacio et al. 1992)(Timsit, Caillet-Zucman et al. 1992). It has been demonstrated that some ICAs stain predominantly β-cells within islets ('β-cell restricted ICA') and these are associated with a lower risk of diabetes than ICAs which stain all types of islet cells ('nonrestricted ICA'). Since β-cell restricted ICA are responsible for immunofluorescence staining in ~25% of ICA-positive first-degree relatives, a proportion of false positives may be eliminated by sub-division of the antibody response. Bingley et al. have calculated that the effect on a 35% risk of progression over 5 years for relatives with ICA >20 JDFU would be marginal (rising to only

49%), however, at the end of 10 years >90% would be expected to develop diabetes(Bingley, Bonifacio et al. *1993)*.

Two other autoantibodies of importance in risk prediction have been identified; insulin autoantibodies (IAA) and autoantibodies to an islet cell antigen of 64,000 molecular weight **(64-kD).** IAAs react specifically with insulin and can be detected before exogenous insulin therapy has been commenced(Palmer, Asplin et al. *1983)*. They appear to have little prognostic significance in the absence of ICA but the combination of these antibodies is highly predictive. In the study of Ziegler et al., for example, family members positive for IAA with ICA >40 JDFU had a 77% risk of diabetes within 5 years, compared with 42% for those with ICA >40 JDFU alone(Ziegler, Ziegler et al. *1989)*. It is of interest that IAAs are inversely related to age, highest levels being reported in children below the age of five years at diagnosis(Vardi, Ziegler et al. *1988)*. This has led to the suggestion that IAAs may appear early in life in susceptible individuals and then slowly disappear over time. Alternatively, this finding might indicate heterogeneity of type 1 diabetes (Section 2.1.4).

The 64-kD autoantigen is defined by the ability of diabetic serum to immunoprecipitate a 64,000-Mr ^{35}S labelled islet cell protein (Baekkeskov, Landin et al. *1987)*. This protein has recently been identified as glutamic acid decarboxylase (GAD), an enzyme expressed in brain and pancreas which is responsible for the synthesis of γ-aminobutyric acid(Baekkeskov, Aanstoot et al. *1990)*. Antibodies to the 64-Kd antigen and to GAD are found in ~80% of relatives studied before diagnosis(Atkinson, Maclaren et al. *1990)*(Barmeier, McCulloch et al. *1991)* and they may persist for a longer period than ICA(Christie, *Daneman* et al. *1990)*. As in the case of ICA, distinct specificities of these antibodies has been

demonstrated(Christie, Vohra et al. *1990).* Antibodies to a 50,000-Mr tryptic fragment are found in most type 1 diabetics; these antibodies are, however, also present in 15% of **MZ** twins who remain discordant for disease and in ICA-positive plyendocrine patients who do not develop diabetes. This has led to the suggestion that GAD may be the autoantigen of the ß-cell restricted ICA, with low **risk** of progression to disease. In contrast, antibodies to 37,000/40,000-Mr tryptic fragments, distinct from GAD, are found in the majority of type 1 patients and correlate well with nonrestricted ICA. These are found in only 2% of discordant twins who have not developed diabetes(Christie, Tun et al. *1992).* To date, large prospective studies have not been performed using these markers and their prognostic utility is not known.

1.4.2. Metabolic tests of β-cell function.

Immunological markers identify those subjects at high **risk** of autoimmune β-cell damage but give no indication as to the stage of this process. Direct tests of ß-cell function are an obvious (and essential) measure of target organ damage in type 1 diabetes. Unfortunately, the available methods are imprecise and there is difficulty in extrapolating from functional abnormalities to an estimation of **ß-cell** mass.

1.4.2.i. Glucose tolerance.

Prospective follow-up of first-degree relatives of type 1 diabetics has shown progressive or intermittent hyperglycaemia many months before the development of overt disease. Indeed, oral glucose tolerance tests diagnostic of diabetes or impaired glucose tolerance (IGT) have been described up to ten years prior to the development of symptoms(Rosenbloom, Hunt et al. *1982)*(Millward, Alviggi et al. *1986).* Abnormalities of glucose tolerance are relatively common in the latter stages

of the prodrome of diabetes; one analysis showed raised random blood glucose levels or IGT in 11/15 ICA+ individuals who later developed diabetes(Tarn, Smith et al. 1987). Using results from intensively studied first-degree relatives with ICA >40 JDFU, the Joslin group described a progressive rise in fasting and 60 minute plasma glucose levels in the intravenous glucose tolerance test (IVGTT) during the 18 months prior to overt diabetes(Bleich, Jackson et al. 1990). A fasting plasma glucose >5.9mmol/l had a positive predictive value of 100% for overt diabetes within 18 months, however, the 60 minute glucose value did not discriminate so well.

1.4.2.ii. Insulin secretion.

The insulin response to an intravenous glucose load, as measured by the IVGTT, is the most widely employed measure of β-cell function used in the prediction of type 1 diabetes. A progressive decline in FPIR prior to diabetes has been shown in prospective twin and family studies(Srikanta, Ganda et al. 1983)(Chase, Voss et al. 1987)(Vialettes, Mattei-Zevaco et al. 1988). It was initially suggested that this decline was linear and an individual's time to diabetes could be estimated on the basis of two tests(Srikanta, Ganda et al. 1984). Unfortunately, the IVGTT has proved to be highly variable both within and between subjects and there are major changes in the insulin response during puberty (Smith, Tarn et al. 1988)(Smith, Archibald et al. 1988). This makes interpretation of changes within the normal range extremely difficult and has limited the application of this finding in individual subjects.

Loss of first phase insulin secretion (usually defined as below the first centile) is claimed to be highly predictive of imminent type 1 diabetes in subjects with detectable ICA. In the largest published series, 1169 family members were screened

for ICA and 66/71 of those with detectable ICA had an IVGTT(Chase, ***Voss et*** al. ***1987).*** Of those subjects with a response below the first centile (sum of 1 minute and three minute insulins <46mUnits/l), 7/16 needed insulin within 2 years. The test was most useful in predicting diabetes in children where 7/10 developed diabetes within 2 years. The positive predictive value of the test was improved by using a lower threshold; below 25mU/I insulin response, 7/7 children developed diabetes within one year and 5/7 were diabetic within one month. The short time lapse between loss of FPIR and the need for insulin therapy implies that that the β-cell mass is only slightly greater than at diagnosis; benefit from intervention at this stage may therefore be marginal.

1.4.3. Combinations of humoral markers and metabolic tests in families.

Elevated titres of IAA and loss of FPIR are each strongly predictive of rapid progression to type 1 diabetes in high-titre ICA-positive relatives. A preliminary report indicated that a model based on combined analyses gave highly specific prediction of end-stage prediabetes, with progression in 90% of high-risk individuals within 4 years(Colmanand Eisenbarth ***1988).*** Five years after it's initial description, this 'dual parameter model' has yet to be validated. Further, investigators have described patients with low insulin secretory responses who have remained stable or improved(Bingley, Bonifacio ***et*** al. ***1993).*** The proper evaluation of various combinations of disease markers is still awaited.

1.4.4. Assessment of risk of type 1 diabetes in the general population using serological and metabolic markers of disease.

Bingley et al. compared the prevalence of **ICA** and the **risk** of diabetes in approximately **3,000** schoolchildren, aged 9-13 years, and 272 age-matched non-diabetic siblings of type 1 diabetics(Bingley, Bonifacio et al. ***1993).*** **ICAs** were detected (threshold of 4 JDFU) in 2.8% of schoolchildren and 6.6% of siblings. Since incidence **data** suggest that 0.2% of these children develop diabetes within 10 years, compared with 2.8% of the siblings, siblings were only twice as likely to have a given titre of **ICA** than healthy children, but are 14 times more likely to develop diabetes. The predictive value of **ICA** in the general population will fall, therefore, to approximately one-seventh of that in siblings. This estimate is consistent with prospective **data** from siblings and schoolchildren in Finland (Knip, Vahasalo et al. ***1992).*** The correction factor implies that progression to type 1 diabetes in 5 years would occur in 2-5% children with detectable **ICA** and 5-10% of those with **ICA** >20 JDFU. Hence, **ICA** alone will have limited prognostic value in the general population.

The predictive value of **ICA/IAA** in combination with metabolic tests is also significantly reduced if used to screen the general population. When combined with a test of **FPIR,** high titre **ICA** and fluid phase **IAA are** estimated to give a specificity of 99.75% and sensitivity of 60%(Palmer and McCulZoch ***1991).*** In the general population these figures yield a positive predictive value of only 42%; per 100,000 population screened there would be 180 true positives, 120 false negatives and 250 false positives.

Irrespective of their ability to detect cases at high risk of diabetes, serological and metabolic tests are less than ideal for screening, even for the purposes of research studies. Whilst the presence of ICA and **IAA** in cohorts of at-risk individuals correlate with future disease development, in individuals they do not always persist ***(Riley, Maclaren et al. 1990)(Thivolet, Beaufiere et al. 1991)(Ziegler, Alper et al. 1991)(Kuller, Becker et al. 1990).*** To date, the time of emergence and the persistence of the autoantibody response in the general population has not been described. Hence, multiple testing may be required and is a feature of many of the published studies. In addition, the antibody analyses are labour intensive, not easily reproduced and, therefore, unsuitable for large-scale use. Metabolic testing is also time-consuming and labour-intensive whilst IVGTTs are too invasive to be acceptable to the low-risk individuals. Finally, methodological differences between centres have hindered comparison of results from IVGTT studies.

1.4.5. Genetic markers.

For population screening, the use of genetic markers has a number of potential advantages over serological and metabolic testing. The test need only be performed once since the inherited genome is invariant, and this screening could take place at the time of birth. Invasive procedures are not necessary as **PCR** analysis can be performed on umbilical cord blood or buccal cell **DNA** from mouthwashes(Lench, ***Stanier et al. 1988).*** **PCR** methodologies are potentially simple, robust and easily transferred between laboratories. There are also theoretical attractions, in that the identification of genetic markers should lead to the isolation of individual genes and the responsible polymorphisms, so-called positional cloning ***(Collins 1992).*** This will allow insights into the pathophysiology of type 1 diabetes and ultimately lead to new therapeutic approaches.

An often cited limitation to genetic testing is the 36% concordance rate in MZ twins. This implies that the highest predictive power that can be achieved by a genetic approach is a risk of only one-in-the. Indeed, given the likely ascertainment bias in twin studies (twins are more likely to be ascertained and, therefore studied, if both are diabetic), assignment of even a 1-in-3 risk may seem optimistic. Calculations of this type are, however, flawed on at least two counts. Firstly, the penetrance of high-risk genotypes is not known; MZ twins who are HLA-DR3,-DQB1*0201/DR4,-DQB1*0302 heterozygotes may have a higher concordance rate than 36%. This is supported by subdivision of data from Johnston et al. which shows ***>50%*** concordance in (the small numbers **of)** HLA-DR3/4 MZ twins(Johnston, ***Pyke et al. 1983).*** Unfortunately, further HLA typing has not been performed on this cohort (Dr R.D.G. Leslie, personal communication) and I have been unable to obtain data on the Joslin twins.

Secondly, the increasing incidence of type 1 diabetes has important implications for genetic screening. Given the relatively fixed genetic background in the UK Caucasoid population over the past 100 years (at least), the increased number of new cases implies one of three things: 1) more individuals with known genetic susceptibility determinants are developing disease, for example, the penetrance of HLA-DR3/4 has been increased; 2) individuals who do not have currently recognised high risk genotypes are becoming diabetic; 3) a combination of both. This issue has important implications for population screening strategies using genetic markers. If the penetrance of known determinants increases, these markers will become more effective in pin-pointing individuals at high risk. Alternatively, if new cases are developing in a 'genetically low-risk group' then current markers will have reduced power and may become obsolete as screening tools. The available evidence is inconclusive. Kontiainen et al. reported that the frequency of HLA-DR3

in children with type 1 diabetes in Finland was increased in patients diagnosed in the 1960s but fell to background levels in the 1970s and 1980s(Kontiainen, Scheinin et al. ***1988).*** In contrast, HLA-DR4 is reported to be significantly increased in frequency in epidemic cases of type 1 diabetes compared to non-epidemic cases(Ziegler, Alper et al. ***1991).*** Prospective studies of diabetic cohorts diagnosed during different periods will be needed to answer this important question.

These provisos aside, the criticism of genetic screening assumes that genetic markers would be used in isolation from other investigations and this is clearly not the case. Individuals identified as being at high genetic risk would be monitored for evidence of active ß-cell autoimmunity (using a combination of autoantibody tests) and, if positive, be subjected to invasive tests of ß-cell function. In this scenario, invasive investigations are only offered to those individuals at greatest risk (in whom the take-up rate is likely to be highest).

Using PCR and oligonucleotide dot blot analysis the most diabetogenic HLA types can be identified. In a study from Finland, a panel of 4 sequence-specific oligonucleotide probes was used to identify susceptibility and protective HLA-DQ alleles. HLA-DQB1*0302, in the absence of a protective HLA-DQB1 allele, was found in 82% of type 1 diabetic patients compared with 3% of controls, giving a life-time absolute risk of 13.7%(Reijonen, Ilonen et al. ***1991).*** These figures cannot be applied to the UK population since the incidence rates of type 1 diabetes are so different from those in Finland (approximately 14/100,000 versus 30/100,000)(Diabetes Epidemiology Research International Group ***1988).*** Similar large-scale studies have yet to be performed in the **UK** but analyses from other countries suggest that HLA-DQ typing may identify individuals at 6-8%

risk(Sheehy, Scharf et al. 1989)(Todd 1991). This figure can probably be improved since studies have not subdivided HLA-DR3 haplotypes into those conferring high and low risk (Section 1.3.1.iii). It is of note that, even at this low level of absolute risk, HLA screening can identify individuals from the general population with a similar risk to siblings of affected individuals. Given the **MZ** twin data, it is reasonable to assume that identification of additional non-HLA susceptibility loci may allow identification of individuals at 15-20% risk of developing diabetes.

1.5. Identification of new genetic markers for type 1 diabetes.

1.5.1. Technological aspects.

The three main strategies for the identification of disease susceptibility loci are: the candidate gene or gene region approach, random screening (exclusion mapping) of the genome using highly polymorphic probes; and identification of subphenotypes that are genetically less complex than the clinical disorder(Todd and Bain 1992). The analysis of candidate genes has already been successful in the study of type 1 diabetes (HLA and **INS)** and remains the method of choice. Virtually any gene that affects β-cell function or activity of the immune system is a potential candidate. Further, it is possible that genes influencing susceptibility to type 2 diabetes are involved in the development of type 1 disease (Sections 3.1.2.ii and 7.2.1).

An additional and potentially more targeted source of candidate genes are being generated from animal models. The large degree of conservation between mouse and human genomes means that susceptibility gene regions identified in the **NOD** mouse have homologous regions in man(Erkkson 1989). To date, there have been

reports of four susceptibility gene regions outside the **MHC** in the NOD mouse and there is preliminary evidence of at least a further seven (J.A.Todd, personal communication). As genes in the NOD mouse are mapped and functionally characterised, candidate biochemical pathways will also be identified.

The task of testing candidate loci has been greatly simplified by the application of the polymerase chain reaction **(PCR).** Most genes are close to segments of tandemly repeated simple DNA sequences (microsatellites) that are highly polymorphic and can be easily amplified by **PCR.** Microsatellite size variation (down to **2** base pairs) can be resolved using non-denaturing polyacrylamide gels stained with ethidium bromide. This method has recently been successful in demonstrating linkage between a subphenotype of non-insulin-dependent diabetes (Maturity Onset Diabetes of the Young) and the glucokinase gene on chromosome ***7(Froguel, Vaxillaireet al. 1992).*** Large numbers of microsatellite primers have been published and further sequences are available from the EMBL and Genbank data bases. For regions where microsatellites are not available or have low information content, new microsatellite markers can be developed by screening Yeast Artificial Chromosome clones from the genetic region of interest.

1.5.2. Statistical aspects.

The analytical process by which susceptibility genes are identified is known as genetic linkage analysis. Genetic linkage reflects the fact that two genes located near to one another on the same chromosome are not inherited independently. If two loci are in close proximity, alleles at these loci will tend to be inherited together. If they are some distance apart, recombination between homologous chromosomes during reproductive events (meioses) creates new combinations of alleles. The frequency at

which recombination occurs (the recombination fraction), increases with the distance between loci; if they are sufficiently far apart, the probability of inheriting recombinant and parental (nonrecombinant) chromosomes is equal *(0.5)*, the same as when loci are on different chromosomes.

The principle underlying the application of classical linkage analysis to the study of single-gene disorders is straightforward (Morton ***1955)***. If two traits (such as a disease and a genetic marker) are Co-inherited more frequently than is expected by chance, this constitutes genetic linkage. For example, in a pedigree, the probability of observing co-segregation of disease and a **DNA** polymorphism in ten informative meioses will equal $(1)^{10}$ if their loci are very tightly linked. If the two loci are not linked, then the probability of observing complete co-segregation in all ten meioses will be $(0.5)^{10}$, since the probability that two randomly selected genes at unlinked loci will cosegregate is equal to 0.5. **Thus,** if complete Co-segregation is observed, the relative probability that the two loci are tightly linked, as opposed to not linked (the null hypothesis), equals **1:** $(0.5)^{10}$ or 1024 to 1. The $\log_{10}$ of this ratio is known as the log of the odds' or **LOD** score. In general, a positive **LOD** score of **3** (or more) is taken as proof of linkage. The strictness of this criterion reflects the low prior probability (approximately 1 in 50) of linkage between any two randomly selected loci; conditional odds of 1000 to 1, therefore, yield an overall probability of 1 in 20 that the observed cosegregation has occurred by chance (equivalent to the standard "$p<0.05$"). **A** negative **LOD** score of **-2** is taken **as** strong evidence against linkage, in line with the high prior probability against linkage (approximately 49.to 1).

In contrast to the situation in monogenic disorders, in complex diseases such as type 1 diabetes, modes of inheritance, penetrances and gene frequencies are unknown. **LOD** score analyses can be performed, using various genetic

models(Lathrop, Lalouel et al. ***1984)***(Lander, Green et al. *1987),* however, simulation studies show that the chance of falsely obtaining a LOD score of **3** is high(Clerget-Darpoux, Babron et al. ***1990).*** The power of such analyses in complex diseases is further reduced by genetic and clinical heterogeneity (Section 2.1.4), and interactions between loci(Risch ***1991).*** In addition, linkage analysis is hampered in type 1 diabetes since large pedigrees are uncommon.

An alternative method is to restrict the analysis to affected relative pairs, especially affected sibling pairs (ASPs). **ASPs** are more readily available than other relative pairs and, as model specification is not required for analysis, the emphasis is on the detection of susceptibility loci. At any one locus, the Mendelian expectation is that ASP share 0, 1 or 2 alleles identical-by-descent (IBD) in the proportions of 1:2:1. If, however, the allele under consideration is involved in disease susceptibility (or is in linkage disequilibrium with a susceptibility locus) one would expect increased allele sharing in ASPs (more '2-sharers' and fewer '0-sharers'). The simplest method of statistical analysis of these tests is to compare the IBD data with the Mendelian expectations using a χ^2 goodness-of-fit test. The formula used is

$$\sum \frac{(O_i - E_i)^2}{E_i}$$

where ***Oi*** and ***Ei*** are the observed and expected values for *i* alleles IBD and ***i=0,*** 1 and 2. This χ^2 test has two degrees of freedom and a value of 13.8 is numerically equivalent to a LOD score of 3.0.

Similarly, from each individual parent, the frequency that **ASPs** share ***0*** or 1 alleles IBD should be equal unless the allele is involved in disease susceptibility. In this case ASPs will receive the same allele more frequently than not, and the proportion of 1-sharers' would be increased. Again, a statistical comparison can be made with

the Mendelian expectations **(1** : 1) using a χ^2 goodness-of-fit test, this time with one degree of freedom.

A requirement for **ASP** analysis is that the parental origins of marker loci can be unambiguously assigned; this requires that parental types are available for analysis. Since type 1 diabetes is a disease of early-onset, living parents are generally available after the diagnosis of disease in their offspring. **A** potential difficulty in the data generated from **ASPs** is the inherent ascertainment bias since the majority of type 1 diabetics are sporadic cases with no first-degree family history of disease (Section ***1.7).***

The collection of a large number of multiplex families gives adequate power to detect linkage, even with genes of moderate effect, provided that there is a high resolution map with markers of polymorphism information content **(PIC)** ***>0.7(Risch 1990)(Hyer, Julier et al. 1991).*** The number of families required to demonstrate linkage to **HLA** can be calculated from previous studies, however, for new genes, the power of the analysis is dependent upon many variables (for example, magnitude of the gene effect, proximity and **PIC** of the genetic marker, disease and locus heterogeneity), most of which can only be estimated. In practice, the more families that are available for analysis, the higher the chance of success. Minimum numbers of pedigrees can be estimated from (recently published) theoretical evaluations ***(Risch 1990)(Risch 1990)(Risch 1990)(Rich 1990)*** but these targets must be flexible and be revised in line with direct experience from handling data generated by the data set ***(Todd and Bain 1992).***

Some methods of linkage analysis demand that disease-associated markers are first identified by means of population association analysis(McGinnis, ***Spielman et al. 1991).*** Also, where disease susceptibility alleles are common, association studies may be more informative than linkage data (Section 5.1). To facilitate these analyses, large numbers of samples from sporadic cases of type 1 diabetes and non-diabetic controls are required. In addition, this resource allows a comparison of familial and sporadic cases since there is a possibility that the aetiology of disease differs in these two groups (as is the case in Creutzfeldt-Jacob disease). Further, the overall contribution of genes identified by linkage analysis of pedigrees needs to be assessed in sporadic diabetics which account for more than 90% of new cases. Again, the precise numbers of subjects required for these analyses is an estimate given the number of unknown parameters(Cox ***and Bell 1989).***

1.6. Conclusions.

Type 1 diabetes is an important condition. **It** is already common, the incidence of new cases appears to be rising and it is responsible for substantial premature morbidity and mortality. The pathogenesis of disease develops over a prolonged period and is influenced by environmental factors. Prevention ***or*** early intervention during the pre-clinical phase is, therefore, feasible. Identification of high-risk individuals is necessary to facilitate such a strategy and, in the general population, this is best achieved by genetic screening in the first instance. Serological and metabolic markers would be employed in the genetically high-risk subgroup to study environmental factors and to target those for intervention therapy.

Alleles of the HLA-DQ loci assign an absolute risk of approximately 1-in-15 and this risk may be increased by consideration of other haplotypic loci (both class I and II). The identification of additional non-HLA genes is, however, essential to raise the predictive power of genetic markers to levels where primary screening is viable. A co-ordinated approach to the mapping of new susceptibility loci in complex diseases such as type 1 diabetes is now possible. Techniques in molecular biology have advanced to a sufficient level to enable rapid testing of candidate genes and, theoretically, exclusion mapping of the entire human genome.

At the time of commencement of this thesis, many features of type 1 diabetes made it an ideal experimental system to develop approaches to the mapping and characterisation of susceptibility genes for complex disease: the disease is relatively unambiguous to diagnose, thus reducing clinical heterogeneity; families with multiple affected siblings and living parents are potentially available due to the high prevalence and low age-at-onset of disease; the HLA region had already been identified as a susceptibility locus; a considerable amount was known about the immunology of type 1 diabetes, enabling a rationale for choosing candidate genes for analysis; and animal models of disease were available.

1.7. Aims.

The major aim of the work documented in this thesis was to provide a resource for the identification of new susceptibility genes for type 1 diabetes. This was to be made up of three components; multiplex families (nuclear families with living parents and at least two affected offspring), sporadic cases of type 1 diabetes and unaffected control subjects. The initial target number of families required (~150) was calculated to be large enough to identify 'important' gene effects, given the

provisos detailed above (Section 1.6). Samples were also be amassed from at least 200 type 1 sporadic diabetics and *200* ethnically-matched non-diabetic controls.

A secondary aim of this work was to make this resource available for posterity. This can be achieved by Epstein **Barr** Virus (EBV) transformation of peripheral blood lymphocytes into immortalised cell lines. These lines have the potential to reproduce indefinitely and *can* be stored in a viable state under liquid nitrogen. They represent **,** therefore, a limitless supply of **DNA** for study and allow the resource to be used by other groups. This means that an extensive data base can be established on one re-usable facility, limiting duplication of effort. In theory at least, this should accelerate research activity in the field of genetics of type 1 diabetes.

Although the idea is by no means new, the scale of this project was novel. Clearly, for such a resource to be accepted by the scientific community, the validity of the project must be beyond question. Validation can be considered in a number of ways: validation of the concept of family studies identifying genes important in a mainly sporadic disease necessitates a detailed comparison of multiplex and sporadic cases(Bingley ***and Gale 1991);*** validation of the family collection requires confirmation of the known genetic involvement of **HLA** in type 1 diabetes; and validation of the resource implies exclusion of sample mix-ups and cases of non-paternity (using **HLA** markers). Validation of the project formed the third aim of my work.

Finally, I set out to make a contribution to the current knowledge of genetic susceptibility by: 1) examination of the controversy surrounding the involvement of the insulin gene locus and susceptibility to type 1 diabetes in this independent

data set; 2) assessment of published reports of parental effects on HLA-mediated susceptibility to disease; **3)** analysis of new candidate gene loci.

CHAPTER 2

THE GENETIC RESOURCE

2.1. Collection of multiplex families with type 1 diabetes.

2.1.1. Ethics.

The collection of multiplex families began in February, 1989 after approval had been granted from the local Ethical Subcommittees of both the East Birmingham Hospital, Birmingham and the John Radcliffe Hospital, Oxford.

2.1.2. Funding.

Establishing a large collection of this kind is an expensive undertaking. Unfortunately, there are a limited number of grant-giving bodies with sufficient resources to adequately fund such an enterprise. Furthermore, most of these prefer to support "the science" which is facilitated by such a resource, rather than it's establishment. This attitude has prevailed, despite a consensus among those working on the genetics of type 1 diabetes in the UK, that a multiplex family collection was essential for future progress (outcome of a British Diabetic Association sponsored Workshop on the Genetics of Type 1 Diabetes, 1988). As a result of this situation, funding has been piecemeal and obtained from a number of different sources, acknowledged at the beginning of this thesis. Three months after the collection began, the British Diabetic Association (B.D.A.) made available **part** of a bequest from Alec and Beryl Warren to fund the collection of samples and immortalisation of cell lines; as a result, the collection is known as the British Diabetic Association - Warren Repository.

2.1.3. Steering Committee.

A Steering Committee was established early in the collection (table 2.1). This comprises representatives of the **B.D.A.** and those individuals involved in the ascertainment, collection, validation and immortalisation of the Repository. Initially the committee established the inclusion and exclusion criteria for the resource. Subsequently, the role of the committee has been to oversee financial and organisational aspects of the collection. Currently, the committee is involved in the distribution of cell lines to other laboratories.

2.1.4. Diagnosis of type 1 diabetes.

Linkage analysis of any disease is most likely to be successful if the pedigrees under study have an identical phenotype; accurate diagnosis is, therefore, essential. Diagnosis remains a major problem in the analysis of many complex diseases. For example, the label 'schizophrenia' may be attached to various psychiatric disorders manifesting in young individuals, whilst in hypertension and type 2 diabetes the clinical syndromes are arbitrarily defined extremes of a 'normal distribution'. In contrast, the diagnosis of type 1 diabetes is usually clear-cut: acute symptoms in a child or young adult leading to ketosis and an absolute dependence upon insulin. Phenocopies of type 1 diabetes are also relatively uncommon and can be easily recognised, for example, insulin-dependent diabetes following an overwhelming viral infection or alcohol-induced pancreatitis (phenocopies are environmentally-induced cases of disease with little or no genetic input). Misdiagnosis, due to confusion with type 2 diabetes or maturity-onset-diabetes of the young **(MODY),** may occur but can be distinguished by close questioning; type 2 diabetes usually manifests over the age of 40 years and is accompanied by weight gain; **MODY** may

Table 2.1. Members of the B.D.A. - Warren Repository Steering Committee.

Chairman	Professor Simon Howell	(B.D.A.Research Committee)
	Dr Moira Murphy (M.M.)	(B.D.A. Research Director)
	Dr John A Todd (J.A.T.)	
	Professor Anthony H Barnett (A.H.B.)	
	Dr Bryan Boulton	(E.C.A.C.C., Porton Down)
	Dr Steve C Bain (S.C.B.)	
	Sister Beth R Bain (B.R.B.)	

present in children or young adults but with mild symptoms; neither condition necessitates immediate and continual insulin therapy.

There is, however, evidence which suggests that genetic heterogeneity might exist in type 1 diabetes. Genetic heterogeneity describes a situation where a number of different alleles can cause the same, or very similar, phenotype. This term can be subdivided into allelic heterogeneity, where the alleles are of the same gene, and locus heterogeneity, where the alleles are of different genetic loci. Reports in the literature suggest that type 1 diabetes with onset after adolescence differs from the classical, juvenile-onset disease; the clinical onset is often more gradual, allowing a prolonged period of oral hypoglycaemic therapy(Tarn, Thomas et al. 1988); subjects are less likely to have circulating insulin autoantibodies at diagnosis(Vardi, Ziegler et al. 1988); stimulated C-peptide levels are higher after five years of disease(The DCCT Research Group 1987) and HLA associations may differ and be quantitatively less important(Karjalainen, Salmela et al. 1989)(Caillat-Zucman, Garchon et al. 1992). Histological studies support this contention; in the largest postmortem study, insulitis was invariably present in children aged 3-9 years at death (19/19), but found in only 70% of cases (23/33) aged 10-19 years at death(Foulis, Liddle et al. 1986). A decreased frequency of insulitis with older age-at-onset has also been documented in other autopsy series(Gepts 1965)(Doniach and Morgan 1973)(Junker, Egeberg et al. 1977).

There are two broad approaches to the problem of genetic heterogeneity;

1) the use of extremely stringent inclusion criteria to produce as homogeneous a data set as possible. This will probably limit genetic heterogeneity but greatly reduces the number of pedigrees available for study.

2) the recruitment of vast numbers of pedigrees by including all likely cases. This provides a large data base (in which major gene effects might be detected), with the potential for subgroup analysis using clinical data or new genetic loci. Small gene effects, however, are likely to be missed and it is difficult to exclude loci using such a data set (since negative findings could always be due to admixture).

We chose to use a combination of these two approaches. Our main inclusion criteria applied to the first or youngest diagnosed diabetic in each multiplex pedigree; these were clinical criteria (acute symptoms, weight loss, immediate requirement for insulin) and an age-at-onset of diabetes less than 17 years of age. Requirements for the second (and third) diabetics were less stringent, namely age-at-diagnosis <29 years and no prolonged period of oral hypoglycaemic therapy. Only families satisfying these criteria were to be included in the **B.D.A.** - Warren Repository (hence, the age-at-onset criteria are referred to as '**B.D.A.** age criteria'). In addition, we also collected pedigrees who did not satisfy the above age criteria (but were clinically type 1) when they were keen to participate and in close proximity to other suitable families.

The rationale for these decisions were as follows:

1) The age-at-onset criterion of less than 17 years for the first-diagnosed or youngest-onset diabetic ensured that each family included a 'juvenile-onset' diabetic. The probability of the second, or third, diabetic (aged <29 years at onset) also having type 1 disease would, therefore, be high.

2) The parents of a type 1 diabetic child are very alert to the same diagnosis in other offspring. As a result, the second diabetic is often diagnosed before the stage when insulin (or any other therapy) is required. Also, these cases are unlikely to develop severe symptoms such as weight loss or ketoacidosis before insulin is commenced.

3) Since the age criteria were arbitrary, we also collected other pedigrees who only satisfied the clinical criteria. These additional pedigrees were to allow an analysis of age-at-onset and linkage of HLA to type 1 diabetes (previous evidence of genetic heterogeneity at HLA had come from association studies only). In addition, age-related genetic heterogeneity at HLA does not ordain the same effect at other susceptibility loci.

4) ICA and C-peptide responses were not used as inclusion/exclusion criteria. Although approximately **80%** of type 1 diabetics are ICA-positive at diagnosis, this proportion falls over time. **A** rational use of this investigation would have limited the number of pedigrees to those diagnosed within a certain period of collection. Similar considerations apply to interpretation of the stimulated C-peptide response.

5) Finally, the age-at-onset criteria further reduced the possibility that subjects with early onset non-insulin-dependent diabetes (but treated with insulin) would be included in the data set.

2.1.5. Racial background.

Attempts to establish an ethnically homogeneous group are mandatory, given the reports of differences in HLA associations between racial groups (Section 1.3.1.ii. and ***Jenkins, Mijovic et al. 1990).*** Hence, all diabetics and controls are white Caucasoids with all four grandparents born within the British Isles.

2.1.6. Source of families.

The first family to be collected live in Cowley, Oxfordshire. They were volunteered by **Dr** David Dunger, who directs the Oxford Regional Prospective Study of Type 1 Diabetes (O.R.P.S.). This study has been gathering data on newly diagnosed type

1 diabetic children in Oxfordshire since 1985; those families known to have two diabetic offspring were 'flagged', yielding eighteen sibling pairs for this study. The notes of type 1 patients attending the East Birmingham Hospital diabetic clinic were scanned, in an opportunistic way, for a positive family history of disease and patients directly questioned about affected siblings and offspring. Over a three year period, this yielded 18 participating families.

A plea for suitable families was made to local diabetologists and consultant physicians in the West Midlands (A.H.B). Consultants farther afield were then canvassed by telephone (A.H.B) and face-to-face at scientific meetings (A.H.B, S.C.B). In 1990, all members of the Medical and Scientific Section of the B.D.A. received a 'circular' concerning the project. An announcement was also made at the Annual General Meeting of the Section in Glasgow, stressing that the collection was truly nationwide. Items published in scientific journals(Bain, *Todd* ***et al. 1990)(Bain,Barnett et al. 1991)(Brown 1991)*** and the B.D.A.'s magazine Balance further publicised the Repository(Rowe, ***Bain et al. 1990),*** but still did not directly appeal to patients and their families. This was due to a reluctance of the Steering Committee to be seen to be by-passing the 'medical establishment'. This contrasts with a French collection of diabetic multiplex families, launched by a 'Telephon' event and maintained using Metro advertising. Indeed, the B.D.A.'s low-key approach was such that journalists writing for Balance in 1992 were apparently unaware of the project; when a multiplex family wrote to the Question/Answer section of the magazine asking whether they could participate in research projects, the reply stated that "the B.D.A. is not directly involved in research projects and you should contact you own hospital doctor".

Each new initiative produced a positive response in terms of family referrals, however, this was variable and often short-lived. Presentations to local B.D.A. patient meetings (S.C.B, A.H.B) and diabetes support groups (S.C.B), were also turning up few additional families. In July 1992, the Steering Committee felt that the Repository was sufficiently established to allow a more widespread and direct approach. Dr Peter Swift, Secretary of the British Paediatric Association (B.P.A.) agreed to canvass all the B.P.A.'s members and to print details of the Repository in the Society's newsletter. A further article was published in Balance, for the first time appealing to families to volunteer themselves for inclusion *(Murphy 1992)*.

By February, 1993 I had been in correspondence with 117 consultant physicians and paediatricians throughout the British Isles. This has generated 451 possible families for inclusion. Our procedure for approaching families and collecting samples developed during the first year of collection and has since become standard.

2.1.7. The approach.

Families who volunteer themselves generally make contact with the B.D.A. They receive a reply from Dr Moira Murphy, thanking them for their interest and explaining that their name and address has been passed on to the collecting team. We (B.R.B and S.C.B) then send details of the project with a form for the family to complete and return in a stamped-addressed envelope. This establishes 1) that both parents and diabetics are alive, available and willing to donate a blood sample; 2) the diabetics satisfy the B.D.A. age criteria; 3) the name and address of the diabetics' hospital consultant(s); and 4) an easy contact point with a telephone number. I then write to their consultant explaining that the family have volunteered

for the study and ask that he/she should contact me if they have any objections to their participation; I also enclose details of the project in the hope that they might volunteer other families from the same area. When the families are referred by medical staff, I contact the family by telephone (if possible) prior to sending out the above package. This gives an early indication of those occasions where members of the family have not been forewarned of our approach (or consented to the release of their personal details). Such matters are best dealt with at an early stage and even disgruntled families are eventually keen to help. In only one case has there been the spectre of legal action.

Families who do not satisfy the inclusion criteria are not troubled further. Those who do are contacted by telephone **(S.C.B)** to check details (especially willingness and availability) and to arrange a possible time for collection. I also attempt to make telephone contact with those families who do not reply to our initial contact; this reveals unsuitable families, incorrect addresses and occasionally, families who do not wish to participate.

2.1.8. Sample collection.

Families are bled in their own homes, usually at weekends, by **B.R.B** and myself. Although this has necessitated **a** good deal of travelling, this arrangement has prevailed for the following reasons; 1) patient compliance is good since they do not have to travel, take time from school or work, and children are more easily bled in their own surroundings; 2) the samples are collected in a uniform way and not exposed to the rigours of courier firm travel; **3)** personal contact with the families improves future compliance if further sampling is required or additional clinical details are sought; **4)** the samples do not go missing.

On arrival in the family home, I explain the concept and aims of the B.D.A. - Warren Repository and field the inevitable questions concerning diabetic care. I then take details regarding the clinical diagnosis of type 1 diabetes, confirming to my satisfaction that the clinical and age-at-onset criteria are met. We also record details of any family history of type 1 and type 2 diabetes and enquire of a personal or family history of other autoimmune diseases, specifically, Addison's disease, coeliac disease, myasthenia gravis, pernicious anaemia, rheumatoid arthritis, thyroid disease and vitiligo. Diabetic complications are recorded if volunteered by patients, but are not directly illicited; complications data obtained from patient questioning is unreliable and would not justify the potential distress of raising the subject in young families. Forty millilitres of blood is collected into a 60ml syringe containing 0.5ml preservative-free heparin (Minihep™, Leo Laboratories Ltd.). The syringe, already labelled with the patient's identification number and Christian name (checked by the patient), is capped and kept at room temperature. The average time spent in a family home (irrespective of the number of individuals to be bled) is one hour.

Peripheral blood lymphocytes (PBLs) are separated from 20mls of blood within 48 hours of venesection (J.A.T., S.C.B.) and then stored at *-80°C.* The remaining 20mls of blood are separated into plasma and cells. Plasma is aliquoted into 1ml vials, one of which is sent for thyroid and anti-adrenal antibody screening at the Regional Immunology Unit, East Birmingham Hospital (Professor R.A. Thompson).

2.1.9. Immortalised cell lines.

The frozen PBLs are subjected to viral transformation at the European Collection of Animal Cell Cultures (E.C.A.C.C.) in Porton Down, Wiltshire. Viral transformation is a technique in which infection of lymphocytes with Epstein Barr Virus (EBV) produces 'immortalised cell lines'. These lines can be stored under liquid nitrogen and, under appropriate conditions, will replicate indefinitely; they, therefore, represent a renewable source of DNA from the diabetic families. Using the above procedure, we achieved successful transformation in more than 98% of cases and further venesection is not necessary.

On occasions, we have attempted to use alternative strategies, for example, bleeding performed by local hospital staff or **G.P.s,** use of the Vacutainer™ (Becton Dickinson) collection system and courier firm transport. These have been more expensive (up to ten times more so) and have led to high rates of failure of EBV transformation (approaching 50%)due to poor patient compliance, transport delays etc. In September 1991, a collaboration was established with Professor E.A.M. Gale (St Bartholomew's Hospital, London) to facilitate the inclusion of multiplex families already recruited into the Bart's-Oxford (BOX) study. This prospective study is using serological markers to assess the risk of type 1 diabetes in siblings of new cases. Since the families are under regular review by BOX field workers, it was decided that bleeding should be performed on their routine visits and samples delivered by courier firm or hospital transport. Despite the undoubted dedication of these staff, after eighteen months less than half of the families volunteered have been successfully transformed, highlighting the difficulties of delegation.

2.1.10. Validation.

The aims of validation of the Repository are to ensure the integrity of all families (i.e., exclude non-paternity) and to confirm that there are no sample mix-ups during the process of EBV-transformation. Lymphoblastoid cell lines are serologically typed for HLA class I and II loci in a microcytotoxicity assay by the Oxford Transplant Centre at the Churchill Hospital, Oxford. HLA haplotypes are then determined (S.C.B) to confirm the relationship between parents and offspring. Using standard techniques, DNA is extracted from blood cell pellets and HLA-DR typing is performed using an allele-specific PCR assay for HLA-DR3 and -DR4 (S.C.B)(Patel, ***Lo et*** al. ***1993).*** Comparison of the HLA-DR typing of EBV and blood from the same individual is used to confirm identity of samples.

2.1.11. Progress: February 1989 - March 1993.

Of the 451 family names passed to me for inclusion in the B.D.A. - Warren Repository, I have been unable to contact 95 (0.21) due to incorrect addresses and/or no available telephone number. Of the remainder, 4 have refused to take part and 110(0.24) did not fit the inclusion criteria. In all but eight cases, exclusions were made without visiting the family home. The commonest reasons for exclusion were as follows; unavailable family members (usually deceased parents); parenusibling affected family members only or identical twins; age-at-onset; race; misdiagnosis. It is of note that families were more likely to be excluded if they were volunteered by computer; two large diabetic departments with 'computerised diabetic clinics' volunteered a total of 45 pedigrees of which only ***5*** were suitable.

The need for samples to be processed within 48 hours imposes a limitation to the number of families that can be collected in a given period. In addition, in 64% of the families collected the diabetics and parents lived in separate homes (reflecting the large input of families from adult diabetic clinics). As a result, in only 48% of families have the diabetics and parents been collected during the same weekend period and 22% of families have required three or more visits.

Samples have been collected from members of 187 multiplex pedigrees. A further 70 suitable families have volunteered to take part in the study and are awaiting collection. With the exception of 15 families from the BOX study and one pedigree from King's College Hospital, London, all venesection has been performed by S.C.B and B.R.B. This has entailed more than 18,000 miles travelling within the British Isles. A total of 895 individuals have been bled in various locations including gardens, public houses, newsagents and a nightclub in Shrewsbury. Immortalised cell lines are available from both parents and at least two diabetics in 160 families. This is the largest collection of it's kind in the world.

2.1.12. Cell line distribution

Immortalised cell lines from the first 45 B.D.A. - Warren families were made available to the scientific community in June 1991 at the International Diabetes Federation Meeting in Washington DC. In May 1992, a catalogue of 100 families was released. To date, two laboratories in North America have purchased 'DNA and cell lines and three others are in the process of placing orders. HLA and insulin data amassed for this thesis has been made freely available to researchers using this resource, in the hope that a large collaborative data base will be generated. The

families are also included in Professor ***G.*** Thomson's **HLA data** base (University of California, U.S.A.).

2.1.13. Follow-up of families.

Clearly, this group of families is a unique resource with the potential to generate much useful information. To facilitate future studies, we (S.C.B, B.R.B) have tried to avert a major source of patient dissatisfaction with medical studies, namely lack of feedback. All participating families (and many of those who were excluded) are sent copies of articles about the repository and relevant B.D.A. press releases. The **B.D.A.** (M.M., B.R.B) also send Christmas cards to the families each year. This has already yielded returns, with families informing us of newly diagnosed cases.

2.2. Collection of sporadic type 1 diabetics and controls.

The importance of genes discovered in multiplex families will need to be confirmed and assessed in sporadic cases using population association studies. In June 1990, I initiated the collection of a large number of 'sporadic' type 1 diabetics. These are selected using the same criteria as the proband or youngest diabetic of the multiplex pedigrees i.e. acute onset of symptoms with weight loss, an immediate requirement for insulin and age less than 17 years at diagnosis. Once again, all patients are white Caucasoid with grandparents born within the British Isles. The sporadic diabetics, however, have no affected siblings at the time of bleeding. Patients are being recruited from adult and paediatric clinics in Birmingham and Oxford. In addition, samples have been collected from almost all the diabetics in the O.R.P.S.

To provide a geographically matched group of controls, I approached the Blood Transfusion Centre in Birmingham and Dr T.J. Aitman contacted the Plasma Donor Centre in the John Radcliffe Hospital. Samples have subsequently been collected from these two centres and EBV lines are being transformed. The controls are all aged more than 25 years at the time of bleeding, they are white Caucasoids with grandparents born within the British Isles and they have no siblings with type 1 diabetes. Questionnaires concerning family history of type 1 and type 2 diabetes and other autoimmune diseases are being completed by both the sporadic diabetics and controls.

2.2.1. Progress: June 1990 - March 1993.

Samples have been collected from over 400 sporadic diabetics and 500 control subjects. PBLs have been harvested from almost all of these subjects and I have prepared DNA from 360 diabetics and 340 controls.

2.3. Summary and conclusions.

This chapter describes the successful establishment of a resource which will facilitate the genetic analysis of type 1 diabetes. In contrast to previous collections of diabetic multiplex families, the Repository is larger, ethnically homogeneous and able to furnish laboratories throughout the world with **DNA.** The potential for many groups to work on one data set is already being realised and should facilitate a more rapid accumulation of knowledge. The following chapters describe my own analyses of data generated from this group of pedigrees.

CHAPTER 3

ANALYSIS OF CORE DATA

3.1. Introduction.

Samples have been collected from members of 187 multiplex pedigrees. Of these, basic data on family size, family history of diabetes, and personal and family history of other autoimmune disease is available on **183** families.

3.2. Family size.

In 65 families, the two diabetics are the only offspring. Sixty two families have **3** children (9 with **3** diabetics) and **34** families have four. Twenty families have five or more offspring. The mean number of children is **3.2 (SD1.4). 166** families have two affected offspring ('duplex families'; 0.91), seventeen have three ('triplex'; 0.09) and none have four; indeed, I have not received details of any pedigrees in the United Kingdom with four diabetic children.

3.3. Non-paternity.

HLA and microsatellite analysis of EBV-transformed **DNA** of three individuals from three separate families have suggested non-paternity. On typing the blood-derived **D.N.A.,** it emerged that two of these samples had been mixed up after EBV-transformation. Further cell pellets grown up from the original transformed cell lines have identical types to the blood **DNA,** confirming paternity in these cases. In the third case, typing of the blood **DNA** is also incompatible with family integrity. This individuai is an unaffected family member and has not been included in any analyses; the sample has been excluded from the Repository.

The low rate of non-paternity contrasts with other studies where rates in excess of 10% have been reported. There are a number of reasons that may explain this difference. First, in the various explanations of the project before a visit is arranged, the families are made fully aware that 'inheritance' is being studied. Further follow-up is generally made through the parents who then have the opportunity to avoid participation without arousing suspicion within the family. Second, the incidence of diabetes is such, that a mother would have to be extremely unfortunate to have two offspring from different fathers who both go on to become type 1 diabetics.

3.4. Family history of diabetes.

The number of diabetic offspring, parents and families with a history of diabetes and other autoimmune diseases is shown in table 3.1.

3.4.1. Type 1 diabetes.

In seventeen multiplex families (0.09), one parent is also an insulin-dependent diabetic; this is the father in 14 cases (0.82). In 9/17 cases (0.53), the parent was affected prior to conception of both of the diabetic children (all fathers). The mean age-at-onset of the parents is significantly higher than that of their offspring; 25.1 (SD11.4) years versus 10.3 (8.5) yrs, $p<0.0001$). In only one pedigree is there a diabetic parent (father) and three affected offspring; there are no cases where both parents are diabetic. Including three generations (grandparents, parents, parental siblings, cousins), 46 pedigrees (0.25) reported a family history of insulin-dependent diabetes.

Table 3.1. Personal and family history of diabetes and autoimmune disease in 183 multiplex families.

	Parent	Diabetic offspring	3 generation
Type 1 diabetes	17	-	**46**
Type **2** diabetes	10	-	81
Addison's disease	0	0	1
Coeliac disease	0	0	2
Myasthenia gravis	0	0	0
Pernicious anaemia	0	0	12
Rheumatoid arthritis	9	*5*	38
Thyroid disease	18	10	**45**
Vitiligo	2	1	**4**

Familial clustering of type 1 diabetes is well documented in the literature. Wagener et al. analysed the family histories of 1280 type 1 diabetics from Pittsburgh and reported 57 families with at least one insulin-dependent parent (0.05)(Wagener, Sacks et al. 1982). Chern et al,, documented a similar finding (0.07) in Minnesota, whilst in the Swedish Childhood study of 3503 cases, the proportion of probands with a diabetic parent was identical to this data set *(0.09)(Chern, Anderson et* al. 1982)(Dahlquist, Blom et al. 1989). In that study, there was also a preponderance of diabetic fathers (0.73) and, of the 339 families responding to a questionnaire, 41% had a three generation history of insulin-treated diabetes. Allen et al. studied the families of 194 diabetic probands resident in Wisconsin(Allen, Palta et al. 1991); parental IDDM occurred in 6.4% of families, half of which were fathers. 29% had a second or third-degree relative with IDDM.

3.4.2. Type 2 diabetes.

In ten families *(0.05),* one parent is known to have type 2 diabetes *(5* fathers: *5* mothers). A three generation family history of type 2 diabetes, however, was documented in 81 pedigrees (0.44). The familial clustering of type 1 and type 2 diabetes has also been described. Gottlieb noted an increased risk of insulin-dependent diabetes among the offspring of NIDDM parents(Gottlieb 1980). In the Pittsburgh study(Wagener, *Sacks* et al. 1982), 55 families had at least one type 2 parent (0.05) and in the study of Chern at al., the risk to siblings of type 1 diabetics was highest when a parent had type 2 diabetes (rather than type 1 disease)(Chern, Anderson et al. 1982). In 1987, however, Tillil and Kobberling found no increase in type 2 diabetes in parents of 554 type I probands(Tilliland Kobberlìng 1987) and in the Swedish Childhood study, non-insulin-dependent diabetes was present in only 1.7% of parents, although 31.8% of families reported a three generation

history of type 2 disease(Dahlquist, ***Blom*** et al. ***1989).*** In the study of Allen et al., 4.3% of 194 newly diagnosed cases had a parent with type 2 diabetes and 34% had a second or third-degree relative with type 2 diabetes(Allen, ***Palta et al. 1991).***

The differences between these studies are difficult to interpret for the following reasons.

1) Type 2 diabetes is often undiagnosed. Hence, designation of 'unaffected status' is unsatisfactory unless an oral glucose tolerance test has been performed and will lead to underestimates of prevalence. In multiplex pedigrees, however, the diagnosis may be more frequently made due to increased concern about diabetes. There is some support for this contention from the Pittsburgh study where 5/7 parental cases of type **2** were diagnosed following the onset of type 1 diabetes in their second sibling(Wagener,***Sacks et*** al. *1982).*

2) Insulin is frequently used in the treatment of type 2 diabetes and this can lead to misclassification of disease. The Swedish Childhood study, for example, reported figures for 'insulin-treated diabetes', rather than type 1 diabetes.

3) Type 2 diabetes is a condition diagnosed in middle and later years. Hence, studies in which parental status is assessed at the time of diagnosis of the type 1 offspring (e.g., the Swedish Childhood study and that of Allen et al.) would be expected to find lower rates of type **2** disease than this study.

3.5. Personal and family history of autoimmune disease.

Only 16 of the 385 diabetics (0.04) from these multiplex pedigrees are known to have another autoimmune condition (table 3.1). In 10 cases this is thyroid disease (0.03), 5 have seropositive rheumatoid arthritis **(all** female) and there is one case of

vitiligo. There are no known cases of Addison's disease, coeliac disease, pernicious anaemia or myasthenia gravis.

3.5.1. Autoantibodies.

To date, 365 plasma samples from 98 different families have been tested for thyroid thyroglobulin and microsomal antibodies and human adrenal antibodies. This total includes 162 parents (80 fathers; 82 mothers), 158 diabetic offspring and 43 unaffected offspring (at the time of venesection). Results are shown in table 3.2. Eight fathers and 9 mothers were positive for both thyroid antibodies, in contrast to only three diabetics (all at low titres).

3.5.2. Addison's disease.

Addison's disease (primary hypoadrenalism) is rare in type 1 diabetes and there were no cases in these multiplex families. Adrenal antibodies are also uncommon, present in less than 1% in the diabetics. This finding is consistent with previous studies of Caucasoid type 1 patients which have found antibodies in 1.5-2.0% of cases(Riley, Maclaren *et* al. 1980)(Bright, Blizzard et *al.* 1982).

3.5.3. Coeliac disease

An association between coeliac disease and type 1 diabetes has been confirmed in a number of populations(Savilanhti, Simell et al. *1986)*(Cacciarì, Salardi et al. 1987)(Ul-Hague Chowdhury, Sher et al. 1992). The reported prevalence of coeliac disease in type 1 diabetics varies from approximately 1.7/1000 in the UK to 3.5% in Finland. Either condition may present first although symptoms of coeliac disease

Table 3.2. Number of subjects with raised and borderline levels of thyroid thyroglobulin and thyroid microsomal antibodies and human adrenal antibodies.

	Thyroid thyroglobulin antibody		Thyroid microsomal antibody		Human adrenal antibody
	Positive	Borderline	Positive	Borderline	Positive
Fathers	8	1	9	1	1
Mothers	9	1	22	4	3
Diabetics	4	4	14	6	1
Unaffecteds	0	0	2	1	0

Positive = antibodies detected at >1:400 dilution, Borderline = 1:400>antibodies>1:100

are sometimes absent in patients who already have type 1 diabetes(Maki, ***Hallstrom et al. 1984).*** The finding of no known affecteds in these multiplex pedigrees, despite the frequency of high-risk HLA-DQA1*0501/DQB1*0201(found in **HLA-**DR3/4 heterozygotes) suggests that; coeliac disease is often undiagnosed in these subjects; it is relatively asymptomatic; or a combination of both.

3.5.4. Rheumatoid arthritis.

The association between rheumatoid arthritis and type 1 diabetes is not well described. In one study from London, 39 of 295 patients (0.13) with classical or definite rheumatoid arthritis had a first or second degree relative with type 1 diabetes(Thomas,***Young et al. 1983).*** In this **data** set, five multiplex diabetics have seropositive disease (all female) and in 38/183 (0.21) pedigrees, there is a three generation family history of rheumatoid arthritis. This is a similar figure to that reported for 'rheumatic' diseases (25%) in families of sporadic type 1 diabetics in Sweden(Dahlquist, ***Blom et al. 1989).***

3.5.5. Thyroid disease.

Ten diabetic offspring **(8** female; 2 male) have received treatment for hyper- or hypothyroidism and 45/182 (0. 25) families volunteered a three generation history of thyroid disease. Fourteen diabetics (7 males; 7 females) are positive (>1:400) for thyroid microsomalantibodies **(TMA),** but only three have significant titres of both thyroglobulin and TMA; none of these patients **are** receiving treatment.

The reported incidence of thyroid antibodies in type 1 diabetic patients varies considerably. Riley et al. examined **643** white Americans who had developed type 1

diabetes before the age of 30 years and found TMA in 131(0.20)*(Riley, Maclaren et al. 1981).* They were then able to follow 117 of these patients for a mean of 20.6 months; 50(0.43) were found be have a clinical thyroid disorder (8 hyper- and 42 hypothyroidism). This led to the authors to suggest that all type 1 diabetics should be screened for TMA and that those with positive results should have yearly checks of thyroid function. In 1982, Bright el. al reported even higher levels of thyroid antibodies; 53/164 (0.32) white type 1 diabetics in Virginia were positive for thyroglobulin antibodies and/or *TMA(Bright, Blizzard et al. 1982).* In contrast, a study from Newcastle-upon-Tyne, UK found thyroid antibodies in 17 of 134 (0.13) type 1 diabetics diagnosed before the age of 20 years. 25 of their patients (0.19) had a positive family history and only four (0.03) had abnormal thyroid function(Court *and Parkin 1982).* These results are extremely similar to those from this multiplex data set and suggest that there is considerable ethnic and/or geographical variation in the incidence of thyroid disease in type 1 diabetes.

3.6. Summary and conclusions.

Clearly, there are limitations to the interpretation of disease recall data; for example, the family recall of diagnoses such as rheumatoid arthritis and thyroid disease is likely to include a number of non-autoimmune conditions; vitiligo may be concealed from relatives; the prevalence of pernicious anaemia and type 2 diabetes will depend upon the parental age; these families are particularly aware of diabetes and are likely to have searched for other family cases of type 1 and type 2 disease. There are also biases inherent in the method of data collection. To gain a disease history, I have questioned at least four members of each family; compare this with data gathered from proband studies, where only one individual completes a questionnaire. Given these provisos, there are two important findings;

1) There appears to be no significant increase in the prevalence of diabetes or autoimmune disease in the families of multiplex diabetics compared with sporadic diabetics.

Since over **90%** of newly diagnosed type 1 diabetics are sporadic, with no first degree family history of disease, it is important to establish whether there are differences between diabetics from multiplex pedigrees and the diabetic population as a whole. For example, it is possible that multiplex diabetics represent a particularly 'autoimmune' form of type 1 disease and such heterogeneity would potentially limit the value of multiplex families in genetic and therapeutic studies. In the above **data,** there is no evidence of a difference in the prevalence of type 1 or type 2 diabetes in this multiplex group compared with large epidemiological studies of (mainly) sporadic cases. Further, the prevalence of other autoimmune conditions in these diabetics and their families is no greater than has been reported for sporadic cases.

2) The use of autoantibodies may not be a practical means of sub-grouping type 1 diabetic pedigrees (or sporadic cases).

One way of approaching the genetic analysis of complex diseases is to concentrate on sub-groups of affected individuals with additional, well-defined characteristics (subphenotypes). These are less likely to show genetic heterogeneity and may have a recognisable mode of inheritance, for example, the **MODY** sub-group of type 2 diabetes. Reports that 20-30% of Caucasoid type 1 diabetics have **TMA** suggested that autoantibody estimation is a useful way of sub-grouping multiplex families for linkage studies(Riley, Maclaren et al. 1981)(Bright, Blizzard et al. 1982). The above results do not support this contention; the prevalence of adrenal antibodies was extremely low (<1%) and only 15/158 diabetics were positive for one thyroid autoantibody (0.10). This latter finding is much lower than **data** published from the

United States but is consistent with reports from the UK (0.13, 0.10) and from preliminary analysis of 111 sporadic diabetics collected for this study (0.14). Subgroups of this size (n~20) severely limit the power of linkage analysis and are consequently of little value. The limitations of thyroid autoantibodies in sub-group determination are further highlighted by the finding that less than 40% of cases who had received or are receiving therapy for thyroid disease had positive titres of TMA (presumably reflecting 'burn-out' of the autoimmune process).

CHAPTER 4

HLA DATA

4.1. Introduction.

This chapter describes the age-at-onset, sex and HLA typing of multiplex diabetics, sporadic diabetics and control subjects. When appropriate, comparisons are made with two other large type 1 diabetes multiplex data sets; access to the Genetic Analysis Workshop no.5 (G.A.W.5) data base was facilitated by Dr **R.S.** Spielman, Department of Human Genetics, University of Pennsylvania; details from the Human Biological Data Interchange (H.B.D.I.) multiplex families are obtained from their 1992 "Catalog of Family Pedigrees". **A** small amount of overlap between these two data sets cannot be excluded.

In medical genetics, the word 'proband' is synonymous with the terms 'propositus' and 'index case' and is defined as the affected person through which a pedigree is discovered and explored. For simplex families, this is an appropriate and useful label, however, this is not the case for multiplex pedigrees. Almost all of the families studied for this thesis were identified because of multiply affected offspring and were, therefore, referred to the collection ***en bloc'.*** This difficulty has also been encountered in other multiplex studies and has led to the use of the term 'proband' to indicate the first affected (or first diagnosed) offspring. It is this definition that I will apply in the following chapters.

4.2. Age-at-onset of diabetes.

Age-at-onset information is available on 183 probands, 198 later-affected diabetics and 357 sporadic diabetics (table 4.1). The mean age-at-diagnosis of the probands of multiplex pedigrees is significantly less than that of later-affected siblings (pelo-4). Very similar results are seen in the other multiplex family data sets;

Table 4.1. Mean age-at-onset of diabetes in multiplex and sporadic diabetics.

Diabetic group (n)	Mean age-at-onset years (SD)	Age range years
Multiplex diabetics (381)		
Proband (183)	8.5 (5.7)	0.5-29
Second diagnosed (182)	13.2 (7.6)	1-41
Third diagnosed (16)	17.2 (8.0)	7-33
Sporadics (357)	10 (3.9)	0.5-16

Table 4.2. Sex of multiplex and sporadic diabetics.

Diabetic group (n)	Sex (M:F)	χ^2, 1df
Multiplex diabetics		
Proband (184)	92:92	0
Second-diagnosed (184)	110:74	7.0, p<0.01
Third-diagnosed (16)	*10 : 6*	3.3, p=0.071
Later-diagnosed (200)	121: 79	8.0, p<0.005
Total multiplex diabetics (384)	212: 173	4.2, p<0.05
Sporadics (418)	227: 191	3.4, p=0.066

G.A.W.5 (94 unrelated families) probands mean age-at-onset 9.4 (6.6) years versus later-affecteds 12.7 (7.6) years, $p<0.002$; H.B.D.I. (99 unrelated families) proband mean age-at-onset 8.1 (4.9) years versus later-affecteds 13.4 (8.6) years, $p<0.0001$.

The multiplex probands in this data set are also significantly younger at disease-onset than the sporadic diabetics ($p<0.002$); this is despite the 16 years upper age limitation on sporadic diabetics which would tend to reduce the mean age of the sporadic group. Also, some of the sporadic diabetics may become 'multiplex' if one or more of their siblings become diabetic. A similar finding has been reported from Scandinavia (multiplex probands mean age-at-onset 7.8 (5.1) years versus sporadics 12.9 (6.4) years, $p<0.001$)(Pociot,Johannesen et al. ***1993).*** It has been suggested that this result indicates a stronger role for environmental triggers and/or additional genetic factors in familial cases (i.e., heterogeneity between multiplex and sporadic disease).

4.3. Sex of diabetics.

Once again, there is a difference between probands and later-diagnosed diabetics in the multiplex data set (table 4.2). Probands have an equal ratio between males and females whilst in the later-diagnosed diabetics, there are significantly more males than expected ($p<0.005$).Comparison of the sex ratios in the two groups confirms that this difference is significant at the 5% level (92:92 **vs** 120:80, χ^2 3.9, 1df, $p<0.05$). Overall, in the multiplex diabetic cases, there is a slight preponderance of males *(0.55)* and this is also seen in the sporadic diabetic group (males 0.54). There is no evidence of heterogeneity between the multiplex and sporadic diabetics with regard to sex (χ^2 0.07, 1df, $p=0.8$).

The difference in the sex ratio in probands and later-affected diabetics in these multiplex families is not replicated in the other data sets. In **G.A.W.5,** there is a significant excess of males in both the probands (57:36, $p<0.05$) and later-affecteds **(67:45,** $p<0.05$). In the H.B.D.I. families, there are more male probands than female probands **(59:40,** $p=0.06$) but an equal sex ratio in the later-affecteds (61:63).

Having examined only two variables, age-at-onset and sex, there appears to be evidence of considerable heterogeneity: between sporadic and multiplex diabetics (age-at-onset); within multiplex families (sex and age-at-onset); and between multiplex data sets (sex). This is difficult to accept, given that the clinical criteria for the diagnosis of diabetes were identical for the multiplex and sporadic cases in this study (and very similar to those used in **G.A.W.5** and H.B.D.I.). Similar findings have, however, been reported in the literature and are generally accepted as evidence of 'environmental factors', 'genetic effects' or a combination of both(Johannesen, Pociot et al. 1993)(Pociot, Johannesen et al. 1993). I have re-examined these data and find that these apparently random and inexplicable differences can be reconciled by consideration of the term 'proband' and age-at-onset effects.

By defining the proband of a multiplex family as the first diabetic offspring, there is an in-built bias towards the proband group being younger at diagnosis. This is due to the fact that later-affecteds (by definition) have zero risk of disease up to the diagnosis of the proband. For example, if the first-born child is diagnosed at an early age (hence, the proband), then siblings have many years to develop disease (up to the age of 28 years the family would still satisfy B.D.A. age criteria). Conversely, if the first-born is not diagnosed until his/her twenties, then a younger

sibling must develop disease before the age of 17 years (to satisfy B.D.A. criteria) and in so doing, becomes the proband.

A comparison of the mean age-at-onset of multiplex diabetics according to order of birth (i.e. oldest sibling in the family to have diabetes, versus second oldest etc.) should obviate this anomaly, and this is indeed the case (table 4.3). There is no significant difference between the mean age-at-onset of the first-born diabetic compared with the second-born ($p=0.6$) or the third-born ($p=0.7$). This result is more consistent with the mean age-at-onset **of** all multiplex diabetics being greater than that of the sporadics (11.1 (7.3) years versus 10 (3.9) yrs, $p<0.01$). The 'proband effect' also disappears from the **G.A.W.5** and **H.B.D.I.** data sets when they are treated in this way and the mean ages-at-onset of the first-born and later-born diabetics do not differ from UK multiplex families: **G.A.W.5;** 12 (7.9) and 10.6 (6.8) years, **H.B.D.I.;** 12.5 (8.3) and 9.9 (7) years, respectively.

Analysis of the UK data set by birth order also diminishes the sex difference observed in the probanflater-affected analysis; first-born diabetic (M;95: F;89 **,** later-born 117:83, χ^2 1.8, 1df,$p=0.18$). Nevertheless, the observed differences in diabetic male: female ratios deserve further consideration. Combining the data from table 4.1 and table 4.2 it appears that there is a relationship between the mean age-at-onset of a diabetic group and the ratio of males to females; specifically, the proportion of male diabetics increases with the mean age-at-onset. **I** have formally examined the multiplex data set for this effect in the following way. All multiplex diabetics were pooled (n=381) and ranked for age-at-onset; the group was then subdivided into tertiles according to the age-at-onset of disease. A comparison was made between the ratio in each tertile and an expected *50:50* split. An identical analysis was performed on the sporadic diabetics (table 4.4).

Table 4.3. Mean age-at-onset of diabetes in multiplex pedigrees according to birth order.

Diabetic group (n)	Mean age (SD)	Age range
Multiplex diabetics		
First-born (183)	**11.3** (6.6)	**1-37**
Second-born (182)	**10.8 (7.6)**	0.5-41
Third-born **(16)**	**12.3 (9.9)**	**1-33**
Sporadics **(357)**	**10 (3.9)**	**0.5-16**

Table 4.4. Male to female sex ratio in diabetic tertiles according to age-at-onset.

	Multiplex diabetics		Simplex diabetics	
Tertile	Mean age (SD)	Male:Female	Mean age (SD)	M : F
Youngest	**3.6 (1.6)**	***60*** : 54	5.6 (2.1)	65 : **59**
Middle	**9.8 (1.7)**	**70** : **68**	**10.7** (1.1)	**64** : **63**
Oldest	**19.1(5.9)**	**81** : **48****	**14.3** (1.1)	***64*** : 42*

*=p<0.05, **=p<0.01

The ratio of males to females does not differ significantly from unity in the youngest and middle age-at-onset tertiles in both multiplex and sporadic diabetics. In the oldest tertiles, however, there is a significant increase in the proportion of male diabetics; this is more pronounced in the multiplex tertile, which has the higher mean age-at-onset. Examination of the G.A.W.5 and H.B.D.I. data sets reveals the same trend (table 4.5).

Since there is now no significant difference between the four data sets with respect to age and sex, it is reasonable to combine them, producing a total of 1,166 diabetics for analysis. Given such large numbers, this group can be split into quintiles according to age-at-onset (table 4.6). These data suggest that males and females have an equal risk of developing type 1 diabetes up to the age of approximately 13 years; thereafter, males are more likely to develop disease. This finding is consistent with reports showing a slight male preponderance of disease, especially in adult-onset diabetes(Wolf,***Spencer et al. 1983)(Karjalainen,Salmela et al. 1989).*** In addition, Bruno et al. have recently published an identical result based on an analysis of 298 type 1 diabetics in the province of Turin, Italy(Bruno, ***Pisu et al. 1993).***

4.4. HLA-DR genotype and phenotype frequencies.

Despite the clear limitations of subdividing multiplex diabetics into proband and later-affecteds (see above), this categorisation of family data has been extensively reported. I will, therefore, continue to employ these terms, so as to allow direct comparison of this data set with results from the literature.

Table 4.5. Male to female sex ratio in diabetic tertiles according to age-at-onset.

	G.A.W.5 multiplex diabetics		**H.B.D.I.** multiplex diabetics	
Tertile	Mean age	MaleFemale	Mean age	Male:Female
Youngest	3.9 (1.8)	36 : 34	4.0 (1.8)	37 : 42
Middle	10.5 (1.7)	43 : 25*	10.1 (1.6)	36:41
Oldest	19.8 (5.3)	46 : 21**	14.3 (1.1)	47 : 20**

*=p<0.05, **=p<0.05,

Table 4.6. Male to female sex ratio in diabetic quintiles according to age-at-onset (combined datasets).

Quintile age range	n	MaleFemale	χ^2, 1df
<4 years	200	104 : 98	0.1
5-8 yrs	248	130 : 118	0.6
9-11 yrs	242	124 : 118	0.2
12-14 yrs	220	122: 98	2.6
>15 yrs	254	169: 85	27.8*

χ^2 16.6, 4df, p<0.003

*=p<10^{-6}

Three hundred and forty multiplex diabetics have been fully HLA typed. I have also typed 369 sporadic type 1 diabetics and 321 controls subjects for HLA-DR3, -DR4 and HLA-DQB1*0201, -DQB1*0301 and -DQB1*0302. Table 4.7 shows the HLA-DR3 and -DR4 genotype frequencies of family and sporadic diabetics. There is no heterogeneity between the proband and later-affected offspring (χ^2 1.64,3df, p=0.65), however, both show a significant difference from sporadic diabetics (proband vs sporadic χ^2 13.57, 3df, p<0.005; later-affected **vs** sporadic χ^2 9.75, 3df, p<0.03). Specifically, there are significantly more HLA-DR3/4 heterozygotes in multiplex families.

It is of interest that a comparison of the HLA-DR types of these multiplex probands (and/or later affecteds) with those of British Caucasoid diabetics studied by Wolf et al. shows no evidence of heterogeneity(Wolf, ***Spencer et al. 1983).*** In that study of 122 mainly sporadic type 1 subjects (diagnosed below twenty years), 0.51 were HLA-DR3/4, 0.71 HLA-DR3 and 0.78 HLA-DR4. This compares with 0.47,0.67 and 0.75 respectively, in the multiplex probands (χ^2 1.2, 3df, p=0.75). One explanation for the difference between the sporadic diabetics in this study and those **of** Wolf et al., is that the HLA-DR3/4 PCR assay which I used to type the sporadic diabetics failed to detect a proportion of HLA-DR3/4 heterozygotes. This is not inconceivable since, at the time **of** this work, the assay was in early stages of development and has only subsequently been published(Patel, ***Lo et al. 1993).*** This hypothesis is not, however, supported by the results of serological typing which has now been performed on more than two thirds of the sporadic diabetics. There were only 10 disagreements between the two assays **(8** missed types by PCR) and a comparison of diabetics typed by both methods with those typed by **PCR** alone shows no evidence of heterogeneity (table 4.8, χ^2 1.65, 3df, p=0.65). Furthermore, the HLA-DR3 and -DR4 results from these sporadic

Table 4.7. HLA-DR genotype frequencies (proportions) in multiplex and sporadic diabetics.

	HLA-DR type				
	X/X	3/X, 3/3	4/X,4/4	3/4	Totals
Proband	8 (0.05)	33 (0.2)	47 (0.28)	79 (0.47)	167
Later-diagnosed	6 (0.03)	33 (0.19)	59 (0.34)	75 (0.43)	173
Total multiplex diabetics	14 (0.04)	66 (0.19)	106 (0.31)	154 (0.45)	340
sporadic	26 (0.07)	91 (0.25)	138 (0.37)	114 (0.31)	369

Table 4.8. Comparison of HLA-DR3 and -DR4 genotype frequencies (proportions) of sporadic diabetics typed by serology and PCR (n=267) and PCR only (n=102).

	HU-DR type				
	X/X	3/X,3/3	4/X,4/4	3/4	Totals
Serology/PCR	17 (0.06)	67 (0.25)	101 (0.38)	83 (0.31)	267
PCR	9 (0.09)	24 (0.24)	37 (0.36)	31 (0.31)	102

diabetics are compatible with those from a large study of French Caucasian diabetics; Caillat-Zucman found the **HLA-DR3/4** genotype in **0.38 of** patients aged less than **15** years at diagnosis **(n=112)** and this fell to **0.24** in those aged **15-30** years at onset of disease(Caillat-Zucman,***Garchon et*** al. ***1992).***

An alternative explanation is that the Barts-Windsor study group were 'preselected' and this produced an excess of **HLA-DR3/4** heterozygotes(Wolf,***Spencer et al. 1983).*** The mechanism by which this could occur is difficult to explain, although it is supported by other evidence of selection; a high proportion of diabetic probands had a first degree relative with disease **(19/123** = 0.15 compared with **<0.1** expected); and the high number of twin pairs ***(6*** verses an expected number of **1-2).**

If one accepts that there is a difference between multiplex and sporadic diabetics, with regard to **HLA** (and Dr E.Tuomilehto-Wolf herself, now insists that this is the case, personal communication), then does this indicate genetic heterogeneity? I think not, since the ability of parental couples to produce an **HLA-DR3/4** heterozygote increases their chances of becoming a multiplex pedigree for at least two reasons. First, for an **HLA-DR3/4** offspring to be conceived, the parents must carry at least two high risk **HLA** haplotypes between them; therefore, three of the four potential inherited **HLA** combinations will be high risk for diabetes **(HLA-DR3/4, -DR3/X** and **-DR4/X).** Second, the risk of diabetes to **HLA-DR3/4** offspring is higher than that of **HLA-DR4/4,4/X** and **HLA-DR3/3,3/X** (Section **4.7).** Hence, families with an **HLA-DR3/4** diabetic child will be more likely to have multiple diabetic offspring than those in which the diabetic is not **HLA-DR3/4.** Given these considerations, one would expect **HLA-DR3/4** to be more frequent in multiplex diabetics than sporadic cases and genetic heterogeneity need not be evoked as an explanation.

4.5. HLA-DR type and sex

Analysis of the male to female sex ratio by HLA-DR type shows no significant heterogeneity in sporadic diabetics, however, there is a significant difference in multiplex diabetics, mainly due to a high proportion of HLA-DR3/3,3/X male diabetics (table 4.9).

4.6. HLA-DR type and mean age-at-onset

Table 4.10 shows the mean age-at-onset according to HLA-DR3 and -DR4 typing. In the multiplex diabetics in this data set, mean age-at-onset of the HLA-DRX/X group is significantly younger than both the HLA-DR3/3,3/X ($p<0.01$) and HLA-DR4/4,4/X diabetics ($p<0.05$). The mean age-at-onset of HLA-DR3/4 diabetics is also significantly less than that of the HLA-DR3/3,3/X multiplex group. In contrast, in the sporadic group, the HLA-DRX/X diabetics are significantly older than both HLA-DR3/3,3/X ($p<0.05$) and -DR3/4 diabetics ($p<0.05$).

Interpretation of these findings is difficult. The number of tests performed means that results significant at the 5% level should be viewed with caution, especially when three of the four 'significant results' involve small HLA-DRX/X groups (n=14 multiplex and 24 sporadic diabetics, respectively). This contention is supported by examination of the G.A.W.5 multiplex data set (table 4.10). Here, the mean ages-at-onset for HLA-DR3/4, -DR4/4,4/X and -DR3/3,3/X are almost identical to those of UK multiplex diabetics and there are no significant differences between them. In this data set, however, the small number of HLA-DRX/X diabetics (n=4) have a higher mean age-at-onset of 18.7 (10.4) years although this difference is not statistically significant.

Table 4.9. Sex of multiplex and sporadic diabetics according to HLA-DR3 and -DR4 typing.

	Multiplex diabetics Male: Female ratio	Sporadic diabetics M:F
DR3/3,3/X	49 : 18*	51 : 39
DR4/4,4/X	52 : 52	74 : 65
DR3/4	83 : 71	58 : 55
DRX/X	7 : 7	16 : 10
	χ^2 9.99, 3df, p<0.02	χ^2 1.21, 3df, p=0.75

*=p<0.001

Table 4.10. Mean age-at-onset of multiplex and sporadic diabetics according to HLA-DR3 and -DR4 typing.

	HLA-DR type			
	X/X	3/X,3/3	4/X,4/4	3/4
Multiplex (U.K.)	8.1 (4.8)	12.8 (8.1)	11.5(6.9)	10.2 (7.3)
Sporadics (U.K.)	11.4 (3.2)	9.8 (3.8)	10.3 (3.6)	9.6 (4.2)
Multiplex (G.A.W.5)	18.7 (10.4)	12.4 (7.8)	11.3 (7.2)	10.4 (7.0)

One constant feature of the three data sets is the lower mean age-at-onset of diabetes in **HU-DR3/4** diabetics. This is consistent with published reports showing that the proportion of **HLA-DR3/4** diabetics is greatest in diabetics diagnosed at an early age ***(Caillat-Zucrnan, Garchon et al. 1992)(Wong, Hibberd et al. 1993).*** Division of multiplex and sporadic diabetics into tertiles by age-at-onset does not show significant heterogeneity in the proportions of **HLA-DR3** and **-DR4** types (data not shown). The number of **HLA-DR3/4** diabetics is, however, highest in the youngest tertiles of both multiplex (youngest tertile 0.57 vs later tertiles **0.40;** p<0.01) and sporadic diabetic groups **(0.36 vs 0.30;** p=NS). The reason that a definitive interaction between **HLA-DR3/4** and age-at-onset cannot be confirmed by this data set almost certainly relates to the age-at-onset of the diabetics under study. In the largest published study ***(Caillat-Zucman, Garchon et al. 1992),*** a significant effect was only seen when comparing diabetics less than 15 years at diagnosis with those aged more than **30** years: in this data set, only the multiplex group includes type 1 diabetics diagnosed at this late age and the numbers are small (n = **8);** all sporadic diabetics are less than **17** years at diagnosis.

One surprising aspect of the above data (table **4.9)** is the male to female sex ratio in **HLA-DR3/3,3/X** diabetics; in the sporadic diabetics there is a male predominance, despite a mean age-at-onset of only **9.8** years, whilst over 73% of the multiplex diabetics are male (mean age-at-onset 12.8 years). To examine sex and age-at-onset according to **HLA-DR3** and **-DR4 type,** I have combined all the diabetics in this data set and analysed them in tertiles (as above). Table 4.11 shows a summary of the findings. In the combined group, there is no difference in the mean age-at-onset according to **HLA-DR3** and **-DR4** type. **As** in the earlier age analysis, there is an increase in male diabetics in the older tertiles, however, this male skewing **is** also seen in the younger tertiles of **HLA-DR3/3,3/X** (M:F, **31:20,** 31:17 and **35:18**

Table 4.11. Sex ratio and age-at-onset of diabetes according to HLA type (multiplex and sporadic diabetics combined).

		HLA-DR type		
	X/X	3/3,3/X	4/4,4/X	3/4
Number	38	152	233	264
Mean age-at-onset **(SD)**	10.2 (4.2)	11.1 (6.3)	10.8 (6.3)	9.9 (6.2)
M : F ratio in Combined youngest & middle tertile ***(<13*** years)	13 : 12	62 : 37*	80 : 78	89 : 96
Oldest tertile (>13 **years)**	9 : 4	35 : 18*	45 : 32	50 : 29*

*=p<0.02

respectively). Unfortunately, this effect cannot be confirmed or refuted by analysis of the other family data sets; HLA-DR data from H.B.D.I. families is not freely available and only 37 diabetics in G.A.W.5 are HLA-DR3/3,3/X although the same trend is seen (M : F younger/middletertiles 12:7, oldest ***9:5).***

4.7. Disease associations with HLA-DR3 and -DR4.

This data set demonstrates the well-recognised population associations between HLA-DR3 and HLA-DR4 and type 1 diabetes. Comparison of the HLA-DR3 and -DR4 genotype frequencies (HLA-DR3/3,3/X, -DR4/4,4/X, -DR3/4 and ***-X/X)*** in diabetics and controls shows highly significant heterogeneity (table 4.12). Relative risks (RRs) are calculated according to the Method of Woolf(Woolf 1955)and are shown in table 4.13. For the 'All DR3' relative risk for probands, for example, a 2x2 contingency table is created with one column comprising the totals of diabetic probands who are HLA-DR3-positive (including -DR3/4) and those who are not whilst the second column contains equivalent totals from controls. It should be noted that the RRs are higher in multiplex offspring than in sporadic diabetics, although the wide 95% confidence intervals indicate that these differences are not significant.

4.8. HLA-DQB1 associations in HLA-DR4-positive diabetics

HLA-DR4 is in linkage disequilibrium with two HLA-DQB1 alleles, HLA-DQB1*0301 and -DQB1*0302 (Section 1.3.1.i); 90% of HLA-DR4 diabetics are reported to be positive for HLA-DQB1*0302 allele(Nepom,***Palmer et al. 1986).*** I examined the disease association of these HLA-DQB1 alleles in multiplex and sporadic diabetics using PCR and oligonucleotide dot-blotting(Bain *and* ***Todd***

Table 4.12. HLA-DR genotype frequencies (proportions) of diabetics and controls (χ^2 vs controls).

		HU-DR type			
	x/x	3/x,3/3	4/x,4/4	3/4	χ^2
Proband (167)	8 (0.05)	33 (0.2)	47 (0.28)	79 (0.47)	164''
Later-affected (173)	6 (0.03)	33 (0.19)	59 (0.34)	75 (0.43)	160.1''
Sporadic (369)	26 (0.07)	91 (0.25)	138 (0.37)	114 (0.31)	176.1''
Controls (321)	151 (0.47)	59 (0.18)	96 (0.3)	15 (0.05)	

*=χ^2>160, 3df, p<10^{-6}

Table 4.13. Relative risks for HLA-DR3, -DR4 and -DR3/4 (shown with 95% confidence intervals).

Probands		Later-affected vs controls	
All DR3 = 6.8 (4.5-10.3)	χ^2 = 90''	All DR3 = 5.5 (3.7-8.3)	χ^2 = 75*
All DR4 = 5.8 (3.8-8.9)	χ^2 = 74''	All DR4 = 6.5 (4.3-9.9)	χ^2 = 83*
DR3/4 = 18.3 (10-33.4)	χ^2 = 128''	DR3/4 = 15.6 (8.6-28.4)	χ^2 = 113''
Sporadic diabetics			
All DR3 = 4.2 (3.0-5.8)	χ^2 = 75''		
All DR4 = 4.1 (3.0-5.6)	χ^2 = 78''		
DR3/4 = 9.1 (5.2-16.0)	χ^2 = 78*		

*=χ^2>74, 1df, p<10^{-10}

1993). HLA-DQB1 typing was performed on 278 HLA-DR4-positive multiplex diabetics and 243 sporadics; 229 (0.93) and 218 (0.9) carry HLA-DQB1*0302, respectively (χ^2 between groups 1.4, 1df, p=0.24). Of the 75 HLA-DR4-positive controls typed for HLA-DQB1,44 (0.59) are HLA-DQB1*0302and 31 (0.41) are -DQB1*0301. Closer examination of the association revealed that the proportion of HLA-DQB1*0302 was highest in HLA-DR3/4 heterozygotes versus controls (χ^2 28.8, 1df, $p<10^{-7}$) and this was significantly increased compared with HLA-DR4/4,4/X diabetics in both multiplex (χ^2 10.4, 1df, p<0.002) and sporadic samples (χ^2 19.5, 1df, pelo-5).

This difference between the association of HLA-DR4 and -DQB1 alleles in HLA-DR314 heterozygotes and HLA-DR4/X diabetics has been noted previously ***(Tait, Mraz et al. 1988).*** The same study also found that HLA-DR1/4 diabetics did not have a significant excess of HLA-DR4, -DQB1*0302 (TA10-) compared with HLA-DR4-positive controls. Although their control population was large (n=583), only 20 diabetics were HLA-DR1/4. Thirty-seven multiplex and 35 sporadic diabetics in this series are HLA-DR1/4 and there is no difference between them regarding the HLA-DR4, -DQB1*0302 association (χ^2 0.013, 1df, p=0.9). Comparison of the total HLA-DR1/4 diabetic cohort with the total DR4-positive controls shows a significant increase of HLA-DQB1*0302 (χ^2 8.3, 1df, p<0.005).

There is no doubt that the association between HLA-DR4 and HLA-DQB1*0302is strongest in HLA-DR3/4 diabetics. This suggests an interaction (leading to increased disease susceptibility) between haplotypes carrying HLA-DR3, DQB1*0201 and HLA-DR4, DQB1*0302, which cannot be explained by alleles at HLA-DQB1 alone. Before this can be confirmed as a diabetogenic association, however, **data** on a large group of HLA-DR3/4-positive controls is required.

Although the HLA-DR4, -DQB1 association is weaker in other HLA-DR4-positive diabetic groups, it is present; the negative report in the literature may reflect the small numbers studied previously. Once again, there is no significant difference between multiplex and sporadic cases.

4.9. Genetic linkage of HLA and type 1 diabetes

Comparison of the HLA haplotype sharing in affected sibling pairs with that expected under a model of random segregation (1:2:1) demonstrates genetic linkage of HLA to diabetes (Section 1.5.2). Of 174 fully informative sibling pairs, **83** (0.48) share two haplotypes identical-by-descent (IBD), 67 **(0.38)** share one haplotype IBD and 24 (0.14) share zero (χ^2 51, 2df, $p<10^{-10}$). The number of zero sharers in this data set is higher than is generally quoted in the literature (~0.07). Comparison of these sharing data with those from the G.A.W.5 data set, however, shows no significant heterogeneity between the multiplex families in these collections (table 4.14).

Using the IBD probabilities from the UK data and the method of Risch one can calculate how much of the observed familial clustering of type 1 diabetes is accounted for by the HLA region (Risch *1987).* The As for HLA is the expected proportion of sibling pairs sharing zero HLA haplotypes (under a hypothesis of no linkage) divided by the observed number of zero sharers, i.e. for this data set, As = 0.25/0.14 = 1.78. This compares with the overall As of 15 (the **risk** of disease to the sibling of a type 1 diabetic compared with that of the general population), implying that an unlinked locus and/or environmental factors are required to fully account for the observed familial clustering. The As of 1.8 for HLA compares with

Table 4.14. Evidence for linkage to HLA to type 1 diabetes in this U.K. dataset vs G.A.W.5 dataset.

Dataset	HLA haplotype sharing IBD			
	0	1	2	Total
U.K. (this study)	**24 (0.14)**	**67 (0.38)**	**83 (0.48)**	**174**
G.A.W.5	10 **(0.07)**	**48 (0.36)**	**77 (0.57)**	**135**

χ^2 heterogeneity **4.3,** 2df, p=0.12

the value estimated by Risch, $\lambda s = 3.42$ (by meta-analysis of all data sets in the literature) and from the G.A.W.5 data, $As = 3.57$.

Given the evidence in support of genetic heterogeneity according to age-at-onset (Section **4.6)** and the low value of As, I have subdivided the fully informative sibling pairs according to age-at-onset of disease(Bain, Aitman et al. ***I993).*** This has been done by dividing the data set into roughly equal tertiles using these four criteria; age-at-onset of the younger diabetic in each sib-pair, age-at-onset of the older diabetic in each sib-pair, age-at-onset of the proband in each sib-pair and mean age-at-onset of the pair. Almost identical results are obtained from each analysis and only the data for ageat-onset of the younger diabetic in each sib-pair is shown (table 4.15).

Clearly, there is evidence of heterogeneity in the linkage of HLA to type 1 diabetes according to age-at-onset, with the oldest tertile showing no significant distortion in haplotype sharing. This result has not been described previously but is consistent with reports of weaker HLA associations according to ageat-onset and the increase in the proportion of HLA-DRX/X - positive sporadic diabetics with age(Caillat-Zucman, Garchon et al. ***I992).***

Examination of the G.A.W.5 sib-pairs by age-at-onset (table **4.16)** does not confirm this effect, however, the G.A.W.5 data set was assembled from families that had already been HLA typed. Knowledge of these data could have affected the selection of families and this is inkeeping with the extremely low proportion of HLA-DRX/X in this data set **(<0.02).** There is further support for this contention from data on French multiplex families **(G.M.**Lathrop, personal communication). Young French multiplex families (at least one diabetic aged less than 18 years at

Table 4.15. Evidence for linkage heterogeneity to HLA by age in all U.K. sib-pairs (tertiles by youngest age-at-onset diabetic in each pair).

Age tertile	HLA haplotype sharing IBD				
	0	1	2	Total	χ^2, 2df
<4.5 years	3 (0.05)	22 (0.38)	33 (0.57)	58	35.0, $p<10^{-7}$
4.5-10 yrs	5 (0.08)	23 (0.38)	33 (0.54)	61	29.4, $p<10^{-6}$
>10 yrs	16 (0.29)	22 (0.4)	17 (0.31)	55	2.2, p=0.33

χ^2 heterogeneity 18.7, 4df, $p<0.001$

Table 4.16. HLA IBD by age in all G.A.W.5 sib-pairs (tertiles by youngest age-at-onset diabetic in each pair).

Age tertile	HLA haplotype sharing IBD				
	0	1	2	Total	χ^2, 2df
<5 years	2 (0.04)	19 (0.42)	24 (0.53)	45	22.6, $p<10^{-4}$
5-11yrs	4 (0.09)	13 (0.30)	26 (0.60)	43	29.2, $p<10^{-6}$
>12 yrs	3 (0.07)	16 (0.35)	27 (0.59)	46	28.9, $p<10^{-6}$

χ^2 heterogeneity= 1.9, 4df, p=0.76

diagnosis) are selected using clinical criteria only; in this group, there is a similar trend in haplotype sharing, this being strongest in the youngest age-at-onset tertile (χ^2 31,2df, $p<10^{-6}$) and weakest in the oldest tertile (χ^2 6.2,2df, $p<0.05$). Older sibling pairs in the French data set are, however, pre-selected; both diabetics must be ICA positive at diagnosis. In this older group (n=23), the evidence for linkage to HLA is extremely strong (χ^2 30,2df, $p<10^{-6}$).

Another consideration in the analysis of the G.A.W.5 data is that both the zero-sharers in the youngest age-at-onset tertile come from one family which has four diabetic offspring. Both of these diabetics share 1 and/or 2 **HLA** haplotypes with other diabetic family members, implying that all four parental haplotypes are high risk. In these circumstances, zero sharing may have less relevance (Section 4.6).

Given that age-related HLA heterogeneity exists (in the UK at least), what is the effect of excluding those sibling pairs that do not satisfy the B.D.A. age criteria? These results are shown in table 4.17. Although there remains a trend towards more zero-sharers in the oldest tertile, there is significant evidence of linkage in each tertile ($\chi^2 > 13.8$, equivalent to LOD score >3) and no statistical heterogeneity between the tertiles. These data show that by use of age-at-onset criteria alone, evidence for genetic heterogeneity at HLA can be reduced (as was anticipated). This finding also highlights the importance of accumulating detailed clinical data on pedigrees, especially when clinical diagnostic criteria only are used for inclusion.

Table 4.17. Lack of evidence for linkage heterogeneity to HLA by age in U.K. sib-pairs satisfying B.D.A. age criteria (tertiles by youngest age-at-onset diabetic in each pair).

Age tertiles	HLA haplotype sharing IBD				
	0	1	2	Total	χ^2, 2df
<4 years	2 (0.04)	20 (0.42)	26 (0.54)	48	25.3, $p<10^{-5}$
4-8 yrs	6 (0.12)	18 (0.35)	28 (0.54)	52	23.5, $p<10^{-5}$
9-16 yrs	9 (0.16)	21 (0.36)	28 (0.48)	58	16.9, p<0.0003

χ^2 heterogeneity 3.8, 4df, p=0.44

4.10. Genetic linkage of HLA to type 1 diabetes in duplex versus triplex families.

The use of all available sibling pairs when calculating identity-by-descent probabilities (for example, three sibling pairs from a triplex family) is common practice. This treatment of all sib-pairs, as if they were independent, is known as the 'all pairs method' and is justified since the **IBD** statistic is based upon no assumptions. Sibships greater than two, however, are neither statistically nor genetically independent; this has led to the development of methods which 'weight' the results according to the number of related sib-pairs included in the analysis(Suarez *and* Hodge *1979).* I have examined the evidence for linkage to **HLA** in duplex and triplex families separately. Given the effect of age-at-onset on linkage to **HLA** (Section **4.9),** I have restricted the analysis to sib-pairs that satisfy **B.D.A.** criteria (table **4.18).** These *data* show no significant distortion of haplotype sharing in the sib-pairs from triplex families.

Since triplex diabetics have similar proportions of **HLA-DR3 (0.79)** and **-DR4 (0.79)** to the multiplex group as a whole, the implication is that parents of triplex sib-ships carry more **HLA** susceptibility haplotypes. This is confirmed by comparison of the **HLA** haplotypes in 'triplex parents' with 'duplex parents' who have at least three children (those with only two offspring are potential triplex parents). The triplex group of parents *are* significantly more likely to *carry* two high risk haplotypes **(HLA-DR3, DQB1*0201** homozygous, **HLA-DR4, DQB1*0302** homozygous or **HLA-DR3/4, DQB1*0201/0302** heterozygous) (χ^2 **7.1,** 1df, **p<0.01).** Further, none of the triplex parents are **HLA-DRX/X** compared with **20**

Table 4.18. Identity-by-descent (IDB) data on sib-pairs from duplex and triplex families satisfying B.D.A. age-at-onset criteria.

Family type	HLA haplotype sharing IBD				
	0	1	2	Total	χ^2, 2df
Duplex	12 (0.1)	41 (0.33)	71 (0.57)	124	70.4, $p<10^{-8}$
Triplex	5 (0.14)	19 (0.54)	11 (0.31)	35	2.3, p=0.32

χ^2 heterogeneity 7.3, 2df, p<0.03

Table 4.19. HLA types of diabetics from duplex families with three or more offspring and from triplex families.

Diabetics	**HLA-DR** type			
	X/X	**3/X,** 3/3	**4/X,4/4**	**3/4**
Duplex	**5 (0.03)**	**40 (0.24)**	**60 (0.35)**	**65 (0.38)**
Triplex	**2 (0.05)**	7 (0.17)	7 (0.17)	**26 (0.62)**

χ^2 **heterogeneity** 9.1, **3df, p<0.03**

duplex parents. Comparison of the diabetic offspring of these two parental groups (table 4.19) highlights the higher proportion of HLA-DR3/4 diabetics in triplex families which is a result of the differences in parental haplotypes.

Once again, this effect is not confirmed by analysis of G.A.W.5 pedigrees where families with more than two affected offspring do show evidence of linkage (χ^2 58.8, 2df, $p<10^{-10}$). There is, however, a similar trend with sib-pairs from these pedigrees having a higher proportion of zero-sharers at HLA.

4.11. Evidence for linkage heterogeneity to HLA in duplex families.

The mean age of the third diabetic offspring in triplex families is significantly greater than that of the proband and second diabetics in this data set (Section 4.2). As a result, sib-pairs from triplex families (in whom linkage to HLA cannot be demonstrated by the IBD statistic) may be over-represented in older cohorts when age-at-onset analyses are performed. The age-at-onset heterogeneity seen in Section 4.9 could, therefore, simply reflect a predominance of sib-pairs from triplex pedigrees in the oldest tertile. The data from duplex sib-pairs only do not support this contention (table 4.20) and confirm age-related heterogeneity at HLA within pedigrees with only two diabetic offspring (χ^2 14.2,4df, $p<0.01$).

Could the difference in IBD distortion in duplex sib-pair tertiles be due to differences in parental HLA types, as appears to be the case in triplex pedigrees? This hypothesis would seem unlikely since it implies that the parents of sibling-pairs in the oldest age-at-onset tertile are most likely to carry high-risk HLA types and hence, they should produce more HLA-DR3/4 offspring. The literature and data from this thesis (Section 4.6), however, suggests that HLA-DR3/4 diabetics

Table 4.20. Evidence for linkage heterogeneity to HLA by age in all duplex sib-pairs (tertiles by youngest age-at-onset diabetic in each pair).

Age tertile	HLA haplotype sharing IBD				
	0	1	2	Total	χ^2, 2df
<5 years	3 (0.07)	14 (0.31)	28 (0.62)	45	34.2, $p<10^{-6}$
5-10 yrs	2 (0.04)	18 (0.37)	29 (0.59)	49	33.2, $p<10^{-6}$
11- yrs	11 (0.28)	13 (0.32)	16 (0.4)	40	6.15, p=0.05

χ^2 heterogeneity 14.2,4df, p<0.01

develop disease at an earlier, rather than later, age. Table 4.21 shows a comparison of the HLA-DR types in diabetics from the youngest and oldest age-at-onsettertiles; this confirms that duplex diabetic offspring in the oldest age-at-onset tertile are less likely to be HLA-DR3/4 heterozygotes, although this is not a significant finding. Furthermore, there is no significant difference in the parents of the youngest and oldest tertiles when comparing the proportions who are homozygous for high-risk HLA-DR,-DQ haplotypes (as performed in Section 4.10). Twenty-six of eighty (0.32) parents of sib-pairs in the oldest tertile are "high-risk homozygotes" compared with 19/88 (0.22) parents in the youngest tertile (χ^2 2.5, 1df, p=0.11).

4.12. Summary and conclusions.

Analysis of this large, ethnically homogeneous data set confirms the well-described associations between HLA-DR3 and HLA-DR4 and type 1 diabetes. Further, the analysis demonstrates linkage between the HLA region and type 1 disease in multiplex pedigrees.

These data also show that heterogeneity, related to age–at-onset of disease, is present in type 1 diabetes. Below the age of approximately 13 years, the risk of type 1 diabetes appears to be equal for males and females but as the age-at-onset increases above this age, males are more likely to be affected This result may direct research towards environmental factors acting around the onset of puberty and whilst studies of this kind do not provide mechanistic hypotheses, it is interesting to speculate on the possible roles of hormonal changes associated with menarche and rubella vaccination. The additional finding that there is a male preponderance of HLA-DR3/3,3/X diabetics, irrespective of age-at-onset, requires confirmation in larger numbers of subjects.

Table 4.21. Comparison of HLA-DR types of diabetic offspring in age-at-onset tertiles of duplex pedigrees.

Diabetics	HLA-DR				
	3/3, 3/x	4/4,4/x	3/4	X/X	Total
Tertile 1	17 (0.19)	23 (0.26)	45 (0.5)	5 (0.06)	90
Tertile 2	16 (0.16)	36 (0.37)	41 (0.42)	5 (0.05)	98
Tertile 3	22 (0.28)	28 (0.35)	29 (0.36)	1 (0.01)	80

χ^2 heterogeneity 9.1, 6df, **p=0.17**

There is also evidence that linkage between **HLA** and **type** 1 diabetes is dependent upon age-at-onset of disease, at least in this multiplex family **data** set. This is consistent with data from population association studies, as well as reports of clinical and 'biochemical' heterogeneity according to age at diagnosis. The lack of support for this finding from the G.A.W.5 **data** set is both surprising and a matter for concern; the possibility that this relates to pre-selection of pedigrees (on the basis of **HLA** typing) needs to be explored. In addition, the mean age-at-onset of disease appears to be lower in **HLA-DR3/4** diabetics and this is consistent with **data** showing a higher proportion of **HLA-DR3/4** heterozygotes in younger diabetics. The age range of subjects in this **data** set, however, precludes firm confirmation of this finding.

There is little evidence of genetic heterogeneity between multiplex and sporadic diabetics. The previously reported differences in mean age-at-onset of disease (between probands and sporadics) are shown to be spurious and the only convincing difference between familial and sporadic diabetics is the higher proportion of **HLA-DR3/4** heterozygotes in multiplex diabetics. This finding is consistent with an increased prevalence of high risk **HLA** haplotypes in the parents of multiply affected offspring and the higher risk of disease to **HLA-DR3/4** individuals. This contention is supported by the even higher proportion of **HLA-DR3/4** heterozygotes in triplex diabetics. The implication of these findings is that genes identified in linkage **analyses** of multiplex families *are* likely to be involved in sporadic type 1 disease. Assessment of the importance of new loci, however, must be performed in sporadic cases (compared with controls) since multiplex cases will tend to over-emphasise the risk afforded by susceptibility alleles (as seen with **HLA-DR3/4** heterozygote relative risk in multiplex versus sporadic cases).

Finally, these data may help in the selection of sibling pairs which are most likely to demonstrate linkage to new susceptibility loci. The ability to detect linkage to HLA using affected sibling pair analysis is greatly reduced by analysis of both older pedigrees and triplex sib-ships; this suggests that young onset, duplex pedigrees may be of most value in linkage analysis of other loci.

CHAPTER 5

INSULIN GENE ANALYSIS

5.1. Introduction.

The insulin gene region (*INS*) on the short arm of chromosome 11 (11p15.5) was initially regarded as a candidate gene for type 2 diabetes. The first polymorphism to be assayed for association studies was described by Bell et al. in 1981 (Bell, Karam et al. 1981); this consists of a length polymorphism created by variation in the number of copies of a 14bp repeat located 5' to the start of the transcription of the insulin gene. This is termed a variable number tandem repeat (VNTR). The number of tandem repeats in Caucasians falls into two main classes; small alleles of approximately 40 repeats, known as Class 1 alleles and larger alleles (~170 repeats) designated Class 3. Intermediate sized alleles (Class 2) are extremely rare in Caucasians.

Initial studies on the *INS VNTR* examined only small numbers of subjects with type 1 diabetes since the focus was on type 2 disease (Bell, Karam et al. 1981) (Rotwein, Chyn et al. 1981) (Owerbach *and* Nemp 1982) (Rotwein, Chirgwin et al. 1983). In 1984, however, Bell at al. analysed a group of 113 type 1 diabetics and reported an association between Class 1 alleles and *disease (Bell,* Horita et al. 1984). This was followed by a study from Hitman et al. (53 patients versus 88 controls) which again showed a significantly higher frequency of Class 1 alleles in diabetic patients (Hitman, Tarn et al. 1985). Linkage analysis of 17 multiplex families (in the same paper), however, showed no evidence of linkage between *INS* and *type* 1 diabetes, despite using various models of inheritance and penetrance. In 1988, Cox et al. analysed 33 affected sibling *pairs* (from 22 multiplex families) for linkage between *INS* and type 1 diabetes (Cox, *Baker* et *al.* 1988); they found no significant distortion in haplotype sharing from the expected 1:2:1 ratio. These authors concluded that either there was no susceptibility locus linked to *INS* and the

association found in unrelated diabetics and controls was an artifact (due to unrecognised population stratification) or a susceptibility locus did exist but it's effects were too weak to be detected by linkage analysis of small numbers of pedigrees.

In 1989, an analysis of the **G.A.W.5 data** set was published but this did little to resolve the issue; **data** was available on **74** sibling pairs and showed that **0.28** shared zero haplotypes IBD, **0.47** shared one and **0.24** shared both(Field 1989); these results do not differ significantly from the expected 1:2:1 ratio. Thomson et al. subsequently applied the affected family based controls **(AFBAC)** method to the **G.A.W.5 data(Thomson, *Robinson* et al. 1989).** This method catagorises parental haplotypes according to whether or not they are transmitted to affected offspring; those that **are** not (DO haplotypes) act as **an** intenial, and therefore racially matched, control group. This analysis showed that significantly more D1 haplotypes (transmitted to diabetics) were Class 1, supporting weak linkage of a locus at **INS.**

The first evidence for linkage between INS and **type** 1 diabetes was established in 1991 by Julier et al.(Julier, Hyer et al. 1991). This group identified new polymorphisms within the INS region which could be analysed by PCR and confirmed an association between these polymorphisms and type 1 diabetes. They then analysed a large, mostly French, group of multiplex families for evidence of linkage. The approach was different, in that analysis of pedigrees was limited to those in which at least one parent was heterozygous at the locus of interest. In effect, this meant that each parent could transmit a high risk (disease-associated) allele ***or*** a low risk (non-disease-associated) allele to affected sibling pairs. Under a model of no linkage, the INS alleles would be transmitted to affected offspring in a ratio of 1:1 and siblings would share the same parental allele ***50%*** of the time.

Distortion of segregation (assessed by the x^2 goodness-of-fit test, with a significance level of 0.05) was taken as evidence for linkage. Since the parents were being analysed separately, information was generated from partially informative families and a comparison of parental segregation was facilitated. Whilst these statistical manoeuvres had been used many times in the past, neither had been been applied to the so-called INS paradox (positive association studies in the face of negative linkage analysis)***(Owerbach, Gunn et al. 1990).***

In the study of Julier et al., evidence for linkage was observed only when the INS loci were paternally inherited. Conventional IBD analysis of the French data set showed no evidence of linkage; indeed, more sibling pairs shared zero INS haplotypes than shared two IBD. Maternal imprinting was considered the most likely explanation for the observed sex difference since this phenomenon appears to be involved in monoallelic expression of other genes on chromosome 11p(Heutink, ***van der Mey et al. 1992)(Zhang and Tycko 1992).*** Imprinting describes a phenomenon where the expression of a gene is affected by the sex of the parent from whom it is inherited. In the case of the INS susceptibility locus, the authors suggested that disease-associated alleles were active only when inherited from a male parent.

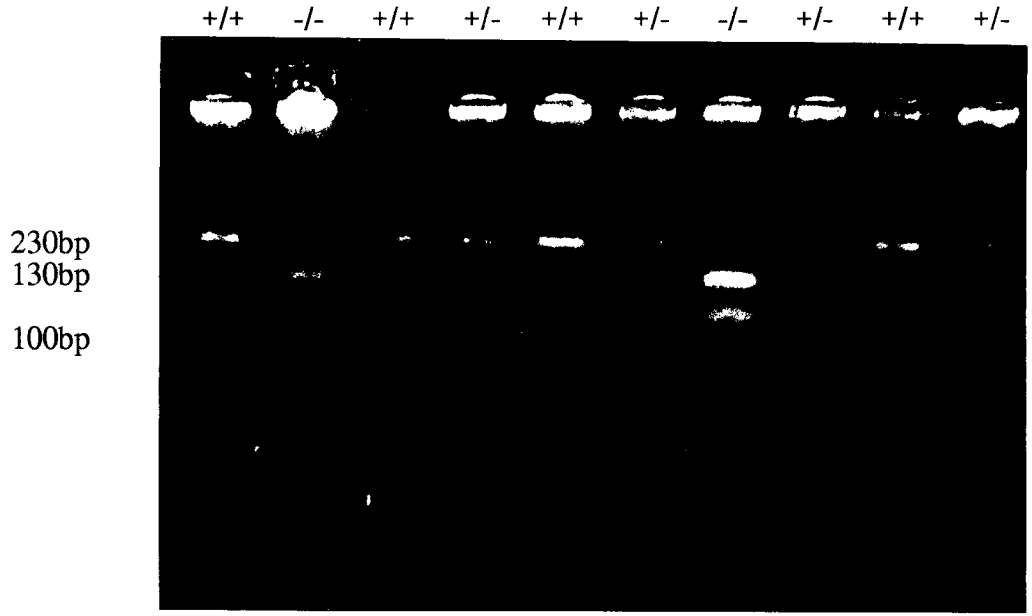
The same study also reported that the INS association was significant only in **HLA-**DR4-positive diabetics, with no additional risk conferred in combination with other **HLA** types. Further, the overall relative risk due to INS was similar to that of **HLA-**DR4 itself, implying that INS is a major susceptibility locus. These results pointed to complex interactions in genetic susceptibility to type 1 diabetes and implied that parental origin of susceptibility loci must be known before risk can be measured. If confirmed, this would have a major impact upon the feasibility of population

screening for type 1 diabetes using genetic markers (Section 1.4.5). I analysed this independent, homogeneous data set for an association between alleles in the INS region and disease. I then went on to search for an interaction between HLA-DR and INS and to assess the effect of parental sex on susceptibility determinants at INS using identical statistical methodology to Julier et al. (Julier, *Hyer* et al. 1991). Given the findings of the previous chapter, the analysis was limited to those families satisfying the B.D.A. age criteria.

5.2. Methodology

I designed new primers to amplify the 1,428/FokI, 1,404/Fnu4H I and 1,127/PstI polymorphic sites of the INS region (Bain, *Prim* et al. *1992).* PCR amplification was performed in a 25µl volume containing 25-250ng DNA, 1.0-1.5mM $MgCl_2$, 10mM TRIS buffer (pH 8.4), 'Tween 20' 0.01%, 200mM of each dNTP, 125ng of each primer and 0.5 units of Taq polymerase (Boehringer). 32-35 cycles (1' at 94°C, 1' at 55°C and 30 seconds at 72°C) were performed in a Perkin-Elmer Cetus Thermal Cycler. Primers SCB1 5'-CCA GCA TGC AGT GGC TCT CC-3' and SCB2 5'-ATT GCT GGA ATG TTC TCA TTC CC-3' amplify a 230 base-pair product which contains the l,428/FokI and 1,404/Fnu4HI restriction sites. Digestion was performed on 8µl of PCR product for 2 hours at 37°C using either 0.5 units of FokI or 3 units of Fnu4HI enzyme. Amplification of the 1,127/PstI polymorphic site was performed using the primers BRB1, 5'-AGC CCT TGG CCC TGG AGG G-3' and BRB2 5'-CTT TAT TCC ATC TCT CTC GGT G-3' under the same conditions and digested with 4 units of PstI enzyme for 2 hours at 37°C. The digested and undigested products are visualised by ethidium bromide staining following electrophoresis in a 4% agarose gel (see figure 5.1). All family members, sporadic diabetics and controls were typed for the 1,428/FokI,

Figure 5.1. 4% agarose gel showing the INS gene product containing 1,428/*Fok*I site following digestion with *Fok*I.



'+'= 1,428/*Fok*I site absent (common variant), '-' = 1,428/*Fok*II site present

1,404/Fnu4HI and 1,127/PstI polymorphic sites of the INS region (Julier, Hyer et al. *1991).* The 1,127/PstI and 1,404/Fnu4HI data do not differ significantly from that generated using FokI digestion due to the strong linkage disequilibrium across the INS region, as described previously (Cox, **Bell** et al. *1988).* For this reason, only the 1,428/FokI data are presented.

Haplotype data for the INS region were generated in 131 sibling pairs by additional typing of the INS VNTR (Bell, Karam et al. *1981),* HRAS VNTR (Owerbach, Gunn et al. *1990)* and a microsatellite within the Tyrosine Hydroxylase gene (Hearne, *Ghosh* et al. 1992). The Southern blotting was performed as part of a laboratory effort to facilitate RFLP analyses on the first 120 multiplex families. I am indebted to the following individuals; J.R.S. **Hall,** C.M. Hearne, J.M. Love, J-**B.** Prins, L.E. Pritchard, R.J. Ritchie and N.R. Rodrigues.

5.3. Analysis of the 1,428/FokI INS locus.

340 control subjects were typed for the 1,428/FokI INS polymorphisms. The common 1,428/FokI allele (allele frequency 492/628 = 0.78) lacks the polymorphic restriction site and is designated '+'; the other allele is '-'. Since only 23 of over 1,300 individuals typed for this thesis were -/- homozygotes, these individuals have been included in the +/- group.

5.3.1. Association data.

Table 5.1 shows that the + 1,428/FokI INS allele is associated with type 1 diabetes. 512/648 (0.79) diabetics are 1,428/FokI INS +/+ homozygotes compared with 0.59 control subjects; this gives an overall relative risk of 2.6 (1.9-3.5) for the

Table 5.1. Numbers (proportions) of +/+ homozygote and +/-, -/- diabetics and controls at the 1,428/*Fok*I INS site.

	+/+	+/- or -/-	Relative Risk (95% confidence intervals)
Controls	186 (0.59)	128 (0.41)	
Diabetics			
Probands	116 (0.81)	27 (0.19)	3.0 (1.8-4.8)*
Later affected	129 (0.82)	28 (0.18)	3.2 (2.0-5.1)*
Sporadics	267 (0.77)	81 (0.23)	2.3 (1.6-3.2)**
Combined	512 (0.79)	136 (0.21)	2.6 (1.9-3.5)***

*=$p<10^{-4}$, **=$p<10^{-5}$, ***=$p<10^{-7}$

homozygous state. The association appears to be stronger in the multiplex diabetics, RR=3.1 (2.1-4.4) versus 2.3 (1.6-3.2), but this difference is not statistically significant (χ^2 2.4, 1df, p=0.12). There is no difference between probands and later-affected multiplex diabetics (χ^2 0.06, 1df, p=0.82).

5.3.2. Sex, age-at-onset and the 1,428/FokI polymorphism.

The status at 1,428/FokI **INS** does not affect the ratio of male to female diabetics in either the multiplex or sporadic groups (table 5.2). Similarly, there is no difference in the mean age of 1,428/FokI INS +/+ homozygotes compared with +/-,-/- diabetics. Sub-division of diabetic groups into tertiles according **to** age-at-onset shows no significant differences in the proportions of 1,428/FokI INS +/+ homozygotes (data not shown: multiplex diabetics; χ^2 3.1,2df, p=0.22, sporadics; χ^2 0.92, 2df, p=0.63).

5.3.3. Interaction between 1,428/FokI INS genotype and HLA-DR.

Given the reported interaction of the 1,428/FokI **INS** +/+ genotype and HLA-DR4, I estimated the relative risk for INS genotypes according to HLA-DR status (table 5.3). There is no difference in relative risk for +/+ 1,428/FokI **INS** homozygosity between individuals who are HLA-DR3/4, HLA-DR3/3,3/X, HLA-DR4/4,4/X compared with pooled controls. Because relative risks can be sensitive to the frequency of alleles in the general population, a χ^2 test of independence was used to compare the proportions of +/+ homozygotes versus +/- (and *4-)* individuals in these groups. This analysis showed no evidence of heterogeneity in either multiplex (χ^2 0.65,3df, **p=0.88)** or sporadic diabetics (χ^2 0.6, 3df, **p=0.90).**

Table 5.2. Mean age-at-onset and sex of multiplex and sporadic diabetics according to 1,428/*Fok*I INS genotype.

	Multiplex diabetics (n=300)		Sporadic diabetics (n=353)	
	+/+	+/- or -/-	+/+	+/- or -/-
Mean age-at-onset (SD)	10.5 (5.9)	11 (5.7)	9.9 (3.9)	10.4 (3.6)
Male diabetics	142 (0.81)	34 (0.19)	148 (0.77)	43 (0.23)
Female diabetics	103 (0.83)	21 (0.17)	124 (0.77)	38 (0.23)

Table 5.3. Relative risks by HLA type for homozygote status at the 1,428/*Fok*I INS site in U.K. diabetics versus pooled controls.

HLA-DR type	Numbers of diabetics with 1,428/*Fok*I INS status		Relative risk (95% confidence intervals)
	+/+	+/-, -/-	
a) Multiplex diabetics			
DR3/X, DR3/3	52	12	3.0 (1.5-5.8)**
DR4/X, DR4/4	72	15	3.3 (1.8-6.0)***
DR3/4	112	27	3.1 (1.8-4.6)***
DRX/X	9	1	6.2 (0.8-49.5)
b) Sporadic diabetics			
DR3/X, DR3/3	65	18	2.5 (1.4-4.4)*
DR4/X, DR4/4	101	30	2.3 (1.5-3.7)**
DR3/4	84	25	2.3 (1.4-3.8)**
DRX/X	17	7	1.7 (0.7-4.1)

*=$p<10^{-2}$, **=$p<10^{-3}$, ***=$p<10^{-4}$

Division of HLA-DR4 types into those that are positive for HLA-DQB1*0301 versus -DQB1*0302 shows no heterogeneity at **INS** between groups (data not shown). Further subdivision of HLA-DR4 types according to the accompanying HLA-DR type shows no significant interactions between HLA-DR genotypes and **INS** but the numbers in each group are small (data not shown).

The relative risk for HLA-DR4 does not differ significantly according to 1,428/FokI **INS** status; RR in 1,428/FokI +/+ sporadic diabetics versus +/+ controls = 4.0 (2.7-6.1), $p < 10^{-4}$; RR in +/- sporadics versus +/- controls = 4.1 (2.2-7.6), $p < 10^{-4}$. Furthermore, the **RR** of HLA-DR4 is consistently higher than that for **INS** +/+ homozygosity in ail groups, implying that HLA-DR4 is a more powerful susceptibility locus than **INS.**

Since the 1,428/FokI **INS** +/+ genotype increases the relative risk of type 1 diabetes in all HLA-DR types, one would expect 1,428/FokI INS +/- parents to preferentially transmit the + allele to their diabetic offspring, irrespective of HLA type. In multiplex families (table 5.4), there is significantly increased transmission of the + 1,428/Fokl allele only to HLA-DR4/4,4/X offspring: the distortion to HLA-DR3/4, HLA-DR3/3,3/X and HLA-DRX/X diabetics is in the same direction but does not reach significant levels. Although this suggests a specific interaction between HLA-DR4 and INS, there is no significant evidence of heterogeneity of segregation of 1,428/FokI INS alleles assessed by χ^2 analysis (χ^2 2.0, 3df, p=0.58).

Table 5.4. Segregation of 1,428/FokI INS alleles in relation to the HLA type of diabetic offspring: HLA genotypes and 1,428/FokI INS allele transmitted to diabetic offspring from informative *(+/-)* meioses.

HLA-DR type							
DRX/X		DR3/3,3/X		DR4/4,4/X		DR3/4	
+	-	+		+	-	+	-
3	1	13	10	31	**13***	34	23

*= p<0.01

Table 5.5. Paternal and maternal identity-by-descent (IBD) of the 1,428/FokI INS alleles in affected sibling pairs.

	Alleles shared from father		Alleles shared from mother		Alleles shared from both parents	
	1	0	1	0	1	*0*
1,428/FokI	19	13	20	7''	39	20''

*= **p<0.05**

5.3.4. Parental sex and segregation of 1,428/FokI INS alleles.

Using the same analytical method as Julier et al.(Julier, Hyer et al. ***1991),*** I examined the transmission of alleles from parents who were +/- at the 1,428/FokI **INS** restriction site to affected sibling pairs in 133 multiplex families. There were 33 heterozygous fathers and 30 heterozygous mothers. In both sexes combined, segregation of alleles to affected sibling pairs differed from the expected 1:1 **ratio** (table 5.5). From paternal meioses, 19 sib-pairs shared the same 1,428/FokI **INS** allele compared with 13 who did not (p=0.29) whilst for maternal alleles 20 sib-pairs shared one allele versus 7 sharing none ***(p<0.05).*** There was no significant difference between the maternal and paternal transmission distortions of the 1,428/FokI INS alleles (χ^2 1.4, 1df,p=0.23).

5.4. Analysis of INS haplotype data.

The reason that Julier et al. used new disease-associated polymorphisms in their analysis was the lack of success in demonstrating evidence for linkage using INS haplotypes. Conventional IBD analysis of their **data** set showed similar results **to** those of the G.A.W.5 **data** set (table 5.6)(Julier, Hyer et al. 1991)(Field,Stephure et al. 1991); i.e. there was no distortion from the expected 1:2:1 ratio and if anything, an **increase** in zero-sharers.

Maternal imprinting was thought to explain why the French IBD **data** showed no evidence of linkage since transmission of 1,428/FokI **INS** alleles to affected sibling pairs showed a significant distortion **from** fathers only. In this UK **data** set I have shown no evidence of a difference in segregation of 1,428/FokI INS alleles from fathers and mothers of diabetic siblings. I therefore examined INS haplotype

Table 5.6. INS haplotype sharing identical-by-descent (IBD) in affected sibling pairs from the G.A.W.5 dataset and Julier et al.

	INS haplotype sharing IBD			
	0	1	2	Totals
G.A.W.5	21	35	18	74
Julier et al. (1991)	33	60	29	122

Table 5.7. INS haplotype sharing identical-by-descent (IBD) in affected sibling pairs from the U.K. (this dataset).

Sibling pairs	INS haplotype sharing IBD				
	0	1	2	Totals	χ^2, 2df
U.K. (B.D.A. criteria, all families)	27	63	39	129	2.3
U.K. (B.D.A. criteria, duplex)	17	46	32	95	4.8

sharing in the B.D.A. families; this showed a slight distortion (χ^2 2.3, 2df, p=0.33), but in the expected direction (table 5.7).

Given the lack of evidence for linkage to HLA in triplex families (Section 4.10), I have extracted sibling pairs from triplex families from this analysis; this removes a sub-group of sibling pairs in whom evidence for linkage to **INS** is lacking (10 zero-, 17 one- and 7 two-sharers). This is consistent with triplex parents having a high prevalence of high risk genotypes at **INS.** Although the 0, 1,2 IBD data is still not significantly distorted (χ^2 **4.8,** 2df, p=0.09), analysis of the parents separately (allowing the use of partially informative pedigrees) shows a significant distortion towards sibling pairs sharing the same parental **INS** haplotype (table ***5.8).*** Once again, there is no significant difference between the parents (χ^2 0.68, 1df, p=0.4). Evidence for linkage of **INS** to type 1 diabetes can, therefore, be demonstrated without the need to define disease-associated polymorphisms within the **INS** region.

5.5. Analysis of INS haplotype data according to age-at-onset.

The data suggest that those factors which I have shown to influence the strength of linkage between HLA and **type** 1 diabetes may be important in the analysis of **INS.** Therefore, I subdivided the B.D.A. duplex families into tertiles, according to age-at-onset (table 5.9). The results of this analysis are consistent with **an** age-at-onset effect; the youngest age-at-onset tertile has the highest proportion of two-sharers, the lowest proportion of zero-sharers and the highest χ^2. The distortion from expected, however, does not differ from expected in any tertile, possibly due to the small cohorts. An alternative way of imposing age-at-onset criteria is to select those fully informative sibling **pairs from** duplex families where both sibs were diagnosed

Table 5.8. Paternal and maternal identity-by-descent (IBD) of INS haplotypes in affected sibling pairs.

	Haplotypes shared from father		Haplotypes shared from mother		Haplotypes shared from both parents	
	1	0	1	0	1	0
INS Haplotype	57	45	61	38*	120	85*

* = $p<0.05$

Table 5.9. Examination of linkage heterogeneity to INS by age-at-onset in U.K. sib-pairs satisfying B.D.A. criteria (tertiles by youngest age-at-onset diabetic in each pair).

Age tertiles	INS haplotype sharing IBD				
	0	1	2	Total	χ^2, 2df
<5 years	4	16	11	31	3.2
5-10 yrs	6	18	12	36	2.0
>11yrs	7	12	9	28	0.6

before the age of 17 years (n=71). In this group, 10 share zero **INS** haplotypes, 36 share one and 25 share two (χ^2 6.3, 2df, $p<0.05$). Analysis of the parental segregation separately provides further evidence for linkage: paternal 1:0;46:31 (χ^2 2.9, 1df, $p=0.09$), maternal; 47:28 (χ^2 **4.8** 1df, $p<0.05$) and combined; 93:59 (χ^2 7.6 1df, $p<0.006$).

5.6. Comparison of linkage to INS and HLA.

Seventy sibling pairs (duplex families with both diabetics aged less than 17 years at diagnosis) are fully informative for both INS and HLA; this allows a comparison of linkage at the two regions in the same families (table 5.10). The IBD distortions at **INS** clearly differ according to HLA haplotype sharing and vice versa (confirmed by χ^2 test of heterogeneity 9.5,4df, $p<0.05$). There is strong evidence for linkage to INS in sibling pairs who share only one HLA haplotype (χ^2 12.9,2df, $p<0.005$) but no significant IBD distortion at INS in pairs sharing two HLA haplotypes (χ^2 5.1, 2df, $p=0.08$). A similar result is seen with HLA; very strong evidence of linkage in INS one-sharers (χ^2 38.0, 2df, $p<10^{-8}$) but no significant distortion in INS two-sharers (χ^2 4.3,2df, $p=0.12$). The implication of these **data** is that there is an interaction between HLA and INS: in sibling pairs sharing two susceptibility haplotypes at HLA, INS haplotype sharing confers little additional risk of disease whilst in siblings sharing two INS susceptibility haplotypes, the requirement for two-sharing at HLA is reduced.

There remains the question of whether an HLA-INS interaction is specifically with one HLA-DR-defined sub-set, as reported by Julier et ***al.(JuZier,*** Hyer et al. 1991). Examination of **INS** haplotype sharing according to HLA-DR **type** does not support a HLA-DR4 - INS interaction. Indeed, INS haplotype sharing is greatest in

Table 5.10. HLA and INS haplotype sharing identical-by-descent (IBD) in duplex families (both diabetics aged less than 17 years at diagnosis) which are fully informative for both regions.

		INS haplotype sharing IBD			
		0	1	2	Totals
HLA haplotype sharing IBD	0	2	3	2	7
	1	3	8	14	25
	2	4	25	9	38
Totals		9	36	25	70

χ^2 heterogeneity 9.5, 4df, $p<0.05$

Table 5.11. INS haplotype sharing identical-by-descent (IBD) in sibling pairs who are HLA-DR identical (by state); duplex pairs both <17 years at diagnosis.

HLA-DR type	INS haplotype sharing IBD			
	0	1	2	Totals
HLA-DR3/4	1	14	9	24
HLA-DR4/4,4/X	4	9	7	20
HLA-DR3/3, 3/X	2	5	0	7

χ^2 heterogeneity 6.6, 4df, p=0.16

siblings that are both HLA-DR3/4 (table 5.11) although this effect is not statistically significant.

5.7. Summary and conclusions.

Analysis of this ethnically homogeneous data set confirms an association between alleles at INS and type 1 diabetes; this finding is, therefore, unlikely to be due to ethnic stratification. The association between +/+ homozygosity at the 1,428/FokI INS locus and disease is seen in both multiplex and sporadic diabetics, and confers an overall relative risk of 2.6 (1.9-3.5). This is similar to that reported by Julier et al. but significantly less than the RRs for HLA-DR3 (5.0,3.7-6.7) -DR4 (4.9, 3.7-6.5) and DR3/4 (12.4,7.2-21.2), implying that the INS locus is less 'diabetogenic' than HLA.

The detection of linkage between INS and type 1 diabetes has been a protracted process and has yet to be formally proven. Based on the above data, I believe that the enigma of INS can be resolved. The analysis of HLA and type 1 diabetes (Chapter 4) has provided a good model on which to examine INS-mediated disease susceptibility; both loci show stronger associations in multiplex diabetics compared with sporadics; both show no evidence of linkage in triplex families (in this data set) and the IBD sharing of each region is greatest in siblings with a young age-at-onset of disease. By limiting linkage analysis to duplex sibling pairs satisfying stringent age-at-onset criteria (both diabetics less than 17 years at diagnosis), this data set provides evidence for linkage of INS to type 1 diabetes. Assuming a similar distortion from the expected 1:2:1 IBD ratio, analysis of approximately 150 fully informative sibling pairs would provide a significance level equivalent to a LOD score of 3, formal proof of linkage.

Examination of haplotype sharing at both INS and HLA in the same 70 families (both siblings less than 17 years at diagnosis) has provided evidence of an interaction between these two loci; linkage between INS and type 1 diabetes is strong in HLA one-sharers, but absent in siblings that share zero- or two-HLA haplotypes. This finding clearly requires confirmation, however, it may direct future analyses of INS and other putative disease loci; based on the data in table 5.10, the number of families required to prove linkage to INS in the HLA one-sharers may be less than thirty.

In this setting, the previous difficulties in demonstrating linkage between INS and type 1 diabetes may be explained. Neither the G.A.W.5 nor the French data set have age-at-onset limitations; both include large numbers of triplex (and greater) pedigrees (10-20%); and more than 50% of the sibling pairs in each collection are two-sharers at HLA. Preselection of families using HLA data may overcome the first two difficulties for HLA loci, but will have little effect on INS analyses. Indeed, HLA-guided selection of pedigrees may hinder the search for non-HLA loci in linkage studies.

I have also tested for a specific interaction between 1,428/FokI INS +/+ homozygosity and HLA-DR types. In multiplex families there is significant preferential transmission of the disease associated l,428/FokI allele to HLA-DR4-positive diabetic offspring only. This is not, however, translated into a difference in INS relative risk to probands (or later diagnosed diabetics, data not shown) subdivided by HLA-DR type. In view of the relative risk of 2.5 for INS +/+ l,428/FokI homozygosity in HLA-DR3/3,3/X probands, the lack of preferential transmission of the + 1,428/FokI INS allele to this sub-group is unexpected and has no obvious explanation.

In sporadic diabetics, the relative risks for **INS** also do not differ significantly according to HLA-DR type. This latter finding has been confirmed by analysis of an independent data set from Norway(Bain, **Prias *et al. 1992*).** Using my primers, **Dr** K.S. Ronningen analysed 151 type 1 diabetic patients, mean age of diagnosis 15.4 years (range 1-37 years), and 186 healthy control subjects (blood donors). In addition, 75 HLA-DR3/4 and 45 HU-DR4-positive controls were selected from the Norwegian bone marrow register to facilitate comparison of **diabetics** and controls matched for HLA-type. The results are shown in table 5.12 and show no evidence of heterogeneity for 1,428/FokIINS status according to HLA-DR genotype (χ^2 0.95,3df, p=0.8). Similar results have been reported by groups analysing **INS** haplotype status according to HLA-DR type(DonaZd, ***Barendse et al. 1989)(Raffel, Vadheim et al. 1991).***

Table 5.12. Relative risks by HLA type for homozygote status at the 1,428/*Fok*I INS site in Norwegian sporadic diabetics versus HLA-matched Norwegian controls.

HLA-DR type	Numbers of diabetics with 1,428/*Fok*I INS status		Number of controls with 1,428/*Fok*I INS status		Relative risk (95% confidence intervals)
	+/+	+/-, -/-	+/+	+/-, -/-	
DR3/4	53	13	51	34	2.7 (1.3-5.7)**
DR4/X, DR4/4	51	8	62	32	3.3 (1.4-7.8)*
DR3/X, DR3/3	16	4	21	14	2.7 (0.7-9.7)
DRX/X	5	1	45	28	3.1 (0.3-28)

*=p<0.05, **=p<0.01.

CHAPTER *6*

ANALYSIS OF HLA TRANSMISSION

6.1. Introduction

In multiplex families, susceptibility haplotypes carrying **HLA-DR3** and **-DR4** are preferentially transmitted from parents to diabetic offspring. This is entirely expected since families are selected on the basis of their multiply affected offspring and **HLA-DR3** and **-DR4** are susceptibility haplotypes for disease. During the past 10 years, however, many studies have reported effects which imply that the inheritance of **HLA** susceptibility may be more complicated than this. There are reports that male diabetics (and unaffected fathers) are more likely to transmit **HLA-DR4** to their diabetic offspring than are diabetic mothers(Vadheim, Rutter et al. *1986).* It has also been suggested that maternal **HLA** susceptibility haplotypes may impart risk to offspring, irrespective of whether or not they are inherited(Rubinstein *1991).* In addition, diabetic **HLA-DR3/4** heterozygotes are said to inherit paternal **HLA-DR4** haplotypes significantly more frequently than maternal (Deschamps, Hors et al. *1990);* this implies a parent-of-origin effect on **HLA** mediated susceptibility.

There are other reports of modulation of **HLA-DR** mediated susceptibility in multiplex diabetics, unrelated to parental effects. **HLA-DR** type has been found to vary according to the affection status of the diabetic offspring (i.e. whether they are the proband or later affected)(Rubinstein, Walker et al. *1986).* It has also been reported that haplotype sharing is dependent upon the **HLA-DR** status of the proband in multiplex families(Rutter, *Vadheim* et al. *1987).* The implication of these findings is that the risk to siblings of type 1 diabetics is dependent upon **HLA-DR** status of the proband.

These results have two important consequences. First, they may provide important mechanistic insights into HLA mediated susceptibility to type 1 diabetes. Second, they imply that population screening for type 1 diabetes using genetic markers (Section 1.4.5) will be fraught with difficulties since risk assessment could not be solely based upon an individual's genotype; one would also need to know the genotypes of parents and siblings (at least). Replication of these findings is clearly essential. In this chapter, I examine this data set for evidence to support these reports.

6.2. Effects of parental sex on HLA-inherited susceptibility to type 1 diabetes

6.2.1. Transmission of HLA-DR4 and HLA-DR3 from parents to diabetic offspring

In 1984, Warram et al. reported that type 1 diabetes was transmitted less frequently to the offspring of diabetic mothers than to those of diabetic fathers (1.3 versus 6.1%, $p<0.05$) (Warram, Krolewski et al. *1984).* Although the numbers reported were small (13 diabetics from 419 offspring), the result was consistent with a review of published data. Degnbol and Green had reported a cumulative risk of diabetes of 4.2% by age 20 for offspring of diabetic fathers and 1.8% for offspring of mothers (Degnbol and Green *1978).* Reanalysis of the prevalence rates of 'juvenile-onset diabetes' in Prince Edward Island, Canada also showed lower rates of diabetes in the offspring of diabetic mothers (Simpson *1969).* Further support for this finding came from prevalence studies of diabetes in parents of newly diagnosed type 1 diabetics; the frequency of diabetes was higher in fathers than in

mothers in both Pittsburgh and Sweden(Wagener, Sacks et al. *1982)*(Dahlquist, Gustavsson et al. 1982).

In 1986, Vadheim et ai. seemed to provide a 'genetic' mechanism for the difference in risk to offspring of diabetic parents(Vadheim,Rotter et *al.* 1986). They reported that fathers with an HLA-DR4 allele were more likely to transmit this allele to their diabetic and non-diabetic children than were mothers (72.1 versus *55.6%,* $p<0.001$). Although this paper was severely criticised(Falk, Field et al. 1987), subsequent examination of the G.A.W.5 data set showed a similar parental difference in the transmission of HLA-DR4-carrying haplotypes to diabetic offspring; namely, healthy HLA-DR4/X fathers were significantly more likely to transmit HU-DR4 than were mothers(Field 1989). Field has subsequently added more data from GAW4 simplex pedigrees and her own *Calgary* data set, confirming this effect (L.L. Field, personal communication).

I analysed 168 multiplex families for an effect of parental sex on the transmission of HLA-DR3 and -DR4 haplotypes to offspring in multiplex families. As expected, there is significant preferential transmission of HLA-DR3-positive haplotypes to diabetic offspring from HLA-DR3/X fathers and mothers (X is any HLA-DR antigen other than DR3 and DR4) (table 6.1). Similarly, transmission of HLA-DR4 to diabetics is significantly increased from both parents. In contrast to the previous reports, however, there is no difference between parents concerning the transmission of these haplotypes (HLA-DR3; $p=0.51$, HLA-DR4; $p=0.44$). Consistent with these findings, the highly significant increase in HLA haplotype sharing in affected sibling-pairs (ASPs) is independent of parental sex; ASPs share 120/176 paternal haplotypes ($p<10^{-5}$) and 118/179 maternal ($p<10^{-5}$). Segregation

Table 6.1. Transmission of HLA-DR alleles to diabetic and non-diabetic offspring in multiplex families (HLA-DRX refers to DR antigens other than -DR3 and -DR4).

Parental genotype	Transmission of HLA	Paternal (P)	Maternal (M)	χ^2 (P vs M)
1. Diabetic offspring				
HLA-DR3/X	-DR3	77/90 (0.86) $p<10^{-10}$	63/77 (0.82) $p<10^{-7}$	χ^2 0.43, p=0.52
HLA-DR4/X	-DR4	69/83 (0.83) $p<10^{-8}$	76/87 (0.87) $p<10^{-10}$	χ^2 0.61, p=0.44
2. Non-diabetic offspring				
HLA-DR3/X	-DR3	11/28 (0.39)	14/28 (0.5)	χ^2 0.65, p=0.42
HLA-DR4/X	-DR4	11/22 (0.5)	8/20 (0.4)	χ^2 0.42, p=0.52

of HLA-DR3 and -DR4 haplotypes to unaffected siblings does not differ from the expected 1:1 ratio in either parent (table 6.1).

These data provide no support for a parental difference in the transmission of HLA-DR4 (or HLA-DR3) to diabetic offspring and implies that, if such a phenomenon does exist, it's effect is minor in this ethnic group. It is of interest that a large study of simplex type 1 diabetic pedigrees in France (n=174) has also failed to confirm a paternal HLA-DR4 effect(Thivolet, Beaufrere et al. ***1988).*** The reasons for these differences between data sets is not clear, however, it is of note that in the G.A.W.5 data set there is a significant difference in HLA-DR susceptibility types in fathers compared with mothers (table 6.2, **$p<0.03$).** A similar trend was also reported in the population studied by Vadheim et al. (Vadheim, Rotter et al. ***1986).***

6.2.2. Analysis of the effects of non-inherited HLA antigens.

Rubinstein et al. have suggested that maternal HLA antigens (specifically HLA-DR4 and -DR6) may affect susceptibility to type 1 diabetes in offspring irrespective of whether or not they are inherited(Rubinstein and Ginsberg-Fellner ***1990)***(Rubinstein ***1991).*** Such an effect of non-inherited maternal antigens (NIMAs) has recently been reported in rheumatoid arthritis, another autoimmune condition associated with HLA-DR4(ten Wolde, Breedveld et al. ***1993).*** It has been postulated that both 'self ' HLA antigens and NIMAs may influence thymic selection of the T-cell repertoire in-utero and hence, susceptibility to disease in later life. Such a hypothesis could account for differences in parental transmission of disease, according to whether NIMAs were protecting or promoting disease susceptibility.

Table 6.2. HLA haplotypes positive for HLA-DR3, -DR4 or neither (-DRX) in parents of the G.A.W.5 families.

Parental sex	Number of HLA haplotypes positive for HLA-DR3 and -DR4			
	DR3	DR4	DRX	Total
Male	38	65	79	182
Female	58	58	60	176

χ^2 heterogeneity 7.1, 2df, $p<0.03$

If NIMAs are exerting an effect, one might expect to see a difference in parental HLA types. Specifically, in multiplex families, mothers would carry fewer susceptibility alleles than fathers (since HLA-DR3 and -DR4 need not be inherited to promote susceptibility) and/or they would be less likely to be HLA-DR2-positive (since this would tend to protect offspring). I analysed 168 multiplex families for differences in parental HLA-DR antigens. For HLA-DR3 and -DR4, the phenotype and genotype frequencies in the parents in this data set are almost identical (table 6.3). There is also no difference between the frequencies of non-inherited paternal antigens (NIPAs) and NIMAs **(p=0.82)** (table 6.4).

For all of the 10 HLA-DR specificities tested by serology, there is no significant difference between parents (data not shown, p=0.15); indeed, HLA-DR2 is more, rather than less, frequent in mothers but this difference is not significant when the number of specificities tested is taken into account (pc=0.1). I also find no significant differences between the proportions of NIMAs and NIPAs not inherited by diabetics (table 6.5). Of the 75 diabetic offspring who were HLA-DR4-negative, twenty-five had fathers with a non-inherited DR4 haplotype and twenty had DR4-positive NIMAs (data not shown, p=0.37); again this is not consistent with non-inherited maternal HLA-DR4 haplotypes influencing susceptibility.

If **NIMAs** were exerting an influence upon disease susceptibility, one would expect a difference in non-inherited HLA types between mothers and fathers. No such differences are seen in this data set. Indeed, the proportions of paternal and maternal antigens which are not transmitted to diabetics are almost identical in all HLA-DR types analysed. Furthermore, diabetics who did not inherit HLA-DR4 are no more likely to have a DR4-positive mother than father. In addition, the specific

Table 6.3. Comparison of HLA-DR3, -DR4 and -DRX phenotype and genotype frequencies in parents of multiplex diabetic offspring.

Phenotype	Paternal (n=336)	Maternal (n=320)
DR3	95	92
DR4	115	107
DRX	126	121

χ^2 0.05, 2df, p=0.98

Genotype	Paternal (n=168)	Maternal (n=160)
DRX/X	19	18
DR3/X	44	39
DR3/3	10	12
DR4/X	44	46
DR4/4	20	16
DR3/4	31	29

χ^2 0.87 , 5df, p=0.97

Table 6.4. Comparison of HLA-DR phenotype frequencies of non-inherited antigens according to parental sex (NIPAs and NIMAs not transmitted to any diabetics).

Phenotype	NIPA (n=102)	NIMA (n=97)
DR3	11 (0.11)	13 (0.13)
DR4	20 (0.2)	17 (0.18)
DRX	71 (0.7)	67 (0.69)

χ^2 0.4, 2df, p=0.82

Table 6.5. Comparison of proportions of non-inherited HLA-DR antigens (NIPAs and NIMAs not inherited by any diabetics).

Phenotype	Paternal	Maternal
DR1	0.43	0.37
DR2 (15,16)	0.9	0.83
DR3 (17,18)	0.12	0.14
DR4	0.17	0.16
DR5 (11,12)	0.8	0.82
DR6 (13,14)	0.55	0.53
DR7	0.72	0.46
DR8	0.38	0.4
DR9	0.5	0.33

(All χ^2 <0.8, 1df, p>0.3)

differences observed by Rubinstein et al (increased HLA-DRw6 and decreased HLA-Bw62,DR4 in mothers compared with fathers) are absent ***(Rubinstein and Ginsberg-Fellner 1990);*** I observe HLA-DR6-positive haplotypes in 25 fathers and 19 mothers and HLA-B62, DR4 haplotypes account for 17/334 (0.05) paternal and 23/320 **(0.07)** maternal haplotypes (data not shown).

6.2.3. Summary and conclusions.

I have examined this data set for parental effects on HLA inherited susceptibility and find no evidence to support either preferential transmission of HLA-DR4 from fathers to diabetic offspring nor an effect of **NIMAs.** If these phenomena exist, then they are of minor importance in this population. It is possible that the differences between these data and previous reports are due to ethnic differences. It is more likely, however, that the original observations were erroneous (at least in terms of magnitude) and due to the study of relatively small numbers. This highlights the need for replication of data, especially when findings are unexpected.

These data may also question the dogma that diabetic mothers are less likely to have diabetic offspring than are diabetic fathers. It is of note that in Warram et al's study, 88 male diabetics produced 244 children compared with 175 children from 99 affected females (χ^2 10.6, 1df, $p<0.001$ assuming an expectation of equal fecundity) ***(Warram,Krolewskì et al. 1984).*** This highlights a possible effect (noted by Warram et al.) of increased spontaneous abortion and high perinatal mortality rates in type 1 diabetic patients. At the time of that retrospective study, a substantial proportion of diabetic pregnancies ended in foetal death. This alone could account for the differences reported (11 diabetics born to type 1 fathers versus 2 to mothers)

and selective loss of 'potentially diabetic' foetuses (postulated by some authors) is not required.

To date, there are no studies comparable to that of Warram et al. The effect of reduced fecundity would not be detected by analyses which assess risk of parental type 1 diabetes in families ascertained by the presence of diabetic offspring. Further, these studies are prone to difficulties relating to the age-at-onset of disease, for example, in this data set, over half of the affected parents were diagnosed after the birth of their diabetic offspring. Furthermore, I have shown that type 1 diabetes developing after puberty is more likely to affect males (fathers) than females (Section 4.3); this can hardly be regarded as 'transmission of disease'.

Improvements in diabetic-obstetric care have led to an outcome in diabetic pregnancy which is now comparable with that in non-diabetics. Further studies under present conditions of improved foetal mortality should resolve the issue of parental transmission of diabetes. Until then genetic counselling of type 1 diabetics should be practised with caution.

6.3. Parental origin of HLA-DR types in HLA-DR3/4 heterozygote diabetics.

In 1990, Deschamps et al analysed the inheritance of HLA-DR3 and **-DR4** haplotypes from fathers and mothers to the probands, later-affected diabetics and unaffected offspring of 246 simplex and 121 multiplex families (including 80 G.A.W.5 families)(Deschamps, Hors et al. ***1990)***. They reported that in the DR3/4-positive probands and affected siblings, the paternal and maternal HU-DR3 and -

DR4 antigens were not distributed randomly; 0.62 and 0.72 respectively received HLA-DR4 from their father and -DR3 from their mother (DR3m/DR4p). This result differed significantly from the expected 1:1 ratio and was not observed in unaffected siblings. Once again, this has important implications for risk assessment, since HLA-DR3/4 siblings with DR3m/DR4p were found to have a significantly higher risk for disease than those who did not. I have analysed 78 probands, 74 later-affected diabetics and 17 unaffected offspring for this effect.

In the family data set, the parental origin of HLA-DR3 (and/or -DR4) in HLA-DR3/4 diabetics does not differ from the expected 1:1 ratio (table 6.6). Any suggestion of a trend towards increased inheritance of paternal HLA-DR4 seen in the probands is also present in the small numbers of unaffected offspring, which act as controls (χ^2 0.5, 1df, p=0.47).

Once again, the results from this multiplex data set do not support a report in the literature(Deschamps, Hors et al. *1990).* Closer examination of the Deschamps' paper, however, shows that the effect described was also not significant in French simplex families (DR3m/DR4p found in 46/81 diabetics, χ^2 versus expected 1.5, 1df, p=0.22, χ^2 vs unaffected offspring 1.28, 1df, p=0.26). In French multiplex families, the effect was seen comparing the total affected offspring with expected (23134, χ^2 4.2,1df, p=0.04) but not when they were compared with unaffected DR3/4 multiplex siblings (χ^2 3.29, 1df, ~0.07) That is to say, the finding was very weak in French subjects. In fact, the major effect came from the G.A.W.5 multiplex data set. As mentioned previously (Section 6.2.1), the HU-DR types of fathers and mothers differ significantly in G.A.W.5, with decreased levels of paternal HLA-DR3 and slightly increased HU-DR4 (table 6.2). Until the effect of these parental differences is controlled for, the relevance of Deschamps' finding can

Table 6.6. Parental origin of HLA-DR3 in HLA-DR3/4 heterozygotes in multiplex families.

HLA-DR3/4 offspring	Paternal HLA-DR3	Maternal HLA-DR3	χ^2, 1df
Proband (n= 78)	35 (0.45)	43 (0.55)	0.82, p=0.37
Later affected (n=74)	36 (0.49)	38 (0.51)	0.05, p=0.82
Total affected DR3/4 (n=152)	71 (0.47)	81 (0.53)	0.67, p=0.42
Unaffected (n=17)	6 (0.35)	11 (0.65)	1.5, p=0.23

be questioned. It is also of note that the increased relative risk of 3.3 for DR3m/DR4p over DR3/4 alone in that paper was calculated by comparing affected multiplex offspring (0.68 G.A.W.5) with only French unaffected offspring, a manoeuvre not justified in the text.

6.4. Differences between proband and later-affected offspring in multipiex families.

6.4.1. HLA-DR status of proband versus later-affected offspring.

A persistent characteristic of virtually all type 1 diabetic samples is a higher than expected frequency of HLA-DR3/4 heterozygotes. It has been observed that in some multiplex samples, the HLA-DR distribution is different for the first affected offspring in a family compared to subsequent affected offspring. Rubinstein reported that the proband in multiplex families showed an excess of HLA-DR3/4 but this was markedly reduced in the second- and later-affecteds (Rubinstein, Walker et al. *1986).* Falk reported that in a combined sample from the Genetic Analysis Workshop 4 (GAW4) and the HLA80 workshop, 55% of the first affected were HLA-DR3/4 compared to 40% of the second affected (Falk *1987).*

Rubinstein et al. went on to examine HLA-DR3/4 excess in multiplex families with so-called "conducive" parents. These are families in which only one DR3/4 combination can be inherited, i.e. parental combinations of HLA-DR3k vs -DR4/X, -DR3/X vs -DR3/4, -DR4/X vs -DR3/4. Their data set was small (n=17 parent pairs) but showed that 13/17 (0.76) probands inherited HLA-DR3/4 compared with 10/25 (0.4) later-affected siblings (χ^2 5.43, p~0.02). It was suggested that this effect was due to "an interaction with environmental factors that

is most noticeable for the proband in a sibship". Although Rubinstein produced support for this finding from the literature, subsequent analysis of 91 multiplex families in the G.A.W.5 data set failed to provide confirmation of the result(Falk 1989); the frequencies of DR3/4 in proband and second affecteds were 0.39 and 0.40 respectively.

In this multiplex data set, 79/167 (0.47) probands and 75/173 (0.43) later-affected diabetics are HLA-DR3/4 heterozygotes (χ^2 0.54, 1df, p=0.46). Indeed, comparison of the genotype frequencies HLA-DRX/X, 3/X,3/3, 4/X,4/4 and 3/4 reveals no evidence of heterogeneity at HLA between the two groups (χ^2 1.6,3df, p=0.65). Considering "conducive" parents only, 55 families satisfy the criteria of Rubinstein et al. (including age-at-diagnosis; proband <16 years, later-affected less than 20 yrs)(table 6.7). In this subgroup, 0.71 probands inherit HLA-DR3/4 compared with 0.59 later-affecteds (χ^2 1.8, 1df, p=0.18); once again, there is no significant difference in HLA-DR3 and DR4 genotype frequencies between the groups (p=0.43).

Although there does appear to be weak support for Rubinstein's finding (there are slightly more HLA-DR3/4 probands than later-affecteds), this may simply reflect age-at-onset effects (Section 4.9). The probands of the conducive parents in table 6.7 have a mean age-at-onset of 7.0 (4.7) years and this is significantly less than that of the later-affecteds, 11.3 (5.6) years, $p<10^{-4}$. Given the trend towards HLA-DR3/4 diabetics being younger at diagnosis, any manipulation which selects a younger age-at-onset subgroup of diabetics is likely to produce an over-representation of the HLA-DR3/4 genotype.

Table 6.7. Comparison of HLA-DR types in probands and later-affected diabetics in multiplex families with conducive parents.

	HLA-DR types				
	X/X	3/X,3/3	4/X,4/4	3/4	Total
Proband	1	8	7	39 (0.71)	55
Later affected	1	9	15	36 (0.59)	61

χ^2 heterogeneity 2.8, 3df, p=0.43

Finally, Rubinstein et al. noticed in their own data and in other published data sets, that if the first affected offspring did not inherit HLA-DR314 then later-affected siblings were never HLA-DR3/4. In the G.A.W.5 sample, there were 10 such instances and in this data set 12 non-DR3/4 probands have affected siblings who are HL,A-DR3/4 heterozygotes.

6.4.2. HLA haplotype sharing according to HLA-DR type of the proband.

In 1987 Rotter et al. reported that the proportion of HLA haplotype sharing in affected sib pairs was dependent upon the HLA-DR phenotype of the proband(Rotter, *Vadheim* et al. *1987).* In an analysis of the GAW4 data they found that, if the proband was HLA-DR3/4, there was a significantly higher frequency of 2-haplotype sharing with affected sibs than if the proband was not HLA-DR314. Falk reported a similar 'trend' towards two-haplotype sharing in 86 sib pairs from the G.A.W.5 data set (proband HLA-DR3/4 versus not -DR3/4: χ^2 4.9, 2df, **p=0.087)**(Falk *1989).*

I have analysed 134 ASPs from duplex families (any age-at-onset) and confirm an effect of proband HLA-DR type, although this is relatively weak (table 6.8). When the proband is HLA-DR3/4, the proportion of affected siblings that share both HLA haplotypes is 0.62 compared with 0.45 in probands who are not HLA-DR3/4 ($p<0.05$).

Table 6.8. HLA haplotype sharing identical-by-descent (IBD) in duplex families according to HLA-DR type of the proband.

Proband HLA-DR type	HLA haplotype sharing IBD		
	0	1	2
HLA-DR3/4	3	20	38
Non-HLA-DR3/4	13	26	34

χ^2 heterogeneity 6.2, 2df, p=0.044

Given the increased risk of diabetes in HLA-DR3/4heterozygotes, this result is not unexpected. Furthermore, the presence of HLA-DR3/4 in a proband may designate a more homogeneous group of families in which HLA susceptibility is important for disease. This contention is supported by analysis of only those sib pairs which satisfy the B.D.A. age criteria (which also reduces genetic heterogeneity at HLA). In this group, proband HLA-DR status has no significant effect on haplotype sharing (HLA-DR3/4 proband versus non-DR3/4: two-sharing 0.59 vs 0.50; χ^2 2.4, 2df, p=0.3).

6.5. Summary and conclusions.

The literature contains many reports which suggest that HLA-mediated susceptibility to type 1 diabetes is more complicated than was first perceived These findings are often unexpected and are based on relatively small sample sizes. Whilst they lack an a priori reason for existence, they quickly generate tenable hypotheses which have become part of the dogma of diabetes. I have examined this data set in order to confirm five such findings; one (the effect of NIMAs) is very recent, two (increased paternal HLA-DR4 transmission to diabetics and specifically to HLA-DR3/4 heterozygotes) are generally accepted and two (proband HLA-DR3/4excess and increased HLA haplotype sharing in HLA-DR3/4 proband pairs) are almost mutually exclusive. I find little evidence to support any of these phenomena. The effects seen are at best weak and *can,* in my opinion, be explained by the well-described risks afforded by different HLA-DR genotypes.

My data do not definitively refute any of the published findings since it is possible that they may all present in different ethnic groups (although I doubt it). They do, however, imply that such effects are minor in this Caucasoid family group. They

also suggest that population genetic screening is a feasible aim since an individual's risk of disease appears to depend upon his/her genotype. In this population at least, we need not concern ourselves with the genotypes of a person's mother or siblings.

Finally, this chapter highlights the need for replication of new and unexpected findings in independent data sets. This data set is the largest and most homogeneous collection of multiplex pedigrees in the world; it has also produced the fewest surprises. It is to be hoped that the 'negative findings' which this collection will generate are received with the same enthusiasm as the novel hypotheses from smaller studies.

CHAPTER 7

CANDIDATE GENE ANALYSIS

7.1.Introduction.

In this chapter, I describe analyses of three candidate loci for type 1 diabetes, the genes encoding glucokinase, apolipoprotein E and Octomer Binding Protein 3A.

7.2. Glucokinase.

7.2.1. Introduction.

There are several reports that relatives of type 2 diabetics are at increased risk of type 1 diabetes(Chern, Anderson et al. 1982)(Wagener, Sacks et al. 1982)(Dahlquist, Blom et al. 1989) and this is the case in this data set (Section 3.1.2.ii). These findings may be due to recall bias (the diagnosis of type 1 diabetes generating a search for diabetes in all family members). They could, however, reflect an overlap of genetic susceptibility to both conditions. Studies of HLA and INS lend support to this contention. Rich and colleagues have presented evidence that **HLA-DR4** contributes to increased susceptibility of type 1 diabetes in families with type 2 diabetic parents(Rich, Panter et al. 1991)(Rich, French et al. 1993) whilst alleles in the **INS** region may be associated with both type 1 and type 2 diabetes in different populations(Himan, Tarn et al. 1985)(Hitman, Kambo et al. 1991). Any genetic locus found to contribute susceptibility to type 2 diabetes, therefore, should be regarded as a candidate gene region for type 1 diabetes (and vice-versa).

In March, 1992 Frougel et al. described linkage of a glucokinase microsatellite polymorphism (GCK 1) to non-insulin dependent diabetes(Froguel, Vaxillaire et al. *1992).* Combined analysis of the sixteen families studied revealed a maximum LOD

score of 11.6 i.e. the odds of linkage versus non-linkage are $10^{11.6}$:1 (Section 1.5.2). Within the 16 pedigrees, however, there was evidence of genetic heterogeneity and a high degree of linkage was only seen in those families with early-onset disease (at least two individuals diagnosed before the age of 25 years). This subphenotype of non-insulin-dependent diabetes is an autosomal dominant disorder, known as maturity-onset diabetes of the young (MODY)(Tattersall and Fajans *1975).* Linkage of GCK1 was subsequently confirmed in a large 5-generation pedigree in the UK, however, this family has many features typical of type 2 diabetes(Hattersley,Turner et *al. 1992).* This suggests that glucokinase gene defects might also be involved in the pathogenesis of type 2 diabetes; glucokinase is, therefore, a candidate gene for both type 2 and type 1 disease.

GCK1 is a polymorphic compound imperfect dinucleotide repeat element located approximately 10 kilobases 3' to the glucokinase gene on chromosome 7p(Matsuani, Janssen et al. *1992).* I tested for an association between alleles at GCK1 and type 1 diabetes in probands of multiplex families(Bain, Barnett et al. *1992).* This approach was adopted following my experience with **INS** (Section 5.3.1) which suggests that it may be easier to demonstrate an association with a candidate locus than linkage. This is especially true when the disease-associated allele has a high frequency in the unaffected population(Todd and Bain *1992).* A positive association clearly requires confirmation by linkage analysis since it may reflect population stratification effects. Linkage studies are, however, unlikely to be positive in the absence of an association. Probands were used in the study since associations with the known susceptibility determinants, HLA and **INS,** are both stronger in multiplex diabetics than in sporadic cases.

7.2.2. GCK1 analysis.

Probands from 137 type 1 diabetic multiplex families were compared with 176 non-diabetic control subjects. The alleles of GCK1 were amplified by PCR using primers 9509 and 9510 as described(Hattersley, Turner et al. 1992). The final product was electrophoresed on a 6% polyacrylamide gel at 100 V for 15 hours and bands were detected by staining with ethidium bromide. Three alleles were detected and these are designated z, z+2 and z+4 . The allelic frequencies of these are shown in table 7.1 and genotype frequencies are shown in table 7.2. There were no significant differences in the allele frequencies (p=0.58) or genotype frequencies (p=0.41) between the groups. Contrary to a recent report(She, *Bui* et al. 1993), the frequencies of heterozygotes with alleles z+4 and z+2 did not differ between diabetics and controls (χ^2 0.64, 1df, p=0.4) and both populations were in Hardy-Weinberg equilibrium.

7.2.3. Summary and conclusion of glucokinase study.

This study shows no evidence for an association between type 1 diabetes and alleles at GCK1, the same microsatellite used to detect linkage between the glucokinase gene and type 2 diabetes(Froguel, Vaxillaire et al. 1992)(Hattersley, Turner et al. 1992). Family studies are unlikely to show linkage of this locus to type 1 diabetes. This finding, however, does not exclude the possibility that mutations in the glucokinase gene may be susceptibility determinants in some individuals with the type 1 diabetes phenotype. As new mutations are identified(Vionnet, Stoffel et al. 1992), they should be analysed in type 1 diabetic pedigrees, especially those with type 2 diabetic parents. This may facilitate the recognition of additional genetic

Table 7.1. GCK1 glucokinase microsatellite allele frequencies (proportions) in multiplex proband diabetics and controls.

	z	z+2	z+4	Totals
Diabetics	185 (0.68)	11 (0.04)	78 (0.28)	274
Controls	239 (0.68)	20 (0.06)	93 (0.26)	352

χ^2 heterogeneity 1.1, 2df, p=0.58

Table 6.7. Comparison of HLA-DR types in probands and later-affected diabetics in multiplex families with conducive parents.

	HLA-DR types				
	X/X	3/X,3/3	4/X,4/4	3/4	Total
Proband	1	8	7	39 (0.71)	55
Later affected	1	9	15	36 (0.59)	61

χ^2 heterogeneity 2.8, 3df, p=0.43

heterogeneity in type 1 diabetes and define possible overlaps in the genetics of clinically different forms of diabetes.

7.3. Analysis of apolipoprotein E genotype in type 1 and type 2 diabetics.

7.3.1. Introduction.

Apolipoprotein E (Apo E) is a ligand for the LDL receptor and this interaction is responsible for removal of triglyceride-rich very-low-density lipoprotein (VLDL) remnants from plasma(Utermann 1987). The Apo E gene is polymorphic with three co-dominant alleles coding for the major isoforms Apo E2, E3 and E4. Apo E2 has a low affinity for the LDL receptor and homozygosity may lead to elevated VLDL remnants. The frequency of the Apo E2/E2 phenotype has been reported to be increased in patients with both type 1 and type 2 diabetes and this has led to the suggestion that the Apo E gene is a candidate susceptibility locus for disease development *(Eto,* Watanabe et al. 1986)(Winocour, Tetlow et al. 1989)(Black, Hewett et al. 1990). In those studies, designation of Apo E phenotype was assessed by isoelectric focusing. This is a difficult, time consuming, method(Wenham, Sedky et al. 1991) and I have previously demonstrated that this assay is affected by raised ambient glucose levels (glycosylation increases the amount of Apo E in the E2 position during isoelectric focusing)(Bain, Guy et al. 1990). It is possible, therefore, that post-translational modification of Apo E may account for the differences reported in diabetics.

In collaboration with E.K. Green and Dr A.F. Jones (Department of Clinical Chemistry, East Birmingham Hospital), we directly assessed the Apo E genotype in

subjects with type 1 and type 2 diabetes and controls(Green, Bain *et* al. ***1991).*** The analysis was performed on 67 type 1 diabetic probands from multiplex families, 89 controls and 122 type 2 diabetics(Bain, Green et al. ***1992).*** The type 2 diabetics were aged over 40 years at diagnosis and were consecutive patients attending the Diabetic clinic at East Birmingham Hospital. Type 2 diabetes was diagnosed according to WHO criteria(WHO Expert Committee on Diabetes Mellitus ***1980).***

7.3.2. Apo E analysis.

The Apo E allele and genotype frequencies are shown in tables 7.3 and 7.4 respectively. The allele frequencies of Apo E2, **E3** and E4 in controls are consistent with previous studies of Apo E phenotype (for example, comparison with Winocour et al. χ^2 0.9, 2df,p=0.63). Genotype frequencies in the controls are also consistent with previous reports of Apo E phenotype. The allele frequencies did not differ significantly from control subjects in type 1 (χ^2 0.6, 2df, p=0.74) or type 2 diabetics (χ^2 1.2, 2df, p=0.55). There was also no difference in genotype frequency in type 1 (χ^2 2.4, 5df, p=0.78) or type 2 diabetics (χ^2 4.1, 5df, p=0.49) compared with controls. Further, the genotype frequencies in all three groups did not differsignificantly from Hardy-Weinberg equilibrium.

7.3.3. Summary and conclusions.

These findings confirm previous phenotypic studies of Apo E in control subjects but do not support suggestionsof allelic or genotypic differences in type 1 *or* type 2 diabetics. Reviewing the only previous study of Apo E in type 1 diabetes(Winocour, Tetlow et al. ***1989),*** it is possible that post-translational modification of Apo E may be responsible for these discrepancies. Glycaemic

Table 7.3. Apolipoprotein E2, E3 and E4 allele frequencies (proportions) in type 1 and type 2 diabetics and controls.

	E2	E3	E4	Totals
Type 1 Diabetics	9 (0.07)	113 (0.84)	12 (0.09)	134
Type 2 Diabetics	16 (0.07)	208 (0.85)	20 (0.08)	244
Controls	16 (0.09)	145 (0.81)	17 (0.1)	178

χ^2 heterogeneity 1.3, 4df, p=0.86

Table 7.4. Apolipoprotein E2, E3 and E4 allele frequencies in type 1 and type 2 diabetics and controls.

	E2/E2	E2/E3	E3/E3	E2/4	E3/E4	E4/E4
Type 1	0	9	46	0	12	0
Type 2	2	12	91	0	14	3
Controls	1	13	59	1	14	1

χ^2 heterogeneity 8.1, 10df, p=0.62

control in the diabetics in that study (assessed by glycosylated haemoglobin) was abnormal (mean 9.4%, normal range 5-8%) and this could account for the increased incidence of E2/E2 homozygosity observed in that group. The finding that allelic and genotypic frequencies are very similar in controls, whether assessed by phenotypic and genotypic methods, is also consistent with an effect of hyperglycaemia. A larger study is required to confirm these findings.

7.4. OFT3 gene polymorphism in type 1 diabetes.

7.4.1. Introduction.

Transcription factors containing the POU-homeo domain have been shown to be important regulators of tissue-specific gene expression in pituitary and lymphoid cells. Using a PCR-based strategy, Takeda et al. looked for similar factors which might be involved in the tissue-specific expression of genes in pancreatic ß-cells(Takeda, Seino et al. 1992). This led to the isolation of two isoforms of the transcription factor Octomer Binding Protein 3 (Oct3A and B). The sequence of human Oct3A shows 87% amino acid identity with mouse Oct3 and polymorphisms of the Oct3 gene have been found to segregate with murine type 1 diabetes in the NOD mouse. The human Oct3 gene (named **OFT3)** is localised to the region of the MHC on chromosome 6. It contains two polymorphisms, one of which can be typed using PCR followed by digestion with HindIII. Takeda et al. typed the HindIII polymorphism in Japanese type 1 diabetics and controls and found a significant association between presence of the restriction site and disease (Dr J. Takeda & Dr G.I. Bell, personal communication).

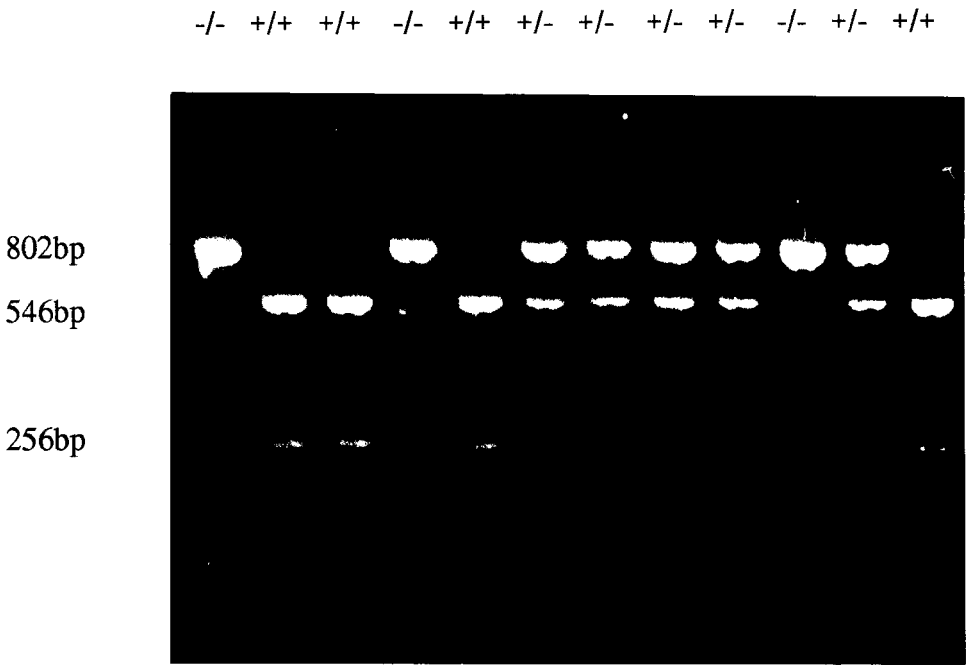
7.4.2. Methodology.

I have examined this Caucasoid population for an association between type 1 diabetes and polymorphism within **OFT3.** Since this gene appeared to be localised within the MHC, it was hoped that a disease associated allele might split HLA-DR3 and /or -DR4- mediated disease susceptibility. I typed 114 probands from multiplex families and 117 control subjects for the **HindIII** polymorphism of the **OFT3** gene. The primers used were; sense 5'-AGC TCA TTG TCT AAT GTC AT-3' and antisense 5'-CAG CTA CAT GGT GAC TGA GT-3'. PCR conditions; denaturation 95°C 1min, annealing 63°C 1min, extension 72°C 1min. The PCR product (802 base pairs) digested to two products (546 and 256bp) in the presence of a **HindIII** site; this is designated the '+' allele. The '-' allele does not digest with **HindIII** (figure 7.1).

7.4.3. OFT3 analysis.

Comparison of the allele frequencies in diabetics and controls shows a significant increase of the OET3+ allele in diabetics (table 7.5, $p<0.01$). The genotype frequencies are shown in table 7.6. This confirms the association observed in Japanese type 1 diabetics ($p<0.01$). Having confirmed the association, I went on to type 402 haplotypes in the parents of multiplex families, allowing analysis of which HLA haplotypes were in linkage disequilibrium with the + and - alleles of the OFT3 gene. Unfortunately, the few HLA recombinants observed (n=9) were all between uninformative homozygotes at OFT3, preventing fine mapping of the gene by linkage analysis in this data set.

Figure 7.1. 4% agarose gel showing the OFT3 gene product (amplified by the polymerase chain reaction) followed by *Hind*III digestion.



'+'= *Hind*III site present, '-' = *Hind*III site absent

Table 7.5. Allelic frequencies of the OFT3 HindIII polymorphism in diabetics and controls.

HindIII allele	Controls	Diabetics
	135 (0.58)	104 (0.46)
+	99 (0.42)	124 (0.54)
	χ^2 heterogeneity 6.75, 1df, $p<0.01$	

Table 7.6. Genotype frequencies (proportions) of the OFT3 HindIII polymorphism in diabetics and controls

HindIII genotype	Controls (n=117)	Diabetics (n=114)
-/-	42 (0.36)	20 (0.18)
+/-	51 (0.44)	64 (0.56)
+/+	24 (0.2)	30 (0.26)
	χ^2 heterogeneity 9.9, 2df, $p<0.01$	

7.4.3.i. HLA-DR3 haplotypes and OFT3.

Concerning HLA-DR3,DQB1*0201 haplotypes, 36 (0.32) carry the - OFT3 allele and 78 (0.68) were +. This appears to be the result of linkage disequilibrium between the OFT3 polymorphism and HLA class I loci. The HLA-B8, DR3, DQBl*0201 haplotypes, for example, carry the **OFT3+** allele in 88/90 cases (0.98 vs expected 0.5, χ^2 82, 1df, $p<10^{-10}$) whilst HLA-B18,DR3,DQB1*0201 haplotypes are **OFT3-** in 23/25 cases (χ^2 17.6, 1df, 10^{-4}). It is, therefore, unlikely that alleles at OFT3 are directly involved in susceptibility associated with HLA-DR3 since both + and - alleles are present on high risk HLA-DR3 haplotypes.

7.4.3.ii. HLA-DR4 haplotypes and OFT3.

HLA-DR4-positive haplotypes are OFT3+ in 65/137 (0.47) cases. There is, however, a significant difference between the HLA-DQB1 subgroups of HLA-DR4. Low risk HLA- DR4, -DQB1*0301 haplotypes are OFT3 + in 6/27 (0.22) cases, compared with 59/110 (0.54) of DR4, -DQB1*0302 haplotypes (χ^2 8.6, 1df, $p<0.005$). Once again, however, there is little to support the contention that the OFT3 locus is directly affecting susceptibility since there is no significant difference in the proportion of **OFT3+** HLA-DR4, -DQB1*0302 haplotypes in multiplex diabetics (84/163=0.52) compared to the parental group (χ^2 0.12, 1df, p=0.73). That is to say, OFT3+ HLA-DR4,-DQB1*0302 haplotypes do not segregate preferentially to the diabetic offspring.

7.4.4. Summary and conclusions.

It is unlikely that the OFT3 gene directly contributes to susceptibility to type 1 diabetes in Caucasians and the disease association probably reflects linkage disequilibrium with true susceptibility determinants. Since the two alleles of OFT3

are both carried on high risk haplotypes, it has little potential for screening (even in combination with other HLA markers).

This polymorphism may, however, be useful in further trans-racial studies. **HLA-DR4** and **-DR9** are the major disease susceptibility determinants for type 1 diabetes in Japanese and this population shows a disease association with the **OFT3+** allele. In this Caucasoid population, the positive association of OFT3+ and disease is due to linkage disequilibrium with a majority of the high-risk **HLA-DR3** haplotypes; the **HLA-DR4,** -DQB1*0302 and **-DR9, -DQB1*0303** haplotypes are almost equally split by the OFT3 polymorphism. Since **HLA-DR3** is rare in Japanese populations, the association with the **OFT3+** allele cannot be due to linkage disequilibrium with this **HLA-DR** type. The implication is that the positive association with **OFT3+** in Japanese is due to linkage disequilibrium with other class II alleles found on **HLA-DR4 and -DR9** haplotypes or that other loci (perhaps class I) are involved in disease susceptibility.

7.4.5. Addendum.

The human OFT3 gene has recently been mapped and is located approximately 90 kilobase pairs telomeric to the class I **HLA-C** locus (Dr J. Ragoussis, personal communication).

CHAPTER 8

CONCLUDING REMARKS AND FUTURE WORK

The successful identification of genes responsible for monogenic disorders, including cystic fibrosis and Duchenne muscular dystrophy, has focused attention on the genetics of so-called complex diseases. The essential feature of these disorders is that both environmental and genetic influences are necessary for disease expression. Complex diseases, which include type 1 diabetes, hypertension, ischaemic heart disease and Alzheimer's disease, are common and account for a major part of morbidity and mortality in the Western world. Elucidation of susceptibility (and resistance) loci will allow identification of individuals at high risk of these conditions; this should facilitate the study of environmental factors and hence the development of preventative strategies. Ultimately, characterisation of genes and their expression may lead to new therapeutic approaches. Technological advances in the fields of molecular biology and statistics suggest that it is now feasible to approach complex disease analysis in a logical and structured fashion.

In many ways, the genetic analysis of type 1 diabetes will be a model for the study of complex diseases. It is clear that disease heterogeneity can be reduced by the use of strict clinical criteria, in a way that is probably not possible in other conditions (for example, psychiatric disorders). Further, two linked susceptibility gene regions have now been identified. These features are in addition to the considerable amount of knowledge concerning the immunology of type 1 diabetes and the availability of animal models. Finally, a large ethnically homogeneous genetic resource is now available to facilitate the necessary experimentation and, if the scientific will exists, a world-wide collaborative effort.

This thesis describes the establishment, validation and use of the largest genetic resource of its kind. Although a large collection of EBV-transformed cell lines from

multiplex families with type 1 diabetes has been regarded as a necessity, it is of note that no salaries were specifically funded to establish this resource. Concerning validation, I find no evidence to suggest that multiplex diabetics differ from sporadic cases in the UK population. Genetic susceptibility at HLA and INS is confirmed in this data set but evidence of parental effects on HLA-mediated susceptibility are not seen, implying caution in their interpretation. The expected influence of age-at-onset on genetic susceptibility is confirmed and an age effect on sex reported. The first 100 multiplex families of the B.D.A. - Warren Repository are now being used in North America by groups led by David Owerbach and Leigh Field and an exchange of data has already taken place. This is promising for the future of type 1 diabetes research.

A larger resource will undoubtedly be required to allow division of pedigrees into potentially more homogeneous subgroups. This will be performed using data such as HLA and INS genotypes, clinical details, family history and possibly humoral markers (plasma is available should persistent antibody responses be discovered). The monies to continue blood sample collection have been made available by the Wellcome Trust through funding of a complex disease initiative based in Oxford. The Steering Committee set up during this project will continue to oversee the collection and will be involved in the distribution of cell lines from the expanding resource.

As far as our own work on the resource is concerned (S.C.B., A.H.B. and J.A.T.), there are two major directions this will take. Initially it is important to examine those associations and linkages for which there is preliminary data (for example, T-cell receptor β chain gene, immunoglobulin genes, CD3ε), as well as new candidate gene regions generated from comparative mapping. In the near

future, automised technologies should enable exclusion mapping of the human genome using microsatellite markers. This latter stategy will be based on the successful Genethon programme in ***France(Weissenbach, Gyapay et al. I992)*** and again will be funded by the Wellcome Trust initiative.

The analysis of complex diseases is **now** one of the highest priorities in basic medical research. Type 1 diabetes is at the forefront of this research effort and will be a major beneficiary if the current programmes are successful. Indeed, given the (apparent) propensity of this condition to genetic analysis, **type** 1 diabetes may be regarded as the crucible for the 'new genetics'.

ABBREVIATIONS

ABBOS	17 amino-acid sequence (position 152-168) of the BSA molecule.
Ala-57	Alanine (at position 57 of the HLA-DQβ chain)
Apo E	Apolipoprotein E
Asp-57	Aspariate (at position 57)
ASP	Affected Sibling Pairs
BB	Bio-breeding (rat)
BCG	Bacillus Calmette-Guerin (anti-TB vaccine)
B.D.A.	British Diabetic Association
BOX	Bart's Oxford Study
B.P.A.	British Paediatric Association
BSA	Bovine Serum Albumin
CMV	Cytomegalovinis
CY-A	Cyclosporin A
df	Degrees of Freedom (for χ^2 test)
DNA	Deoxyribonucleic Acid
E.C.A.C.C.	European Collection of Animal Cell Cultures
EBV	Epstein Barr Virus
EMBL	European Molecular Biology Laboratory
FPIR	First Phase Insulin Response
GABA	y-aminobutyricacid
GAD	Glutamic acid dehydroxelase
G.A.W.5	5th Genetic Analysis Workshop
GCK1	Glucokinase gene microsatellite
Gm	Immunological heavy chain
H.B.D.I.	Human Biological **Data** Interchange
HLA	Human Leukocyte Antigen
H-2	Major Transplantation Complex (Mouse)
HRAS	c-Ha-ras-1 proto-oncogene
IAA	Insulin Autoantibodies
I-Aß	Murine homologue of HLA-DQβ
IBD	Identity-by-Decent
ICA	Islet Cell Antibodies
I-E	Murine homologue of HLA-DR
IGT	Impaired glucose tolerance

INS	Insulin gene region
Ir	Immune Response
IVGTT	Intravenous Glucose Tolerance Test
JDFU	Juvenile Diabetes Federation Units
Km	Immunological κ light chain
LCMV	Lymphocytic Choriomeningitis Virus
LDL	**Low** density lipoprotein
LOD	'log of the odds'
MHC	Major Histocompatibility Complex
MHV	Mouse Hepatitis Virus
MODY	Maturity Onset Diabetes of the Young
MZ	Monozygotic
Mr	Molecular weight
NIMA	Non-inherited **maternal** antigen
NIPA	Non-inherited **paternal** antigen
N.I.H.	National Institute of Health
NOD	Non-Obese Diabetic (Mouse)
OCT3	Octomer Binding Protein **3** gene
OFT3	Human Octomer Binding Protein **3** gene
OGTT	*Oral* Glucose Tolerance Test
O.R.P.S.	Oxford Regional Prospective Study
PCR	Polymerase Chain Reaction
PBL	Peripheral Blood Lymphocyte
PIC	Polymorphism Information Content
PPV	Positive Predictive Value
RFLP	Restriction Fragment Length Polymorphism
Ser-57	Serine (at position 57)
SPF	Specific-pathogen Free
TNF	Tumour Necrosis Factor
TCR	T-cell receptor
TCRA	T-cell receptor *α* chain gene
TCRB	**T-cell** receptor β chain gene
TMA	Thyroid Microglobumin Antibody
VLDL	Very low density lipoprotein

VNTR	**Variable Number Tandem Repeat**
Wor	**Worstar** (rat)
YAC	**Yeast Artificial Chromosome**

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