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A Study of the Molecular Immunogenetics of Type 1 Diabetes in Man

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To

Mariam, Abdul-Ghani and Ibrahim

And to

Farzaneh and Kawthar

"If we stop asking the question 'what breaks tolerance' and begin to focus on the vast array of possibilities for each disease, we may begin to understand their multigenic nature" (Matzinger, 1998).

ABSTRACT

A Study of the Molecular Immunogenetics of Type 1 Diabetes in Man

Mohamed Mirza Jahromi

Type 1 diabetes is caused by immune destruction of pancreatic β cells. There is increasing evidence that genes outside the MHC region contribute to the pathogenesis of type 1 diabetes. Cytokines due to their role in immune regulation seem to play a crucial role in the pathogenesis of the disease. Three hundred and eight patients with type 1 diabetes and 150 normal controls were genotyped for polymorphism in the genes for IFN-γ, IL-4, IL-6, and TGF-β1. All assays employed in this study were PCR based. The IFN-γ CA repeats was an octa-allelic repeat and the 3 / 3 genotype showed a significant association with type 1 diabetes (p=0.0001). The IL-4 C (-590) T polymorphism did not show a significant association with type 1 diabetes. The GG genotype of G (-174) C of the IL-6 gene polymorphism showed a strong association with the susceptibility towards type 1 diabetes (p= 0.002). The TC genotype of the TGF-β1 T (+869) C polymorphism also showed a significant association with type 1 diabetes (p= 0.003). The association of the 3 / 3 genotype of the IFNG CA repeats and no association of IL-4 C (-590) T polymorphism may support the idea of dominance of the TH1 cytokine profile and type 1 diabetes suggesting a cell mediated disease. The IL-6 G (-174) C result attests an existing hypothesis of the important role of IL-6 in the onset of type 1 diabetes and its development. Immunosuppression of the TGF-β1 may have been initiated after deviation of the TH1/TH2 cytokine milieu. The GC of the IL-6 G (-174) C and the TC of the TGF-β1 T (+869) C showed strong association with diabetic nephropathy. Haplotype studies showed that cytokine function might be as a result of a cytokine network rather than individual cytokines. Further, the genetic susceptibility may be influenced not only by genetic composition but by the gender of patients as well as age at onset of type 1 diabetes. In conclusion these results suggest a contribution of the IFNG CA repeats, the TGF-β1 T (+869) C, and the IL-6 G (-174) C to the genetic susceptibility of type 1 diabetes and may have future therapeutical values.

LIST OF CONTENTS .....Page No

LIST OF TABLES..... Page No

LIST OF FIGURES.....Page No

LIST OF ABBREVIATION

γ^{32P} dATP	gamma phosphate-32 deoxyadenosine
µL	Microlitre
µM	Micromolar
APC	Antigen presenting cell
ARMS	Amplification refractory mutation system
ASA	Allele specific amplimer
BB	Bio-breeding
BDA	British diabetic association
bp	Base pair
BP	Blood pressure
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complimentary Deoxyribonucleic Acid
cM	Centimorgan
CMP	Cows milk protein
cpm	Counts per minute
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DC	Diabetic complication
DCCT	Diabetes control and complications trial
DEC	Dendritic epidermal cell
DIG	Digoxygenin
DN	Diabetic nephropathy
DNA	Deoxyribonucleic Acid
dNTP	2'Deoxyribonucleotide 5' triphosphate
DNu	Diabetic neuropathy

DR	Diabetic Retinopathy
EAE	Experimental allergic encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylene Diamine Tetra Acetic acid
EDTA	Ethylene diamine tetra acetic acid
ESRD	End-stage renal disease
g	Gram
GABA	γ amino butyric acid
GAD	Glutamic acid decarboxylase
GADA	Glutamic acid decarbocylase
GLUT1	Brain type glucose transporter
GM-CSF	Granulocyte/macrophage colony stimulating factor
HbA1c	Glycated haemoglobin
HCl	Hydrochloric Acid
HLA	Human Leukocyte antigen
HSP	Heat shock protein
IA2	Tyrosene phosphate
IAA	Insulin autoantibodies
ICA	Islet cell antibody
ICAM	Intercellular adhesion molecule
IDDM	Insulin-dependent diabetes mellitus
IDF	International diabetes federation
IFN	Interferon
IFNG	Interferon-gamma gene
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin

IMS	Industrial Methylated spirit
INS	Insulin gene
Kb	Kilo-base
kDa	Kilo-dalton
L	Litre
Mϕ	Macrophages
MCP	Monocyte chemotactic peptides
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mmHg	Millimetres of mercury
MODY	Maturity onset diabetes of the young
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
n	Number of subjects
NaCl	Sodium chloride
NC	Normal controls
NIDDM	Non-insulin dependent diabetes mellitus
NK	Natural killer
NOD	Non-obese diabetic
ns	Not significant
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-SSOP	Sequence specific oligonucleotide probing
PDGF	Platelet derived growth factor
RFLP	Restriction fragment length polimorphism
RSPs	Restriction site polymorphisms

SD	Standard deviation
SLE	Systemic lupus erythamatosus
SNS	Self-non-self
SSC	Standard saline citrate
SSCP	Single stranded conformational polymorphism
SSO	Sequence specific oligotyping
SSP	Sequence specific primers
TAP	Transporter associated with antigen processing
TAP	Transporters associated with antigen processing
TBE	Tris-borate acid-ethylene diamine tetra acetic acid
Tc	Cytotoxic T-cell
TCGF	T-cell growth factor
TCR	T cell receptor
TDT	Transmission disequilibrium test
TE	Tris EDTA buffer
TEMED	n,n,n '' Tetramethylediamine
TGF	Transforming growth factor
TH	T helper cell
TMAC	Tetra-methyl-ammonium chloride
TMED	Tetra-methyl-ethylene-diamine
TNF	Tumour necrosis factor
UK	United Kingdom
USA	United states of America
UV	Ultraviolet
UV	Ultraviolet
V	Volts
vs	Versus

VNTR	Variable number of tandem repeats
W	Watts
WHO	World health organisation
χ^2	Chi square

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

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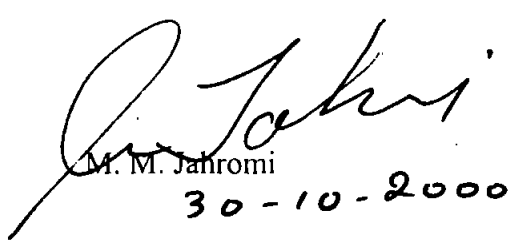

M. M. Jahromi

30-10-2000

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PREFACE

About the Author- I did my BSc at my home country, Bahrain, majoring in Biology and Chemistry as a minor at the University of Bahrain. I then joined the quality control laboratories in Bahrain Petroleum Company (Bapco) to gain work experience. Within less than 3 years I reached the level of quality control laboratory shift supervisor with full responsibilities for the Laboratory quality control operation as well as fire and safety of the area during the shift. I also was responsible for a group of 6 technicians and 3 chemists who worked under my supervision. After 5 years working for Bapco I decided to leave the Company to continue my studies. From September 1995 to June 1997 I have been a MSc student at the University of Manchester. From June 1997 to the present I have been a PhD student at the University of Plymouth.

PUBLICATIONS

Abstracts &Presentation:

Jahromi MM, Millward BA, Demaine AG. Strong Association of the G (-174) C polymorphism in the human interleukin-6 promoter region with type 1 diabetes. BDA Ann Prof Meet 2000; **17/1**: A46, pp 15.

Jahromi MM, Millward BA, Demaine AG. Investigation of interferon-γ and interleukin-4 gene polymorphism in type 1 diabetes. Diabatologia 1999; **42/1** (Suppl) A90.

Jahromi MM, Millward BA, Demaine AG. Polymorphisms of interferon-γ gene are associated with type 1 diabetes. Diabetologia 1998; **41/1**: A79.

Manuscripts arising from this work

Nicola Cartwright, Andrew Demaine, **Mohamed Jahromi**, Hilary Sanders, and Edward Kaminski. A study of cytokine protein secretion, frequencies of cytokine expressing cells and IFN-G gene polymorphisms in normal individuals. J Transplantation 1999; 68/10: 1546-1552.

Mohamed M Jahromi, B Ann Millward, Andrew G Demaine. Analysis of T (+869) C polymorphism at codon 10 of TGF-$\beta_1$gene in type 1 diabetes pathogenesis (to be submitted to Diabetes; 2000).

Mohamed M Jahromi, B Ann Millward, Andrew G Demaine. A polymorphism in the promoter region of the gene for interleukin-6 is associated with the susceptibility to type 1 diabetes (accepted for publication in October 2000; J Interferon and Cytokine Research).

Mohamed M Jahromi, B Ann Millward, Andrew G Demaine. A CA repeat polymorphism of the interfron-γ gene is highly associated with type 1 diabetes: IFNG and IL-4 gene polymorphism and type 1 diabetes. J Interferon and Cytokine Research 2000; **20**: 187-190.

In collaboration with the combined laboratory in Derriford Hospital, Pancreatic Research Group of the University of Plymouth as well as Molecular Medicine in Edinburgh Royal Infirmary few projects were conducted manuscripts of of which have been published or in preparations.

1 INTRODUCTION

Immunogenetics is that branch of medical science which deals with the genetics of the components which together form the immune system: the major histocompatibility complex (MHC), cytokines, cytotoxic T-cells (T_C), and autoantibodies. Immunogenetics is the study of immunity to diseases and the relationship to the genetic make-up of the immune response elements with regard to the pathogenesis of immune regulated diseases.

A major feature of the immune system is its ability to inflict damage upon cells. Therefore, it must confine this activity to cells that are a potential threat, such as cells in the body that have become infected or neoplastic, whilst remaining unresponsive or tolerant to the normal cells of the body. As the regulation of the immune system is very important in the context of human disease, a disordered response may result in a breakdown of tolerance leading to autoimmune disease or immune deficiency.

1.1 Categories of immune diseases

In general, autoimmune diseases can be divided into two categories: organ-specific and systemic autoimmune disease. In organ-specific autoimmune disease, the immune response is directed towards a target antigen unique to a single organ or gland, so that the manifestations are largely limited to that organ. In systemic autoimmune disease, the response is directed towards a broad range of target antigens and involves a number of organs and tissues (Andre et al., 1996).

1.1.1 Organ-specific auto-immune diseases

Damage to the target organs in organ-specific auto-immunity can occur as the result of direct cellular damage by humoral or cell-mediated mechanisms or by stimulating or blocking autoantibodies. In this instance, the immune system usually targets discrete antigens present on the tissue. Gradually, the cellular structure of an affected organ is replaced by connective tissue, hence, the function of the organ declines. Type 1 diabetes mellitus and Hashimoto's thyroiditis are good examples of this type of autoimmune disease (Andre et al., 1996).

1.1.2 Systemic auto-immune diseases

Autoimmune diseases with systemic manifestations reflect a generalised defect in immune regulation that results in hyperactive T-cells and B-cells. Tissue damage is widespread, both from cell-mediated immune responses and from direct cellular damage caused by autoantibodies or by accumulation of immune complexes. One of the best examples of a systemic autoimmune disease is systemic lupus erythematosus (SLE). Rheumatoid arthritis and multiple sclerosis (MS) are also important systemic autoimmune diseases (Andre et al., 1996).

1.2 Elements of the immune response

Functional or structural damage to self-cellular components caused by the immune system is not fully understood. Tolerance to self may be broken because normally occurring autoimmunity is not controlled, either due to deficient elimination or lack of functional inactivation or inhibition of autoreactive lymphocytes in the thymus or in the periphery (Kroemer and Martinez, 1992). However, there is no evidence to suggest that the initiating events in the immune response are different whether the antigen is self or non-self derived. Specific recognition of antigens is dependent on several cells, the following are the most important:

1.2.1 Lymphocytes

Lymphocytes are wholly for the specific immune recognition of pathogens, so they initiate adaptive immune responses. All lymphocytes are derived from bone-marrow stem cells, but T cells then develop in the thymus, while B cells develop in the bone marrow.

1.2.1.1 B cells

Each B cell is programmed to encode a surface receptor specific for a particular antigen. Having recognised its specific antigen, the B cells multiply and differentiate into plasma cells, which produce large amounts of the receptor molecule in a soluble form that can be secreted, termed as antibody. B cells are essentially little antibody factories (Park and Weigert, 1995), able to switch on high-rate synthesis and secretion of antibody molecules when stimulated by recognition of the "right" antigen.

Recognition and response in B cells are perfectly coordinated, because their surface antigen-receptor has antibody the same as they will secrete when stimulated. Thus only those antibody molecules that are made which can bind to the stimulating antigen and help in its disposal will be produced; thus the production of unwanted antibodies is avoided. It is the formation of plasma antibody that forms the foundation of the so called humoral immune response (McPherson et al., 1999).

1.2.1.2 T cells

Resting T cells look very much like resting B cells, but when they respond the difference becomes apparent. T cells have no specificity towards particular antigens. There are several different types of T cells, and they have a wide variety of actions. One group interacts with B cells and helps them to divide, differentiate and make antibody. Another group interacts with mononuclear phagocytes and helps them destroy intracellular pathogens. These two groups of cells are called T-helper (TH1, TH2) cells which carry the CD4 marker and mainly helps or induces immune responses (Chen et al., 1999). A third group of T cells is responsible for the destruction of host cells which have become infected by viruses or other intracellular pathogens. This kind of action is called cytotoxicity and these T cells carry the CD8 marker and are predominantly cytotoxic (T_C). $CD4^+$ T cells recognise antigen in association with the MHC class II molecules, whereas $CD8^+$ T cells recognise antigens in association with the MHC class I molecules (Croft et al., 1994).

The T cell receptor has similarities to the antibody molecule, but with important differences. Unlike the antibody on the B cell, which can be thought of as a sample of what that cell can produce, the T cell receptor can be thought of as a hand reaching out to feel the surface of neighbouring cells, with some fingers devoted to contacting MHC molecules and others probing for peptide bound to them (Yawaker et al., 2000).

1.2.1.2.1 T-cell receptor

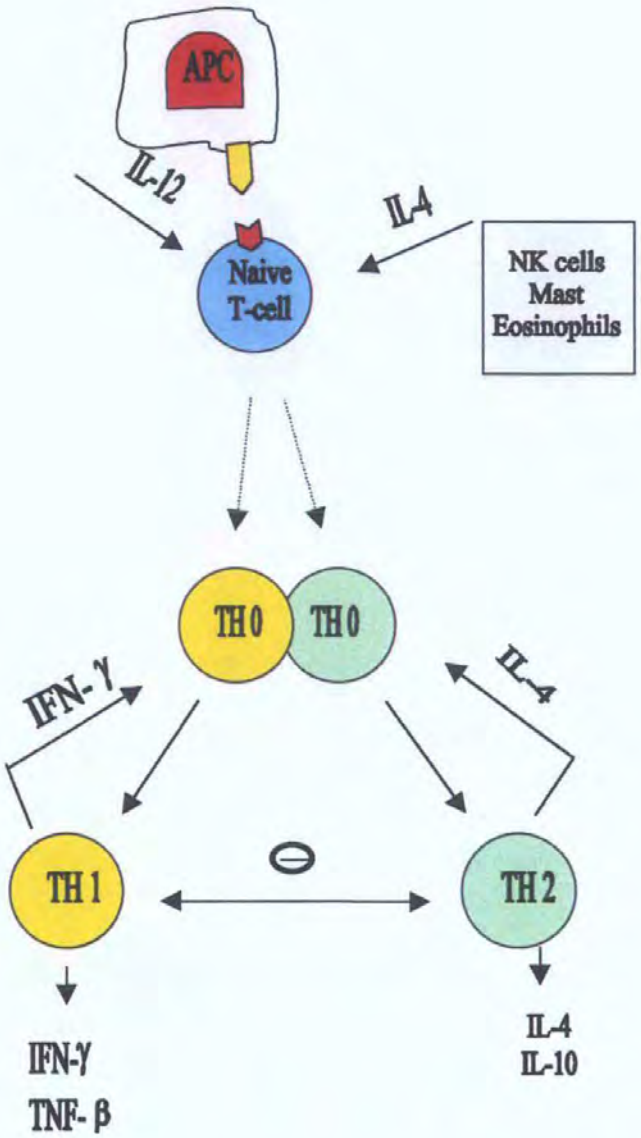
The definitive T-cell lineage marker is the TCR. There are two types of TCR: one is a heterodimer of two disulphide-linked polypeptides α and β; the other is structurally similar, but consists of γ and δ polypeptides . Both receptors are associated with a set of five polypeptides, the CD3 complex, and together form the T-cell receptor complex

(TCR-CD3). Approximately 90-95% of blood T cells are αβ T cells and only 5-10 % are γδ T cells. The αβ T cells are subdivided into two distinct non-overlapping populations: CD4 and CD8 (Housset et al., 1997).

The CD4 further divides into TH1 and TH2 subsets. Different factors may be responsible for this differentiation. Cytokines, which are produced and secreted by T-helper cells, might be responsible for the distinction of CD4 differentiation. The two subsets of CD4 T cells, TH1 and TH2 cells, have very different functions: TH2 cells are the most effective activators of B cells, especially in primary responses, whereas TH1 cells are crucial for activating macrophages. It is also clear that the two CD4 T-cell subsets can regulate each other. Once one set becomes dominant, it is often hard to shift the response to the other subset. One reason for this is that cytokines from one type of CD4 T cell inhibit the activation of the other. Thus, IL-10, a product of TH2 cells, can inhibit the development of TH1 cells by acting on the antigen-presenting cell, whereas IFN-γ, a product of TH1 cells, can prevent the activation of TH2 cells (Figure 1.01). Such cross-regulatory function of TH1 and TH2 maintains the balance between TH1/TH2 (Chen et al., 1999).

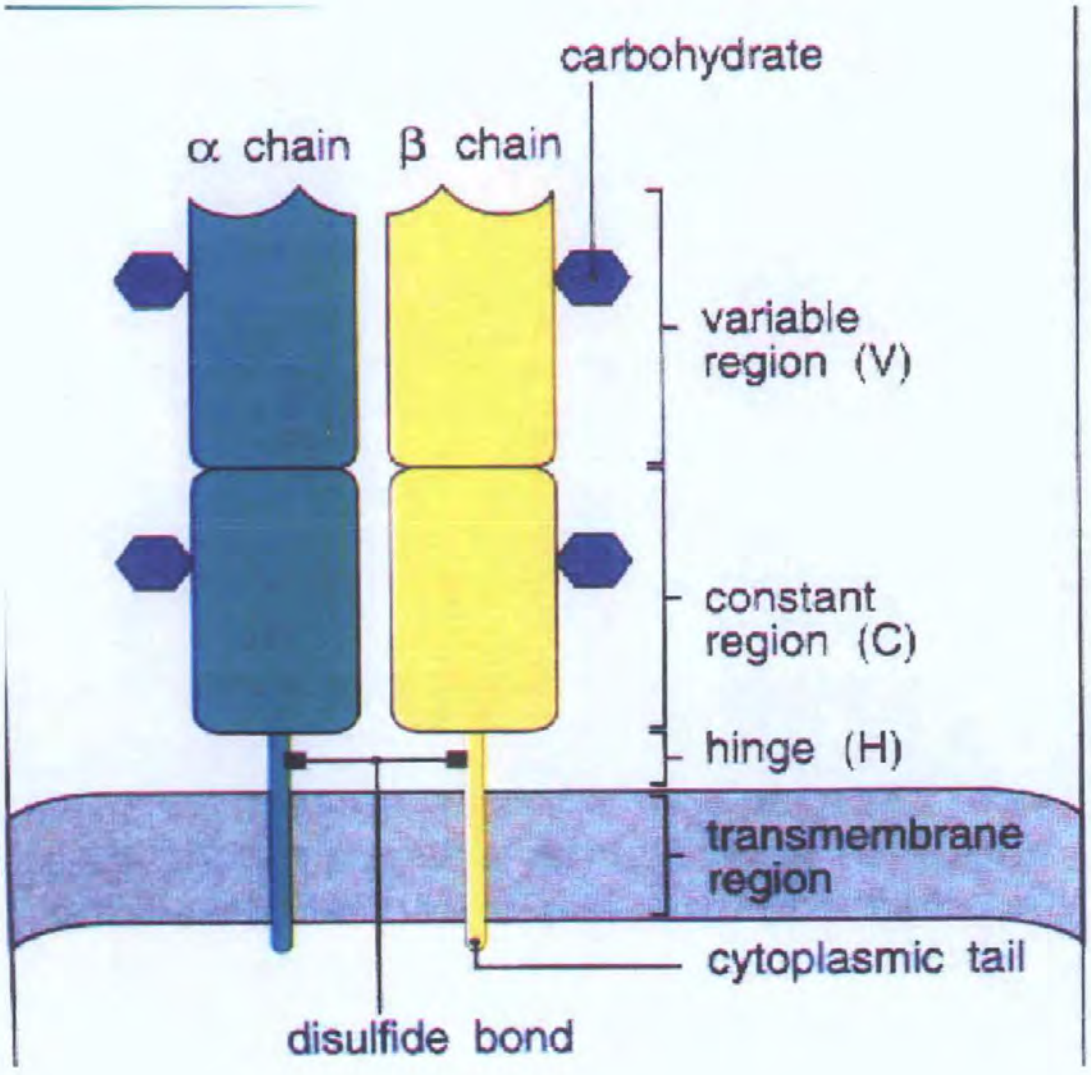
As might be expected from their function as highly variable antigen-recognition structures, T cell receptors are closely related to antibody molecules in structure (Zamoyska, 1998). Like immunoglobulins, TCR are encoded by V, D, and J gene segments, which rearrange to form a complete variable-region exon. There are, however, important differences between TCRs and immunoglobulins that reflect the unique features of antigen recognition by the TCR (Zamoyska, 1998) (Figure 1.02).

Figure 1.01: Illustration of CD4 T cell differentiation



Generation of TH1 and TH2 cells from naïve T cells. Commitment to either subset is determined by the microenvironmental conditions that predominate at the time of initial stimulation (Adapted from Lafaille, 1998 with modification).

Figure 1.02: Illustration of structure of T-cell receptor



The T cell receptor heterodimer is composed of two transmembrane glycoprotein chain α and β. The external portion of each chain consists of two domains, resembling immunoglobulin variable and constant domains, respectively (Adapted from Janeway, Travers, Walpoert, and Capra, 1999).

1.2.2 Major histocompatibility complex

The molecules that display peptide antigens to T cells are membrane glycoproteins encoded in a cluster of genes bearing the cumbersome name of major histocompatibility complex. The human MHC molecules were first discovered as the result of attempts to use skin grafts from donors to repair badly burned pilots and bomb victims during World War II (Klein, 1986). The patients rejected the grafts, and eventually genetic experiments on inbred mice led to the identification of genes causing the rapid rejection of a skin graft between mice that differed only at these genetic loci and at no other. Because they control the compatibility of tissue grafts, these genes became known as "histocompatibility genes". Later, it was found that several closely linked genes specify histocompatibility, which led to the term major histocompatibility complex (Marsh, 1998[a];1998[b]). The MHC is referred to as the HLA in human, as the H-2 in mice, and as the RTI in rat (Klien, 1986).

The function of the MHC molecules is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells (Fremont et al. 1996). The consequences of such presentation are almost always deleterious to the pathogens. Virus-infected cells are killed, macrophages are activated to kill bacteria in intracellular vesicles, and B cells are activated to produce antibody molecules capable of eliminating or neutralising extracellular pathogens (Fremont et al.,1996). Thus, there is strong selective pressure in favour of any pathogen that can mutate its structural genes to escape presentation by the MHC molecules.

Two separate properties of the MHC make it difficult for pathogens to evade immune responses in this way. First, the MHC is polygenic. There are several MHC class I and MHC class II genes, encoding proteins with different ranges of peptide-binding

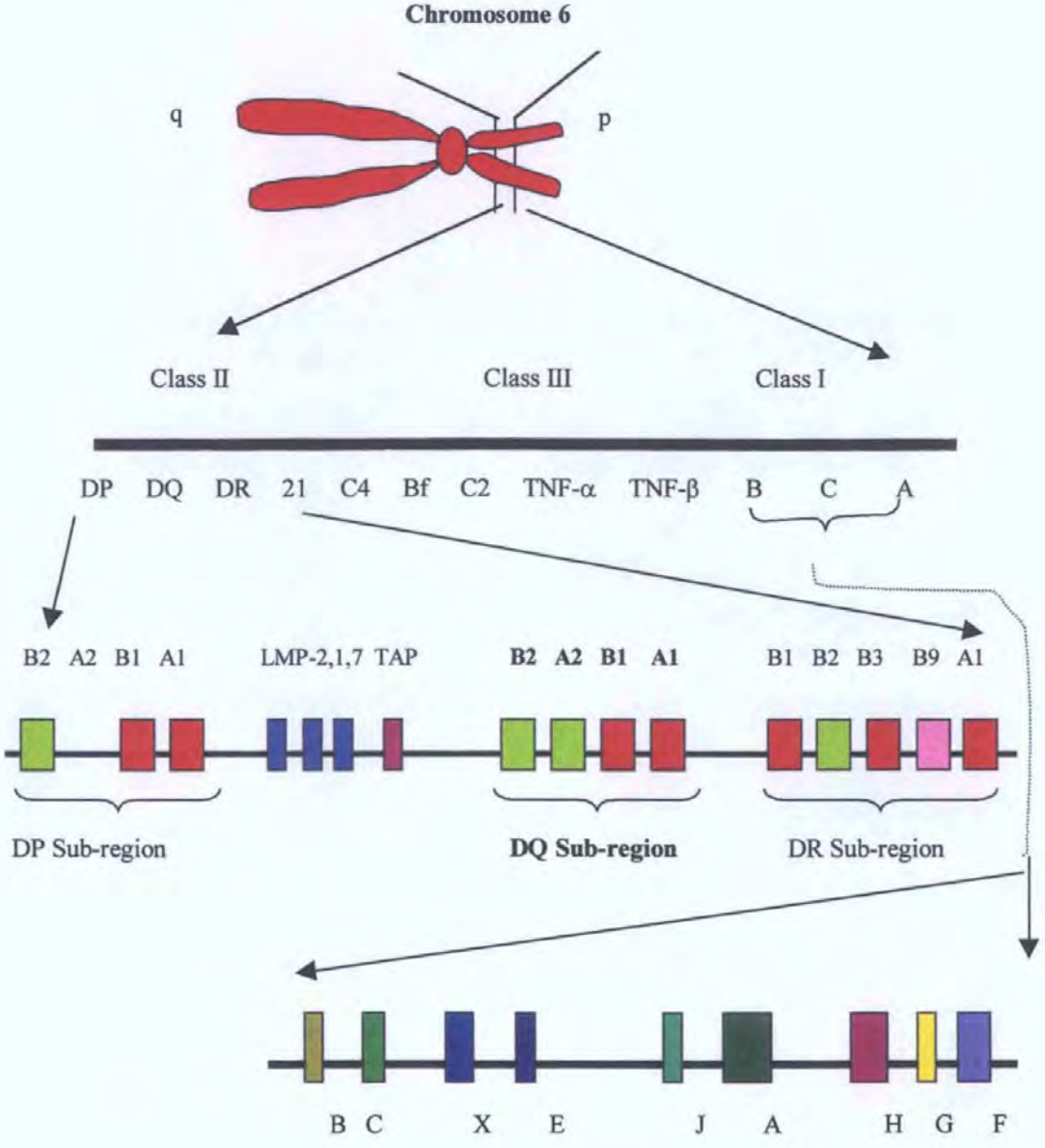
specificities. Second, The MHC is highly polymorphic. There are multiple alleles for each gene. The MHC genes are, in fact, the most polymorphic genes known.

1.2.2.1 The structure of the MHC molecules

The MHC extends over approximately 4 centimorgans of DNA, or about 4×10^6 base pairs, and contains more than 200 genes in humans (Herberg et al., 1998). As work continues to define the genes within and around the MHC, both its extent and numbers of genes involved are likely to grow. Recent studies suggest that the MHC may span at least 7×10^6 base pairs (Herberg et al., 1998). The genes encoding the α chains of MHC class I molecules and the α and β chains of MHC class II molecules are within the complex. The genes for β_2-microglobulin and the variant chain lie on separate chromosomes. Figure 1.03 shows the general organisation of these genes in the MHC in man. The particular combination of MHC alleles found on an individual chromosome is known as a MHC haplotype (Herberg et al., 1998; Marsh, 1998[b]).

There are three class Iα-chain genes in humans, called HLA-A, -B, and –C. There are also three pairs of MHC class II α- and β-chain genes, called HLA DR, -DP, and –DQ. However, in many haplotypes the HLA-DR cluster contains an extra β-chain gene whose product can pair with the DRα-chain. This means that the three sets of genes give rise to four types of MHC class II molecules (Herberg et al., 1998; Marsh, 1998). All the MHC class I and class II molecules are capable of presenting antigens

Figure 1.03: A simplified map of the MHC region on the short arm of chromosome 6



on the short arm of the chromosome 6, showing the major genes of class II, III, and I. The class II is relatively well studied. Among the genes contained in each sub-region, there are some that encode functional molecules (in red) such as DPB1 and DPA1, genes that are not functioning i.e. pseudogenes or not yet characterised (in green). The DRB9 gene has not been well studied yet but seems to be able to encode for a non-functionally stable protein chain. Transporter associated with antigen processing LMP and TAP are all functional and shown in blue and purple colour respectively. TNF-α and β genes among the rest of class III genes are shown next to the genes for class II. The class I genes are shown next to class III, and are not been well studied (Williams and Pickup, 1998; Friday et al., 1999 with slight modification).

to T cells (Fremont et al., 1996). Each protein binds to a different range of peptides, the presence of several loci meaning that any one individual is equipped to present a much broader range of different peptides than if only one MHC protein of each class were expressed at the cell surface (Fremont et al., 1996).

The MHC class I and MHC class II molecules are cell-surface glycoproteins closely related in overall structure and function, although they have different subunit structures. Both molecules have two domains that resemble immunoglobulin domains, and two domains that fold together to create a long cleft that is the site where peptides bind. However, differences in their structures allow them to serve distinct functions in antigen presentation, binding peptides from different intracellular sites and activating different subsets of T cells (Smith et al., 1996;Murthy et al., 1997).

The MHC class I structure is outlined in Figure 1.04. The MHC class I molecules consist of two polypeptide chains, an α or heavy chain encoded in the MHC, and a smaller non-covalently associated chain, β2-microglobulin, which is not encoded in the MHC (Smith et al., 1996). The most remarkable feature of the MHC class I molecules is the structure of the α1 and α2 domains, which pair to generate a cleft on the surface of the molecule that is the site of peptide binding (Smith et al, 1996; Marsh, 1998[b])

The MHC class II molecules consists of a non-covalent complex of two chains, α and β, both of which span the membrane (Figure 1.05). The crystal structure of the MHC class II molecule shows that it is folded very much like the MHC class I molecule. The major differences lie at the ends of the peptide-binding cleft, which are more open in MHC class II molecules (Murthy et al. 1997; Marsh, 1998[b]). The main consequence of this is that the ends of a peptide bound to an MHC class I molecule are substantially buried

within the molecule, whereas the ends of peptides bound to MHC class II molecules are not (Murthy et al.,1997).

A third region of the MHC, denoted class III, codes for several molecules having a variety of functions, namely complement components (C4A, C4B, factor B and C2), tumour necrosis factor (TNF-α and -β), heat shock protein (HSP 70), and 21 hydroxylase genes (CYP21P and CYP21) (Marsh, 1998[b]) (Figure 103). The class III region of the MHC is less polymorphic (Marsh, 1998[a]; 1998[b]).

The two TAP genes(transporters associated with antigen processing) lie in the MHC class II region in close association with the LMP (subunit of proteasome) genes that encode components of the proteasome (Figure 1.03). The gene for tapasin, which binds to both TAP and empty MHC class I molecules, lies at the centromeric edge of the MHC (Ortmann et al., 1997; Bang et al., 2000; Ferlazzo et al., 2000; Zhang et al., 2000).

1.2.3 Antigen presenting cells

T cells do not generally bind to, nor are activated by, peptide antigen in solution, but recognise antigen only when it is physically associated with MHC molecules on the surface of another cell. The primary function of the MHC is to alert the immune system to the presence of pathogens by binding short pathogen-derived peptides and expressing them on the cell surface (Braciale et al., 1987, Germain and Margulies, 1993; Bang et al., 2000; Zhang et al., 2000).

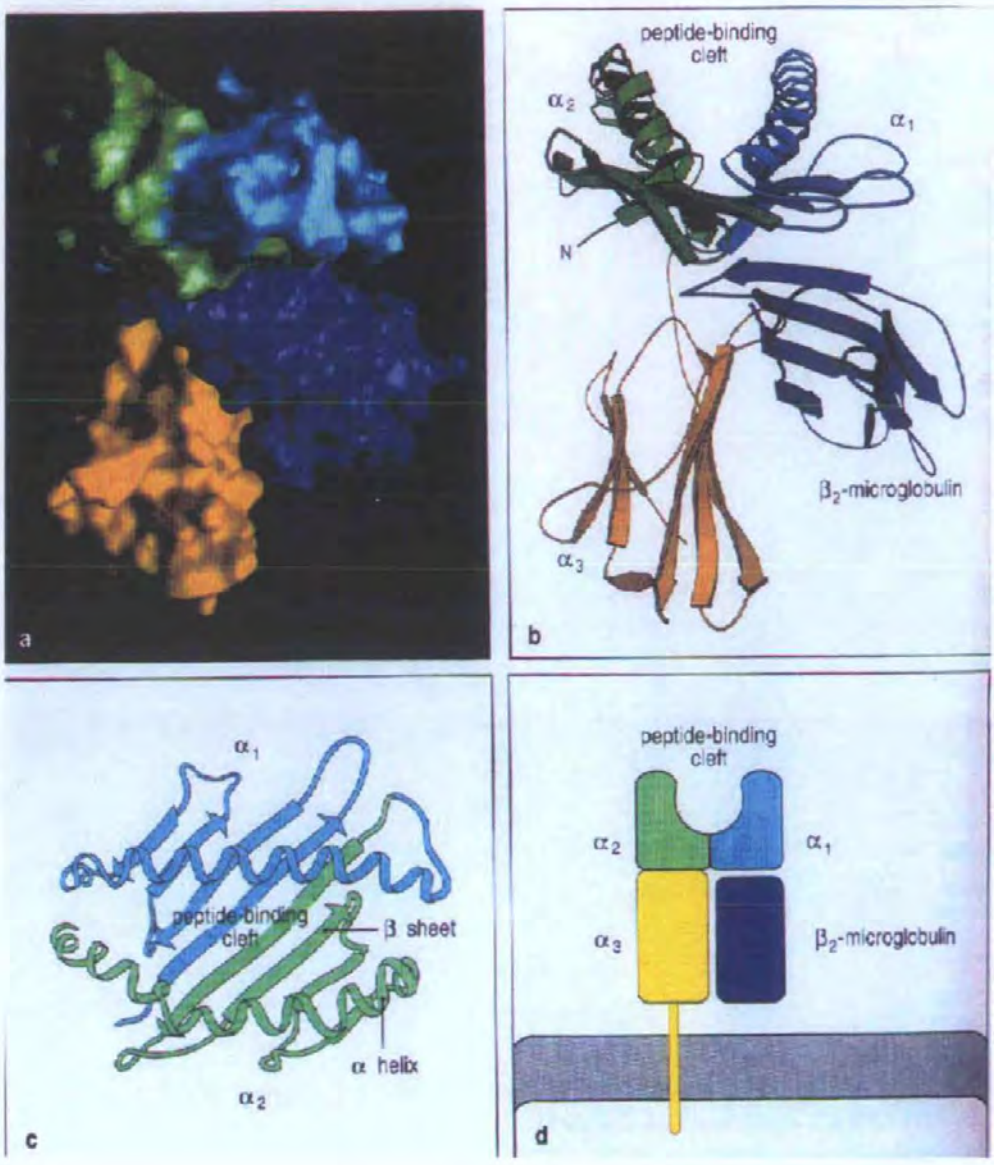
Antigen processing refers to the degradation of antigen into peptide fragments which become bound to MHC class I or class II molecules. These are critical fragments

involved in triggering T cells, the receptors on which recognise sequences of amino acids in the MHC groove, rather than the conformational determinants recognised by antibodies (Fremont et al., 1996).

The interaction between T cells and the heterogenous group of cells collectively regarded as "antigen-presenting cells" are the most extensively studied example of cell: cell interaction in the immune system. It is the first such interaction to occur after antigen challenge, and its outcome largely influences the following series of events. If a sufficient number of $CD4^+$ T-helper cells are triggered, then activation of B cells or the development of cell-mediated immunity almost certainly follows. If T-helper cells did not trigger, a form of immunological tolerance can occur, so that no proper immune response can take place (Germain and Murgulies, 1993; Bang et al., 2000).

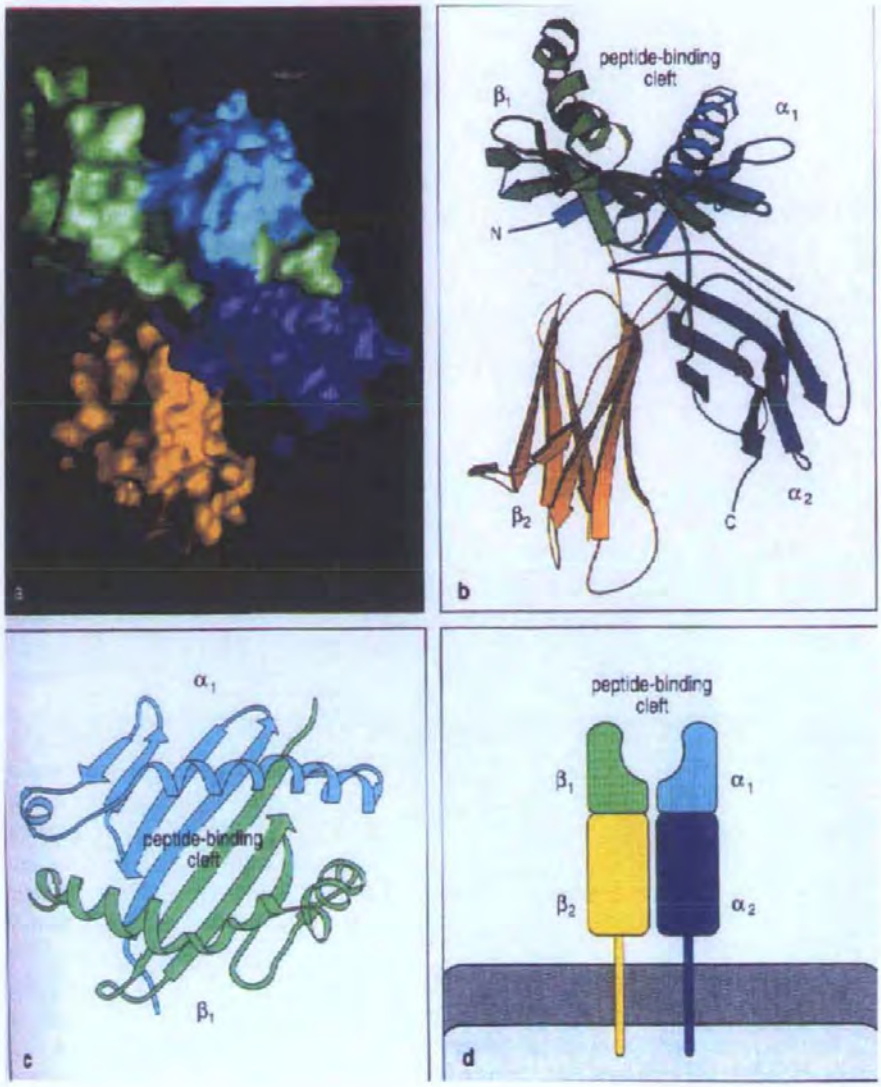
A wide spectrum of cells can present antigen, depending on how and where the antigen first encounters cells of the immune system. Monocytes/macrophages, B cells, and dendritic cells are for all professional APCs (Brodsky and Guagliardi, 1991; Liszewski et al., 1996; Zerrahn et al., 1997).

Figure 1.04: Illustration of structure of the MHC class I



The structure of MHC class I (HLA-A2), determined by X-ray crystallography. a) shows a molecular graphics, b) show a ribbon diagram, c) looking down on the molecule from above, and schematic representation of the molecule (Adapted from Janeway, Travers, Walport, and Capra, 1999).

Figure 1.05: Illustration of structure of the MHC class II



The structure of MHC class II (HLA-DR1) determined by X-ray crystallography. a) shows a molecular graphics, b) show a ribbon diagram, c) looking down on the molecule from above, and schematic representation of the molecule (Adapted from Janeway, Travers, Walport, and Capra, 1999).

1.2.4 Monocyte/macrophage

Macrophages are mature monocytes. Maturation involves an increase in size, number of organelles, secretory capacity and phagocytic activity. Class II MHC antigens are present on some of the monocytes/macrophages and are important in the presentation of antigens to T cells (Steimle et al., 1994; Yrlid et al., 2000).

The most distinguishing feature of monocytes and macrophages is that they possess receptors for cytokines such as IL-2, IL-4, IFN-γ. The functions of monocyte and macrophages can be enhanced by T-cell derived cytokines through their receptors. Such activated monocytes and macrophages also produce cytokines themselves, including IL-12, IL-1, IL-6, and TNF-α, which will be discussed in the forthcoming sections (Chen et al., 1999).

1.2.5 Dendritic cells

These are called dendritic epidermal T-cells which are a specialised class of γδT-cells present in the tissues (Banchereau and Steinman, 1998; Yrlid et al., 2000). Their function is to capture antigen and to migrate to the local lymph nodes and spleen, where they are particularly active in presenting the processed antigen to T cells (Yrlid et al., 2000). The mature dendritic cells found in lymphoid tissues are the most potent stimulators of naïve T cells. This ability is not shared, however, by the immature dendritic cells found under most surface epithelia and in most solid organs such as heart and kidneys. Dendritic cells arise from myeloid progenitors within the bone marrow, and emerge from the bone marrow to migrate via the blood to their peripheral sites (Bang et al., 2000). In these sites, they have an immature phenotype that is associated with low levels of MHC proteins, lacking expression of co-stimulatory molecules. Thus, they are not yet equipped to stimulate naïve T cells. However, they are very active in taking up

antigen by phagocytosis using receptors such as DEC (dendritic epidermal cell), whereas other extracellular antigens are taken up non-specifically by macropinocytosis, a process in which large volumes of surrounding fluid are engulfed (Banchereau and Steinman, 1998; Bang et al., 2000; Yirlid et al., 2000).

1.3 Cytokines, as molecules which signal between lymphocytes, phagocytes, and other cells of the body

Cytokines can act on the cells that produce them (autocrine), on other cells in their immediate vicinity (paracrine), or on cells at a distance (endocrine) after being carried in the blood or tissue fluids. Cytokines comprise a collection of small proteins (~20 kDa) which act on a specific receptor; many of these cytokine receptors use a particularly rapid and direct signalling pathway. They are involved in signalling between cells during immune responses (Bach, 1996; Adorini and Sinigalia, 1997; Rabinovitch, 1998; Yrlid et al., 2000).

The development of an effective immune response involves lymphoid, inflammatory, and other hematopoietic cells. The complex interactions among these cells are mediated by a group of low molecular weight proteins that are collectively designated cytokines to denote their role in cellular and molecular communication. Cytokines assist in regulating the development of immune effector cells and some cytokines possess direct effector functions of their own. Just as hormones serve as messengers of the endocrine system, so cytokines act as messengers of the immune system ((Bach, 1996; Adorini and Sinigalia, 1997; Rabinovitch, 1998; Yrlid et al., 2000). Howéver, unlike endocrine hormones, which exert their effects over large distances, the cytokines generally act locally (Fischer, 2000).

Cytokines are secreted by various cells involved in the immune response and act on target cells bearing membrane receptors that are specific for a given cytokine. Binding of a cytokine to its membrane receptor transmits a signal into the cell that leads to changes in the activation and expression of genes. Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting the proliferation of various cells or their secretion of antibodies or other cytokines (Fenton et al., 1992; Bach, 1996; Diamond et al., 2000).

The action of cytokines is pleotropic; that is, cytokines elicit different biological activities at different target cells (Walley and Cookson, 1996; Bach, 1996; Fukaura et al., 1996; Adorini and Sinigalia, 1997; Groux et al., 1997; Smith and Bluestone, 1997; Yrlid et al., 2000; Fischer, 2000,Diamond et al., 2000). Cytokines' action can also be "redundant", that is the action of certain cytokines can be mediated by another cytokine, making it difficult to ascribe a given activity to a single cytokine. Adding to the complexity of cytokine action, two cytokines may exhibit synergy; that is, the combined effect of two or more cytokines on a biological activity of an organ is greater than the additive effects of the individual cytokines (Warren, 1996). In other cases two cytokines might exhibit antagonism, the effects of one cytokine inhibiting the effects of another cytokine (Kiniwa et al., 1992; Karlsson and Ludvigsson, 2000). The attributes of pleiotrophy, redundancy, synergism, and antagonism permit cytokines to regulate biological activity in a co-ordinated interactive way. The actions of one cytokine on a responsive target cell generally regulate expression of cytokine receptors and expression of new cytokines, which in turn can modulate other cells. Thus, the antigen-specific response of a single lymphocyte can influence the activity of a variety of cells necessary to generate an effective immune response. For example, cytokines produced by activated TH cells can influence the activity of B cells, cytotoxic T-cells, NK cells, macrophages, granulocytes, and hematopoietic stem cells, thereby activating an entire network of interacting cells (Walley and Cookson, 1996; Bach, 1996; Fukaura et al., 1996; Adorini and Sinigalia, 1997; Groux et al., 1997; Smith and Bluestone, 1997; Yrlid et al., 2000; Fischer, 2000,Diamond et al., 2000; Karlsson and Ludvigsson, 2000; Fischer, 2000).

Cytokines act on receptors that can be grouped into equivalent families on the basis of their structure (Figure 1.06). These families of cytokines and their receptors are also characterised by functional similarities and genetic linkage. For instance, among the

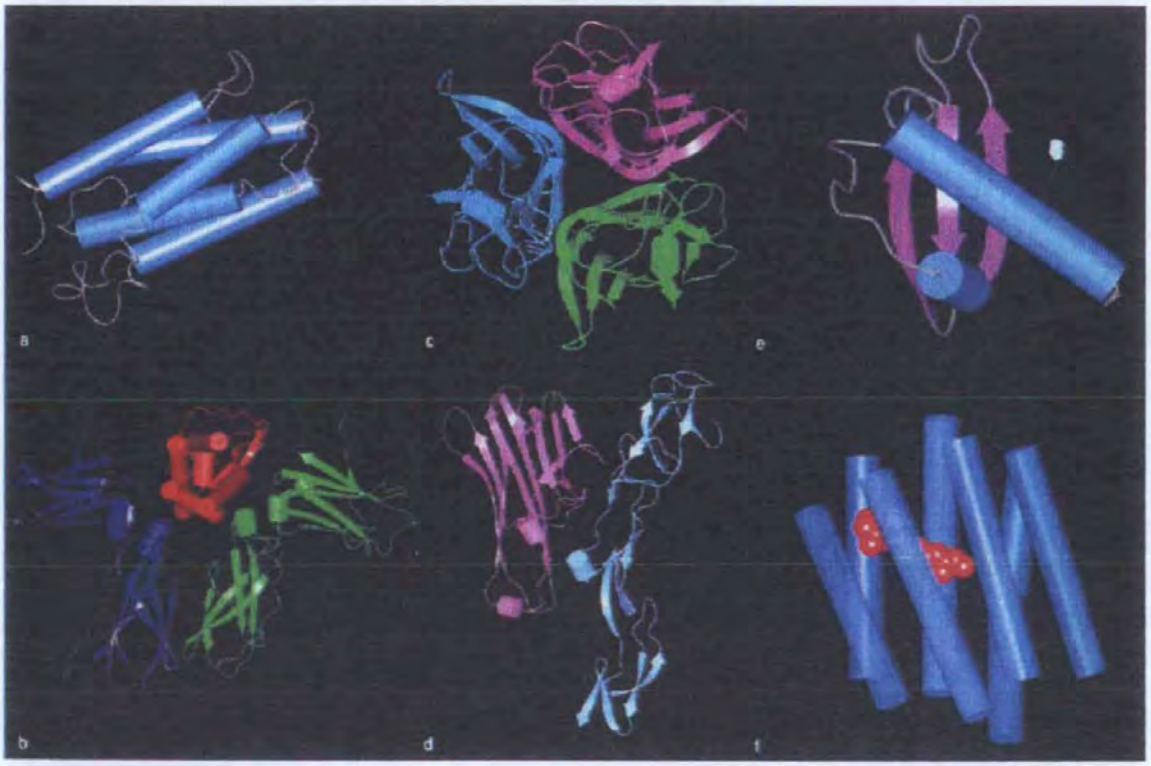
hematopoeitins, IL-3, IL-4, IL-5, IL-13 are related structurally, their genes are closely linked in the genome, and all are major cytokines produced by TH2 cells (Di Santo et al., 1995; Chomarat and Banchereau, 1997; Thompson, 1998). In addition, they bind to closely related receptors: the IL-3, IL-4, IL-5, and IL-13 share a common β chain (Chomarat and Banchereau, 1997; Thompson, 1998). Another subgroup of hematopoietin receptors is defined by their use of the γ chain of the IL-2 receptor; this is shared by receptors for the cytokines IL-4, IL-7, IL-9, and IL-15 and is called the γ common chain (γ_c) (Di Santo et al., 1995; Chomarat and Banchereau, 1997).

These specific functional effects depend on intracellular signalling events that are triggered by the binding of cytokines to their specific receptors. Cytokine signalling will be discussed in section 1.6.6.

1.3.1 Classification of cytokines

Cytokines can be divided according to their T-cell of origin. That is, correlating with two subunits of T-helper cells with contrasting and cross-regulating cytokine profiles (Mosmann et al., 1986; Bach, 1996; Groux et al., 1997). The TH1 and TH2 patterns of cytokine production were originally described in mouse $CD4^+$ T-cell clones (Mosmann et al., 1986, Cherwinski et al., 1987) and later in human T-cells (Del Prete et al., 1991).

Figure 1.06: illustration of cytokines and their receptor categories



Representatives of the main families of immunological interest except the interferons and their receptors are shown here. Cytokines are in the top row and their receptors below (adapted from Janeway, Travers, Walport, and Capra, 1999).

a) IL-4
b) IL-4 receptor
c) TNF
d) TNF receptor
e) IL-8
f) IL-8 receptor

Mouse TH1 cells produce IL- 2, IFN-γ and TNF-β, also termed lymphotoxin, whereas TH2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13.

The function of TH1 and TH2 cells correlates well with their distinctive cytokine peptides. TH1-type cytokines (IL-2, IFNγ and TNF-β) activate cell-mediated immunity, i.e. cytotoxic and inflammatory responses mediated by T-cells, NK cells and macrophages. TH2 cell type cytokines (IL-4, Il-5, IL-9, IL-10, and IL-13) activate antibody production by B-cells, particularly IgE responses, and enhance eosinophil proliferation and function. Importantly, the characteristic cytokine products of TH1 and TH2 cells are mutually inhibitory for the differentiation and effector function of the reciprocal phenotype. Thus IFN-γ selectively inhibits proliferation of TH2 cells (Mosmann and Coffman, 1989; Farrar et al., 1993; Grewall and Flavell, 1997; Delvitch and Singh, 1997; Rabinovitch, 1998; Suri and Katz, 1999; Proud'homme and Piccirillo, 2000; Karlsson and Ludvigsson, 2000), and IL-10 inhibits cytokine synthesis by TH1 cells (Fiorento et al., 1989; Bach, 1996;Adorini and Sinigalia, 1997; Rabinovitch, 1998; Matzinger, 1998; Kalsson and Ludvigsson, 2000). This cross regulation may partly explain the strong biases towards TH1 or TH2 responses during many infections in mice and man (Rabinovitch, 1998). TH1 and TH2 responses are not the only cytokine patterns possible. Immature T-cells have been termed TH0 cells (Mosmann and Coffmann, 1989), cells producing large amounts of transforming growth factor- (TGF-) β have been termed TH3 (Chen et al., 1994; O'Garra et al., 1997; Rabinovitch, 1998; Matzinger, 1998; Sedden and Mason, 1999;Proud'homme and Piccirillo, 2000). These cytokines and their characteristics are listed in Table 1.01.

However, Clerici and Shearer defined cytokines on the basis of the response they modulate rather than on the cell type that produces them. Thus type 1 cytokines (IFN-γ,

Table 1.01: Characteristics of cytokines

Cytokine	*Molecular Weight/ kDa*	*Chromosome human*	*Source*	*Target cells*
IL-1α	17	2q	Macrophages	T cells, B cells, macrophages and others
IL-1β	17	2q	Macrophages Epithelial cells	T cells, B cells, macrophages and others
IL-2	15.5	4q	TH1	T cells, B cells, NK cells
IL-4	18-20	5	TH2, basophils/mast cells	T cells, B cells, mast cells, monocytes
IL-6	21-26	7p	Macrophages Fibroblast, mast cells, TH2	T cells, B cells, hepatocytes, osteoclasts
IL-8	8-10	17q	Monocyte macrophages, T cells, fibroblasts, NK cells	Neutrophils, T cells, B cells, basophils, monocytes, endothelial cells
IL-10	17-21	1	TH2	T cells, B cells, mast cells
IFN-γ	20-25	12q	TH1, NK cells	Many cell types
TNF-α	17	6p	Macrophages, T cells, B cells	Many cell types
TNF-β	25	6p	TH2	T cells, B cells
TGF-β1	25	19q	T cells, chondrocytes, monocytes	Powerful immunosuppressant, inhibits cell growth

The major sources of major cytokines, in light of this thesis, with characteristics, target and principal effects are listed. Some cytokines have several sources and most cytokines act on more than one cell types (Derynck et al 1987; Male, Cook, Owen, Trowsdale, Champion 1996; Roitt, Brostoff, Male 1998; Janeway, Travers, Walport, Capar 1999).

IL-2, TNF-β, and IL-12) primarily stimulate cell mediated immunity; type 2 (IL-4, Il-5, IL-9, IL-10, and IL-13) primarily induce humoral immunity and diminish cellular immunity (Clerci and Shearer, 1994).

On the other hand, TGF-β triggers the IgA isotype switch (Stavnezer et al., 1997), and can help to shut down a TH1 response (Seder et al., 1998). In addition to cytokines that influence B cell maturation but are not involved in isotype switching are IFN-γ, IL-1, IL-2, IL-5, IL-6, IL-12, and IL-15 which are also involved in humoral immunity. In this thesis cytokines are classified on the basis of their origin of secretion. The biological actions of some cytokines that have been studied are as follows:

1.3.1 Macrophage derived

Cytokines that are primarily derived from macrophages are effective in promoting the cellular infiltration and damage to resident tissue, characteristics of the inflammatory response. The sequence of events leading to the activation of the cytokine cascade in the early stages of acute inflammation includes the recruitment of T and B cells by the release of cytokines by macrophages. Both proinflammatory (IL-1, IL-6, and TNF-α) and anti-inflammatory IL-ra are cytokines secreted by macrophages.

1.3.1.1 Tumour necrosis factor (TNF)

TNF comprises two proteins of 17 and 25 kilodaltons (kDa) that are primarily derived from mononuclear phagocytes (TNF-α) and lymphocytes (TNF-β) respectively (Beuller and Cerami 1989; Zhang and Tracey, 1998). In addition to mononuclear phagocytes, TNF-α may be produced by neutrophils, activated T or B cells, NK cells, endothelial cells, and smooth muscle cells. Furthermore, many cytokines including IL-1, IL-13, granulcyte-macrophage colony stimulating factor (GM-CSF), and interferon may also induce transcription of the TNF gene. The genes coding for TNF-α and -β are located in the human MHC. TNF loci have been shown to be extremely polymorphic (Zhang and Tracey, 1998). Recent studies have shown that TNF polymorphisms are associated with increased susceptibility to autoimmune disease. TNF microsatellite alleles (TNF a2, b3, d2) are significantly associated with susceptibility to SLE, RA, and type 1 diabetes. These TNF Polymorphisms have also been shown to be associated with RA in an age and gender dependent manner (Hajeer et al., 1997). Certain TNF polymorphisms are more frequent in female than in male patients (Hajeer et al., 1997). These differences are more obvious in patients stratified according to age at onset. Gender and age at onset may be important factors in the immunogenetic studies of autoimmune disease.

Cytotoxic effect of TNF-α in the destruction of β cell in diabetic patients has been intensively studied (Soldevila et al., 1990; Soldevila et al., 1991; Pociot et al., 1993).

1.3.1.2 Interleukin (IL) –1

The IL-1 family represents three peptides (IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra or IL-γ) (Oppenheim et al., 1986, Dinarello et al., 1993). One of the most prominent biologic activities of IL-1 is its ability to activate lymphocytes. T-helper cell activation requires the interaction of antigen-MHC complex with the TCR. However, an

additional non-cognate signal is also required for optimal T-cell activation and proliferation, which is generally provided by IL-1.

IL-1ra is secreted naturally in inflammatory responses. Its production is up-regulated by many cytokines IL-4, IL-13, transforming growth factor (TGF-β), and IL-1 itself. Alleles of the IL-1ra genes are associated with several autoimmune and inflammatory diseases, where they seem to have a role in the severity of the diseases rather than in susceptibility to the disease itself.

Several polymorphisms have been identified in the IL-1 genes. Hexa-allelic intron 5 microsatellite or hexa-allelic intron 6 tandem repeat polymorphisms were found in the IL-1α gene (Danarello, 1991). There are contradicting reports about their association with autoimmune diseases (Eisenburg et al., 1990; Re et al., 1994).

IL-1 ra has been shown to be polymorphic in intron 2 (Danis et al., 1995). Its relationship with different autoimmune diseases has also been investigated.

Finally, another interesting bi-allelic polymorphism in the IL-1 gene is based on a C → T transition in exon 1B of the IL-1RT I gene. It is associated with susceptibility to type 1 diabetes (Pociot et al., 1994, Bergholdt et al., 1995). Polymorphisms of IL-1R II gene have not been reported to date and only limited sequence information concerning this gene is available.

1.3.1.3 Interleukin (IL) -6

Mononuclear phagocytic cells are probably the most important source of IL-6. However, IL-6 is also produced by T and B cells, fibroblasts, keratinocytes, hepatocytes, neuroglial cells, and bone marrow stromal cells (Akina et al., 1993). Its synthesis is induced by IL-1, IL-2, and INF-γ; however, it is inhibited by IL-4 and IL-13. An important biological activity of IL-6 is its ability to stimulate the final stages of B cell

maturation. IL-6 shares several activities with IL-1, including the induction of pyrexia and the acute phase response. IL-6 may have anti-viral activity and a capacity, shared with INF-γ, to induce class I MHC expression. Like IL-1 and TNF, IL-6 may display anti-tumour effects. Finally, IL-6 is a neutrophils activator and synergies with other cytokines to support bone marrow stem cell maturation.

Interleukin- (IL-6) is a pleotropic cytokine that recently has been reported in different autoimmune diseases (Foulis et al., 1987, Hivano et al., 1989, Ohno et al., 1993, Peters et al., 1998, Fishman et al., 1998, Autsushi et al., 1999; Fernandez-Real et al., 2000).

Interleukin-6 may take part in the destruction of pancreatic β cells. The role of IL-6 in type 1 diabetes has been studied in vitro or in animals (Cavallo et al., 1991, Jiang and Woda, 1991, Cambell et al., 1991, Ohno et al., 1993). IL-6 along with IFN-γ have been reported in the late stage of insulitis of recently diagnosed patients with type 1 diabetes mellitus who died shortly after diagnosis (Foulis et al., 1987). However, IL-6, IL-4 and IL-10 have not been detected in recently diagnosed patients with type 1 diabetes (Cavallo et al., 1991, Hussain et al., 1996) or in prediabetes (Hussain et al., 1996). Urinary IL-6 is known as a useful indicator of mesangial proliferative glomerulonephritis, especially IgA nephropathy (Shikano et al., 1999). Urinary and protein levels of IL-6 have been reported in diabetic nephropathy (Shikano et al., 1999).

Recently, it has been reported that a polymorphism in the 5′ flanking region of the IL-6 gene alters the transcriptional response to stimuli such as endotoxin and IL-6 (Fishman et al., 1998).

In addition to its role in acute response, IL-6 has been recently shown to be released by adipose tissue, and release is greater in obese subjects (Mohamed-Ali et al., 1997).

Furthermore, IL-6 increases in parallel to glucose and insulin levels in the interstitial fluid of subcutaneous adipose tissue (Orban et al., 1999). This increase suggests that IL-6 might modulate adipose glucose metabolism (Orban et al., 1999; Clarke and Mohamed-Ali, 2000).

1.3.1.4 Interleukin (IL) -15

IL-15 is a 14-15 kDa peptide that has activity similar to that of IL-2. This molecule has a predicted tertiary structure similar to that of IL-2 and has been shown to act as a potent growth factor for activated T- and NK cells (Grabestein et al., 1994). Its potential role in regulation, proliferation of human NK cells has been investigated (Warren et al., 1996; Warren et al., 1996; Carson et al., 1997; Lin et al., 1997).

The IL-2 receptor system plays a pivotal role in regulatory and effector functions of the immune response. Disorders of this system have been observed in association with a broad array of human diseases (Waldmann, 1993). Several diseases are associated with high serum level of the IL-2Ra subunit (CD25) which may not only be due to IL-2 but also IL-15 stimulation. This fact was investigated by Treibar-Held and co-workers on native human peripheral blood mononuclear cells who found that IL-15 stimulated more IL-2Ra than IL-2. The production of IL-15 by macrophages, and possibly other cell types, in response to environmental stimuli and infectious agents suggests that IL-15 may play a role in protective immune responses, allograft rejection, and pathogenesis of autoimmune diseases (Kennedy and Park, 1996).

The IL-15 gene has been mapped to chromosome 4 region q25-35. It is 14968 bp in length and consists of 6 exons (Krause et al., 1996). IL-15 and IL-15 receptor expression was measured in retinal pigment epithelial cells (RPE) and shown to play an important

role in ocular immune and inflammatory responses by stimulating infiltrated T cells and RPE cells via paracrine and autocrine loops, respectively (Kumaki et al., 1996).

IL-15 stimulates the proliferation of γδ T cells (Nishimura et al., 1996) as well as the CD8+ T cell-mediated response (Kohn & Kasper, 1996). It has been shown that IL-15 is a tumour promoting factor for human non-hemapoietic cells. IL-15 may play an important role in T cell-mediated human renal rejection (Pavlakis et al., 1996) as well as a role in the host response to HIV infection (Seder et al., 1995). Interleukin 15 may also play an important role in autoimmune diseases. Increased expression of IL-15 has been found in autoimmune diseases (Kirmann et al., 1996; Ajjan et al., 1997). IL-15 has been shown to recruit and activate T cells into the synovial membrane, and may contribute to the pathology of Rheumatoid arthritis (McInnis et al., 1996).

To date no polymorphism has been reported in the IL-15 gene although its serum level and expression has been investigated in different autoimmune diseases including type 1 diabetes (Hussain et al., 1996).

1.3.1.5 Interleukin (IL) -12

IL-12 is a mononuclear phagocytic cell-derived peptide originally described as NK stimulatory factor (Bellone & Trinchieri, 1994). It is a heterodimer composed of unrelated subunits. The larger subunit (p40) is homologous to the soluble receptor for IL-6, whereas the smaller subunit (p35) is homologous to both IL-6 and GM-CSF.

IL-12 is predominantly derived from monocytes and macrophages, but also from B cells and connective tissue type mast cells. It exerts its effects on resting and activated T cells and NK cells by enhancing IFN-γ and TNF-α production (Adorini et al., 1995).

IL-12 suppresses the synthesis of immunoglobulin E by IL-4 stimulated human T cells (Kiniwa et al., 1992). IL-12 has the capacity to induce TH1 CD4+ cells and its potential role in the mediating TH1 cell-mediated autoimmune diseases has made it an attractive target for immunointervention (Adorini et al., 1995). Although IL-12 plays a very important function in immune regulation and TH1 cell mediated autoimmunity its genetic structure is not well known and to date there is no reported polymorphism in the IL-12 gene.

As type 1 diabetes is postulated to be a TH1 cell mediated auto-immune disease, IL-12 may play an important role in the development of the disease. Szelawska and colleagues have shown that IL-12 is involved in the pathogenesis of type 1 diabetes.

1.3.1.6 Chemokines (IL-8 and the small cytokine family)

The chemokines are a small chemotactic cytokine family of structurally and functionally related proteins that include at least 14 distinct members (Oppenheim et al., 1991, Horuk, 1994, Baggioliolini et al., 1994; Terun, 2000). In addition to mononuclear phagocytic cells, chemokines are secreted by T cells, NK cells, neutrophils, keratinocytes, hepatocytes, fibroblasts, endothelium, and epithelial cells. The peptides are small (8-10 kDa) secreted proinflammatory cytokines that exhibit between 20-50 % homology in their amino acid sequences (Baggioliolini et al., 1994; Nelsson et al., 1998). The chemokines are characterised by the presence of four conserved cysteine residues and are subdivided into two families on the basis of positioning of these cysteines. The C-X-C subfamily is located on chromosome 4q, and its members are characterised by the separation of the first two cysteines by a variable amino acid. The C-C subfamily has the first two cysteine residues adjacent to each other, and its genes are located on chromosome 17q. These two families may be distinguished by their

primary target cells: the C-X-C subfamily primarily targets neutrophils, and the C-C family targets monocytes and T cells. Recently, a new family of chemokines, which lacks the first and third C, has been identified and is referred to as the "C family" (Nelson et al., 1998; Terun, 2000).

IL-8 is the most extensively studied member of chemokine superfamily (Leonard et al., 1990, Oppenheim et al., 1991, Horuk, 1994, Nelson et al., 1998). IL-8 is primarily derived from mononuclear phagocytes and endothelial cells but also from T cells, eosinophils, neutrophils, fibroblasts, keratinocytes, hepatocytes and chondreocytes. IL-8 synthesis may be induced by IL-1, TNF, and viruses. IL-8 on a molar basis is one of the most potent chemoattractants for neutrophils. It also stimulates polymorphonuclear neutrophils degranulation, the respiratory burst, and adherence to endothelial cells through CD11b/CD18. Other members of chemokine family-including platelet factor-4, macrophage inflammatory protein (MIP)-1α, and neutrophil-activating protein-2 share to a lesser extent a capacity to activate polymorphonuclear neutrophils. Other members of the chemokine C-C family, including RANTES, MIP, and monocyte chemotactic peptides (MCP)-1 and MCP-3 have unique contributions towards allergic inflammation (Baggiolini et al. 1994; Nelson et al. 1998), leading to the promotion of the infiltration into tissues of a range of leukocyte cell types including effector T cells. Further, tissue phagocytes initiate host responses in tissues, and their numbers are soon augmented through the action of chemokines, which recruit large numbers of circulating phagocytic and immunocompetent cells to sites of infection and tissue damage. Why there are so many chemokines is not yet known; neither is the exact role of each one in host defence and in pathological responses (Leonard et al., 1990, Nelson et al., 1998, Ward et al., 1998; Terun, 2000). Recently, however, polymorphic variants in the IL-8 (Emi et al., 1999) and RANTES peptides (Al Sharif et al., 1999, Hajeer et al., 1999) have been

characterised which could be used in the investigations of the role of chemokines in the pathogenesis of different diseases.

1.3.2 T helper 1 (TH1) cytokines

1.3.2.1 Interleukin (IL) -2

IL-2 is 15.5 kDa glycoprotein originally called T cell growth factor (TCGF). It is produced by CD4+ TH1 cells and some CD8+ T cells. Stimulation of T-cells by antigen in the presence of IL-1 induces the simultaneous secretion of IL-2 and the expression of high affinity IL-2 receptors (Taga and Kishimoto, 1995). The binding of secreted IL-2 to its receptor on T cells induces clonal IL-2 production and IL-2R expression for T cells to proliferate and assures that only T cells specific for the antigen inciting the immune response will be activated.

IL-2R consists of three chains, IL-2α, IL-2β, and L-2γc, (Minami et al., 1993). One of the IL-2 receptor chains (γc) is found to be common between different cytokines, IL-4, -5, -7, -9, -13, and IL-15 (Waldmann, 1991; Taniguchi et al., 1993; Santo et al., 1995). This phenomenon plays a critical role in the interaction between the latter cytokines and their receptors, which acts as a major factor in the development and regulation of the hematopoietic system (Santo et al., 1995). The high serum level of IL-2R in most diseases is perhaps due to this fact. Absence of γc chain is responsible for one form of X-linked severe immunodefficiency (Clark et al., 1995).

With regard to the importance of IL-2 in the regulation of the immune system, little is known about polymorphism in its gene although a microsatellite marker has been identified (Denny et al., 1997).

1.3.2.2 Interferon (IFN) -γ

Interferon-γ is produced by T- and NK cells. IFN- γ is a cross regulator between TH1 and TH2 CD4+ cells. It down-regulates Th2 cell activity and also possesses many stimulatory activities. IFN-γ is considered by many to be the most important cytokine responsible for activation of macrophages. Although IFN-γ is a member of cell-mediated cytokines it plays an important role as a pro-inflammatory cytokine. Interferon-γ is a potent immunoregulatory molecule with additional anti-proliferative effects (Farrar et al., 1993). IFN-γ stimulates antigen presentation and cytokine production by monocytes and stimulates monocyte effector functions including adherence, phagocytosis, secretion, the respiratory burst, nitric oxide production, and anti-tumour activity. The net result is the accumulation of macrophages at the site of the cellular immune responses, and their activation of macrophages makes them capable of killing intracellular pathogens. As discussed later, IFN-γ has been proposed as an inhibitor of allergic responses through its ability to inhibit IL-4 mediated effects on B cells, hence, inhibit IgE secretion and expression of low-affinity IgE receptors.

Interferon-γ is located on chromosome 12 (Overbach et al., 1981; Weissman, 1981). However, IFN-γ is quite unrelated to other IFNs and human IFN-γ contains three introns and repetitive DNA sequence which is not highly polymorphic (Patrick et al., 1982). The cytokine IFN-γ, alone or in combination with IL-1, TNF-α /β or both and/or IL-6 was suggested to be cytotoxic to beta cells (Meuller et al. 1995; Rabinovitch et al., 1996). This has been shown subsequently to be the case in rat, mouse, and human pancreatic islets in vitro (Nagota and Yoon, 1992; Bradley et al., 1992; Eduard et al., 1993; Kelso, 1995; Peterson and Haskins, 1996; Miner and Craft, 1998), at least in part via generation

of nitric oxide (Craft and Swain, 1995; Peterson and Haskins, 1996). IFN-γ is a product of T cells and NK cells, possesses unique immunomodulatory properties, and can affect nonlymphoid tissues as well. In addition to the direct effect on beta cells, IFN-γ may play a role in the pathogenesis of type 1 diabetes for the following reasons. IFN-γ upregulates MHC class I (Nicholson and Kuchroo, 1996), class II (Soldevila et al., 1990; Soldevila et al., 1991; Meuller et al., 1996; Wilson et al., 1998), and adhesion molecules (Meuller et al., 1997; Szelachowska et al., 1998) on various cells, including pancreatic beta cells. It may also modulate the processing of exogenous (MHC class II) antigens by human monocytes (Nadler et al., 1994). Transgenic mice expressing IFN-γ by pancreatic β cells develop autoimmune diabetes (Sarvetnick et al., 1990; Ciampolillo et al., 1993; Rabinovitch et al., 1995), and postnatal anti- IFN-γ treatment prevents pancreatic inflammation in this model (Wogensen et al., 1994). Antibodies to IFN-γ also protect against diabetes development in NOD mice (Campbell et al., 1991; Debrays-Sachs et al., 1991; Rabinovitch et al., 1995; Almawi et al., 1999), and in BB rats (Nicholetti et al., 1990; Almawi et al., 1999). Finally, IFN-γ is expressed in late-stage insulitis in patients with a recent-onset of type 1 diabetes (Foulis et al., 1991; Rabinovitch et al., 1995). The reported actions if IFN-γ are consistently diabetes promoting, and, therefore, the IFN-γ gene (IFNG) is an obvious candidate gene for conferring susceptibility to type 1 diabetes.

1.3.3 T helper 2 (TH2) cytokines

1.3.3.1 Interleukin (IL) -4

IL-4 was originally identified as a B-cell growth factor, obtained from the optimal stimulation of B cells by antigen (Coffman et al., 1986; Paul, 1987). In addition to T-helper cells, IL-4 may be derived from cytotoxic T cells, mast cells, and basophils.

Furthermore, IL-4 induces the immunoglobulin isotope switch from IgM to IgE (Coffman et al., 1986). IgE production occurs after the IL-4 mediated initiation of transcription of IgE heavy chain transcripts. The presence of the signal triggers the expression of the CD40 molecule and leads to genomic splicing and synthesis of IgE transcripts. The additional presence of B cell-activating cytokines, including IL-2, IL-5, and IL-6 synergises IL-4 to increase the secretion of IgE (Vercelli et al., 1993).

In addition to the effect on B cells, IL-4 is a growth factor for T cells (TH2) and suppresses TH1 activity. IL-4 activates cytotoxic T cells as well as NK and LAK cells. It has some macrophage-activating activities, enhancing the expression of MHC class I and II molecules, leukocyte function associated antigen-1, and low affinity IgE receptors (Paul et al., 1991).

In contrast to these proinflammatory effects on monocytes, IL-4 inhibits antibody-dependent cellular cytotoxicity, inhibits the expression of Fcγ receptors, down-regulates production of nitric oxide, IL-1, IL-6, and TNF-α while stimulating the production of IL-1ra (Fenton et al., 1992).

Apart from the latter characteristics of IL-4, it plays a critically important role in allergic diseases through its ability to induce the differentiation of IL-4-producing T helper cells. IL-4 along with IL-13 (has approximately 30% homology to IL-4 and shares much of IL-4's biologic activities on mononuclear phagocytic cells and B cells), and IFN-γ play crucially important roles in allergic and inflammation. IFN-γ acts as an inhibitor of allergic responses through its ability to inhibit IL-4-mediated expression of low affinity IgE receptors and isotope switch to IgE. The capacity of T cell clones to support IgE production is inversely proportional to their IFN-γ production, and clones producing both IL-4 and IFN-γ will support IgE production only in the additional presence of anti-

IFN-γ antibodies (Romagnani, 1990; Vercelli et al., 1993). Additional cytokines that have been reported to inhibit IL-4 induced IgE production are IL-12, IL-8, and TGF-β (Kiniwa et al., 1992; Gauchat et al., 1992). However, it should be noted that at least one study has found no correlation between IL-4 expression and serum IgE levels (Van der Pouw-Kraan et al., 1994).

Genetic markers within the IL-4 encoded region (5q31-33) have been shown to be linked to total serum IgE concentration (Marsh et al., 1994; Xu et al., 1995). Since IL-4 has been located in that region, it could be a candidate for the reported genetic linkage.

Consequently, there have been a number of studies investigating IL-4 gene polymorphisms with raised serum IgE levels. Several polymorphisms in the IL-4 gene have been identified and have been shown to be associated to IgE concentration (Borish et al., 1994; Song et al., 1996; Walley and Cookson, 1996; Dreckback et al., 1997; Beghe et al., 1999). Song and his co-workers have analysed the sequence of "allele 2", which was reported to exhibit higher transcriptional activity than all of the other IL-4 alleles.

Administration of IL-4 to NOD, non-obese diabetic mice for a period of 14 weeks, starting at 6 weeks of age, a marked reduction of diabetes incidence was observed (Rapport et al., 1993). Mueller and colleagues (Mueller et al., 1996) generated NOD mice in which IL-4 was expressed in the pancreas. Although peri-insulitus was observed in NOD-IL-4, it never progressed to insulitis and all mice were protected from diabetes. Moreover, sublethally irradiated NOD-IL-4 transgenic recipients, but not their non-transgenic littermates, were protected from diabetes when injected with splenocyes from diabetic NOD mice. However, in co-transfer experiments, they did not protect NOD-SCID recipients from diabetes caused by splenocyte transfer from diseased NOD donors

(Mueller et al., 1997). An intriguing result was observed when NOD-IL-4 mice were crossed with NOD-TCR transgenic mice specific for a pancreatic antigen. While neither the NOD-IL-4 nor the NOD-TCR transgenic mice developed diabetes, NOD-IL-4-TCR transgenic mice developed diabetes with a higher frequency than non-transgenic NOD mice (Mueller et al., 1997). Thus, in this restricted T cell repertoire model, pancreatic expression of IL-4 has a deleterious effect on the development of diabetes.

Recently, however, Leech and colleagues showed that a significant drop in secretion of IL-4 and IL-10 in type 1 diabetes (Leech et al., 1999). The increase in IL-4 and IL-10 production in recently *in vivo* activated T cells in newly diagnosed diabetic subjects may represent an attempt at immunoregulation of active insulitis (Leech et al., 1999).

1.3.3.2 Interleukin (IL) -10

IL-10 has a predominantly anti-inflammatory effect. IL-10 or cytokine inhibitory factor was initially identified in mice, in which it is a product of TH2 cell that inhibits both TH1 proliferation and production of IFN-γ and IL-2 (Fiorentino et al., 1989). Subsequent studies have extended these activities to man and have shown that IL-10 is also a product of other cells, B cells, mast cells, and mononuclear phagocytic cells. IL-10 contributes to isotype switching to IgG1 and IgG3 (Briere et al., 1994). IL-10 also functions as a growth factor for cytotoxic T cells. Thus, IL-10 inhibits cytokines associated with cellular immunity and allergic inflammation while stimulating cytotoxic immune responses. The kinetics of IL-10 production differs from those of other cytokines (Yassel et al., 1992). The appearance of IL-10 thus correlates with and may contribute to the down-regulation of the pro-inflammatory cytokines which may be a signal for IL-10 synthesis (Wanidworanun et al., 1993; Eskada et al., 1997).

TNF-α secretion is a potent stimulator for IL-10 secretion. These studies suggest a homeostatic mechanism whereby an inflammatory stimulus induces TNF-α secretion, which in turn stimulates IL-10 secretion, which acts as a negative feedback for TNF-α secretion.

Interleukin-10 is an important regulatory cytokine whose involvement extends into diverse areas of the human immune system. Recent studies have shown elevated serum levels of IL-10 in several auto-immune diseases; such changes may have a genetic background. Genetic influence on IL-10 production is an area that is currently being studied. To date there are few reports about polymorphisms in the IL-10 gene (Eskada et al., 1997; Turner et al., 1997; Hobbs et al., 1997). Some of these polymorphisms have been associated with autoimmune diseases (Eskada, 1997).

1.3.4 T-helper 3 (TH3) cytokine

A third type of T cell has been identified following oral immunisation with ovalbumin (OVA) (Del Prete et al., 1991; Chen et al., 1994; Rabinovitch, 1998; Chen et al., 1998; Suri and Katz, 1999; Prud'homme and Picirrollo, 2000). TH3 secretes large amounts of TGF-β but low amounts of IL-4, IL-10, and IFN-γ.

1.3.4.1 Transforming growth factor (TGF) -β

In view of the vast literature on TGF-β biology, and the publication of recent reviews (Derynck and Choy, 1998; Letterio et al., 1998; McCarthy et al., 1998), only the most salient points will be mentioned here. TGF-β is a family of polypeptide growth factors that has a fundamental role in growth and differentiation. TGF-β is synthesised as an

inactive precursor that requires proteolytic cleavage, in a high salt and low pH environment to become active. Activation can occur in macrophages (Nunes et al., 1995) which are present in inflammatory lesions.

The TGF-β superfamily consists of more than 25 molecules isolated from a number of species (Kitamura and Suto, 1997). The main subgroup includes three mammalian isoforms termed TGF-β1, TGF-β2, and TGF-β3. Differential expression of these isoforms may be critical during embryogenesis, carcinogenesis, and in chronic fibrotic diseases (Border, 1994[a]). The three isoforms are 60-80 % homologous with virtually indistinguishable biological activities (Ohta et al., 1987). They are structurally similar and act through the same receptors to stimulate a wide variety of cellular responses (Basile, 1999). The TGF-β1 is produced by many cell types, usually in its latent form, but the highest amounts are found in human platelets and mammalian bone (Fontana et al., 1992). Antigen specific T cells and activated macrophages produce both active and latent TGF-β1 (Kehrl et al., 1986). Chen et al. report that TH0, TH1, and TH2 clones can all be induced to secrete TGF-β1 through cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). Clearly, TGF-β1 production cannot be considered a feature unique to a subset of TH cells. At this point, the persistence of TGF-β1 production by TH0/TH1/TH2 clones is unclear, and the significance of this observation in terms of immunoregulation has not been defined. However, it seems that CTLA-4, acting partly or primarily through TGF-β1, counterbalances CD28 co-stimulation of TH cells (Chen et al., 1998). The TGF-β1-producing phenotype was enhanced by adding IL-4 to cultures, or by neutralising TH1 cytokines with anti-IFN-γ plus anti-IL-12 antibodies. The presence of IL-10 in cultures also favoured TGF-β1 production. In addition, Seder et al. reported that TGF-β1 strongly stimulated its own production (Seder et al., 1992).

From this study it appears that IL-4, IL-10, and TGF-β1 promote differentiation of TGF-β1-producing T cells, while IFN-γ and/or IL-12 have the opposite effect. However, from the literature, it does not appear that either IL-4 or IL-10 is essential for inducing TGF-β1 production in T cells, while IFN-γ does not necessarily prevent its production. Notably, these regulatory T cells can be generated in IL-4 knockout mice (Seder et al., 1992). Moreover, memory T cells can produce TGF-β1 upon activation independently of exogenous cytokine stimulation (Seder et al., 1992).

Since TGF-β1 protected efficiently against antigen-induced EAE (Chen et al., 1994) and various other autoimmune diseases, it is of great interest to learn more about the conditions leading to the generation of TGF-β1. Receptors for TGF-β1 are present on virtually all cells, and it mediates a wide range of biologic activities. In general, it is an important stimulant for fibrosis, including formation of the extracellular matrix. TGF-β1 has been invariably associated with a decrease in immune responses and improvement of autoimmune conditions (Kuruvilla et al., 1991; Racke et al., 1991; Karpus and Swanborg, 1991; Thorbecke et al., 1992; Ruscetti et al.. 1993; Groux et al., 1995; Poerie et al. 1996; Santambrogio et al. 1997). In contrast to these immunosuppressive effects, TGF-β is a chemoattractant for macrophages and supports the α isotype switch to IgA by B cells (Sonoda et al., 1989; Stavnezer et al., 1997). TGF-β1 knockout mice do not survive because of diffuse inflammation of numerous organs by mononuclear cells (Shull et al., 1992). In allergic inflammation the expression of TGF-β1 may be associated with the fibrosis observed in long-standing asthma and the subendocardial fibrosis associated with the hypereosinophilic syndrome. TGF-β1 may lessen allergic inflammation through a capacity to inhibit ε gremlin transcription and IgE synthesis in IL-4-treated human B cells and through inhibition of mast cell proliferation. Recently,

Han's group showed that TGF-β1 prevents spontaneous autoimmune diabetes and recurrent diabetes in syngeneic islet-transplanted NOD mice (Han et al., 1996).

A considerable amount of evidence supports the pathogenic role of TH1 cells and related cytokine production in type 1 diabetes, and this topic has been recently reviewed (Delovitch and Singh, 1997; Rabivitch, 1998; Suri and Katz, 1999; Prud'homme and Picirrollo, 2000). In an islet antigen-specific TCR transgenic model, adoptive transfer of TH1 cells induced diabetes rapidly in recipients, whereas transfer of TH2 cells did not induce diabetes (Kaltz et al., 1995). In NOD mice, gene therapy with a vector encoding a soluble IFN-γ receptor/ IgG fusion protein (Prud'homme and Young, 1999), or null mutation of the IFN-γ receptor (O'Garra et al., 1997) protect from autoimmune diabetes. IL-12 administration provokes disease in NOD mice, while diabetes is prevented with IL-12 antagonists (Trembleau et al., 1995; Trembleau et al., 1997). Expression of IFN-γ or IL-4 in the β-islet cells of transgenic mice either promotes or prevents the onset of type 1 diabetes, respectively (Delovitch and Singh, 1997, Grewall and Flavell, 1997; Rabinovitch, 1998; Suri and Katz, 1999; Prud'homme and Piccirillo, 2000). Administration of IL-4 by protein injection (Delovitch and Singh, 1997; Rabinovitch, 1998; Suri and Katz, 1999) or gene therapy (Chang and Prud'homme, 1999) is also protective. However, islet-reactive TH2 clones injected into NOD-SCID mice can induce insulitis and diabetes (Pakala et al., 1997). Thus, the concept that TH2 cells are unequivocally protective is not entirely satisfactory. It is possible that the immunoprotective effect of IL-4 is mediated indirectly by induction of TGF-β1 (King et al., 1998; Moritani et al., 1998), as well as by the injection of TGF-β1 –producing T-cell clones (Han et al., 1996) or gene therapy with latent TGF-β_1-encoding vector (Piccirillo et al., 1998). However, locally produced TGF-β_1 in transgenic mice can induce chronic pancreatitis and fibrosis (Sanvito et al., 1995).

Interestingly, Han and colleagues found that a protective regulatory T cell clone (responsive to self-MHC class II determinants) produced substantial amounts of TGF-β_1, IL-10, and IFN-γ, and that the suppressive activity could be abrogated by anti- TGF-β1 antibodies, but not by anti-IL-10 antibodies (Han et al., 1996). Evidently, simultaneous production of TGF-β1 and the inflammatory cytokine IFN-γ can occur in regulatory clones, and the latter cytokine does not stimulate inflammation in that context (Zekzer et al., 1997).

Further, oral administration of insulin induces TGF-β1-producing protective cells against diabetogenic factors (Polanski et al., 1997; Maron et al., 1998). Diabetes in Bb/Wor rats was prevented by injection of peritoneal exudate cells incubated with TGF-β1 and islet cells (Keovary, 1994).

Glucose intolerance is the hallmark of diabetes mellitus. Several studies have shown that high glucose induces increase in TGF-β1 (Wolf et al., 1992; Bollineni and Sholey, 1993; Nakamura et al., 1993; Shankland ans Sholey, 1994; Ziadeh et al., 1994; Moitani et al., 1998). Glomerular TGF-β1mRNA expression measured by northern blot analysis after 12-15 weeks of diabetes was increased 2-3 fold in diabetic rats compared to non-diabetic rats (Bollineni and Sholey, 1993; Nakamura et al., 1993; Shankland ans Sholey, 1994; Ziadeh et al., 1994). An increase in TGF-β_1mRNA was detected after 24 h of the induction of diabetes (Ziadeh et al., 1994). Plasma level of TGF-β_1 in diabetic rats was three times higher than in non-diabetic rats (Nakamura et al., 1993). The increase of glomerular TGF-β_1mRNA expression in diabetic rats was attenuated by insulin treatment (Bollineni and Sholey, 1993; Ziadeh et al., 1994). Elevation of urinary TGF-

β1 has been reported in patients with diabetes mellitus, with or without renal insufficiency (Kapoor et al., 1997; Fagerudd et al., 1997).

Further, autoimmune diabetes in NOD mice could be prevented by transgenic paracrine TGF-β1 in the islet compartment through protection against CD4 (+) and CD8 (+) (Pankeycz et al., 1992; Han et al., 1996; Zekzer et al., 1997; Han et al., 1997).

All of these findings strongly support a causative role of high glucose increasing the TGF-β_1 levels. An increase in TGF-β_1 concentration has been proposed to be a factor for the excessive production of extracellular matrix (Ziyadeh et al., 1994; Border and Noble, 1994; Sharma et al., 1997) proteins seen in diabetes (Bollineni and Reddi, 1993; Bollineni and Sholey, 1993; Ziadeh et al., 1994; Sharma et al., 1997). TGF-β_1 stimulates glucose uptake by enhancing the expression of Glut 1 in mesangial cells, which leads to intracellular metabolic abnormalities in diabetes (Inoki et al., 1999).

1.4 Immunoregulatory mechanisms

Autoimmunity, that is, immune reactivity against self, is a normal part of the immune system. What causes autoimmunity to progress to autoimmune disease, with functional and structural damage to self-cellular components, is not fully understood. Tolerance to self may be broken because normally occurring autoimmunity is not controlled, either due to deficient elimination or lack of functional interactaction/inhibition of autoreactive lymphocytes in the thymus or in the periphery (Kreomer and Martinez, 1992).

An antigen, typically soluble proteins or glycoproteins are taken up by APC, for example, tissue macrophages, dendritic cells, or B-cells by binding to antigen receptors on the APC. The antigen/receptor complexes are internalised into the endosomal compartment where the antigen is cleaved by sequential proteolysis and trapped in the antigen-binding groove of MHC class II molecules found in the endosomes. When the endosomes fuse with the cell membrane, the antigen peptide/MHC class II molecule complex is presented on the surface of the cell. The presented antigen/MHC class II complex can be recognised by specific T-helper cells carrying the appropriate T-cell receptor.

Antigen recognition in the absence of secondary signals usually gives rise to tolerance to the presented antigen. Secondary signals to elicit an immune response include soluble factors and specific cell surface antigens. Soluble secondary signals encompass certain cytokines which are non-immunoglobulin signal proteins synthesised by immune and a number of non-immune cells when appropriately stimulated. Thus, the cytokine IL-1 is produced mainly by activated macrophages but also by many other cells (Dinarello, 1994) and provides an important activating signal particularly to resting T-cells by binding to specific cell surface receptors. Several cell surface expressed receptor/ligand-

pairs increase the adhesion between the APC and antigen recognising cells; for example, lymphocyte function associated antigen 1 (LEA 1) that binds to inter-cellular adhesion molecules 1,2, and 3 (ICAM 1-3), LEA-3 binding to CD2 on T-cells and NK cells, CD8 expressed by cytotoxic T-cells binding to the MHC class I and CD4 expressed on T-helper cells binding to the MHC class II molecules on the APC (Dustin and Springer, 1991).

When the antigen is recognised by the T-helper cell in the context of appropriate cell adhesion and soluble secondary signals, the T-cell is activated primarily via the production of a number of cytokine genes. The synthesis and release of cytokines mediate leukocyte chemotaxis, endothelial activation, and differentiation of recruited leukocytes, and many other biological activities. Interferon-gamma (IFN-γ) positively feeds back on macrophage IL-1 and tumour necrosis factor alpha (TNF-α) secretion, whereby the immune response is amplified. Together, IL-1, TNF-α, and IFN-γ are central mediators of the acute phase response of inflammation, infection and tissue trauma, and have overlapping, but also distinct, biological effects.

Thus, the early phase of the immune response is characterised essentially by interaction between one APC and one antigen recognising cell, which will lead to high concentrations of cytokines with paracrine effects on the immediate environment, and endocrine actions on more remotely situated tissues. Late stages of the immune response are characterised by build-up of an inflammatory reaction encompassing most active immunocompetent cells, the secretion of cytokines, proteases, free oxygen and nitric derived radicals, and other immune mediators.

1.4.1 Cytokines involved in peripheral tolerance

Immunosuppressive cytokines participate in creating a state of tolerance. In the NOD mouse model for insulin-dependent diabetes, transfer of a certain insulin-specific T-cell clone can prevent the destruction of pancreatic β cells by autoreactive T cells (Han et al. 1996). This suggests that insulin-specific T cells can suppress the activity of other autoaggressive T cells in an antigen-dependent manner. They do this by homing to the islet, where they react with insulin peptides presented on the β cells. This stimulates the secretion of cytokines, prominent amongst which is TGF-β, a known immunosuppressive cytokine (Chen et al., 1994; Rabinovitch, 1998; Chen et al., 1998; Suri and Katz, 1999; Prud'homme and Picirrollo, 2000). There are interesting hints that such cells naturally affect the course of the immune response that causes human diabetes. β-cell destruction in humans occurs over a period of several years before diabetes is manifest, yet when pancreas was transplanted from an identical twin into his or her diabetic co-twin, they were destroyed within weeks (Kida et al., 1998). This suggests that, in the normal course of the disease, specific T cells protect the β cells from attack by effector T cells and the disease therefore progresses slowly. It might be that after the host islets have been destroyed, these protective mechanisms decline in activity, but that the effector cells responsible for β cell destruction do not (Kida, 1999; Graves and Eisenbarth, 1999).

Furthermore, experimental allergic encephalomyelitis (EAE) is normally caused by TH1 cells that produce IFN-γ in response to myelin basic protein. In mice fed with this protein, CD4+ T cells that produce cytokines such as TGF-β1 and IL-4 are found in the brain instead. TGF-β, in particular, suppresses the function of inflammatory TH1 lymphocytes. In both of these cases, the protection seems to be tissue-specific rather than antigen-specific. Thus, feeding with insulin protects against diabetes, yet insulin is

not thought to be the target of autoimmune attack on the β cells. Likewise, feeding with myelin basic protein will protect against EAE elicited by immunisation with other brain antigens (O'Garra et al., 1997). Systemic administration of TGF-β1 in mice infected with *L. amozonia* led to increased production of IL-4 and decreased synthesis of IFN-γ (Barral-Netto et al. 1992). TGF-β1 may favour TH2 development by attenuating IL-12Rβ2 expression (Szabo et al., 1997). IL-12 signal transduction, and cytokine signalling will be discussed in section 1.6.9 (Bright and Stiram, 1998). This ultimately reduces IL-12 responsiveness in T cells (Gorham et al., 1998), and results in decreased T cell proliferation and IL-12-induced IFN-γ production, increase T cell apoptosis, and suppressed TH1-mediated responses (Szabo et al., 1997; Gorham et al., 1998).

An anergic CD8+ T-cell islet-infiltrating clone that releases an inhibitory factor (IL-X) able to block IL-2 and IL-4-dependent proliferation of T cell, abrogates diabetogenic autoimmunity in an accelerated NOD model (Diaz-Gallo et al., 1992). Furthermore, it was shown that this soluble factor was neither IL-10 nor TGF-β. These data, together with the aforementioned studies, would support the existence of cytokine driven immunoregulatory which may participate in self tolerance.

1.5 Theories of immunity

Innate immunity is the system of cells and molecules that make up the body's first line of defence against pathogens. In other words, the immune system may be the ultimate controller of adaptive immune responses through its regulation of the co-stimulatory molecules necessary for T and B cell responses (Janeway, 1989; Janeway, 1992), which leads to an expansion of the longstanding self-non-self (SNS) model of immunity. According to the expanded SNS model the definition of non-self now includes not only the foreign molecules recognised by B and T cells, but also those recognised by the innate immune system (both by cells, such as macrophages and dendritic cells, and by humoral molecules such as those of the complement cascade) (Fearon and Locksley, 1996).

The Danger model (Matzinger, 1994) suggests that immune responses are initiated by tissue injury, not by the recognition of non-self. Looking at primitive systems dealing with tissue injury aids finding the origin of immunity. In many cases there may be nothing wrong with the immune response. The primary cause is in the presentation of antigen and the activation of APCs (Matzinger, 1994).

1.5.1 Autoimmune diseases in light of the "SNS" and "Danger" models

In any autoimmune disease there are two important questions: a) what initiates the destructive response? b) What maintains it? Matzinger has classified autoimmune diseases into five categories according to the type of immune responses (Matzinger, 1998). The immune response in each case is explained in light of both the SNS and Danger models.

1.5.1.1 When there is an unrecognised ongoing infection in the target organ

In this category there is not true autoimmunity and there is nothing wrong with the immune response. The effector cells are clearing the pathogens, however, they damage the target organ in the process. *Helicobacter Pylori* (Frobes et al. 1994), a bacterium in upper gastrointestinal tract, turns out to be a common cause of peptic ulcers that were thought to be due to autoimmunity mimic which this category. Mouse Theiler's virus, which also infects the brain, can closely mimic MS (Miller et al., 1997).

1.5.1.2 Molecular mimicry

Molecular mimicry describes cross reaction of self-tissue with an environmental antigen. It has often been suggested that a reaction to a self antigen might be initiated by a cross-reactive environmental antigen (Steinman and Conlon, 1997). In this type a cell normally expresses only a subset of its potential peptide/MHC complexes and a change in the cell might allow the expression of a peptide that is normally hidden, leading to autoimmunity (Cobbold et al., 1992).

Once tolerance is broken, the SNS models, original and expanded model, have no problem with the maintenance of the response. In the belief that, once initiated, an immune response continues until the target antigen is cleared, there is no way to stop the autoimmune response.

T and B cells are probably functioning normally to clear pathogen. For example, in type 1 diabetes the islets are not efficient at tolerating themselves (Von Herrath et al., 1996). Individuals with certain alleles of MHC and of tissue specific self antigens will be

susceptible to immune damage by crossreactions with environmental pathogens (Vidovic and Matzinger, 1988; Matzinger, 1998). Recurring infections will cause chronic and/or progressive disease (Matzinger, 1998).

1.5.1.3 Bad death

This category is unique and a direct prediction of the Danger model, based on the assumption that normal, programmed, cell death does not activate APCs but the cells that are stressed in a certain way and the cells that die abnormally send signals which can initiate an immune response (Matzinger, 1998). In this category of autoimmune disease, as in previous categories, the immune response is normal. What is abnormal is the way in which a cell dies and activates local APCs.

For example, in SLE, the autoantibody response seems to be directed against DNA, but where does all this antibody come from? It could come from poorly handled cell death which has occurred due to mutation in one of the immune response elements which lead to a general defect in T cell tolerance (Vyce and Kotzin, 1998). Nevertheless, the SNS models have no other explanation for the role of such defect in one of the immune response elements. The Danger model does have an alternative solution where the alarm signals activate local APCs, and initiate a response to exposed self antigens captured by those APCs (Matzinger, 1998).

1.5.1.4 Whenever the response is a result, not a cause, of a disease

This category is a subset of the "bad death" category, described by Livia and Anthony Rosen, 1999. The idea was that the disease is unrelated to the immune system. The immune response is triggered by the damage and points to the relevant antigens. Rosen's idea grew from their study of patients with SLE and with Scleroderma, another

connective tissue disease in which a number of autoantibodies appear (Rosen, et al. 1997; Casciola-Rosen, et al., 1997). They suggested that Scleroderma may not be an autoimmune disease. The damage is not due to antibodies but perhaps the immune system reacting to the released antigens by producing autoantibodies. These antibodies do not influence the course of the disease, though they might help clean up the mess, and give insights into the molecules involved (Matzinger, 1998).

1.5.1.5 The response switches to a harmful class

The antibodies and cytokines released during an immune response are highly potent molecules that can wreak enormous havoc, and some tissues are more sensitive to certain cytokines than others. For example, in diabetes the response could be directed against a self component, perhaps as a cross-reaction only made by individuals with certain MHC haplotypes, or strictly against a pathogen (in which case the autoantibodies would be pointers, as in Scleroderma). In either case, it could be a response made by many individuals without destroying their islets. The problem in diabetes may be that the response is shifted to a class that destroys the islets, which are sensitive to TNF-β and IFN-γ but not to the cytokines made by responses of a different class, such as a TH2 or TH3 (Chen et al., 1997).

This category of autoimmune disease does indeed involve a defect in the immune response. It could be a defect in the tissue itself, in a neighbouring cell, in a regulatory cell that resides in it, in the signal sent and/or received by APCs, or in the immune history of the individual, etc. In short, the defect could be anywhere (Matzinger, 1998).

1.6 Type 1 diabetes

Type 1 diabetes is an autoimmune disease characterised by T lymphocyte-mediated destruction of insulin producing β cells of the pancreatic islet of Langerhans. According to the International Diabetes Federation (IDF) and the World Health Organisation (WHO) there were 30 million people with diabetes in 1985. Today, the figure is an estimated 143 million. By 2025 there are expected to over 300 million diabetic patients worldwide (IDF, 1997). The incidence of type 1 diabetes is from about 5 to 43 per 100,000 births per year in Caucasians of European descent (Green et al., 1992; Kavonen et al. 1993; Buzzetti et al., 1998). It has clearly been established that diabetes is not a single disease but a genetically heterogenous group of disorders with glucose intolerance as a common feature. The concept of genetic heterogeneity, i.e. that different genetic and/or environmental factors can result in similar phenotypes, has significantly altered the genetic analysis of this common disorder. It is now apparent that diabetes and glucose intolerance are not diagnostic terms but simply describe symptoms and/or laboratory abnormalities which can have a number of distinct aetiologies (Graves and Eisenbarth, 1999; Friday et al., 1999).

1.6.1 Classification of diabetes mellitus

According to the modified WHO criteria, diabetes mellitus can be classified into two main categories: Insulin dependent diabetes mellitus, type 1 diabetes or IDDM and non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes. Apart from glucose intolerance, diabetes is associated with certain conditions and syndromes such as pancreatic disease, endocrine problems, certain drug or chemical-induced conditions, abnormalities of insulin or its receptors, or certain genetic syndromes and other syndromes which are out of the scope of this thesis (NDDG, 1979; WHO, 1980; WHO, 1985). Distinguishing features of these two categories are listed in Table 1.02.

Table 1.02: Characteristics of type 1 and type 2 diabetes mellitus

Characteristics	Type 1 Diabetes Mellitus	Type 2 Diabetes Mellitus
Age of onset	Generally in pre-adolscent or adolescent; may appear at any age	Typically older than 35 years; may appear at any age
Nature of onset	Often sudden	Slow, insidious
Genetic predisposition	Related to specific HLA factors DR3, DR4, DQB	Strongly familial; unrelated to HLA
Secondary environmental factors	Viruses, toxins	Obesity
B-cell autoimmunity	Present in initial episode	Not present
Insulin secretion	Generally absent or reduced	Reduced in amount with insulin resistance
Body habitus	Thin, can appear cachectic	Usually normal or obese, 50 % obese
Symptoms at onset	Poyuria, polydipsia, weight loss, hunger; ketoacidosis common	Often none, or mild ; no ketoacidosis
Long-term complication	Retinopathy, nephropathy, neuropathy; onset after 5 years	Similar to Type 1 complications
Insulin dependency	Absolute	Occasionally

Table indicates features of the major types of diabetes mellitus.

1.6.2 Long term complications of diabetes

The pathogenesis of long-term complications of diabetes remains a subject for debate, with the genetic hypothesis and the metabolic hypothesis each receiving support and criticism. It has been assumed by some that a genetic predisposition must be necessary (Deckert, 1960; Bennett, 2000), although the Diabetes Control and Complications Trial (DCCT) results strongly support the over-riding importance of hyperglycaemia for the specific complications (DCCT, 1993). Evaluation of the incidence of microvascular and macrovascular complications in diabetes secondary to acquired pancreatic disease is of interest because it may shed light on the relative contributions of heredity and hyperglycaemia. Experimental pancreatectomy is associated with development of vascular changes similar to those found in spontaneous genetic diabetes (Dulin et al., 1983). Recent work in diabetic microvascular complications in man supports the idea that genetic factors are important agents for these complications (Tiengo et al., 1988; Heesom et al., 1997; Pociot et al., 1998; Chowdhury et al., 1998, Bennett , 2000).

1.6.2.1 Diabetic Nephropathy

Diabetic nephropathy is characterised by thickening of the glomerular basement membrane, expansion of mesangial matrix with an altered composition of extracellular matrix (ECM), such as the accumulation of fibronectin and collagen, and increasing degrees of nodular or diffuse glomerular sclerosis and interstitial fibrosis. Kidney changes related to diabetes involve alterations of the glomerular capillaries and associated arterioles that lead to changes in filtration, proteinuria, and eventually impaired renal function (Border et al. 1998). Nephropathy is a serious late complication of diabetes. The disease can be diagnosed clinically with the fulfilment of additional criteria including the presence of diabetic retinopathy, and no clinical or laboratory evidence of kidney or urinary tract disease other than glomerulosclerosis. Only 20-40 %

of patients with type 1 diabetes will develop nephropathy (Krolewski et al., 1985; Kofoed-Enevoldsen et al., 1987; Yokoyama et al., 1994). Besides poor glucose control, genetic susceptibility seems to be an important pathogenic factor. However, once developed, this subgroup of patients with type 1 diabetes demonstrates a mortality rate 40 times that of the background population and a higher mortality than type 1 diabetes patients without nephropathy (Borch-Johnsen and Kreiner, 1987). Patients with nephropathy also suffer from an extremely high prevalence of other diabetic microvascular complications, invariably retinopathy and neuropathy (Jensen et al., 1987). Therefore patients with diabetic nephropathy are characterised to be at extremely high risk for severe micro- and macrovascular problems. Diabetic nephropathy is now the most common and important cause of end-stage renal disease (ESRD) in the United States and Europe (Breyer, 1992; Striker et al., 1993; Pociot et al., 1998; Bennett, 2000).

1.6.2.2 Diabetic Neuropathy

Peripheral and autonomic nerves are significantly affected by the abnormal metabolism of type 1 diabetes, resulting in pathological change, functional disturbance and clinical morbidity. Diabetic neuropathy is the clinical expression of structural and biochemical alterations to peripheral somatic or automatic nerve fibres (Sima, 1992; Nathan, 1993). Changes in nerve electrophysiology, such as decreased sensory and motor nerve conduction velocity, can be detected even in newly diagnosed diabetic patients. However, nerve function often improves after hyperglycaemia is corrected by treatment. These observations suggest that the changes in nerve function may be secondary to metabolic changes in the nerve, such as changes in myelin lipid composition, reduction in tissue myoinositol, increased sorbitol, and increased glycosylation of neural proteins. Neuropathies are more likely to develop if blood glucose levels are poorly controlled.

On average the onset of symptoms occurs 10 to 20 years after diabetes has been diagnosed. However, some caution should be used in relating hyperglycaemia to diabetic somatic and autonomic neuropathy, because alcohol consumption may be common in these individuals. Therefore, the true pathogenic mechanism(s) of neuropathy in diabetic patients with pancreatic disease is difficult to define (Zeigler, 2000).

1.6.2.3 Diabetic Retinopathy

Diabetic retinopathy involves both morphological and functional changes in the retinal capillaries, including basement membrane thickening, loss of pericytes, increased permeability and vascular dysfunction (Bennett, 2000). Long-term studies have shown that after 20 years of diabetes, 90 % of patients will have evidence of retinopathy (Klien et al., 1984, Bloom et al., 1994, Anonymous et al., 1998, Klein et al., 1998). Incidence of retinopathy is correlated with the duration of the hyperglycaemia (Tiengo et al., 1988). Factors that have been associated with the development and progression of diabetic retinopathy include gender, HLA type, age at onset, duration of disease, degree of metabolic control, presence and treatment of hypertention and relation to puberty (Kohner et al., 1996).

1.6.3 Mechanisms underlying the anti-β cell autoimmune response

It is well established that type 1 diabetes results from destruction of pancreatic β-cells by the autoimmune mechanisms in which both environmental and genetic factors are involved (Kida et al., 1998;Graves and Eisenbarth, 1999).

1.6.3.1 Environmental factors

Several lines of evidence point to a major role of environmental factors in the pathogenesis of type 1 diabetes. First, more than 70% of identical twins are discordant for the disease, and it is quite unlikely that this is all due to differential somatic

rearrangement of TCR. Second, disease frequency varies enormously from country to country (Diabetes Epidemiology Research International Group, 1988; Leslie and Elliott, 1994). These differences cannot simply be explained by ethnic genetic differences since migrants from countries with a low frequency of type 1 diabetes to countries with a high frequency are more susceptible than their compatriots (Patrick et al., 1989; Leslie and Elliott, 1994). Intriguingly, northern countries are more exposed to the disease than southern countries (Diabetes Epidemiology Research International Group, 1988; Leslie and Elliott, 1994); it will be critical to discover the factor(s) responsible for this North/South gradient. Finally, disease incidence is on the increase in most countries (IDF, 1997) strongly pointing to an environmental influence; this holds true even in areas with a distinct genetic background such as Sardinia, where the incidence has increased dramatically, to values much higher than those in surrounding regions (Songini and Mountoni, 1991; Leslie and Elliott, 1994).

Not only do environmental factors seem to influence the onset of type 1 diabetes but they may also alter the course of the disease. These factors can be shared by the whole population (climate factor, hygiene, etc.), or by a given family (e.g. eating habits), or be specific to the individual (e.g. foreign travel and sexual partners) (Buzzetti et al., 1998; Knip and Akerblom, 1999).

1.6.3.1.1 Viruses

A viral origin of type 1 diabetes was the first etiological hypothesis (Harris, 1898; Jenson et al., 1980; Patterson et al., 1981; Jenson and Rosenburg, 1984; MacDonald et al., 1987; Yoon and Ihm, 1990; Munoz and Figueredo, 1992 ; Von-Herrath et al., 1998), but the data on which it was based are more complex than initially thought and must now be interpreted in light of data on the autoimmune pathogenesis of the disease. Nonetheless, the viral origin of type 1 diabetes remains a central point in the aetiology of the disease.

It has been known for some time that mumps, rubella, and coxasackie B viruses (Jenson et al., 1980; Notkins and Yoon, 1984; Parkkonon et al., 1992; Pato et al., 1992; Von Herrath et al., 1998) can infect islet cells *in vitro* and in the pancreas *in vivo*. This evidence has been derived from studying children with acute onset early childhood diabetes, but there is not enough clarity about the potential association of viral infections with most cases of autoimmune diabetes (Harris, 1898; Jenson et al., 1980; Patterson et al., 1981; Jenson and Rosenburg, 1984; MacDonald et al., 1987; Yoon and Ihm, 1990; Cambell and Harrison, 1990; Munoz and Figuerodo, 1992; Batch, 1994, Leslie and Elliott, 1994; Von-Herrath et al., 1998). Further, immunologically mediated damage can occur after the causative viral infection has been cleared and viral "footprints" are no longer detectable in the affected organ. The concept that viruses can cause the disease continues as a distinct possibility, especially with the inclusion of recent observation. A more mechanistic understanding of events leading to virally mediated type 1 diabetes has evolved from studies using animal models (Ohashi et al., 1991; Von-Herrath et al., 1994, Forster et al., 1995;Von Herrath et al., 1998).

There are two major processes through which viruses can activate potentially autoreactive lymphocytes without directly destroying the pancreas and/or islets through

activation involving inflammatory cytokines (Tough et al., 1996). The other is the concept of "molecular mimicry". In this instance, autoreactive lymphocytes are directly "cross activated" because they recognise both viral and self determinants (Oldstone, 1987; Barnett and Fujinami, 1992; Leslie and Elliott, 1994; Von Harrath et al., 1994; Baum et al., 1996; Von Herrath et al., 1996; Von Harrath and Oldstone, 1996; Von Harrath et al., 1998).

1.6.3.1.2 The cows' milk hypothesis

The theory that diabetes incidence is affected by infant diet was stimulated by Scandinavian studies showing correlation between the decline in breast-feeding and increase in diabetes in diabetes incidence (Borch-Johnson et al., 1984; Pozzilli, 1999). It was suggested that exposure to certain cows' milk proteins (bovine serum albumin, casein, or β-lactoglobulin) at an early age interrupted the normal development of tolerance to these common dietary antigens (Pozzilli, 1999; Graves and Eisenbarth, 1999). This theory was strengthened by the observation that there were antibodies in diabetes patients which reacted with both a BSA peptide and with islet-cell antigens (Karjalainen et al., 1992; Norris and Pietropaolo, 1999; Pozzilli, 1999; Graves and Eisenbarth, 1999). While it is true that reduction in breast feeding often leads to exposure to potential diabetogens in cows' milk-based formula, this may occur concomitantly with introduction of other foods in the infant diet (Graves and Eisenbarth, 1999). Numerous subsequent case control studies gave conflicting results about the association between breast-feeding and type 1 diabetes, but a meta-analysis of these studies indicated a significant but moderate effect of exposure to breast-milk substitutes or cows' milk-based substitutes (Norris and Scott, 1996; Graves and Eisenbarth, 1999). However, recall bias as well as differential participation rates by case controls may have

affected these studies, and the relatively weak associations observed may have had methodological explanations (Graves and Eisenbarth, 1999).

In summary, despite the conflicting data in this field, the underlying message is that exclusive breast-feeding for at least 4 months would be an easy way to reduce the risk of type 1 diabetes (Pozzilli, 1999).

1.6.4 Genetic susceptibility

Genetic susceptibility to type 1 diabetes appears to be inherited as a polygenic trait (Kida, 1988; Buzzetti et al., 1998; Friday et al., 1999; Graves and Eisenbarth, 1999; Todd, 2000). The presence of specific "genes disease" can be on the basis of two main observations:

1.6.4.1 Twin studies

Twin-studies generally provide an idea of which genetic or environmental factors are dominantly involved in the development of the disease. The concordance rate for type 1 diabetes between identical twins is estimated to be 35-50%, being significantly lower than that in dizygotic twins (Barnett et al., 1981; Olmos et al., 1988; Atkinson and Maclaren, 1994; Lorenzen et al., 1994; Todd and Farrall, 1996; Buzzetti et al., 1998; Hawa et al., 2000). Nevertheless, the concordance in identical twins does not reach 100%, underlining the importance of environmental factors, whether they be nutritional factors to which an individual was exposed early in life, such as cows' milk (Cavallo et al., 1996; Szopa et al., 1993; Leslie and Elliott, 1994; Graves and Eisenbarth, 1999), or viral infection (Szopa et al., 1993; Von Herrath et al., 1998). However, two significant factors should be taken into consideration:

- Identical twins may be genetically different for certain genes because of postzygotic events. The TCR and immunoglobulin (Ig) molecules result from both random and

ordered recombination of the genes encoding them and may differ between identical twins (Tonegawa, 1993; Buzzetti et al., 1998; Friday et al., 1999; Todd, 2000). For example, a study in twins affected by multiple sclerosis (MS) indicates that the expressed TCR repertoire differs within twin pairs discordant for the disease but not within concordant pairs (Utz et al., 1993).

- It is agreed that monozygotic twins are predisposed to type 1 diabetes more than dizygotic twins and non-twin siblings. The concordance rate for the disease is significantly higher in monozygotic twins possessing a susceptible HLA type of DR3/DR4 than those not possessing it (Wolf et al., 1983; Thompson et al., 1988; Buzzetti, 1998; Graves and Eisenbarth, 1999; Todd, 2000; Puglies and Eisenbarth, 2000). Therefore the genetic component may be responsible for the susceptibility to type 1 diabetes.

1.6.4.2 Familial clustering

Epidemiological studies support the hypothesised genetic predisposition to development of diabetes with strong evidence for familial clustering of the disease. The overall risk for developing type 1 diabetes in North American Caucasian sibling, parents, and offspring of individuals with type 1 diabetes ranges from 1% to 15% (Wagenger et al., 1982; Warram et al., 1984; Warram et al., 1988; Bleich et al., 1993; Warram et al., 1994; Buzzetti, 1998; Friday et al., 1999; Todd, 2000; Puglies and Eisenbarth, 2000) as compared to less than 1% for individuals without affected relatives, and 1.2/1000 of the general population (LaPorte et al., 1995). It is interesting that an increased risk for type 1 diabetes seems to be present also in first degree relatives of individuals with type 2 diabetes mellitus (Groop et al., 1986; Landin-Olsson et al., 1992; Tuomi et al., 1993; Rewers et al., 1995; Zimmett et al., 1994; Turner et al., 1997). Specific HLA haplotypes may account for the susceptibility to both type 1 and type 2 diabetes mellitus. This latter

finding is supported by the occurrence of β-cell humoral autoimmunity in 10-33% of Caucasian adult onset diabetic patients not treated with insulin (Groop et al. 1986; Landin-Olsson et al., 1992; Tuomi et al., 1993; Rewers et al., 1995). Therefore some forms of type 2 diabetes manifest a similar genetic susceptibility and immunologic abnormalities to those characteristics of type 1 diabetes.

Siblings sharing identical HLA haplotypes have a 3 or more times higher risk of the disease (Cavender et al. 1984; Todd, 1997; Graves and Eisenbarth, 1999;Todd, 2000).

Furthermore, studies conducted in the United States of America and Scandinavia have shown that the offspring of parents with type 1 diabetes have a higher risk for progression to the disease if the father, rather than the mother, is affected by the disease (Tillil and Kobberling, 1987; Warram et al., 1988; Dahlquist et al., 1989; Allen et al. 1991; Dahlquist et al., 1991; Tuomelehto et al., 1992; Buzzetti et al., 1998). The risk is, in fact, 1/40 among offspring of father's with type 1 diabetes and 1/60 of offspring of type 1 diabetic mothers (Nepom, 1995). Different explanations have been proposed for this finding:

- Spontaneous abortion by diabetic mothers of foetuses that might develop type 1 diabetes (Graves and Eisenbarth, 1999).

- Maternal environmental factors determine the foetus' level of tolerance to islet autoimmunity (Warram et al., 1984; Graves and Eisenbarth, 1999).

- There is a paternal transmission of HLA DR3/X category of patients suggesting a HLA-associated genetic heterogeneity linked to chromosome Xp in type 1 diabetes (Vadheim et al., 1986; Cucca et al., 1998).

1.6.5 Dissection of the genetic components

According to the above evidence type 1 diabetes appears to have a genetic component. Segregation analysis of the genetic component of type 1 diabetes indicates that the disease cannot be categorised as a classic recessive, dominant, or intermediate inheritance. There are three main differences with respect to Mendelian diseases:

- Type 1 diabetes caused as a result of the interaction between environmental agents and different genetic factors (Von Herrath et al., 1998; Pozzilli, 1999).

- More than one gene is involved in the pathogenesis of the disease, the disease is polygenic rather than monogeneic (Buzzetti et al., 1998; Friday et al., 1999; Todd, 2000; Puglies and Eisenbarth, 2000).

- The genes responsible for the disease do not represent rare variants or a rare mutation, but are polymorphic genes presenting different allelic variants, each with a frequency greater than 1% in the general population and which differ from among each other in some functional characteristics. Although it has been suggested that multiple genes play a role in disease susceptibility, until recently only two genetic regions had consistently shown linkage to, and association with, type 1 diabetes in man. To identify the susceptible genetic region the abbreviation *IDDM* and a number, e.g. *IDDM1*, *IDDM2*, etc, with the number usually reflecting the order in

which such genetic region was reported. Of these, by far the greatest effect is encoded by the MHC region on chromosome 6p21.31 (*IDDM1*) (Marton et al., 1983; Todd, 1994; Buzzetti, 1998; Friday et al., 1999; Graves and Eisenbarth, 1999; Todd, 2000; Pugliese and Eisenbarth, 2000). To a lesser degree, the insulin gene (INS) region on chromosome 11p15.5 *(IDDM2)* seems to be associated (Spielman et al., 1993; Lucassen et al., 1993; Metcalfe et al., 1995; Buzzetti, 1998; Graves and Eisenbarth, 1999; Friday, et al., 1999; Todd, 200;Pugliese and Eisenbarth, 2000). The contribution of these two loci to familial inheritance is approximately 42% for *IDDM1* and 10% for *IDDM2* (Davies et al., 1994; Friday et al., 1999). There are two main hypotheses about genetic susceptibility of type 1 diabetes with regard to the MHC and non-MHC loci. The first theory results from genome-wide scans for type 1 diabetes susceptibility genes and have excluded other genes with an equally strong effect to the MHC (Concannon et al., 1998; Mein et al., 1998). On the other hand, many other studies demonstrate a strong association of non-MHC loci to type 1 diabetes (Buzzetti et al., 1998; Friday et al., 1999; Kida, 1999; Todd, 2000; Pugliese and Eisenbarth, 2000).

Further, Verdaguer and colleagues have explained how non-MHC-linked polymorphisms can override the susceptibility to type 1 diabetes provided by pathogenic MHC haplotypes, and demonstrated that protective non-MHC genes may selectively target specific lymphoid cell types in complex autoimmune responses (Verdaguer et al., 1999).

Relationships between many other non-MHC genes and a susceptibility to type 1 diabetes has been proposed. An up to date, comprehensive list of the known susceptible gene regions is shown in Table 1.03.

1.6.5.1 The MHC genes, HLA cluster (*IDDM1*)

Many factors may combine the risk of progression to type 1 diabetes. These include a family history of diabetes, genotype (HLA haplotype), environmental factors, residual insulin secretory capacity islet cell antibody (ICA) on the islet sections, and antibodies reacting with characterised islet autoantigens (Pietropaolo and Eisenbarth, 1994; Tisch and McDevitt, 1996). The presence or absence of the others may modify the prognostic significance of any of these risk factors (Bingley, 1996). With respect to genotype, polymorphic variants of the class II antigens DQ and DR are recognised as the major contributing genes to HLA-linked susceptibility to autoimmune diabetes (Trucco, 1992; Conrad et al., 1994; Aitman and Todd, 1995; She and Marron, 1998). This hypothesis is supported by transracial studies that have consistently shown the influence of HLA genotypes on the risk of developing diabetes (Aitman and Todd, 1995), and the observed conservation of the MHC class II effect in both human and murine autoimmune diabetes (Owerbach and Gabbay, 1996).

The association of type 1 diabetes with HLA antigens has been known for many years. Indeed, 90-95% of Caucasian patients with type 1 diabetes, compared to 45-55% of a control population, possess the HLA DR3 or DR4 or both antigens. However, individuals with heterozygosity for DR3/4 appeared to be the most susceptible to progression to type 1 diabetes (Wolf et al. 1983; Thompson et al., 1988). In contrast, expression of the HLA-DR2 allele is associated with protection from developing type 1 diabetes (Erlich et al., 1991; Graves and Eisenbarth, 1999).

Studies involving a variety of racial and ethnic groups have revealed that the presence of a specific human DQβ chain variant encoding a neural amino acid (alanine, valine, or serine) other than an aspartic acid residue at codon 57 (non-Asp-57) is strongly

associated with type 1 diabetes (Todd et al., 1987; Todd et al., 1988; Morel et al., 1988; Khalil et al., 1990). In contrast, a negatively charged aspartic acid at position 57 of the DQβ chain (Asp-57) appears to confer resistance to type 1 diabetes (Trucco, 1992; Leslie and Elliott, 1994; Todd, 1997).

Apart from the importance of DQβ non-Asp-57 in disease susceptibility, the Arg-52 amino acid residue of the DQα chain also was shown to be important in the susceptibility to the disease (Khalil et al., 1990). These two disease associated HLA polymorphisms represent a refinement of the type 1 diabetes relative risk assessments for the HLA-DR3 and HLA-DR4 disease associations. Because of linkage disequilibrium, the class II MHC extended haplotype inherited with the HLA-DR3 allele includes DQα alleles with the Arg-52 polymorphism and the HLA-DR4 haplotype associates with non-Asp-57 DQβ alleles. A question subsequently raised is whether HLA DQα (52)-Arg and DQβ (57)-non-Asp are simply the genetic markers for the group of people with a risk of type 1 diabetes; or whether the changes in the chemical structure of HLA DQα and β-chains are directly involved in initiation of the autoimmune responses against pancreatic β cells. The latter possibility by a study of the three-dimensional structure of the MHC class II molecule. The MHC class II molecule consists of 2 α helices and 1 β-sheet (Brown et al., 1988; Todd, 1997). The amino acid residue 52 of the α-chain and amino acid residue 57 of the β-chain are located at the tolerance of the alpha helices. The substitution of the amino acids at these residues of the α- and β-chains is thought to alter the three dimensional structure of the HLA class II molecule. This makes it easier for helper T-cells to recognise an antigen peptide sitting on a β-sheet together with the MHC class II antigen which initiates the immune responses of the MHC class II of helper T-cells against the pancreatic β-cells (Figure

1.07). Regarding the HLA-DQα chain, a substitution of its residue 52 being modally serine with argenine (DQA1*0301) predisposes to type 1 diabetes through the different ethnic groups including Caucasian, black and Japanese populations (Khalil, 1990; Buzzetti et al., 1993; Rowe et al., 1994; Sanjeevi et al., 1995; Leech et al., 1995; Nepom and Kwok, 1998). Recently, some other polymorphic amino acid variations in HLA-DQ and HLA-DR are suggested to be involved in susceptibility to type 1 diabetes; DQ-69 (Leu) (Sanjeevi et al., 1995; Nepom and Kwok, 1998), DQ-47(Gly) (Buyse et al., 1994; Zamani and Cassiman, 1998), and DR 71(Lys) (Harfouch-Hammound et al., 1996; Zamani and Cassiman, 1998).

HLA-DQ8 and HLA-DQ2 have been associated with the predisposition to type 1 diabetes (Thorsby and Ronningen, 1993). These DQ molecules occur in linkage disequilibrium with DR4 and DR3, respectively (Thorsby, 1995). Epidemiological studies have shown that individuals carrying the extended haplotype DR4/DQ8 and DR3/DQ2 give the highest predisposition to type 1 diabetes. Several autoantigens have been implicated in the aetiopathology of this disease, and these include insulin, GAD 65, Hsp60, and IA-2 among others. Most of the efforts have focused on GAD 65 and insulin with some work being done on the tyrosine phosphate, IA-2 (Herman et al., 1999; Graves and Eisenbarth, 1999).

1.6.5.1.1 HLA and cytokines

One of several possible mechanisms for the HLA-disease association is HLA-related polymorphism of cytokine expression. However, with the exception of TNF, no evidence has been found for a relationship between HLA alleles and cytokine expression. This may be because cytokine responses to commonly employed mitogens are neither antigen nor HLA dependent, and responses to recall antigens are dominated by the effect of prior antigen exposure. The effect of HLA on secretion level of TNF-α/β and IFN-γ has been studied since 1990 (Soldevila et al., 1990; Sachs et al., 1990; Soldevila et al., 1991). Recently, however, the association of HLA polymorphism to IFN-γ production has become a well documented issue (Petroviski and Harrison, 1997; Weigle and Romball, 1997; Rabinovitch, 1998; Perry et al., 1999). Various authors have reported the association between the elevation of TNF-α and IFN-γ production to polymorphisms in the HLA- DR and DQ haplotypes, whereas, TNF-β production differs. This is at first surprising, given that TNF-α and -β genes are adjacent on the MHC whereas the IFN-γ gene is located on chromosome 12. A possible explanation is that the TNF-α and IFN-γ genes have a common regulatory factor (Petroviski and Harrison, 1997).

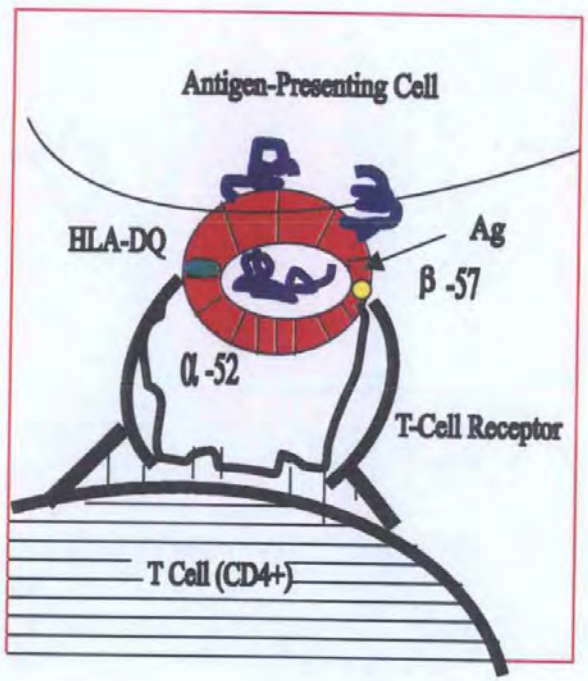
It is not known whether the association between HLA class II alleles and diabetes susceptibility is the result of linkage disequilibrium with other as yet uncharacterised loci, or is a direct result of the gene products themselves. However, given the involvement of class II in peptide presentation to T cells and subsequent immune regulation, it is a likely possibility that their involvement in initiating autoimmune disease is direct (She, 1996), for example by recognition and presentation of self-peptides, or by altering the developing T cell repertoire and influencing in the production of IFN-γ, a major diabetogenic cytokine (Rabinovitch, 1998).

Table 1.03: Summary of human type 1 diabetes susceptibility loci

Locus	Chromosome Location	Candidate genes
IDDM1	6p21.3	HLA-DR/DQ
IDDM2	11p15.5	INS VNTR
IDDM3	15q26	?
IDDM4	11q13.3	MDU1, ZFM1, RT6, ICE,LRP5, FADD,CD3
IDDM5	6q25	MnSOD
IDDM6	18q12-q21	JK(Kidd), ZNF236
IDDM7	2q31-q33	NEUROD
IDDM8	6q25-27	?
IDDM9	3q21-25	?
IDDM10	10p11-q11	?
IDDM11	14q24.3-q31	ENSA, SEL-1L
IDDM12	2q33	CTLA-4
IDDM13	2q34	IGFBP-2, IGFBP-5, NEUROD, HOXD8
IDDM15	6q21	?
IDDM17	10q25	?

The IDDM nomenclature is assigned to a locus after linkage has been formally demonstrated, replicated, and confirmed in at least three different studies (For details refer to Buzzetti et al., 1998; Friay et al., 1999; Pugliese and Eisenbarth, 2000).

Figure 1.07: Illustration of structure of HLA-DQ molecule and antigen recognition



This figure illustrates the process of antigen presentation by APC using HLA-DQ-α and -β chains which are directly involved in initiation of the autoimmune responses against pancreatic β-cells (Adapted from Kida et al., 1999 with slight modification).

1.6.5.2 The insulin gene region (*IDDM2*)

It has previously been demonstrated that the chromosomal region encoding the insulin gene in locus 11q15.5 confers susceptibility to type 1 diabetes (Bell et al., 1984; Julier et al., 1991; Metcalfe et al., 1995; Metcalfe et al, 1996). This locus is a 20 kb region including the structural genes for tyrosine hydroxide, insulin-like growth factor II (IGFII) and insulin.

Further studies restricted the susceptibility to a region of 4.1 kb containing a cluster of highly associated polymorphisms (Lucassen et al. 1993; Owerbach and Gabbay, 1993), one or more of which could be responsible for the (11q15.5) type 1 susceptibility. Finally, Bennett et al, by "cross-match" haplotype analysis, mapped the *IDDM2* within the VNTR at the 5′ end of the insulin gene (Bennett et al. 1995). The polymorphism results from a variable number of tandemly repeated (VNTR) 14 bp oligonucleotides located at 363 nucleotides from the transcriptional start site. Alleles of VNTR are grouped into three classes according to their lengths (number of repeats): class I with an average of 570 bp, class II with an average of 1200 bp, and class III with an average of 2200 bp (Lucassen et al., 1993).

The class I VNTR allele from a parent to offspring is related to the number of repeats (Bennett et al., 1995). *IDDM2* mapped to the VNTR minisatellite region by a genome wide scan of affected sib-pair families is estimated to account for 10% of family clustering of the disease (Davies et al., 1994). It is strongly suggested that the susceptibility gene to type 1 diabetes in *IDDM2* is the VNTR locus itself (Owerbach and Gabbay, 1993; Bennett et al., 1995; Undlien et al., 1995), although it has not been proven definitely (Doria et al., 1996).

Homozygosity for short class I VNTR confers a two fold increase in type 1 diabetes risk (Pugliese et al., 1994), while class III VNTR alleles are dominantly protective (Pugliese et al., 1994; Pugliese et al., 1997). The VNTR locus is known to regulate the transcription and expression of the insulin gene in the pancreas. The class III VNTR alleles, protective for type 1 diabetes, are associated with lower levels of steady-state expression of insulin mRNA in human pancreas *in vivo* (Bennett et al., 1995; Vafiadis et al., 1996) and *in vitro* (Kennedy et al., 1995). However, it seems difficult to find a link between the lower mRNA levels of insulin in the pancreas and the protective effect of class III VNTR against type 1 diabetes in reference to the autoimmune mechanisms directed to pancreatic cells. It has been found that insulin is expressed in the human thymus (Pugliese et al., 1997; Vafiadis et al., 1997). More interestingly, class III VNTR alleles are found to be associated with 2-3 fold higher levels of insulin mRNA expression in the thymus than class I VNTR alleles (Pugliese et al., 1997; Vafiadis et al., 1997).

The INS VNTR locus is close to the IGF-II gene. IGF-II is known to promote T cell survival preventing apoptosis, and also has an immunomodulatory activity (Polychronakos et al., 1995; Metcalfe et al., 1995). Furthermore, IGF-II is the dominant thymic peptide of the insulin-superfamily sharing a high homology to proinsulin and therefore may help induce immune tolerance to proinsulin by negative selection of T cells in the thymus (Green et al., 1993). It is to be noted that the class I VNTR is highly associated with the higher steady-state levels of IGFII mRNA expression in human placenta, showing that the VNTR may act as a long range control element affecting the expression of both insulin and IGF-II (Paquette et al., 1998). Inconsistently, no associations of INS VNTR variations with IGF-II expression are found in the human foetal pancreas and thymus (Vafiadis et al., 1998).

1.6.5.3 Other possible genes

IDDM1 and *IDDM2* are the major susceptibility genes for type 1 diabetes, and the contribution of these two loci to familial inheritance is approximately 42% for *IDDM1* and 10% for *IDDM2* (Davies et al., 1994; Friday et al., 1999; Todd, 2000). As a result of genome wide searches, relationships between many other non-MHC genes and susceptibility to type 1 diabetes have been proposed (Table 1.03). Some of them have been confirmed by subsequent studies, but others have not. The *IDDM12* region, includes a gene for CTLA-4 (cytotoxic T-lymphocyte associate-4), that maps to the long arm of chromosome 2 (2q33) (Donner et al., 1997). As CTLA-4 is a T cell receptor which negatively regulates T cells and induces apoptosis of T cells. It is of interest to see if the polymorphisms of CTLA-4 are involved in the development of autoimmune diseases caused by a lack of negative selection of T cells, namely apoptosis of autoreactive T cells, in the thymus. The polymorphisms of the CTLA-4 gene are reported to be linked to and associated with type 1 diabetes (Nistico et al., 1996; Donner et al., 1997; Marron et al., 1997), but a recent family-association study showed no association of CTLA-4 polymorphisms with type 1 diabetes (Owerbach et al., 1997). A recent genome-wide search for type 1 diabetes susceptibility genes in 356 affected sibpair families from the UK (Warren 1) suggests that chromosome 16q22-q24, chromosome 10p13-q11, chromosome 14p12-q21, and chromosome 19p13-q13 may be potential susceptibility candidates genes for type 1 diabetes, outside the HLA region (Bain et al., 1990; Todd, 1997; Mein et al., 1998; Todd, 2000).

1.6.6 Humoral immunity

Organ specific autoantibodies, such as anti-thyroglobulin antibodies are often detected in patients with type 1 diabetes (Kaino et al., 1994). The autoantibodies specifically directed to pancreatic β cells are known to be present in the circulating blood of patients with type 1 diabetes and, furthermore, to emerge before the clinical onset of the disease. These comprise islet cell antibodies (ICA), insulin autoantibodies (IAA), and some others, such as autoantibodies against tyrosine phosphate (IA2), and membrane glycoprotein (Glima 38) (Baekkeskov et al., 1990; Aanstoot et al., 1996; Graves and Eisenbarth, 1999). Today, these antibodies are not believed to be directly involved in the destruction of pancreatic β cells but to be secondary immunological phenomena subsequent to destruction of pancreatic β cells (Graves and Eisenbarth, 1999).

Some studies have shown that administration of the autoantigens GAD or insulin, prevent or slow down the development of diabetes, which suggests that these autoantigens play a key role in the development of the autoimmune response and destruction of pancreatic β cells (Tisch et al., 1994).

Such antibodies may be detected in a prediabetic stage several months or years prior to the clinical onset of type 1 diabetes. In a study, changes in ICA were noticed before and after the onset of type 1 diabetes in a 9 year old girl. ICA was already positive after 12 months before the clinical onset of the disease and its titre increased steeply thereafter reaching its peak at the time of its clinical onset. The changes in ICA titres were inversely correlated with insulin secretion and glucose tolerance expressed by HbA1c (Kida et al., 1994). Autoantibodies are, therefore, very good predictors of the development of type 1 diabetes in high risk populations, particularly when in high titre.

It has been reported that almost all subjective autoantibody positive develop type 1 diabetes within 5 years, if they were positive for 3 auto-antibodies (Verge, 1996).

Although diagnosis of type 1 diabetes rests on the detection of humoral antibodies detected against beta-cell antigens, it is now recognised that initiation of beta-cell autoimmunity is a T-cell mediated process (Wegman et al., 1996). This conclusion is based on evidence of cellular infiltration (insulitis) in the islets of NOD mice long before the development of overt diabetes, T-cell adoptive transfer experiments and isolation of islet specific T-cell clones. However, the sequence of development of autoantibodies can be revealing in terms of the primary initiating targets of the immune response. Until recently, it was thought that GAD might be the primary antigen to be recognised, but now attention is shifting towards insulin (Eisenbarth, 1994).

1.6.6.1 Cytokines involved in humoral immunity

At least two cytokines contribute to B cell maturation in the bone marrow; the lymphoid stem cell growth factors IL-7 and IL-11. Once B cells egress from the bone marrow, isotype switching, the activation of mature B cells, and their final differentiation into plasma cells are processes that are under T-cell control (Finkelman et al., 1990). Switching mechanisms involve at least two factors: T cell-B cell cognate interactions and secretion of interleukin molecules (Cyster et al., 1994). These interleukins make accessible the 5′ switch regions of the heavy chain DNA sequence so that the γ, ε, or α genes can be transcribed. Contact signals provided by the T cells are triggered through the CD40 ligand on the surface of the B cells by the CD40 ligand on T cells (Cyster et al., 1994). Cytokines that trigger the isotype switch include IL-4 and IL-13, which induce switching to the IgE isotype (Vercelli et al., 1993; Borish and Rosenwasser, 1996). There are many reports that categorise cytokines of TH2 origin as mediators of

humoral immunity. However, there are cytokines of macrophage or TH1 origin that take part in humoral immunity. For example, TGF-β, which triggers the IgA isotype switch. IL-10 contributes to the generation of IgG isotypes. Other cytokines that influence B-cell maturation but are not involved in isotype switching are IFN-γ, IL-1, IL-2, IL-5, and the recently described B-cell growth factors IL-4, IL-6, IL-12, and IL15 (as discussed earlier) are also involved in humoral immunity.

1.6.7 Cellular immunity

It is believed that cellular immunity plays a primary role in the destruction of pancreatic β-cells. Infiltration of pancreatic islets, known as "insulitis" or "insulinitis" is found in patients with type 1 diabetes. Immunostaining of biopsies of pancreatic islets from patients with type 1 diabetes reveals that most of the infiltrating T cells are $CD8^+$ and a few are $CD4^+$ cells (Itoh et al., 1993). Conversely, in NOD mice, which are an animal model of type 1 diabetes, most of the infiltrating lymphocytes on pancreatic islets are $CD4^+$ cells and approximately 10% are $CD8^+$ cells (Ito et al, 1996). The CD45RB low T cells are predominant among CD4+ cells infiltrating a pancreatic islet in NOD mice (Shimada et al. 1996). The balance between TH1 and TH2 is thought to be involved in cellular immunity, leading to destruction of the pancreatic β cell (Rabinovitch, 1994). A shift of TH1/TH2 balance towards TH1 dominance seems to be closely related to cellular autoimmunity against pancreatic β cells in patients with type 1 diabetes and animal models of diabetes. There is a sex difference in the incidence of diabetes in NOD mice, providing a clue for understanding the underlying basis for autoimmunities to pancreatic β cells in man.

More mRNA for TH1 cytokines than for the TH2 seemed to be produced by T cells infiltrating the pancreatic islets in female NOD mice that are diabetes-prone. The opposite was fewer for male NOD mice, which are more diabetes resistant (Fox and Danska, 1997; Hirai et al., 1998). CD45Rblow CD4 splenocytes produce more TH2 cytokines when NOD mice are young. They seem to produce more TH1 cytokines just before the onset of diabetes (Shimada et al., 1996). Female NOD mice have more CD45Rblow CD4+ splenocytes (considered to be the memory/activated cells) and more CD4+CD25+splenocytes (considered to be activated cells) than male NOD mice (Hirai et al., 1998). Administration of peptides of insulin or GAD decreases TH1 cytokine production while TH2 cytokines production increased in NOD mice, associated with the prevention of diabetes in this model (Muir et al., 1995; Sai et al., 1996). These findings indicate that dominance of TH1 to TH2 plays a primary role in initiation of the autoimmune responses against pancreatic β cells leading to its destruction in NOD mice.

1.6.8 Cytokines in autoimmune diseases

Several clinical and experimental observations suggest that autoimmune diseases develop as a result of abnormalities in the immune response mediated by T cells and T cell-derived cytokines (Paul and Seder, 1994; O'Garra et al., 1997; King and Sarvetnick, 1997). Evidence is accumulating in both experimental animal models and human pathological conditions to suggest that the relative contribution of either TH1- or TH2-dominated reactions can determine the development of a particular autoimmune response (Liblau et al., 1995). In most cases, it appears as a consequence of the development of self-reactive CD4+ TH1 cells (reviewed in King and Sarvetnick, 1997). The frequently proposed counter-regulatory or protective role of TH2 cells is still not clearly established, since TH2 clones can induce autoimmune diseases such as EAE and type 1 diabetes under some circumstances (O'Garra et al., 1997). Moreover, in NOD

mice co-transfer of islet-reactive TH2 clones with diabetogenic TH1 clones is not protective, and in this strain IL-4 knockout does not exacerbate the disease (Suri and Katz, 1999).Recently some authors have proposed that immuno-protection, although related in some way to IL-4 production, is mediated by different (non-TH2) subsets of TH0, TH1, and TH2 clones can all be induced to secrete TGF-β1-producing regulatory T cells (TH3) (Del Prete et al., 1991; Chen et al., 1994; O'Garra et al., 1997; Rabinovitch, 1998; Matzinger, 1998; Sedden and Mason, 1999; Proud'homme and Piccirillo, 2000).

There is a strong association between infection and the onset of autoimmunity, suggesting that infectious agents, genetic factors and environmental factors, might cause a breakdown in tolerance that will progress to an autoimmune disease. Diseases such as type 1 diabetes, autoimmune thyroiditis (Graves' disease and Hashimoto's thyroiditis) and so forth are examples of such chronic syndromes. Much interest has gone into the analysis of their pathogenic mechanisms, and there is now a consensus that these diseases are due to a complex interaction between genetic and environmental factors, with more knowledge of the former (Foldmann 1989; Buzzetti et al, 1998; Graves and Eisenbarth, 1999; Friday et al., 1999; Kida, 1999).

An essentially random process of gene rearrangement generates antigen receptors on the surface of lymphocytes. Consequently, individual lymphocytes have no inherent capacity to make the distinction between what is self and what is foreign, and clones with self-reactive antigen receptors are generated. Although the initiating event(s) remains poorly defined, certain genes have been considered as prerequisite for allowing the autoimmune response to occur. Among these, prime consideration has been given to the immunologically relevant genes, i.e. Ig, TCR, and those encoded within the MHC.

However, there is increasing evidence that the genes coding for cytokines and their receptors may be involved in controlling the normal and aberrant immune response. Recent studies have shown that certain cytokine genes are polymorphic and these genetic variants may have a functional role (Tax, et al., 1995; Song et al., 1996; Rosenwaser, 1997; Gurjit, et al, 1997; Awad et al., 1998; Yamada et al., 1998; Perry et al., 1999; Dizier et al., 1999; Yamada et al., 1999; Bathgate et al., 2000; Aziz et al., 2000). Further, cytokine and/or receptor genes are found in areas where susceptibility genes have been mapped. To date, very little is known about cytokine gene polymorphism and susceptibility to type 1 diabetes as well as the long-term complication of diabetes.

1.6.8 TH1/TH2 cytokine profile

One of the most important points in understanding the cause of autoimmune disease is to determine the basis for TH1/TH2 differentiation in response to antigen. The most critical element in determining TH differentiation is the cytokine milieu in which the T cell is activated. The major determinant of TH1 differentiation has been shown to be IL-12. Thus, IL-12 induces, in a dose-dependent fashion, increasing concentrations of INF-γ and decreasing concentrations of IL-4 in the responding TCR lymphocytes (Manetti et al., 1993). IL-12 mediated IFN-γ may be required for the full expression of the TH1 phenotype (Schmitt, et al., 1994). IL-4, on the other hand, is the major inducer of TH2 activation (Abehsira-Ama et al., 1992). This has been best established in transgenic mice in which the concomitant exposure of the animal to IL-4 and the relevant antigen induces, in a dose-dependent fashion, increasing production of IL-4 in the responding T cells. In summary, elevation of TH1 cytokines or reduction of TH2 cytokines are followed by TH1 CD4+ activation. Processes mediated by TH2 cells would be accelerated by TH2 cytokines and attenuated by responses of TH1 type. This cross-

regulation may partly explain the strong biases towards TH1 and TH2 responses during many infections and autoimmune diseases. It may not be the perfect profile model for demonstrating autoimmune mechanisms, however, it is the only one available now (Matzinger, 1998; Rabinovitch, 1998).

Type 1 diabetes is a disease that develops in genetically susceptible individuals due to a breakdown of self-tolerance that results in destruction of the insulin producing β-cells. It is characterised by lymphocytic infiltration of the pancreatic islets of Langerhans that eventually, cause the specific destruction of insulin producing β-cells (Bedossa et al., 1989; Mandrup Poulsen et al., 1989; Castano and Eisenbarth, 1990; Foulis et al., 1991; Meuller et al., 1995; Robinovitch et al., 1996). Several factors may contribute to the destruction of pancreatic β cells, including the profile of the cytokines secreted by TH1 and TH2 subsets of T-helper cells ($CD4^+$), macrophage antibodies against the islet β cells and cytotoxic T-cells.

Disrupted immune regulation and the balance of TH1 to TH2 cells have been postulated to be crucial in the pathogenesis of type 1 diabetes (Kelso, 1995; Nicholson and Kuchroo, 1996). Cytokines, due to their nature, could be considered to be one of the most favourable agents in the latter process, as they are crucially important in the regulation and maintenance of immune responses (Fitzpatrick and Kelso, 1998).

Th1 cells secrete interleukin (IL)-2, interferon (IFN)-γ, and tumour necrosis factor (TNF)-β whereas TH2 cells secrete IL-4, IL-5, IL-10, and IL-13. Both types of T-helper cells secret IL-3 TNF-α, and granulcyte macrophage colony stimulating factor (GM-CSF).

Previous studies have shown that $CD4^+$ T cells play a primary role in the pathogenesis of the disease. For instance, purified $CD4^+$ splenocytes from diabetic NOD mice or a single $CD4^+$ T cell clone can transfer diabetes into immunodeficient NOD-*scid* recipients

(Christianson et al., 1993; Peterson and Haskins, 1996). In contrast, CD 8^+ T cells are dependent on a signal from $CD4^+$ cells before they can destroy pancreatic β-cells (Nagota and Yoon, 1992; Bradley et al., 1992; Edouard et al., 1993; Rabinovitch, 1998).

It is apparent that the ratio of TH1/TH2 cells can have an important role in the initial stage of the aberrant immune response. These reports suggest that TH0 cells can be as prevalent as TH1 or TH2-like cells after naive CD4 activation, that the relative levels of autocrine IL-4 and IFN-γ are important to the lack of commitment, and that not all cells are predestined to have TH1 or TH2 phenotypes early in the response (Craft and Swain, 1995). Previous studies have shown that during the pre-diabetes stage of the disease there is an imbalance of TH1/TH2 cells due to an increase in number of uncommitted T cells. This could be due to abnormal secretion of either IFNG or the cytokine IL-4.

It is becoming clear that the ratio of TH1/TH2 cells can have an important role in the initial stage of the aberrant immune response. It has been suggested that uncommitted TH0 cells can be as prevalent as TH1 or TH2 cells, and this may be due to the abnormal levels of autocrine IL-4 and IFN-γ (Craft and Swain, 1995; Nicholson and Kuchroo, 1996). An imbalance of TH1 and TH2 cells due to an increase in number of uncommitted T cells has been demonstrated during the prediabetes stage of the disease. Upon secretion of IL-12 from macrophages, TH0 produces IFN-γ, which acts as a positive feed back to deviate the TH1/TH2 balance towards TH1 dominance (Szelachowska et al., 1997; Miner and Croft, 1998).

1.6.9 Cytokine signalling

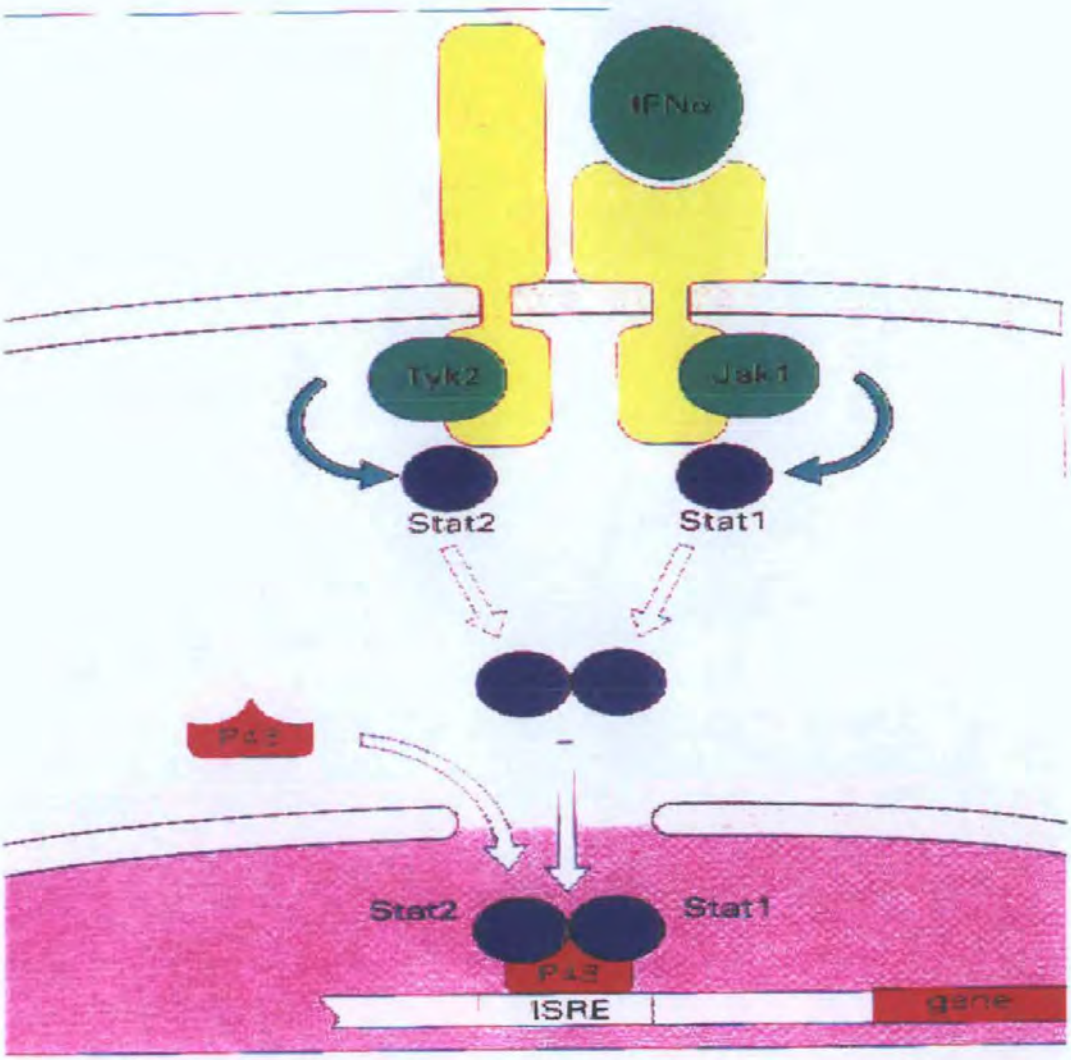
Cytokines have pleiotropic and overlapping effects. Cells of different types, stages of activation and differentiation may utilise distinct signalling pathways, leading to the hope that tissue- and disease-specific intervention strategies based on tissue-specific cytokine action may be developed (Diamond et al. 2000).

Many cytokine receptors signal by rapid pathway using receptor-associated tyrosine kinases of the Janus Kinase family (JAKs), so-called because they have two symmetrical kinase-like domains, and thus resemble the two-headed mythical Roman god Janus. These kinases then phosphorylate cytosolic proteins called signal transducers and activators of transcription (STATs). Phosphorylation of STAT proteins leads to their homo- and heterodimerisation; STAT dimers can then translocate to the nucleus, where they activate various genes (Figure 1.08). The proteins encoded by these genes contribute to the growth and differentiation of particular subsets of T cells (Liu et al., 1998, Leonard and O'Shea, 1998).

In this pathway, gene transcription is activated very soon after the cytokine binds to its receptor, and specificity is achieved by using different combination of JAKs and STATs. This signalling pathway is used by most of the cytokines that are released by T cells in response to antigen. Although cytokines are not in themselves antigen-specific, their effects can be targeted in an antigen-specific manner by their directed release in antigen-specific cell-cell interactions and their selective action on the cell that triggers their production (Liu et al., 1998; Leonard and O'Shea, 1998; Diamond et al., 2000). Figure 1.09 shows a basic model for cytokine action.

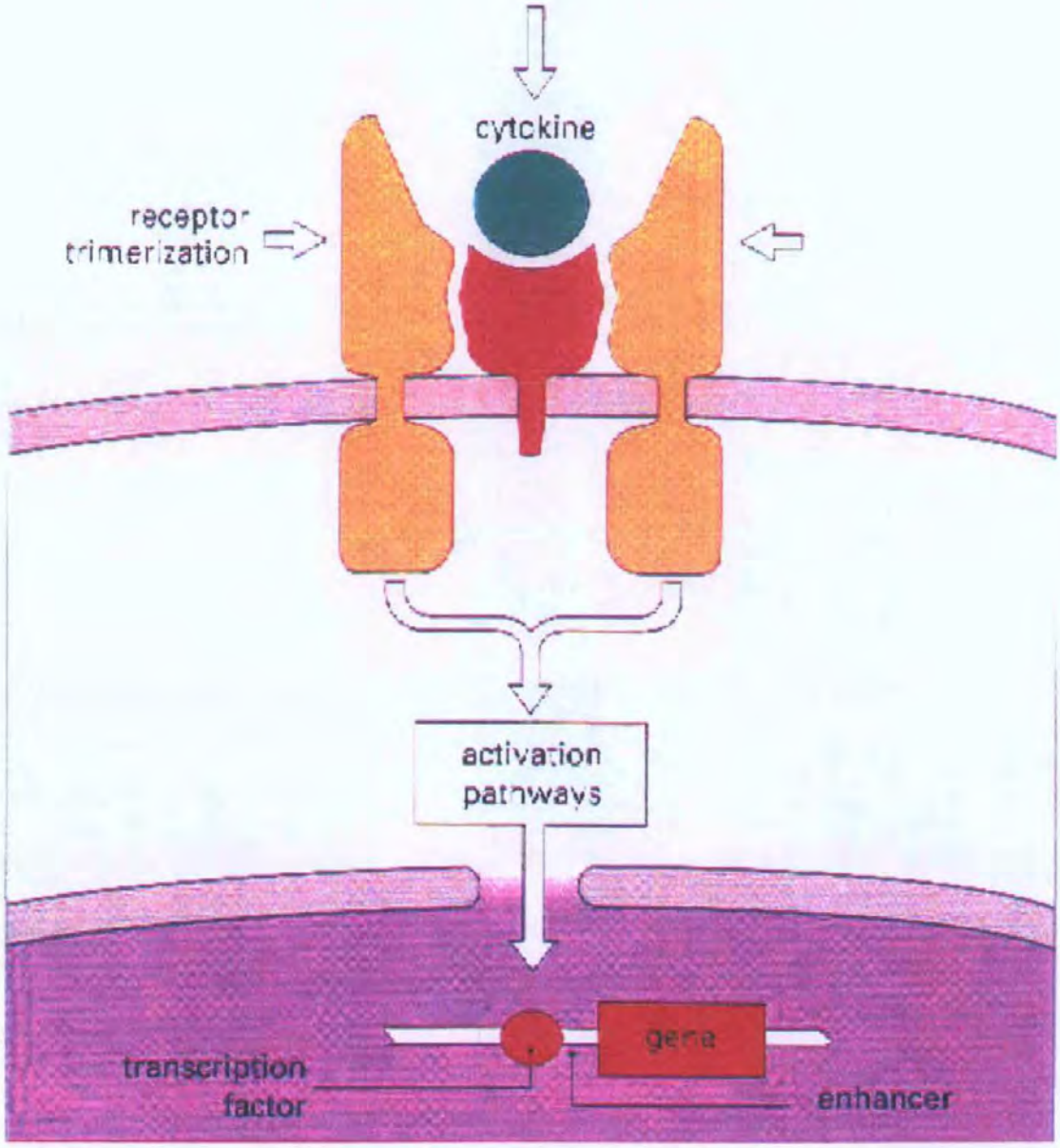
Most work on cytokine signalling in β-cells has been performed with IL-1 and studies are summarised in the following. For comprehensive reviews see (Sandlers et al., 1991; Corbett and McDaniel, 1992).

Figure 1.08: Illustration of intercellular signalling pathways



The intercellular signalling pathways activated by IFN-α are illustrated diagramitacally. IFN-α binding aggregates the two subunits of the receptor. This lead to activation and polyphorylation of two Jak kinases, Jak1 and Tyk2, which then phosphorylate Stat1 and Stat2. These two transcription factors from a complex with a DNA-binding protein called p48 (Adapted from Roitt, Brostoff, and Male, 1998)

Figure 1.09: Illustration of a basic model for cytokine action



A simple model for cytokine activation of a cell is shown, (the structures illustrated are based on those of the IL-6 receptor). Cytokine binds to its receptor on the cells and induces dimerisation or polymerisation of receptor polympeptides at the cell surface. This causes activation of intercellular signalling pathways, resulting in the production of active transcription factors which migrate to the nucleus and bind to the enhancer region of gene induced by that cytokine (Roitt, Brostoff, and Male, 1998).

1.7 Cytokine genes polymorphism

Most studies to date have been performed *in vitro* or in animal models such as the rat (Rabinovitch et al., 1996; Szelachowska et al., 1997; Kallmann et al., 1997) which may not be representative of type 1 diabetes in man. Evidence from present studies in man on the state of cytokines in the pathogenesis of type 1 diabetes is incomplete. However, the role of cytokine genes and type 1 diabetes susceptibility can be summarised as following:

1.7.1 The IL-1 gene cluster

The genes encoding IL-α and -β and IL-1ra as well as the two receptors for IL-1 binding (IL-RTI and II) have been mapped to the long arm of the human chromosome 2 and located to the *IDDM7* susceptibility locus in humans (Copeman et al., 1995). Several polymorphisms have been investigated for their association with type 1 diabetes in case-control studies.

No association has been found between type 1 diabetes and a hexa-allelic intron 6 tandem repeat polymorphism of the IL-1a gene (Copeman et al., 1995). In contrast, the 13.4 kb IL-1B*1 allele of a diallelic polymorphism due to a silent T to C transition in exon 5 of the IL-β gene was found to be significantly associated with type 1 diabetes in familial cases. The strongest association was found in patients with type 1 diabetes not carrying the classic HLA DR3 or DR4 haplotypes (Pociot et al., 1992; Pociot et al., 1994[1]). Interestingly, patients and control subjects carrying type 1 diabetes associated IL-1 β had higher LPS stimulated IL-1 β secreted from isolated PBMNC compared with patients homozygous or heterozygous for the allele not associated with type 1 diabetes (Pociot et al., 1992; Pociot et al., 1994[1]).

The A1/A1 genotype of the penta-allelic 86 bp tandem repeat polymorphism in intron 2 of the IL1Ra gene was also found to be significantly associated with type 1 diabetes, most strongly in familial cases (Pociot et al., 1994[1]). Homozygous IL-1Ra was significantly associated with lower circulating levels of IL-1Ra compared to heterozygous individuals (Mandrup-Poulsen et al., 1994).

Finally, the genotype frequencies of a biallelic polymorphism based on a C → T transition in exon 1B of the IL-1RTI gene were found to be significantly different in patients with type 1 diabetes compared to healthy controls (Pociot et al., 1994[1]; Metcalfe et al., 1995).

1.7.2 TNF-α and β loci

The gene for TNF-α is located to the MHC class III region on the short arm of chromosome 6. There is strong linkage disequilibrium between the TNF locus and the MHC class II susceptibility loci. However, when patients who are identical for HLA DR3/4 haplotypes are compared there appears to be an independent contribution to type 1 diabetes susceptibility from the TNFa2 microsatellite allele (Pociot et al., 1993).

1.7.3 The IFN-γ locus

The IFNG is a single copy gene that contains three introns and four exons encoding a polypeptide of 166 amino acids, 20 of which constitute the signal peptide (Gray and Goedel, 1982; Taya et al., 1982), and has been assigned to chromosome 12q24.12 (Trent et al. 1982). A CA repeat polymorphism in the first intron of the INF-γ gene was reported to be associated with type 1 diabetes in Japanese population of patients (Awata et al., 1994). This association was confirmed in a Spanish (Gallart et al., 1998) and Finnish, but not in a Danish population (Pociot et al.,1997). Previous studies have

suggested that the polymorphism in IFNG may be related to the secretion of the protein (Parvica et al., 1999).

1.7.4 The IL-4 locus

The human IL-4 gene possesses 4 exons and 3 introns localised on the long arm of chromosome 5 on bands q23-31 together with the genes for IL-3, IL-5, IL-9, IL-13, and GMCSF (Arai et al., 1989). Binding sites on the IL-4 promoter region were shown to be critical for its gene regulation. A C to T transition at –285 bp in the promoter region (Song et al., 1996) and another C (-590) T counting from the first ATG codon (Arai et al., 1989) polymorphism have been claimed to be associated with high IgE production (Walley and Cookson, 1996).

Two nucleotide substitutions were identified in allele 2. The first polymorphism is a C to T transition at –285 position. The second polymorphism, which is unique to allele 2, is an A to G transition at –81. Another polymorphism in the IL-4 promoter region is a C to T change at –590 counting from the first ATG codon, which has been investigated for its association with different autoimmune diseases (Walley and Cookson, 1996; Rosenwaser, 1997; Vandenbroeck et al., 1997; Beghe et al., 1999). Recently, a mutation in a sub unit of the IL-4 receptor has been reported which may predispose to allergic diseases by altering the signalling of the receptor (Hershey et al., 1997; Rosenwaser, 1997).

1.7.5 The IL-6 locus

To date there is no genetic study that confirms the role of IL-6 in the pathogenesis of type 1 diabetes. In fact, there has been no report on the association of a polymorphism in the human IL-6 gene to autoimmune disease. Recently, however, a single base change variation at position –174 in the 5′ region of the human IL-6 gene (Yakusha et al., 1987) was characterised and its association to systemic-onset juvenile chronic arthritis has been investigated (Fishman et al., 1998). The association of the IL-6 G (-174) C with insulin sensitivity with regard to white blood cell count in type two diabetes has been studied (Fernandez-Real et al., 2000).

The G (-174) C polymorphism in the IL-6 gene has been found to be associated with different transcription rates. Specially, subjects with the CC genotype showed lower plasma IL-6 levels compared with GC or GG subjects (Fishman et al., 1998).

1.7.6 The TGF-β_1 locus

The gene for TGF-β1 is on chromosome 19q13. TGF-β_1 gene comprises of 7 exons and 8 introns with 390 amino acids (Derynk et al., 1987). Inter-individual variations in the TGF-β_1 gene sequence might be associated with different effects of TGF-β_1 cellular functions (Cambien et al. 1996). Several polymorphisms have been reported in the TGF-β_1 gene (Cambien et al., 1996; Langdahl et al., 1997; Awad et al., 1998).

Pociot et al 1998 have studied a C (76) T in exon 5 resulting in a change from threonine to isoleucine in the position 263 of the peptide and a deletion of C in the intron sequence eight bases prior to exon 5. They have reported no association between the two TGF-β_1

sequence variations with type 1 diabetes. However, a significant association was found in patients with diabetic nephropathy and C (76) T in exon 5 (Pociot et al., 1998).

Recently, Awad and colleagues reported a T (+ 869) C and a G (+ 915) C in exon 1 of the TGF-β_1 gene which lead to a Leucine → Proline and Arginine → Proline substitution, respectively, in codons 10 and 25 of the TGF-β_1 signalling sequence (Awad et al. 1998). These two polymorphisms have been associated with the concentrations of circulating TGF-β_1 protein (Awad et al., 1998; Bathgate et al., 2000; Aziz et al., 2000). The genetic susceptibility of the latter also have been well studied in different organ transplantation (Awad et al., 1998; Bathgate et al., 2000; Aziz et al., 2000). Recently, however, the association of T (+ 869) C has been investigated in other diseases and related to the circulating TGF-β protein (Yamada et al., 1998; Yamada et al., 1999).

1.8 Aims of this study

The aim of this study is to investigate the role of cytokine gene polymorphisms in the susceptibility to type 1 diabetes and the long term complications of diabetes.

To date little is known about cytokine gene polymorphisms in this disease. However, it is becoming clear that the polymorphism in these genes can modulate their function. Therefore, polymorphism of cytokine genes could have a dramatic effect on the regulation of the immune system, hence the pathogenesis of the disease.

In the present study three hundred and eight patients with type 1 diabetes and one hundred and fifty healthy Europid controls were genotyped for polymorphism in the genes encoding for key cytokines from TH1, TH2, TH3 cell, and macrophage origin.

The objectives of the project will be:

- Since IFN-γ and IL-4 play a critical role in the initiation of TH1 and TH2 cytokine profile, respectively, the status of TH1/TH2 cytokine milieu will be studied.

- For the first time the association of the IL-6 G (-174) C polymorphism with type 1 diabetes will be studied.

- The immunosuppression and control on the TH1/TH2 cytokine profile has been under debate for a long time and has mostly been attributed to IL-4. The association of the TGF-β1 gene polymorphism with type 1 diabetes will clarify this issue, and also be the first report of genetic susceptibility to type 1 diabetes.

- In order to confirm the results of case control studies, familial clustering of the marker gene for the cytokine gene polymorphisms will be studied.

- To examine the impact of cytokine gene polymorphism in patients with type 1 diabetes with regard to age at onset and gender of patients, the susceptibility cytokine genes polymorphism with regard to the forementioned factors will be assessed.

Ultimately these results will provide an insight into the role of cytokine gene polymorphism in this disease. It may possibly allow new preventative regimes to be derived for the treatment of this disease and its complications.

2 MATERIALS AND METHODS

2.1 Study subjects

2.1.1 Patients

2.1.1.1 Patients with type 1 diabetes

Three hundred and eight unrelated British Caucasoid patients with type 1 diabetes as, defined by the National Diabetes Group 1979, who had attended the diabetic clinic at Derriford Hospital, Plymouth (Dr B. A. Millward) were studied. Local ethical committee approval had been obtained. At presentation the ages ranged from 1 to 52 years (mean ± SD = 15.3± 9.9 years). The duration of diabetes in the patients ranged from less than 1 to more than 72 years (mean ± SD =21.1± 16.3 years). Although age is a continuous variable, the patients studied were divided into three groups depending on the age at onset of type 1 diabetes. There are definite peaks of incidence of type 1 diabetes shown to exist at different ages (National Diabetes Data Group, 1979; Williams and Pickup, 1998), which are determined as follows:

- <10 years pre puberty at onset of the disease
- 10-20 years time of puberty at onset of disease
- >20 years post puberty at onset of the disease

The characteristics of the patients are presented in Table 2.01.

2.1.1.2 Long term complications of diabetes

In order to study the possible impact of cytokine profiles in the long term complications of diabetes, i.e. retinopathy, nephropathy, and neuropathy, one hundred and twenty three patients with type 1 diabetes with long-term complications were included in the patients with type 1 diabetes (Table 2.02).

2.1.1.3 Familial clustering

Fifty-three multiplex families were used in this study. The families were obtained from the BDA-Warren Repository, UK. The classification of the families is as follows: each family has at least two children with type 1 diabetes, at least one child being diagnosed with type 1 diabetes before the age of seventeen, and both parents are alive (Bain et al., 1990).

2.1.2 Control subjects

2.1.2.1 Normal controls (NC)

One hundred and fifty cord blood samples were taken following normal healthy obstetric deliveries at Derriford Hospital, Plymouth. None of the subjects had a personal or family history of type 1 diabetes or any other autoimmune disease.

2.1.2.2 Diabetic controls

A group of patients with type 1 diabetes who have no documented long term diabetic complications after at least 20 years of duration of diabetes have been used as controls in the studies of long term diabetic complications (n=33) (Table 2.02).

Table 2.01: Characteristics of patients with type 1 diabetes and normal controls

Patient Subgroup	Male : Female n	Age at onset of diabetes mean ± SD/years (range)	Duration of diabetes mean ± SD/years (range)
Total	140 : 168	15.3± 10.0 (0-52)	21.1±16.3 (1-72)
< 10 years	45 : 54	5.9 ± 2.6 (1-9)	20.3±16.0 (2-72)
10-20 years	58 : 52	14.1±3.1 (10-20)	21.5±15.9 (2-72)
>20 years	46 : 42	30.2±6.9 (21-52)	24.6±16.7 (2-67)
NC	61 : 89		

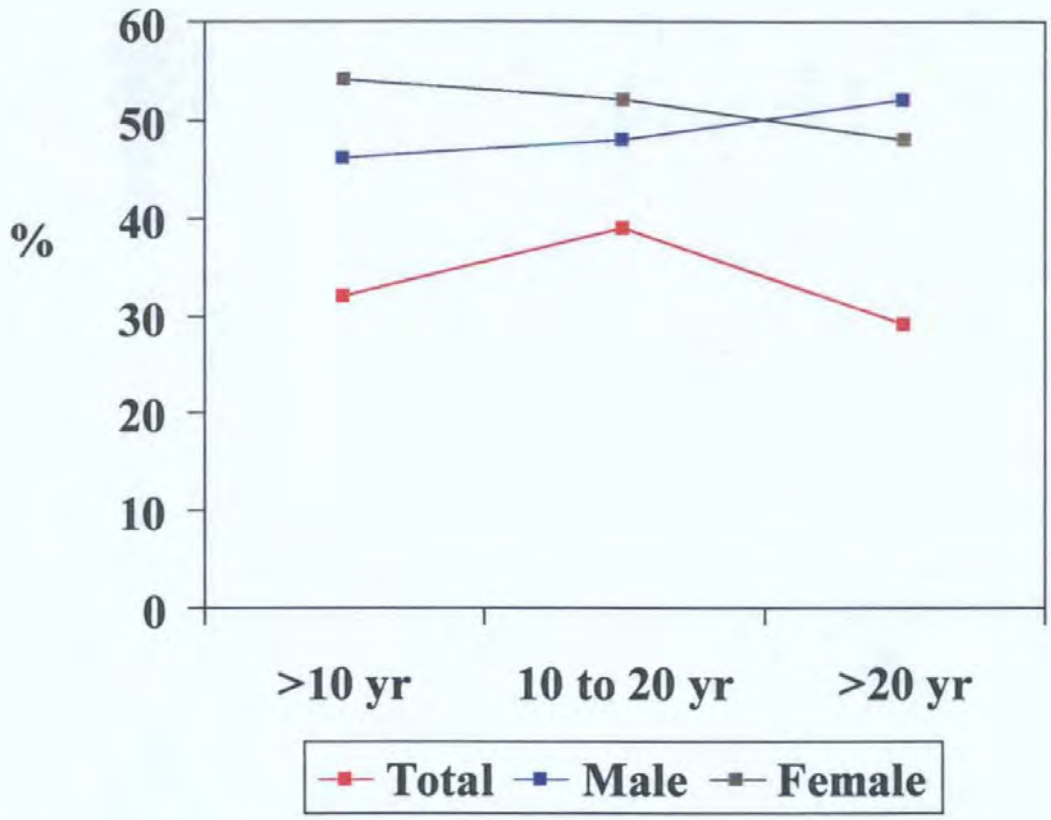
The patient subgroups are expressed as mean ± Standard deviation together with the range in brackets. All patients had type 1 diabetes as defined by the National Diabetes Data Group.

Table 2.02: Patients with the long term complications of diabetes and diabetic controls

Long term complications of diabetes	No.	%
Diabetic control (DC)	33	10.7
Diabetic nephropathy (DN)	50	16.2
Diabetic neuropathy (DNu)	42	13.6
Diabetic retinopathy (DR)	50	16.2

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

Figure 2.01: The distribution of patients with type 1 diabetes used in this thesis with respect to their gender and age at onset of type 1 diabetes



This figure illustrates the frequencies gender of the patients with respect to age at onset of type 1 diabetes. Both male and female groups of patients with type 1 diabetes were distributed almost equally according to age at onset of type 1 diabetes.

2.2 Materials

2.2.1 Water

Double distilled water was prepared using Barnstead thermoline (Triple Red Limited, Oxford, UK), and used in all stock preparations. Sterile water (Baxter Healthcare, Thetford, UK) was used in PCR reaction solutions and oligonucliotide digest solutions.

2.2.2 Reagents

All reagents used in this study were of analytical grade or equivalent. Acetic acid, disodium ethylene diamine tetra-acetic acid, glycerol, hydrochloric acid, magnesium chloride, orthoboric acid, sodium chloride, sodium citrate, sodium dodecyl sulphate, Sodium hydroxide, sucrose, Tris (hydroxymethyl) amenomethane and Tween 20 were purchased from BDH Laboratory Supplies-Merck Ltd (Poole, UK). Choloroform, ethanol, and M were purchased from Rathburns Ltd (Walkerburn,UK). Ammonium persulphate, ethidium bromide, and xylene cyanol purchased from Sigma Chemicals (Poole, UK).

2.2.3 Enzymes and buffers

- Endonuclease restriction enzymes: NheI, BsmFI, and NlaIII were purchased from New England Biolab, Herts, UK.
- Taq polymerase and super Taq buffer were purchased from HT Biotechnology, Cambridge, UK.
- Deoxynucleoside 5'-triphosphates (NTPs) and T4 polynucleotide kinase (T4 PNK), and $\gamma\ ^{32}P$ was purchased from Pharmacia Biotech, Herts, UK.
- 50 x 30 cm filter paper was purchased from Heto Laboratory Equipment, Surrey, UK.

- Ultra pure agarose was purchased from Life Technologies, Paisly, UK.
- TEMED and formaldehyde were ordered from Sigma Chemicals, Poole, UK.

Sequagel ™ Cocentrate, Diluent, and Buffer were purchased from Biorad, Herts, UK.

2.2.4 Stock solutions

- Tris/borate electrophoresis buffer (TBE)

10×solution: 0.89 mM tris base, 0.89 mM boric acid, 2mM EDTA (pH8.0)

- Ethidium bromide

10 mg/ml

- Xylene cyanol track dye/loading buffer

0.25% w/v xylene cyanol, 10% v/v glycerol in 10×TBE

- Sodium chloride/sodium citrate, SDS

20×solution: 3M NaCl, 0.3 M tris-sodium citrate

- TE (Tris-EDTA) 0.1

10 mM Tris, pH 7.6, 0.1 mM EDTA pH 8.0

2.3 Autoclaving

To prevent bacterial infection all solutions, glassware, and plasticware used in this study were autoclaved at 121°C under 15 psi for 30 minutes in a steam autoclave (Prior Clave Limited , London, UK).

2.4 Genomic DNA isolation from peripheral blood leukocytes

Ten to 15 ml of peripheral blood venous blood was collected into 5% disodium ethylene diamine tetra-acetic acid (Na_2EDTA), and stored at –20°C in polypropylene containers prior subsequent DNA extraction.

For DNA extraction a Nucleon®BACC (blood and cell culture) genomic DNA extraction kit was used (Scotlab Ltd, Coatbridge, UK). The manufacturer's protocol was followed with a slight modification, as below.

The kit reagents supplied (Reagents A & B, sodium perchlorate, Nucleon® resin) were used.

The peripheral blood was thawed at room temperature, and an aliquot of 10-15 ml transferred to a 50 ml Falcon tube (Phillip Harris Scientific, Cardiff, UK). To the sample a 4x volume of Reagent A (10 mM Tris-HCL, 320 mM sucrose, 5mM $MgCl_2$, 1% Triton X-100, pH 8.0) was added and mixed on a Luckham R100/TW Rotatest shaker for 4 minutes. After centrifugation at 1300 g for 4 minutes on a MSE Mistral 1000 (MSE Scientific Instruments, Leicester, UK), supernatant containing lysed red cells was separated from the pellet and discarded.

The pellet was resuspended in 2 ml Reagent B (40 mM tris-HCl pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% SDS) and incubated at 37°C for 15 minutes, enabling nuclear membrane disruption to occur. The suspension was transferred to a 15ml falcon tube

(Phillip Harris Scientific, Cardiff, UK), and 500 µl of sodium perchlorate added and mixed by inversion.

Three ml of chloroform (-20°C) (Rathburn Ltd, Walkerburn, UK) was added and mixed to emulsify the 2 phases, which were separated by centrifugation at 1300 g for 3 minutes. The aqueous phase containing the DNA was carefully transferred to a fresh tube ensuring that the interphase with silica resin and the underlying organic phase was not disturbed. The solution was centrifuged for 1 minute at 1300g to pellet any residual silica and after centrifugation, the supernatant was transferred to a clean 15 ml falcon tube and twice the amount by volume of 100% ethanol (-20°C) (Rathburn Ltd, Walkerburn, UK) was added to it to precipitate out the DNA. The 15 ml falcon tube was inverted for several times and resulting "DNA strand" was hooked out using a sterilised glass pipette with a sealed tip was used to hook out the precipitated DNA which was washed in 70% ethanol, dissolved in 500 µl of sterile water. To ensure that all of the DNA went into solution, the sample was kept at 4°C over 24 hours.

2.4.1 Determination of DNA concentration and purity

To ensure that the DNA was in a homogenous solution the samples were stored at 4°C over 24 hours before taking an optical density reading (OD). Five µl of stock DNA was mixed with 495µl of TE buffer in a 1.5 ml microcentrifuge tube. OD's were taken at 260 nm and 280 nm and compared with a TE reference sample using optically matched quartz cuvettes employing a Cecil spectrophotometer (Cecil Instruments Limited, Cambridge, UK). The concentration (µg/µl) of the DNA in the stock solution was determined by multiplying the absorbance reading at 260 nm by a factor of five. The purity of DNA was assessed using the ratio of Ods at λ260 nm/λ280 nm. DNA yields

ranged from between 200μg and 250μg DNA per 10 ml blood. DNA stocks were stored in the DNA Bank of the Molecular Medicine Laboratory. An aliquot of DNA samples were diluted accordingly to give a concentration between 50-150 ng/μl for use in PCR amplification as will be described in subsection 2.6.1.

2.5 Linkage markers

2.5.1 Polymerase chain reaction

It is possible to obtain large quantities of a particular DNA sequence merely by selective replication. The method for selective replication is called the polymerase chain reaction (PCR). The development of the PCR, which allows sequences of DNA to be amplified easily, has revolutionised genetic mapping. It is a cell-free method of DNA cloning. The technique was first described in its initial format in 1971 (Kleppe et al. 1971) and over the next twenty six years, appreciation of its potential became widespread (Mullis and Faloona, 1987; Saiki et al. 1988). To permit such selective amplification, some prior DNA sequence information from the target sequences is required, enabling the construction of two oligonucleotide primers, usually about 20 nucleotides in length, from each end of the target DNA sequence. Such so-called amplimers, when added to denatured genomic DNA, will bind specifically to complimentary DNA sequence immediately flanking the desired target region. They are designed so that, in the presence of a suitably heat-stable DNA polymerase and DNA precursors (the four deoxynucleoside triphosphates, dTP, dCTP, dGTP, dTTP), they can initiate the synthesis of new DNA strands which are complimentary to the target DNA segment.

PCR is a chain reaction because newly synthesised DNA strands will act as templates for further DNA synthesis in subsequent cycles. Starting with a mixture containing as little as one molecule of the fragments of interest, repeated rounds of DNA replication increase the number of molecules exponentially. After about 30 rounds of DNA

synthesis, the PCR product will include, in addition to the starting DNA, about 10^7 copies of the specific target sequence, an amount which is easily visualised as a discrete band of specific size when submitted to agarose gel electrophoresis. An outline of the polymerase chain reaction is shown in Figure 2.02. A heat stable DNA polymerase is used because the reaction involves sequential cycles composed of three steps:

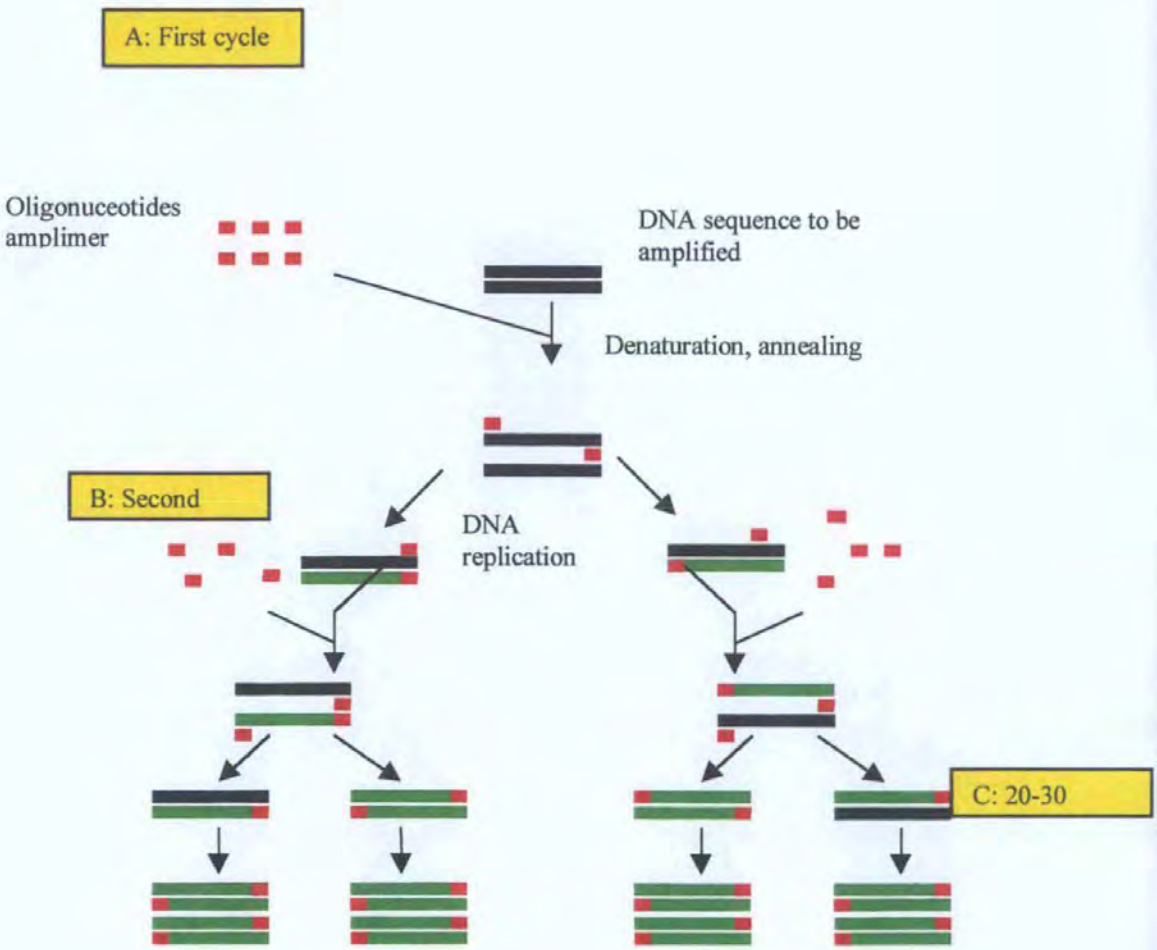
- Denaturation- typically at about 93-95°C for human genomic DNA.

- Annealing- at temperatures usually from 48 to 65°C, depending on the T_m of the expected duplex (the annealing temperature is typically about 5°C below the calculated T_m).

- Elongation (DNA synthesis)- typically about 70-74°C.

All PCR reactions implemented in this study were run in PTC-200 Thermocycler (MJ Research, Essex, UK).

Figure 2.02: Illustration of polymerase chain reaction (PCR)

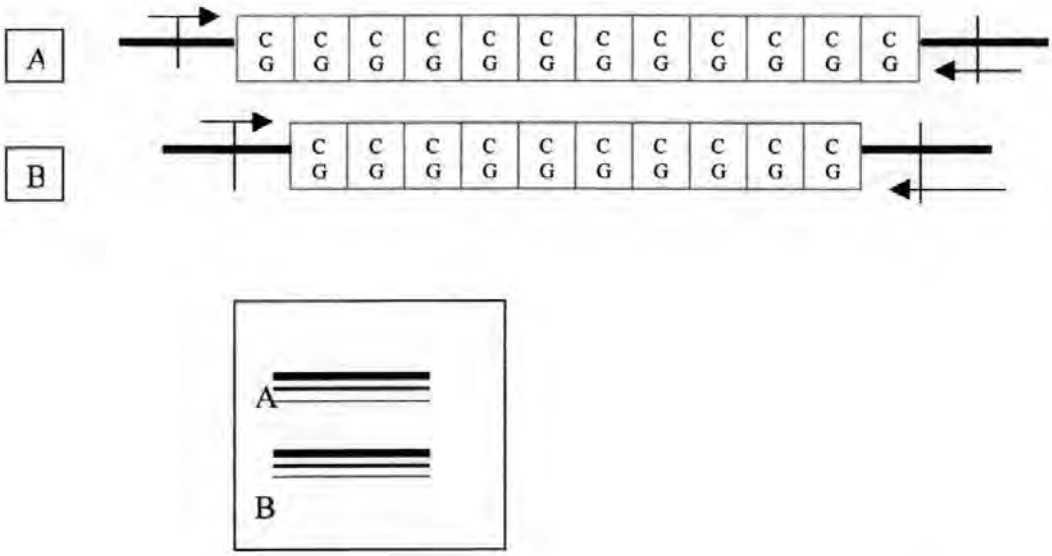


Polymerase chain reaction (PCR) for amplification of particular DNA sequences. Amplimers (red) that are complimentary to the ends of the sequence of interest (black) are used in repeated rounds of denaturation, annealing, and DNA replication. Copies of the target sequence are shown in green. The number of copies of the target sequence doubles in each cycle of replication, eventually overwhelming any other sequences that may be present (Adapted from Hartl and Jones, 1998 with slight modifications).

2.5.2 Microsatellite analysis

Short tandem repeat polymorphisms (STRPs) or microsatellite markers are polymorphic genetic markers. Such markers were first described in 1982 (Hamada et al, 1982). Microsatellites consist of short sequences, typically 1 to 4 bp in length, that is tandemly repeated several times, and often characterised by many alleles. The most useful repeat element is the $(CA)_n$ repeats are often polymorphic if they exceed 12 repeats, although runs of single A's and T's are common as they represent 0.3 % of the genome. Microsatellites are repeated frequently, 10 to 60 times, and can readily be amplified by PCR. They are widely dispersed throughout the genome being found on average every 6 kb and because they have many alleles they are very informative (Weber and May 1989). An example of use of CA repeat marker is shown in Figure2.03.

Figure 2.03: Illustration of microsatellite markers

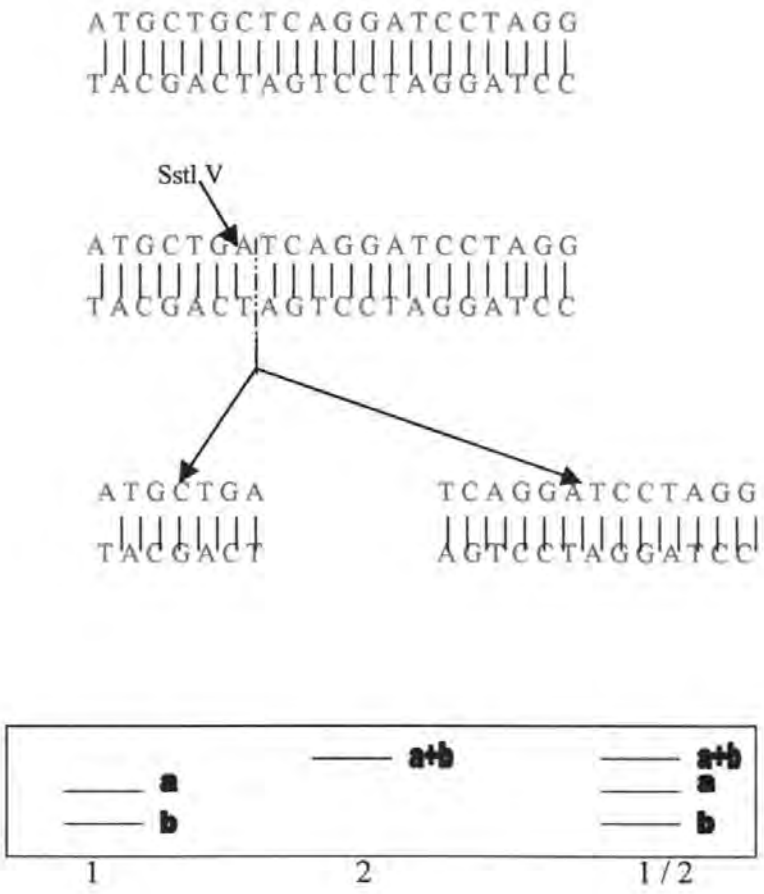


This figure illustrates typing of a CA repeat polymorphism that has two alleles as a result of variation in the number of the CA. In this figure the C= CA and G= GT. On the autoradiograph each allele is represented by a major upper band and two minor 'shadow bands'. Individual 1 has genotype as 1 / 3 (according to this study nomenclature) (Adapted from Strachan and Read, 1996 with slight alteration)

2.5.3 Endonuclease restriction reaction

Restriction Site Polymorphisms (RSP)-RSPs are polymorphisms resulting in alleles possessing or lacking a specific restriction site. Such polymorphisms can be typed using an endonuclease restriction enzyme, which signifies the polymorphic allele by cutting the PCR products into different sized fragments depending upon the polymorphism. The RSP is an alternative to RFLP, a technique which was typed by using Southern blot hybridisation (Southern, 1975). Amplimers for RSPs are designed using sequences which flank the polymorphic restriction site, amplifying from genomic DNA, then cutting the PCR product by an appropriate restriction enzyme and separating the fragments by agarose gel electrophoresis (Figure 2.04).

Figure 2.04: Illustration of restriction site polymorphisms (RSPs)

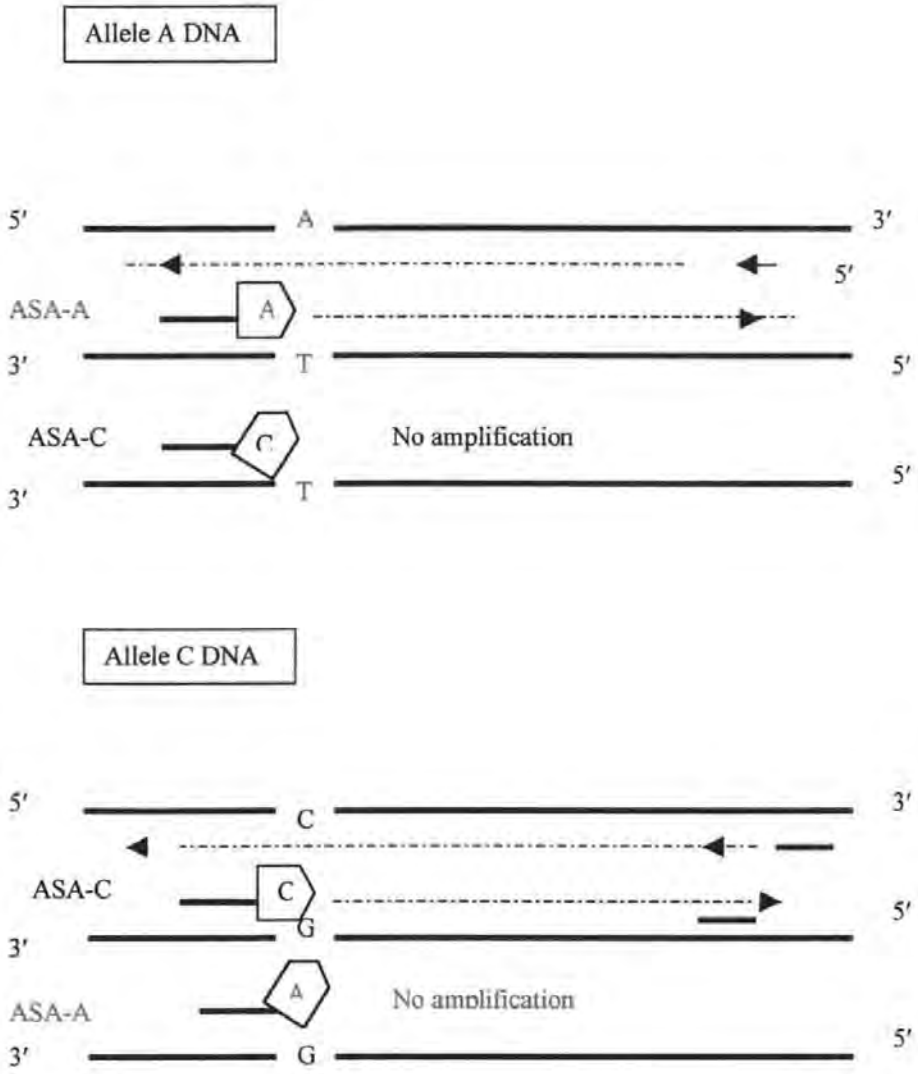


This figure illustrates restriction site polymorphisms which cut PCR product with a suitable restriction enzyme eg Sst1 V that cuts A and T in ATCAGG sequence. Allele1 is cut into a and b fragments as result of restriction reaction and allele 2 has no cutting site, a+b; so genotype for an individual with such alleles is 1 / 2 (Adapted from Strachan and Read, 1996 with slight modifications)

2.5.4 Amplification refractory mutation system (ARMS)

Amplification refractory mutation system (ARMS) is an improved PCR technique which permits rapid analysis of any known mutation in genomic DNA. This system allows genotyping solely by inspection of reaction mixtures after agarose gel electrophoresis. It will clearly distinguish heterozygous from homozygous forms of the allele. The ARMS-PCR system was introduced to molecular biology in 1989 (Newton et al. 1989). The system requires neither restriction endonuclease enzyme digestion, allele specific oligonucleotides (ASO) as conventionally applied, nor sequence specific analysis of PCR. This is the PCR equivalent of the allele-specific hybridisation which is possible with ASO probes. In the case of allele specific hybridisation, alternative ASO probes are designed to have differences in a central segment of the sequence (to maximise thermodynamic instability of mismatched Duples). However, in the case of allele-specific (ARMS) PCR, ASO amplimers are designed to differ at the nucleotide that occurs at the extreme 3′ terminus. This is so because the DNA synthesis step in the PCR reaction is crucially dependent upon the correct base pairing at the 3′ end (Figure 2.05). This method can be used to type specific alleles at a polymorphic locus, particularly to detect a specific pathogenic mutation.

Figure2.05: Illustration of ARMS-PCR



ASA-A: allele specific amplimer-"A" allele

ASA-C: allele specific amplimer-"C" allele

This figure illustrates correct base pairing at 3′ end of PCR amplimers (Adapted from Strachan and Read, 1996 with slight modification).

2.6 Association studies

Association studies, either in the form of a "case-control" or "family-based TDT" (transmission disequilibrium test), have proved to be the most useful approaches (Clerget-Darpoux, 1998). These methods of association studies will be explained in the following subsections.

2.6.1 Case control studies

There are two types of case-control studies: the prospective cohort study and cross-sectional study. Cohort studies suffer from having to follow patients up for many years and are difficult to fund, and so cross-sectional studies are generally accepted as good study designs. Cross sectional case-control studies are performed by comparing the frequency of marker alleles, typically from a selected candidate gene, in a group of affected individuals (cases) to the frequency of marker alleles in a group of unaffected individuals (controls). An allele at a gene of interest is associated with the disease if it occurs at a significantly higher frequency among cases compared to control subjects, $p= \leq 0.05$. The statistical analysis is the 2×2, 3×2, and 8×2contingency table, where applicable, and chi square test, χ^2. In general, it is preferable to select a functional polymorphic marker from a candidate gene which is believed to be implicated in disease pathology. Therefore, markers, for RSPs and ARMS, are typically bi-allelic as opposed to microsatellite.

2.6.2 Family based transmission disequilibrium test

The transmission disequilibrium test (TDT) was introduced by Spielman et al in 1993 as a test for linkage between a complex disease and a genetic marker. This test assesses the possibility of linkage between families with one or more affected offspring, where at least one parent is heterozygous at the marker near the candidate gene, in cases where

disease associations had already been found. However, even if prior evidence for association is absent, the TDT is valid and can be used to test any marker (or set of markers) for which data are available from parents of one or more affected offspring (Spielman and Evans 1996). Family-based association studies will detect marker/disease associations only if the marker and disease genes are in linkage disequilibrium, and in addition, are linked, in contrast to case-control studies (Evans and Spielman 1995).

The advantage of the TDT is that it does not require data from either multiplex families, affected or unaffected offspring (Spielman et al 1993). The TDT involves analysis of the frequency of transmission of a designated allele from heterozygous parents to an affected offspring, with the knowledge of parental genotype, therefore, being mandatory. Each parent transmits one allele to each affected offspring, with the non-transmitted allele acting as the control samples. According to Mendelian law, for heterozygous parents there is a 50% chance for transmission of each allele to the offspring. Deviation from the expected 50 % transmission ratios of marker alleles from heterozygous parents to affected offspring can be analysed by constructing a contingency table of parental transmitted versus non-transmitted parental alleles (see Table 2.03). The statistic is calculated by application of the chi-square statistic (McNemar test) (Thomson 1995).

The TDT is a powerful method of detection of association which will be positive only if the loci are linked to the disease locus. TDT analysis is a valid test for multiplex families studies (Ewens and Spielman 1995).

Table 2.03: Transmission disequilibrium test

		Not transmitted allele
Transmitted	Allele 1	Allele 2
Allele 1	a	b
Allele 2	c	d

TDT formula, χ^2 (1 degree of freedom) = $(b-c)^2/(b+c)$

This table shows TDT calculation steps and the formula for its caculation.

2.7 Analysis of the IFN-γ gene polymorphism

2.7.1 Oligonucleotide amplimers design and production

A pair of oligonucleotide primers which flank a 122 bp region of interferon-γ gene that contains $(CA)_{12}$ repeats (Awata et al 1994) were constructed (Figure 2.06). Amplimers used were commercially produced on an oligonuceotide synthesiser (Pharmacia Biotech, Herts, UK), and diluted for use to concentration of 10 μM. All oligonucleotide amplimers used in this study were obtained from the same company.

2.7.2 5'-end labelled amplimer

The sense amplimer (Table 2.05) was labelled with γ-phosphate group ATP, [^{32}P] dATP (Pharmacia Biotech, Herts, UK) using T4 Polynucleotide kinase (T4 PNK) (Pharmacia Biotech, Herts, UK). For 5'-end labelling the protocol was followed with a slight modification. 25 μl of sterile water was added to the tube containing the T4 PNK, incubated at room temperature for 5 minutes and then mixed. 50 μM of the forward amplimer, 1 μM of [γ^{35}P] ATP (10 μCi/μl) was added to the mixture. The volume was made up to 50 μl with sterile water prior to brief centrifugation followed by incubation at 37 °C for 45 minutes. The reaction was stopped by immersing the mixture in an ice bath.

5 μl of 5M NaCl, 2 μl of *Quick-Precip*® (Advanced Genetic Technologies Corp, USA), 3× volume of ethanol (-20)(Rathburn Ltd, Walkerburn, UK) was added to the reaction tube to precipitate the oligonucleotide. Centrifugation at 1300 rpm in the MSE Mistral 1000 (MSE Scientific Instruments, Leicester, UK) pelleted the oligonucleotide from which the supernatant was pipetted off. The pellet was washed in 70 % ethanol prior to being dissolved in 50 μl sterile water.

One μl of the labelled oligonucleotide was counted in a scintillation counter (LS6500 multipurpose scintillation counter, BECKMAN, USA). This gave a reading around 25,000 cpm.

A 20 μl PCR reaction was carried out in 0.2 ml thin walled microtubes (Advanced Biotechnologies, Surrey, UK) containing the following (Table 2.04).

Table 2.04:The PCR contents for amplification of the IFNG CA repeats

Concentration	µl	Final Concentration
^{35}P radiolabled forward amplimer (1µM)	1.0	0.05µM
Forward amplimer (10 µM)	1.0	0.5 µM
Reverse amplimer (10 µM)	1.0	0.5 µM
10 × Buffer	2.0	1 × Buffer
$MgCl_2$ (10mM)	1.5	0.75 mM
DNTP (20mM)	0.8	0.8 mM
Taq DNA polymerase (5 U/µl)	0.2	1.0 Unit
*DNA (50 ng/µl)	3.0	150 ng
Sterile H_2O	10.5	----------
Total	20	

DNA was added separately to individual tubes.

Table 2.05: The set of amplimers and PCR condition for amplification of the IFNG CA repeats

Amplimers	PCR condition	Fragment size/bp	Rference
1: 5′-TCA CAA TTG ATT TTA TTC TTA C-3′	95°C 5 min	122	Awata et
2: 5′-TGC CTT CCT GTA GGG TAT TAT T-3′	95°C 30 sec 48°C 40 sec } 35 cycles 72°C 40 sec		al 1994
	72°C 5 min		

1=sense amplimer and 2=antisense amplimer

Fig 2.06: Illustration of the IFNG CA repeats on the IFN-γ gene

TTTAAAATTT TATTGATGGT TAATGAAAAG TTTTTACATT TTAAATATTT CATTATTTGT TTAAAACTTA
GCTGTTATAA TTATAGCTGT CATAATAATA TTCAGACATT CACAATTGAT TTTATTCTTA CAACACAAAA
TCAAATCTCA CACACACACA CACACACACA CACTCGCACA TGTTTGGAAC TATCTTTTAA AGCTCGTATA
ATAATACCCT ACAGGAAGGC ACAGTAGATG TAATAGAAAC CTGTACCATT GGGGGGCAGT ATTTTATAGT
GGGGTGGCTT TGCTGTTTTT TGTTTTTGTA TTTTTTAGCC TAGCTTGAAA ATACTTTCTT TAGCTTACTA
TAGTTTTTGG

The CA repeat microsatellite is located at position 1249 to 1373bp. The position of sense and antisense amplimers as well as CA repeat is shadowed (Hum IFNG,DNA star 1997).

2.7.3 Agarose gel electrophoresis of the PCR products

Following PCR amplification, the products were monitored for quality and quantity by electrophoresis of an aliquot mixed with tracking dye on a 2% agarose gel (1% w/v agarose in 0.5 × TBE buffer) containing 0.01% ethidium bromide. The agarose gels were prepared as follows. Two grams of ultra pure agarose (Life Technologies, Paisely, UK) was added to 100 ml of 0.5 TBE buffer. The mixture was boiled in a microwave and allowed to cool to about 65°C. Ten µl of ethidium bromide (10 mg/ml) (Sigma-Aldrich, Dorset,UK) was added and the molten agarose/ethidium bromide gently mixed and poured into a level gel template. Two 24 well combs were inserted into the template and the gel allowed to set for 1 h at room temperature. The combs were removed and the gel/template submerged in an electrophoresis tank containing 0.5 TBE buffer to a depth of 2-3 mm above the surface of the gel. Seven µl of each PCR product was combined with 1.5 µl of 5× gel loading buffer (5× GLB; 50 mM Tris, pH 7.6, 50 mM EDTA, pH 8.0, 0.1 bromophenol blue (Sigma-Aldrich, Dorset, UK), 40% sucrose (Promega, Southampton, UK) was loaded into the appropriate gel well. In addition, 5 µl of 123 bp DNA size marker (Life Technologies, Paisely, UK) was loaded in the first well associated with each row of samples.

The samples then underwent electrophoresis at 200 V (constant voltage) for 45 minutes (Biorad Power Pac, BioRad,Herts, UK). After electrophoresis, the PCR products were viewed under UV transillumination (λ302 nm) and photographed using Syngene Genesnap (Genetic research Instrumentation (GRI) Ltd., Essex, UK) loaded with type IV UPP-11-HA film (Sony Corporation, Tokyo, Japan).

During each run a positive and a negative control samples were run on each gel.

2.7.4 Microsatellite analysis of the PCR products

Following gel electrophoresis of the PCR products, genotyping was performed for the microsatellites (Weber and May, 1989) on an ABI Prism™377 DNA sequencer (Perkin Elmer Applied Biosystems, Warrington, UK) as described below.

2.7.4.1 Gel preparation

Two *sequi-Gen* ™ GT Electrophoresis Cells, (one front, one back) were washed thoroughly with detergent solution, rinsed well with warm water and then allowed to air dry. The outside of the cells were cleaned with ddH_2O using clean white tissue. The *sequi-Gen* ™ cells were reversed and the inside of the cells sequentially washed with ddH_2O followed by 70% isopropanol (Rathburn Ltd, Walkerburn, UK) and then cleaned with white tissue. In order to ease detachment of the gel from the cells, inside of the top cell was introduced to Rapel-saline (Pharmacia Biotech, Herts, UK) and left to dry at room temperature to form a fine silicon film. The cells were assembled in a *sequagel* ™ cassette separated by two 0.2 mm gel spacer aligned along their length. The plates were firmly clamped with a cassette support to aid gel pouring.

A 6%, 6M urea/formamide, polyacrylamide gel mix was performed using 36 ml of sequagel concentrated[1] , 99 ml sequagel diluent[2], 15 ml sequagel buffer[3], 12 ml sequagel formamide[4], 65 µl of TEMED[5], and 1 ml freshly prepared 10% ammonium persulphate (APS) [6] (Sigma-Aldrich, Dorset, UK[1-5]) in a 250 ml conical flask. The gel mix was drawn into a syringe and carefully injected into the space between the plates. The flat edge of a 48 well sharks tooth comb was inserted into the top of the gel left for 2 h to allow complete polarisation. Alternatively, the gel was wrapped in a Saran Wrap™ and left overnight at room temperature.

2.7.4.2 Pre-run

The shark's tooth comb was carefully removed from the gel, rinsed with warm water, dried and reinserted, teeth first, into the gel to a depth of approximately 3mm. The drip tray was removed. The cassette was then placed in the bottom tank and connected to a powerpack (Biorad-Powepac, BioRad, Herts., UK). The top buffer tank attached to the back of the back *sequi-Gen* ™ cell and the bottom tank were filled with 1× TBE buffer. A constant current 1600-2000 V was run through 1× TBE buffer and the gel was allowed to reach and maintain a temperature of 50 °C.

2.7.4.3 Sample loading and electrophoresis

Prior to the loading of samples, the gel temperature was checked to ensure that it was at 50°C. The instrument was paused and a mixture of 3µl of stop solution (Promega, UK) and 6µl of PCR product was loaded in the appropriate well number. The run module was initiated and the samples electrophored for 3 hours.

By completion of the electrophoresis process the current was disconnected and the buffer tanks were discharged from 1× TBE buffer. The *sequi-Gen* ™ cells were gently separated. The gel was detached and washed in a 5% Acetic acid, 15% Methanol solution (Rathburn Ltd, Walkerburn, UK). The gel was supported by a 50 × 30 cm filter paper (Heto Laboratory Equipment, Surrey, UK) and dried. The dried gel was exposed to a Kodak XLS5 X-ray film (Scientific Imaging Systems, Cambridge, UK) and incubated at –85 °C for 4h.

DNA samples with known genotype were incorporated as controls in each microsatellite analysis.

Analysis of the IL-4 gene polymorphisms

2.8.1 The C (-285) T polymorphism

2.8.1.1 Analysis of the C (–285) T polymorphism

DNA samples were taken from the DNA bank in the Molecular Medicine Laboratory. Briefly, a pair of oligonucleotide amplimers, amplimer 1 and 2 of Table 2.07, was constructed which flanks the position of C (-285) T counting from the first ATG codon (Arai et al., 1989). A 20 µl PCR reaction consisted of (Table 2.06) was used.

2.8.1.2 Agarose gel electrophoresis of the PCR products

Following the PCR amplification of 289 bp fragment of the IL-4 gene containing the C (-285) T polymorphism, the efficiency and size of the products were measured prior to endonuclease digestion process. The agarose gel was prepared in exactly the same manner that described in section 2.8.3 with the exception that the gel concentration was 1 %.

2.8.1.3 Precipitation of DNA

Since the IL-4 C (-285) T polymorphism PCR product did not digest straight after the PCR reaction, the product was then precipitated aiming to maximise the chance of endonuclease digestion:

1/10 of the total volume of sample of 3.2 M NaCOOH was added to the sample,

2 × vol of absolute ethanol was added and mixed thoroughly,

Snap frozen in liquid N_2 for 10 min,

Spun in a centrifuge for 15 minutes (13000/min),

The supernatant was decanted and the tube was air dried for 15 min,

The pellet was dissolved in 20 µl TE.

Table 2.06: The PCR contents for amplification of the IL-4 gene polymorphisms

Concentration	µl	Final Concentration
Forward amplimer (10 µM)	1.0	0.5 µM
Reverse amplimer (10 µM)	1.0	0.5µM
10× Buffer	2.0	1× Buffer
$MgCl_2$ (10 mM)	6.0	3mM
DNTP (20 mM)	0.5	0.5mM
Taq DNA polymerase (5 U/µl)	0.2	1.0 Unit
Sterile H_2O	4.3	---------
DNA (50 ng/µl)	3.0	150 ng
Total	20	

DNA was added separately to individual tube.

Table 2. 07: The set of amplimers and PCR conditions for amplification of the IL-4 loci

Amplimers Sense and antisense = 1 and 2 for C (-285) T; 3 and 4 for C (-590) T	PCR condition	Fragment Size/bp	Restriction endonuclease	Reference
	95°C 5 min			
1: 5′- GAG TCT GCC TGT TAT TCT GCC TC- 3′	95°C 30 sec 55°C 40 sec 72°C 40 sec } 35 cycles	258	NheI / C (-285) T	
2: 5′-AAT CAG CAC CTC TCT TCC AGG AG-3′			5′...G▼CTAG C...3′	Song et al. 1996
	72°C 5 min		3′...C GATC▲G...5′	
	95°C 5 min			
3: 5′-ACT AGG CCT CAC CTG ATA CG-3′	95°C 30 sec 55°C 40 sec 72°C 40 sec } 35 cycles		BsmFI / C (-590) T	
4: 5′-GTT GTA ATG CAG TCC TCC TG-3′		252	5′...GGGAC $(N)_{10}$ ▼...3′	Walley and
	72°C 5 min		3′...CCCTG $(N)_{14}$ ▲...5′	Cookson, 1996

This table shows the set of amplimers, PCR condition, size of the fragmentas, well as restriction enzymes implemented for amplification of the IL-4 loci. References are also quoted.

2.8.1.4 The C (–285) T digestion via NheI endonuclease enzyme

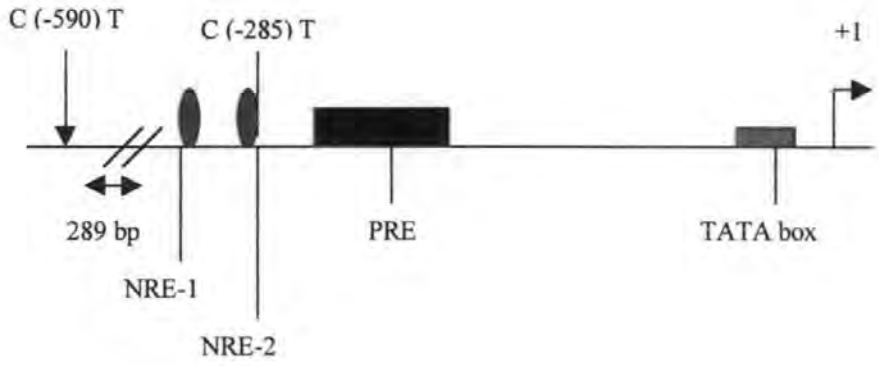
The PCR products were digested using NheI endonuclease restriction enzyme (New England Biolabs, Hertfordshire, UK). 5 µl of enzyme mix [2.0 µl of Buffer B, provided by the supplier, 0.5µl (5 units) of NheI and 2.5µl of sterile H_2O (Baxter Healthcare, Thetford, UK)] was combined with 15µl of each one of PCR products and incubated at 37 °C, the optimal temperature for NheI endonuclease activity.

However, the reaction did not take place even by using excess amount (5-25 U) of NheI enzyme.

2.8.1.5 Agarose gel electrophoresis of the PCR products following digestion

Following endonuclease digestion of PCR products, the resulting fragments were resolved using a 2% agarose gel as described in section 2.8.3.However, the electrophoresis was carried out by running a constant current of 70 V for 1.5 hour after which the gel was visualised and photographed as described above.

Figure 2.07: Illustration of the IL-4 C (-285) T and C (-590) T polymorphisms on the IL-4 gene



This figure illustrates position of the IL-4 C (-285) T and C (-590) T on the IL-4 gene (Adapted from Cookson and Walley, 1996 with slight modifications).

2.8.2 The C (-590) T polymorphism

2.8.2.1 Analysis of the C (-590) T polymorphism

DNA samples were taken from the DNA bank in the Molecular Medicine Laboratory. Briefly, a pair of amplimers, amplimers 3 and 4 of Table 2.07, were constructed which flank the position of the C (–590) T counting from the ATG codon (Arai et al., 1989). A 20 µl PCR reaction as Table 2.06 was used.

The PCR conditions for the C (-590) T polymorphism in the IL-4 region was the same as above.

2.8.2.2 Agarose gel electrophoresis of the PCR products

Following PCR amplification of 252 bp spanning positions 522 to 774 in the interleukin-4 promoter sequence (Genebank accession number M23442), the efficiency and size of the products were measured prior to the endonuclease digestion process. The agarose gel was prepared in exactly the same way as described in section 2.8.3, with the exception that gel concentration was 1 %.

2.8.2.3 The C (-590) T digestion via BsmFI endonuclease enzyme

A total of 15 µl of PCR product was added to 2.5 µl of sterile water (Baxter Healthcare, Thetford, UK) together with 2.0µl of Buffer 4 and 0.5 µl of BsmFI (5 unit) (New England Biolabs™, Hertfordshire, UK) to give a final volume of 20 µl. The restriction digest mixture was then incubated at 37°C for an hour. The reaction was stopped by the addition of 5µl of agarose gel loading buffer (5xTBE, 0.5 mol/l EDTA, 10% v/v glycerol, 0.05 % w/v Bromophenol Blue). The restriction digest was carried out at 37°C rather than the optimum temperature for BsmFI activity, 65°C, because of undesirable

Taq DNA polymerase activity at this temperature. At 37°C the restriction enzyme retained 50% of its activity.

15 µl of the restriction digest mixture was loaded onto a 2% w/v agarose gel and electrophoresed at a constant current of 150 V for 90 minutes. DNA molecular weight markers were also loaded on the gel for reference (Boehringer Mannheim Marker VIII). Fragments of DNA were then visualised by UV transillumination and photographed using video image capture system and thermal printer as described in above

Genotypes were assigned on the basis of the pattern of fragments of different size obtained following restriction endonuclease digestion of PCR products.

2.9 Analysis of the IL-6 gene polymorphism

2.9.1 The G (-174) C polymorphism

DNA samples were taken from the DNA bank in the Molecular Medicine Laboratory. The G (-174) C polymorphism of the IL-6 promoter region was amplified by PCR (Table 2.08) using a pair of appropriate amplimers (Table 2.09).

Table 2.08: The PCR contents for amplification of the IL-6 G (-174) C

Concentration	µl	Final Concentration
Forward amplimer (10 µM)	1.0	0.5 µM
Reverse amplimer (10 µM)	1.0	0.5µM
10× Buffer	2.0	1× Buffer
$MgCl_2$ (10 mM)	6.0	3mM
DNTP (20 mM)	0.5	0.5mM
Taq DNA polymerase (5 U/µl)	0.2	1.0 Unit
Sterile H_2O	4.3	---------
*DNA (50 ng/µl)	3.0	150 ng
Total	20	

*DNA was added to individual tube separately to individual tube.

Table: 2. 09: The set of amplimers and PCR condition for amplification of the IL-6 G (-174) C

Amplimers	PCR condition	Fragment size/ bp	Restriction endonuclease	Reference
1:5′-TTG TCA AGA CAT GCC AAA GTG C-3′	95°C 5 min	243	Nla III	Fishman et al. 1998
2:5′-GGG AAA ATC CCA CAT TTG ATA A-3′	95°C 60 sec 55°C 60 sec } 30 cycles 72°C 60 sec		5′...CATG▼...3′ 3′...▲GTAC...5′	
	72°C 5 min			

This table shows the set of amplimers, PCR condition, size of the fragment, as well as implemented restriction enzymes. The reference is quoted.

2.9.2 Agarose gel electrophoresis of the PCR products

Following PCR amplification of the 243 bp fragment of the IL-6 gene spanning the C (-285) T polymorphism, the efficiency and size of the products were measured before the restriction site digestion process by Nla III. The agarose gel was prepared in exactly the same way as described in section 2.8.3, with the exception that the gel concentration was 1 %.

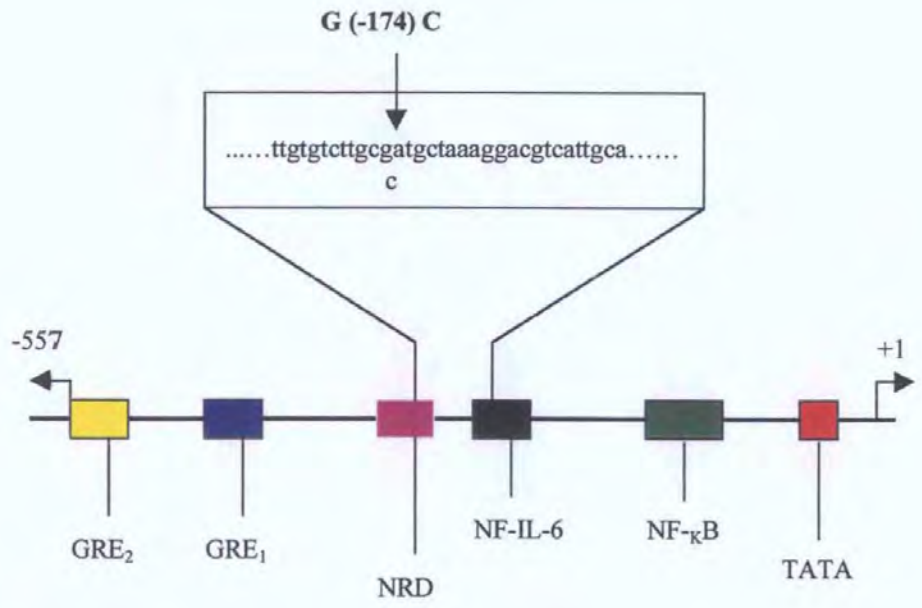
2.9.3 The G (-174) C digestion via NlaIII endonuclease enzyme

The PCR products were digested using NlaIII endonuclease restriction enzyme (New England Biolabs, Hertfordshire, UK). 5 μl of enzyme mix [2.0 μl of Buffer B, provided by the supplier, 0.5μl (5 units) of NlaIII and 2.5μl of sterile H_2O (Baxter Healthcare, Thetford, UK)] was combined with 15μl of each one of the PCR products and incubated at 37 °C for 16 h, overnight. The reaction was stopped by the addition of 5μl of agarose gel loading buffer (5XTBE, 0.5 mol/l EDTA, 10% v/v glycerol, 0.05 % w/v Bromophenol Blue). The restriction digest was carried out at 37°C rather than the optimum temperature for Nla III activity, 65°C, because of undesirable Taq DNA polymerase activity at this temperature. At 37°C the restriction enzyme retained 50% of its activity.

15 μl of the restriction digest mixture was loaded onto a 2% w/v agarose gel and electrophoresed at a constant current of 150 V for 90 minutes. DNA molecular weight markers were also loaded on the gel for reference (Boehringer Mannheim Marker VIII). Fragments of DNA were then visualised by UV transillumination and photographed using video image capture system and thermal printer as described in section 2.8.3.

Genotypes were assigned on the basis of the pattern of fragments of different size obtained following restriction endonuclease digestion of PCR products.

Figure 2.08: Illustration of the IL-6 G (-174) C



This figure illustrates the location of the IL-6 G (-174) on the IL-6 gene and the G→C sequence (Adapted from Fishman et al., 1998 with slight modification).

2.10 Analysis of The TGF-β1 gene polymorphism

2.10.1 The T (+869) C polymorphism

DNA samples were taken from the DNA bank in the Molecular Medicine Laboratory. In order to investigate genetic susceptibility of the TGF-β1 gene to type 1 diabetes, the T (+869) C polymorphism at codon 10 in exon 1 of the TGF-β1 gene was amplified using ARMS-PCR methodology 241 bp fragment of the TGF-β1 gene signalling region spunning the T (+ 869) C polymorphism in codon 10 which substitute Leucine to Proline amino acid (Table 2.10). The reverse amplimers were designed such that they differred at their most extreme 3′ terminus, C substituted to T (Table 2.11).

Table 2.10: The ARMS-PCR contents for amplification of the TGF-β1 T(+869)C

Concentration	µl	Final Concentration
Forward amplimer (15 µM)	1.0	0.75 µM
Reverse amplimer (9 µM)	1.0	0.45 µM
Reverse amplimer (8 µM)	1.0	0.4 µM
10× Buffer	2.0	1× Buffer
$MgCl_2$ (10 mM)	1.5	0.75 mM
dNTP (20 mM)	0.5	0.5 mM
Taq DNA polymerase (5 U/µl)	0.2	1.0 Unit
Sterile H_2O	4.3	---------
*DNA (50 ng/µl)	3.0	150 ng
Total	20	

*DNA was added to individual tube separately to individual tube.

Table 2.11: The set of amplimers and PCR condition for amplification of the TGF-β1 T (+869) C

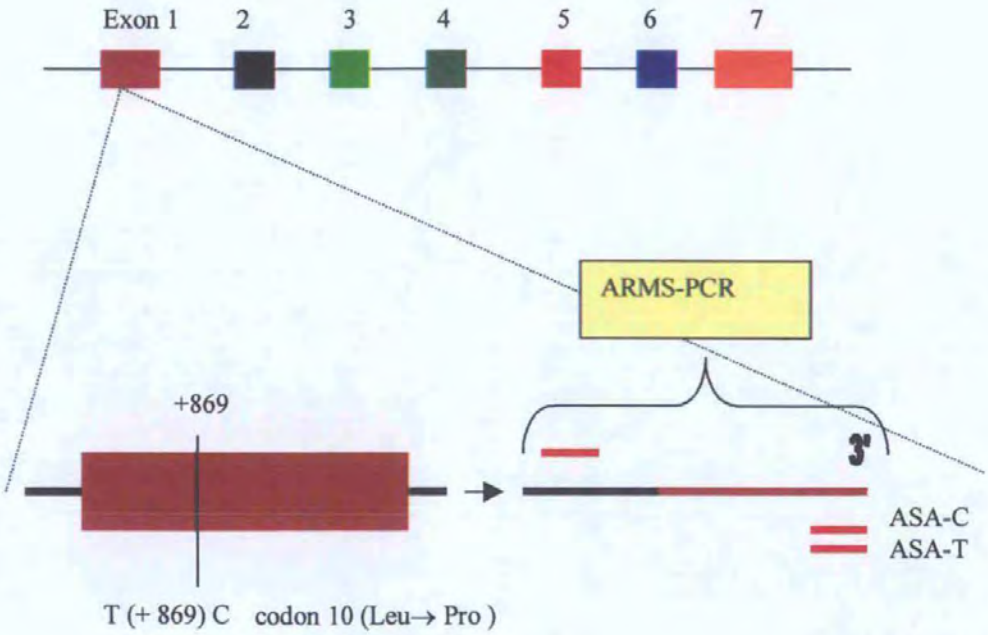
Ampliers	ARMS-PCR condition		Fragment Size/ bp	Reference
	95°C 1 min			
1:5′-TCC GTG GGA TAC TGA GAC AC-3′	95°C 15 sec 65°C 50 sec	10 cycles	241	Awad et al., 1998
2 T:5′-AGC AGC GGT AGC AGC AGC A-3′	72°C 40 sec			
2 C:5′-GCA GCG GTA GCA GCA GCG-3′	95°C 20 sec 58°C 50 sec 72°C 50 sec	20 cycles		

This table shows a set of amplimers, ARMS-PCR condition, as well as size of the fragment. The reference is quoted. The T and C amplimers are allele specific amplimers (ASA-).

2.10.2 Agarose gel electrophoresis of the PCR products

Following PCR amplification, the products were monitored for quality and quantity by electrophoresis of an aliquot mixed with tracking dye on a 2% agarose gel (2 % w/v agarose in 0.5 × TBE buffer) containing 0.01% ethidium bromide. The gel was electrophoresed at a constant current of 150 V. for 90 minutes. DNA molecular weight markers were also loaded on the gel for reference (Boehringer Mannheim Marker VIII). Fragments of DNA were then visualised by UV transillumination and photographed using video image capture system and thermal printer as described in section 2.8.3. Genotypes were assigned on the basis of the pattern of expression of the fragments as a result of each allele specific reaction.

Figure 2.09: Illustration of the TGF-β1 T (+869) C



This figure illustrates the position of the TGF-β1 T (+869) C in the TGF-β1 gene and its amplification using ARMS-PCR technique.

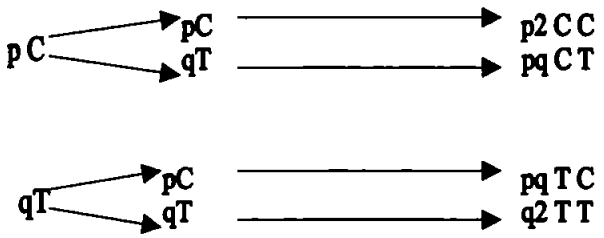
2.11 Statistical analysis

2.11.1 Allele and genotype frequencies

The frequency of allele and distribution of genotype of the studied cytokines in patients and healthy controls were calculated using χ^2 with Yates' correction with significance level set at $p< 0.05$, as appropriate. The p values were corrected for the number of comparisons made (Pc) was applied where appropriate using the Bonferroni inequality method (Tiwari and Terasaki, 1985).

2.11.2 Hardy-Weinberg principle

According to Hardy–Weinberg, principal allele frequencies remain constant from generation to generation (Hardy, 1908; Hartl and Jones, 1998). The genotype frequency of a gene with two alleles can be derived from known allelic frequencies which pairs in random mating. Considering a gene with two alleles, C and T, that have frequencies p and q, respectively. With random mating, the frequencies of genotypes CC, CT, and TT among zygotes expected:



CC:p^2 CT: 2pq TT:q^2

The expected genotype frequency can be derived from the above formula and compared to the observed genotype frequencies using chi square test. Hardy-Weinberg equilibrium occurs if the observed frequency is not significantly different from the expected

frequency (p value > 0.05). This equation can be modified for polymorphisms consisting of 2+n alleles (Strachan and Read, 1996).

2.11.3 Genetic profile

In addition to analysing the genotypes for each gene, the genotypes of different genes were combined to give cytokine profile and analysed to determine whether the genes were linked and hence influence each other's expression. Individuals were considered for genetic pro file analysis provided they were not heterozygous at more than 1 locus. The double heterozygotes were omitted from the study. The subjective removal of the heterozygotes may introduce a bias if a large number of double heterozygotes are present, however, since this bias is likely to be consistent in both controls and patients the "error" is not significant.

TDT was applied to Transmission of pathogenic alleles to offspring. Genetic profile frequencies were estimated using gene counting. All analyses were performed in an Excel spreadsheet (Microsoft, UK) using EPI-Info (EPI-Info version 6, Centres for Disease Control and Prevention, Atlanta, Georgia, USA).

3 RESULTS

3.1 Analysis of the IFN-γ locus

3.1.1 Genotype frequency of the IFNG CA repeats

A total of eight different sized IFNG CA repeat alleles were identified. Each of these alleles differed in a CA repeat (Table 3.01) and corresponded to 10-17 CA repeat respectively. From a total of 29 CA repeats genotypes the 3 / 3 genotype was significantly increased in the patients compared to the normal controls (33.6% vs 13.1% respectively, χ^2=15.0, p= 0.0001, and Pc= 0.02) (Table 3.02). There was a significant decrease in the 1 / 2 in the patients compared to the normal controls (0% vs 6.5% respectively, p= 0.0002, and Pc= 0.006, Fisher exact test). A significant decrease also was found in the 2 / 4 and the 4 / 5 genotypes in the patients compared to the normal controls (0 % vs 5.9 % respectively with p=0.0007, and 0.4% vs 4.7% respectively with p= 0.01, Fisher exact test). There were no other significant differences between the patients and the normal controls (Table 3.02). To avoid complications the 3 / 3, 3 / 5, and 5/ 5 genotypes which occurred more frequently in the patients and normal controls were considered in the following analyses.

3.1.2 Allelic frequency of the IFNG CA repeats

The allelic frequency of the IFNG CA repeats were calculated using χ^2 and p values were corrected to the number of variables (Table 3.03). The allele 3, 122 bp, which consists of 12 CA repeats compared to the normal controls (51.4 % vs 31.3 % respectively, χ^2= 23.61, p= 0.000001, and Pc= 0.00007). There was a significant decrease in the frequency of the allele 2 in the patients compared to the normal controls (6.7 % vs 14.0 % respectively, χ^2= 8.40, p= 0.004, and Pc= 0.03). There was no other significant differences between the patients and the normal controls.

Figure 3.01: Photographic presentation of the IFNG CA repeats

In this photo lane1= 122 / 122, lane 2= 126 / 120, lane 3= 126 / 122, lane 4= 120 / 120, lane 5= 120 / 120, lane 6= 126 / 122, and lane 7= 126 / 122.

Table 3.01: Different allele size and their CA repeat position

Allele	Size (bp)	CA repeat position
1	118	10
2	120	11
3	122	12
4	124	13
5	126	14
6	128	15
7	130	16
8	132	17

8 different alleles of IFNG whose sizes differ by a CA repeat unit.

Table 3.02: The genotype frequency of the IFNG CA repeats in the patients and the normal controls

Genotype	Patients (n= 247) n	%	NC (n= 107) n	%	χ^2	p value	Pc value
3 / 3	83	33.6	14	13.1	15.0	0.0001	0.002
1 / 2	0	0	7	6.5		0.0002	0.006
2 / 4	0	0	6	5.9		0.0007	0.02
4 / 5	1	0.4	5	4.7		0.01	ns
1 / 5	2	0.8	2	1.9		ns	ns
2 / 2	8	3.2	5	4.7	0.4	ns	ns
2 / 3	15	6.1	4	3.9		ns	ns
2 / 5	2	0.8	3	2.8		ns	ns
2 / 6	1	0.4	0	0		ns	ns
3 / 4	17	6.9	3	2.8		ns	ns
3 / 5	35	14.2	18	17.1	0.2	ns	ns
3 / 6	4	1.6	4	3.9		ns	ns
3 / 7	6	2.4	3	2.8		ns	ns
3 / 8	3	1.2	4	3.9		ns	ns
4 / 4	8	3.2	3	2.8		ns	ns
4 / 6	1	0.4	2	1.9		ns	ns
4 / 7	0	0	1	0.9		ns	ns
4 / 8	2	0.8	0	0		ns	ns
5 / 5	27	10.9	8	7.5	1.0	ns	ns
5 / 6	3	1.2	2	1.9		ns	ns
5 / 7	6	2.4	4	3.7		ns	ns
5 / 8	1	0.4	0	0		ns	ns
6 / 6	1	0.4	1	0.9		ns	ns
6 / 7	1	0.4	1	0.9		ns	ns
6 / 8	0	0	1	0.9		ns	ns
7 / 7	0	0	1	0.9		ns	ns

Where 'n' for the patients and the normal controls (NC) is less than 5 Fisher exact test was used.
'ns'= not significant which stands p and Pc values more than 0.05.
The 3 / 3 was significantly increased in the patients compared to the NC, χ^2= 15.0, p= 0.0001, and Pc= =0.002.
The 1 / 2, the 2 / 4, and the 4 / 5 were significantly decreased in the patients compared to the NC, p= 0.0007, and Pc= 0.002; p= 0.0007 and Pc= 0.02; p= 0.01 and Pc= ns.

Table 3.03: The allelic frequency of IFNG CA repeat in the patients and the normal controls

Alleles	Patients (n=494) %	NC (n=214) %	χ^2	p value	Pc value
3	51.4 (254)	31.3 (67)	23.61	0.000001	0.00007
2	6.7 (34)	14.0 (30)	8.40	0.004	0.03
4	7.7 (38)	10.7 (23)	1.8	ns	ns
5	21.3 (105)	23.4 (50)	1.4	ns	ns
6	2.8 (14)	5.1 (11)	1.7	ns	ns
7	2.6 (13)	3.7 (8)	0.3	ns	ns
8	1.6 (8)	2.8 (6)	0.6	ns	ns

NC= normal controls
'ns'= not significant which stands p and Pc values more than 0.05.
The allele 3 was significantly increased in the patients compared to the NC, χ^2= 8.4, p= 0.004 and Pc= 0.003.

3.1.3 The impact of gender of patients on the genetic susceptibility of the IFNG CA repeats

The relationship of gender of the patients to the IFNG genotype and allelic frequencies was calculated (Table 3.04,3.05 respectively). No significant association was found between the genotype or allelic frequencies of the IFNG CA repeats and gender of the patients compared to the normal controls.

3.1.4 The impact of age at onset on the genetic susceptibility of the IFNG CA repeats

In order to find out the relationship of the age at onset of type 1 diabetes to the IFNG CA repeat polymorphism the patients were grouped according to the age at onset (that is, those under 10 years, between 10-20 years, and over 20 years). There was a significant increase in the frequency of the 3 / 3 of the IFNG in the patients with <10 and 10-20 compared to >20 years age at onset (37.0 % vs 24.0 % respectively, χ^2= 3.75, p= 0.05; 38.5 % vs 24.0 % respectively, χ^2= 7.10, p= 0.008). No other significant association was found between age at onset of type 1 diabetes and the IFNG CA repeats genotype frequency (Table 3.06). No significant association was found between the allelic frequency of the IFNG and the long term complications of diabetes (Table 3.07).

Table 3.04: The genotype frequency of the IFNG CA repeats with respect to gender of the patients and the normal controls

Genotype	Male		Female	
	Patients (n=117) %	NC (n=47) %	Patients (n=130) %	NC (n=60) %
3 / 3	34.2 (40)	12.8 (6)	33.1 (43)	13.3 (8)
3 / 5	12.8 (15)	17.0 (8)	15.4 (20)	16.7 (10)
5 / 5	12.0 (14)	8.5 (4)	10.0 (13)	6.7 (4)
X / X	41.0 (48)	61.7 (29)	41.5 (54)	63.3 (38)

No significant association was found between the IFNG CA repeats and gender of the patients.

Figure 3.05: The allelic frequency of the IFNG CA repeats with respect to gender of the patients and the normal controls

Allele	Male		Female	
	Patients (n= 234) %	NC (n= 58) %	Patients (n= 262) %	NC (n= 76) %
3	46.6 (109)	21.3 (20)	55.3 (145)	21.7 (26)
5	23.5 (50)	17.0 (16)	19.1 (50)	15.0 (18)
X	29.9 (70)	61.7 (58)	25.6 (67)	63.3 (76)
χ^2	4.13			
p value	ns			

No significant association was found between the frequency of the IFNG CA repeats and gender of the patients with type 1 diabetes.

Table 3.06: The genotype frequency of the IFNG CA repeats with respect to age at onset of type 1 diabetes

Age at onset	Genotype			
	3 / 3 (n= 83) %	3 / 5 (n= 35) %	5 / 5 (n= 27) %	X / X (n= 102) %
< 10 years	36.1 (30)	28.6 (10)	25.9 (7)	33.3 (34)
10-20 years	42.3 (35)	37.1 (13)	44.4 (12)	30.4 (31)
> 20 years	21.8 (18)	34.3 (12)	29.6 (8)	36.3 (37)
χ^2	3.31			
p value	ns			

No significant association was found between the distribution of the IFNG CA repeats genotypes and age at onset of patients with type 1 diabetes.

Table 3.07: The allelic frequency of the IFNG CA repeats with respect to age at onset of type 1 diabetes

Age at onset	Allele 3 (n= 254) %	5 (n= 105) %	X (n= 135) %
< 10 years	35.4 (90)	40.0 (42)	33.3 (45)
10-20 years	38.6 (98)	36.2 (38)	41.5 (56)
> 20 years	26.1 (66)	23.8 (25)	25.2 (34)
χ^2	0.60		
p value	ns		

No significant association was found between age at onset of type 1 diabetes and distribution of the IFNG alleles.

3.1.5 The role of the IFNG CA repeats in the long term complications of diabetes

To study the possible effects of the IFNG CA repeats polymorphism on the long term complications of diabetes, the genotype and allelic frequencies of the IFNG CA repeats were studied with respect to long term complications of diabetes. No significant association was found between the genotype or allelic frequencies of the IFNG CA repeats and long term complications of diabetes (Table 3.08, 3.09 respectively).

3.1.6 The familial clustering analysis for the 3 / 3 genotype of the IFNG CA repeats

In order to study the chance of familial clustering of the linked genotype of the IFNG CA repeats, the 3 / 3, 53 trios from the BDA-Warren family collection were genotyped for the IFNG CA repeats polymorphism. Forty one families were found to have at least one heterozygous parent for the allele 3 of the IFNG CA repeats. From 60 cases of allele 3, 38 cases were transmitted to the offspring (Table 3.10). The TDT analysis showed a slight trend for preferential transmission of the allele 3 (63.3% vs 36.7%, χ^2= 1.66,p=ns).

Table 3.08: The genotype frequency of the IFNG CA repeats with respect to the long term complications of diabetes

Long term complications of diabetes	IFNG CA repeat genotype 3 / 3 (n=47) %	3 / 5 (n=18) %	5 / 5 (n=20) %
DC	17.8 (7)	22.2 (4)	15.0 (3)
DN	28.6 (14)	33.3 (6)	30.0 (6)
DNu	25.0 (12)	16.7 (3)	30.0 (6)
DR	28.6 (14)	27.8 (5)	25.0 (5)

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

No significant association was found between the IFNG genotype 3 / 3, 3 / 5, and 5 / 5 distribution among different patients with long term complications of diabetes.

Table 3.09: The allelic frequency of the IFNG CA repeats with respect to the long term complications of diabetes

Long term complications of diabetes	IFNG CA repeat allele 3 (n=173) %	5 (n=76) %
DC	22.0 (38)	21.1 (16)
DN	9.1 (45)	22.4 (16)
DNu	8.5 (42)	27.6 (21)
DR	9.7 (47)	28.9 (22)

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

No significant association was found between the IFNG alleles 3 and 5 and patients with long term long term complications of diabetes.

Table 3.10: TDT for the linkage analysis of the 3 / 3 of the IFNG CA repeats

Transmitted	Non-transmitted
38	22
30	30
χ^2= 1.66	
p=ns	

The TDT result showed that out of 60 alleles transmitted from parents to offspring 38 (63 %) were allele 3 which means there is a slight trend towards transmission of allele 3 from parents to offspring, slight preferential transmission, 38/60 vs 22/60, χ^2= 1.66 and P= ns.

3.2 Analysis of the IL-4 loci

3.2.1 The C (–285) T polymorphism

Forty five DNA samples were analysed for the C (-285) T polymorphism. Several different assay conditions were used in an attempt to identify the polymorphism. For instance, samples were incubated with the restriction endonuclease NheI for between 1 and 16 hours with increasing amounts of enzyme (5-25 units). No digestion was found and this it appears that this is a cloning defect.

3.2.2 C (-590) T polymorphism

3.2.2.1 Genotype and allelic frequencies of the IL-4 C (-590) T polymorphism

The DNA samples (Table 2.01), after amplification of the PCR for the IL-4 gene polymorphism, were analysed for C (-590) T polymorphism. Samples were subjected to incubation at 37°C due to undesirable Taq DNA polymerase activity at 65°C, the optimum temperature for BsmFI activity.

After 90 minutes incubation, 252 bp fragments of the IL-4 PCR product resulted in two fragments of 193 and 60 bp as a result of cleavage with BsmFI. The 60 bp fragment could not be detected due to slow rate of electrophoresis.

As a result of cleavage of the 252 bp fragment of the IL-4 gene, flanking the C (-590) T polymorphism, with BsmFI restriction enzyme three genotypes were obtained: CC, 252 bp; TT, 193bp; and CT, 252 bp/193 bp (Fig 3.02).

The expected and observed genotypes of the IL-4 C (-590) T meet Hardy-Weinberg principle (Table 3.12).

Allelic and genotype and allelic frequencies of the IL-4 C (-590) T polymorphism in the patients with type 1 diabetes were compared to normal controls (Table 3.11).

No significant association was found between allelic and genotype distribution of the IL-4 C (-590) T gene polymorphism and type 1 diabetes (Table 3.11).

Figure 3.02: Photographic presentation of the IL-4 C (-590) T

1 2 3 4 5 6 7

In this photograph lane1= DNA ladder, lane 2= CT, lane 3= TT, lane 4= CT, lane 5= TT, lane 6= CT, and lane 7= CC.

Table 3.11: The genotype and allelic frequencies of the IL-4 C (-590) T in the patients and the normal controls

Genotypes	Patients (n= 261) %	NC (n= 120) %	$\chi 2$	p value
CC	20.7 (54)	20.3 (27)	1.31	ns
CT	31.8 (83)	31.6 (42)	0.25	ns
TT	47.5 (124)	42.5 (51)	0.64	ns
Alleles	(n= 522)	(n= 240)		
C	36.6 (191)	40.0 (96)	0.02	ns
T	63.5 (331)	60.0 (144)		

No significant differences were found between the genotype or allelic frequencies of the patients and the normal controls.

Table 3.12: Comparison between the expected and the observed genotypes of the IL-4 C (-590) T according to Hardy-Weinberg principle

Hardy-Weinberg principle $P^2+2pq+q^2=1$	IL-4 C (-590) T					
	NC	C (n=96) T (n=144)		Patients	C (n=191) T (n=331)	
	CC	TC	TT	CC	TC	TT
Expected Genotype	28	48	44	40	103	118
Observed Genotype	27	42	51	54	83	124
χ^2		0.93			3.68	
p value		ns			ns	

The expected and observed genotypes of the IL-4 C (-590) T polymorphism in healthy controls and patients with type 1 diabetes meet Hardy-Weinberg principle.

3.2.2.2 The impact of gender of patients on the genetic susceptibility of the IL-4 C (-590) T polymorphism

The genotype and allelic frequencies of the IL-4 C (-590) T were calculated among gender of the patients. No considerable association was found between the IL-4 C (-590) T genotype or allelic frequencies and gender of the patients (Table 3.13 and Table 3.14 respectively).

3.2.2.3 The impact of age at onset of type 1 diabetes on the genetic susceptibility of the IL-4 C (-590) T polymorphism

In order to find out the relationship of age at onset of type 1 diabetes to the IL-4 C(-590) T polymorphism the patients were categorised according to section 3.1.4. No significant association was found between the age at onset and either of genotype or allelic frequencies (Table 3.15 and 3.16 respectively).

3.2.2.4 The role of the IL-4 C (-590) T polymorphism in the long term complications of diabetes

To study the possible effects of the IL-4 C (-590) T on the long term complications of diabetes the frequencies of the IL-4 C (-590) T genotype as well as alleles were calculated (Table 3.17, 3.18 respectively). No significant association was found between the genotype or allelic frequencies of the IL-4 C (-590) T polymorphism.

Table 3.13: The genotype frequency of the IL-4 C (-590) T with respect to gender of the patients and the normal controls

Genotype	Gender				χ^2	p value
	Male		Female			
	Patients %	NC %	Patients %	NC %		
CC	19.7 (25)	17.6 (9)	20.9 (28)	27.5 (19)	1.14	ns
CT	31.5 (40)	35.3 (18)	32.8 (44)	31.9 (22)	0.01	ns
TT	48.8 (62)	46.6 (24)	46.3 (62)	40.6 (28)	0.09	ns

No significant association was found between the IL-4 C (-590) T polymorphism genotype and genders of patients with type 1 diabetes.

Table 3. 14: The allelic frequency of the IL-4 C (-590) T with respect to gender of the patients and the normal controls

Allele	Gender				χ^2	p value
	Male		Female			
	Patients (n= 253) %	NC (n= 102) %	Patients (n= 268) %	NC (n= 139) %		
C	35.6 (90)	39.2 (40)	37.3 (100)	40.3 (56)	1.45	ns
T	64.4 (164)	60.8 (62)	62.7 (168)	59.7 (83)	0.15	ns

No significant association was found between the allelic distribution of the IL-4C(-590) T polymorphism and gender of patients with type 1 diabetes.

Table 3.15: The genotype frequency of the IL-4 C (-590) T with respect to age at onset of type 1 diabetes

Age at onset	Genotype		
	CC (n= 54) %	CT (n= 83) %	TT (n= 124) %
< 10 years	35.2 (19)	33.7 (28)	33.9 (42)
10-20 years	33.3 (18)	36.1 (30)	35.5 (44)
> 20 years	31.5 (17)	30.1 (25)	30.6 (38)
χ^2	<0.01		
p value	ns		

No significant association was found between genotypes of the IL-4 C (-590) T polymorphism and age at onset of the patients with type 1 diabetes.

Table 3.16: The allelic frequency of the IL-4 C (-590) T with respect to age at onset of type 1 diabetes

Age at onset	Allele C (n= 191) %	Allele T (n= 331) %
< 10 years	35.6 (66)	33.8 (112)
10-20 years	35.6 (66)	35.6 (118)
> 20 years	28.8 (59)	30.6 (101)
χ^2	0.01	
p value	ns	

No significant association was found between allelic distribution of the IL-4 C (-590) T polymorphism and age at onset of patients with type 1 diabetes

Table 3.17: The genotype frequency of the IL-4 C (-590) T with respect to the long term complications of diabetes

Long term complications of diabetes	IL-4 C (-590) T genotype CC (n= 31) %	CT (n= 44) %	TT (n= 52) %
DC	9.7 (3)	15.9 (7)	15.4 (8)
DN	22.6 (7)	25.0 (11)	26.9 (14)
DNu	29.0 (9)	31.8 (14)	25.0 (13)
DR	38.7 (12)	27.3 (12)	32.7 (17)

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

No significant association was found between the IL-4 C (-590) T and patients with long term complications of diabetes.

Table 3.18: The allelic frequency of the IL-4 C (-590) T with respect to the long term complications of diabetes

Long term complications of diabetes	IL-4 C (-590) T allele T (n= 106) %	C (n= 158) %
DC	12.3 (13)	14.6 (23)
DN	23.6 (25)	24.7 (39)
DNu	30.2 (32)	31.6 (50)
DR	33.9 (36)	29.1 (46)

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

No significant association was found between allelic distribution of the IL-4 C (-590) T with respect to the patients with long term complications ofdiabetes.

3.3 Analysis of the IL-6 Locus

3.3.1 Genotype and allelic frequencies of the IL-6 G (-174) C polymorphism

The DNA samples (Table 2.01) after amplification for the IL-6 gene polymorphism, the 243 bp fragment of the PCR product was analysed for the G (-174) C polymorphism.

After an overnight incubation, the 243 bp fragment of the IL-6 5′ flanking resulted in fragments of 119 and 111 and a 13bp as a result of cleavage with Nla III. Obviously 13 bp could not be detected due to rate of electrophoresis and both of 119 and 111 bp fragments were represented as a single band due to 8 bp difference in length.

As a result of cleavage of 243 bp fragment of the 5′ flanking polymorphism of the IL-6 gene with Nla III three possible genotypes were resulted. The GG as a homozygous for wild type allele, the CC as a homozygous for the lower allele, and the GC as a hetrozygout for both wild type and the lower allele (Fig 3.03).

The GG genotype was significantly increased in the patients comparing to the normal controls (50.6 % vs 33.3 % respectively , χ^2= 9.83, p= 0.002, and Pc=0.004). The CC genotype frequency was decreased in the patients comparing to the normal controls (12.5 % vs 24.2 % respectively, χ^2= 8.28, p= 0.004, and Pc= 0.008) (Table 3.19).

The allelic frequencies of the IL-6 G (-174) C polymorphism was calculated (Table 3.20). The G (-174) was significantly increased in the patients while the C (-174) was decreased compared to the normal controls (69.1 % vs 54.6 %, and 30.9 % vs 45.4 % respectively, χ^2= 14.98, p= 0.0001). The allelic distribution of the IL-6 G (-174) C polymorphism showed to be significantly associated with type 1 diabetes.

The expected and the observed genotypes of the IlL-6 G (-174) C polymorphism meet Hardy-Weinberg principle (Table 3.20).

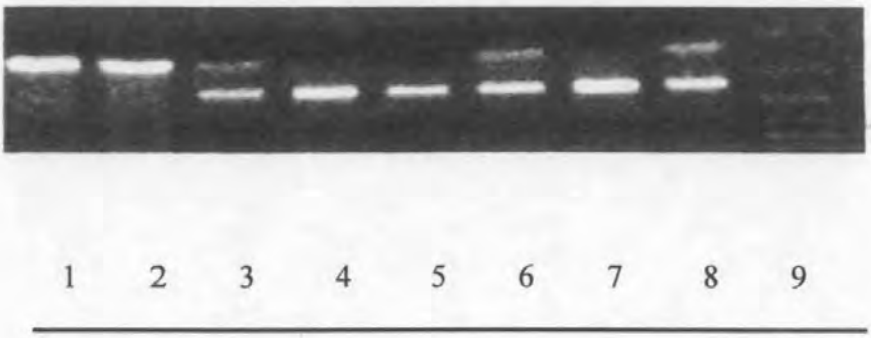
3.3.2 The impact of gender of patients on the genetic susceptibility of the IL-6 G (-174) C polymorphism

Association of the IL-6 gene G (-174) C polymorphism to gender was investigated. Gender of the patients was compared to normal controls with regard to the genotype and allelic frequencies of the IL-6 G (-174) C (Table 3.21, 3.22). No significant association was found between either of the genotype frequency of the IL-6 G (-174) C and gender of patients with type 1 diabetes. However, there was a significant decrease in the frequency of the C (-174) in female patients compared to male (36.0 % vs 30.5 %, χ^2= 5.80, p= 0.02). No other significant association was found (Table 3.22).

3.3.3 The impact of age at onset on the genetic susceptibility of the IL-6 G (-174) C polymorphism

In order to find out relationship of age at onset to the IL-6 G (-174) C polymorphism the patients were grouped according to section 3.1.3. No considerable association was found between the IL-6 G (-174) C genotype or allelic frequency and age at onset of type 1 diabetes (Table 3.25, 3.26 respectively

Figure 3.03: Photographic presentation of the IL-6 G (-174) C



In this photo lane 1 = CC, lane 2= CC, lane 3= GC, lane 4= GG, lane 5= GG, lane 6= GC, lane 7= GG, lane 8= GC, and lane 9= DNA ladder

Table 3.19: The genotype and allelic frequencies of the IL-6 G (-174) C polymorphism

Genotype	Patients (n= 258) %	NC (n= 120) %	χ^2	p value
GG	50.6 (130)	33.3 (40)	9.83	0.002
GC	37.0 (96)	42.5 (51)	1.06	ns
CC	12.5 (32)	24.2 (29)	8.28	0.004
Allele	(n= 516)	(n= 240)		
G	69.1 (356)	54.6 (131)	14.98	0.0001
C	30.9 (160)	45.4 (109)		

A significant difference between the patients with type 1 diabetes and the normal controls. The GG genotype is considerably increases in the patients whereas the CC genotype is favoured, to an important degree, in the normal controls.

Table 3.20: Comparison of the expected and observed genotypes of the IL-6 G (-174) C according to Hardy-Weinberg principle

Hardy-Weinberg principle $P^2+2pq+q^2=1$	IL-6 G (-174) C					
	NC G (n=131) C (n=109)			Patients G (n=356) C (n=160)		
	GG	GC	CC	GG	GC	CC
Expected Genotype	36	59	25	125	110	25
Observed Genotype	40	51	29	130	96	32
χ^2	1.09			ns		
p value	0.58			ns		

Both the expected and observed genotypes for the IL-6 G (-174) C for healthy controls and patients with type 1 diabetes meet Hardy-Weinberg principle.

Table 3.21: The IL-6 genotype frequency of the IL-6 G (-174) C with respect to gender of the patients and the normal controls

Genotype	Male		Female		χ^2	p value
	Patients (n= 117) %	NC (n= 45) %	Patients (n= 141) %	NC (n= 75) %		
GG	51.3 (60)	35.6 (16)	49.6 (70)	32.0 (24)	0.3	ns
GC	34.2 (40)	42.2 (19)	39.7 (56)	42.7 (32)	0.1	ns
CC	14.5 (17)	22.2 (10)	10.7 (15)	25.3 (19)	1.4	ns

No significant association was found between the IL-6 G (-174) C and genders of patients with type 1 diabetes.

Table 3.22: The allelic frequency of the IL-6 G (-174) C with respect to gender of the patients and the normal controls

Allele	Gender				χ^2	p value
	Male		Female			
	Patients (n= 250) %	NC (n= 90) %	Patients (n= 282) %	NC (n= 150) %		
G	64.0 (160)	56.7 (51)	69.5 (196)	53.3 (80)	1.18	ns
C	36.0 (90)	43.3 (39)	30.5 (86)	46.7 (70)	5.80	0.02

There was a considerable decrease in the C (-174) in female patients compared to female normal control.

No other significant association was found between the IL-6 G (-174) C polymorphism alleles and genders of patients with type 1 diabetes.

Table 3.23: The genotype frequency of the IL-6 G (-174) C with respect to age at onset of type 1 diabetes

Age at onset	Genotype GG (n= 130) %	GC (n= 96) %	CC (n= 36) %
< 10 years	41.5 (54)	31.3 (30)	25.0 (8)
10-20 years	28.5 (37)	41.7 (40)	46.9 (15)
> 20 years	30.0 (39)	27.0 (26)	28.1 (9)
χ^2	0.82		
p value	ns		

No significant association was found between the genotype frequency of the IL-6 G (-174) C and age at onset of type 1 diabetes.

Table 3. 24: The allelic frequency of the IL-6 G (-174) C with respect to age at onset of type 1 diabetes

Age at onset	Allele G (n= 356) %	Allele C (n= 160) %
< 10 years	38.8 (138)	28.8 (46)
10-20 years	32.0 (114)	43.8 (70)
> 20 years	29.7 (104)	27.5 (44)
χ^2	0.70	
p value	ns	

No significant association was found between allelic frequency of the IL-6 G (-174) C polymorphism and aga at onset of type 1 diabetes.

3.3.4 The role of the IL-6 G (-174) C polymorphism on the long term complications of diabetes

To study the possible effects of the IL-6 G (-174) C in the long term complications of diabetes the genotype and allelic frequencies of the IL-6 G (-174) polymorphism among diabetes long term complications were calculated (Table 3.25, 3.36 respectively).

No significant differences was found between the genotype or allelic frequency of the IL-6 G (-174) C in the patients with diabetes long term complications (Table 3.25, 3.26 respectively).

3.3.5 The familial clustering of the GG of the IL-6 G (-174) C polymorphism

In order to study the chance of the familial clustering of the GG genotype of the IL-6 G (-174) C 53 trios from the BDA-Warren family collection were genotyped for the IL-6 G (-174) C polymorphism. Forty one families had at least one parent as heterozygous for the IL-6 G (-174) C polymorphism. Out of 55 cases of the G allele 32 were transmitted to the offspring (Table 3.30). The TDT analysis showed a trend towards the transmission of the G (-174) (58.2 % vs 41.8 % respectively, χ^2= 0.44, p= ns).

Table 3.25: The genotype frequency of the IL-6 G (-174) C with respect to the long term complications of diabetes

Long term complications of diabetes	IL-6 G (-174) C genotype GG (n= 66) %	GC (n= 58) %	CC (n= 20) %
DC	12.1 (8)	12.1 (7)	10.0 (2)
DN	30.3 (20)	32.8 (19)	25.0 (5)
DNU	25.8 (17)	24.1 (14)	20.0 (4)
DR	31.8 (21)	31.0 (18)	45.0 (9)

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.
No significant association was found between the IL-6 G (-175) C genotype and diabetic long term complications

Table 3.26: The allelic frequency of the IL-6 G (-174) C polymorphism with respect to the long term complications of diabetes

Long term complications of diabetes	IL-6 G (-174) C allele G (n= 170) %	C (n= 98) %
DC	13.5 (23)	11.9 (11)
DN	22.9 (39)	29.6 (29)
DNU	28.2 (48)	22.4 (22)
DR	35.3 (60)	36.7 (36)

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.
DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.
DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.
DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

No significant association was found between IL-6 G (-174) C allelic distribution with different diabetic long term complications.

Table 3.27: TDT for the linkage analysis of the GG genotype of the IL-6 G (-174) C

TDT	
Transmitted	Non-transmitted
32	23
27	27
χ^2= 0.67	
p = ns	

Out of 55 cases of the G (-174), 32 (58.2 %) cases was transmitted from parents to the offspring. There was a trend for preferential transmission of the G (-174), χ^2= 0.67 and p= ns.

3.4 Analysis of the TGF-β1 locus

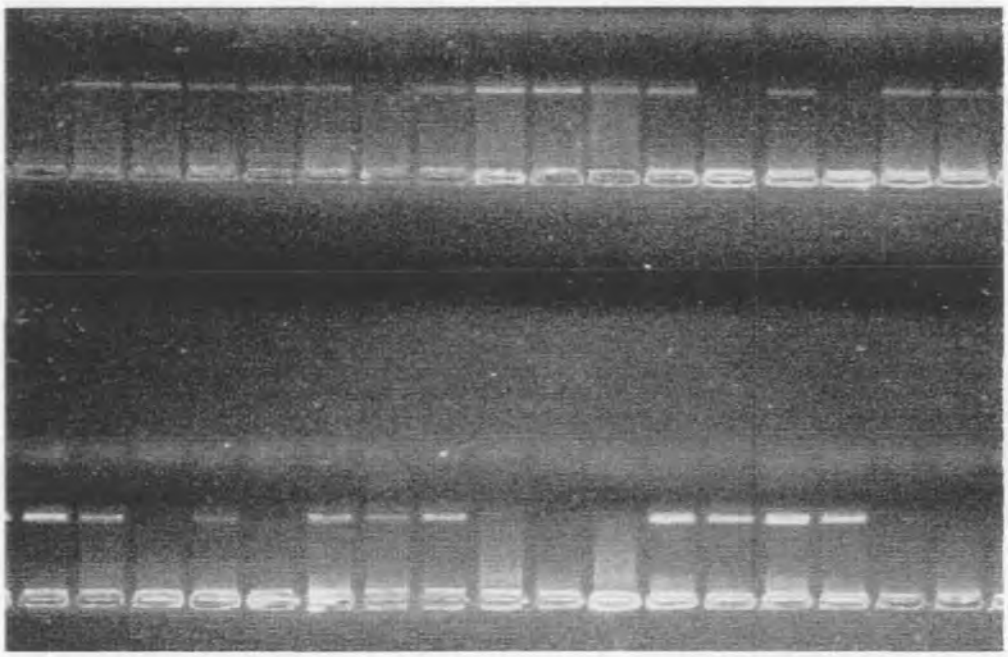
3.4.1 The genotype and allelic frequencies of the TGF-β1 (+869) C

The T (+869) C in the polymorphic region in exon 1 of the TGF-β1 gene that results in a transitions of Leucine to Proline in codon 10 was accurately detected using the ARMS-PCR methodology (Fig 3.04). The frequencies of the TT, the TC, and the CC in the patients with type 1 diabetes were 26.0 %, 56.0 %, and 17.5 % respectively (Table 3.28).

The expected and observed genotypes of the normal controls and the patients with type 1 diabetes meet Hardy-Weinberg principle (Table 3.29).

The genotype and allelic distribution of the T (+869) C in the TGF-β1gene were calculated. The frequency of TC was significantly increased in the patients comparing to the normal controls (56.3 % vs 39.8 % respectively, χ^2= 8.71, p= 0.003, and Pc= 0.006). No other significant differences were found between the frequency of the IL-6 genotype in the patients as well as the normal controls (Table 3.28). No significant difference was found between the allelic frequency of the TGF-β1 T (+869) C in the patients and the normal controls (Table 3.28).

Figure 3.04: *Photographic presentation of the TGF-β1 C (+869) T*



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

In this photo lane 1 represents the CC, lane 2= TC, lane 3= TT, lane 4= TC, lane 5= TT, lane 6= TC, lane 7= CC, lane 8= TC, lane 9= TT, lane 10= TT, lane 11= TT, lane 12= TC, lane 13= CC, lane 14= TC, lane 15= CC, lane 16= TT, lane 17= TT.

Table 3.28: The genotype and allelic frequencies of the TGF-β1 T (+869) C polymorphism

Genotype	Patients (n= 229) %	NC (n= 123) %	χ^2	p value
TT	(60) 26.2	(44) 35.8	3.52	ns
TC	56.3 (129)	39.8 (49)	8.71	0.003
CC	26.2 (40)	24.4 (30)	2.41	ns
Alleles	(n= 458)	(n= 256)		
T	54.4 (249)	55.7 (137)	0.07	ns
C	45.6 (209)	44.3 (109)		

The TC genotype show a significant association with type 1 diabetes (p=0.003). Although the TT genotype frequency is more in the patients than the normal controls but was not significant. Allelic distribution of the T (+869) C polymorphism in codon 10 of TGF-β does not show a significant association with type 1 diabetes.

Table 3.29: Comparison between the expected and observed genotypes of the TGF-β1 T (+869) C according to Hardy-Weinberg principle

Hardy-Weinberg principle $P^2+2pq+q^2=1$	TGF-β1 T (+869) C					
	NC T (n=137) C (n=109)			Patients T (n=249) C (n=209)		
	TT	TC	CC	TT	TC	CC
Expected genotype	38	60	25	67	114	48
Observed genotype	44	49	30	60	129	40
χ^2	2.00			2.00		
p value	ns			ns		

The expected and observed genotypes of the TGF-β1 T (+869) C for the healthy controls and the patients with type 1 diabetes meet Hardy-Weinberg principle.

3.4.2 The impact of gender of patients on the genetic susceptibility of the TGF-β1 T (+869) C polymorphism

The relationship of gender of the patients to the genotype and allelic frequencies of the TGF-β1 T (+869) C polymorphism was calculated (Table 3. 30, 3.31 respectively). No significant association was found between gender of the patients and the genotype or allelic frequencies of the TGF-β1 T (+869) C polymorphism (Table 3.30, 3.31 respectively).

3.4.3 The impact of age at onset of type 1 diabetes on the genetic susceptibility of the TGF-β1 T (+869) C polymorphism

In order to find out the relationship of the age at onset of type 1 diabetes to the TGF-β1 T (+869) C polymorphism, the patients were grouped according to the age at onset according to section 3.1.3. No significant association was found between the genotype and allelic frequencies of the TGF-β1 T (+869) C polymorphism and age at onset (Table 3.32, 3.33 respectively).

The correlation between genotype frequency of the T (+869) C polymorphism in exon 1 of the TGF-β1 gene and gender of the study subject was studied. There has been no significant difference between the genotype frequencies of the T (+869) C polymorphism in the TGF-β1gene with regard to the gender of patients with type 1 diabetes (Table 3.32).

Table 3.30: The genotype frequency of the TGF-β1 T (+869) C with respect to gender of the patients and the normal controls

Genotype	Male		Female		χ^2	p value
	Patients (n= 93) %	NC (n= 55) %	Patients (n= 136) %	NC (n= 68) %		
TT	20.4 (19)	34.5 (19)	30.1 (41)	36.8 (25)	1.00	ns
TC	61.3 (57)	40.0 (22)	53.0 (72)	39.7 (27)	0.01	ns
CC	18.3 (17)	25.5 (14)	16.9 (23)	23.5 (16)	0.01	ns

No significant association was found between the frequency of the TGF-β1 T (+869) C genotype with respect to gender of the patients with type 1 diabetes.

Table 3.31: The allelic frequency of the TGF-β1 T (+869) C with respect to gender of the patients and the normal controls

Allele	Gender				χ^2	p value
	Male		Female			
	Patients (n= 186) %	NC (n= 100) %	Patients (n= 272) %	NC (n= 136) %		
T	51.1 (95)	60.0 (60)	56.6 (154)	56.6 (77)	0.95	ns
C	49.9 (91)	40.0 (40)	43.3 (118)	43.3 (59)	0.16	ns

No significant association was found between the allelic frequency of the TGF-β1 T +8629) C with respect to gender of the patients with type 1 diabetes.

Table 3.32: The genotype frequency of the TGF-β1 T (+869) C with respect to age at onset of type 1 diabetes

Age at onset	Genotype TT (n= 60)	TC (n= 129)	CC (n= 40)
< 10 years	31.7 (19)	33.3 (43)	32.5 (13)
10-20 years	41.7 (25)	37.2 (48)	40.0 (16)
> 20 years	26.6 (16)	29.5 (38)	27.5 (11)
χ^2	0.02		
p value	ns		

No significant association was found between genotype frequencies of the TGF-β1 T (+869) C with respect to age at onset of the patients with type 1 diabetes.

Table 3.33: The allelic frequency of the TGF-β1 T (+869) C with respect to age at onset of type 1 diabetes

Age at onset	Allele T (n= 249) %	C (n= 209) %
< 10 years	32.5 (81)	33.0 (69)
10-20 years	39.4 (98)	38.3 (80)
> 20 years	28.1 (70)	58.7 (60)
χ^2	0.01	
p value	ns	

No significant association was found between the allelic frequency of the TGF-β1 T (+869) C with respect to age at onset of type 1 diabetes.

3.4.4 The role of the TGF-β1 T (+869) C polymorphism on the long term complications of diabetes

To study the possible effects of the TGF-β1 T (+869) C on the long term complications of diabetes, the genotype and allelic frequencies of the TGF-β1 T (+869) C were calculated (Table 3. 34, 3. 35 respectively).

There was a significant decrease in the frequency of the TC in the patients with diabetic nephropathy compared to the diabetic control patients (17.9 % vs 29.9 % respectively, χ^2= 3.89, p= 0.05). No other significant association was found between the genotype of the TGF-β1 T (+869) C and the long term complications of diabetes (Table 3.34).

No significant association was found between the allelic distribution of the TGF-β1 T (+869) C and the diabetes long term complications (Table 3. 35).

3.4.5 The familial clustering of the TGF-β1 T (+869) C polymorphism

In order to study the chance of familial clustering of the TC of the TGF-β1 T (+869) C, 53 trios from BDA-Warren family collection were genotyped for the TGF-β1 polymorphism. Out of 58 transmitted alleles from heterozygous parents the T (+869) transmitted 35 times and the C (+869) transmitted 23 times. The TDT analysis show a significant increase in the T (29) comparing to the C (29) (60.3 % vs 39.7 % respectively,χ2=4.17,p=0.04).

3.5 Association of individual cytokine gene polymorphisms

In order to aid studying individual cytokine gene polymorphisms with type 1 diabetes, genotype frequencies of the IFNG CA repeats, the IL-6 G (-174) C, the TGF-β1 T (+869) C, and the IL-4 C (-590) T were grouped in Figure 3.05.

Table 3.34: The genotype frequency of the TGF-β1 T (+869) C with respect to the long term complications of diabetes

Long term complications of diabetes	TGF-β1 T (+869) C TT (n= 39) %	TC (n= 67) %	CC (n= 15) %
DC	20.5 (8)	29.9 (20)	6.7 (1)
DN	33.3 (13)	17.9 (12)	33.3 (5)
DNu	20.5 (8)	23.8 (16)	33.3 (5)
DR	25.7 (10)	28.4 (19)	26.7 (4)

DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinoria over 12 months in the absence or haematoria or infection.

DNU= patients with overt neuropathy defined as loss of ankle jerks, sensations of pain, foot ulcer and/or autonomic neuropathy.

DC= patients who have had diabetes for at least 20 years but remain free of retinopathy and proteinuria.

There was a significant decrease in the frequency of the TC of the TGF-β1 T (+869) C in the patients with nephropathy compared to the diabetic control patients (χ^2= 3.92, p= 0.04). No other significant association was found.

Table 3.35: The allelic frequency of the TGF-β1 T (+869) C with respect to the long term complications of diabetes

Long term complications of diabetes	TGF-β1 T (+869) C T (n= 145) %	C (n= 97) %
DC	24.8 (36)	22.7 (22)
DN	26.2 (38)	22.7 (22)
DNu	22.1 (32)	26.8 (26)
DR	26.9 (39)	27.8 (27)

DR= patients with retinopathy defined as more than 5 blots or blots per eye. Hard or soft exudates, new vessels or flourescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy.

DN= patients with nephropathy defined as patients who had diabetes for more than 10 years with persistent proteinuria over 12 months in the absence or haematuria or infection.

DNU= patients with overt neuropathy defined as loss of ankle jerks, loss of pain sensation, foot ulcer and/or autonomic neuropathy.

DC= patients who have had diabetes for at least 20 years but remain free fromof retinopathy and microalbuminoria.

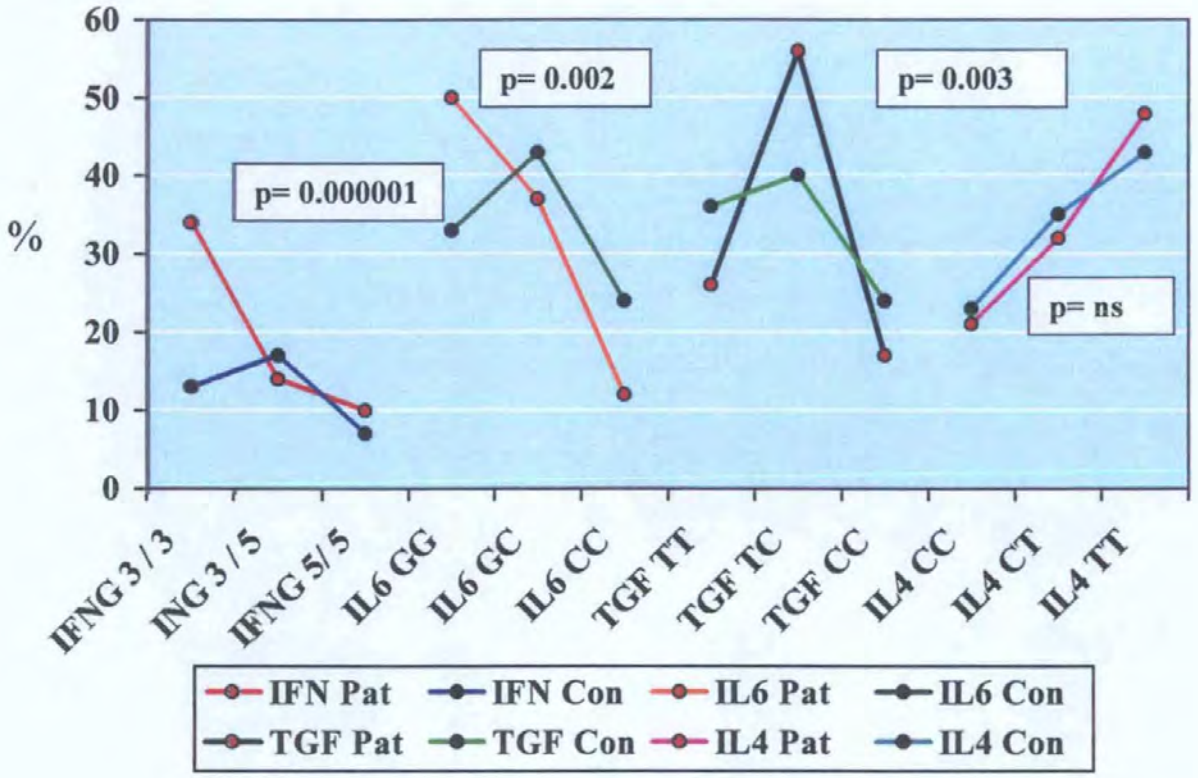
No significant association was found between the allelic frequency of the TGβ-1 T (+869) C and the long term complications of diabetes.

Table 3.36: TDT for the linkage analysis of the TC of the TGF-β1 C (+869) T

Transmitted	Non-transmitted
35	23
23	35
χ^2= 4.17	
p= 0.04	

From 58 transmitted alleles of the TGF-β1 T (+ 869) C 35 (61.0 %) were allele T (+ 869) and 23 were allele C (+ 869). The TDT result shows that there was significant preferential transmission of the TGF-β1 T (+ 869) allele (χ^2= 4.17, p= 0.04).

Figure 3.05: Illustration of the association of the IFNG, IL-4 C (-590) T, IL-6 G (-174) C, and TGF-β1 T (+869) C polymorphisms with type 1 diabetes



This figure illustrates the individual association of this thesis candidate genes with type 1 diabetes

3.6 Analysis of genetic profile

In order to determine the relationship between cytokine gene polymorphisms with respect to each other, the incidence the genotypes in the patients as well as the normal controls were correlated.

3.6.1 The IL-4 C (-590) T vs The IFNG CA repeats

There was a significant increase in the frequency of the patients with the TT of the IL-4 C (-590) T and the 3 / 3 of the IFNG CA repeats compared to the normal controls (37.2 % vs 19.5 % respectively, χ^2=3.86, p= 0.05). On the other hand, there was a significant decrease in the frequency of the patients with the TT and the X / X compared to the normal controls (36.4 % vs 61.0 % respectively, χ^2= 6.41, p= 0.01). No other significant association was found (Table 3.37).

The IL-4 C (-590) T and the IFNG CA repeats were analysed with respect to gender of the patients and the normal controls. No significant difference was found (Table 3.38).

The IL-4 C (-590) T and the IFNG CA repeats were analysed with respect to age at onset of type 1 diabetes. There was a significant increase in the frequency of the patients with the TT and the 3 / 3 genotype in the patients with < 10 and 10-20 years compared to > 20 years age at onset (48.6 % and 45.8% vs 22.2 %, χ^2= 5.81, p= 0.05). No other significant association was found (Table 3.39).

Table 3.37: Correlation between the IL-4 C (-590) T and the IFNG CA repeats genotypes

IFNG CA Genotype	IL-4 C (-590) T genotype CC Patients (n= 46) %	CC NC (n= 26) %	CT Patients (n= 75) %	CT NC (n= 26) %	TT Patients (n= 110) %	TT NC (n= 41) %
3 / 3	32.7 (15)	11.7 (3)	33.3 (25)	15.4 (4)	37.2 (41)	19.5 (8)
3 / 5	13.0 (6)	15.4 (4)	10.7 (8)	23.1 (6)	15.5 (17)	12.2 (5)
5 / 5	15.2 (7)	7.8 (2)	9.3 (7)	7.8 (2)	10.9 (12)	7.3 (3)
X / X	69.1 (18)	65.3 (17)	46.7 (35)	53.7 (12)	36.4 (40)	61.0 (25)

There was a slight increase in the frequency of the TT of the IL-4 C (-590) T with the 3 / 3 of the IFNG CA repeats.

Table 3. 38: Association of the IL-4 C (-590) T and the IFNG genotype frequency with respect to gender of patients with type 1 diabetes

IFNG CA repeat	IL-4 C (-590) T genotype											
	CC				CT				TT			
	Male		Female		Male		Female		Male		Female	
	Patients (n= 23) %	NC (n= 12) %	Patients (n= 23) %	NC (n= 14) %	Patients (n= 34) %	NC (n= 14) %	Patients (n= 41) %	NC (n= 13) %	Patients (n= 51) %	NC (n= 22) %	Patients (n= 59) %	NC (n= 19) %
3 / 3	30.4 (7)	8.3 (1)	34.8 (8)	14.3 (2)	32.4 (11)	21.4 (3)	34.1 (14)	7.6 (1)	39.2 (20)	18.2 (4)	35.6 (21)	21.1 (4)
3 / 5	17.4 (4)	16.7 (2)	8.6 (2)	14.3 (2)	11.8 (4)	14.3 (2)	9.8 (4)	30.8 (4)	15.7 (8)	13.6 (3)	15.3 (9)	10.5 (2)
5 / 5	17.4 (4)	- (0)	13.0 (3)	14.3 (2)	8.8 (3)	21.4 (3)	9.8 (4)	15.4 (2)	11.8 (6)	9.1 (2)	10.2 (6)	5.3 (1)
X / X	34.8 (8)	75.0 (9)	43.6 (10)	57.1 (8)	47.1 (16)	42.9 (6)	46.3 (19)	46.2 (6)	33.3 (17)	59.1 (13)	38.9 (23)	63.1 (12)

No Significant association was found.

Table 3. 39: Association of the IL-4 C (-590) T and the IFNG genotype frequency with respect to age at onset of patients with type 1 diabetes

IFNG CA repeat	IL-4 C (-590) T								
	CC			CT			TT		
	< 10 years %	10-20 years %	> 20 years %	< 10 years %	10-20 years %	> 20 years %	< 10 years %	10-20 years %	> 20 years %
3 / 3	46.7 (7)	27.7 (5)	21.4 (3)	33.3 (8)	39.1 (9)	28.6 (8)	48.6 (18)	40.58 (15)	22.2 (8)
3 / 5	6.7 (1)	16.7 (3)	14.3 (2)	12.5 (3)	8.7 (2)	10.7 (3)	16.2 (6)	13.5 (5)	16.7 (6)
5 / 5	13.3 (2)	16.7 (3)	21.4 (3)	12.5 (3)	- (0)	14.3 (4)	8.1 (3)	10.8 (4)	13.9 (5)
X / X	33.3 (5)	28.9 (7)	42.9 (6)	41.7 (10)	52.2 (12)	46.6 (28)	27.1 (10)	35.2 (13)	47.2 (17)

There was a significant increase in the frequency of the TT and the 3 / 3 in <10 and 10-20 years compared to the patients with >20 years age at onset (48.6 % and 45.8 % vs 22.2 %, χ^2= 5.81, p= 0.05). No other significant association was found.

3.6.2 The IL-6 G (-174) C vs the IFNG CA repeats

The IL-6 G (-174) C genotype frequency was analysed with respect to the IFNG CA repeats genotype. There was a significant increase in the frequency of the patients with the GG of the IL-6 G (-174) C and the 3 / 3 of the IFNG CA repeats compared to the normal controls (41.6 % vs 18.9 % respectively, χ^2= 4.99, p= 0.02. This was accompanied by a significant decrease in the frequency of the patients with GG and the X / X in the patients compared to the normal controls (28.0 % vs 54.1 % respectively, χ^2= 8.25, p= 0.004). In addition, there was a significant increase in the frequency of the patients with GC and the 3 / 3 genotype in the patients compared to the normal controls (39.4 % vs 10.0 % respectively, χ2= 9.21, p= 0.002). Finally, there was a significant decrease in the frequency of the patients with the GC and the X / X genotype in the patients compared to the normal controls (34.9 % vs 72.5% respectively, χ^2= 11.64, p= 0.0006) (Table 3.40).

The IL-6 G (-174) C and the IFNG CA repeats genotypes were analysed with respect to gender. No significant association was found between the frequencies of the IL-6 G (-147) C and the IFNG in the patients and the normal controls with respect to gedender (Table 3.41).

The IL-6 G (-174) C and the IFNG CA repeats genotype were analysed with respect to age at onset of type 1 diabetes. There was a significant increase in the frequency of the patients with the GG and the 3 / 3 with <10 and 10-20 compared to >20 years age at onset (50.9 % and 43.9 % vs 22.6 % respectively, χ^2= 6.61, p= 0.03). No other significant association was found (Table = 3.42).

Table 3.40: Correlation between the IL-6 G (-174) C and the IFNG CA repeats genotypes

IFNG CA repeat genotype	IL-6 G (-174) C					
	GG		GC		CC	
	Patients (n=125) %	NC (n= 36) %	Patients (n= 66) %	NC (n= 40) %	Patients (n= 31) %	NC (n= 17) %
3 / 3	41.6 (52)	18.9 (7)	39.4 (26)	10.0 (4)	29.0 (9)	18.8 (3)
3 / 5	15.2 (19)	16.2 (6)	13.6 (9)	12.5 (5)	9.7 (3)	25.0 (4)
5 / 5	15.2 (19)	10.8 (4)	12.1 (8)	5.0 (2)	9.7 (3)	6.3 (1)
X / X	28.0 (35)	54.1 (19)	34.9 (23)	22.5 (29)	51.6 (16)	49.9 (8)

There is a significant association between the GG of the IL-6 G (-174) C and the 3 / 3 of the IFNG CA repeat (χ^2= 4.99, p= 0.02); there was a significant increase in the patients with the GC and the 3 / 3 genotype compared to the normal controls (χ2= 9.21, p= 0.002). The majority of the GG genotyped patients had the 3 / 3 of the IFNG CA genotype.

Table 3. 41: Association of the IL-6 G (-174) C and the IFNG genotype frequency with respect to gender of patients with type 1 diabetes

IFNG CA repeat	IL-6 G (-174) C											
	GG				GC				CC			
	Male		Female		Male		Female		Male		Female	
	Patients (n= 60) %	NC (n= 20) %	Patients (n= 65) %	NC (n= 17) %	Patients (n= 33) %	NC (n= 20) %	Patients (n= 33) %	NC (n= 20) %	Patients (n= 16) %	NC (n= 8) %	Patients (n= 15) %	NC (n= 8) %
3 / 3	41.7 (25)	20.0 (4)	41.5 (27)	17.6 (3)	36.4 (12)	10.0 (2)	42.4 (14)	10.0 (2)	31.3 (5)	37.5 (3)	26.7 (4)	- (0)
3 / 5	16.7 (10)	15.0 (3)	13.8 (9)	17.6 (3)	15.2 (5)	10.0 (2)	12.1 (4)	15.0 (3)	6.3 (1)	25.0 (2)	13.3 (2)	25.0 (2)
5 / 5	13.3 (8)	10.0 (2)	16.0 (11)	11.8 (2)	12.1 (4)	10.0 (2)	12.1 (4)	- (0)	12.5 (2)	- (0)	6.7 (1)	12.5 (1)
X / X	28.3 (17)	55.0 (11)	27.8 (18)	53.0 (9)	36.4 (12)	70.0 (14)	33.4 (11)	75.0 (15)	49.9 (8)	37.5 (3)	53.3 (8)	74.9 (5)

No significant association was found.

Table 3. 42: Association of the IL-6 G (-174) C and the IFNG genotypes frequency with respect to age at onset of patients with type 1 diabetes

IFNG CA repeat	IL-6 G (-174) C								
	GG			GC			CC		
	< 10 years (n= 53) %	10-20 years (n= 41) %	> 20 years (n= 31) %	< 10 years (n= 22) %	10-20 years (n= 23) %	> 20 years (n= 21) %	< 10 years (n= 7) %	10-20 years (n= 10) %	> 20 years (n= 14) %
3 / 3	50.9 (27)	43.9 (18)	22.6 (7)	40.9 (9)	39.1 (9)	38.1 (8)	28.6 (2)	30.0 (3)	28.6 (4)
3 / 5	13.2 (7)	14.6 (6)	19.4 (6)	13.6 (3)	17.4 (4)	9.5 (2)	- (0)	10.0 (1)	14.3 (2)
5 / 5	11.3 (6)	12.2 (5)	25.8 (8)	9.4 (2)	13.1 (3)	14.3 (3)	14.3 (1)	10.0 (1)	7.1 (1)
X / X	24.6 (13)	29.3 (12)	32.2 (10)	36.4 (8)	30.4 (7)	38.1 (8)	57.1 (4)	50.0 (5)	50.0 (7)

There was a significant increase in the frequency of the patients with the GG and the 3 / 3 in <10 and 10-20 years compared to the patients with age at onset of >20 years (50.9 % and 43.9 % vs 22.6 % repectively, χ^2= 6.61, p=0.03). No other significant association was found.

3.6.3 The IL-6 G (-174) T vs IL-4 C (-590) T

The IL-6 G (-174) C genotype was analysed with respect of the IL-4 C (-590) T genotype. No significant difference was found between the frequencies of the patients and the normal controls (Table 3.43).

The IL-6 G (-174) C and the IL-4 C (-590) T were analysed with respect to gender of the patients and the normal controls. No significant difference was found (Table 3.44).

The IL-6 G (-174) C and the IL-4 C (-590) T genotype were analysed with respect to age at onset of the patients. No significant association was found (Table 3.45).

Table 3.43: Correlation between the IL-6 G (-174) C and the IL-4 C (-590) T genotypes

IL-4 C (-590) T Genotype	IL-6 G (-174) C genotype					
	GG		GC		CC	
	Patients (n= 60)	NC (n= 36)	Patients (n= 129)	NC (n= 42)	Patients (n= 40)	NC (n= 27)
CC	13.1 (17)	18.4 (7)	29.2 (28)	23.5 (12)	25.0 (8)	27.6 (8)
CT	30.0 (39)	31.6 (12)	36.5 (35)	39.2 (20)	28.1 (9)	34.5 (10)
TT	56.9 (74)	50.0 (19)	34.3 (33)	37.3 (19)	46.9 (15)	37.9 (11)

No significant association was found between the frequency of theIL-6 G (-174) C with respect to the IL-4 C (-590) T genotype.

Table 3. 44: Association of the IL-6 G (-174) C and the IL-4 (-590) T genotype frequency with respect to gender of patients with type 1 diabetes

IL-4 C (-590) T genotype	IL-6 G (-174) C genotype											
	GG				GC				CC			
	Male		Female		Male		Female		Male		Female	
	Patients (n= 62)	NC (n= 18)	Patients (n= 68)	NC (n= 20)	Patients (n= 45)	NC (n= 24)	Patients (n= 51)	NC (n= 27)	Patients (n= 14)	NC (n= 16)	Patients (n= 18)	NC (n=13)
CC	14.5 (9)	16.7 (3)	11.8 (8)	20.0 (4)	22.2 (10)	25.6 (6)	35.3 (18)	22.2 (6)	21.4 (3)	31.3 (5)	27.8 (5)	23.1 (3)
CT	29.0 (18)	38.9 (7)	30.8 (21)	25.0 (5)	40.0 (18)	37.5 (9)	33.3 (17)	40.7 (11)	28.6 (4)	37.5 (6)	27.8 (5)	30.7 (4)
TT	56.5 (35)	44.4 (8)	57.4 (39)	55.0 (11)	37.8 (17)	37.5 (9)	31.4 (16)	37.1 (10)	50.0 (7)	31.2 (5)	44.4 (8)	46.2 (6)

No significant association was found.

Table 3. 45: Association of the IL-6 G (-174) C and the IL-4 C (-590) T genotype frequency with respect to age at onset of type 1 diabetes

IL-4 C (-590) T genotype	IL-6 G (-174) C genotype								
	GG			GC			CC		
	<10 years (n=51) %	10-20 years (n= 45) %	>20 years (n=34) %	<10 years (n= 31) %	10-20 years (n= 31) %	>20 years (n= 34) %	<10 years (n= 11) %	10-20 years (n= 12) %	>20 years (n= 11) %
CC	9.8 (5)	13.3 (6)	17.6 (6)	32.3 (10)	35.4 (11)	20.6 (7)	18.2 (2)	16.7 (2)	36.5 (4)
CT	23.5 (12)	28.9 (13)	41.2 (14)	38.7 (12)	32.3 (10)	38.2 (13)	27.3 (3)	33.3 (4)	18.2 (2)
TT	66.7 (34)	57.8 (26)	41.2 (14)	29.0 (9)	32.3 (10)	41.2 (14)	54.5 (6)	50.0 (6)	27.3 (3)

No significant association was found.

3.6.4 The TGF-β1 vs the IFNG CA repeats

The TGF-β1 T (+869) C genotype frequency was analysed with respect to the IFNG genotype. There was a significant increase in the frequency of the TC in the patients weth the 3 / 3 genotype compared to the normal controls (44.7 % vs 17.1 % respectively, χ^2= 10.60, p= 0.001). This was accompanied with a significant decrease in the patients with the TC and the X / X genotype compared to the normal controls (29.0 % vs 57.2 % respectively, χ^2= 14.37, p= 0.0001). No other significant association was found (Table 3.46).

The TGF-β1 and the IFNG genotypes were analysed with respect to gender. No significant association was found (Table 3. 47).

The TGF-β1 and the IFNG genotypes were analysed with respect to age at onset of the patients. No significant association was found (Table 3. 48).

Table 3.46: Correlation between the TGF-β1 T (+869) C and the IFNG CA repeats genotypes

IFNG CA repeat Genotype	TGF-β1 T (+869) C genotype					
	TT		TC		CC	
	Patients (n= 60) %	NC (n= 30) %	Patients (n= 128) %	NC (n=41) %	Patients (n= 36) %	NC (n= 20) %
3 / 3	30.6 (19)	13.9 (5)	44.5 (57)	17.1 (6)	19.4 (7)	18.2 (2)
3 / 5	16.1 (10)	19.4 (7)	15.6 (20)	17.1 (6)	13.9 (5)	27.3 (3)
5 / 5	8.1 (5)	8.3 (3)	10.9 (14)	8.6 (3)	19.4 (7)	19.1 (1)
X / X	45.2 (26)	58.4 (15)	29.0 (37)	57.2 (26)	47.3 (16)	45.4 (5)

There was a significant association between the TC of the TGF-β1 T (+ 869) C and the 3 / 3 of the IFNG CA repeat genotypes (χ^2= 10.60, p= 0.001). This was accompanied by a significant decrease in the CA and the X / X genotypes (χ^2= 14.37, p= 0.0001). No other significant association was found.

Table 3. 47: Association the TGF-β1 T (+869) C and the IFNG genotype frequency with respect to gender of patients with type 1 diabetes

IFNG CA repeat	TGF-β1 T (+869) C genotype											
	TT				TC				CC			
	Male		Female		Male		Female		Male		Female	
	Patients (n= 29) %	NC (n= 15) %	Patients (n= 31) %	NC (n= 15) %	Patients (n= 62) %	NC (n= 20) %	Patients (n= 66) %	NC (n= 21) %	Patients (n= 17) %	NC (n= 9) %	Patients (n= 19) %	NC (n= 11) %
3 / 3	30.0 (9)	15.8 (3)	32.3 (10)	10.5 (2)	43.5 (27)	10.5 (2)	45.5 (30)	17.6 (3)	33.3 (3)	- (0)	21.1 (4)	40.0 (2)
3 / 5	16.7 (5)	21.1 (4)	16.1 (5)	15.8 (3)	14.5 (9)	21.1 (4)	16.7 (11)	11.8 (2)	33.3 (3)	40.0 (2)	10.5 (2)	20.0 (1)
5 / 5	10.0 (3)	5.3 (1)	6.5 (2)	10.5 (2)	14.5 (9)	5.3 (1)	7.6 (5)	11.8 (2)	11.8 (2)	- (0)	26.3 (5)	- (0)
X / X	43.3 (12)	57.8 (7)	45.1 (14)	63.2 (8)	27.5 (17)	63.1 (12)	30.2 (20)	58.8 (14)	21.6 (9)	60.0 (7)	42.1 (8)	40.0 (8)

No significant association was found.

Table 3. 48: Association of the TGF-β1 T (+869) C and the IFNG genotype frequency with respect to age at onset of patients with type 1 diabetes

IFNG CA repeat	TGF-β1 T (+869) C genotype								
	TT			TC			CC		
	< 10 years (n= 21) %	10-20 years (n= 17) %	> 20 years (n= 22) %	< 10 years (n= 41) %	10-20 years (n= 48) %	> 20 years (n= 39) %	< 10 years (n= 12) %	10-20 years (n= 12) %	> 20 years (n= 12) %
3 / 3	31.8 (7)	38.9 (7)	20.8 (5)	40.0 (18)	49.0 (25)	33.3 (14)	16.7 (2)	23.1 (3)	15.4 (2)
3 / 5	9.1 (2)	16.7 (3)	20.8 (5)	26.7 (8)	15.7 (5)	23.8 (7)	8.3 (1)	23.1 (3)	7.7 (1)
5 / 5	9.1 (2)	- (0)	12.5 (3)	8.9 (4)	9.8 (5)	11.9 (5)	25.0 (3)	7.7 (1)	23.1 (3)
X / X	50.0 (10)	44.4 (7)	45.9 (9)	24.4 (11)	25.5 (13)	31.0 (13)	50.0 (6)	46.1 (5)	53.8 (7)

No significant association was found.

3.6.5 The TGF-β1 T (+869) C vs the IL-4 C (-590) T

The TGF-β1 T (+869) C genotype was analysed with respect to the IL-4 C (-590) genotype. No significant association was found (Table 3.49).

The TGF-β1 T (+869) C and IL-4 C (-590) T genotypes were analyse with respect to gender. No significant association was found (Table 3.51).

The TGF-β1 T (+869) C and the IL-4 C (-590) genotypes were analysed with respect to age at onset of the patients. No significant association was found Table 3.52).

Table 3.49: Correlation between the TGF-β1 T (+869) C and the IL-4 C (-590) T genotypes

IL-4 C (-590) T Genotype	TGF-β1 T (29) C genotype					
	TT		TC		CC	
	Patients (n= 60) %	NC (n= 38) %	Patients (n= 129) %	NC (n= 46) %	Patients (n= 40) %	NC (n= 30) %
CC	21.7 (13)	18.4 (7)	22.5 (29)	25.0 (11)	27.5 (11)	20.7 (6)
CT	25.0 (15)	28.9 (11)	37.2 (48)	31.8 (14)	25.0 (10)	37.9 (11)
TT	53.3 (32)	52.7 (20)	40.3 (52)	43.2 (19)	47.5 (19)	41.4 (12)

No significant association was found between the TGF-β1 T (+869) C and the IL-4 C (-590) T genotype frequencies.

Table 3. 50: Association of the TGF-β1 T (+869) C and the IL-4 (-590) genotype frequency with respect to gender of patients with type 1 diabetes

IL-4 C (-590) T Genotype	TGF-β1 T (+869) C genotype											
	TT				TC				CC			
	Male		Female		Male		Female		Male		Female	
	Patients (n= 26)	NC (n= 17)	Patients (n= 34)	NC (n= 21)	Patients (n= 62)	NC (n= 19)	Patients (n= 67)	NC (n= 25)	Patients (n= 19)	NC (n= 13)	Patients (n= 21)	NC (n=16)
CC	19.2 (5)	17.6 (3)	23.5 (8)	19.1 (4)	24.2 (15)	26.3 (5)	20.9 (14)	24.0 (6)	26.3 (5)	15.4 (2)	28.6 (6)	25.0 (4)
CT	26.9 (7)	35.3 (6)	23.5 (8)	23.8 (5)	35.5 (22)	31.6 (6)	38.8 (26)	32.0 (8)	21.1 (4)	38.5 (5)	28.6 (6)	37.5 (6)
TT	53.9 (14)	47.1 (8)	53.8 (18)	57.1 (12)	40.3 (25)	42.1 (8)	40.3 (27)	44.0 (11)	52.6 (10)	48.1 (6)	42.8 (9)	37.5 (6)

No significant association was found.

Table 3. 51: Association of the TGF-β1 T (+869) C and the IL-4 C (-590) T genotype frequency with respect to age at onset of type 1 diabetes

IL-4 C (-590) T genotype	TGF-β1 T (+869) C genotype								
	TT			TC			CC		
	<10 years (n= 20) %	10-20 years (n= 18) %	>20 years (n= 22) %	<10 years (n= 32) %	10-20 years (n= 44) %	>20 years (n= 43) %	<10 years (n= 13) %	10-20 years (n= 14) %	>20 years (n= 13) %
CC	25.0 (5)	22.2 (4)	18.2 (4)	28.1 (9)	22.8 (10)	23.3 (10)	27.1 (3)	28.6 (4)	30.8 (4)
CT	25.0 (5)	27.8 (5)	22.7 (5)	43.8 (14)	38.6 (17)	39.5 (17)	30.8 (4)	21.4 (3)	23.1 (3)
TT	50.0 (10)	50.0 (9)	59.1 (13)	28.1 (9)	38.6 (17)	37.2 (16)	46.1 (6)	50.0 (7)	46.1 (6)

No significant association was found.

3.6.6 The TGF-β1 T (+869) C vs IL-6 G (-174) C

The TGF-β1 T (+869) C genotype was analysed with respect to the IL-6 G (-174) C genotype. There was a significant increase in the frequency of the TC in the patients with the GG compared to the normal controls (64.3 % vs 40.5 % respectively, χ^2= 6.48, p= 0.01). This was accompanied by a significant decrease in the frequency of the TC genotype in the patients with the CC genotype comparing to the normal controls (6.2 %, vs 23.8 % respectively, χ^2= 8.64, p= 0.003). No other significant association was found (Table 3.52).

The TGF-β1 and the IL-6 G (-174) C genotypes were analysed with respect to gender. No significant association was found (Table 3.53).

The TGF-β1 and the IL-6 G (-174) C genotypes were analysed with respect to age at onset of the patients. There was a significant increase in the frequency of the TC and GG in the patients with <10 years compared to 10-20 and >20 years age at onset (70.7%vs 63.2 % and 54.5 % respectively, χ^2= 5.90, p=0.05). No significant association was found (Table 3.54).

Table 3.52: Correlation between the TGF-β1 T (+869) C and the IL-6 G (-174) C genotypes

IL-6 G (-174) C Genotype	TGF-β1 T (+869) C TT		TC		CC	
	Patients (n= 60)	NC (n= 36)	Patients (n= 129)	NC (n= 42)	Patients (n= 40)	NC (n= 27)
GG	36.7 (22)	33.3 (12)	64.3 (83)	40.5 (17)	22.5 (9)	22.2 (6)
GC	42.4 (28)	36.1 (13)	29.5 (38)	35.7 (15)	62.5 (25)	44.5 (12)
CC	20.9 (10)	30.6 (11)	6.2 (8)	23.8 (10)	15.0 (6)	33.3 (9)

There was a significant increase of the TC genotype of the TGF-β1 T (+ 869)C in the patients with the GG of the IL-6 G (-174) C compared to normal controls (χ^2= 6.48, p= 0.01). There was a significant decrease in the frequency of the TC genotype in the patients with the CC genotype of the IL-6 comparing to the normal controls (6.2 %, vs 23.8 % respectively, χ^2= 8.64, p= 0.003). No other significant association was found. No other significant association was found.

Table 3. 53: Association of the TGF-β1 T (+869) C and the IL-6 G (-174) C genotype frequency with respect to gender of patients with type 1 diabetes

IL-6 G (-174) C genotype	TGF-β1 T (+869) C genotype											
	TT				TC				CC			
	Male Patients (n= 30)	NC (n= 18)	Female Patients (n= 30)	NC (n= 18)	Male Patients (n= 57)	NC (n= 19)	Female Patients (n= 68)	NC (n= 22)	Male Patients (n= 21)	NC (n= 13)	Female Patients (n= 19)	NC (n=14)
GG	33.3 (10)	33.3 (6)	40.0 (12)	33.3 (6)	63.2 (36)	42.1 (8)	69.1 (47)	40.9 (9)	19.0 (4)	23.1 (3)	26.3 (5)	21.4 (3)
GC	53.3 (16)	44.4 (8)	40.0 (12)	27.8 (5)	28.1 (16)	36.8 (7)	26.5 (18)	36.4 (8)	66.7 (14)	38.5 (5)	57.9 (11)	50.0 (7)
CC	13.4 (4)	22.3 (4)	20.0 (6)	38.9 (7)	8.7 (5)	21.1 (4)	4.4 (3)	22.7 (5)	14.3 (3)	38.4 (5)	15.8 (3)	28.6 (4)

No significant association was found.

Table 3. 54: Association of the TGF-β1 T (+869) C and the IL-6 G (-174) C genotype frequency with respect to age at onset of type 1 diabetes

IL-6 G (-174) C genotype	TGF-β1 T (+869) C genotype								
	TT			TC			CC		
	<10 years (n=22) %	10-20 years (n= 21) %	>20 years (n=17) %	<10 years (n= 58) %	10-20 years (n= 38) %	>20 years (n= 33) %	<10 years (n= 16) %	10-20 years (n= 11) %	>20 years (n= 13) %
GG	45.5 (10)	33.3 (7)	29.4 (5)	70.7 (41)	63.2 (24)	54.5 (18)	18.8 (3)	18.2 (2)	30.8 (4)
GC	40.9 (9)	47.6 (10)	52.9 (9)	24.1 (8)	28.9 (11)	39.4 (13)	62.4 (10)	72.7 (8)	53.8 (7)
CC	13.6 (3)	19.1 (4)	17.7 (3)	5.2 (3)	7.9 (3)	6.1 (2)	18.8 (3)	9.1 (1)	15.4 (2)

No significant association was found.

4 DISCUSSION

Type 1 diabetes is a chronic autoimmune disease caused by the complete destruction of pancreatic β cells, resulting in an absolute lack of insulin. Both genetic and environmental factors might be involved in the pathogenesis of type 1 diabetes (Buzzetti et al., 1998;Rabinovitch, 1998; Kida, 1998; Friday et al., 1999).

Several environmental factors such as infectious agents, dietary components, e.g. cow's milk, viruses and others exert their action on the genetically susceptible individuals and promote diabetes. It seems that environmental factors are associated with a large number of diseases. This is certainly the case for infectious diseases, even if the genetic background can strongly influence disease expression. The situation is very different in the case of diseases in which the environmental factors essentially modulate the expression of predisposing genes, either positively (predisposing factors) or negatively (protective factors). In the case of triggering factors, the disease onset is directly related to the encounter with the environmental factor, which can then be considered as the cause of the disease. In the modulation hypothesis, the disease appears only in the fraction of the population at genetic risk and it is on this population that environmental factors exert their positive or negative effects. According to the available data type 1 diabetes is of the second category (Songini and Mountoni, 1991).

In addition, evidence has been collected which suggests that genetic factors may play a primary role in the onset of diabetes and its progression (Buzzetti et al., 1998;Rabinovitch, 1998; Kida, 1998; Friday et al. 1999): Twin studies, familial clustering, HLA association, and novel non-MHC susceptible genes for the disease all

provide evidence for a genetic background to the disease by investigating the whole genome approach. It is well recognised that type 1 diabetes is a polygenic disease and the disease cannot be classified according to specific model of recessive, dominant, or intermediate inheritance (see also chapter one, 1.6.4). In addition, the disease-susceptibility genes are highly polymorphic, with a number of different allelic variants that are associated with the disease.

Genome-wide scans leading for linkage of certain chromosome regions with type 1 diabetes in affected sib-pair families have revealed that the major susceptibility locus resides within the major histocompatibility complex (MHC) or the HLA on chromosome 6p21. It is recognised that the HLA contains multiple susceptibility loci- *IDDM1*, including the class II genes, which control the major pathological features of the disease: T cells mediated autoimmune destruction of the insulin producing pancreatic β-cells. .However, a second locus, the insulin gene minisatellite VNTR, on chromosome 11p15 known as *IDDM2* also offers susceptibility. Although the association of certain HLA alleles with type 1 diabetes is very strong, this genetic locus is estimated to account for less than 50 % of genetic contributions to the disease susceptibility (Todd,2000). The search for non-HLA susceptibility genes has received great attention in recent years. Although genome wide searches are frought with controversy, such studies have suggested the association of numerous non-MHC loci with type 1 diabetes that will require careful follow-up investigation (Todd, 1998; Todd, 2000; Pugliese and Eisenbarth, 2000).

Non-MHC polymorphisms have been shown to override the susceptibility to an autoimmune disease provided by pathogenic HLA haplotypes (Verdaguer et al., 1999).

X

Cytokines, due to their role in the immune system, are thought to be an important in the destruction of pancreatic islet β cells and immune regulation (Rabinovitch, 1998). There is now clear evidence that cytokine gene polymorphisms may alter the production of that particular cytokine.

Consequently polymorphisms can alter the functions of these genes. As cytokines play an important role in the pathogenesis of type 1 diabetes, given the relative position of type 1 diabetes susceptibility towards cytokine gene polymorphism, major hurdles in understanding the role of the identified genes in defining genetic susceptibility, as well as their function, lie a head.

Further, as cytokines form part of a signalling network and gene polymorphisms can alter their function, it can be postulated that genetic polymorphism in one cytokine can alter the function of that cytokine which may lead to interruption of the immune system, hence the disease susceptibility.

X

4.1 Amplification of cytokine gene polymorphism

Signalling between cells of the immune system takes place both by direct cell-cell interactions involving surface molecules on the interacting cells and by cytokines. In association with hormones and neurotransmitters, cytokines form a chemical signalling system that regulates development, tissue repair, and the immune response (Thompson, 1998). In parallel with other signals arising from cell-cell or cell-antigen contacts cytokines provide a network controlling both innate or specific immune responses, including inflammation, defence against virus infection, proliferation of specific T- or B-cell clones, and regulation of their functions.

More than thirty years ago investigations were stated into finding polymorphisms in the genetic structure of cytokines (Rabinovitch, 1998; Thompson, 1998). To date there are many repeats of cytokine gene polymorphisms. Some of these gene polymorphisms have been shown to influence genetic susceptibility and progression of diseases. It is well documented that cytokines may play a great role in the onset of type 1 diabetes and its progression. The role of IL1 β and α, IL-1 ra, and TNF-α/β is well documented, section 1.7. In this study the genetic susceptibility of the IFN-γ CA repeat, the IL-4 C (-590) T, the IL-6 G (-174) C, and the TGF-β1 T (+869) C polymorphisms in patients with type 1 diabetes were investigated.

4.2 Cytokines as the immunogenetic markers for type 1 diabetes

4.2.1 IFN-γ, the TH1 cytokines profile inducer

In the present study a strong association within the first intron of the IFN-γ gene and type 1 diabetes was found . The association between the IFN-γ gene and type 1 diabetes had previously been studied by Awata and colleagues (1994).

Evidence was found for an association of the CA repeat polymorphism within the first intron of the IFN-γ gene and type 1 diabetes. Of 3 different studies investigating the association of the IFNG CA repeat and type 1 diabetes, only one, Pociot and colleagues have reported positive association in a Finnish, but no association in a Danish population (Pociot et al., 1997). This study confirmed that the IFNG CA repeat is an octa allelic polymorphism. The allele 3 showed a significant association with type 1 diabetes.

This was in contrast to the original report describing the polymorphism (Ruiz-Linares, 1993), where 6 alleles were reported with the apparent lack of the allele numbers 5 and 8, according to the present nomenclature. The study was based on analysis of 54 chromosomes of unrelated individuals of African, Asian, and European origin (Ruiz-Linares, 1993). In the Japanese population the presence of 8 alleles were reported (Awata et al., 1994). However, the exact length of PCR-identified alleles, or the number of CA repeats of different alleles were not reported, and comparison of data, including evaluation of the type 1 diabetes association, from different studies is, therefore, difficult. In Danish and Finnish populations 5 alleles were reported where alleles number 1, 7, and 8 (of the present nomenclature) were not identified (Pociot et al., 1997). Pociot and his co-workers have reported a significant association between allelic and genotype frequencies of the IFNG CA repeats polymorphism and type 1 diabetes in Finnish population. However, they claimed no association between the IFNG CA repeat polymorphism and type 1 diabetes in Danish population. Further, they have concluded

that this CA repeat polymorphism can differ according to ethnic groups. In Spanish population 8 alleles were identified and this polymorphism was associated with type 1 diabetes (Gallart et al., 1998). Recently the IFNG has been studied in British patients with pancreatitis and 8 alleles were identified. The allele 3 and 5 has been associated with the disease (O'Reilley et al., 2000).

Although Awata and colleagues reported a significant association of the IFNG CA repeat with type 1 diabetes in young-onset patients (<10 years) no significant difference was found between the age at onset of the patients with type 1 diabetes and the IFNG CA repeat polymorphism. The results may reflect differences in the incidence of long term complications.

The TDT result from the 3 / 3 genotype of the IFNG CA repeats showed a trend of transmission of the allele 3 from the parents to the offspring. However, a larger study is required to draw a familial clustering conclusion.

Further, the presence of IFN-γ in diabetes is well documented and it is believed that IFN-γ plays a role in the pathogenesis of type 1 diabetes. This genetic susceptibility confirms the existed reports on the genetic susceptibility of the IFNG CA repeat and attest that the 3 / 3 genotype and the allele 3 is significantly associated with the disease. One should be cautious about the positive association, since multiple factors could give erroneous associations. However, a sufficient number of patients with type 1 diabetes and healthy controls were analysed.

The IFN-γ production was associated with the IFNG CA repeat polymorphism (Pravica et al., 1999). Pravica and colleagues studied this microsatellite region of the IFN-γ gene in 164 healthy individuals and showed that IFN-γ production was significantly associated with allele 3.

4.2.2 IL-4, the TH2 cytokine profile inducer

There was no NheI restriction endonuclease site in the PCR products at –285 bp.

Arai and colleagues have previously reported that the 5′-flanking sequence plays a pivotal role in regulated expression of IL-4 during T cell activation. Since –285 bp position is in this region, any polymorphisms here could effect the expression of the IL-4 gene (Arai et al., 1989). The results presented here suggests that there is no polymorphism at –285 bp. It has been suggested by other groups that this is due to a cloning artefact (Professor P Mathesien, University of Bristol, 1997).

There has been no prevoius studies between the IL-4 C (-590) T polymorphism and type 1 diabetes. Since the IL-4 C (-590) T has been reported to be linked to the IL-4 function it suggests that IL-4 may not have a significant role in the pathogenesis of type 1 diabetes. However, IL-4 has been implicated in the pathogenesis of experimental diabetes (Rapport et al., 1993; Mueller et al., 1996; Mueller et al., 1997; Rabinovitch, 1998). This can be explained by the cross regulatory effect of TH1/TH2 phenomenon where TH2 or IL-4 and IL-10 production would be initiated as a result of the increase in TH1 cytokine profile. Recently, Leech and colleagues have studied *in vivo* activated circulating T cells in type 1 diabetes. They reported the lack of TH2 cytokines in type 1 diabetes and concluded that IL-4 and IL-10 production in newly diagnosed type 1 diabetic patients may represent an attempt at immunoregulation of active insulitis (Leech et al., 1999).

Although several earlier studies (Copeman et al., 1995; Pociot et al.,1992; Pociot et al., 1993; Pociot et al., 1994[1]; Mandrup-Poulsen et al., 1994; Metcalfe et al.,1995) reported possible association of TNF-α/β , IL-1α and β, IL-1 ra, and IFN-γ gene polymorphisms, this is the first report about the IL-4 C (-590) T polymorphism in patients with type 1 diabetes. No significant association was found between the IL-4 C (-590) T polymorphism and type 1 diabetes.

4.2.3 IL-6, a multifunctional cytokine released by macrophages

These results suggest that the IL-6 gene may be involved in the susceptibility to type 1 diabetes. This is the first study investigating the IL-6 gene in this disease. The in vitro production of IL-6 protein following the stimulation of monocytes from patients with type 1 diabetes with lipopolysaccharide has been found together with low levels of IL-1. It has been suggested that this may be due to an immunological defect (Ohno et al., 1993). In the NOD/Wehi mouse model of type 1 diabetes the disease can be prevented by treatment with anti-IL-6 antibodies whilst IL-6 can be detected in the islets of the animals (Campbell et al., 1991).

The G (-174) C polymorphism has been studied in patients with juvenile chronic arthritis where an increase in the frequency of the GC genotype was found compared to normal controls (Fishman et al., 1998). Functional studies using luciferase reporter assays with the IL-6 promoter containing either the G (-174) or C (–174) allele showed that the G (–174) was far more efficient than the C (–174) construct. This was reflected in the plasma levels of IL-6 in normal controls where those individuals with the CC genotype had the

lowest level whilst those with the GG genotype had the highest. In this study it was not possible to measure plasma levels of IL-6 in either the patients or normal controls. Also, there is a suggestion that hyperglycaemia may stimulate IL-6 release and consequently measuring the levels of this cytokine in plasma is difficult (Kato et al., 1997). In conclusion, in this thesis the results suggest rthat the IL-6 G (-174) C may be implicated in the genetic susceptibility to type 1 diabetes.

4.2.4 TGF-β1, the immunosuppressor cytokine

TGF-β1 is a multifunctional cytokine that regulates the proliferation and differentiation of a variety of cell types *in vitro*. The importance of TGF-β1 in immune regulation and tolerance has been increasingly recognised. It is now proposed that there are populations of regulatory T cells (T-reg) which are also categorised as TH3 that exert their action primarily by secreting this cytokine.

Type 1 diabetes is an immune derived disorder which is caused by the destruction of pancreatic β cells. It is well documented that diabetes is a cell mediated disease in which TH1 cells are dominant. TH3 or immunosuppressive cytokine, namely TGF-β1, is a possible cytokine to play its role in the maintenance of TH1 / TH2 cytokine profile.

The possible genetic association of the TGF-β1 T (+869) C polymorphism in the intron 1 of the gene was studied by screening the patients with type 1 diabetes and the normal control subjects for the latter polymorphism. There was a significant association between the TC genotype with type 1 diabetes. Allelic frequencies of the TGF-β1 T (+869) C polymorphism showed no considerable association with type 1 diabetes. The TDT result for the TC genotype of the TGF-β1 T (+869) C showed a significant increase in the transmission of the T (+869) than the C (+869). However, a larger study is required to draw a familial linkage conclusion.

Pociot et al., 1998 have studied the association of the C (76) T and 713-8delC polymorphism in the TGF-β1 gene with type 1 diabetes. These researchers have shown that there has been no genetic association between the latter polymorphisms and type 1 diabetes in general. However, they have reported a weak but significant association of the C (76) T polymorphism in the TGF-β1 gene with diabetes nephropathy (Pociot et al., 1998).

In order to examine the strength of the susceptibility of the T (+869) C polymorphism of the TGF-β1 gene with long-term diabetic complications, this polymorphism has been studied in patients with diabetic nephropathy, retinopathy and neuropathy. Meanwhile, a group of patients with type 1 diabetes who show no complications after 20 years of the disease, was also studied. A significant decrease was found in the frequency of the TC in the patients with diabetic nephropathy compared with the diabetic controls. This decrease was counteracted with a slight increase in the frequency of the TT. No other significant association was found between the T (+869) C polymorphism in the TGF-β1 diabetes long term complications. These findings reflect the a possible role of the TGF-β1 T (+869) C gene polymorphism in the diabetic nephropathy that confirms the available expression studies. However, a large scale study is required to draw such conclusion.

TGF-β1 is synthesised as a latent protein composed of 390 amino acids (Derynck et al., 1985; Lyons et al., 1990). The amino acid sequence of the active TGF-β1 is highly conserved across mammalian species, indicating a strong selection against variant forms of the protein. However, variations in the constitutive or induced expression of the protein as a consequence of variability with different effects of TGF-β1 on cellular functions (Cambien et al., 1996). The Leu (+869) Pro polymorphism of TGF-β1 is

located in the 29-residue signal peptide sequence which functions to translocate newly synthesised proteins (Verner, 1988). An increase in glucose serum level, which is the hallmark of diabetes, would enhance TGF-β1 production in order to stimulate glucose uptake. However, the T (+869) C polymorphism in the signal sequence of the TGF-β1 gene which results in a Leu→Pro substitution in amino acid 10 possibly affects the function of the signal peptide in the TGF-β1 gene, hence, TGF-β1 protein synthesis.

To date there has been no association study on the TGF-β1 gene polymorphism with type 1 diabetes except the C (73) T and 713-8del C polymorphisms in the TGF-β1 gene (Pociot et al., 1998). Since the CT genotype of the T (+869) C polymorphism in exon 1 of the TGF-β1 gene is significantly associated with type 1 diabetes it is unlikely that there is a linkage disequilibrium between the T (+869) C polymorphism and either 713-8del C or C (76) T.

4.3 Correlation studies between cytokine gene polymorphisms studied in this thesis

The function of cytokines in the immune system is a complex process. Although several studies provided evidence for the influence of gene polymorphism in cytokine genes and their function (Cookson and Walley, 1996; Fishman et al., 1998; Awad et al., 1998; Pravica et al., 1999) genetic profile in this thesis provide evidence where the production and function of an individual cytokine may not be only influenced by their genetic structure. However, cytokines are a complex network which seem to influence one another function.

The frequency of the TT of the IL-4 C (-590) T showed a significant increase in the patients with the 3 / 3 of the IFNG CA repeats compared to the normal controls, and a remarkable decrease in the TT with the X / X. The frequency of the patients with the GG and the GC of the IL-6 G (-174) C was increased significantly in the patients with the 3 / 3 compared to the normal controls; a remarkable decrease was found in the frequency of the GG and the GC in the patients with the X / X. There was also a significant increase in the frequency of the TC of the TGF-β1 T (+869) C in the patients with the 3 / 3; and a remarkable decrease in the frequency of the TC in the patients with the X / X compared to the normal controls. No significant association was found between the IL-6 G (-174) C and the TGF-β1 T (+869) C with the IL-4 C (-590) T gene polymorphisms genotype. A significant increase was found between the TC of the TGF-β1 and the GG of the IL-6. On the other hand, the frequency of the TC decreased significantly in the patients with the CC of the IL-6. A significant increase was found in the frequency of the patients with the GG of the IL-6 and the 3 / 3 of the IFNG with <10 and 10-20 compared to >20 years age at onset. A significant association was found between the frequency of TC of

the TGF-β1 in the patients with the GG of the IL-6 with <10 compared to 10-20 and >20 years age at onset. No significant association was found between the genotypes of the TGF-β1 and the IFNG, the TGF-β1 and the IL-4, and the IL-6 and the IL-4 genotype frequencies according to age at onset.

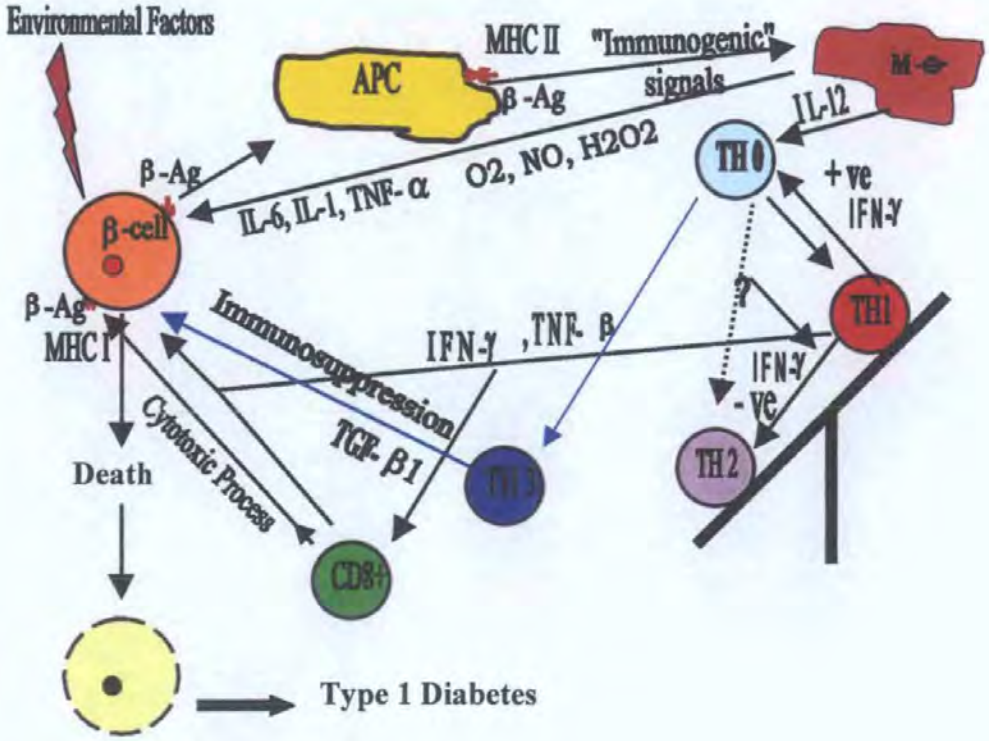
The results of the correlation studies signify that the genetic susceptibility of cytokines to the disease might be influenced by age at the onset of type 1 diabetes of the patients. The factor of age which were reported by Japanese (Awata et al., 1994; Kida et al., 1999) showed to influence the genetic susceptibility of cytokine gene polymorphism in this study as well, though it did not show a significant association in individual cytokine gene polymorphism studies in this thesis.

4.4 Type 1 diabetes pathogenesis model based on this thesis findings

Based on the results of this study and the evidence reviewed in this thesis a model for the pathogenesis of type 1 diabetes is depicted (Figure 4.01).

The process of destruction of β-cells which results in type 1 diabetes is believed to be the result of a disorder of immunoregulation. It is hypothesised that the environmental factors trigger the pathogenetic process by causing a restricted and focal beta-cell damage leading to the release of either modified or previously secluded beta-cell antigen. These antigens are taken up by APC recruited to the islets and accordingly the antigen is introduced to the TH0 which according to the immunogenic signals provided by the APC would deviate the TH1 / TH2 cytokine profile towards TH1 cytokines production. Accordingly, TH1 cytokines-IFN-γ, TNF-β, dominate over TH2 cytokines IL-4 and IL-10. This allows TH1 cytokines to initiate a cascade of immune/inflammatory processes in the islet (insulitis). Upon the interruption of the TH1 / TH2 balance TH3 or regulatory T cells are recruited to produce the immunosuppressive cytokine, TGF-β1. Association of the TC genotype of TGF-β1 T (+869) C polymorphism with type 1 diabetes and its association with low production of TGF-β1 could give a clue for this deviation of the TH1 / TH2 cytokine profile. IFN-γ activates cytotoxic T cells that interact specifically with β-cells and destroy them, and initiates the production of IL-1, TNF-α, and IL-6 as well as oxygen and nitrogen free radicals by macrophages that are highly toxic to islet β-cells.

Figure 4.01: Presentation of the immunogenetic of cytokines in the pathogenesis of type 1 diabetes



Modifications of the existing model for type 1 diabetes pathogenesis. β-Ag: the β-cell antigen is presented through APC: antigen presenting cells via the MHC class II to TH0: the precursor T-helper cells which would be producing TH1 or TH2: T-helper 1 and T-helper 2 subsets. The immunogenic signals produced by the β-cell antigens induce production of TH1 which lead to production of TH1 cytokine profile and deviation of the TH1 / TH2 cytokine profile balance. This deviation initiates TH0 to produce regulatory T subset, TH3. TH3 produce mainly TGF-β1 cytokine which acts as the immunosuppressor or regulator cytokine. Meanwhile TH1 cytokines, IFN-γ, TNF-β would be released. IFN-γ initiates macrophages to produce its cytotoxic activity by production of IL-1, TNF-α, and IL-6. At the same time, produce O^{-}, NO^{-} which are highly toxic to the islets β-cells. IFN-γalso initiates cytotoxic T-cells, $CD8^{+}$, which attacks β-Ag via the MHC class I. All of these factors contribute to the destruction of pancreatic β-cells and progression of type 1 diabetes (Rabinovitch, 1994 with modification).

4.5 Conclusion

Type 1 diabetes is associated with altered humoral and cellular immunity. This is demonstrated by the presence of autoreactive antibodies targeting β-cell constituents and other autoantigens, circular autoreactive T cells, and expression of adhesion molecules. In particular, type 1 diabetes was associated with altered regulation of cytokine expression manifested in part by reduced levels of serum cytokine inhibitors coupled with sustained expression of proinflammtory and immunoregulatory cytokines. The development of hyperglycaemia, a hallmark of type 1 diabetes, appears later in the course of the disease, frequently following months or years of the initiation of the T cell-targeted autoimmune destruction of islet β cells.

The exact role of T cell and macrophages derived cytokines play in the pathogenesis of type 1 diabetes remain the subject of intense investigation. Conclusions reached were largely based on studies on the genetically type 1 diabetes-predispose animals. Based on these and other studies, cytokines were shown to induce or exacerbate type 1 diabetes through direct and indirect mechanisms.

Although it is accepted that type 1 diabetes resulted from altered balance between TH1 / TH2 cells, the exact roles of TH1 and TH2 as well as TH3 subsets play in type 1 diabetes pathogenesis remain to be established.

In this study, association of human TH1-, TH2-, macrophage-, as well as TH3-derived cytokines gene polymorphisms with type 1 diabetes were studied. The CA repeat polymorphism in the first intron of IFN-γ has shown a significant association with type 1 diabetes. The IL-4 C (-590) T polymorphism showed no association with type 1 diabetes. The G (-174) C polymorphism in the IL-6 gene has also shown to be

significantly linked to type 1 diabetes. The T (+869) C gene polymorphism in the TGF-β gene has shown to be significantly associated with type 1 diabetes.

IFN-γ is the initiator of TH1 cytokine profile and also it inhibits TH2 cytokine profile. The association of the CA repeat in the IFN-γ gene with type 1 diabetes defines the role of TH1 in the onset of type 1 diabetes. The IL-4 C (-590) T gene polymorphism did not show a significant association with type 1 diabetes. From the point that IL-4 plays an important role in the initiation of TH2 cytokine profile and inhibition of TH1 cytokines, it seems that TH2 has hardly to do anything in the type 1 diabetes. However, due to the cross regulatory effect of TH1 / TH2 cytokine profile it seems that IL-4, which is the initiator of TH2 cytokines and induces TH1 cytokine, could be released to maintain the balance during the early phases of type 1 diabetes, which would be suppressed later in the course of the disease[1]. The IL-6 G (-174) C gene polymorphism was significantly associated with type 1 diabetes. Last but not least, the T (+869) C polymorphism in the TGF-β1 gene also showed a significant association with type 1 diabetes. According to the mentioned hypothesis, TGF-β1which is a regulatory or a immunosuppressor cytokine produced by TH3 may get into action when the cross regulatory effects of IL-4 is suppressed.

Although familial clustering towards the transmission of the linked cytokine gene polymorphisms studied in this thesis suggest a relative association with the susceptibility to type 1 diabetes in order to draw a conclusion a larger scale study is required.

Genetic profile studies showed to be significantly valid to verify genetic linkage between genes located on different chromosomes. The correlation of the TT genotype of the IL-4

[1] A proposal was submitted to Dr AG Demaine in June 1999 referred to the postulated issue.

C (-590) T with the 3 / 3 genotype of the IFNG but not with the IL-6 G (-174) C and the TGF-β1 T (+869) C suggests that, in spite of lack of association between the IL-4 C (-590) T polymorphism with type 1 diabetes, its genotype is correlated to the IFNG polymorphism. The lack of correlation between the IL-4 C (-590) T and the IL-6 G (-174) C as well as the TGF-β1 T (+869) C may be due to some immunological processes which must be investigated.

Age at onset of type 1 diabetes was not related to individual cytokine gene polymorphisms. It may be related to certain cytokine gene profile.

No association was found between cytokine gene polymorphisms and gender of the patients.

Cytokine gene polymorphisms, which were studied in this thesis, were shown to have a functional impact on their expression (Cookson and Walley, 1996; Fishman et al., 1998; Awad et al., 1998; Pravica et al., 1999). However, genetic profile results of this thesis provide evidence for the impact of age at onset on the frequency of cytokine gene polymorphism genotype, hence cytokine function. Gender of the patients did not show any impact on the frequency of individual cytokine gene polymorphism in the patients apart from a significant increase of the C (-174) of the IL-6 in the females normal controls. The correlation studies in this thesis provide further evidence of the impact of cytokine gene polymorphism on each other, ie the TT of the IL-4 C (-590) T and the 3 / 3 of the IFNG CA repeats. This may explain cytokine network system. These results could add a new edition to the impact of cytokines in type 1 diabetes pathogenesis.

These results provide a human case evidence for the role of TH1-, TH2-, TH3-, and macrophages-derived cytokines in the onset of type 1 diabetes and its progression. The association of the IFN-γ CA repeat gene polymorphism with type 1 diabetes was under debate, this data could confirm the association of the IFNG with type 1 diabetes and the lack of ethnicity which was raised by Pociot and colleagues (Pociot et al., 1997).

The association studies of the IL-4 C (-590) T, the IL-6 G (-174) C, and the TGF-β1 T (+869) C gene polymorphisms with type 1 diabetes are the first case study reports. One should be cautious about the positive association, since multiple factors could give erroneous associations. However, enough normal controls and patients with type 1 diabetes were analysed.

"If we stop asking the question 'what breaks tolerance' and begin to focus on the vast array of possibilities for each disease, we may begin to understand their multigenic nature" (Matzinger, 1998).

4.6 Future considerations

It is well known that type 1 diabetes is caused by an immune-mediated destruction of pancreatic islet β-cells. Recent studies have shown that genes outside of the HLA region are involved in determining susceptibility to type 1 diabetes. It is now known that polymorphisms in the coding and non-coding regions of the genes encoding cytokines may contribute to the susceptibility to autoimmune diseases as well as influencing the immune response to foreign antigens. There are few studies on the role of cytokines in human type 1 diabetes. Candidate genes implicated in the susceptibility to type 1 diabetes have been selected on the basis of our knowledge of the pathogenesis of type 1 diabetes and its long term complications.

Investigating the role of cytokine gene polymorphisms of TH1, TH2, TH3, and macrophages origin in the understanding of the pathogenesis of type 1 diabetes and its long term complications is an urgent need in the understanding of the pathogenesis of type 1 diabetes. Single nucleotide polymorphisms (SNPs) are major contributors to genetic variation as they comprise more than 80 % of all known polymorphisms and their density in the human genome is about one in every 300 to 500 base pairs (Wang et al., 1998; Collins, 1999). They are mostly biallilic and consequently less informative than mocrosatellite markers, although they are more frequent and mutationally more stable which makes them suitable for association studies. Some SNPs may contribute directly to a trait or disease phenotype by altering gene expression or protein function. The susceptibility of the IFNG CA repeats, the IL-4 C (-590) T, IL-6 G (-174) C, and the TGF-β1 T (+869) C have been accomplished using case control and family based parent offspring trios studies.

Interleukin-12 seems to play important role in initiating the cytokine network in type 1 diabetes. Recently a polymorphism is reported in the promoter region of the IL-12 gene (Pravica et al., 2000). Investigating the association of this polymorphism with type 1 diabetes can help in better understanding of type 1 diabetes pathogenesis.

To correlate the cytokines gene polymorphism association to type 1 diabetes and its complications it seems necessary to investigate the latter genetic association to cytokines protein secretion. To determine the role of cytokines in type 1 diabetes the elevation in bioactivity of each cytokine at different stages of the disease ie newly diagnosed, patients with history and each of complications could be defined by measuring their protein levels in serum.

To accomplish such a project the following is require:

- Peripheral blood of the follwing.

a) newly diagnosed patients

b) patients with Nephropathy

c) patients with Retinopathy

d) patients with Neuropathy

- Immunoassays to measure IL-4, IL-6, IL-12, IL-1, TGF-β, and IFN-γ.

Further, to fulfil this study it seems necessary to find out how the cytokine network is initiated and how antigen switches on Th1 mediated cytokine profile in type 1 diabetes. For example, the role of IL-12 in initiation of Th1 cytokine network or the role of IL-6 in preceding insulitis by measuring NFκB.

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